The Molecular Cloning and Expression of Human Placental Matrix and Surface Proteins

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by

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I wish to dedicate this thesis,

most of all to my wife

for all her help and support

and to our daughter Zahraa

and to our parental families,

especially my **brothers.**

Declaration

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

Sirous Zeinali

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Summary

Fibronectin protein isoforms have several functions in the body and play a vital role in maintaining the integrity of human placenta. The multifunctional properties of FN may have been mediated by the ability of FN isoforms to bind to cells and other extracellular matrix components such as collagens and fibrin. These and other functional properties which have been assigned to FN are thought to have been made possible by the existence of multiple FN isoforms.

In order to investigate the presence of multiple FN isoforms, human placental FN was investigated at molecular and protein level. At the molecular level a placental cDNA library was screened by molecular probing. At the protein level three monoclonal antibodies (McAbs) were used in immunohistological studies.

Screening a cDNA library from the chorionic plate of a human placenta resulted in the isolation of several clones of which two were analysed further. Both clones extended toward the regions of alternative splicing (e.g. ED-I and IIICS). Both regions of alternative splicing were present in one of the clones (HCF24), whilst in the second clone (HCF17), both regions were absent.

Exclusion or inclusion of these regions has not yet been reported and may account for some of the varaibilities seen at the protein level in human placenta.

Immunohistological studies were also carried out to investigate the pattern of alternative splicing at the protein level. The results were not as clear cut as the cDNA findings. This may have been caused by the nature of the work, or possibly by differential level of alternative splicing in different placentae.

As part of a team organized by the WHO to isolate and characterize trophoblast cell-surface determinants, our group was asked to provide research and expertise at the molecular level. The second part of this work was therefore concerned with investigating toward that objective.

Our aim was to identify placental specific cDNA clone(s) which could be expressed as membrane proteins. The expressed proteins could be identified by a number of monoclonal antibodies (McAbs) provided to us by other members of the above team. The obvious choice was to use a mammalian expression system which allows the expression of the surface determinants as native proteins, and also allows the proper post-translational modification to take place. The plasmid based expression system of CDM8 which uses a mammalian host cell (COS) developed by Seed and colleagues (1987), seemed most attractive since it had previously been successful in the isolation of several cell surface determinants. The cDNA clone coding for a cell surface determinant would be able to express the determinant after transfection into COS cells. However, the COS system did not lead to any isolation of trophoblast specific clone.

Lack of progress with the COS expression system meant that other methods of approach had to be investigated. One such approach was to amplify DNA sequences by PCR or to use another expression system such as lambda gt11.

PCR approach did not amplify the FDO161G clone. The main complication is thought to have been caused by the Cys/His difference in the amino acid sequence of the FDO161G antigen. The 3' end of the upstream primer was designed to be complementary in part to the codons for cysteine (based on the partial amino acid sequence of FDO161G recognised polypeptides), but later this residue was found to be histidine (Chapter 7). This codon difference would be enough to de-stabilize the 3' end of the upstream primer and may account for lack of specific PCR product.

The lambda expression system could only be used for FDO161G recognised antigen when polyclonal antibody against FDO161G antigen became available.

Using lambda gt11 cDNA library a non-surface protein was isolated by using FDO161G polyclonal antibody, however, this could not be repeated using other Abs as no polyclonal Abs were available for isolating other determinants (GB17, GB25, and GB24). DNA sequencing and *in situ* hybridisation indicated that the FDO161G isolated clone codes for 3 beta-hydroxy-5-ene steroid dehydrogenase, HSD. Immunofluorescence staining of COS cells transfected with the HSD clone showed that acetone fixed cells stained with FDO161G, while viable COS cells from the same experiment did not. These findings suggest that the HSD might not be present in the cell membrane. The positive staining of fixed cells may have been due to the delipidation of the membrane resulting in the lumen end of the HSD becoming exposed to the Ab.

Contents

Abbreviations

Ab	- antibody.
Ag	- antigen.
Amp	- ampicillin.
ATP	- adenosine triphosphate.
Bis-acrylamide	- N,N'methylene-bis-acrylamide.
BSA	- bovine serum albumin.
IIICS	- connecting segment III.
cccDNA	- covalently closed circular DNA.
cDNA	- complementary DNA.
CIP	- calf intestinal phosphatase.
DAB	- 3,3'diaminobenzidene.
dATP	- deoxyadenosine triphosphate.
ddNTPs	- dideoxy nucleotide triphosphates.
DEAE	- diethylaminoethyl.
dH_2O	- distilled water.
DMF	- dimethylformamide.
DNA	- Deoxyribonucleic acid.
DNase	- deoxyribonuclease.
dNTPs	- deoxyribonucleotide triphosphates.
DTT	- dithiothreitol.
DMSO	- dimethyl sulfoxide.
EDTA	- ethylenediaminetetra-acetic acid.
ED-I (-A)	- extra domain-I (-A).
ED-II (-B)	- extra domain-II (-B).
FCS	- fetal calf serum.
FN	- fibronectin.
g	- gravity.
Ig	- immunoglobulin.
IAA	- isoamyl alcohol.
IPTG	- isopropylthio-B-D-galactoside.
LMP	- low melting point.
mRNA	- messenger RNA.
MW	- molecular weight.
McAb	- monoclonal antibody.

oligo	- oligonucleotide.
OD	- optical density.
pfu	- plaque forming units.
PAGE	- polyacrylamide gel electrophoresis.
PEG	- polyethylene glycol.
RE	- restriction enzymes.
RNA	- ribonucleic acid.
RNase	- ribonuclease.
SDS	- sodium dodecylsulphate.
TCA	- trichoracetic acid.
TE	- tris-EDTA.
TEMED	- N,N,N',N'-tetramethylenediamine.
T_m	- termal denaturation (melting temprature).
Tris	- tris (hydroxymethyl) aminoethane.
U	- unit.
UV	- ultraviolet.
X-gal	- 5-bromo-4-chloro-3-indolyl-B-galactoside.
PCR	- polymerase chain reaction

Units

A	- amps.
mA	- milliamps.
A_{260}	-absorbance at 260 nm.
bp	- base pairs.
kp	- kilo base pairs.
^oC	- degrees Centigrade.
Ci	- curies.
mCi	- millicuries.
uCi	- microcuries.
CPM	- counts per minutes.
dal	- daltons.
kDa	- kilo daltons.
g	- grammes.
hr	- hour.

mg	- milligrammes.
ug	- microgrammes.
ng	- nanogrammes.
l	- litres.
ml	- millilitres.
ul	- microlitres.
m	- metres.
cm	- centimetres.
mm	- millimetres.
M	- molar.
mM	- millimolar.
uM	- micromolar.
min	- minutes.
pH	- acidity ($-\log_{10}[H^+]$).
rpm	- round per minute.
sec	- seconds.
V	- volts.
mV	- millivolts.

CHAPTER 1

INTRODUCTION

1.1 The Human Placenta

Placenta is the part of the conceptus that mammalian evolution has selected as the best means of ensuring secure developmental conditions for the fetus and allowing gestation to be beneficial to the fetus. After the blastocyst has implanted into the endometrium, the trophoblast derivatives from the embryo combine with maternal uterine decidual tissues to form the placenta (Wegmann & Gill, 1983). The human placenta's role, in its limited life span of forty weeks, is to nourish the fetus and maintain the fetal environment and thus the structure and function of the placenta changes during its period of growth as it is required by the developing embryo and fetus. It acts as a physical barrier separating the maternal and fetal blood circulations but allowing the exchange of substances such as metabolites and hormones between the mother and fetus. The placental microvilli, which effectively increase the surface area of the placenta in contact with the maternal blood, are thought to facilitate this transfer (Jones & Fox, 1979).

The placenta is a complex organ composed of different types of trophoblast and a variety of other fetal tissues such as stroma, endothelium, and blood elements. Furthermore, the junction area of the human placenta with the uterus comprises a mixture of fetal and maternally derived cells in direct contact.

1.1.1 Decidualization and Preparation for Implantation

Implantation and subsequent development of the human placenta depend on certain changes in the endometrium that culminate in the decidual cell formation. Extensive studies have been concerned with the endocrine control of the generation of uterine sensitivity to decidual stimuli and the extent of decidualization (Glasser, 1972; O'Grady & Bell, 1977; Bell, 1983). It has been shown that decidualization is induced spontaneously, after hormonal preparation during

the late secretory phase of the menstrual cycle and does not proceed further unless pregnancy ensures a maintained production of progesterone. The decidua directly beneath the site of implantation forms the decidua basalis (Fig. 1.1). Surrounding the fertilized ovum and separating it from the rest of the uterine cavity in the early months of gestation is the decidua capsularis, which forms as a result of deep implantation of the human fertilized ovum. The remainder of the uterus in pregnancy, is lined by decidual parietalis (O'Grady & Bell, 1977; Fig. 1.1). Since the fertilized ovum does not occupy the entire uterine cavity in the early months of pregnancy, there is a space between the capsular and parietal portions of the decidua. By the fourth month, the growing fertilized ovum hits the uterine cavity, leading to the fusion of both the capsularis and parietalis, obliterating the endometrial cavity (Wynn, 1975).

1.1.2 Placental Formation, Development and Structure

After ovum fertilization and the subsequent blastocyst formation, the human blastocyst loses its surrounding zona pellucida (Sacco, 1987). The outer layer of the blastocyst proliferates to form the trophoblastic cell mass, from which cells infiltrate between the endometrial epithelium and thus the blastocyst becomes completely embedded in the endometrium by the eleventh or twelfth day (Fox, 1978; Fig. 1.2). This is followed by the differentiation of the trophoblast into a peripheral layer of primitive syncytotrophoblast and an inner layer of cytotrophoblast, which starts proliferating to form the precursors of the primary villi (Boyd & Hamilton, 1970). Between the 9th to 25th day of gestation there is a period of intense growth and differentiation, which results in the chorionic villi becoming established. The primary villi are solid trophoblast, which arise from the Langhans cells. After developing a mesenchymal core,

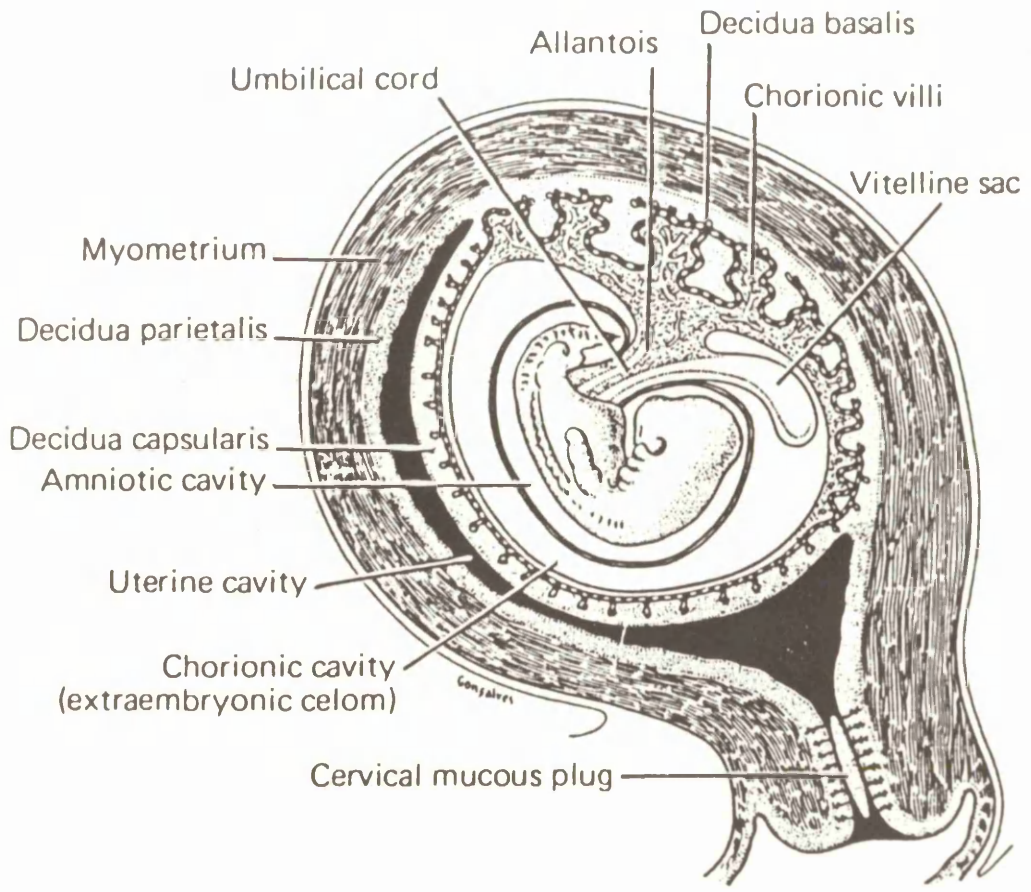


Figure 1.1 Schematic drawing showing formation of the regions of the decidua and the chorionic villi (from Junqueira et al, 1989).

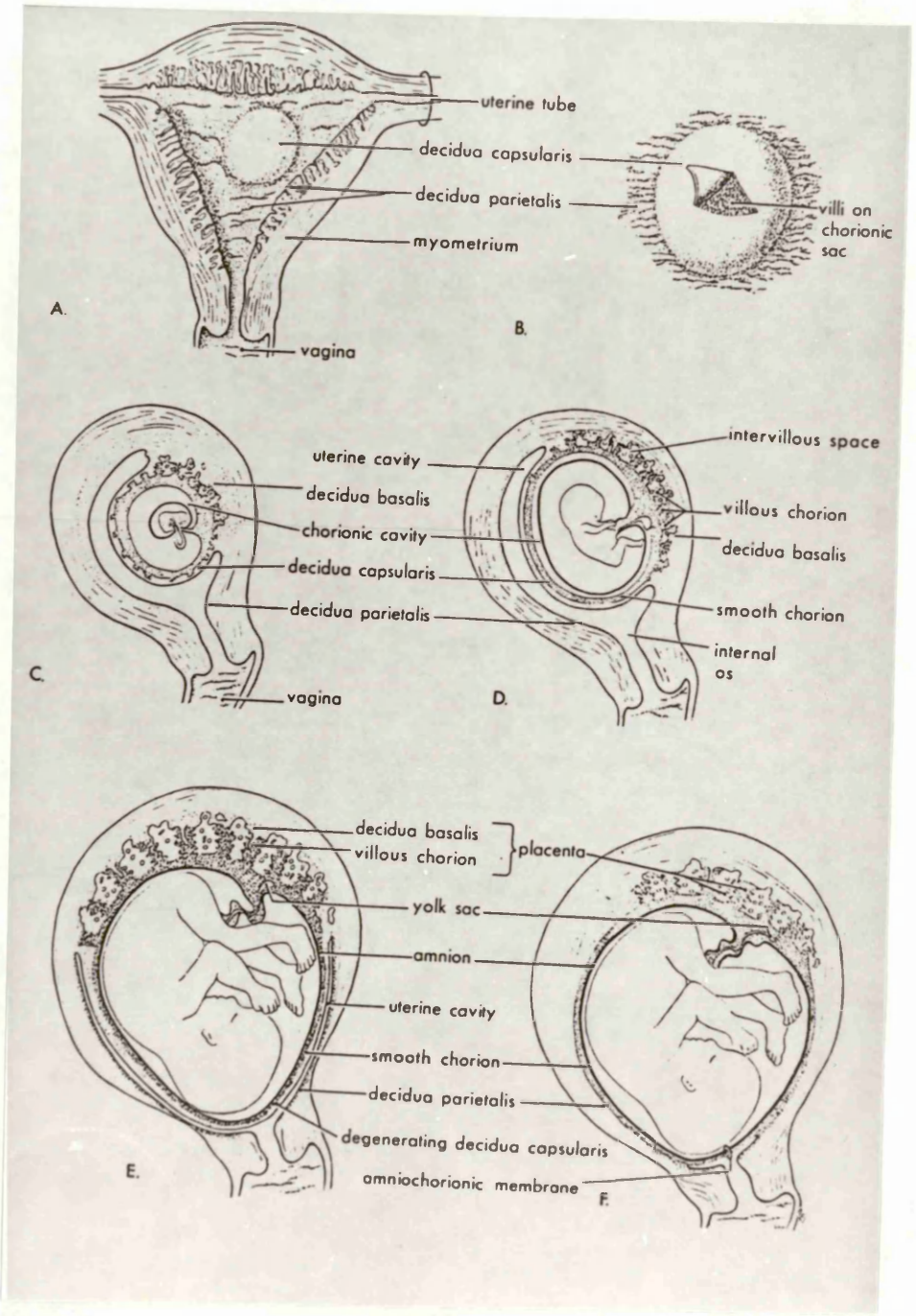


Figure 1.2 Schematic representation placental formation and development. The figure shows the implanted four-week embryo (A) and enlarged implantation site (B) and changing relations of the fetal membranes to the decidua from the fourth to twenty-second weeks of pregnancy (C-F). (From Moore, 1973).

which contains fibroblasts, phagocytotic Hofbauer cells and collagen fibers, the villi, with the development of fetal capillaries in the mesenchymal cores, are classed as secondary villi. The mature villi have an outer layer of syncytiotrophoblast, a layer of cytotrophoblast and an inner layer of connective tissue containing the fetal capillaries. The diameter of the villi decreases throughout pregnancy, from 170 um in the first trimester to 40 um at term (Fox, 1978). In addition to the chorionic villi, there is another class of villi, whose function would seem to be to anchor the placenta to the maternal endometrium. These villi are composed of solid trophoblast and are structurally identical to the primary chorionic villi. These cells subsequently proliferate to form the cytotrophoblastic shell (Clint *et al.*, 1979). The cytotrophoblastic cell columns which extend through the peripheral syncytium and join together to form the cytotrophoblastic shell, are derived from the cytotrophoblasts found in the Langhans layer of the placenta (Boyd & Hamilton, 1970; Wynn, 1972).

Later, after mesodermal cores growth leads to the secondary and tertiary villi, the cellular trophoblast, at the tips of the villi, forms the cytotrophblastic cell columns, which are not invaded by mesenchyme and are not vascularised, but are anchored to the decidua of the basal plate. These cells, subsequently, proliferate and spread laterally, to form a continuous cytotrophblastic shell which divides the syncytiotrophoblast into two layers, the definitive syncytium on the fetal aspect of the shell and the peripheral syncytium between the shell and the decidua. The definitive syncytium persists as the limiting layer of the intervillous space, whereas, the peripheral syncytium gives rise to masses of syncytium-like giant cells that extend through the decidua basalis and into the myometrium, which together form the placental bed (Boyd & Hamilton, 1970; Wynn, 1975; Fox, 1978; Pijnenborg *et al.*, 1981a). Furthermore, the cytotrophblastic cells invade and partially replace the

endothelium of the decidual portion of the decidual spiral arteries; trophoblasts cause considerable disruption of the arterial wall, with the deposition and formation of fibrinoid material (Pijnenborg *et al.*, 1981b; Fig. 1.2).

The establishment of the trophoblastic shell would allow rapid growth of the developing placenta. This, subsequently, leads to an expansion of the intervillous space, into which sprouts of syncytiotrophoblasts extend from the primary villous stems. These villi, as the placental development proceeds, become orientated towards the uterine cavity, degenerate and form the chorion laeve which comes into contact with the parietal decidua of the opposite wall of the uterus. Moreover, those villi on the side of the chorion, towards the decidua basalis, proliferate to form the chorion frondosum, which develops into the definitive placenta (Fig 1.3). During this period, some regression of the cytotrophblastic elements in the chorionic plate and in the trophoblastic shell take place, where the cytotrophoblastic cell columns degenerate and become largely replaced by fibrinoid material (Rohr's Layer), clumps of cell remain to form the 'cytotrophoblastic cell islands' (Boyd & Hamilton, 1970; Fox, 1978).

The placental septa (Fig. 1.4) appear during the third gestational month (Boyd & Hamilton, 1970). The cytotrophoblastic cells which predominate in both the basal plate and the septa, have been referred to as 'X-cells', however, their fetal origin has been clearly confirmed by the presence of Y chromosome, if the fetus is male (Fox, 1978).

By the end of the fourth month of pregnancy, the placenta has attained its definitive form and undergoes no further anatomical modifications. It is composed of the chorionic plate, which is on one side covered with the amnion and on the other side is attached to the fetal cotyledons. These project towards the decidua basalis, together with the cytotrophblastic shell,

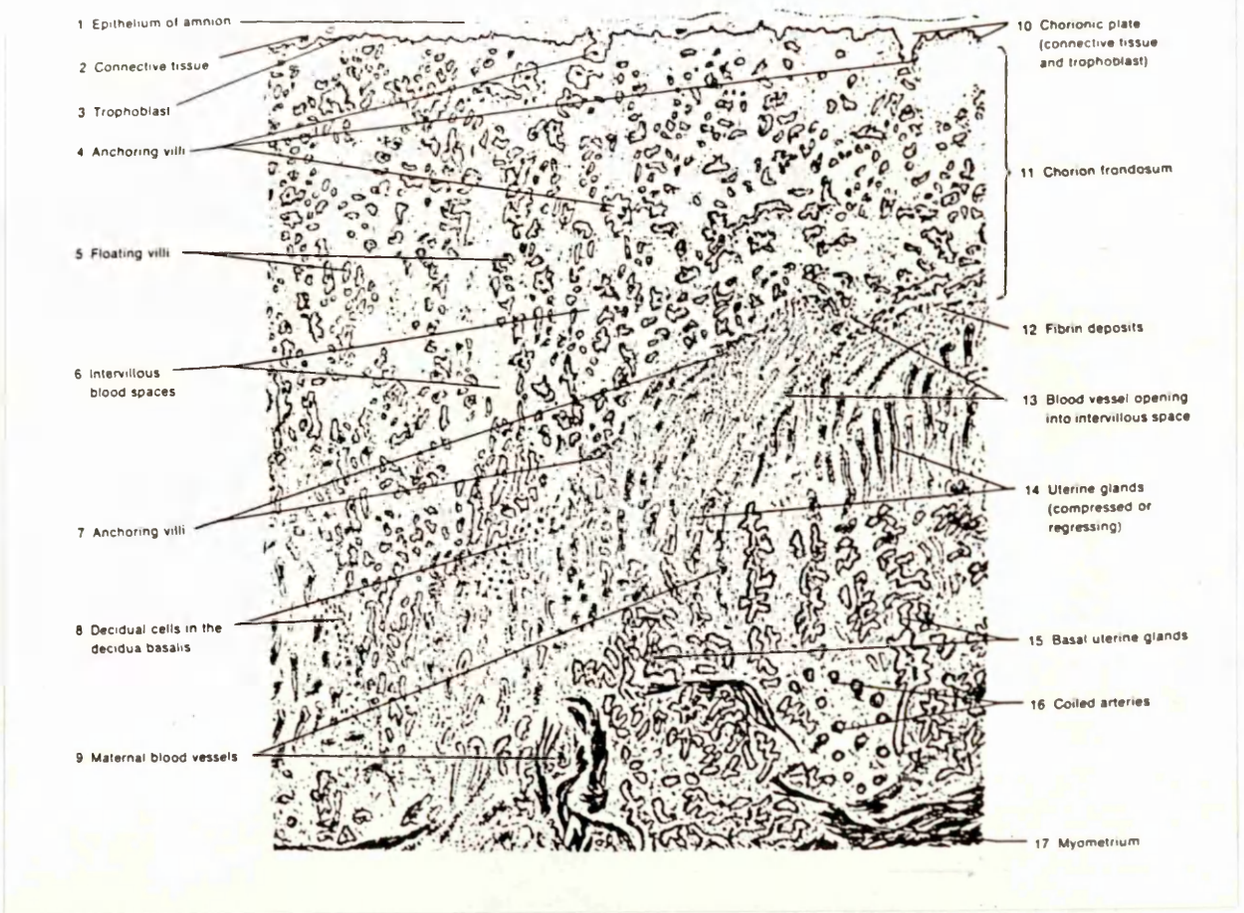


Figure 1.3 Five months pregnancy, a panoramic view. The upper part of the figure represents the fetal portion of the placenta. The figure shows surface of the amnion (1), merged connective tissue of amnion and chorion (2), trophoblast of chorion (3, 10), anchoring villi arise from the chorionic plate (4) and extend to the uterine wall, and embed in the dicidua basalis (7) and floating villi (chorion frondosum), sectioned in various planes (5, 11) due to their growth in all directions from the anchoring villi; these villi "float" in the intervillous space (6), which are bathed in maternal blood. The maternal portion of the placenta or dicidua basalis shows embedded anchoring villi (7), groups of large dicidual cells (8), uterine glands (14), maternal blood vessels (9), and maternal blood vessel is seen opening into an intervillous space (13). Fibrin depositews appear on the surface of the decidua basalis (12) and increase in volume and extent as the pregnancy continues and shown to be mainly in the form of fibrin (Surcliffe et al., 1982). The figure is from Fiore (1989).

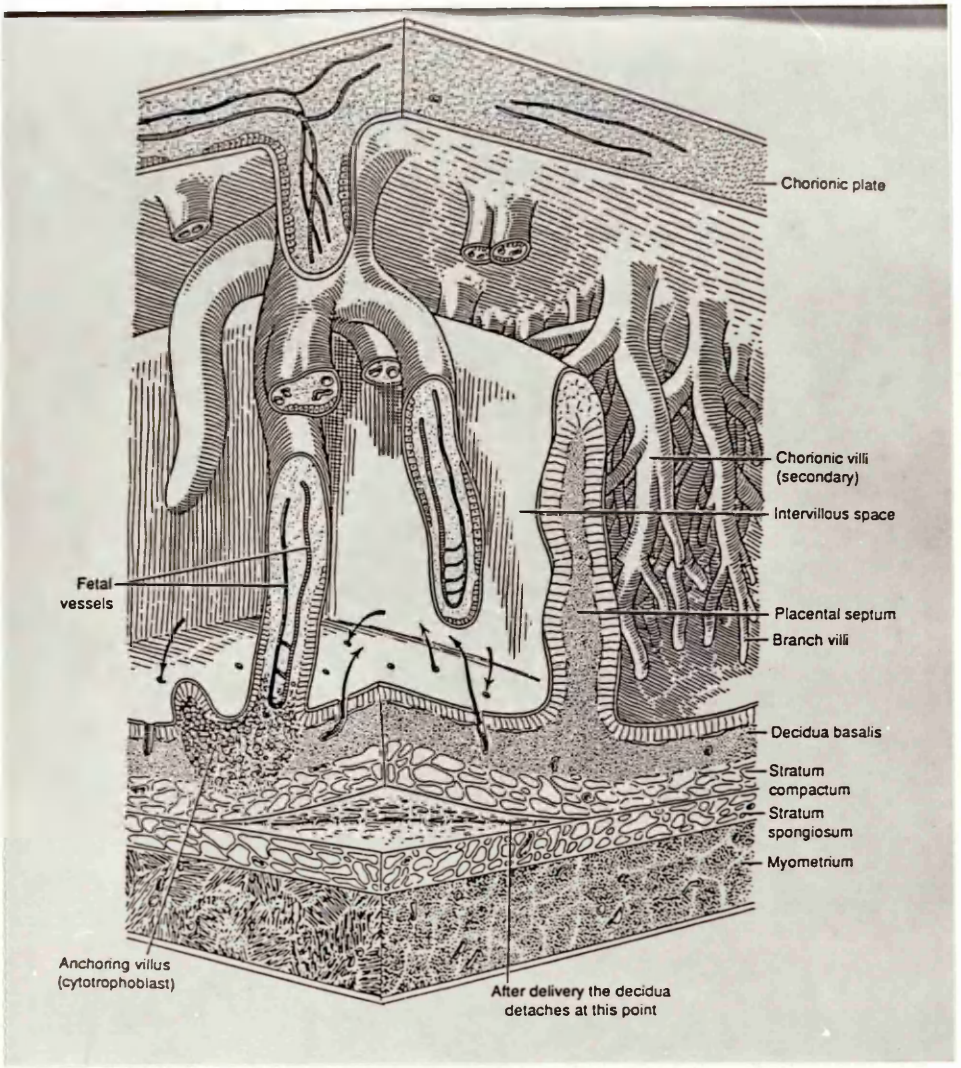


Figure 1.4 Schematic drawing of placental structure. Arrows indicate the blood flow from decidual arteries to intervillous space and back to decidual veins. The chorionic plate, chorionic villi section and the basal plate are also shown. (From Junqueira et al., 1989).

forms the basal plate (Fig. 1.4). The smallest fetal cotyledons form groups of villi, near the chorionic plate. The terminal villi are formed from the side branches of the anchoring villi, which float in the intervillous space, some of which are united by syncytial bridges or by deposits of fibrinoid substances on the surface of the adjacent villi (Fox, 1978).

These terminal villi which are the functional units of the placenta are composed of an outer syncytiotrophoblastic layer, an inner cytotrophoblastic layer (Langhans cells) and the villous stroma which is composed of fibroblasts, Hofbauer cells (which appear to be macrophages) (Wynn, 1975; Fox, 1978), endothelial cells of the fetal capillaries and other mesenchymal elements (Fig. 1.5). As the placenta ages, these form numerous subdivisions. The more obvious histologic changes that are consistent with increased efficiency of transfer include an increase in the ratio of the villous surface to volume, a decrease in thickness of the syncytium, discontinuity of the Langhans layer and reduction in the proportion of villous connective tissue relative to the trophoblast (Fig. 1.5; Boyd & Hamilton, 1970; Wynn, 1975).

1.1.3 Syncytiotrophoblast and Cytotrophoblast

There are considerable morphological differences between the syncytiotrophoblast and cytotrophoblast cells. The cytotrophoblast cells show considerable variation in shape, which is dependant on their positioning in the placental structure and the gestation period. The uninucleated cytotrophoblast cells fuse to form the syncytiotrophoblast cells. These syncytiotrophoblast cells form the outer multinucleated syncytial layer of the placenta and they invade the maternal endometrium. The syncytiotrophoblast of the term placenta is the predominant trophoblastic component, as the ratio of cytotrophoblast to syncytiotrophoblast decreases throughout gestation. The

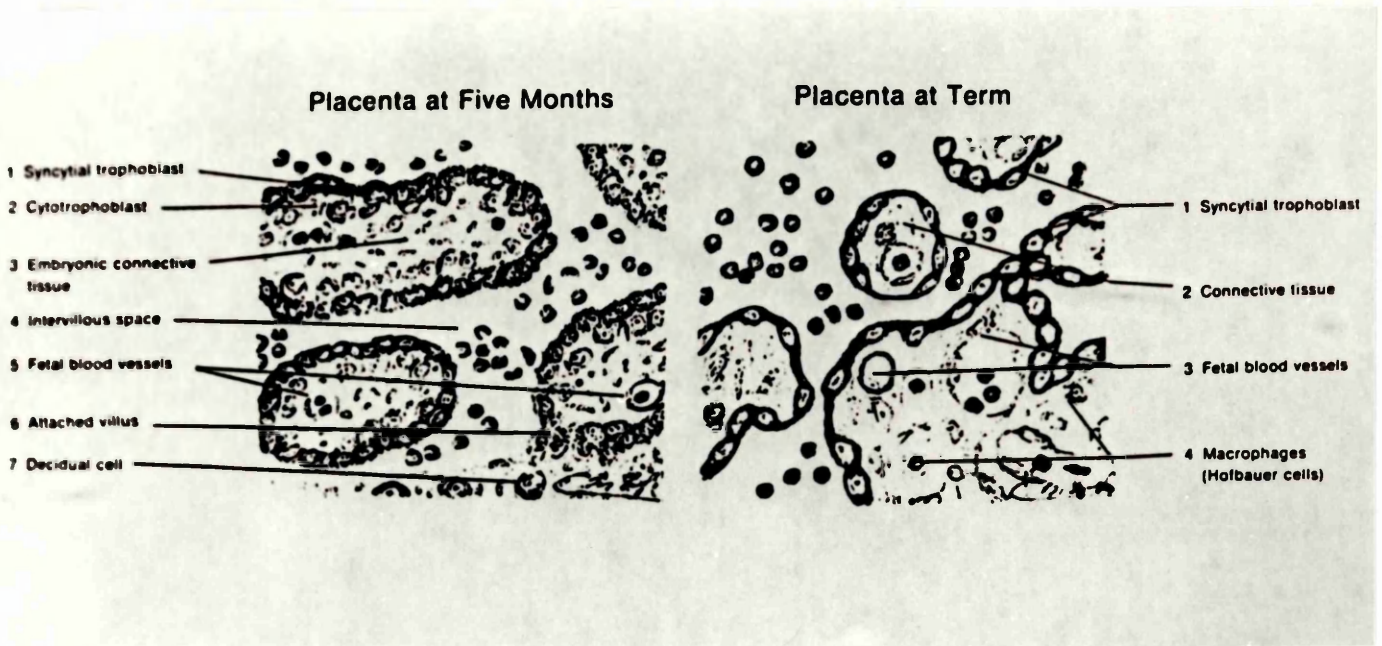


Figure 1.5 Chorionic villi at 5 months of pregnancy and at term. In contrast to villi in early stages of pregnancy (A), the chorionic epithelium is only synsytiotrophoblastic type. Another feature of term placenta is the presence of numerous fetal blood vessels (from di Fiore, 1989).

external surface of the syncytiotrophoblast is invested with a thick coat of microvilli, bathed by extra-endothelial maternal blood, which is outside the confines of the endothelium of the mothers vascular system (Hamilton & Hamilton, 1977; Truman & Ford, 1984).

1.2 Placental Proteins and Markers

The placental proteins fall into three groups, (1) proteins with hormonal functions, (2) proteins with enzymatic functions and (3) proteins with unknown function. Many of these placental proteins have not been shown to be essential for fetal or placental well being during pregnancy and apparently normal pregnancies have come to term without the synthesis of one or more of the placental proteins (Gordon & Chard, 1979; Chard, 1982; Reshef *et al.*, 1990). Though many of the proteins produced by the placenta have no proven function, they are studied with the hope that the more is known about the structure of the proteins, the closer one will be in deducing their function.

1.2.1 Monoclonal Antibodies (McAbs) Against Human Placental Determinants

A wide variety of McAbs have been raised against human placental trophoblast preparations (Sunderland *et al.*, 1981; Johnson & Molloy, 1983; Loke *et al.*, 1984; Bulmer & Johnson 1985; Hsi *et al.*, 1987 and some other references later in this chapter). Several of the McAbs raised against human placental trophoblast cells are non-specific, being raised against surface membrane proteins, common to both trophoblast and other tissues. These non-specific McAbs raise the possibility of shared membrane complexes between the placenta and other tissues.

Thiede & Choate (1963) and Fox & Karkhongor (1970), using immunofluorescent staining, have found that human chorionic gonadotrophin (hCG) is localised in both cytotrophoblast- and syncytiotrophoblast and in cytotrophoblastic cells tissue culture. Since then hCG has been localised in different cells of placenta (Gaspard *et al.*, 1980; Tabarelli *et al.*, 1983; Kurman *et al.*, 1984). Some of the other proteins include hPL, SP1, PAPP-A, etc (Lin & Halbert, 1976; McIntyre *et al.*, 1981; Tabarelli *et al.*, 1983; for a fuller review of the early pregnancy factors, see Ellendorff & Koch, 1985).

1.2.2 Trophoblast Surface Markers

Different studies have been directed towards the trophoblast, since the placental cells originate from trophoblast cells and these cells subsequently cover the surface of the placenta and extraembryonic membranes, coming into direct contact with meternal tissues over a wide area during gestation. Most attention has been focussed on the syncytiotrophoblast because it is easy to purify its microvillous brush border which is directly exposed to maternal blood circulation. Transferrin and IgG receptors were identified on syncytiotrophoblasts (Faulk & Johnson, 1977; Faulk & Galbraith, 1979; Johnson & Brown, 1980; Johnson *et al.*, 1982) in addition complement component C3 and trophoblast antigen 1 (TA1) have been detected in this layer as well (Faulk & Johnson, 1977; Faulk *et al.*, 1978).

Recently, the advent of hybridoma technology has opened up new avenues of approach, to the study of trophoblast-specific or trophoblast associated molecules. Some of these monoclonal antibodies (McAbs) have been used to differentiate morphologically, very similar trophoblast cell populations of human first-trimester chorionic villi from each other (Butterworth & Loke, 1985) and three separate fetal trophoblast populations were identified within the term

amniochorionic membrane (Bulmer & Johnson, 1985). Some other placental trophoblast-specific monoclonal antibodies and their specificity will be discussed in section 1.4 (contraception).

1.3 Fibronectin at the Protein Level

1.3.1 Extracellular Matrices

Extracellular matrices are composed primarily of three types of macromolecules: collagens, proteoglycans and glycoproteins. Collagens constitute a group of genetically distinct, but related molecules with unique tissue distribution (Bornstein & Sage, 1980; Nimni, 1983). Similarly, proteoglycans are a highly polymorphic group of molecules that also have specialized distributions in tissues and different types of extracellular matrices (Hascall & Hascall, 1981). Glycoproteins are also major components of the extracellular matrices and several functions have been assigned to them.

The interaction of cells with one another and with extracellular materials (matrices, solid surfaces, etc) are of vital importance to cell function. These interactions have major effects on the proliferation, differentiation and organization of cells. Our understanding of these interactions has advanced considerably in recent years and it is now clear that they are often mediated by a class of high molecular weight glycoproteins that are involved in both interaction and in the actual structure of extracellular matrices. The most intensively studied of these glycoproteins is fibronectin (FN), but there is a set of proteins with analogous properties (laminin, von Willebrand protein, thrombospondin, vitronectin, etc), the analysis of which is also progressing apace (Hynes, 1985).

1.3.2 Cell-Matrix Interactions

The most obvious consequence of cell matrix interactions is cell attachment, that results in anchoring of cells. However, the effects of cell-matrix interactions clearly go well beyond mere anchoring of cells and include cellular responses such as increased migration, differentiation, and stimulated or arrested growth (Ruoslahti *et al.*, 1988). In considering the effects of extracellular matrix on cells, it is important to note that there are three essentially independent aspects of the relationship of a given cell with the extracellular matrix: 1) the production of extracellular matrix proteins by the cell, 2) the deposition of the proteins produced into an insoluble matrix and 3) the ability of the cells to interact with matrices made by other cells. Normal cells typically participate in all three of these aspects, while tumuor cells are frequently deficient in one or more of these areas (Ruoslahti *et al.*, 1988).

1.3.3 Glycoproteins

During embryogenesis, morphogenetic processes involve interactions among cells and between cells and their immediate environment, the extracellullar matrix (Dufour *et al.*, 1988). The components of the extracellular matrix and their cell-surface receptors that are involved in adhesive systems have been identified and extensively studied in many laboratories. These adhesive processes, encountered during embryogenesis, malignancy, hemostasis, wound healing, host defense and maintenance of connective tissue integrity, involve the participation of glycoproteins with a subunit molcular weight of about 250 kDa (Dufour *et al.*, 1988). The glycoproteins, which have been isolated, purified and were originally named according to their sources or their biological activities, come from a family now known as fibronectin (reviewed by Hynes & Yamada, 1982; Dufour *et al.*, 1988).

1.3.4 Distribution of Fibronectin Proteins

As mentioned above, cell-cell and cell-substrate interaction play a fundamental role in cell behaviour, and differentiation. Among the extracellular components involved in these events, fibronectin is probably the most important and widely distributed. This high molecular weight glycoprotein is present in blood plasma, extracellular matrices, basal lamina, and at cell surfaces (Owens *et al.*, 1986). It has been identified not only in vertebrate species such as amphibia (Heasman *et al.*, 1981; Akiyama & Johnson, 1983), fish (Natali *et al.*, 1981; Duband *et al.*, 1987), birds (Vaheri & Mosher, 1978) and higher mammals (Mosessin & Umfleet, 1970) but also in invertebrates such as the sea urchin (Iwata & Nakano, 1981) and in insects such as Drosophila (Gratecos *et al.*, 1988). It has been implicated in a variety of cell contact processes, including cell attachment and migration, opsonization and wound healing. Expression of fibronectin appears to be developmentally regulated and is modulated by malignant transformation of cells (Owens *et al.*, 1986).

1.3.5 FN Subunits

FN is a disulphide-linked dimer comprised of subunits of approximately 230-250 kDa molecular weight. Two major forms of the molecule have been distinguished: a plasma form (pFN), which is a soluble heterodimer and a cell surface-associated (or cellular) form (cFN), which consists of dimers and multimers. The latter have a fibrillar structure and are highly insoluble (Owens *et al.*, 1986). Differences between the subunits of the two major fibronectin types have been identified using monoclonal antibodies (Atherton & Hynes, 1981) and peptide mapping (Hayashi & Yamada, 1982; Sekiguchi *et al.*, 1985). Each subunit of plasma and cellular fibronectin also shows considerable heterogeneity in charge and size which is only partly accounted for by

vaiable post-translational modification (e.g. glycosylation, phosphorylation, and sulphation) (Paul & Hynes, 1984). This implies that there are primary structural differences, not only between the subunits of plasma and cellular fibronectin, but also between subunits within each type of the molecule. FN may, therefore, represent a family of very closely related glycoproteins (Owens *et al.*, 1986)

1.3.6 Molecular Structure of FN Subunits

Individual FN molecules are dimers of similar but not identical polypeptides. The plasma FN (pFN) is synthesized by hepatocytes and then secreted into the bloodstream. On the other hand the cellular FN (cFN) is made by many cell types, including fibroblast and epithelial cells, which after secretion can be deposited as long insoluble fibrils in the extracellular matrix. Primary structural differences exist, not only between plasma and cellular FNs, but also among the subunits of each type. Despite their heterogeneity, all FN subunits show a common modular organization in their primary sequence, which contains a series of homologous repeating units, the so-called homology types I, II and III (about 40, 60 and 90 amino acids in length, respectively) (Petersen *et al.*, 1983). In addition to these structural similarities, the various form of FN subunits share common functional properties that correspond to domains for bonding other extracellular matrix macromolecules and the cell surface (Owens *et al.*, 1986).

1.3.7 Homology Types I, II, III

The fibronectin chain presents three types of internal homologies (types I, II, and III). As shown in Figure 1.6, most of fibronectin is formed by type III units. The protein also contains four regions which have no homologous counterparts within the molecule. These

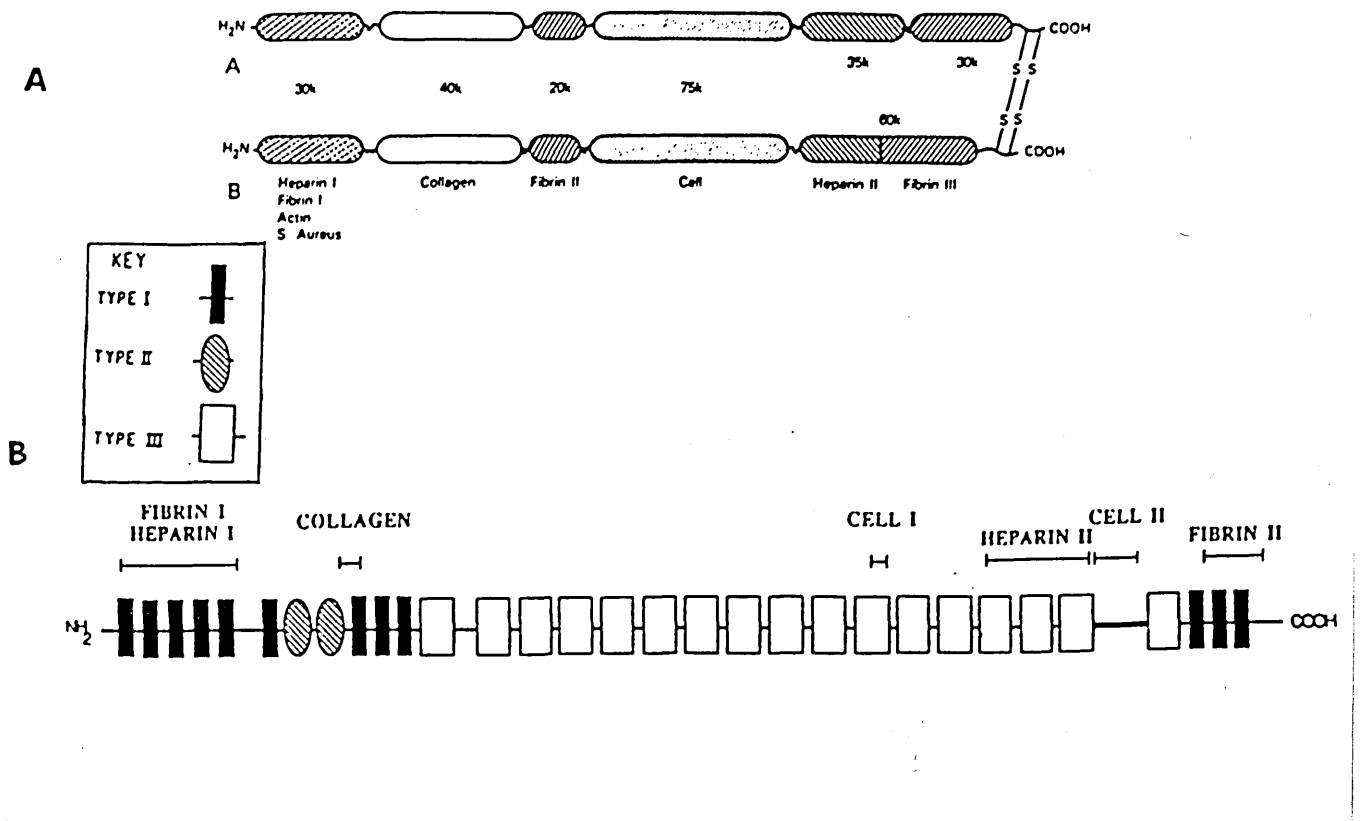


Figure 1.6 Functional domain mode for fibronectin (A) and structure of mature protein (B). In A the two polypeptide chains, the disulfide bonds, the approximate sizes of the binding domains and the binding ligands are indicated. In B Type I homologies (black boxes), Type II homologies (dashed boxes) and Type III homologies (empty boxes) are shown.

occur at the amino- and carboxy - termini of the protein and between the first and last two type III units. The protein sequence defines the exact location of the only two free sulphydryl groups known to exist in each subunit of fibronectin. One of them is the same as the SH group identified in bovine plasma fibronectin (Vibe-Pedersen *et al.*, 1982). The location of the second one is consistent with the mapping of a free sulphydryl to a position close to the C-terminal fibrin- binding domain of human plasma fibronectin (Smith *et al.*, 1982; Owens *et al.*, 1986).

The two subunits are joined by disulfide bonds very near their C-terminal. Within each subunit there is a series of tightly folded globular domains, each specialized for binding to other molecules or to cells (Fig 1.6; Hynes, 1985). The two Type II homologies are about 60% identical within species and each is 98% identical between bovine and human FNs (Hynes, 1985). Also the amino acids sequence of individual Type III repeats in rat (Schwarzbauer *et al.*, 1985), human (Kornblihtt *et al.*, 1985) and bovine (Skorstengaard *et al.*, 1986) FN are better than 90% conserved, while different repeats within a species are much less similar (typically 20-40%) (Schwarzbauer *et al.*, 1987). These similarities may suggest that the process of endoduplication and divergence of Type III units occured long before the divergence of mammals (Schwarzbauer *et al.*, 1987).

1.3.8 Subunit Variations Due to Alternative Splicing

It has been known for years that plasma FN contains subunits of two different mobilities on SDS-polyacrylamide gels and that FN from fibroblasts shows a different subunit pattern (Yamada & Kennedy, 1979; Tamkun & Hynes, 1983; Paul & Hynes, 1984). As FNs are glycoproteins, it was unclear whether this different subunit pattern might be due to differential glycosylation, which is known to occur or other factors

were involved such as presence of more than one gene (Fukuda *et al*, 1982; Paul & Hynes, 1984). To test the role of glycosylation in the FN variation FN was synthesized in the presence of tunicamycin, which blocks addition of asparagine-linked carbohydrates, the major type on FN. Even such carbohydrate-free FN, showed multiple subunits on 2D gels and differences between FNs from different sources (Paul & Hynes 1984; Price & Hynes, 1985). Furthermore, proteolytic fragments, thought to be carbohydrate-free, showed differences among different FNs (Hayashi & Yamada 1981, 1983; Sekiguchi *et al.*, 1981, 1985; Sekiguchi & Hakomori 1983a, b), and a McAb was isolated that shows higher binding to fibroblast than to plasma FN (Atherton & Hynes 1981). All of these results suggested the possibility of structural differences betweeen subunits, although it was still possible that they arose by post-translational modifications. It was only when cDNA clones were isolated that it could be shown conclusively that there exist FN subunits with different primary sequences (Schwarzbauer *et al.*, 1983; Kornblihtt *et al.*, 1984a, b) which might account for most of the variations seen above.

1.3.9 Alternative Splicing Regions in FN mRNA

The accumulated evidence from cDNAs isolated from human and rat fibronectin indicates that there are at least three regions of variability in the fibronectin transcripts. One region is located between the cell-binding and heparin-binding domains of the protein and involves the presence or absence of a 270 bp segment in fibronectin mRNAs refered to as the extra domain (ED-I or ED-A, Kornblihtt *et al.*, 1984a) (Fig. 1.7). The second and more recently characterized region maps between the collagen- and the cell I-binding domains of the protein, and involves another full type III repeat (ED-II or ED-B) that can be included or be absent in

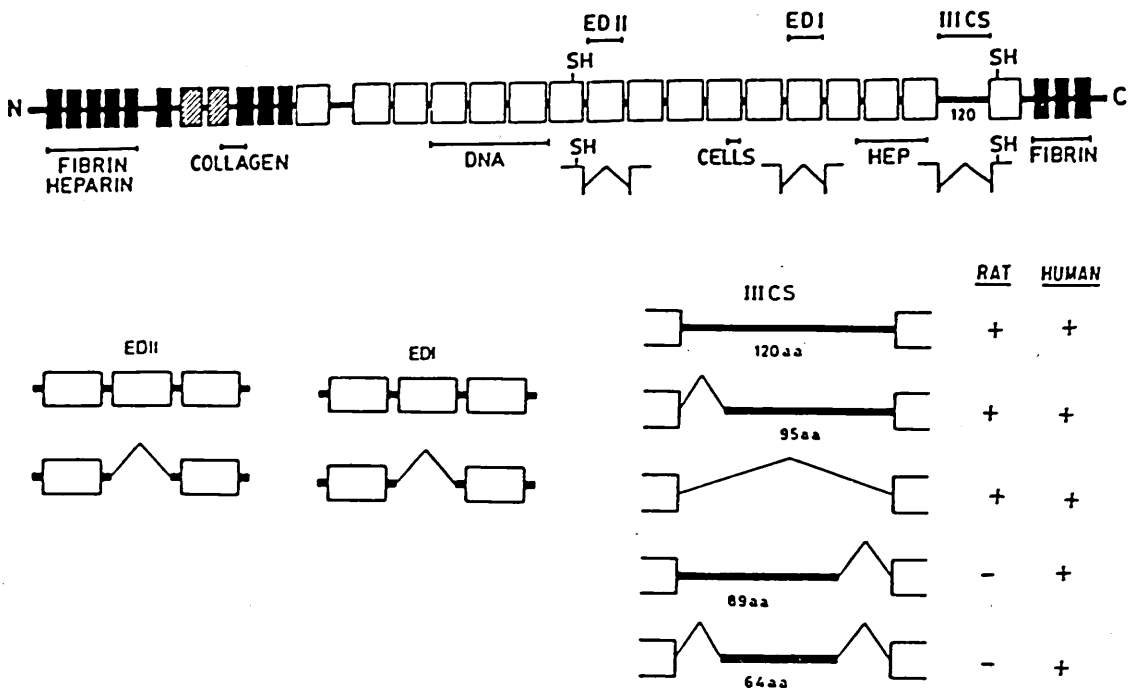


Figure 1.7 Variations of the fibronectin primary structure. The primary sequence of fibronectin is largely composed of repeats of three types of homology. The locations of the various functional domains are shown. The location of the three regions of variations caused by alternative splicing are also shown (IICS, ED-I and ED-II). The possible type of variations in the IIICS region in human and rat are also indicated.

the mature mRNA (Gutman & Kornblihtt, 1987; Schwarzbauer *et al.*, 1987). The third region, corresponds to the non-homologous segment which connects the last two type III units at the C-terminal end of the protein (connecting segment III, IIICS) (Kornblihtt et al., 1984a,b), which appears to be of variable length in different mRNAs (Owens *et al.*, 1986; Dufour *et al.*, 1988).

1.3.9.a Alternative Splicing in the IIICS Region

When cDNA clones were isolated from rat liver, three classes were found, which differed in sequence at a point between the penultimate and final Type III repeats, in the central region of FN (Schwarzbauer *et al.*, 1983). In clones of one class, these two Type III repeats were contiguous, whereas clones of the other two classes had inserts of 285 and 360 bases encoding 95 or 120 amino acids, respectively. The 120 amino acid segment consisted of the 95 residue segment plus an extra 25 amino acids at the N-terminal (Fig. 1.7). S1 nuclease analyses showed that all three clones represented genuine mRNAs found in rat liver and in other cell types. The 95 and 25 amino acid segments were completely different from any of the three types of repeating homology and they were shown to be extremely proline-rich (Hynes, 1985; Dufour *et al.*, 1988).

The first human cDNA clone covering the IIICS region showed a further variation. It contained an insert encoding, 89 amino acids homologous with the 25 amino acid segment plus the first 64 residues of the 95 amino acid segment (Kornblihtt *et al* 1984a,b). Another human cDNA clone, from a different cell source encoded the entire 120 amino acid segment (Bernard *et al.*, 1985) and other clones have now been isolated which contain no insert, or an insert encoding only the 64 amino acid segment. These variants are all diagrammed in Figure 1.7. It appears that 3 variants are possible in rat and 5 in human FN for this region (Hynes, 1985; Dufour *et al.*, 1988).

1.3.9.b Alternative Splicing in the ED-I (ED-A) Region

A second region of variation was first detected in human cDNA's by Kornblihtt *et al.*, (1984a, b), who isolated two classes of FN cDNA clones from a human cell line (Fig. 1.7). The clones differ as to the presence or absence of a segment of 270 bases encoding an entire Type III repeat. S1 nuclease analysis confirmed that these both represent genuine mRNAs. While the extra Type III repeat is not encoded by any of the cDNA clones from rat liver (Schwarzbauer *et al.*, 1983), it is present in the rat gene, and is expressed in other cell types (Odermatt *et al.*, 1985). Therefore, there are (at least) two positions of subunit variation at which protein-encoding segments can be present or absent.

1.3.9.c Alternative Splicing in the ED-II (ED-B) Region

Variability is produced by the inclusion or exclusion of a full repeat of type III homology, named ED-II, by virtue of its similarity to the previously described ED-I (Vibe-Pedersen *et al.*, 1984; Kornblihtt *et al.*, 1984b; Gutman & Kornblihtt, 1987; Schwarzbauer *et al.*, 1987). The ED-II repeat is encoded by exactly one exon in the human genome and alternative splicing seems to occur by an exon-skipping mechanism (Vibe-Pedersen *et al.*, 1984; Oddermatt *et al.*, 1985). Similarities between ED-I and ED-II regions are evident both structurally and functionally (Fig. 1.7). In both cases full type III repeats are encoded by single (fused) exons. This is in contrast with the other nine type III repeats analyzed so far, in which two smaller exons are needed for each unit (Vibe-Pedersen *et al.*, 1984; Padgett *et al.*, 1986). Most interestingly, alternative splicing of the ED-II exon seems to have the same cell specificity as that of ED-I. S1 nuclease mapping experiments have revealed that the ED-II is present in FN mRNAs from cells known to produce cFN,

indicating that this type III repeat is restricted to cell surface FN, as happens with ED-I.

Combination of all the possible patterns of splicing may generate, from a single gene, up to 20 distinct FN polypeptides in humans, 12 in rat and 8 in the chick (Dufour *et al.*, 1988). This data also suggest that, while most cells offer a pool of 20 FN polypeptides in humans (12 in the rat and 8 in the chick) to make up FN dimers or multimers, hepatocytes have a more restricted pool of five subunits (three in the rat and two in the chick) to offer. It should be noted that, even though most cells seem able to transcribe all possible FN mRNA variants, the relative proportions of each variant differ among different cell types. However, the expression pattern of each FN variant in embryonic development is not yet known (Dufour *et al.*, 1988).

1.3.10 Alternative Splicing and Subunit Complexity

1.3.10.a ED-I$^-$ Isoforms

The primary transcript of the fibronectin gene is processed to produce a variety of different mRNAs and the heterogeneity of fibronectin subunits may be observed at the protein level. Various lines of evidence indicate that the presence of the ED-I may account for a difference between cellular and plasma fibronectin. This evidence may be summarized as follows:

1. ED-containing fibronectin mRNAs are synthesized by various human cell lines but not by liver cells. It has been shown that hepatocytes are the principal source of plasma fibronectin (Tamkun and Hynes , 1983), consistent with the synthesis of exclusively ED-I$^-$ mRNA by these cells.
2. The ED-I has not been seen at the polypeptide level in bovine plasma fibronectin.
3. Among other structural differences, it has been shown that heparin-binding fragments are approximately 10000u

larger in cellular, than in plasma fibronectin, in both the chicken (Hayashi & Yamada, 1981) and human (Sekiguchi *et al.*, 1985). The size and location of the polypeptide differences are coincident with ED-I.

1.3.10.b Significance of ED-I$^+$ Isoforms

The presence of an extra type III unit (ED-I) between the cell-binding and heparin-binding domains of one type of fibronectin, but not the other, may be functionally significant. For example, it has been reported that cellular fibronectin is considerably more active than plasma fibronectin in restoring normal morphology to a transformed fibroblast cell line (Yamada & Kennedy, 1979). It is possible that the function of ED-I is to increase the distance between the cell-binding and heparin- binding sites, resulting in an enhanced binding activity of the cellular fibronectin molecule (Owens *et al.*, 1986).

1.3.10.c Significance of IIICS Isoforms

Direct evidence has been obtained that alternative splicing in the IIICS region accounts for some of the fibronectin subunit variants. Antibodies were raised to the 95 amino acid (285 bp) sequence of the IIICS synthesized as a beta-galactosidase fusion protein in lambda gt11 (Schwarzbauer *et al.*, 1985). In immunoblotting experiments the antiserum reacted only with the larger subunit of rat and hamster plasma fibronectin. The serum also recognized all subunits of cellular fibronectin. The results, thus indicate that some of the molecular weight differences between the subunits of plasma fibronectin arise from alternative splicing of the IIICS. The antibody data also show that cellular fibronectin are characterised by the presence of part, or all of, the IIICS sequence. This is consistent with the results of the analysis of fibronectin cDNAs from human epithelial cells and

fibroblasts. However, the functional implications of this and the more subtle variations in this region remain unclear (Owens *et al.*, 1986).

The alternative splicing of FN mRNAs in the IIICS region means that the CS1 sequence (a cell-binding site located at the amino-terminal end of the IIICS, see section 1.3.11) that promotes cell adhesion of melanoma or neural crest cells is present in certain, but not all, FN variants. Thus, some FNs can possess all sites, while others may have only the RGDS (see section 1.3.11) and high affinity site region, and the different FNs could thereby, specifically promote the adhesion, or migration of one cell type rather than another. It is conceivable that different kinds of FNs, carrying some or all of the total complement of adhesion sites, are secreted at specific regions of the embryo where they modulate cellular behavior according to the environment of the region or its degree of differentiation. This scenario might explain for the observation that some areas of the embryo are never occupied by migrating neural crest cells, even though they contain high amounts of FN (Dufour *et al.*, 1988).

1.3.10.d Alternative Splicing and Tissue Specificity

Alternative splicing of both the ED-I and ED-II exons is cell specific and it accounts for the primary structural differences between cellular and plasma FNs. In fact, hepatocytes make only FN mRNAs lacking both EDs, while fibroblasts and many other cells make FN mRNAs both with and without the EDs (Tamkun *et al.*, 1984; Paul *et al.*, 1986). These results are consistent with experiments in which antibodies, raised against ED-specific sequences, recognised extracellular matrix FN, but failed to react with plasma FN (Borsi *et al.*, 1987).

It has been shown that the percentage of FN molecules containing the ED-I segment is about 10x higher in transformed human cells than in normal human

fibroblasts (Borsi *et al.*, 1987). FN from transformed cells is also composed of a population of molecules in which the IIICS sequence is more expressed than in FN from normal cells (Castellani *et al.*, 1986). This may suggest that in malignant cells the mechanisms that regulate the splicing of mRNA precursors are altered.

The ED-II repeat is rarer than ED-I in established cell lines but, in early passage cultures of fibroblasts and astrocytes, the prevalence of ED-II is similar to the ED-I; about 50% in each case (Schwarzbauer *et al.*, 1987).

1.3.11 Binding Properties

As a major component of the extracellular matrix, fibronectin probably interacts indirectly with cell surfaces via other matrix components, for example heparin and type III collagen. In contrast to the direct cell-binding activity of fibronectin, much less is known about its binding site to glycosaminoglycans and collagens. FN interacts with a number of polyanionic substances. Heparin, hyaluronic acid, dextran sulphate and DNA show binding to fibronectin, while dermatan sulphate and chondroitin sulphates do not (for review see Ruoslahti *et al*, 1981). Two heparin- and one DNA-binding site have been mapped to different domains of the molecule (Fig. 1.6; Owens *et al.*, 1986).

The interaction of fibronectin with collagens appears to be fundamental to the organization of extracellular matrices and the behavior of cells on these substrates (Pearlstein 1976; McDonald *et al.*, 1982; Nagata *et al.*, 1985). Of the genetic types of collagen, type III collagen has the highest affinity for fibronectin (Engvall *et al.*, 1978).

Another major binding function of fibronectin is for fibrin, which has been mapped to two sites, one at the amino terminus, the other at the carboxy terminus of the molecule (Fig. 1.6). Both binding activity domains

comprise type I units, which strongly implicates them in the binding activity (Owens *et al.*, 1986).

FN molecules act as bridges between the cell surface and extracellular material because FN molecules contain a cell-binding site and binding sites for collagen, heparin, gangliosides and fibrin. These multiple binding sites enable the FNs to play an important role in diverse biological phenomena including cell adhesion, cell migration, hemostasis and thrombosis, wound healing and the ability to induce a more normal phenotype in transformed cells (Hynes & Yamada, 1982; Mosher, 1984; Ruoslahti *et al.*, 1981).

As indicated above several binding activities have been assigned to different regions of the fibronectin molecule. However, so far only in the case of the ability to bind cells has the actual binding site been identified. Pierschbacher & Ruoslahti (1984a) demonstrated that the minimum sequence required for cell binding was the tetrapeptide Arg-Gly-Asp-Ser (RGDS). Conservative substitutions in position one to three completely abolish activity, whereas the fourth position can be varied with any other amino acid (except proline) without loss of cell-binding activity (Pierschbacher & Ruoslahti, 1984b). The RGDS tetrapeptide is present only once on the fibronectin sequence within one of the type III units, and appears outside the blocks of conserved residues in this region. Interestingly, there is another potential cell-binding site in the IIICS area of rat but not human fibronectin (RGDV in rat compared with REDV in human). This sequence occurs in the amino acid segment at the 3' end of the IIICS which is spliced out of fibronectin mRNA in liver (Schwarzbauer *et al* 1983).

The discovery that the FN tetrapeptide RGDS mediated adhesion of FN to cell surface was a turning point in the field of cell adhesion. RGDS appears only once in the FN sequence and is embedded in a type III repeat which is constitutive to all FN variants. RGDS

sequences have also been found in other adhesive proteins, including vitronectin, von Willebrand factor, thrombospondin and collagens. Interestingly, all these adhesive molecules interact with the surface of cells through distinct receptors, that nevertheless, share common structural properties. These receptors have been grouped into a single family called RGD receptors or integrins (Owens *et al.*, 1986; Hynes, 1987; Buck & Horwitz, 1987).

1.3.11.a Integrins

Integrins are composed of two noncovalently linked alpha and beta chains which are both integral membrane glycoproteins. The integrin family has been tentatively subdivided into three groups of proteins, each with a common beta subunit but with variable alpha subunits (Hynes, 1987; Buck & Horwitz , 1987). The different alpha chains are denoted by the nature of the ligand or by the original cell type. Even though both subunits are required to provide the binding activity of the receptor, recent studies indicate that the beta subunits apparently participate in the recognition of the RGD sequence, while the alpha subunits determine the specificity of the binding to each particular type of adhesive protein (Hynes, 1987; Dufour *et al.*, 1988).

1.3.11.b High Affinity and CS1 Binding Sites

Besides RGDS, other sites in the FN molecule may mediate or participate in cell binding. In particular, the low affinity of RGDS-containing peptides for fibroblasts, compared with that of intact FN, led to the hypothesis that a site, distinct from RGDS but in association with it, might be required for high affinity binding. This 'high affinity site' has recently been localized within a type III unit, approximately 300 amino acids away from RGDS and toward the amino terminus (Fig. 1.8; Obera *et al.*, 1987). The high affinity site

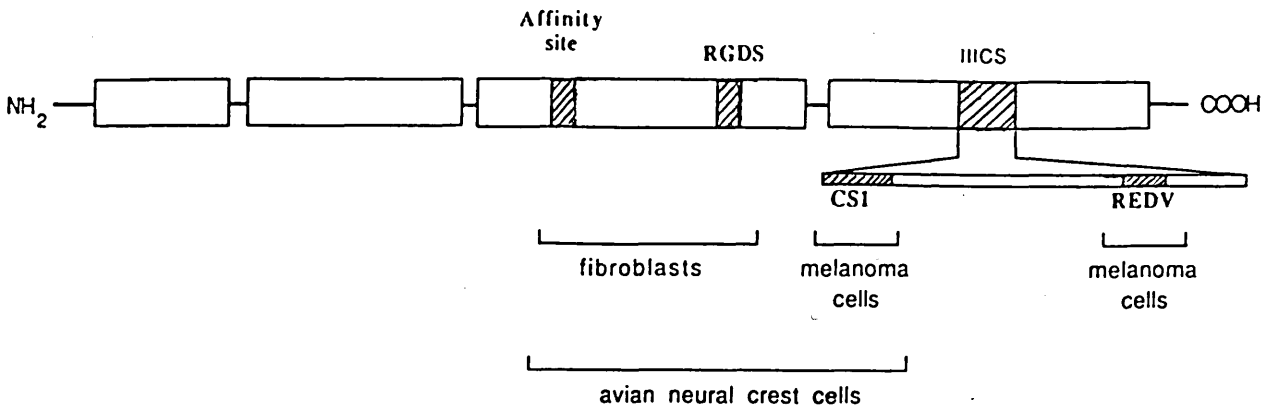


Figure 1.8 Different cell-binding sites identified on fibronectin. A high affinity site is localised towards the amino-terminal from the RGDS sequence. Both the high affinity and the RGDS sites are used for attachment and spreading of fibroblasts. Another cell-binding site carried by the CS1 sequence of the IIICS domain promotes specific adhesion of melanoma cells

alone is not sufficient to mediate cell adhesion, which also requires the presence of RGDS. Two other cell-binding sites map in the IIICS segment: one is located at the carboxy-terminal end of IIICS and has been identified in humans as the REDV (Arg-Glu-Asp-Val) tetrapeptide (RGDV in rats). The other cell-binding site, termed CS1, is located at the amino-terminal end of IIICS (Fig. 1.8; Humphries *et al.*, 1986, 1987). Interestingly, these sites, whose presence is regulated by alternative splicing, specifically promote the adhesion of melanoma cells (Humphries *et al.*, 1986, 1987). Receptors for the IIICS-binding sites have not been isolated, but preliminary data indicate that they also belong to the integrin family. In conclusion, the presence of considerable flexibility in the control of cell behavior by FN, a fact which may have important implications during development (Dufour *et al.*, 1988).

1.3.12 Structure of the FN Gene

Human fibronectin gene has been assigned to chromosome 2 using human-mouse hybrids and species-specific anti-human fibronectin monoclonal antibodies (Zardi *et al* , 1982), somatic cell hybrids and cDNA probing (Prowse *et al.*, 1986) and by in-situ hybridisation (Jhanwar *et al.*, 1986). Despite the existence of multiple forms of FN, there is only one copy of the FN gene in the human and rat genomes. This gene is about 75 kb long, contains around 50 exons and is transcribed from a single promoter into a single primary transcript, which gives rise to the different FN mRNAs, through a complicated pattern of alternative splicings, occurring in three separate regions (Dufour *et al.*, 1988).

The sequence of the longest possible FN mRNA molecule, deduced from several cDNA and genomic clones, is composed of 8418 nucleotides, 7431 of which code for the 2477 amino acids of the primary translation product. A 31 amino acid segment comprising the signal and

propeptides, precedes the amino terminus of the mature polypeptide. The same pre- and pro-coding sequences were found in all mRNAs encoding the different subunits of plasma and cellular FNs (Dufour *et al.*, 1988). This rules out control at the membrane translocation site as a mechanism to explain the differential localization and behavior of the two forms.

Most of the Type I and both of the Type II units are encoded by a single exon each, while 14 of the 17 Type III units are coded for by two exons each. The other three Type III repeats are encoded by a longer (fused?) single exon each. This strict correlation between the intron/exon organization of the gene and the repetitive structure of the protein, together with the fact that other, apparently unrelated, genes carry and express segments which are homologous to FN Type I and Type II sequences, strongly reinforce the theory of exon shuffling for the origin of multidomain proteins (Owens *et al.*, 1986; Dufour *et al.*, 1988; see also section 1.3.14).

1.3.13 Regulation of FN Expression

There are several potential transcriptional control elements in the 5' flanking region and in the first intron of the gene. The fibronectin gene is subject to complex control mechanisms, being regulated by cell density and growth rate, oncogenic transformation, differentiation state and glycocorticoids (Adams *et al.*, 1982; Senger *et al.*, 1983; Oliver *et al.*, 1983; Allebach *et al.*, 1985; Patel *et al.*, 1987).

1.3.14 Evolutionary Implications

There are several evolutionary implications of the structure of the FN gene and protein. The first and most obvious is that the repeating homologies strongly suggest that the FN gene arose by endoduplication of

several primordial minidomains or modules, corresponding with the present day homology Type I, II, and III. This supposition is supported by analyses of the intron-exon structure of the gene, which shows clearly that each Type III repeat is encoded by a basic repeating unit in the gene. Seven Type III repeats in the rat FN gene are precisely separated, in each case by an intron (Odermatt *et al.*, 1985). Type III repeats that can be alternatively spliced, the twelfth, is encoded by a single exon (Vibe-Pedersen *et al.*, 1984; Odermatt *et al.*, 1985), but all the others analysed thus far are interrupted by an intron (Odermatt *et al.*, 1985).

It seems very likely that the single or double exon units encoding each repeat are the modules that underwent duplication and divergence to generate the FN gene. This duplication and divergence must have occurred well before the divergence of vertebrates, while individual repeats show only 20-60% homology within species (i.e. are quite widely diverged), the homology between vertebrate species in a given repeat is more than 90% (Hynes, 1985). That is, once the duplication and divergence had occurred, the gene became highly conserved. This is true, not only at the level of amino acid sequence, but also at the nucleotide level and even applies to several untranslated segments of the gene. For example, there is a highly conserved stretch of 200 bases in the 3' untranslated region, immediately preceding the poly-A addition signal (Kornblihtt *et al.*, 1983; Schwarzbauer *et al.*, 1983). Similarly, the 6 intron-exon boundaries that have been determined in the human gene (Vibe-Pedersen *et al.*, 1984) are very similar to those in the corresponding position in the rat gene (Odermatt *et al.*, 1985; Hynes, 1985).

Another fasinating aspect of the evolution of the FN gene also has come to light. Banyai *et al.* (1983) noted that tissue plasminogen activator (t-PA) has an N-terminal Type I repeat homologous with those in FN. The related plasminogen activator, urokinase (u-PA), lacks this homology. t-PA binds to fibrin whereas u-PA does

not, which is consistent with the idea that Type I homology confers affinity for fibrin and as it is also suggested by the presence of 3 and 5 Type I homologies in the two fibrin-binding sites of FN. More recently, McMullen and Fujikawa (1985) determined the sequence of human Factor XIIa (activated Hagemann factor). This protease contains one copy of each of the Type I and II homologies, as well as other conserved structural domains characteristic of serum protease. The Type II homology is identical with one or both Type II repeats of FN at more than 50% of the positions, and is also extremely similar to two Type II repeats found in a bovine seminal plasma protein (McMullen & Fujikawa, 1985). The Type I repeat of Factor XIIa is closely related to those of FN and t-PA. Thus, the two disulfide-bonded minidomains present in multiple copies in FN are also found in other proteins. They presumably moved, from one gene to another, by a process of exon reassortment of shuffling (Gilbert, 1978). This hypothesis gains support from the observation that the Type I repeat in t-PA is encoded precisely by one exon (Ny *et al.*, 1984). Therefore, these minidomains that appear to be involved in protein-binding functions in FN may subserve similar roles in other proteins. Furthermore, Patthy *et al.* (1984) noted that Type II homologies are related in sequence to the core of "kringles", which are protein-binding modules characteristic of proteolytic enzymes; this suggests even more distant evolutionary relationships among these various minidomains (Hynes, 1985).

The single large gene that encodes this modular structure is composed of a series of multiple small exons. Some of these exons appear to have found their way into other genes, where they presumably carry out similar functions (Hynes, 1985).

1.3.15 Human Placental FN

Placental extracellular matrix, in addition to the

trophoblast differentiation, supports many other cellular migratory and developmental changes occurring during embryogenesis and the integrity of the placental membrane which is necessary for maintenance of pregnancy (Aplin anf Foden, 1982). Its role and presence in the placenta, amniotic fluid and fetal tissues has been investigated (Zhu *et al.*, 1984; Matsuura & Hakomori, 1985; Sekiguchi *et al.*, 1986; Molnar-G. *et al.*, 1988; Robert *et al.*, 1988 and other references in this section).

FN is one of the major components of the human placenta and it has been localised by immunohistological methods around the fetal blood vessels, stroma of placental villi (Isemura *et al.*, 1985; Kurosawa *et al.*, 1985) and in the placental chorionic plate (Khalaf *et al.*, 1985). FN has been shown to be abundant in human placenta and in fact it constitutes between 1.8-2.9% of the dry weight of the placental villi (Bray, 1985).

Human plasma fibronectin contains 75% biantennary asparagine-linked glycopeptides and 25% triantennary structures (Fisher & Laine, 1979). In contrast, fetal placental fibronectin contains substantial amounts of polylactosamine-containing glycopeptides, many in the form of tetraantennary asparagine-linked oligosaccharides of 7-10 kDa size (Zhu *et al.*, 1984). The presence of these large oligosaccharides weakens the binding of fibronectin to denatured collagen (gelatin) (Zhu & Laine, 1985). The phenomenon of weakened binding to denatured collagen, however, for the type of fibronectin found in term placenta, has not yet been related to any difference in the functional capacity of the fibronectins. Human fetal plasma fibronectin (Yamaguchi *et al.*, 1984) and amniotic fluid fibronectin (Krusius *et al.*, 1985) have been reported to contain a smaller amount of polylactosamine structure and a higher carbohydrate content than adult plasma fibronectin. However, placental tissue fibronectin is much less soluble than the plasma or amniotic fluid forms,

indicating chemical differences there are as yet undefined. To see whether polylactosaminyl fibronectin was unique to the term placenta, a developmental study of the carbohydrates of placental tissue fibronectin during gestation was conducted by Zhu and Laine (1987). They found that a steady increase in the size, carbohydrate quantity, and polylactosamine glycosylation on tissue fibronectin occurs throughout the lifetime of the placenta attaining its final form only during the last month before birth. This increase in carbohydrate is accompanied by a steadily diminishing binding affinity to gelatin.

1.4 Alternative Splicing

Eukaryotic cells have a characteristic feature in that protein isoforms which are structurally distinct, cell type specific and developmentally regulated are produced by a regulated manner. Thus far at least two broadly categorised systems have been identified to be responsible for the generation of this protein diversity. One of these systems involves the selection of one gene among the member of a multigene family for expression in a particular cell, developmental stage or other conditions (Breitbart et al., 1987). Two examples are the expression of haemoglobin (Hb) and immunoglobulin (Ig), the former one is developmentally regulated and the latter one can be affected by different physiological conditions. There are at least three different Hb molecules, embryonic, foetal and adult forms (Antonarakis et al., 1985). The Ig molecules are much more diverse and the light chain has 3 segments and the heavy chain has 4 segments but because there are usually more than one copy for each segment (e.g. the variable gene cluster has about 200 members) the combination of these gene clusters can result in thousands of different possibilities (Rabbits 1984). These forms of protein diversity are achieved by DNA rearrangement. There is another mechanism which also give rise to different protein isoforms as above but the

main difference in here is that there is only one gene involved and the diversity is caused by alternative pre-mRNA splicing.

The protein isoforms generated by alternative splicing usually share extensive regions of identity and only vary in specific domains. The domain variability, caused by alternative splicing, affects almost all aspects of protein function, ranging from localisation to the modulation of enzyme activity (Smith et al., 1989; Latchman 1990).

Alternative splicing, as a mechanism to regulate gene expression and generate protein diversity, has several advantages over gene rearrangement and extensive multigene families. In this way protein isoforms are made without any need for additional members of a gene family or any change in the transcriptional activity. In other word the role of alternative splicing is to increase the coding power of the genome (Smith et al., 1989).

The mechanism involved in the regulation of alternative splicing has been intensely examined (for recent reviews see Leff et al., 1986; Breitbart et al., 1987; Smith et al., 1989; Latchman 1990) in recent years and major advances have been made in understanding the biochemical basis of the splicing reaction since the discovery of pre-mRNA splicing. There are still areas of splicing (as well as alternative splicing) which remain obscure such as the basis for the extremely high precision of the splicing reaction and the selective advantages and evolutionary origin of introns (Smith et al., 1989). Meanwhile alternative pre-mRNA splicing has become a subject of major interest in two ways, firstly as an important biological regulatory mechanism and secondly for providing insights into some fundamental aspects of splicing.

By 1986 more than 50 genes were known to generate protein diversity through the use of alternative

splicing but their number has expanded so rapidly since then (Breitbart et al., 1987; Smith et al., 1989). Thus far neither the experimental data available nor the hypotheses put forward for the removal of introns in pre-mRNA splicing can fully explain the cell- and developmental-specific patterns of alternative splicing seen in some mRNA splicings (Breitbart et al., 1987).

When compared with *constitutive* splicing (where each and every one of the exons present in the gene are incorporated into one mature mRNA; Breitbart et al., 1987) the alternative splicing often appears to be generated by subtle changes in the basic splicing mechanisms (Smith et al., 1989). This is not to say that the consensus sequences or splice-site elements in this case differ significantly from elements involved in constitutive splicing (Breitbart et al., 1987) Nevertheless there are cases in which extreme variants in the *cis* elements are associated with utilisation of differential splice-site (Smith et al., 1989). There are, however, cases where novel *cis* elements, unrelated to the type of consensus elements seen in constitutive splicing, are involved (Breitbart et al., 1987; Smith et al., 1989). The major cause of alternative splicing, where no apparent *cis* element variation can be seen, may be caused by differences in cellular *trans* factors as seen in cell-specific and developmentally regulated splicing (Smith et al., 1989). This is not to say that the constitutive splicing does not utilise cellular *trans* factors because they have been shown to be involved in apparently constitutive splicing (Breitbart and Nadal-Ginard 1987).

Manifestation of Alternative Splicing

Unlike their prokaryotic counterparts, most eukaryotic genes in addition to exons contain another type of sequences (introns) which are present in the pre-mRNA transcripts but are absent in the mature mRNA. Introns are not therefore represented in the coded protein (Breitbart et al., 1987). The intron sequences

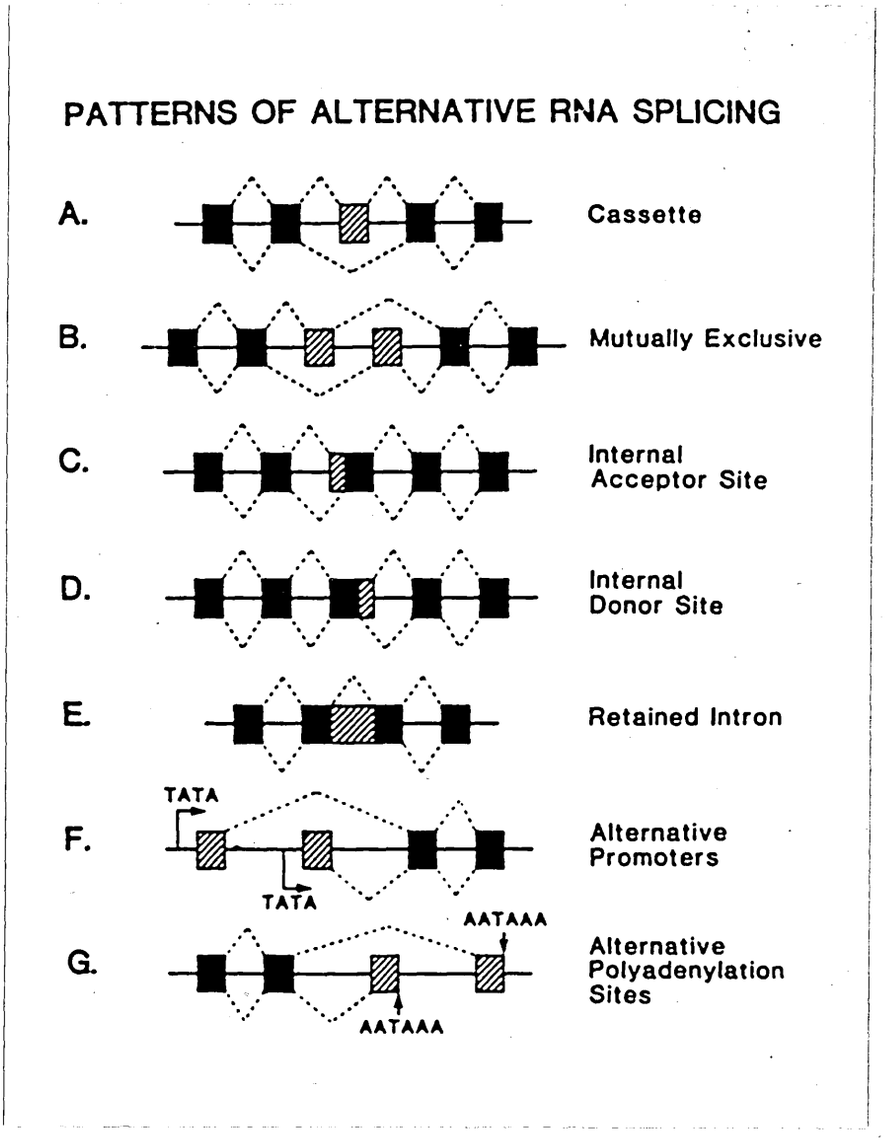


Fig 1.9 Pattern of alternative splicing. Constitutive exons (black boxes), alternative sequences (striped boxes) and introns (solid lines) are spliced according to different pathways (dotted lines), as described in the text. Alternative promoters (TATA) and polyadenylation signals (AATAAA) are indicated (picture from Breitbart et al., 1987).

are posttranscriptionally removed from the mRNA in always at the same position (Breitbart et al., 1987). This type of splicing contributes no diversity to gene expression and one pre-mRNA transcript is converted to one mature mRNA (Solnick 1985). In most genes studied so far the mature mRNA is generated by all the exons present in the gene being joined together through ligation of consecutive pairs of donor and acceptor splice sites present in the exon-intron boundaries (Breitbart et al., 1987).

Constitutive splicing as mentioned above yields a single mature mRNA from each transcriptional unit in spite of exons being interrupted as many as 50 times by introns (Solnick 1985). Though generally constitutive splicing is regarded to be precise, efficient and apparently universal (i.e. in higher eukaryotes), but sometimes all the exons are not joined in consecutive manner (Breitbart et al., 1987). This pattern of splicing is termed *alternative* splicing and can exclude all or part of an individual exon from the mature mRNA in some transcripts but include them in others (Fig. 1.9; Breitbart et al., 1987; e.g. alternative pattern of splicing of EDs and IIICS regions in FN, see section 1.3.9).

It is now known that alternative splicing occurs in a wide variety of metazoans ranging from *Drosophila* to human (for full list and references see Breitbart et al., 1987). The protein products of alternative splicing have a wide variety of functions and distributions. These products include proteins of the contractile apparatus, extracellular matrix, cytoskeleton, membrane receptors, hormones and enzymes involved in the intermediary metabolism and DNA transposition (for full references see Breitbart et al., 1987).

1.4.2 Pattern of Alternative Pre-mRNA Splicing

In some genes such as contractile protein genes

alternative splicing is a prevalent mechanism which generates isoform diversity (Breitbart et al., 1987). Alternative splicing has also been shown to be present in several of sarcomeric proteins such as myosin heavy chain, alkali myosin light chain, tropomyosin, skeletal and cardiac troponin T and FN (for full list of references see Breitbart et al., 1987 and for FN see section 1.3).

Splicing usually occur when a donor and acceptor site are present in the pre-mRNA transcript and are joined together to form mature mRNA (Padgett et al., 1986). Whenever this process does not occur all the time and in some mRNA a particular pair of donor and acceptor sites fail to join the result would be formation of at least two mRNAs with different primary sequences (Breitbart et al., 1987). There are various ways in which alternative splicing can occur and they are summarised in Figure 1.9. These patterns of splicings have been given similar names by Breitbart et al. (1987) and Smith et al. (1989) and for convenience same names will be used here. These include combinatorial exons, internal donor and acceptor sites, mutually exclusive exons, retained introns and alternative 5'- and 3'-terminal exons. Some of these patterns of splicing, due to their relevance to the pattern of alternative splicing seen in FN, will be described in more detail than others.

1.4.2.a Combinatorial Exons

Some exons function as cassettes and can be included or excluded from the mature mRNA independently, that is to say such exons are sometimes present and in other time absent from the mRNA. Therefore if *n* number of cassette exons are present in a given gene then 2^n is the number of potential isoforms which might arise from such a gene at the level of mRNA modification (Breitbart et al., 1987; Smith et al., 1989). A very high diversity can be generated when several cassette exons are present

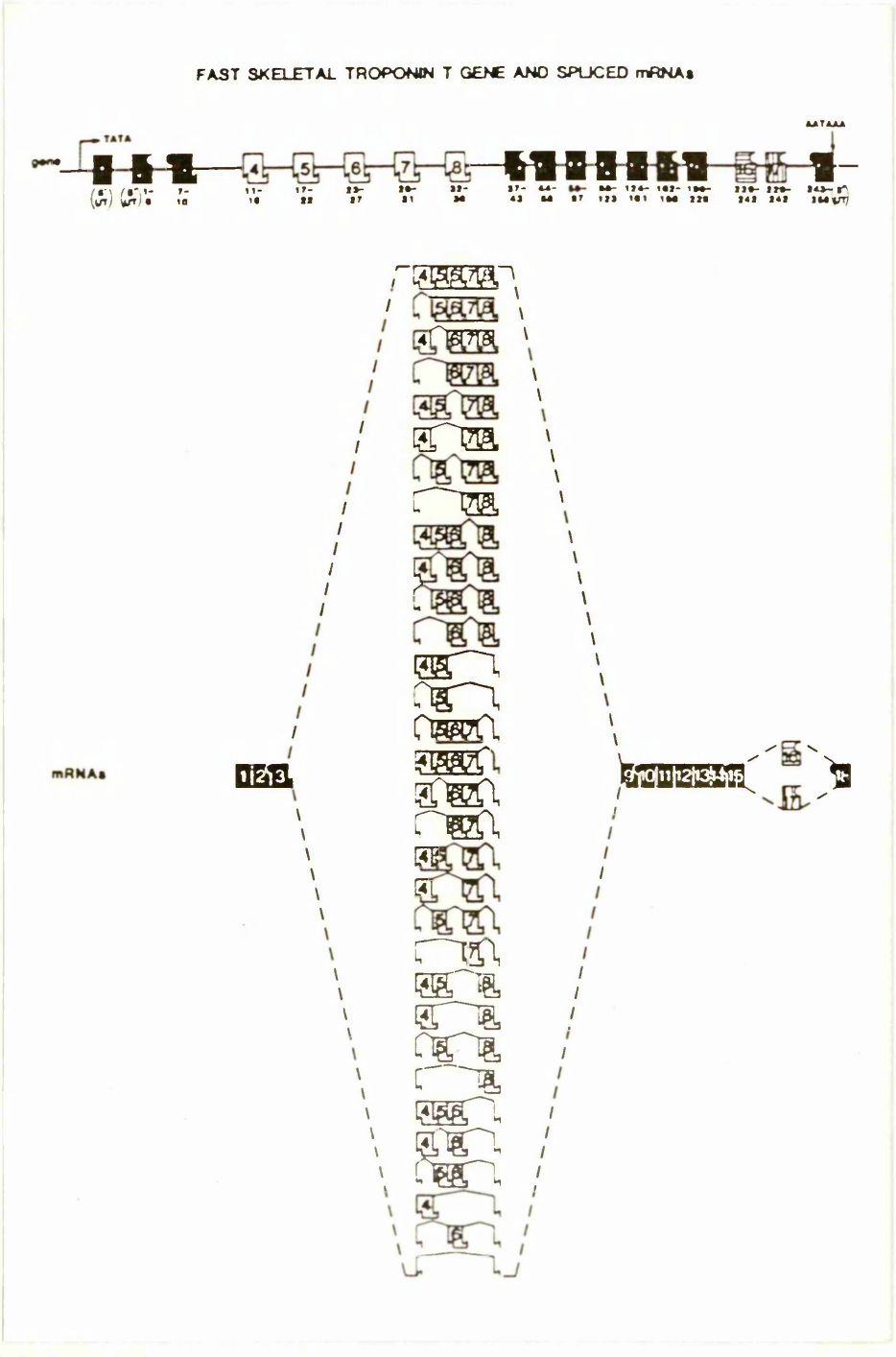


Figure 1.10 Fast skeletal troponin T gene organization in rat and 64 possible mRNAs. There are 32 variations caused by alternative splicing of exons 4-8 and two by exons 16 or 17, making the total 64 isoforms (picture from Breitbart et al., 1987).

in a gene and the best example of such system is the pattern of alternative splicing seen in fast skeletal troponin T (TnT) gene (Breitbart et al., 1987). In TnT gene there are five consecutive cassettes exons (4-8) which are spliced in a combinatorial fashion and in theory as many as 32 different protein isoforms can be generated (Breitbart et al., 1987; Fig. 1.10). The generation is, however, tissue-specific or developmentally regulated. Presence of yet another pair of cassette exons at the 5' end can increase this diversity even further (2^6) (Breitbart et al., 1987; Smith et al., 1989).

1.4.2.b Internal Donor and Acceptor Sites

In most cases the donor and the acceptor sites for splicing are located in the exon/intron boundaries but in other times there may be donor and acceptor sites within the coding sequences which alternatively can cause such sequences be included or excluded from the mature mRNA (Breitbart et al., 1987; Smith et al., 1989; Fig 1.10C and D). With most other types of alternative splicing this type can also cause a frameshift, or premature termination of reading frames (Smith et al., 1989). The best example is the pattern of splicing seen in the IIICS region of FN. Presence of one internal donor and one acceptor sites in combination of those in the exon/intron boundaries can generate up to 5 different isoforms from a single exon (see section 1.3.9.a).

1.4.2.c Mutually Exclusive Exons

Sometimes there are pairs of exons one of which is always present and the other one is skipped (Breitbart et al., 1987; Smith et al., 1989). That is to say internal exons are sometimes mutually exclusively incorporated into the mature mRNA. Each mutually exclusive cassette encodes an alternative version of the protein. Mutually exclusive cassette exons can be seen in rat alpha and beta-tropomyosin genes (Breitbart et al., 1987).

1.4.2.d Retained Introns

As the name suggests in this case introns can be retained in the mature mRNA and the protein product of such mRNA would contain peptide segment from such intron (Smith et al., 1989; Fig. 1.10E). This is apparently caused by the failure of the splicing mechanism to splice an intron and it can be seen in gamma-fibrinogen gene transcripts (Breitbart et al., 1987).

1.4.2.e Alternative 5'- and 3'-Terminal Exons

In some cases different promoters or cleavage/polyadenylation sites are utilised resulting in alternative 5' and 3' end sequences (Breitbart et al., 1987; Smith et al., 1989). This may be caused by presence of multiple promoters or poly (A) sites (Breitbart et al., 1987). This kind of utilisation leads to variability in the protein sequence in the N or C termini and may cause differential regulation of gene expression via effect on other processes dependent on such a protein (Smith et al., 1989).

1.4.3 Evidence for the Existence of Alternative Splicing Factors

It has been long believed that tissue-specific or developmentally regulated patterns of alternative splicing need more than just presence of for example internal donor or acceptor sites or presence of multiple promoter or poly (A) sites (Leff et al., 1986). It has been therefore suggested that some kind of regulatory factors might be involved. To provide evidence for the existence of such factors, many workers have used minigene constructs containing the alternative spliced exons and monitored their pattern of splicing by introducing them to various cell types or conditions. In many such experiments the correct pattern of tissue specific splicing have been obtained with two different types of cells (Paul et al., 1986; Castellani et al., 1986; Schwarzbauer et al., 1987; also see Breitbart et al., 1987; Smith et al., 1989 and Latchman 1990).

The fact that different patterns of splicing were obtained in different cell types using identical constructs (Leff at al. 1987; Crenshaw *et al.*,. 1987; Balza *et al.*, 1988; Barone *et al.*, 1989) provides clear evidence for the existence of alternative splicing factors. The findings in these studies and the identification of cis-acting sequences with which the alternative splicing factors interact have led to many attempts to identify these factors. The investigations in Drosophila have been most promising and several candidates have been identified (for reviews see Padgett *et al.*, 1986; Maniatis & Reed, 1987; Baker, 1989; Latchman, 1990).

1.4.4 On/Off Regulation of Gene Expression at the Level of Splicing

Alternative splicing may even act as on/off regulator. Recent studies in Drosophila indicate that splicing is used to turn the expression of protein gene products on and off. A large proportion of Drosophila genes have at least one intron of the large size class suggestive of additional function. The available sample of genes suggests that a significant fraction of these large introns may function in on/off regulation of gene expression at the level of splicing (for review see Bingham *et al.*, 1988).

1.5 WHO and Fertility Regulation

1.5.1 Special Programme of Research, Development and Research Training in Human Reproduction

As shown in Fig. 1.11, the population of the world is increasing in an exponential manner. Demographers recently reported that there are now more than 5 billion people on this planet. Information about the dramatic increase in world population, with its serious consequences regarding the quality of all human life,

has been known for several decades. In the early 1970's, several organizations, including the World Health Organization (WHO) instituted large-scale multidisciplinary programmes of research, with the goal of developing new methods of contraceptive technology. In addition, the special programmes of these organizations instituted studies regarding effectiveness, utilization and continuation rates of existing methods of contraception as well as studies of acceptance of various methods of contraception by groups of different socio-economic backgrounds and ethnic origins (Mishell, 1986).

For the above purpose a Special Programme of Research, Development and Research Training in Human Reproduction was set up by the WHO on request from the member states in early 1970's. The programme has a broad mandate from the World Health Assembly. This mandate encompasses a spectrum of activities, such as research on and the development of new and improved methods of fertility regulation, extensive studies on the safety and efficacy of existing and newly developed methods etc. Subsequently, a multicentred research team was developed into a unique multinational and multidisciplinary collaborating mechanism, the Task Force, which together with the network of centres conducting clinical research constitutes the backbone of the Programme's activities in research and development (reviewed by Diczfalusy, 1986).

One area of the Programme's research and development has been focused on making new and improving the existing fertility regulation methods. These new and some of the existing methods of fertility regulation include: long-acting systemic methods, oral contraceptives, post-ovulatory methods, intrauterine devices, plant extracts for fertility regulations, regulation of male fertility, sterilization (of male or female), natural regulation of fertility or natural family planning and anti-fertility vaccines (such as antisperm, anti-beta-hCG and anti-trophoblast vaccines).

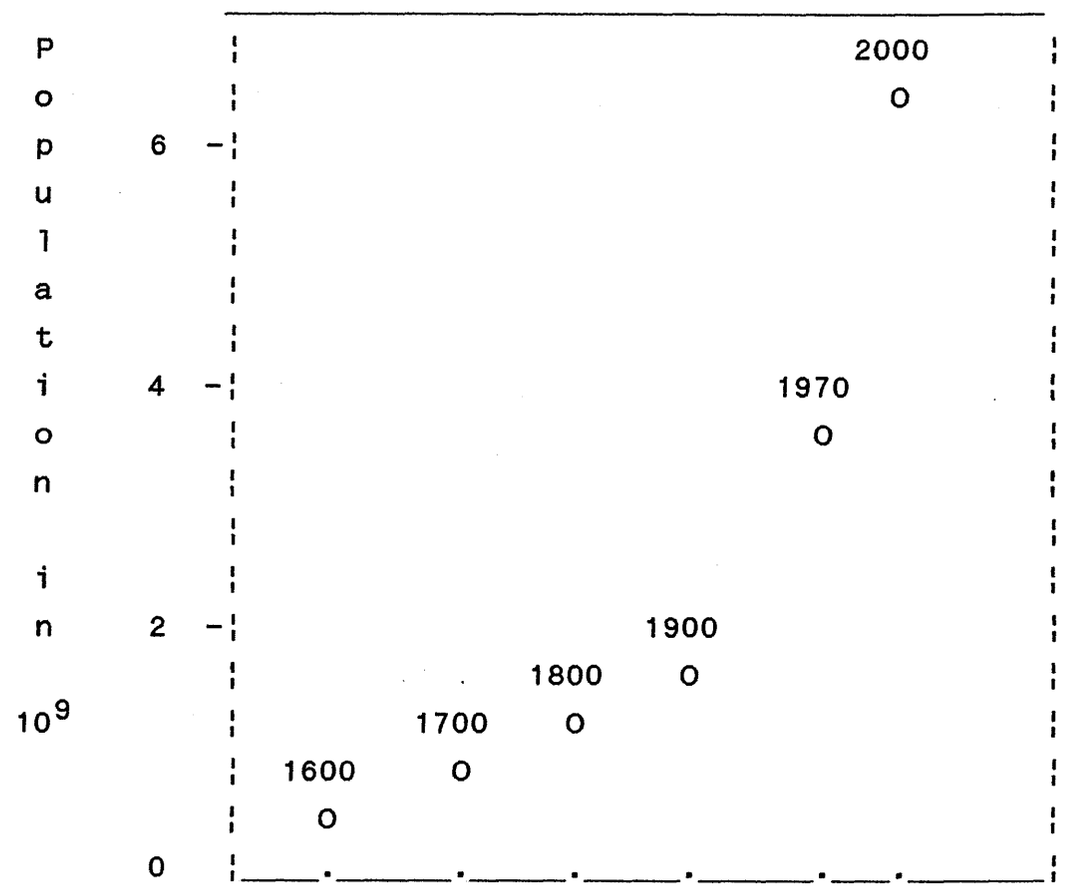


Figure 1.11 World population growth. As it can be seen the world population is growing in exponential manner and there will be over $6x10^9$ people in year 2000.

1.5.2 Contraceptive Vaccines

A safe, effective and reversible birth control vaccine would be a greatly needed addition to the currently available fertility regulating methods and an attractive proposition to family planning programmes. The advantages of such a vaccine would be numerous: its effects would be of long duration and devoid of metabolic side-effects; it could be administered by paramedical personnel as part of the primary health care system; and its synthetic components could be manufactured on a large scale and at a low cost. The potential hazards of such a method include cross-reactivity with non-target antigens, the formation of immune complexes, possible fetal damage, adverse genetic selection, some toxic effects of the carriers and/or adjuvants used and the possibility of irreversibility.

1.5.3 The Task Force on Immunological Methods of Fertility Regulation

The Task Force on Immunological Methods of Fertility Regulation (Task Force) initiated its studies as early as 1973. The original objectives were to develop immunological methods to: (a) prevent or disrupt implantation; (b) prevent sperm transport and fertilization; (c) prevent blastocyst hatching through interference with the zona pellucida; and another objective was added later as: prevention of placental formation by inhibition of trophoblast growth.

1.5.4 Placenta and Contraceptive Vaccines

The human placenta contains and secretes a number of biologically active proteins which are necessary for the maintenance of pregnancy and for the development of the fetus. It should be possible to block their action by immunochemical techniques and thus interfere with implantation or early development of the embryo.

Antibodies raised against placental surface determinants may also have a cytotoxic effect and lead to the destruction or immunological rejection of the conceptus.

The initial work was, however, concentrated on several placental hormones or proteins and also sperm enzymes. Due to several reasons the Task Force concentrated primarily on beta-hCG and sperm isoenzyme lactic dehydrogenase (LDH-C4) and some of these methods have reached the clinical trial stage (for a thorough review see Diczfalusy, 1986).

1.5.5 Placental Trophoblast-Specific Cell-Surface Determinants

Searching other avenues for the above purpose, in 1987 the Task Force organized a multinational team of scientists to work toward isolating human placental trophoblast-specific cell-surface determinants which might be used for developing anti-fertility vaccines. The team's tasks were to isolate and identify as many antibodies to the placental specific cell-surface determinants as possible and to test these antibodies against other human tissues. Those antibodies which proved to be placental specific could be used for isolating the relevant determinant. This involves transfecting, for example COS, cells with cDNA from human placenta (see below). The cells expressing such determinants could be used for isolating the gene (or cDNA clone) coding for such determinants. Molecular cloning therefore provides powerful methods for the investigation of the structure and function of such proteins and expressing such proteins for large-scale antigen production. These antigens could be used for the production of an anti fertility vaccine and the clinical trials which need to be followed. As a part of the above investigation team our group was asked to define, at the molecular level, the placental specific cell-surface determinants and isolate the cDNA clones coding for such determinants.

1.5.6 Molecular Studies of Placental Specific Determinants

The isolation of placental cell-surface determinants at the molecular level involves several steps, such as preparation of placental cDNA expression library, expression of the cDNA clones, immunological isolation of the cells expressing the target Ag and amplification and characterizing the isolated clone(s).

1.5.7 Antibodies Specific to Human Placenta

Several antibodies which had been raised against human placental tissues and found to be placental specific were chosen by the Task Force to be investigated and their relevant determinants be identified. Some of the antibodies which are going to be used in this work include FDO161G, GB17, GB25, FT10 McAbs.

1.5.7.a FDO161G

The FDO161G McAb was prepared by immunizing Balb/c mice with freshly prepared syncytiotrophoblast cell sheets of human first trimester placentae (Anderson *et al.*, 1987; Mueller personal communication). FDO161G is an IgG1 subclass and has kappa light chain. Immunoperoxidase stainings have shown that FDO161G McAb stained strongly both villus syncytiotrophoblast and non-villous cytotrophoblast of human first trimester placental sections. FDO161G also has been shown to react strongly with cultured first trimester trophoblast and villous syncytiotrophoblast of term placenta.

The Ag recognised by the FDO161G McAb migrates on SDS-PAGE as a single entity having an apparent molecular weight of 43 kDa for the dithiothreitol reduced protein. The unreduced protein migrates similarly, indicating that the isolated protein possesses neither inter nor

intrachain disulphide bonds (Mueller personal communication).

1.5.7.b GB17

GB17 McAb was prepared by using term human placental syncytiotrophoblast microvilli as the immunogen (Hsi *et al.*, 1987). The isotype of GB17 was determined to be IgG1 kappa (Hsi *et al.*, 1987).

Immunohistological studies demonstrated the GB17 antigen to be present on syncytiotrophoblast of human term, first- and second trimester trophoblast. The Ag recognized was shown to migrate as a single protein band of 175 kDa on SDS-PAGE (Hsi *et al.*, 1987).

1.5.7.c GB25

The GB25 McAb was prepared by using term human placental syncytiotrophoblast microvilli as the immunogen (Hsi & Yeh, 1986). Its isotype was shown to be IgG1 kappa. It recognizes syncytiotrophoblast, villous cytotrophoblast of the first trimester placentae and a majority of the residual cells of cytotrophoblastic shell in the basal plates and the cells in the cytotrophoblastic columns (Hsi & Yeh, 1986). The size of the Ag recognized by GB25 McAb is not known.

1.5.7.d GB24

GB24 McAb is not a Task Force recomended Ab but the Ag it recognizes might be the TLX antigens (see below) and it is investigated alongside the above McAbs.

The GB24 is a mouse McAb (IgG1) raised against human term placental microvilli (Hsi *et al.*, 1988). This Ab recognizes a trophoblast-lymphocyte cross-reactive Ag. In addition, it recognizes normal peripheral

leukocytes, fresh sperm after the spermatozoa have been fixed and permeabilized and acrosomal region of intratesticular. GB24 antigen seems to be the MCP (membrane cofactor protein) which is involved in the cascade events leading to the complement activation (Hsi personal communication). Two protein bands of 62 kDa and 75 kDa have been immunoprecipitated by this Ab from placental microvilli (Hsi *et al.*, 1988).

1.5.8 TLX

The existence of trophoblast-lymphocyte cross-reactive antigens (TLX) was first proposed as trophoblast Antigen 2 (TA2) by Faulk *et al.* (1978). Later, based on the cytotoxicity data of rabbit anti-trophoblast sera to lymphocytes, McIntyre and Faulk (1982) extended this TLX Ag system into a hypothesis to explain how the fetus survives as an allograft through pregnancy. This hypothesis formed the scientific basis for the initial clinical trial of lymphocyte immmunization for the chronic aborters of unknown etiology (Taylor & Faulk, 1981). The basic criteria of the TLX Ags defined by the hypothesis are: (1) the Ag is present on both trophoblast and lymphocyte, although expression of this Ag is not necessarily restricted to only these two types of cells; (2) the Ag is allotypic and variable from individual to individual; and (3) the Ags have a role in the maternal recognition of fetal Ag and can initiate a specific protective response from the mother (McIntyre & Faulk, 1982). The Ag of GB24 fulfills the first criteria, in that GB24 is present on both Trophoblasts and lymphocytes. Recognizing two different peptides (i.e. 62 kDa and 75 kDa) may suggest that GB24 Ag is allotypic and so fulfills the second criteria of TLX Ags.

The demonstration of three different types of placentae with two independent groups A and B (Hsi *et al.*, 1988), fits into the prediction by McIntyre and Faulk (1986) for the TLX Ag system using statistical

analysis of lymphocyte cytotoxicity data of rabbit antitrophoblast sera (McIntyre & Faulk, 1986). However, the clear identification and characterization of a TLX Ag by GB24 McAb does not resolve the basic mystery of maternofetal immunobiology and further data on the functional properties of GB24 is needed (Hsi *et al.*, 1988).

1.6 Technical Aspects of the Investigation

The screening for placental specific cell-surface determinants, using some of these McAbs, involves several techniques such as making placental cDNA library, cloning the library into a suitable vector, transfecting the library into a mammalian cell line, screening these cells with antibodies, isolating those cells which express the target determinants, isolating the cDNA clone(s) from these cells, back-transfecting the cDNA clones for further testing as above as well as by immuno-staining methods and finally sequencing and characterising the isolated clone(s) (some of the techniques which are going to be used are described in detail in next chapter and are only described briefly here).

1.6.1 Expression Libraries

Generation of an expression cDNA library is similar to that of other cDNA libraries with only one main difference, the choice of vector is limited. The library is generated by a series of steps, starting with the synthesis of cDNA, its ligation into vector, and the transformation or transfection into bacterial or mammalian cells respectively. Only when the library DNA is in cells as a set of independent and replicable elements can the effective size of the library be estimated. The major advantage of using lambda-derived cloning vector (e.g. lambda-gt11), is that the recombinants can be packaged into phage *in vitro* with an

efficiency of 10^{-1} (Maniatis *et al.*, 1982). Packaged phage heads can transform *E. coli* with an efficiency of close to unity. This is a very much higher frequency than can be achieved for plasmids, where with the best transformation efficiencies (10^8/ug DNA) only 0.1% of the ligated vector ends up as transformants (Maniatis *et al.*, 1982).

The second requirment is to identify specific clones within the library that encode the required polypeptide. These requirements have been most frequently met, to date, by using lambda vectors and by screening with nucleic acid probes (notably in lambda-gt10; see Chapter 3) or with specific antibodies to detect expressed protein epitopes (notably in lambda-gt11).

1.6.1.a Lambda Base Expression Library

The significance of phage lambda has already been discussed in that its highly efficient DNA packaging system enables the creation of large and representative cDNA libraries. In this section, the use of lambda gt11 as an expression vector is reviewed and later it is compared with a mammalian expression system.

Lambda gt11 (Young and Davis, 1983) can except cDNA sequences of up to about 7 kp in length into a unique EcoRI site (Fig 1.12), 53 bp before the stop codon of the *lacZ* gene which encodes beta-galactosidase. Any adjacent open reading frame in a cDNA insert can be expressed as a fusion polypeptide with the beta-galactosidase protein. Cloning is not directional, and the frequency with which a cDNA will be expressed in the correct frame and orientation will therefore be 1/6. Lambda gt11 must be phosphorylated before ligation of cDNA to reduce the yield of parental phage. There is no system for supressing parental phage as is possible with for example lambda gt10 (Young & Davis, 1983). Thus, it is important to have a low frequency of parental

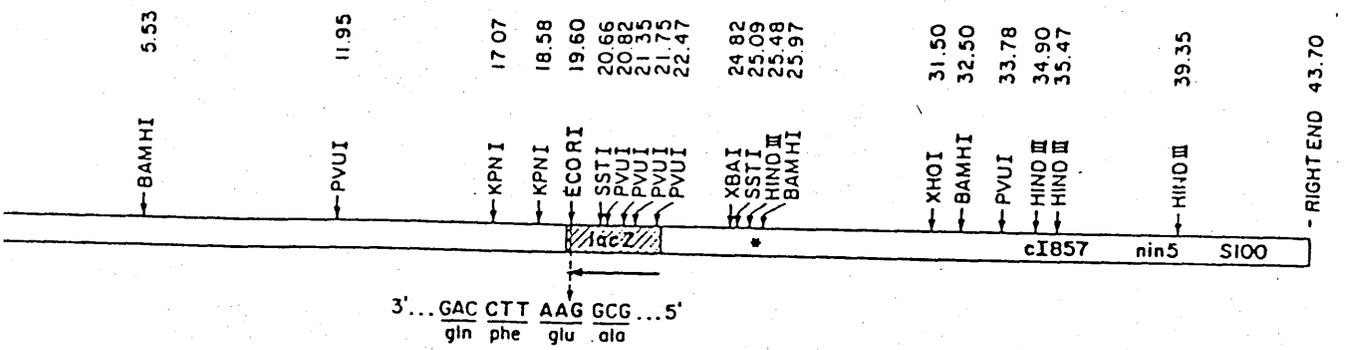


Figure 1.12 Map of lambda gt11. Restriction endonuclease cleavage sites are designated in kilobase pairs. The transcription of *lac Z* is indicated by the horizontal arrow. The sequence of the unique EcoRI site, the nucleotides that immediately surround it, and the amino acids encoded are shown. DNA sequences inserted in the EcoRI site can be expressed as fusion protein (from Young and Davis, 1983).

(nonrecombinant) phage, otherwise, very large screenings are necessary to prevent rare recombinants being obscured by the overgrowth of the parental phage.

The phages are grown on *E. coli* (e.g. protease-deficient lon^- Y1090) cells and after few hours of incubation, filter membrane impregnated with the *lac* inducer IPTG, is layed over the plate and incubated continued.

The filter then needs to be stained with antibody (Mierendorf *et al.*, 1987). The prime requirements are for a low background and high sensitivity. Because an incomplete length of polypeptide is being expressed as a fusion protein in *E. coli*, it is wise to screen for as many epitopes on the protein as possible. Monoclonals that react with antigen in Western blots are more likely to combine with stable epitopes. Mixtures of monoclonal antibodies are preferable to single ones. Polyclonal antibodies are probably most useful, especially if there is a source of purified antigen to act as an adsorption control. It will often be necessary to adsorb antisera with *E. coli* cells, and prefilteration through 0.2 um filters can also reduce background staining, possibly because of the removal of serum lipoproteins (Sutcliffe *et al.*, 1990). A wide variety of commercial antibody detection systems are available which uses ^{125}I-labeled protein A or protein G, biotin-avidin-horseradish peroxidase, or alkaline phosphatase.

1.6.1.b Plasmid Base Expression Library

The use of mammalian cells for the expression and screening of cDNA clones is an attractive alternative to lambda cloning. The polypeptide can be expressed in its native conformation with the appropriate pattern of post-translational modification One such a system is the CDM vector system of Seed and colleagues, which has been successful in the cloning of a series of membrane proteins, most of which were defined by single or by a

few McAbs (Seed & Aruffo, 1987; Aruffo & Seed, 1987; Seed, 1987; Simmons & Seed, 1988).

The CDM8 plasmid (Fig. 1.13) is a shuttle vector with an SV40 origin, which allows the plasmid to replicate episomally in COS cells (Seed & Aruffo, 1987). COS cells contain a chromosomal copy of the gene for the SV40 T antigen, which derives plasmid replication (Gluzman, 1981). Transient expression of cDNA sequences, cloned into CDM8, is initiated by the CMV promoter and enhancer 5' to the cDNA cloning site, enabling transient expression of cDNA sequences. In contrast to the lambda gt11 system, no fusion protein is formed and the cDNA should therefore, contain the entire coding sequence of the polypeptide. The cDNA is therefore size selected before the library is made (Aruffo & Seed, 1987), because small cDNA inserts are unlikely to contain a complete coding sequence. A useful detail in the design of two BstXI cloning sites (separated by 350 bp "stuffer" DNA sequence) increases the efficiency of ligation of cDNA into the vector. BstXI sites are interrupted palindromes, of sequence 5'CCAN(5)NTGG3'. The middle six bases are in inverted orientation in the two sites, yielding identical and noncomplementary sticky ends after cleavage with BstXI restriction enzyme. These will not religate without an insert sequence, obviating the need to phosphatase the vector. The cDNA is then ligated into the vector via BstXI adaptor.

Another feature of CDM8 plasmid is the presence of a synthetic tyrosine suppressor tRNA gene (supF gene); such a plasmid can be selected in a nonsupressing host containing a second plasmid, p3, which contains amber mutated ampicillin and tetracycline drug resistance elements (Seed, 1983). The p3 plasmid is derived from RP1, is 57 kb in length, and is a stably maintained, single copy episome. The ampicillin resistance of this plasmid reverts at a high rate, so that amp^r plasmids usually cannot be used in p3-containing strains. Selection for tetracycline alone is almost as good as

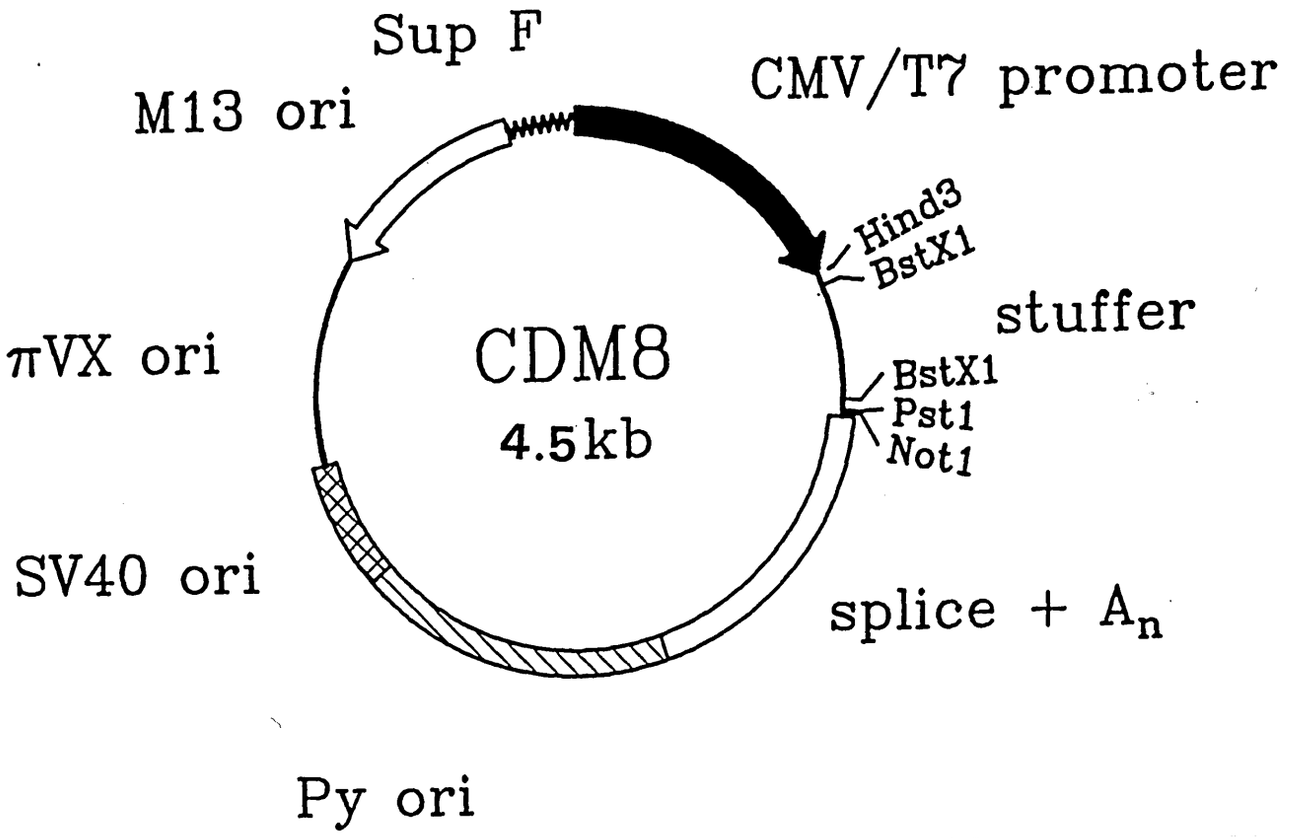


Figure 1.13 Map of CDM8 cloning vector. The 4512 bp vector is made of 8 segments. PiVX is from pBR322 origin. M13 ori is from M13 origin. Sup F is from synthetic tyrosine suppressor tRNA gene (sup F gene). CMV/T7 are from human cytomegalovirus and T7 RNA polymerase promoter. The two polylinker regions are separated by a replaceable fragment called stuffer. Splice $+A_n$ is from pVS2 and are splice and polyadenylation signals. Py ori is from polyoma origin. Finally SV40 ori is from simian virus 40 origin. Some of the restriction endonuclease sites flanking the BstX1 cloning sites are shown. The direction of transcription are indicated by the arrows.

selection for amp+tet resistance. However, spontaneous background appearance of chromosomal suppressor tRNA mutation presents an unavoidable background (frequency of about 10^{-9}) in this system (Seed personal communication).

1.6.2 Transformation and Amplification of the cDNA Library

The cDNA library, in CDM8, is amplified by transforming them into a p3 containing strain such as *E. coli* MC1061/p3 (Casadaban *et al.*, 1983) cells and growing them up under antibiotic selection. To maintain sequence representation, it is important to use a high efficiency transformation method. For this purpose Seed and colleagues have made the *E. coli* MC1061/p3 cells competent, by a variation of the $CaCl_2$ procedure which yields a transformation efficiency of $3\text{-}5x10^8$ colonies per ug of supercoiled vector DNA (Aruffo & Seed, 1987).

1.6.3 Selection of Recombinant Clones Through Transient Expression in Mammalian Cells

After amplification in *E. coli*, the library is expressed in COS cells so that clones can be expressed and immunoselected. For the immunoselection procedure, transfected COS cells are mixed with the specific antibody of interest and kept on ice in the presence of NaN_3. The cells are then pelleted through Ficoll, to remove unbound antibody and then placed on bacteriological dishes coated with a second antibody. Cells coated with primary antibody attach to the dish through the second antibody bridge and the unbound cells are discarded. This procedure is known as *panning* (Fig. 1.14; Seed & Aruffo, 1987). The attached cells are then lysed to yield their plasmids, which are then purified and amplified by re-transformation into MC1061/p3 cells (Seed & Aruffo, 1987).

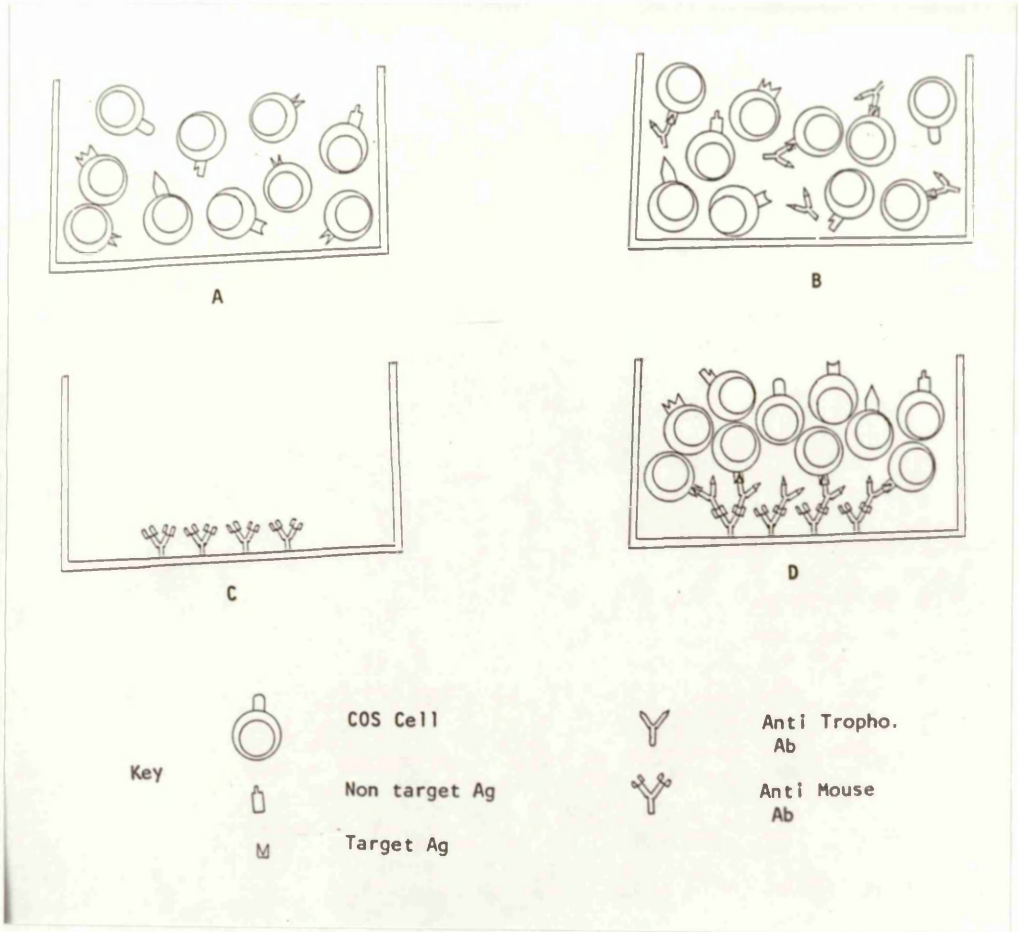


Figure 1.14 Immunoscreening of a cDNA library for cell surface determinants using a mammalian expresion system (panning). A cDNA library is transfected into COS cells and these cells express various determinants (A) (only one type is shown for each cell, though each may receive several copies of different plasmids, hence expressing more than one type of protein). Specific McAb is added to the cells (primary Ab, e.g. mouse anti-human McAb) in the culture dish (B). In another dish (panning dish) the secondary Abs (e.g. sheep anti-mouse Ab) are made stationary in the dish (C). The cells are then transferred into the panning dish and the cells bounded to the primary Abs (expressing the target determinant) bind to the stationary Abs and the free cells are washed away. Plasmids from the cells remained in the panning dish are made (Hirt DNA) and amplified in *E. coli* cells.

To obtain specific clones, three cycles of expression, immunoselection, and amplification are usually required. In the first cycle the COS cells can be transformed with the library in the form of purified DNA, using DEAE-dextran and DMSO shock (Lopata *et al.*, 1984; Selden, 1987). The COS cells are transfected at a high frequency (10-40%) and this allows a library to be panned from about $5x10^6$ cells. However, with this approach the cells receive a mixture of plasmids, so that cells that are then panned with a specific antibody will contain a large number of different "passenger" clones as well as the cDNA encoding the required epitope. Further cycles of DEAE-dextran transfection and panning will not reduce the complexity of these clones beyond 10^2 to 10^3 (A. Aruffo, personal communication). Transfection by spheroplast fusion (Sandri-Goldin *et al.*, 1981) has a lower efficiency (1-5%), and transfectants are most likely to result from the fusion of a single *E. coli* cell with a single COS cell. This method is, therefore, used in the second and third rounds of selection (Seed & Aruffo, 1987).

The advantage of the COS cell system is that antigens of receptors can be expressed in their native state as full-length polypeptides on the surface of mammalian cells. Thus, monoclonal or polyclonal antibodies, or other ligands, can be used to select cells expressing the clones.

1.6.4 Amplification of DNA Sequences by PCR

Polymerase chain reaction (PCR) is a method whereby microgram quantities of a short sequence of DNA (commonly up to few kilobases) can be amplified from an initial template of nanogram quantities of high complexity DNA (White *et al.*, 1989). This permits genetic analysis on single diploid cell and single sperm (Li *et al.*, 1988). PCR has been used in several ways to assist in cDNA cloning, both to create new clones and to extend the cloning of rare cDNAs to the full length

of their corresponding transcripts (Frohman *et al.*, 1988; O'Hara *et al.*, 1989; Lee & Caskey, 1990). Using the genetic code, PCR primers can be designed from protein sequence. The degeneracy of the code can lead to a large number of possible oligonucleotides. Degenerate oligonucleotides have been successfully used to clone protein coding sequences from cDNA libraries of genomic DNA and in the case of the 54 kDa subunit of signal recognition particle, from a single sequence of 30 amino acids (Bernstein *et al.*, 1989; for methods and further references see Compton, 1990). Some base pair mismatching between primer and template may be tolerated (Gould *et al.*, 1989), provided there is a stable duplex at the 3' end of the primer so that DNA synthesis can occur.

The amplification of DNA (genomic or cDNA) by PCR method requires the presence of two primer sites on the same piece of template DNA. After the DNA has been rendered single stranded by heating to 94^{o}C, the temperature is reduced so that the primer can anneal to the template DNA. If the primers anneal close enough together at sites on complementary strands of DNA and with their 3' ends facing inward (Fig. 1.15), then DNA synthesis can extend those 3' ends past the opposite primer site, thereby creating a new template for a further round of PCR. The result is the amplification of a short piece of DNA whose ends are precisely defined by the primers employed (White *et al.*, 1989). DNA synthesis is catalyzed by Taq polymerase, which is sufficiently thermostable to withstand 40 cycles at a temperature of up to 94^{o}C and, hence, yield microgram quantities of amplified DNA (Gelfand & White, 1990). The inclusion of restriction sites at the 5' ends of the PCR primers allows amplified DNA to be readily cloned into vectors for sequencing and other work (Scharf, 1990).

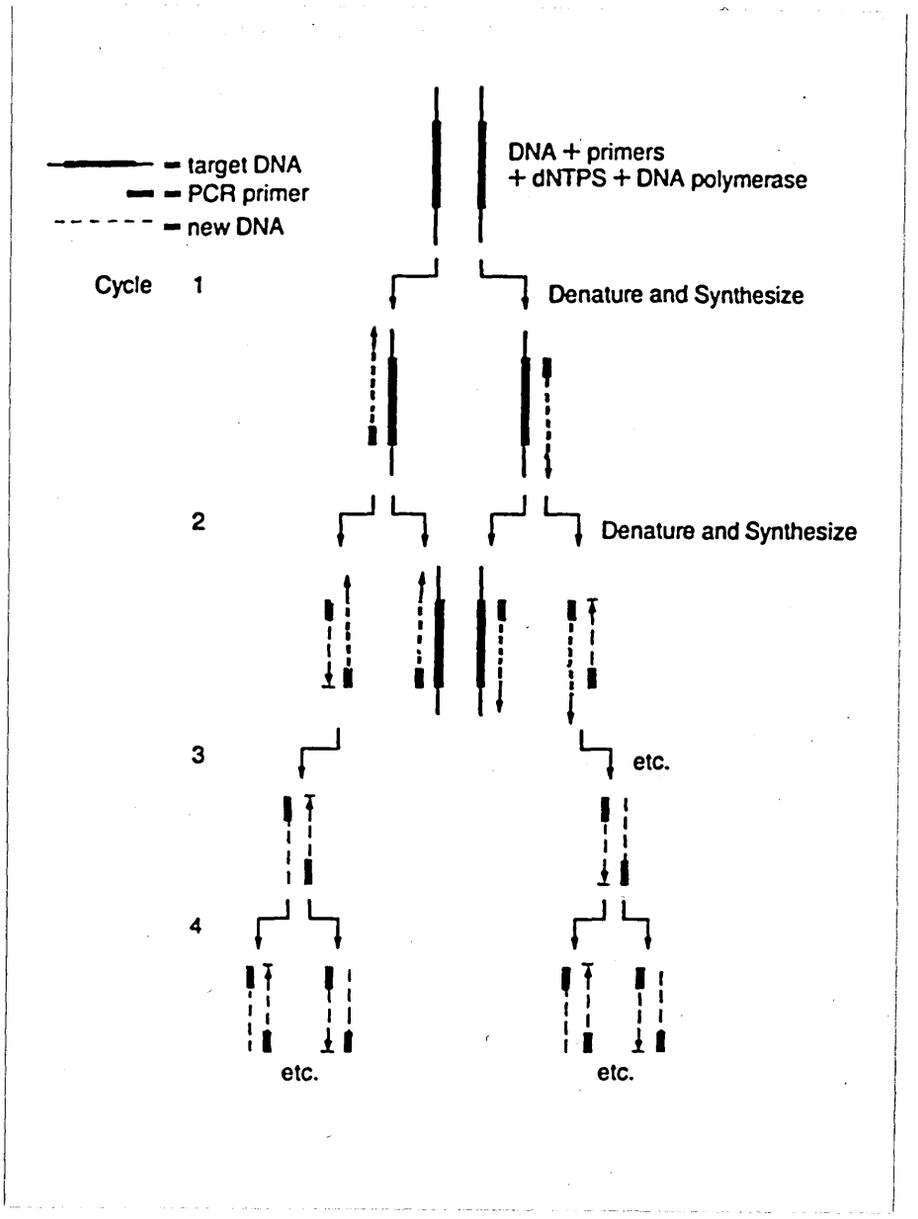


Figure 1.15 PCR amplification of DNA. PCR is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridise to opposite strands of the target sequence. Repeated cycles of heat denaturation of the template, annealing of the primers to their complemetary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the primers.

CHAPTER 2

MATERIALS AND METHODS

2.1.1 Prokaryotic and Eukaryotic Strains

The prokaryotic strains were all derivatives of *Escherichia coli* (*E. coli*) and the eukaryotic strain was COS-7 an SV40-transformed monkey cell line and these are listed in Table 2.1 and 2.2. Nomenclature follows the proposals of Demerec *et al* (1966), using the symbols for genotype recomended by Bachman *et al* (1976).

Table 2.1 Plasmid Free Strains

Name	Genotype	References/Source
DS941	*lacZ M15, lacI^q, thr-1 recF143, leuB6, hisG4, thi-1, ara-14, argE3, galK2, supE44, xyl-5, mtl-1, tsx-33, rpsL31*	D.J. Sherratt Dept of Genetics Glasgow Univ.
JM101	/\ lac Pro, SupE, Thi, (rk$^+$,mk$^+$)/F' traD36, ProAB, LacI^q,Z/\15	Messing *et al.*, 1981, N.A.Res. 9, 309
/\HFL	hsdB$^-$, hsdM$^+$, supE, thr, leu, thi, lacY1, tonA21, hflA150 [chr::Tn10]	Young and Davis 1983
COS-7	Contains SV40 T antigen	Gluzman 1981

Table 2.2 Plasmid Containing Strain

Name	Host genotype	Plasmid	Reference/Source
MC1061/P3	/\(LacPOZYA)x74 gal U gal K str.A	p3	Casadaban *et al.*, 1983

2.1.2 Plasmids and Bacteriophages

The plasmids used in this study are listed in Table 2.3. Nomenclature of plasmids follows Novic *et al.* (1976). The symbols for genotype are after Warren, Twigg and Sherratt (1978) and Tacon *et al.* (1981). The nomenclature of the M13 bacteriophage clones follows the recommendation of Staden (1982).

2.1.3 Chemicals, Organic Compounds, Immunochemicals, Radiochemicals, Enzymes etc.

Chemicals, organic chemicals, immunochemicals. radiochemicals, enzymes and some other materials or reagents used are listed in Table 2.4 with the name of main suppliers.

Table 2.3 Plasmids and Bacteriophages

Name	Relevant marker	Reference/Source
pUC 18	Mob-, Bom-, Amp^r, Rom-	Yanish-Perron *et al.*, 1985
M13 mp18	cloning vector drived from M13	Yanish-Perron *et al.*, 1985
mHCF17	M13 mp18+HCF17	this work Chapter 4
pBR322	Amp^r, Tet^r	Sutcliffe 1978
\gt 10	\srI\1°, b527,srI\3° imm^{434} (sr 434^+), srI\4°, srI\5°	Haynh, Young & Davis 1984
pFH 1	as pBR 322	Kornblihtt *et al.*, 1983
pFH 23	as pBR 322	Kornblihtt *et al.*, 1984b
pFH 154	as pBR 322	Kornblihtt *et al.*, 1984b
pHCF 11	as pUC 18	this work Chapter 4
pHCF 17	as pUC 18	this work Chapter 4
pHCF 24	as pUC 18	this work Chapter 4
\HCF 17	as \gt 10	this work Chapter 4
\HCF 24	as \gt 10	this work Chapter 4
CDM 8	Sup F	Aruffo & Seed 1987
CD 2	as CDM8	Seed and Aruffo 1987

* The symbol \ means lambda e.g. \gt10 means lambda gt10

Table 2.4 Chemicals, General Chemicals, Organic Compounds, Immunochemicals, Radiochemicals, Enzymes and etc.

Material	Source
Chemicals, general chemicals, organic compounds	B.D.H., BRL, Hopkins & Oratoreis, May and Baker and Sigma
Media	Difco and Oxoid
Agar	Davis and Difco
Biochemicals	Sigma
Antibiotics	Sigma
Agarose	BRL and Sigma
BSA (for general use)	Sigma
BSA (for DNA work)	Pharmacia
Sequencing kit	BRL
Gama ^{32}P ATP and alpha ^{32}P dNTP	Dupont
Alpha-^{35}SdATP	Amersham
Lamda DNA marker	Dr. K. Kaiser, Dept. Genetics, Glasgow Univ.
IPTG and X-Gal	BRL
tRNA and Glycogen	Boehringer-Mannheim
Lysozyme	Sigma

Table 2.4 cont^{d}.

Material	Source
Restriction enzymes	BRL & New England Biolab
Proteinase K and RNase	Boehringer-Mannheim & BRL
T4 ligase	BRL and NBL
T4 kinase	NBL
Calf intestinal alkaline phosphatase (CIP)	BRL
Nylon membrane	Pall Process Filtration
Taq polymerase	Cambio
Propidium iodide	Sigma
H_2O_2	Fisons
Haematoxylin	Ortho Diagnostics
Diaminobenzodine (DAB)	Sigma
Periodic Acid	Sigma
Dynabeads	Dynatech
DEAE-dextran	Sigma
Chloroquine diphosphate	Sigma
DMSO	Sigma

2.1.4 Antibiotics

The concentrations of antibiotics used were as in Table 2.5. All the antibiotics were kept at -20°C, Tetracycline was kept in light proof tubes. When required, antibiotic stock solutions were added to molten agar pre-cooled to 55°C.

Table 2.5 Antibiotics

Name	Abr	Selective con.	Stock soln.
Ampicillin	Amp	50 ug/ml	50 mg/ml (dH2O)
Chloramphenicol	Chlm	50 ug/ml	10 mg/ml (ethanol)
Tetracycline	Tet	12.5 ug/ml	12.5 mg/ml (v/v of ethanol/H2O)

2.1.5 Antibodies

Antibodies (Abs) with their specificity and type are listed in Table 2.6 The antibodies were used in different concentrations; and were usually diluted in TBS. For further details see the relevant results sections (chapters 4 and 7).

Table 2.6 Antibodies.

Name	Specificity	Type	Source/Reference
IST 2	M anti-H FN	IgG	Borsi *et al.*, 1987
IST 8	M anti-H FN	IgG	Zardi *et al.*, 1988
IST 9	M anti-H FN	IgG	Carnemolla *et al.*, 1987
W6/32	M anti-HLA-A,-B,-C	Ig	Barnstable, 1978
CD2	M anti-CD2	Ig	Seed & Aruffo, 1987
GB 17	M anti-H Syncytio.	Ig	Hsi *et al.*, 1987
GB 24	M anti-H TLX	Ig	Hsi *et al.*, 1988
GB 25	M anti-H V. Troph.	Ig	Hsi *et al.*, 1986
FDO161G	M anti-H place.	Ig	Anderson *et al.*,1987
Peroxidase conjugated G or S anti-M Ab		IgG	Sigma
FITC conjugated second Ab G or S anti-M Ab		IgG	Dako, Sigma, Seralab SAPU (Scotish Ab PU)

H=human, M=mouse, S=sheep, G= goat and FITC=Fluorescein isothiocyanate ; FN= fibronectin, troph= trophoblast, V= villi and Syn= syncytitrophoblast

2.1.6 Oligonucleotides

Oligonucleotides used for ligation as adaptors, as probes for hybridisation, as primers to run PCR and as size markers to run on polyacrylamide gel electrophoresis are listed in the Table 2.7.

Table 2.7 Oligonucleotides Used for Ligation, Hybridisation, PCR and Size Markers.

No	Name	Size mer	Tm min	Tm max	Specificity	Sequence 5' 3'
1	189	8	22	22	adapt.	CTC TAA AG
2	192	12	34	34	adapt.	ACA CGA GAT TTC
3	380	17	52	52	CDM8	TAT AGG GAG ACC GGA AG
4	382	17	50	50	CDM8	ACT GGT AGG TAT GGA AG
7	496	26	76	88	161G	CTGCAGACNGGNTGGACNCACCTNGT TG TT
8	497	26	78	92	161G	CTGCAGGCNGGNGGNTTCCTNGGNCA TT
9	736	26	78	92	161G	AAGCTTTGNCCNAAAAANCCNCCNGC GG
10	CD2	15	50	50	CD2	CCA CCA GCC TGA GTG
11	391	54	-	-	-	size marker
12	395	54	-	-	-	size marker
13	HSB-T	54	-	-	-	size marker

2.1.7 Microbiological Culture Media and Supplements

L-B Broth: 10 g Bacto tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose and 20 mg thymine; made up to 1 litre in dH_2O and adjust the pH to 7.0 with NaOH.

2YT Broth: 10 g Bacto tryptone, 10 g yeast extract, 5 g NaCl; made up to 1 litre in dH_2O.

TYM Broth: 2% Bacto tryptone, 0.5% yeast extract, 0.1 M NaCl and 10 mM $MgSO_4$

Soft Agar (0.6%): 6 g agar in 1 litre dH_2O.

Minimal Medium: 17.5 g agar, 0.25 g trisodium citrate, 7 g K_2HPO_4, 2 g KH_2PO_4, 4 g NH_4SO_4 and 0.1 g $MgSO_4.7H_2O$, made to 1 liter in dH_2O. Alternatively to 300 ml water agar add 100 ml DM salts, 4ml 20% glucose and 2 ml 1% vitamin B1 was added.

Water Agar: 2% agar in distilled water.

SOC Medium: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

Indicators: X-gal (5-Bromo-4-chloro-3-indolyl-B-galactoside) was used in conjuntion with IPTG (isopropyl B-D thiogalactopyranoside). These two were used for strains (i.e. DS941 and JM101) which were transformed by pUC or M13 vectors which provided a screen for plasmids with inserts in the polylinker region. Colonies containing inserts were generally white, as opposed to clones lacking inserts, which were usually blue. X-gal was stored at a concentration of 25 mg/ml in dimethylformamide (DMF) at -20°C and added to pre-cooled L-agar (48°C) to a final concentration of 50 ug/ml. IPTG was stored at a concentration of 25 mg/ml in water at -20°C and was used in conjunction with X-gal to a final concentration of 25 ug/ml.

Thiamine Vitamin B1: Stock solution of Vitamin B1 was made to 20 mg/ml in dH_2O and kept at -20°C.

2.1.8 Sterilization

All growth media were sterilized by autoclaving at 120°C for 15 min, buffer solutions at 108°C for 10 min and $CaCl_2$ at 114°C for 10 min. Heat sensitive reagents were sterilizes by filtration using 0.22 um disposable filters.

2.1.9 Plating

The agar media, for plating, was melted in a steam bath and cooled down to 48°C in a water bath before adding supplements such as antibiotics, X-gal, IPTG or vitamin B1 whenever required. On minimal medium plates supplements were usually added to the top agar istead of minimal medium agar. When agar was set, the plates were usually let to dry for 1 hr at 37°C in a hot room. The drying was particularly important for quick absorbtion of liquid when large volumes of transformants were plated.

2.1.10 Electrophoresis Buffers and Dyes

10X TBE Buffer pH 8.0: 108 g Tris base, 55 g Boric Acid and 20 ml 0.5M EDTA (pH 8.0); made up to 1 litre in dH_2O.

50X TAE Buffer pH 8.0: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) made to 1 litre in dH_2O.

5X Final Sample Buffer (FSB): 10% ficol, 0.05% bromophenol blue, 0.25% orange G and 0.5% SDS made in 1X TBE.

Formamide Loading Buffer (for sequencing gels): 95% deionized formamide, 0.1 g xylene cyanol FF, 0.1 g Bromophenol Blue and 10 mM 0.5 M EDTA made into 100 ml in dH_2O.

Ethidium Bromide (EthBr): 10 mg/ml made in dH_2O and kept in a dark bottle at 4^oC.

2.1.11 Stock Solutions

1 M Tris: 121.1 g Tris base made up to 1 litre in dH_2O after adjusting the pH by adding concentrated HCl (pH 7.4, 7.5 and 8.0 need approximately 70, 65 and 42 ml concentrated HCl respectively).

0.5 M EDTA (pH 8.0): 186 g of Na_2-EDTA.$2H_2O$ (disodium ethylene diamine tetra acetate) made up in dH_2O after adjusting the pH by 10 N NaOH.

10X TE Buffer: 100 mM Tris.Cl (pH 8.0) and 10 mM EDTA (pH 8.0) made in distilled water.

10X E Buffer pH 8.2 (for making single colony gel buffer): 48.4 g Tris, 16.4 g Na acetate and 3.6 g Na_2EDTA.$2H_2O$, made up to 1 litre in dH_2O, adjust the pH by acetic acid.

10% SDS pH 7.2: 100 g of electrophoresis-grade SDS (sodium dodecylsulfate), made up to 1 litre in dH_2O after adjusting the pH to 7.2 by concentrated HCl.

20X SSC pH 7.0: 175.3 g NaCl and 88.2 g sodium citrate, made up to 1 litre after adjusting the pH to 7.0 by 10 N NaOH.

20X SSPE pH 7.4: 174 g NaCl, 27.6 g $NaH_2PO_4.H_2O$ and 7.4 g EDTA, made up to 1 litre in dH2O after adjusting the pH to 7.4 by 10 N NaOH.

2.1.12 DNA Manipulation Buffers

Restriction Enzyme Buffers: The restriction enzyme buffers which were mostly used are listed in **Table 2.8.** Other restriction enzyme buffers which were used are listed after **Table 2.8.**

REact No	Tris-HCl mM	PH	MgCl2 mM	NaCl mM	KCl mM
1	50	8.0	10	-	-
2	50	8.0	10	50	-
3	50	8.0	10	100	-
4	20	7.4	5	50	
5	10	8.2	8	-	-
6	50	7.4	6	50	50
7	50	7.4	10	50	50
8	20	7.4	10	-	-
9	50	8.5	5	-	-
10	100	7.6	10	150	-
11	10	9.0	12	-	100

Table 2.8 Restriction Enzyme Buffers (REact) Buffers (from BRL): The REact buffers were 10X concentration.

10X XhoII Buffer: 6 mM Tris.Cl (pH 7.5), 6 mM $MgCl_2$, 7 mM B-mercaptoethanol and 0.01% V/V X-100 Triton.

10X Bstx1 Buffer: 100mM Tris (pH 7.6), 1.5 M NaCl, 60 mM $MgCl_2$, 10mM dithiothreitol (DTT) and 1 mg/ml BSA.

10X Low Salt: 60 mM Tris (pH 7.5), 60 mM $MgCl_2$, 50 mM NaCl, 2.5 mg/ml BSA and 70 mM Mercaptoethanol(ME).

10X Ligation Buffer I: 20 mM DTT, 1 mg/ml BSA, 10 mM spermidine and 1 mM ATP.

10X Ligation Buffer II: 10mM ATP, 20 mM DTT, 10mM spermidine, 1 mg/ml BSA and 10 mM $MgCl_2$.

10X Dephosphorylating Buffer (10X CIP Buffer): 0.5 M Tris.Cl (pH 9.0) 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM spermidine.

Sequencing Annealing Buffer: 0.1 M Tris.Cl (pH 8.0); 0.1 M $MgCl_2$.

10X Taq Polymerase Buffer: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tri:HCl (pH 8.0) and 0.1 mg/ml gelatin.

2.1.13 DNA Labelling and Hybridization Buffers

10X End Labelling (Kinaseing) Buffer: 0.5 M Tris (pH 7.5), 10 mM ATP, 20 mM DTT, 10 mM spermidine, 1 mg/ml BSA and 10 mM $MgCl_2$.

Radio-Labelling of DNA Probes by Random-Primed Synthesis: One ml of reaction mix is made of 200 ul solution A, 500 ul solution B and 300 ul solution C.

Solution A: 1 ml 1.25 M Tris.Cl (pH8.0), 0.125 M $MgCl_2$, 18 ul B-mercaptoethanol and 5 ul each of 100 mM stock of dCTP, dGTP and dTTP (in 1x TE).

Solution B: 2 M Hepes titrated to pH 6.0 with 4 M NaOH.

Solution C: Hexadeoxyribonucleotides are dissolved in 1 mM TE pH 7.5 at 90 O.D. units per ml.

Sephadex Column Buffer: 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 50 mM Tris.Cl (pH 7.5).

Denaturing Solution: 1.5 M NaCl and 0.5 M NaOH.

Neutralizing Solution 1: 3 M Na acetate pH 5.5.

Neutralizing Solution 2: 1.5 M NaCl, 0.5 M Tris.Cl (pH 8.0).

DNA Pre-Hybridisation Buffer: 5X Denhardt's solution, 5X SSPE, 0.2% SDS and 100 ug/ml denatured nonhomologous DNA (salmon or herring sperm DNA) made in water.

100X Denhardt's Solution: 2g ficoll, 2g BSA and 2g polyvinylpyrolidone, made up to 100 ml in dH_2O.

Low Stringent Wash Buffer: 1X SSPE and 0.1% SDS made in dH_2O.

High Stringent Wash Buffer: 0.1X SSPE and 0.1% SDS made in dH_2O.

Probe Removal Buffer I: 0.4 M NaOH.

Probe Removal Buffer II: 0.1X SSC, 0.1% SDS and 0.2 M Tris pH 7.5.

2.1.14 DNA Preparation, Purification and General Purpose Solutions

Single Colony Gel Buffer: 2% Ficoll, 1% SDS, 0.01% Bromophenol Blue and 0.01% Orange G, made up in buffer E.

Birnboim Doly Solution I: 50 mM Glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA and add freshly made lysozyme to final concentration of 1 mg/ml immediately before use each time.

Birnboim Doly Solution II: 0.2 M NaOH and 1% SDS, freshly made each time.

Birnboim Doly Solution III: To 60 ml of 5 M KOAc added 11.5 ml of glacial acetic acid and 28.5 ml dH_2O.

Lytic Mix Solution: 2% Triton X-100, 50 mM Tris pH 8.0 and 60 mM EDTA pH 7.5.

STET Buffer: 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 8% sucrose, 5% Triton X-100 and add freshly made lysozyme to final concentration of 1mg/ml immediately before use.

Phage Dilution Buffer: 7 g NaHPO4, 3 g KH2PO4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$ and 1% gelatin solution, made up to 1 litre in dH2O.

PEG Solution: 20% PEG and 2.5 M NaCl.

DNA Resuspension Buffer (TNSE Buffer): 1X TE, 0.1 M NaCl, 1% SDS and 1mM EDTA.

TFBI Buffer: 10 mM KOAc, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$ and 15% glycerol.

TFBII Buffer: 10 mM Na_mops pH 7.0, 75 mM $CaCl_2$, 10 mM KCl and 15% glycerol.

Linear Polyacrylamide for DNA Precipitation: 1% acrylamide was made from 40% stock solution of acrylamide:bisacrylamide (19:1 W/W).

Linear Acrylamide for DNA Precipitation: 1% acrylamide was made in dH_2O and had no bisacrylamide.

2.1.15 Staining Materials

Antibodies: See section 2.1.

Human Serum: Human serum had been obtained from pregnant women and was diluted in TBS.

BSA: 5% BSA fraction V made in TBS. BSA was made fresh each time and any excess was kept at $4^{o}C$ and usually discarded after a week.

Haematoxylin: Harris Haemotoxylin was filtered before use and kept in light proof bottle at $4^{o}C$ for several months but after 3-4 months it had to be filtered again.

Saline: 9.5 g NaCl in 1 litre of dH_2O.

TBS: 100 ml 0.5 M Tris pH 7.5 and 900 ml saline.

Periotic Acid: 1 ml 50% periotic acid in 500 ml TBS (0.1% soln.).

Diaminobenzodine (DAB): 5 g DAB was dissolves in 50 ml dimethyl formamide overnight in the dark. Once dissolved 50 ml PBS was added to it to give 5% stock solution, then made into 2 ml aliquots and kept at $-20^{o}C$. Working solution of DAB was made by adding 2 ml of 5% DAB solution into 500 ml PBS and 330 ul H_2O_2 (hydrogen peroxide).

Acid Alcohol: 99 ml ethanol and 1 ml 1 N HCl (1%).

Scot Tap Water: 500 ml dH_2O and 100 ul ammonium hydroxide or alternatively 500 ml dH_2O, 1 g potassium bicarbonate and 10 g $MgSO_4$.

Propidium Iodide: 0.1% was made in dH_2O and final dilution was made in TBS.

2.1.16 Tissue Culturing Media and Reagents: The mammalian cell line, COS-7, was maintained in GMEM medium supplemented with 10% fetal calf serum (FCS) and made up to 400 ml as follows:

GMEM Medium (Glasgow Modified Eagle Medium and Suplements:

300	ml	dH_2O
40	ml	10X Glasgow Modified Eagle Medium
13.5	ml	Sodium bicarbonate (7.5%)
40	ml	FCS (filter sterile during addition)
4	ml	Sodium pyruvate (100 mM)
4	ml	L-glutamate (200 mM)
4	ml	Penicillin/Streptomycin (10^3 U:10^3 ug/ml)
4	ml	Non essential growth acids (50 mM asparagine and glutamate, 10 mM alanine, aspartate, glycine and serine).

10X PBSA: To make 500 ml, 125 ml 1 M Tris:HCl (pH 7.4), 25 ml 1 M KCl, 6 ml 0.5 M Na_2HPO_4, 70 ml 50 mM $CaCl_2$, 2.5 ml 1 M $MgCl_2$ and 134.5 dH_2O were added together.

10X STBS: To make 500 ml stock solution suspension TBS (STBS) was made by 125 ml 1M Tris:HCl (pH 7.4), 25 ml 1 M KCl, 6 ml 0.5 M Na_2HPO_4, 70 ml 50 mM $CaCl_2$ and 134.5 ml dH_2O.

5X PEA: 100 ml PEA (PBS, EDTA and Azide) was made by adding 50 ml 10X PBSA, 0.5 ml 0.5 M EDTA, 1 ml 10% Azide and 48.5 ml dH_2O.

10% DMSO: Dimethyl sulfoxide (DMSO) was made in PBSA.

DEAE-Dextran: 10 mg/ml DEAE-dextran was made in STBS.
Chloroquine: 100 mM stock solution of chloroquine was made in STBS.

2.1.17 Polymerase Chain Reaction (PCR) Reagents and Apparatus

PCR reagents: DNA polymerase Thermus aquaticus Type 3 (Taq 1 polymerase) 5 units/ul and 10X Taq polymerase buffer were purchased from Cambio Ltd. (UK). dNTPs (BRL) were diluted to 200 uM and the final mix for PCR had 1.25 uM of each of the dNTPs. Oligonucleotide primers were made in the Dept. of Biochemistry, Glasgow University.

PCR Apparatus: The apparatus to run PCR reactions was an air cooled programmable cyclic reactor from Cambio (UK).

2.2 General Methods

2.2.1 Transformation of *E. coli* by $CaCl_2$ Procedure

Plasmid or phage DNA can be introduced into *E. coli* cells by a process known as transformation. *E. coli* is not naturally competent to take up DNA from the environment, but can be made to do so by treatment with CaCl2 or other methods. Sterile plastic lab-ware was used throughout because competent cells are easily lysed by detergents.

Preparation of Competent DS941 Cells:

In order to make competent cells 1 ml of a fresh stationary growing overnight culture of cells was diluted in 100 ml L-Broth and grown with vigorous shaking at 37°C to a density of $5x10^7$ cells/ml, which takes approximately 2-4 hrs. The culture was then rapidly chilled on ice and spun down at 4000g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml of ice cold, sterile 50 mM CaCl2 and 10mM Tris-HCl (pH 8.0). The cell suspension was placed on ice for 15 min and then centrifuged as above. After discarding the supernatant, the pellet was resuspended in 5 ml of ice cold 50 mM CaCl2 and 10mM Tri-HCl (pH 8.0). The cell suspension was divided into 200 ul aliquots in prechilled tubes. The cells were either used immediately or stored at 4°C overnight to maximize the transformation efficiency.

Transformation of DS941 Cells:

The DNA for transformation was diluted to 10 ng/ul and 1 ul was added to 200 ul competent cells. After mixing, the cells were kept on ice for 30 min. and then transferred to either 42°C for 2 min or 37°C for 5 min. After the heat shock, 1 ml L-Broth was added to each tube and incubated at 37°C for 30 minutes to one hour for antibiotic expression (1 hour for tetracycline and 30 min for ampicillin selection) without shaking. For antibiotic selection, a 100 ul of the transformation mix was spread on to selective media plates and the plates were incubated at 37°C inverted, overnight.

2.2.2 Precipitation of DNA

Ethanol precipitation of DNA: DNA was ethanol precipitated for wide variety of reasons in this work (see text for detail). Precipitation DNA is enhanced at low temp. (e.g. -20° C -70° C) in the presence of moderate concentration of monovalent cations (e.g. NaOAc, NaCl or NH_4OAc or carriers (e.g. tRNA, glycogen or linear polyacrylamide or linear acrylamide; for concentration of each monovalent cations or carriers see Table 2.9). The DNA is recoverd by centrifugation and redissolved in an appropriate buffer. To the measured volume of DNA solution, the desired salt or carrier was added and then 2 vol ice cold ethanol was added. In most cases NaOAc or NaCl were used in the ethanol precipitations as monovalent cations. The DNA/ethanol solution was stored at either -20° C for >30 min. or at -70° C for >15 min. The DNA was pelleted down by centrifugation at 12000 g in a microfuge for 15 min or in a Beckman centrifuge in a JA2-21 or JA20 rotor for 30 min at 18 krpm (40000 g) at 4°C. After decanting the supernatant, traces of precipitated salt were removed by washing the pellet with cold 70% ethanol and then spinning the tube for 1-5 min to remove most trapped salt. The tubes were inverted to remove most of the 70% ethanol and then the pellet

was dried in a vacuum dessicator for 5-30 min. Finally the DNA pellet was dissolved in the desired vol of buffer by vortexing and warming at 37_oC for few minutes.

Salt/Carrier	Name	Final conc.
Salt	NaCl	0.1 M
Salt	NaOAc	0.25M
Salt	NH_4OAc	2.0 M
Carrier	tRNA	10-20 ug/ml
Carrier	glycogen	50-200 ug/ml
Carrier	lin. polacryl.	10-100 ug/ml
	lin. acryl.	10-100 ug/ml

Lin. acrylamid = linear acrylamide
Lin. polacryl. = linear polyacrylamide
[acrylamide plus bisacrylamide (19/1)]

Table 2.9 Salts and Carriers for Ethanol Precipitation. The carriers were mostly used for work described in Chapter 4 (see text for detail).

Ethanol Precipitation of Oligonucleotides: This procedure was found to give high yield of salt free oligonucleotides longer than 20 bases in a quantity of greater than 0.1 OD. For compounds which were shorter or in low concentration, the yield was variable. For extremely small quantities of DNA a carrier such as tRNA was used.

Usually the oligonucleotides were supplied either in ammonium hydroxide (NH_4OH) or as a dried pellet. If it was in NH_4OH then a measured volume was evaporated under vacuum while spinning until a dried DNA pellet was formed. The pellet (from either the supplier or made above) was dissolved in 200 ul of ammonium acetate (2.5 M pH 7.0) or sodium acetate (0.3 M pH 5.2). The oligonucleotide was resuspended either in dH_2O or TE. To the resuspended oligonucleotide 3 volumes ethanol (-20°C) was added and stored at -20°C for at least 2 hrs. The centrifugation, washing and drying of the DNA was as described in ethanol precipitation of DNA above. The pellet was resuspended in 1xTE and kept at -20°C. Usually an aliquot was end labelled and run on a 20% polyacrylamide gel to check the size and impurities.

Isopropanol Precipitation of DNA: The procedure for isopropanol precipitation of DNA was as in ethanol except in following steps. Only one volume of Isopropanol was used instead of two. The precipitation was carried out at room temprature. The DNA was spun for only 7 minutes in a microfuge and the pellet was washed with 70% ethanol as in ethanol precipitation.

2.2.3 DNA Preparations

Large Scale DNA Preparation by Alkaline-SDS Extraction: The method was a modified version of the procedure described by Birnboim and Doly (1979). An overnight culture of the plasmid-bearing *E. coli* was grown in selective media overnight at 37°C in a shaking water bath. The o/n culture was transferred to 500 ml L-Broth and cells were grown at 37°C overnight with vigorous shaking. The cells were divided into two 250 ml large tubes and the cells were pelleted by centrifugation at 5 krpm (3000 g) for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in the remaining traces of media by vortexing. Then 8 ml of lysis solution (Birnboim Doly I) was added into each tube, vortexed briefly, then

incubated on ice for 5 min. 16 ml alkaline-SDS solution (Birnboim Doly II) was added to the DNA mix and left on ice for 5 min, then 12 ml of ice cold 5 M KOAc (Birnboim Doly III) was added and mixed gently and incubated for a futher 10 min. The viscous solution was centrifuged at 18 Krpm (40,000g) for 30 minutes at 4°C.

The cleared supernatant (18 ml approx.) was precipitated at room temperature with 12 ml of isopropanol for 15 min. (to avoid excessive coprecipitation of SDS which may occur at -20°C). The DNA was centrifuged at 18 Krpm (40,000g) for 30 min at 20 °C. After discarding the supernatant, The DNA pellet was rinsed in ice-cold 70% ethanol. After drying, the DNA was resuspended in 8 ml 1x TE for the next step.

The DNA was further purified from RNA, protein and host chromosomal DNA by banding on CsCl/EtBr (Cesium Chloride/Ethidium Bromide) gradient (density of 1.58-1.61 g/ml). After dissolving CsCl, 0.6 ml EtBr (10 mg/ml)was added to the DNA/CsCl solution. The gradient was then centrifuged at 5 Krpm for 10 min to precipitate the purple aggregates of EtBr-bacterial protein complex. This step dramatically reduced the protein contamination of the DNA in CsCl/EtBr gradient. The next step was to separate covalently closed circular plasmid DNA (CCC DNA) from other remaining contamination, such as RNA, protein and chromosomal DNA, by centrifuging in an ultra high speed centrifuge. For this the CsCl solution was transferred into a polyallomer (Quick-Seal Beckman) 10 ml ultracentrifuge tube, then the remainder of the tube was filled with light paraffin oil, avoiding any air bubbles traped. The tubes were centrifuged in a Beckman Ti-70 fixed angle rotor at 49 Krpm (200,000 g) at 22°C for 16 hrs.

Usually two bands were visible under long wave UV light; a lower supercoiled plasmid (ccc DNA) and an upper chromosomal band with some nicked circular plasmid DNA. The lower band was recovered with a hypodermic syringe with a 21 gauge needle by inserting the needle

just beneath the band and puncturing the top of the tube with the same size needle (for easy drainage of the band into the syringe). The DNA mix was transferred into a 20 ml siliconised universal glass tube.

EtBr and CsCl were removed in one of two ways: either extracted with isoamyl alcohol (IAA) several times until all the pink colour from the EtBr had disappeared from the aqueous phase. The DNA solution was then dialyzed against several changes of 1x TE (pH 8.0), to remove CsCl. An aliquot was diluted and the concentration was determined by spectrophotometry at 260 nm wave length.

Alternatively two volumes of dH_2O was added to the DNA band and this solution was ethanol precipitated with 6 volume of ethanol without adding any more salt. The DNA was pelleted, washed with 70% ethanol and dried. The DNA was then resuspended in 250 ul DNA resuspension buffer (TNSE buffer). The extracted DNA was extracted once with phenol/chloroform and once with chloroform. The extracted DNA was ethanol precipitated and resuspended usually in 1x TE or dH_2O. Then an aliquot was diluted and the concentration was determined by spectrophotometry at 260 nm wave length.

Cleared Lysate DNA Preparation: This method has the advantage of being a quick way of making a large amount of plasmid DNA in shorter time than alkaline-SDS extraction method. This method was extensively used for the work described in chapter 6. The procedure was as follow: A 100ml culture of the plasmid bearing *E. coli* was grown in selective media overnight at 37°C with vigorous shaking. The cells were pelleted at 10K rpm for 5 minutes at room temperature. The pellet was dislodged by vortexing and resuspended in 1.65 ml 25% sucrose and 50mM Tris-Cl pH 8.0 and kept on ice. 350 ul freshly made lysozyme (20 mg/ml in 250mM tris-cl pH 8.0) was added and mixed gently on a rotating platform, while on ice, for 15 minutes. Then 650 ul 250 mM EDTA (pH

8.0) was added to the solution and mixed for further 15 minutes as above. 2.5 ml lytic mix solution was added to lyse the cells, as well as 50 ul 10 mg/ml RNase, the content was mixed rapidly but gently after adding the lytic mix and kept on ice for 20 min. The crude lysate was cleared by centrifugation at 18K rpm (40000g) at 4°C for 40 min. This separated the cell debris as a spongy pellet from the supernatant which contained the plasmid DNA. 50 ul proteinase K (20mg/ml) was added to the supernatant and heated at 65°C for 10 minutes. The volume was adjusted to 8 ml by addition of 0.6 ml EthBr (10 mg/ml) and 1 x TE. CsCl was then added to a final density of 1.58-1.61 g/ml. The solution was centrifuged at 5K rpm for 15 min to separate the EthBr-bacterial protein complexes as a thin film on top and side of the tube. The DNA solution was then transfered into a 10 ml polyallomer Quick-Seal (Beckman) ultracentrifugation tube and treated as described in the alkaline-SDS DNA extraction method. The plasmid band was collected and purified from EthBr and CsCl in either of the methods described above.

Mini DNA preparation: This method is also known as STET DNA prep (Holmes and Quigley, 1981). This technique enables one to test several recombinant transformants in one experiment and the purified DNA produced, has the advantage of being restrictable, which means that large plasmid DNA preparations are only necessary after the initial characterization of the clones.

The following procedure was used: 1.5 ml overnight culture of plasmid-bearing *E. coli* was pelleted in a microcentrifuge for 20 sec. The pellet was resuspended in 350 ul STET buffer and 25 ul freshly made (10 mg/ml) lysozyme was mixed in. The tube was placed in boiling water for 40 seconds to lyse the cells. Then the tube was centrifuged immediately at 4°C for 15 min in a micro centrifuge (12000g). The sticky, spongy pellet which was formed was carefully removed

using a tooth pick and discarded. 40 ul 3M NaAc and 400 ul isopropanol was added to the tube, mixed and then centrifuged for 15 minutes at 4°C. The pellet was washed with 70% ethanol and dried briefly in a vacuum desiccator. The pellet was resuspended in 50 ul 1 x TE buffer. Alternatively, after removal of sticky pellet, RNase and proteinase K can be added. If desired 20 ul RNase (10mg/ml) was added and heated at 37°C for 10 min then added 20 ul proteinase K (20 mg/ml) and heated at 65°C for 10 min. The DNA solution was then Phenol extracted and then followed the steps for ethanol precipitation as above.

Single Colony Gels (SCG) DNA Preparation: This technique enables the plasmid content of an isolated colony to be observed with unparalleled ease, and it was developed, in this Institute a few years ago, by Dr. Arthur. The technique, like STET mini DNA preparations, enables one to test several recombinant transformants in one experiment. The only limitation of the technique is that the DNA can not be restricted. For making single colony gel (SCG) DNA, each single colony was patched out (1 cm^2) on a selective agar plate and grown overnight. Cells were scraped, with a toothpick, and transferred to a small microfuge tube containing 100 ul of SCG buffer. The cells were mixed well in the buffer and left at room temperature for 15 minutes to lyse. Cell debris and chromosomal DNA were separated by centrifugation for 15 minutes (12000g). If the original cell mass was too much, longer centrifugation was necessary. The supernatant containing the plasmid DNA were run on an agarose gel with some parental plasmid to identify the recombinant clones by the size shift.

Liquid Lysate Phage DNA Preparaation: Phage DNA was made to perform restriction analysis on the positive clones. Several methods are available for making phage DNA. The method described below is similar to the method described in the Molecular Cloning, a Laboratory

Manual (Maniatis *et al.*, 1982) which was modified by Dr P. Balf (University of Edinburgh, personal communication).

The host *E. coli* cell /\Hfl C600, was grown in L. Broth with vigorous shaking at 37°C to log phase. 250 ul of stationary growing cells was inoculated with phage to a multiplicity of infection (MOI) of 0.001, that is for $1.25x10^{10}$ cells $1x10^{7}$ plaque forming units (PFU) was inoculated. The mixture was incubated at 37°C for 30 min. To the tube 1 ml L-Broth/$MgSO_4$ was added and the mix was poured into a 500 ml flask containing 99 ml L-Broth. The flask was incubated at 37°C with vigourous shaking (300 rpm) for 24 hrs. The culture was spun down at 4 K rpm for 10 min. The supernatant was transfered into fresh JA-20 (Beckman) tubes and DNase and RNase (both crude) were added to the tube to final concentration of 1 ug/ml and 2 ug/ml respectively and incubated at room temp. for 1 hr. After this incubation period, NaCl was added to a final concentration of 1 M. The JA-20 tubes were spun at 19.5 K rpm for 2.5 hrs at 4°C. The glossy pellet was resuspended in 2 ml phage dilution buffer (PDB) by whirlimixing fo 1 min (if viable phage was needed, resuspen ion had to be carried out overnight with gentle rolling of the tube and whirlimixing had to be avoided).

The tube content was transfered into a 10 ml tube and SDS and proteinase K were added to the final concentration of 0.01% and 100 ug/ml respectively.. The tube was incubated at 60°C for 1 hr. To this hot solution an equal volume phenol/chloroform was added and whirlimixed for 1 min (gentle rolling if viable phage was needed). The tube was spun at 5 K rpm for 20 min. The aqueous phase was transfered to fresh tube and extracted with chloroform and treated as above. To the aqueous phase NaOAc (pH 5.2) was added to a final concentration of 0.25 M and the DNA was precipitated by ethanol. The DNA pellet was resuspended in 1xTE and was ready to be resticted.

2.2.4 Screening cDNA Library

To screen the cDNA library which had been cloned into \gt10, usually, $1X10^4$-100 PFU (plaque forming units) were added to 150 ul $MgSO_4$ treated /\HFL *E. coli* cells, prepared specially for plating phage particles. After 15-20 minutes of incubation at 37°C which the phage particles had been adsorbed to the cells, 5 ml top agar (kept at 42°C to stay warm) was added into each tube. The tube content was then immediately poured onto each agar plate. The agar plates contained no antibiotics and were dried in a 37°C room for 1 hour prior to plating. The drying usually speeded up the absorption of the liquid from the plated material. Plates were gently but quickly swirled for better spreading of the plated material. After setting of the top agar, on the plate (about 10 minutes), the plates were inverted and incubated at 37°C overnight.

The plaques were counted and recorded and then the plates were used for plaque lifting. To perform plaque lifting, plates and filters were marked for later orientation. After denaturation, neutralising and baking, the plaque lifts were hybridised with the appropriate probes. Positive plaques were identified and picked by alignment of plates, filters and autoradiograph of hybridised filters. After picking of positive plaque by an end of pasture pipette, it was transferred into a tube containing 500 ul PDB (phage dilution buffer) and 10 ul chloroform. In the primary screening usually more than one plaque (i.e., one positive and few negative) were transfered but the negative plaques were elimenated in the subsequent rounds of screenings (i.e., 2° and 3° screenings). The tube containing the phage stock was kept at 4°C.

Each plaque from an overnight plate had 10^{8-9} PFU. For secondary screening usually a titration was worked out (in PDB) from the phage stock to have 200-300 PFU for an 10 X 10 cm agar plate. Plating, plaque lifts, hybridisaton, etc. in the secondary screening were as in

the primary screening except in that the screening was done at a lower density to get individual plaques. The individual positive plaques which were picked were treated as above.

The positive plaques were screened for another round to ascertain even further that the right clone(s) had been picked. The main differences between the secondary and tertiary screenings were that in the tertiary screening the $MgSO_4$ treated cells and the top agar were not mixed with the phages, instead they were poured on to the agar plate and then, after setting, 5 ul from phage stock from secondary screening was added into each box of matrix. The matrix was either drawn on the back of the agar plate or on a piece of paper and taped, temporarily, under the plate. After absorption of phages, the plates were inverted and incubated as above. The plaque lifts, hybridisation, etc were as for primary and secondary screenings.

DNA from positive plaques were made mainly by cleared lysate phage DNA prep method. The DNA was used for restriction analysis or for further screening of cDNA library for more clones as above.

2.2.5 Growth and Storage of Bacterial Host Strains for Plating of Phage Particles

The following method is an adaptation of a method described by Maniatis *et al.* (1982). To ensure optimal absorption of phage lambda to the bacteria, the bacterial strain *E. coli* are grown in the presence of 0.4% maltose but in the absence of glucose. Maltose induces phage receptors on the *E. coli* surface, by inducing maltose operon on *E. coli*, which contains the gene (lamb) coding for the lambda receptor. The *E. coli* are resuspended in 10mM $MgSO_4$ to enhance the interaction of the phage tail and the Lamb receptor of the bacteria. To do this an overnight culture of a single colony of *E. coli* was grown in LB broth containing 0.4% maltose.

100ul of overnight culture was then added to 20ml LB broth containing 0.4% maltose and grown at 37°C with shaking for about 2hrs (OD_{600}=0.6).

The cells were centrifuged at low speed (4000g) for 10 min. The cells were then resuspended in 5ml of sterile 10mM $MgSO_4$. Usually 150ml of indicator cells were used per plate. The remaining cells were stored at 4°C and were stable for 2 weeks.

2.2.6 Transfer of DNA on to Nylon Membrane and DNA Hybridisation

Colony and Plaque Lifts: Plaque lifts are the ideal way of screening cDNA libraries, and colony lifts are used mainly where single colonies could be patched out on a matrix plate. For this purpose single colony of *E. coli* were patched out (1 cm^2) on a selective agar plate. On the other hand plaque forming phages after being absorbed by the /\Hfl *E. coli* cells, were grown on L-agar media plate. Both of these plates, for lifting colonies or plaques were grown overnight. For plaque or colony lifts the right nylon membrane (filter) size (e.g. for 10x10 cm plate a 9.5x9.5 cm filter was used) was selected and labelled. The filter was placed on agar surface and the plate and the membrane were marked for later orientation of colonies or plaques. The membrane was carefully lifted after one minute and placed colony/plaque side up, on denaturing solution for 5 minutes. The membrane was then transferred on to neutralizing solution for 5 minutes. Then the membrane was air dried on Whatman 3MM paper for 30 minutes. The membrane was then baked at 80°C for 1 hr and hybridised with the appropriate probe.

Southern Transfer of DNA: Hybridisation of labelled DNA to filter bound plasmid born DNA followed the procedure of Southern (1975) as modified by Reed and Man (1985).

Horizontal agarose gels were run with the required samples. The gel was photographed after ethidium bromide staining. The gel was soaked in denaturing solution for 30 min, and then neutralised for 30 min. in neutralising solution 1. The gel was then placed on a glass plate covered by 2 layers of 3MM paper which overhung the edges and dipped into a tray containing transfer buffer (20x SSC). Pall Biodyne Nylon membrane (filter) cut to the size of the gel. The filter was soaked in 20x SSC and placed on top of the gel ensuring that no air bubbles were trapped underneath the gel or the filter by rolling a glass pipette over it. The edges of the filter (0.5-1 cm) were shrouded with clear tape to prevent transfer of buffer other than through the filter. The filter was covered with 2 layers of 3MM paper then by stack of paper towels or nappies to about 5-8 cm height. The stack was topped with a glass plate weighed down with a 500 ml bottle. Transfer was allowed to proceed overnight. The filter was let to dry at room temprature and then baked at 80^{o}C for 2 hrs. The baked filter was either kept in a sealed plastic bag for later use or pre-hybridised the same day.

DNA Hybridisation: The DNA bounded filters from Southern transfers or colony/plaque lifts were hybridised as follows. The filter(s) was first prehybridised and then hybridised with the probe. Prehybridisation was carried out using prehybridisation solution containing 100 ug/ml heat denatured (boiled for 10 min then on ice for 5 min) non-homologous (salmon or herring sperm) DNA. For every 4 cm^{2} filter 1 ml solution was added to a bag containing the filter(s). The filter was sealed after removal of all trapped air bubbles. The bag was placed in a box and incubated in a water bath with gentle agitation usually set at 60-65^{o}C for 1-2 hrs.

Hybridisation was carried out at the same conditions and in the same solution as in prehybridisation. After the end of the pre hybridisation a corner of the bag was cut and the random primed

radiolabelled probe was added to the bag. Usually $1x10^6$ cpm probe was added for each 10x10 cm filter. The bag was then sealed after removal of trapped air bubbles. The solution was mixed by agitation prior to incubation. The bag was incubated as in pre hybridisation with gentle agitation overnight.

The filter was washed once in low stringent wash buffer at room temperature for 15 min then washed in the same stringency at the hybridisation temperature 3x15 min. If at the end of the last wash the signal was high (i.e. >20 cpm) the filter was washed under more stringent conditions. It was washed in either higher temperature or in high stringent wash buffer or combination of temperature and more stringent wash buffer until the signal from the filter was less than 10 cpm, measured by a Geiger-Mueller counter. The filter was sealed wet in a plastic bag and autoradiographed for a variable length of time depending on the number of counts retained on the filter. The X-Omat X-ray film was then devaloped in the X-Omat machine (Kodak).

Re-Hybridisation: The filters which had already been hybridised once or more can be rehybridised again by first removing the old probe. The filters, for this purpose, were washed 2x with 200-300 ml probe removal buffer I and incubated at 45^oC for 15 min each time. Then the filters were washed 2x with probe removal buffer II at 45^oC for 15 min. The filters were then ready for hybridisation as above.

In Situ Hybridisation of Bacterial Colonies: A plasmid base cDNA library can be screened by in situ hybridisation. The cDNA clones are, in this case, transformed in bacterial cells and the bacterial colonies are hybridised with a specific probe. Colony hybridisation is accomplished by transferring bacteria from a master plate to a membrane (e.g. nylon filter). The colonies on the filter are let to grow for few hours

then they are lysed and the liberated DNA is fixed to the filter by baking. After hybridisation to a ^{32}P-labeled probe, the filter is monitored by autoradiography. A colony whose DNA gives a positive signal may then be recovered from the master plate and analysed further (Grunstein and Hogness 1975; Maniatis, Fritsch and Sambrook 1982).

The procedure outlined below is used when large number of colonies are screened (i.e. plasmid library screening). In this case bacteria are plated directly from a transformation mixture on to nylon filters; replica filters are prepared, after initial bacterial growth, by filter to filter contact. The plasmids need to have an antibiotic marker because most of the transfers can not be done under sterile conditions, it was however necessary to keep everything as sterile as possible. This procedure was adapted for plasmids bearing antibiotic marker (i.e. Tet. and Amp.).

A 9.5 X 9.5 cm nylon filter was marked and places marked side down on a day-old agar plate containing appropriate antibiotic. The filter was peeled off and inverted then replaced it on the same plate, marked side **up**. A small volume of the bacterial stock (transformed plasmid library) containing 10^4 bacteria was plated on the filter and spread with a strile glass rod, leaving 2-3 mm of the edge free from bacteria. When all the liquid was adsorbed the plate was inverted and incubated at 37^oC until very small colonies (0.1 mm in diameter) appeared (about 8-10 hours). When colonies reached the right size three replica filters were made one at a time. A marked filter (marking corresponding to those on the master filter), was wetted by touching it to the surface of an agar plate containing the appropriate antibiotic. Meanwhile the master filter was removed (with a blunt-ended forceps) from the plate and placed it on a stack of 3 MM paper, colony side up. The wetted filter for replica filter was removed and placed carefully (marked side **down**) on top of the master filter, being careful not to move the filters at this

stage. Once contact was made the two filters were pressed together evenly but gently, with a soft sponge. A characteristic pattern was made by keying holes in the filters by a needle (for later orientation) while they were sandwiched together. The filters were gently peeled apart and the replica filter was returned to its plate, colony side up.

Up to 3 replicas were usually made as above, each with its own characteristic holes. The plates (i.e. master and replicas) were incubated as above. The master plate was incubated for 6-8 hours but replica plates were incubated 8-10 hours (if colonies were ovecrowded) or overnight (if colonies were not so dense that over growth would be a problem). the master plate was sealed and kept at 4^{o}C until the result of hybridisation was available.

To liberate the DNA the cells had to be lysed and then the DNA be bound to the filter permanently. To do this three pieces of 3 MM paper were cut to 20 X 20 cm in size and placed three of the filters on the bottom of three different trays. The first filter was saturated with 10% SDS, the second one with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and the third one with netralising solution (1.5 M NaCl, 0.5 M Tris:Cl [pH 8.0]).

Each filter (colony side up) was placed on the SDS try for 3 minutes, on the denaturing tray for 5 minutes and on the neutralising tray for 5 minutes. The filters were then layed on a sheet of dry 3 MM paper and allowed to air dry for 1 hour. The filters were then baked at 80^{o}C for 2 hours and then hybridised as in colony hybridisation with ^{32}P-labeled probe (random primed or PCR labelled).

2.2.7 Radio-Labelling of DNA Probes by Random-Primed Synthesis

Random prime (RP) DNA labelling gives high specific activity probe even with unpurified DNA (Feinberg and Vogelstein 1983). There are several advantages in using this method of labelling over nick translation. One advantage is that as little as 10 ng DNA can be used. The second advantage is that high specific activity is achieved by this very little amount of DNA. This high specific activity is suficient for hybridisation of up to ten 10x10 cm filters. The other advantage is that unpurified DNA (i.e. DNA recovered from LMP agarose) can efficiently be labelled almost as well as purified DNA.

Usually 50ng DNA, after denaturation by boiling for 5 min. and rapid cooling on ice, (DNA from LMP was treated as explained below), was mixed in with 10 ul reaction mix-dATP (e.g. if the radiolabel for example was ^{32}P dATP then reaction mix was -dATP), 2 ul BSA, nuclease free, 1 ul (5 units) Klenow fragments of DNA polymerase I, and 3 ul (30 uCi) ^{32}P dATP and the total volume was adjusted to 50 ul dH2O. The tube was left at room temperature overnight and the reaction was stopped by addition of 2 ul 0.5 mEDTA. It was found however that maximum reaction was achieved by incubation at 37^{o}C overnight.

The efficiency of radiolabelling could be checked by addition of 1 ul of R.P. mix, before addition to the column, on to 2 cm filter paper discs (Whatman) and treat the discs by TCA. The TCA precipitation can also be applied to radilabelled oligonuleotides as well. In this case oligonucleotides of 20bp length or more remain bounded to the disc but unincorporated nucleotides would be washed away.

One ul of the labelled DNA before separation through Sephadex column was added on to two Whatman 2 cm

filter paper discs. The discs were allowed to dry at room temprature. Only one of the discs was submerged in 10% of trichloroacetic acid (TCA) on ice for 30 minutes then washed in methanol and allowed to air dry. Both discs, TCA treated and untreated, were counted in scintillation counter. The difference in the two values was interpreted as the percentage of incorporation (i.e. TCA treated discs was showing incorporation and untreated one was showing total counts of unincorporated and incorporated label). Random prime usually gave up to 70% incorporation of ^{32}P-dATP.

2.2.8 Chromatography on Sephadex G50-150 Columns

Sephadex columns were used to separate incorporated radiolabelled nucleotides from unincorporated nucleotides in random prime radiolabelling mix. Due to the mechanism of sephadex beads, smaller molecules lag behind the larger ones and one can collect fractions in separate tubes and count them to see which one has the incorporated DNA. Addition of column dye (phenol red and dextran blue) can help in collection of fractions without use of any monitor. The DNA and free nucleotides can also be monitored be a Geiger-Mueller counter; (usually column dye was used in this work). The incorporated DNA comigrates with dextran blue (the blueish colour) and free nucleotides comigrates with phenol red (the pinky colour).

To prepare the column equal amounts of Sephadex G50, G150 were soaked in 1 X TE (pH 8.0) overnight at room temperature. The borosilicate glass column was plugged with glass wool and packed with Sephadex G50-150 beads. The column was washed few times with Sephadex column buffer. The column was also washed several times after each use to free it from an unincorporated nucleotides and after few chromatography the sephadex beads were discarded and the column was packed with fresh Sephadex G50-150 beads.

The incorporated probes were separated from unincorporated nucleotides by passing the mix through a Sephadex 9-50-150 column after addition of 10 ul dye to ther tube. The incorporated nucleotides travel faster than unincorporated nucleotides. To collect the incorporated nucleotides 4 drops fractions were collected in each tube and the tubes were counted by Cerenkov counting the tubes containing the incorporated DNA (i.e. 1st peak) were pooled and 1 ul was spotted on a Whatman paper filter disc and counted on scintillation counter to measure specific activity.

2.2.9 Restriction of DNA

For several purposed DNA from either lambda or plasmids had to be digested with various restriction enzymes. Restriction of DNA was performed in a total volume ranging from 10 ul to 100 ul depending on the amount of DNA being restricted and the type of experiments for which the restricted DNA was needed for. As a rough guide 0.5 to 2 ug of DNA for Southern blotting and 2-5 ug for insert isolation and purification was restricted in one tube. Usually 5 units of restriction enzymes for 1 ug of DNA was used. Appropriate restriction buffers was added to the DNA reaction mix and the volume was made up with dH20. The tubes were incubated at the appropriate temperature recommended for that particular enzyme for 1 to 3 hours after which time digestion was usually complete. If the DNA needed to be cut with more than one enzyme with different restriction buffer requirements, then usually all enzymes were added together and the restriction buffer which had lowest salt concentration was added first and incubated for one hr. After one hour of incubation, the salt(s) concentration was adjusted to suit the other enzyme(s). If more than one enzyme was used and they had different temperature requirement, again all enzymes were added together, the reaction was allowed to be carried at lower temperature for 1 hour then, after adjusting the salt concentration, if

needed,the temperature was raised as required for other enzyme(s). If neither of these two could be done or the one enzyme had any inhibitory effect on the restriction of the other enzyme then the following treatment was carried out. The DNA after being digested with first enzyme was extracted with phenol/chloroform and ethanol precipitated and resuspended in 1 X TE for the digestion to be carried out with other enzyme. For example in an experiment which pFH1 DNA was restricted with 4 different enzymes (HaeIII, BstEII, EcoR1, BamH1 with salt condition of Reacts 2,2,2,4 and Temp 37^{o}, 60^{o}, 37^{o}, 37^{o}C respectively), all 4 enzymes were added together and were cut in React 4 for 1 hr then 30 mM Tris-Cl (pH 8.0) and 5 mM $MgCl_2$ was added to make it as REact 2 and incubated for another hour. The tube was then transferred to 60^{o} for BstEII to be fully functional. Restriction was arrested by either the addition of FSB loading buffer or freezing at -20^{o}C.

2.2.10 Ligation

T4 DNA ligase catalyzes the formation of phosphodiester bound between 5' phosphate and 3' hydroxyl termini in duplex DNA. This enzyme will join both blunt-ended and cohesive ended restriction fragments of duplex DNA. Ligations of both sorts (i.e. blunt ended and cohesive ended DNA) were carried out in this work. The plasmid or bacteriophage DNA was first digested with the desired restriction enzyme. The digested DNA was mixed with compatible oligonucleotides or restriction fragments of the target DNA. The concentration of fragment (insert or adaptor) to vector was adjusted to approximately 2 to 1 for cohesive ends, 5-10 to 1 for blunt ended, and 100 to 1 for adaptor ligation. The cohesive ended ligation was incubated at 12^{o}C; and the blunt ended ligation was incubated at 16^{o}C and ligation was allowed to proceed for 12-16 hrs. A typical of 20 ul reaction mix comprised between 50-500 ng DNA (vector and insert), 2 ul 10X ligation buffer II (for DNA ligations carried out in Chapter 4) or 2 ul

10X ligation buffer I, 2 ul 10X Low salt (for DNA ligations carried out in Chapter 5) and 0.5- 1 unit of T4 DNA ligase, the volume was made up in dH_2O. The incubation temperature was as indicated above.

2.2.11 Phosphatase Treatment

In most cloning experiments (i.e. single cut or non-forced ligation) self ligation of cloning vector molecules reduces the efficiency of cloning dramatically. To avoid this kind of problem the terminal 5' phosphates on linearised vector can be removed prior to ligating to the target DNA to minimise self ligation of vector DNA. This can be done by removing the terminal 5' phosphate by calf intestinal alkaline phosphatase (cip) treatment.

The restricted vector DNA was dephophorylated by adding 0.01 unit CIP enzyme and 1/10 vol CIP buffer. The DNA mix was incubated for 15 min at 37^{o}C. The enzyme was usually heat inactivated at 68^{o}C for 10 min; and then extracted the DNA with phenol/chloroform followed by ethanol precipitation. The DNA was resuspended in 1x TE buffer and then an aliquot was used for ligation.

2.2.12 Phosphorylation and/or End labelling of DNA

Dephosphorylated DNA or unphosphorylated oligonucleotides can be treated with either ^{32}P-gamma-ATP or cold ATP. The gamma labelled DNA could be used either as a tracer in the ligation reaction or can be used as a probe. In this work the primary use of either labelled ATP or cold ATP was to phosphorelate the adaptors for ligation purposes (Chapter 5). The end labelled oligoucleotides were used as probes for hybridisation experiments (Chapter 7).

The DNA was usually end labelled by adding 2-5 ug DNA in a microfuge tube with 1/10 vol 10x end labelling

(kinasing) buffer, 30 uCi gamma-^{32}P-ATP and 10 unit of T4 kinase enzyme The volume was usually made to 20 ul with dH_2O. For end labelling oligos for adaptors the reaction was chased by addition of 1 ul 10 mM ATP and For labelling oligos for probe more label was used but was not chased with cold ATP. The intensity of labelling and size of oligos was checked by running 1 ul of end labelled oligo on a 12-20% polyacrylamide gel. The end labelled reactions were kept at -20^{o}C.

2.2.13 Phenol extraction

Phenol extraction was used to remove proteins contaminations from DNA. Most of the time phenol was used in conjunction with chloroform because of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Phenol was equilibrated with Tris.Cl (pH 8.0) containing 0.1% hydroxyquinidine and 0.2% B-mercaptoethanol. Chloroform was a mixture of chloroform and isoamyl alcohol (24:1 v/v). The DNA mixture was mixed with equal volume of phenol/chloroform (1/1 v/v) and vortexed. The tube was centrifuged briefly and the aqueous layer was transferred to a fresh tube and either extracted again with phenol/chloroform or chloroform alone and treated as above. After extractions the DNA was ethanol precipitated.

2.2.14 Extraction of DNA From Agarose Gels

Recovery of DNA from Low Melting Point Agarose: The main purpose of this technique was to prepare digested fragments of DNA for random prime radio-labelling. For this purpose the DNA after being restricted with appropriate restriction enzyme was electrophoresed on LMP agarose gel containing 5 ul EthBr per 100 ml agarose, at 4^{o}C (the percentage of agarose gel was determined by the size of the DNA fragment to be recovered but it was kept to a minimum possible

recovered but it was kept to a minimum possible percent). When the electrophoresis was complete then the DNA fragment was visualized under long wave UV light and the DNA fragment was excised with a scalpel. Care was taken to minimize the amount of extraneous agarose. Agarose piece containing DNA was transferred to a pre-weighed microfuge tube and weighed again to measure the amount of agarose. Usually 2-3 ml dH2O per each gram of agarose was added to the tube (amount of dH2O should be more for larger size or higher % agarose) and placed the tube in a boiling water for 10 minutes. The tube was transferred to 37^{o}C and incubated for at least 10 minutes before transferring the DNA to the random prime mix for radiolabelling. This boiling-cooling step was repeated each time the DNA was used for random priming.

Gene Clean

The recovery of nucleic acids after electrophoretic separation is an essential part of the technology required for nucleic acid manipulations. Gene Clean is a very effective method of cleaning and purifying DNA, from salts, protein, agarose, etc. The method is efficient for DNA of small quantity and sizes. The basic principal is that DNA will bind to glass beads but not to the salt, proteins and agarose. After binding the DNA the impurities are washed out, then DNA is recovered from glass beads in 1x TE.

The DNA was restricted with the enzyme and after staining, the gel was placed on a long wave transilluminator (300 nm-360 nm) and the band of interest was excised. The agarose chip was added into a microfuge tube. Then 2-3 volumes (usually 800 ul) of "NaI" solution was added to the tube.. The tube was then heated to 45-55^{o}C for 5-10 min or until the agarose had completely dissolved. The tube was vortexed and 5 ul "Glassmilk" suspension was then added, rapidly mixed and then placed on ice for 5 min. The tube was spun for 5 seconds and the supernatant was carefully discarded. The

pellet was washed three times in ice-cold "New" solution by adding about 700 ul. The pellet was then resuspended by vortexing and spun for 5 sec and discarded the supernatant. After final wash almost all the "New" solution was removed. The DNA was eluted from the glass beads by adding 25 ul 1xTE (PH 8.0), and incubating at 50°C for 5 min. The tube was spun for 10 secs to pellet glass beads. The supernatant was carefully removed avoiding any contamination with glass beads. The tube was however spun for 20 sec to remove any trace of glass beads which had been carried over. The DNA was used after this step for either restriction, ligation or lablling. The composition of the "NaI","Glassmilk" and "New" solution are not disclosed.

Isolation of DNA Fragments from Gels using NAE-DEAE Membrane: DEAE cellulose in membrane from Schleicher & Schuell NA-45 provides a convenient medium for simple and rapid techniques for the recovery of DNA from gels. The purity of the recovered material is adequate for virtually any subsequent biochemical manipulation, including restriction digest, ligation, labeling, etc. The technique outlined below has been adapted from Young *et al.* (1985). The principle behind this method is that DNA is recovered from agarose gels, after their electrophoretic separation, by placing a strip of S&S NA-45 DEAE membrane in an incision just in front of the band of interest. Electrophoresis is continued until binding is complete as observed by ethidium bromide fluorescence. The strip is then washed of residual agarose, immediately eluted or stored at 4°C (the strip must not be allowed to dry since this leads to irreversible binding.

To recover the restricted DNA, it was run on agarose gel alongside with a small amount of DNA from the same sample as marker. The DNA was electrophoresed until fragments separated from each other then a small strip of S&S NA-45 DEAE membrane was placed in an incision just behind the fragment of interest. The

membrane was lowered as much as possible because DNA usually travels at the bottom of the gel. The electrophoresis was continued until the same size marker band in the adjacent track had passed the membrane mark.

The membrane was removed and binding of DNA to the membrane was checked by observing under UV light (for ethidium bromide to fluoresce). The membrane was washed briefly with 1 X TE and transfer into a tube containing 400 ul 1 M NaCl and 0.05 M argenin. The tube was heated at 70^{o}C for 2 hours and then the membrane was removed and stained with ethidium bromide to check if the DNA had been released. The DNA was ethanol precipitated and resuspended in either TE or dH_2O for subsequent use.

Isolation of DNA from Gel Slices by Electro-elution: DNA fragment, after electrophoresis, was isolated from the gel as a slice of gel. The gel slice was sealed into dialysis tubing with a minimal amount of 1 X TBE and a voltage gradient of 100 volts was applied. After approximately 1 hour the current was reversed for 1-5 minutes, depending on the size of the tubing and gel slice, to dislodge the DNA from the wall of the dialysis tubing. The gel slice was removed and the DNA was washed over into a microcentrifuge tube. The DNA solution was either extracted with phenol/chloroform and ethanol precipitated or dialysed in 1XTE for 1 hour and then passed through siliconised glass wool to remove any agarose residue.

Recovery of DNA by Centricon Column: Centricon column (Amicom UK) was used to separate small size oligonucleotides from larger size fragments. The column is said to retain larger fragments but can not keep smaller size adaptors or linkers. The filter in the column was blocked with denatured salmon sperm DNA, making sure that all of free DNA had been washed away (by doing OD to the washed material).

The DNA to be separated was loaded on to the column in 2 ml volume made in 1 X TE. The column was spun down for 30 minutes at 5K rpm. The volume was readjusted to 2 ml again and spun as above for 1 hour then repeated this step again every time discarding the spun liquid (containing free oligonucleotides. Finally the remaining liquid was spun into the lid and the DNA was recovered by ethanol precipitation.

2.2.15 Staining Tissue Sections

Preparing Placental Sections for Staining: Fresh human placentae were obtained from Queen Mother's Hospital, Glasgow. The placentae were washed with dH_2O and the amnion was peeled off and 1x1 cm pyramid like sections were cut deep in three areas (chorionic plate (CP), Nitabuch's layer or uteroplacental interface (N) by a sharp scalpel. The sections from feto-maternal margin or chorionic plate/Nitabuch's layer margin (CPN) were obtained by cutting the feto-maternal margin (CPN) as such that the section would contain chorionic plate, Nitabuch's layer and the tissue in between. The cut sections were washed in TBS and then snap frozen by dipping into a labelled tube containing liquid nitrogen. The sections were either immediately transferred into a $-70^{o}C$ freezer, or were taken (still in liquid nitrogen) to the Dept. of Pathology, Glasgow University, to be sectioned. The cryostat sections were air dried and then fixed in acetone for 10 min and then every 24 slides were kept in a sealed bag at $-20^{o}C$ for future use.

The sections were brought to room temperature whenever needed, while still in the sealed bag to avoid condensation. After the equilibration of temp. the bags were cut open and the sections were treated for staining.

Immunoperoxidase staining: Most immunoperoxidase stainings were done on cryostat sections of human placenta. Placental sections on labelled slides were

washed gently with TBS with a wash bottle and were let stand for 1 min at room temperature. The sections were treated with periodic acid for 10 min. by adding 100-200 ul 0.1% periodic acid on each slide. Sctions were then washed with TBS for 1 minnute. The excess TBS was wiped off and 100-200 ul 5% BSA was added to each section and were let to stand for 1 min. The BSA was however left on the control slides until the time for addition of Ab. Excess BSA was drained and the periphery of the sections were wiped off with no further washing to follow. 100 ul diluted 1st Ab (in TBS) was added onto each section and then let to incubate, inside a humid chamber, at room temperature for 1 hour. The antibody was washed 2x1 min with TBS. The excess TBS was wiped off, then 100 ul diluted peroxidase conjugated antibody (in TBS) was added on to each section and incubated as above for 45 minutes. The slides were then washed 3x1 min in TBS. The slides were then dipped into a dish filled with diaminobenzidine (DAB) for 5 to 15 minutes depending to the freshness of DAB and the thickness of the section. The slides were washed under running water and the sections were observed under microscope. If staining was not complete the DAB incubation was continued for longer. After DAB treatment the slides were dipped, a few time, into Haematoxylin dish and then washed for 5 minutes under running water. The slides were then dipped few times into acid alcohol followed by washing as above. The slides were then dipped into Scots tap water a few times, washed as above. The slides were then dipped in methanol few times then in 70% ethanol and after that in absolute ethanol to dehydrate the tissues. After dehydration the slides few at a time were dipped into xylin and after evaporation of xylin a drop of DPX mountant was added to each section then covered with a clean coverslip.

2.2.16 Photographying Immuno-Stained Tissue Sections

The pictures from sections were taken under tungsten light built in the microscope, using Leitz

Universal microscope fitted with an automatic camera system using Kodak colour films (200-400 ASA). The films were developed and printed by the Glasgow University Photography Unit.

2.2.17 DNA Electrophoresis Through Gels

Two gel matrices were used in this work, for DNA of 1Kb and >1Kb 0.8% agarose gels, for DNA of 0.5Kb to 1Kb 1% agarose gels and for DNA of 0.1Kb to 0.5Kb 1.2-2% agarose gels were used. For DNA of very small sizes (i.e. oligonucleotides 8-60 bases) and also DNA sequencing reaction, polyacrylamide gels of 20%, 12% and 8% were used respectively. The agarose gels were of different sizes of horizontal perspex gel former of 5X7.5 cm (baby gel), 7.5X11 cm (mini gel), 11X16.5 cm and 16.5X22 cm (large gels).

The polyacrylamide gels were 2 different sizes of vertical gel kits (15.5X18 cm and 23X42 cm for oligonucleotides and 23X42 cm for sequencing reaction).

Ordinary Agarose Gels (in TBE): The buffer mostly used for making agarose gels was 1 X TBE unless otherwise specified. Agarose powder (gelling temperature 36-42^{o}C) was added to a measured volume of electrophoresis buffer. The powder was heated to boil in a microwave oven until it was completely dissolved. The solution was cooled in a 50^{o}C water bath then 5ul of stock solution of ethidium bromide (10mg/ml) was added to every 100ml of agarose solution. The ends of the gel former were sealed by teflon end sealer or autoclave tape and sealed completely by a few drops of agarose. The gel former was placed on a flat surface and a teflon well former (comb) was placed on its position. Combs of different teeth sizes were used depending to the need. When placing the comb usually 1-1.5 mm of space was allowed between the bottom of the teeth and the base of the gel. Malton agarose with EtBr solution was poured and allowed to solidify completely at room temperature.

Low melting point (LMP) agarose was, however, poured in a 4°C room. After the gel was set, enough buffer was added to cover the gel to a depth of 1-5mm, then the comb was carefully removed as well as the sealer ends or autoclave tapes. To the DNA sample $^1/_4$ vol of FSB loading buffer was added and then loaded into the wells. DNA size marker(s) was/were also loaded for estimation of fragments size. Gels were electrophoresed for different lengths of the time or current strength depending on the size of the DNA and the amount of separation desired but usually it was electrophoresed at about 5V/cm of agarose. DNA mobility was usually monitored by a hand held UV transilluminator (260nm) while the gel was still in the tank.

After the electrophoresis was completed, gel was photographed with 260nm UV transillumination using a Polaroid camera loaded with Polaroid 4X5 Land Film (57 or 667, ASA 3000). For more permanent pictures the gel was photographed with a Pentax 35mm SLR camera loaded with Ilford HP5 black and white film; in either case using Kodak Wratten Filter 9 (red).

The interpretation of restricted and untreated DNA were based on Dugaiczyk *et al.* (1975) Birnboim and Doly (1979) and Maniatis *et al.* (1982). Restricted DNA migrate according to their sizes and the bigger the size the slower it migrates. Untreated DNA have several forms; supercoiled (SC) plasmid DNA migrates fastest, then is the open circular plasmid (OC) and often comigrates with supercoiled plasmid dimeric DNA (2^d SC). Linear plasmids run between the SC and OC plasmids. The chromosomal DNA (if present) run slowest. Restricted DNA run as linear DNA and its size is estimated by comparing with the mobility of size marker DNA fragments.

The distance migrated is related to the size of a molecule of given conformation; small molecules migrate the furthest. Restricted fragments were so interpreted. In few cases (see Chapter 4 for detailes) the mobilities of the bands were measured by making a size calibration

curve. This was done by measuring DNA size using origin of each track at the top of the gel and the known size standards on the same gel and plotting the values on a log paper. The vertical axis was representing fragment size and the horizontal axis was representing fragment distance to the top of the gel. By joining the points together a graph was produced which was used to measure the size of the unknown fragments.

Low Melting Point Agarose (LMP) Gel: LMP agarose gels do behave almost as general purpose agarose gel but because of its low melting temperature it is mainly used for recovery of DNA for random prime radio labelling of DNA. The LMP gels were mainly made in mini gels (7.5X11 cm) and the percentage of gel, amount of EtBr and DNA mix were as in ordinary agarose gels. LMP gels were usually poured at $4^{o}C$ and run in a $4^{o}C$ room as well. At the end of the run gel was let to cool to become firmer and then after taking photograph, the appropriate fragment was cut with minimal amount af agarose and put in a pre-weighted tube.

Low-LEO Agarose (in TBE) for Recovery of DNA: Low-LEO agarose (Sigma) also behaves as general purpose agarose. Its advantage is that its impurities is much less and is ideal for recovery of DNA. Low-LEO agarose was used for recovery of DNA on NEA-DEAE membrane and by Gene Cleaning.

Low-LEO Agarose Gels (in TAE): Due to the inhibitory effect of borate in TBE in the purification of DNA by Gene Cleaning method, usually Low-LEO agarose was made in 1XTAE buffer. The TAE gels otherwise behaves as general purpose agarose.

Polyacrylamide Gels: Two different types of polyacrylamide gels were used, denaturing and nondenaturing. The nondenaturing gels did not have any urea and were electrophoresed at lower watts (45-50 watts versus 60-70 watts for denaturing gels).

Polyacryamide gels were cast in a two different size apparatus (15.5X18 cm or 23X42 cm and 1.5 mm thick). Polyacrylamide concentrations varied from 8% to 20% (W/V) depending on the size of the fragments to be resolved. The 8% gel was however used mainly for sequencing reactions. Gels of appropriate concentrations were made from a 40% stock solution of polyacrylamide (acrylamide:bisacrylamide 19:1, W/W) and were polymerized by the addition of TEMED and ammonium persulphate. Urea was only added to the denaturing gel mix before addition of polymerizers. The concentration of each component was as in **Table 2.10.**

The glass plates for casts were usually cleaned using 20% SDS as detergent. After rinsing the plates thoroughly under tap water they were wiped with lint free tissue and ethanol. One of the plates were usually trated with replecoat for easier separation of gel after electrophoresis.

Dried plates were sealed together by water resistant tape, after placing two 1.5 mm spacer between the two plates on either sides and a narrow strip of 3MM paper at the bottom of the plates. The narrow strip of 3MM paper was placed to slow down any possible polyacrylamide leakage during polymerization as well as retaining most of the free gamma-^{32}P-ATP for easier discarding. One of the plates were usually trated with repl coat for easier separation of gel after electrophoresis.

The gel mix after addition of ammonium persulphate and TEMED was poured between the two plates by a 50 ml syringe without any needle. Usually very clean glass plates did not produce much air bubbles. The air bubbles were removed by tapping the plates. The comb (shark tooth combs for sequencing and conventional combs for other uses) was placed on top of the plates and the cast was laid flat at 10 degree wrapped by cling film to stop excess drainage of polyacrylamide. After the gel was set the tape from the bottom of the cast was removed

and was pre-electrophoresed in 1X TBE for 30 minutes to 1 hour at 60-70 watts (denaturing gels only) after removal of the comb.

For electrophoresing the DNA, the DNA sample was heat denatured (only sample which needed to be denatured such as sequencing material and PCR product). The DNA samples were mixed with $^1/_4$ volume of sequencing formamide loading buffer prior to loading the samples. For sequencing sample usually four sample were loaded at a time and the electrophoresed for a short time until samples had entered the gel then another four samples were loaded and so on until all samples had been loaded. PCR and oligonucleotide samples were all loaded together and then electrophresed until the front dye had travelled $^1/_4$ to 5 cm to the bottom for PCR/oligonucleotide or sequencing gel respectively.

Ingredients	Denaturing			Nondenaturing
	8%	12%	20%	20%
40% polyacrylamide	20 ml	30 ml	50 ml	50 ml
Ultra pure urea	42 ml	42 ml	42 ml	--
10X TBE buffer	10 ml	10 ml	10 ml	10 ml
dH_2O	35 ml	25 ml	7 ml	39 ml

Table 2.10 Concentraction of Different Ingredients for Making Poyacrylamide Gels. Urea was allowed to dissolve in the above mix by warming at 37^o and the volume was made to 99 ml. The mix was filtered through 0.45 um filter and then, just before pouring, 1 ml 10% ammonium persulphate and 30-50 ul TEMED was added.

The gel was separated from one of the glass support and was fixed by treating with 5% methanol/ 5% acetic acid (vol/vol) for 15 min. The gel was transferred on to 3 layers of 3MM Whatman paper and after separating the other side from glass plate it was covered with cling film. The gel was then dried under vacuum for at least 1.5 hrs (higher percentage gels usually took longer to dry). The dried gel was set for autoradiography with or without intensifying screen at room temperature or -70°C (sequencing and PCR/oligonucleotide gels respectively). Type of the film and developing was as described in DNA hybridisation.

2.2.18 Preparation of Competent JM 101

JM 101 *E. coli* cells were grown overnight with vigorous shacking at 37°C. A tube containing 40 ml of 2XTY broth was innoculated with 0.5 ml of the overnight culture. The cells were grown as above for approximately 2 hours. The cell culture was then transferred on ice and after cooling it was spun down in JA-20 (Beckman) rotor at 7 Krpm at 4°C for 2 minutes. The cell pellet was resuspended in 20 ml ice cold 50 mM $CaCl_2$ and stored on ice for 20 minutes. The cells were respun as above and the cell pellet was resuspended in 4 ml ice cold 50 mM $CaCl_2$ and left on ice for 5 min. For every transformation of JM 101 with M13 DNA fresh competent cells were used.

2.2.19 Transformation of JM 101 Competent Cells with M13 DNA

Parental or recombinant M13 DNA were transformed into JM 101 competent cells mainly for obtaining single stranded M13 DNA for sequencing. 50-200 ng of M13 DNA in 10 ul was mixed with a 300 ul aliquot of competent JM 101 cells in a tube and the tube was stored on ice for at least 40 minutes. The cells were heat-shocked at 42°C for 2 minutes. 200 ul of a fresh culture of JM 101 (on a

same growth stage as competent cells) and 3 ml of soft agar which was maintained at 42°C containing X-gal and IPTG (20 ug/ml each), were mixed together. The mixture was plated onto minimal medium plate contaning vitamin B1 (20 ug/ml) and incubated overnight at 37°C.

Clear plaques were picked for recombinant clones and blue plaques for M13 parental clones. Each plaque was transferred into a tube containing 500 ul 2XYT media as a M12 stock sorce. An aliquot from M13 phage stock was transferred into starter culture of JM 101 for mini-preparation of double stranded/replicating form (ds) or single stranded (ss) DNA.

2.2.20 Single-Stranded M13 DNA Preparations

The following method for single stranded M13 DNA preparation is a modification of the method described in BRL Sequencing Manual (1987) which yiedls more DNA.

In 20 ml 2XYT media 200 ul overnight culture of E.coli JM 101 was mixed with 100 ul of M13 phage stock and grown for 6-7 hours at 37°C with vigorous shaking. The cells were then spun down at 10 Krpm (15000 g) for 10 minutes at 4°C. The pellet was usually used to make double stranded M13 DNA by mini-DNA preparation (see mini DNA preparation) to allow the identification of recombinant clones by restriction analysis.

The supernatant containing M13 virus particles was transferred to a fresh tube and 4 ml 20% polyethylene glycol (PEG 600)/ 2.5 M NaCl was added to the tube. The mixture was stored at room temperature for 15 minutes and virus particles were sedimented by centrifugation for 10 minutes at 4°C at 15 Krpm. The pellet was resuspended in 1 ml 1X TE and 200 ul PEG/NaCl (20%/2.5 M) was added to the tube and left to stand at room temperature for 10 minutes. The mixture was spun for 10 minutes in a microfuge at 4°C. After the supernatant was removed, the pellet was re-centrifuged briefly and all traces of

supernatant were removed. The pellet was resuspended in 100 ul of 1X TE and was extracted once with phenol, once with phenol/chloroform and once with chloroform. Single stranded M13 DNA was ethanol precipitated and the pellet was resuspended in 50 ul 1X TE. The DNA was directly used as a template for DNA sequencing.

2.2.21 DNA Sequencing

M13 recombinant clones single stranded DNA were sequenced by the dideoxy chain termination method of Sanger (1977).The reactions were performed using reagents supplied in the BRL M13 Sequencing kit and according to instructions described in the manual supplied with the sequencing kit.

Primer Annealing: 0.5-1.0 ug (9.4 ul) of single stranded template DNA was mixed with 2 ng of the 17 base M13 universal primer and 1 ul 10X primer anealing buffer. The mixture was denatured by boiling, allowed to cool slowly in a beaker of hot water at room temprature.

Preparation of Nucleotide Stocks: An aliquot of each of the 10 mM dNTP stock solutions was diluted to 1:20 in dH_2O to give a final concentration of 0.5 mM. The N^O mixes were prepared from the 0.5 mM dNTP stock solutions as outlined next page.

	A^O	C^O	G^O	T^O
0.5 mM dCTP	20 ul	1 ul	20 ul	20 ul
0.5 mM dGTP	20 ul	20 ul	1 ul	20 ul
0.5 mM dTTP	20 ul	20 ul	20 ul	1 ul
10X polymerase reaction buffer	20 ul	20 ul	20 ul	20 ul

These N^{o} mixes were designed for the use of dATP as the radioisotope. The N^{o} mixes was replaced approximately every two weeks and kept at $-20^{o}C$.

Several different dilutions of ddNTPs from the 10 mM stock solutions were made and the dilutions which gave the best sequencing results are outlined below.

ddATP 1:200 to 1:300 dilution of 10 mM stock

ddCTP 1:150 to 1:250 dilution of 10 mM stock

ddGTP 1:100 to 1:150 dilution of 10 mM stock

ddTTP 1:100 to 1:150 dilution of 10 mM stock

These ddNTPs were replaced every two weeks and the diluted solutions were kept at -20^{o}.

Sequencing Reactions: For the sequencing reactions four tubes were made as follow:

Tube A: 1 ul A^{o} mix, 1 ul diluted ddATP

Tube C: 1 ul C^{o} mix, 1 ul diluted ddCTP

Tube G: 1 ul G^{o} mix, 1 ul diluted ddGTP

Tube T: 1 ul T^{o} mix, 1 ul diluted ddTTP

After temprature equilibration, to the anealing primer to template (hybridised primer/template), 3 ul [alpha ^{35}S]dATP, 1 ul 0.1 M DTT and 1 ul Large Fragment DNA Polymerase I (Klenow fragments; 1.5 U/ul) were added and mixed gently. 3 ul was dispensed into each of the

four reaction tubes, A, C, G and T which contained the nucleotides. After spinning briefly, the tubes were incubated at 30°C for 20 minutes. The sequencing reactions were cold chased by addition of 1 ul 0.5 M dATP solution and incubating for 15 minutes at 30°C. The reactions were usually stopped by addition of 5-10 ul sequencing formamide loading buffer. The sequencing reactions were denatured by boiling for 3-5 minutes and quickly transferred on ice. Usually 3 ul from each of the reaction mixes was loaded per lane. Unused proportion of the reactions were stored at -20°C. The samples were run on 8% polyacrylamide gels.

2.2.22 Methods for COS Expression System

The methods which follow were exclusively used for COS expression system experiments. These techniques were used for experiments described in Chapter 6.

The protocols are listed in sequence for convenient reference and include preparation of (adaptors), (2) DNA size fractionation, (3) preparation of vector for cloning, (4) tissue culture conditions, (5) spheroplast fusion, (6)recovery of clones by panning, (7) recovery of clones by Dynabeads, (8) Hirt DNA supernatant, (9) high efficiency transformation of *E. coli* by $CaCl_2$, (10) high efficiency transformation of *E. coli* by electroporation, (11) DEAE-dextran transfection, (12) transfering cells on to slides by cytospinning and (13) immunofluorescence staining.

2.2.22.1 Preparation of Adaptors

The ethanol precipitated oligonucleotides for the adaptors were resuspended in 1 X TE at a concentration of 1 mg/ml. 25 ul of resuspended oligos were phosporylated by the addition of 3 ul 10x kinasing buffer and 20 units of T4 kinase followed by incubation at 37°C overnight. Sometimes oligonucleotides for making

adaptors were end labelled to trace the ligation of the adaptors to cDNA and cDNA to the vector DNA. The end labelling was as described in the end labelling section above and was chased by cold ATP.

2.2.22.2 DNA Size Fractionation

cDNA can be fractionated as well as separated from free adaptors or linkers by KOAc gradient. The gradient can also be used to separate different size fragments from a DNA digest. The method below is from Seed group (Aruffo and Seed 1987; Seed group, personal communication) and was mainly used to separate either stuffer from digested vector or to fractionate pBR322 digested fragments.

To make gradient a 20% KOAc, 2mM EDTA, 1 ug/ml EthBr solution and a 5% KOAc, 2mM EDTA, 1 ug/ml EthBr solution were prepared. 2.6 ml of 20% KOAc solution was added to a back chamber of a small gradient maker. Air bubbles were removed from tube connecting the two chambers by allowing solution to flow into the front chamber and then tilting it back. The passage between the two chambers was closed and 2.5 ml of the 5% solution was added to the front chamber. If there was any liquid in the tubing from a previous run, the 5% solution was allowed to run just to the end of the tubing, and then returned to the chamber. The apparatus was placed on a stirplate, the stir bar was set to move as fast as possible. The stopcock connecting the two chambers was opened and then opened the front stopcock. A polyallomer SW55 (Beckman) tube was filled from the bottom with the KOAc solution.

Overlaid the gradient with 100 ul of cDNA or restricted DNA solution. A balance tube was prepared and spun the tubes for 3 hr at 50 Krpm at 22^{o}C. To collect fractions from the gradient the SW55 tube was pierced with a butterfly infusion set (with the luer hub clipped off) close to the bottom of the tube and

collected three 0.5 ml fractions and then six 0.25 ml fractions into microfuge tubes (about 22 and 11 drops respectively). The fractions were ethanol precipitated by adding linear polyacrylamide to 20 ug/ml or other carriers (see Chapter 5 for detail) and filled the tube to the top with ethanol. After cooling the tubes (-20°C for 2 hours) the tubes were spun in a microfuge for 3 minutes then vortexed and respun for 1 min. The pellets were washed with 70% ethanol and respun briefly. Resuspended each pellet in 10 ul of TE and run 1 ul on a 1% agarose. The tubes containing the desired fragments were pooled and used in the subsequent experiments (e.g. ligation to vector).

The CDM8 vector DNA was separated from stuffer by the above fractionation method (for detail see next procedure "preparation of vector for cloning"). The vector DNA was prepared either as above or recovered by inserting an 18 gauge needle to the side of spun KOAc gradiant tube under UV light and removed the vector band manually. The DNA in either case was ethanol precipitated as above.

2.2.22.3 Preparation of Vector DNA for Cloning

20 ug of vector in a 200 ul reaction was cut with 50 units of BstXI at 37°C overnight in a circulating water bath. Two KOAc 5-20% gradients were prepared in SW55 tubes (Beckmann) as described above. 100 ul of the digested vector was added to each tube and run for 3 hours at 50 Krpm (Beckmann Ultracentrifuge) at 22°C. The tubes were under 300nm UV light. The desired band usually had migrated 2/3 of the length of the tube by the end of 3 hours (forward trailing of the band means the gradient was overloaded). The band was removed with a 1 ml syringe and 20 gauge needle into a fresh tube. Linear polyacrylamide was added to the final concentration of 20 ug/ml and the DNA was precipitated by adding 3 volumes of ETOH. The pellet was resuspended in 50 ul of 1XTE. Ligations were set up by using a

constant amount of vector and increasing amounts of cDNA. On the basis of these trial ligations large scale ligation was set up. Different ratio of vector and cDNA (or cDNA like DNA) which were used are given in the result section (Chapter 5).

2.2.22.4 Tissue Culture Conditions

Mammalian Cells (COS) culture were maintained in 2,5or 10 cm^3 plastic tissue culture flasks (Falcon) under 10 or 20 ml (respectively) of appropriate medium (GMEM plus 10% FCS) at 37°C in an atmosphere containing 5% CO_2. The cells were subcultured when confluent, approximately every 3 or 4 days, marking the date and number of passes on the flask.

All the tissue culturings were done in a positive flow sterile cabinet. In order to pass the cells the medium was aspirated off and the cells were washed with 10 ml PBSA. The PBSA was aspirated off and the cells were detached by treatment with 1 ml trypsin-versene (TV) for 1 minute. The cells were dislodged by tapping the flask and TV was inactivated by addition of 10 ml medium. Fresh cultures were initiated with 1/5th to 1/20th dilution made up to 10 ml with fresh medium (20 ml for larger flasks).

In Preparation for transfection experiments, cells after being detached as above, were counted using a haemacytometer and usually $1X10^5$ cells were used to initiate fresh cultures in a 10 cm diameter tissue culture plates with 5 ml of fresh medium, 24 hours before transfection.

2.2.22.5 Recovery of Clones by Panning

Cells expressing a particular Ag can be separated from non-expressing or nonspecific expressing cells by first bounding specific Ab to the target cells and then

adhering them to a dish which had been coated with Abs (Seed and Aruffo, 1987 modified from Wysocki and Sato, 1978).. These Abs are against the Abs which have been raised against cell surface Ags (e.g. if the primary Ab which has been raised against the cell surface Ag was made in mouse then the coated Ab would be anti-mouse Ab).

The procedure is divided into two parts, the first step is to coat the dish with Ab and the second step is panning the cells.

(a) Antibody-Coated Dishes: For coating dishes with Ab, Falcon bacteriological 6.0cm or 10 cm plates, or equivalent were used. The Ab for coating was goat anti mouse Ig affinity purified. The antibody (goat anti mouse Ig affinity purified) was diluted to 10 micrograms per ml in 50 mM Tris HCl (pH 9.5). 3 ml of diluted antibody was added to each of 6 cm dish or 10 ml per 10 cm dish (Falcon bacteriological 6.0 cm or 10 cm plates or equivalent). The Ab was left to stand in the first plate for 1.5 hrs and then removed to the next dish for 1.5 hrs as well and finally to the 3rd dish. The plates were washed 3x with 0.15M NaCl (a wash bottle was convenient for this), and then incubated with 3 ml 1 mg/ml BSA in PBS overnight. Next day the BSA was aspirated from the plates and the unused plates were kept at -20^{o}C for later use.

(b) Panning Cells: Cells were usually grown in 6 cm dishes for this purpose. The medium was aspirated from the dish, and then 2 ml PBS/0.5mM EDTA/0.02% azide (PEA) was added to each dish and incubated the dishes at 37^{o}C for 30 min to detach cells from dish. The cells were pipetted up and down vigorously with short pasteur pipet and the cells were collected from each dish and transferred to a 15 ml centrifuge tube (Falcon). The cells were spun for 4 min at 200g (setting 3.5 in MSE Minor 5). The cells were resuspended in 0.5-1.0 ml PEA/5% FCS (i.e. about 2 x 10^{6} cells per ml). The cells were resuspended so that single cell suspension was

achieved because it is important that the cells be a single cell suspension for immunoadsorption to be really effective.

The antibodies (i.e. primary Abs to the target Ag on the cell surface) were added to the whole of cell suspension (usually Abs were used $^{1}/10$ dilution for supernatants or $^{1}/100$ for asities) and incubated for > 30 min on ice. The cell/Ab mix was occationally mixed gently because cells usually tend to settle. An equal volume of PEA was added to cell/Ab mix and it was layered carefully on 3 ml PEA/2% Ficoll in a tube. The tube was spun for 4 min setting 3.5 in the MSE Minor 5. The supernatant meniscus was aspirated in one smooth movement. The cells resuspended in 0.5 ml PEA and aliquots of these cells were added to each antibody coated dishes containing 3ml PEA/5% FCS for 6 cm dish (5 ml PEA/5% FCS for 10 cm dish). Usually 1 X 10^6 cells were added to each 6 cm antibody coated dish (2 X 10^6 cells/10 cm dish). The dishes were left to stand at room temperture for 1-3 hours.

The excess cells not adhering to dish were removed by gentle washing with PBS/5% FCS (2 or 3 washes of 3-5 ml are usually sufficient). The cells adhering to the 6 cm dish were resuspended in 0.4 ml of 0.6% SDS and 10 mM EDTA (0.8 ml for 10 cm plate) for making DNA by the Hirt method for electrotransformation (electroporation).

2.2.22.6 Hirt DNA Supernatant

DNA can be made from recovered cells from either panning or Dynabeads selections and the DNA made in this way can be transformed into bacterial cells for amplification and further rounds of selection. The DNA can be made in a simple method called Hirt supernatant and it provides transformable DNA for amplification in bacterial cells. To make Hirt DNA the cells are treated with SDS/EDTA and a viscous supernatant is formed. the more viscous the supernatant the more cells had been

selected at either panning or Dynabeads step. The procedure was as follow:

0.4 ml 0.6% SDS and 10 mM EDTA were added to a 6 cm panned plate (0.8 ml to a 10 cm panning plate or Dynabeads selected cells in the tube). The cells were left to stand for 20 minutes which at the end a viscous mixture was formed. The viscous mixture was transferred into a microguge tube. 0.1ml 5M NaCl was added per 0.4 ml Hirt, and mixed by inverting tube (3 x). The mix was left on ice for at least 5 hours (usually the ice incubation was carried out overnight at $4^{o}C$ and it was noticed that keeping the mixture as cold as possible seemed to improve the quality of the Hirt). The Hirt mix was then spun 4 minutes at $4^{o}C$ and the supernatant was carefully removed and transferred into a fresh tube. The mixture was extracted 2 X with phenol and then with phenol/chloroform and finally with choloroform.

To concentrate the DNA 10 ug linear polyacrylamide was added to the mix and ethanol precipitated it. The tube was spun down and the pellet was resuspended in 0.1 ml dH_2O by placing the tube at $65^{o}C$ and vortexing. Usually the DNA was ethanol precipitated for better purified DNA. After the second precipitation the pellet was resuspended in 100 ul dH_2O and the excess was kept at $-20^{o}C$. The DNA was usually transformed into MC1061/p3 *E. coli* cells electroporation.

2.2.22.7 High Efficiency Transformation of MC1061/p_3 *E. Coli* Cells by $CaCl_2$ Method

(a) Making Competent MC1061/P_3 *E. Coli* Cells: The following method has been found to give high frequency of transformation and has been adapted for MC1061/P_3 *E. coli* cells (Seed and Aruffo 1987). A single colony was innoculated into 20 ml TYM broth in a 250 ml flask. the cells were grown to midlog phase (OD_{600} 0.2-0.8), then transferred into a 2 litre flask containing 100ml TYM, and continued incubation with vigorous agitation until

cells were grown to OD_{600} 0.5-0.9. 300 ml TYM media was added to the cells and continued the incubation to OD_{600} 0.6. The flask was transferred on a rocking platform with gentle shaking to assure rapid cooling. When culture was cool the cells were spun down at 4.2 Krpm for 15 minutes. The pellet was resuspended in 100 ml cold TfB I by gentle shaking on ice. The cells were spun down at 4.2 krpm for 8 minutes. The pellet was resuspended in 20 ml of cold TfB II by gentle shaking on ice. Aliquots of 0.1 to 0.5 ml were made in prechilled microfuge tubes, and were frozen in freeze in liquid nitrogen, and stored at $-70^{o}C$.

(b) Transformation of MC1061/P_3 *E. Coli* Cells: The cells were made competent as above. For transformation, an aliquot, was thawed at room temperature until just melting, then placed on ice. DNA was added to the cells and kept on ice for 15-30 minutes, then the cells were shocked by incubating at $37^{o}C$ for 5 minutes (6 minutes for 0.5 ml aliquots). The transformed cells were diluted 1:10 in L-broth and grew for 90 minutes before plating or applying antibiotic selection.

2.2.22.8 High Efficiency Transformation of MC1061/P_3 *E. Coli* Cells by Electroporation

Foreign genes can be transformed into bacterial cells by electro-transformation (electroporation) method (Potter *et al.*, 1984; Ausubel *et al.*, 1987). Cells are placed in suspension and put into an electroporation cuvette and then DNA is added. The cuvette is connected to a power supply, and the cells are then allowed to recover briefly before they are replaced in normal growth medium. The transformation efficiency was found to be higher than other methods (e.g. transformation by $CaCl_2$. The method involves making cells and electroporating them with DNA made from selected cells (i.e. panning or Dynabeads selection) either in form of Hirt DNA or DNA prepared by large plasmid prep.

(a) **Preparation of Cells:** 10 ml of overnight culture of *E. coli* cells (MC1061/P_3) was added to 1 litre of L-broth and incubated at 37°C with vigorous shaking until it reached OD_{600} of 0.6-0.7. To harvest the cells, the culture was chilled on ice for 15 to 30 minutes, and then centrifuged in a cold rotor at 4,000 x g for 15 min at 4°C. The cells pellet was resuspended in a total volume of 1 litre of a cold, low ionic strength wash medium (e.g. 1 mM *HEPES*, pH 7.0). The cells were centrifuged again as above and the cell pellet was resuspended in 0.5 litre of cold, wash medium (1 mM *HEPES* pH 7.0) and the cells were spun down as above. The cells were resuspended in 20 ml 10% glycerol, and then centrifuged as above. The cells were resuspended finally in 10% glycerol in 2 ml aliquots to give either 3 X 10^{10} cells/ml if used fresh or 3 X 10^9 cells/ml if cells were stored frozen (cell suspension was frozen in aliquots on dry ice and stored at -70°C).

(b) **Electroporation (Electro-transformation):** In order to transform cells either fresh or frozen aliquots were used. The 2 ml frozen aliquot was first let to thaw at room temperature to almost melting then transferred on ice. The cells (i.e. 2 ml aliquot) was spun down at 6.5 Krpm in a microfuge at 4°C. The cells were resuspended in 200 ul 10% glycerol. 40 ul of the cell suspension was mixed in a cold microfuge tube with 1 to 2 ul of 10 ug/ml DNA (DNA was usually in dH_2O). The tube content was mixed well and kept on ice for 1 minute.

Gene Pulser apparatus (Bio Rad) was set at 25 uF and 2.5 kV and the Pulse Controller was set to 800-1000 ohms. Each batch of cells made were tested to see which ohm setting gave the greatest number of transformants. The mixture of cells and DNA was transferred to a cold, 0.2 cm electroporation cuvette, and the suspension was shaken to the bottom of the cuvette. The cuvette was placed in the chilled safety chamber slide, and pushed the slide into the chamber until the cuvette was seated between the contacts in the base of the chamber and then pulsed at once at the above settings. Both red buttons

were pressed and held together until the tone sounded, which signalled the delivery of a pulse. The cuvette was removed from the chamber and immediately 1 ml of SOC medium was added to the cuvette and quickly resuspended the cells with a pasteur pipette. This rapid addition of SOC after the pulse was very important in maximizing the recovery of transformants. The cell suspension was transferred into a Larger tube and incubated at 37°C for 1 hour. 10ul and 100ul of the transformed cells were plated on selective medium.

2.2.22.9 Transfection of Foreign Genes into Mammalian Cells Using DEAE-Dextran

Foreign gene can be expressed in mammalian cell line and those cells expressing a novel determinant can be selected by either panning or Dynabeads. The DNA to be expressed has to be transfected into mammalian cell line either by variation of the electroporation method or by DEAE-dextran.

The DEAE-Dextran procedure works very efficiently in transient expression systems. However, it cannot be applied for the production of stably transfected cell lines (because plasmid is eliminated from the growing cells in the subsequent generations). The DNA/DEAE-dextran mixture with chloroquine (chloroquine is believed to increase transfection efficiency) is prepared and incubated with the cells in culture for up to 4 hrs. The cells are then exposed to DMSO to increase DNA uptake. The cells are incubated for up to 48 hrs to allow expression of the introduced gene. The protocol has been optimized for Cos-7 cell line (Ausubel *et al.*, 1987; Seed and Aruffo 1987).

Usually 5 x 10^5 Cos cells were plated out in a 10 cm tissue culture dish. The cells were allowed to grow overnight, at 37°C in 5% CO_2. The cells were usually 30-50% confluent the next day. The DNA for transfection

was usually kept in ethanol at -20°C and an aliquot (usually 5 ug DNA for each dish) was spun down on the same day as the transfection. The precipitated DNA was washed with 70% ethanol and the ethanol was aspirated off in the culture hood. The pellet then was air dried in the culture hood. Meanwhile DEAE-Dextran, chloroquine, TBS, GMEM medium with 10% FCS (filter sterilized) were placed at 37°C water bath.

DNA pellets were resuspended according to table below (for any variations in DNA, cell or DEAE-dextran concentration see Chapter 6).

DEAE-Dextran con. ug/ml	DNA pellet resuspended in TBS* (ul)	DEAE-Dextran (10mg/ml) (ul)	Chloroquine (100mM) (ul)	Total
75	15	30	4	49
100	20	40	4	64
200	40	80	4	124
400	80	160	4	244

* for control omited DNA and added corresponding volume of TBS

After addition of the correct amount of DNA, DEAE-Dextran, TBS and chloroquine, the tube contents were mixed and spun down very briefly to concentrate the liquid at the bottom of the tube. The media was aspirated from dishes of overnight grown cell culture (usually 3 plates at a time), and washed the cells twice with 5 ml PBS(A). The PBS(A) was aspirated off and 4 ml of media (+ 10% FCS) was added into each dish.

The DNA/dextran mixture was added dropwise but evenly over the dish and swirl each dish gently to mix. A minimum of 10 drops/plate was usually carried out (where each drop touched the media it changed the colour of the

media from red to yellow; the dish was swirled until a uniform colour returned which meant that the DNA/Dextran had been mixed sufficiently).

Alternatively, when a single concentration of DEAE-dextran/DNA (i.e. when several dishes were transfected with a constant amount of DEAE-dextran/DNA) was used the DEAE-dextran mix was added to a stock of medium and then 4 ml was added to each 10 cm dish. This was of course more convenient and all dishes were treated identically.

The plates were usually incubat for 3 hours in a CO_2 incubator. To check that the DNA was being taken up by the cells, every $^1/_2$ hour the dishes were looked under microscope; appearance of vacuoles within the cells confirmed the up take of DNA. The DEAE-dextran treatment was followed by DMSO treatment when the number of vacuoles within the cells became so great that further incubation in DEAE-dextran would increase the rate of cell death.

The DEAE-dextran treatment was followed by DMSO shocking. Although DMSO is very toxic to cells, it significantly enhances the uptake of DNA. For DMSO shocking, the media was aspirated and cells were shocked by addition of 5 ml 10% DMSO (in PBSA). DMSO was usually left for 1 minute in the dishes and then the cells were washed with 5 ml of 1 X PBSA. The PBSA was aspirated and 10 ml media was added to each dish and incubated in a CO_2 incubator at 37^oC overnight.

After 24 hours of incubation the media was aspirated and the dishes were washed 2 X with PBSA. 1 ml trypsin/versene was added into each dish and evenly spread then let to stand for 1-4 minutes. The dishes were tapped several times with the palm of the hand to lift the cells. Once about half of the cells had been lifted (by checking under the microscope) 9 ml media was added into each dish to inactivate trypsin. The cells were pipetted up and down to ensure all cells had

been lifted and that the cells had not clumped. The cells were transferred into a fresh dish and incubated as above for 24 hours (for full discussion about the need for the transfer see Chapter 6).

After the 24 hours incubation the media was aspirated and the cells were washed 2 x with 5 ml PBSA and then after removal of PBSA 5 ml PEA (PBS, EDTA 0.5mM Azide 0.02%) was added into each dish to lift the cells. The dishes were usually incubated at $37^{o}C$ for 30 minutes and then moved the cells up and down using a Pasteur pipette to resuspend them and break up cell clumps. Once the cells were resuspended the cell suspension was transferred into a 15 ml Falcon tube. The cells were spun down for 5 minutes at 3.5 in MSC bench-top centrifuge. The cells were resuspended in 2 ml 5% FCS/PBS (FCS was necessary for cells to stick to the slide during cytospinning) and cells were cytospun for staining. If the cells were required for panning or dynabeads selection the cell pellet was resuspended in 5% FCS/PEA or 1% FCS/PBS to a final cell concentration 2 x 10^{6} cells/ml.

2.2.22.10 Transfering Cells on to Slide

(a) Cytspinning: Transfected cells in order to be stained with antibody had to be transferred on to a solid support. Slides were chosen as solid support and the best method of transfering cells on to slides was found to be by cytospinning (cytocentrifugating) the cells on to slides. The cells were harvested as described in D-D transfection section and were resuspended usually 2 ml of PBS/5% FCS. Density of cultured cells was measured by counting them in a heamocytometer under a microscope. The cell concentration then was adjusted to have $1X10^{4}$/ml ($3X10^{3}$/slide). Cells were declumped after centrifugation by pipetting up and down by a pasture pipette or one ml syringe with a 21 gauge needle. The cells were kept on ice during cytospinning. Slides and cytospinning chambers

were labelled and assembled as recommended (Shandon Cytospin 2 users manual). For every slide usually 200-300 ul cell suspension was cytospun at 500 rpm for 5 min on Shandon Cytospin 2 cytocentrifuge. The slides, at the end of cytospinning, were removed from slide chamber and immediately were placed in acetone and fixed the cells for 1-10 minutes.

(b) Cleaning Slides and Coverslips: Slides and coverslips were found to contribute to some of the background seen in FITC stainings (see Chapter 6 for details). Slides and coverslips therefore had to be washed in a special way. The best washing condition for slides was achieved by soaking the slides in ethanol (bulk) overnight, then washing them briefly under hot water. The slides were then washed in washing up liquid for few minutes then washed for several minutes under hot tap water. The slides were then dried by placing them in acetone after draining excess water.

If slides were a better quality (BDH) then simple wiping with lint free (Kimwipe) paper tissue moisten with ethanol was usually enough to clean them. The slides, in either case, were then looked at against light and if they were not clean enough, cleaning with Kimwipe moisten with ethanol was repeated.

Cleaning coverslips was only possible by soaking in ethanol overnight and wiping with ethanol weted lint free tissue as above.

The background caused by cover slip was different than caused by slides. The background caused by dirty coverslip could be seen moving, under microscope, when coverslip was moved and it was avoided by replacing the coverslip. But the background caused by dirty slides was not moving by moving the coverslip and could not be washed away or removed by any other way and only using very clean slide was the answer.

2.2.22.11 Immunofluorescence Staining

Immunofluorescence staining (FITC staining) was mainly used for work described in Chapter 6. FITC staining proved to be the ideal staining method for COS cells. FITC stainings were done on cytospun COS cells. The slides were marked and placed inside a humid chamber. The cells were allowed to rehydrate in TBS for at least 5 minutes before applying the first antibody. Meantime the primary (first) antibody was diluted in TBS. The correct dilution for a particular antibody had to be determined by carrying out a pilot experiment using a dilution series (e.g. $^1/_5$, $^1/_{10}$, $^1/_{50}$, $^1/_{100}$ etc.). Usually TBS was drained off from 3-4 slides at a time by wiping the periphery of the cells. 100 ul of diluted primary antibody was added on to each slide and the slides were incubated for 15-30 minutes at room temperature in a humid chamber. The slides were first washed with a wash bottle then transferred to a dish of circulating TBS buffer (the buffer was circulated by a magnetic stirrer). The circulating buffer was found to be more effective than washing with a jet of buffer from a wash bottle. The cells were washed 2 X 5 minutes in the same way. One slide, at a time, was taken out from the dish and after wiping excess TBS off, 100 ul diluted second antibody (FITC conjugated) was added on to the cells. Several slides could be wiped and then diluted antibody be added to them but care had to be taken not to let the cells dry (i.e. rehydrated cells may burst if become dry). The antibody was incubated as above for 20-40 minutes. The slides were washed as above and after final wash, 3-4 slides, at a time, were placed at a 60^o angle and were rinsed with wash bottle. It was noticed that this last washing treatment would remove most of unbound FITC conjugated antibody floating on the slide which had not been washed away. After wiping excess TBS off about 30 ul wet mountant (glycerol/TBS v/v) was added on to each slide. The glycerol had to be nonfluorescent, ultra pure for staining purposes only.

Nuclear stainer (counter stainer), propidium

iodide usually was either added to the second antibody (i.e. 1 ul stock solution of propidium iodide in 40 ul diluted second antibody) or directly to the wet mountant (5 ul stock solution of propidium iodide was added to 100 ul wet mountant). If propidium iodide was added to the second antibody it needed washing but if it was added to the mountant no washing was needed. Adding propidium iodide to the mountant was found to be more convenient and was used more often specially after the aduption of new washing method (i.e. in circulating buffer). Cells were covered by a clean coverslip and examined under a Litz Universal microscope fitted with an epi-fluorescence condencer filter equipped with UV light source.

Immunofluorescence pictures were taken on the Litez Universal microscope fitted with an epi-fluorescence condenser filter and UV light sorce, using Kodak Ectar 1000 colour film (Ectar 1000 film was found to be superior to any other speeds or brands). The films were developed and printed by the Glasgow University Photography Unit.

2.2.22.12 Spheroplast Fusion

Spheroplast fusion was used in the tertiary screening and it involves fist making the spheroplasts and then fusing them into COS cells. In brief *E. coli* cells containing amplifiable plasmid are grown to OD_{600} of 0.5 and then chloromphenical is added and incubated for 10-16 hours. Cells are then kept at $4^{o}C$ for 10 min. and then spun down at 9,000 rpm for 5 min. The cell pellet is resuspended in ice cold 20% sucrose and 50 mM Tris-HCl (pH 8.0). To these cells ice cold lysozyme (5 mg/ml) is added and stored on ice for 15 min. and then ice cold 0.25 M EDTA is added to them and incubated for 5 min. After this cold 50 mM Tris-HCl was added and incubated for 5 min as above.

The cells are then transferred to 37°C and incubated for 5 min. and then transferred back on ice and kept there until needed.

Second part involves in preparing the COS cells. In this case COS cells are plated the day before the spheroplast fusion. Usually $3x10^5$ cells per dish was needed. 24 ml Glasgow MEM (no FCS), 10% sucrose and 10mM $MgCl_2$ was added (dropwise) to the *E. coli* cells and allowed to diffuse with very gentle rocking. The media from COS cells plated the day before was removed. Using gentle pipetting 4.5 ml of spheroplast suspension was added to each dish and then plates well spun at 1000 g. The media was gently aspirated and 1.5-2.0 ml 50% (w/w) PEG 1450/50% GMEM was added and after gentle mixing the PEG solution was removed. After 2 minutes of PEG contact, 2 ml of GMEM was added to each dish and after swirling aspirated the media and repeated the wash once more then added 3 ml GMEM with 10% FCS and incubated for 4-6 hours at 37°C. After this the media was removed and fresh media was added and incubated to 36-48 hours.

2.2.22.13 Recovery of Cells Using Dynabeads

Dynabeads (M-450 Dynal Inc.)work in the principle that the cells which are bounded to the primary Ab are mixed with Abs (i.e. Second Ab) which have affinity to the primary Ab. The second Ab has been conjugated to magnet beads. The cell bounded Abs (primary Ab) would attract the second Abs and these cells are controlled by using magnetic particle concentrator and discarding the supernatant containing the non-Ab bounded cells and free Abs. The bounded cells are used for making Hirt DNA and the rest is as in panning.

CHAPTER 3

MOLECULAR STUDIES OF PLACENTAL FIBRONECTIN

3.1 Introduction

Numerous studies have shown that fibronectin (FN) is a major protein in human placenta and in the past few years FN has been intensively studied at the molecular level (see Chapter 1). Consequently several different mRNA splicing patterns of FN pre-mRNA transcripts, in human and rats, have been identified (see Chapter 1). Despite these findings not much is known about the molecular structure of FN mRNA transcripts in the human placenta. The aim of the work described in this chapter was therefore to investigate the pattern of alternative splicing in the FN mRNA from human placenta. By screening a placental cDNA library, the study was aimed at isolating human FN cDNA clones and characterising them by restriction analysis and sequencing.

3.2 Screening Placental cDNA library

The placental cDNA library which was used for this investigation was made by Dr. D.A. Nickson, in this laboratory, prior to the start of this work (Nickson 1985). The library had been prepared from the mRNA from chorionic plate of human term placenta and the cDNA clones had been inserted into lambda gt10 (\gt10) (Nickson, 1985).

In order to screen the library the phages, bearing the cDNA clones, were grown on E. coli c600 delta hf1 cells (Young and Davis 1983). For phage DNA transfer (plaque lifts), hybridisation and washing the filters, the method used was an adaptation of the Benton and Davis (1977) protocol (Chapter 2). The Plaque lifts, after denaturing, neutralising and baking, were prehybridised for 1-2 hours (Chapter 2). After prehybridisation the filters were hybridised with human FN cDNA clones, pFH1, pFH23 and pFH154 (Kornblihtt *et al.*, 1983; 1984b)(Fig 3.23)

These clones were prepared for radiolabelling in

two ways. In both methods the FN cDNA were double digested with BamHI/EcoRI restriction enzymes in order to release the insert (Fig 3.1). In the first method, the digested DNA was run on a low melting point (LMP) agarose gel and the insert DNA fragments were excised from the gel and kept in separate tubes (Feinberg and Vogelstein 1983; Chapter 2). An aliquot of these fragments were usually radiolabelled by random priming technique (Feinberg and Vogelstein 1983). The other method involved in running some of the digested DNA on an agarose gel and after the completion of the electrophoresis, the gel slices containing the DNA fragments, were electroeluted in order to free the DNA fragments (Maniatis *et al.*, 1982; Chapter 2). The electroeluted DNA were run on agarose gel to check the recovery of the DNA (Fig. 3.2). The filters were hybridised with the above probes. After hybridization the filters were washed and then left for autoradiography.

3.2.1 Primary Screening

For the primary screening of the library usually $5x10^3$-$1x10^4$ pfu (plaque forming units) from the phage library was incubated with 150ul of specially prepared host stock, delta hf1 cells (Chapter 2). After the adsorption of the phage particles to the cells, they were plated and incubated at 37°C overnight. Duplicate plaque lifts were performed from these plates and the filters were hybridised with the pFH1 FN probe, The filters were then washed in low stringency condition (55° and 1x SSPE and 0.1% SDS). The autoradiographs showed the presence of several positive plaques (frequency of 1.57% positives). The filters were then washed at the high stringent washing condition (65°C and 0.1x SSPE and 0.1% SDS) and set for autoradiography again. This time the frequency of positives was only 0.18%. The stronger looking positive plaques (Fig. 3.3) were removed from the master plates and kept as phage stock.

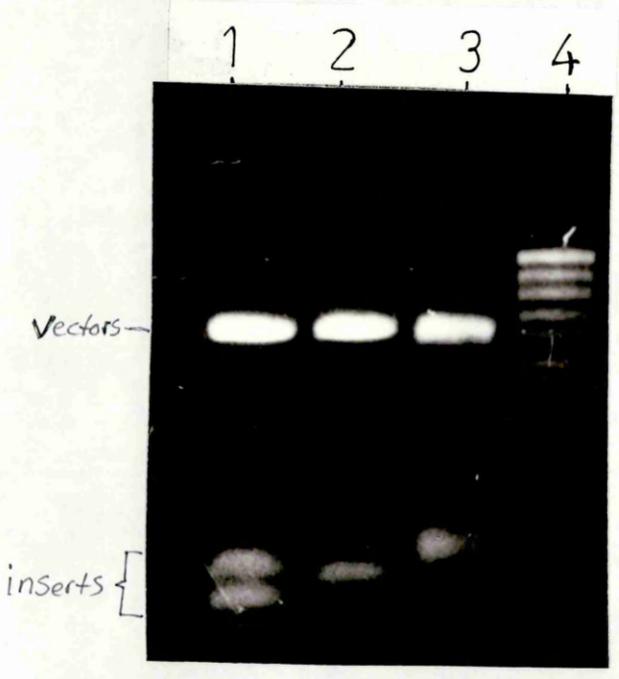


Figure 3.1 Fibronectin cDNA clones digested to release the inserts. The pFH1, pFH23 and pFH154 were double digested with EcoRI and BamHI restriction enzymes.

1-pFH1 digested with EcoRI/BamHI
2-pFH23 digested with EcoRI/BamHI
3-pFH154 digested with EcoRI/BamHI
4-pBR322 DNA digested with TaqI

Figure 3.2 Fibronectin cDNA inserts recovered by electroelution. The gel clearly shows that the inserts are free from vector DNA.

1-3' end of pFH1
2-5' end of pFH1
3- pFH23 insert
4- 3' end of pFH154
5- 5' end of pFH154
5- pBR322 digested with TaqI

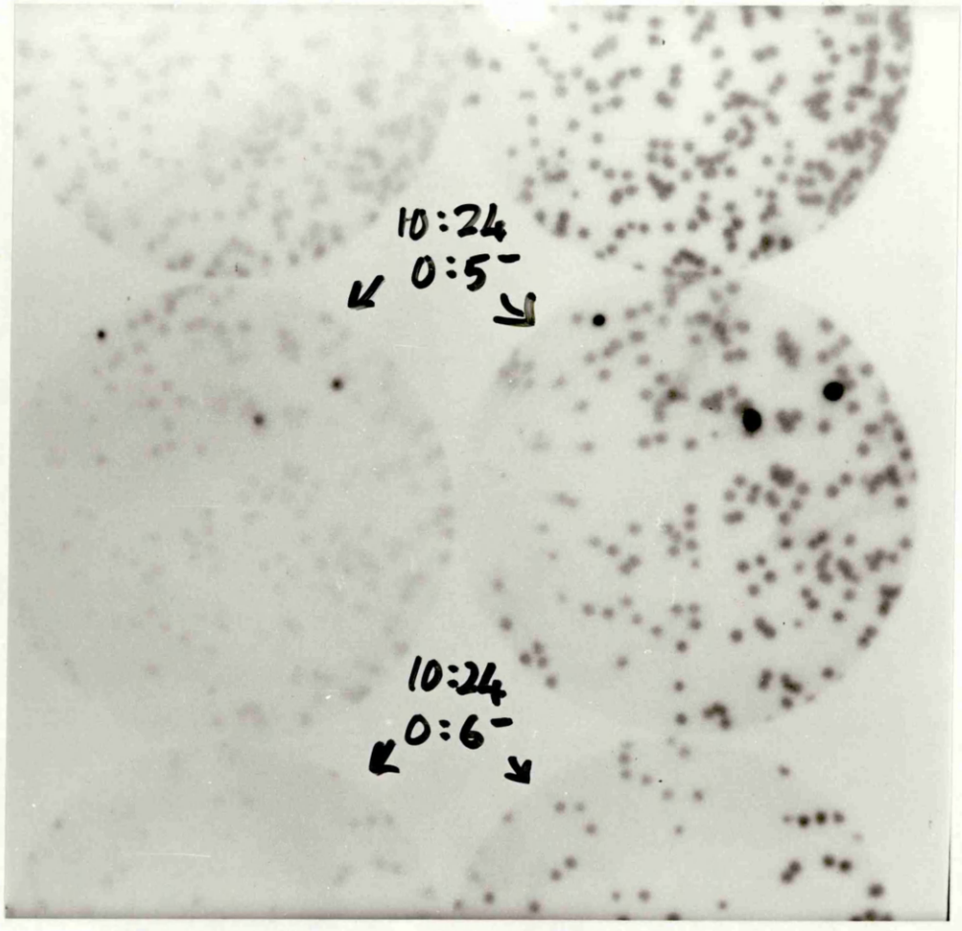


Figure 3.3 Primery screening of the placental cDNA library for FN. The figure shows two duplicate filters which both have been hybridised with the same probe (arrows). The positive signals are clearly distinguishable from the background.

3.2.2 Secondary Screening

Whenever a positive signal was identified from the autoradiograph, the corresponding area in the agar plate was marked and the selected plaques were picked as BBL-agarose plugs and transferred into fresh tubes with some phage dilution buffer. Each agarose plug would usually have few plaques and normally only one plaque would be the positive clone. In order to identify the positive plaque the picked plaques had to be screened further (secondary screening).

For the secondary screening the titration of each phage stock had to be determined as in the primary screening (Chapter 2). The main difference between the primary and secondary screening was that the titrations were worked out to give only around 200 plaques per plate (10x10 cm). For the secondary screening, the plating, plaque lifting, hybridisation, etc. were as in the primary screening. The autoradiographs showed that only three sets of identical plaques were still positive (ferquency of 0.18% in the original library). These three plaques populations (called Lambda Human placental Cytotrophoblast Fibronectin 11, 17 and 24; \HCF11, 17 and 24) and also plaques from weaker signals were grown in a matrix plate. Several individual copies of the positive plaques were picked and treated as above. These positive clones were screened once more (tertiary screening) to ensure the isolation of positive ones.

3.2.3 Tertiary Screening

The FN positive clones, which so far had been positive to the FN probe, were still screened further with the pFH1 and other FN probes recovered from the LMP gel above. For this purpose the bacterial cells, instead of being infected with the phage particles and then being plated (as in the primary and secondary screening), they were poured on to the agar plate and

then the plate was marked, on the back, into 49 square matrices (Fig. 3.4). 5 ul from each phage stock was spoted on to each square on the matrix and the plate was then incubated. Dublicate plaque lifts were prepared as above and the filters were hybridised with several different FN probes (i.e. the 3' of pFH1, pFH1, pFH23 and the 5' end of the pFH154). See also Fig 3.23 For this purpose the filters were hybridised with one probe at a time and after each autoradiography, the filters were freed from the probe and re-hybridised with another probe and so on (Chapter 2).

The hybridisations results showed that three clones (\HCF11; \HCF17 and \HCF24) were hybridising to the pFH1 (Fig. 3.5). The result of hybridising these filters with probe 3' to the EcoRI site in pFH1 showed that only one of the clones (\HCF11) was hybridising to the probe (Fig. 3.6). None of the clones hybridised to the 5' to the EcoRI site in the pFH154 probe suggesting that \HCF17 and \HCF24 may not extend toward the 5' end of the pFH154 clone. Further analysis of these clones were not easily possible at this stage and DNA had to be made from them to allow restriction analysis to be carried out.

3.3 Restriction Analysis of the HCF Clones

The hybridisation of the filters from the tertiary screening with different probes indicated that only two clones were extending beyond the 5' end of the pFH1 (\HCF17 and \HCF24) and only one was extended toward the 3' to the EcoRI site in the pFH1 (\HCF11). None of these finding could confirm the existence of the two splicing regions, (i.e. ED-I [ED-A] or IIICS, Chapter 1 and Fig. 3.7), in \HCF17 and \HCF24 and the IIICS in \HCF11. Liquid lysate phage DNA from each clone was therefore prepared (Chapter 2) to allow further analysis of these clones.

	1	2	3	4	5	6	7
A	-	17/1	17/2	17/3	17/4	17/5	-
B	17/6	13/1	13/2	13/3	13/4	-ve	-
C	14/1	14/2	14/3	-ve	11/1	11/2	11/3
D	11/4	11/5	11/6	11/7	-ve	19/1	19/2
E	19/3	21/1	21/2	21/3	21/4	23/1	23/2
F	23/3	-ve	24/1	24/2	24/3	24/4	-ve
G	-	-ve	-ve	-ve	-ve	-ve	-

Figure 3.7 The tertiary screening matrix plate. The figure shows the lay out of the plaques plated onto the agar plate. The filters from the corresponding plates were hybridised with several probes (see text and Figures 3. and 3.). The number before the virgule (e.g. 17/) is the number given to the plaque picked in the primary screening and the number after the virgule (e.g. /4) designates the plaque picked after the secondary screening. squares without any phage and negative (turbid) plaques are also shown (- and -ve respectively).

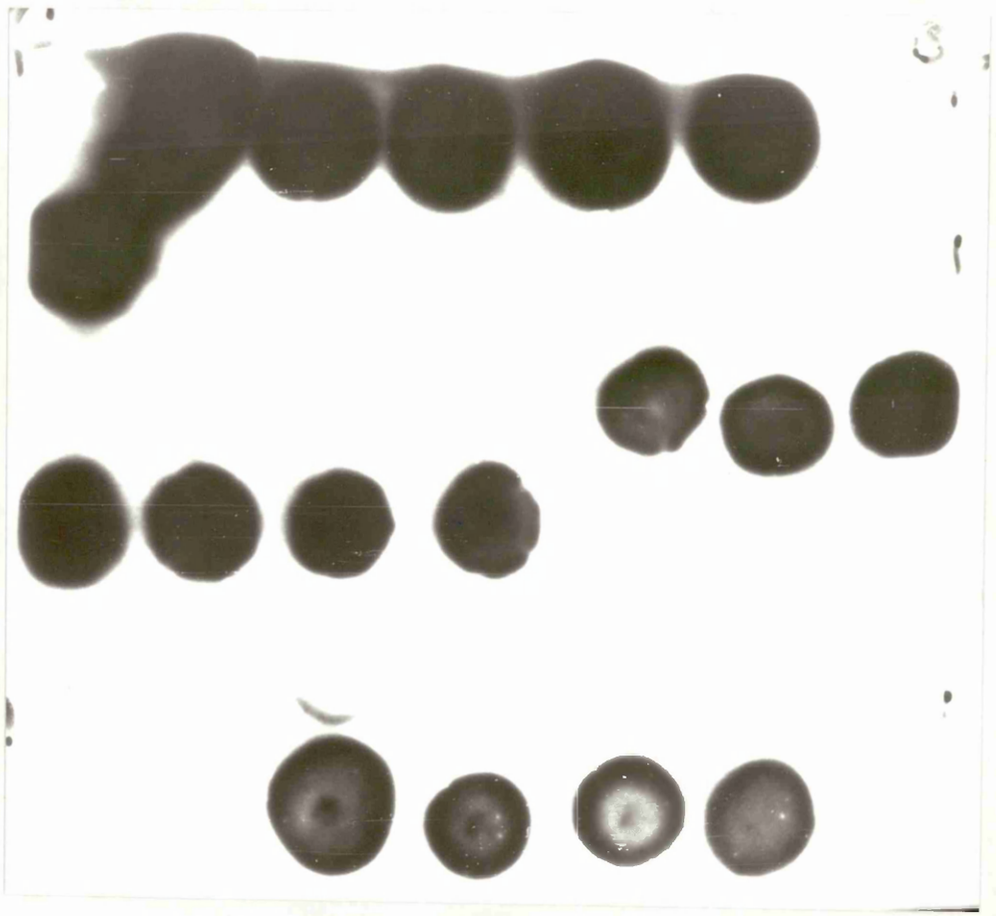


Figure 3.5 The tertiary filters hybridised with the pFH1 insert (both 3' and 5' mixed). Three clones were strongly hybridising to this probe. These were \HCF11, 17 and 24 (compare with the matrix in figure 3.4).

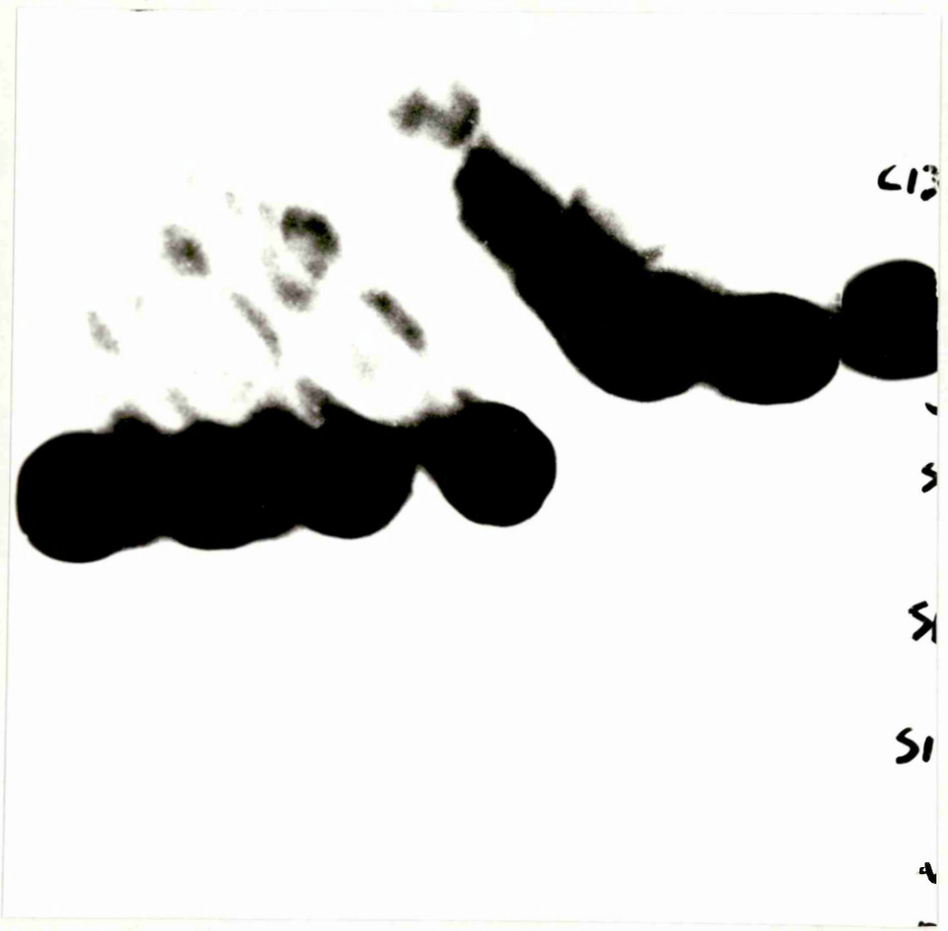


Figure 3.6 The tertiary filters hybridised with the 3' end fragment of the pFH1 insert. Only on clone hybridises to this probe, \HCF11. The other two (\HCF17 and 24) did not which indicated that they did not extend toward the 3' end of the sequence.

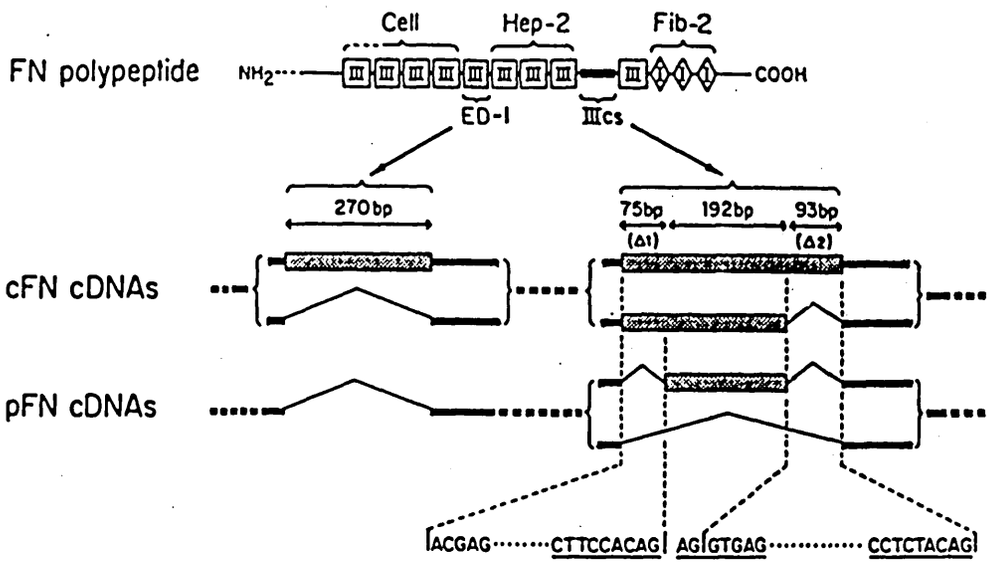


Figure 3.7 The splicing pattern of ED-I and IIICS region of plasma and cellular form of fibronectin gene. The partial nucleotide sequence at the 5' and 3' boundaries of splicing regions are shown. ED-I is always absent in plasma FN but may be absent or present in the cellular FN.

The restriction analysis of these clones was aided by referring to the restriction map of the vector which the cDNA had been cloned into (i.e. \gt10, Fig. 3.8). As it can be seen from the figure the EcoRI digest of the cDNA clones would release the insert and BglII\HindIII double digests would generate several fragments including a 1.14kb fragment containing the EcoRI site (Figs. 3.8-9). The lambda (\ symbol will be used to designate lambda hereafter) DNA from these clones (\HCF11, 17 and 24) were digested with several restriction enzymes. The digested DNA were run on an agarose gel and after photography, the gel was Southern blotted (Figs. 3.10-11). Among several digestions which were carried out in order to determine the sizes of the inserts, the result of the BglII/HindIII digests for \HCF11 and \HCF17 are given here (Fig. 3.10). At this stage the size of each clone was determined by drawing a calibration curve. Combining the results obtained from hybridisation of the Southern blot with pFH23 probe and the calibration curve it was concluded that the sizes of \HCF11, \HCF17 and \HCF24 would be 0.62kb, 2kb and 2.6kb respectively (Figs. 3.10-11). These figures also show that both \HCF17 and \HCF24 clones contain internal BglII or HindIII sequence. Searching through the FN sequence available (Kornblihtt *et al.*, 1985), it became apparent that there are two BglII sites within the boundaries of the two EcoRI sites; one is within the IIICS region and the other one between the IIICS and the ED-I splicing regions (Appendix 2). Therefore the internal digestions of the two clones most probably were generated by BglII restriction enzyme. The exact position of the internal sites and presence of either of the splicing sites could not be confirmed, accurately, at this stage and more analysis was needed.

3.3.1 Restriction Analysis of \HCF17 Clone

In order to determine whether the two splicing regions were present in the \HCF17 clone, probes specific for these regions were needed. After several experiments, which were done to recover the ED-I and

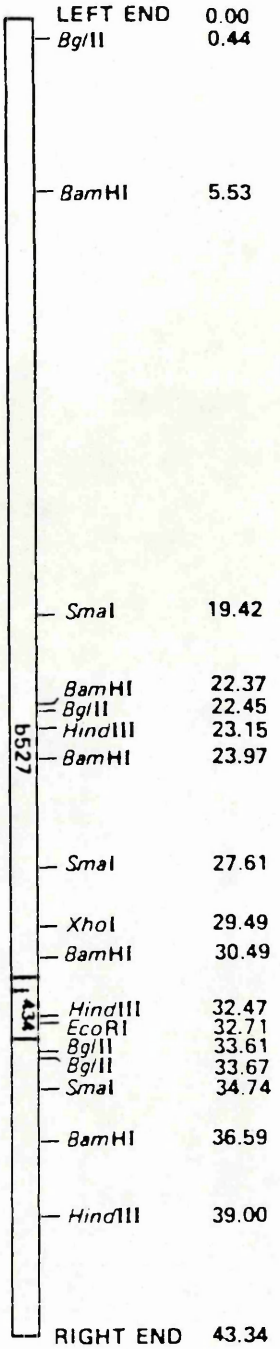


Figure 3.8 Map of lambda gt10. Restriction endunuclease cleavage sites are designated in kilobase pairs from the left end. There is a unique EcoRI cloning site. Recombinant cI^- phage containing insertions at the EcoRI site can be distinguished easily from the cI^+ parental phage on the basis of their clear plaque morphology.

EcoRI	BglII	HindIII	BglII/HindIII
32.71kb	22.05kb	23.15kb	22.01kb
10.63kb	*11.16kb	9.32kb	9.32kb
	9.67kb	*6.53kb	5.33kb
	0.44kb	4.34kb	4.34kb
	0.06kb		*1.14kb
			0.7 kb
			0.44kb
			0.06kb

Figure 3.9 The restriction fragments which would be generated when /gt10 is digested with EcoRI, BglII, HindII and BglII/HindIII. The star (*) indicates that the fragments shown contains the EcoRI site.

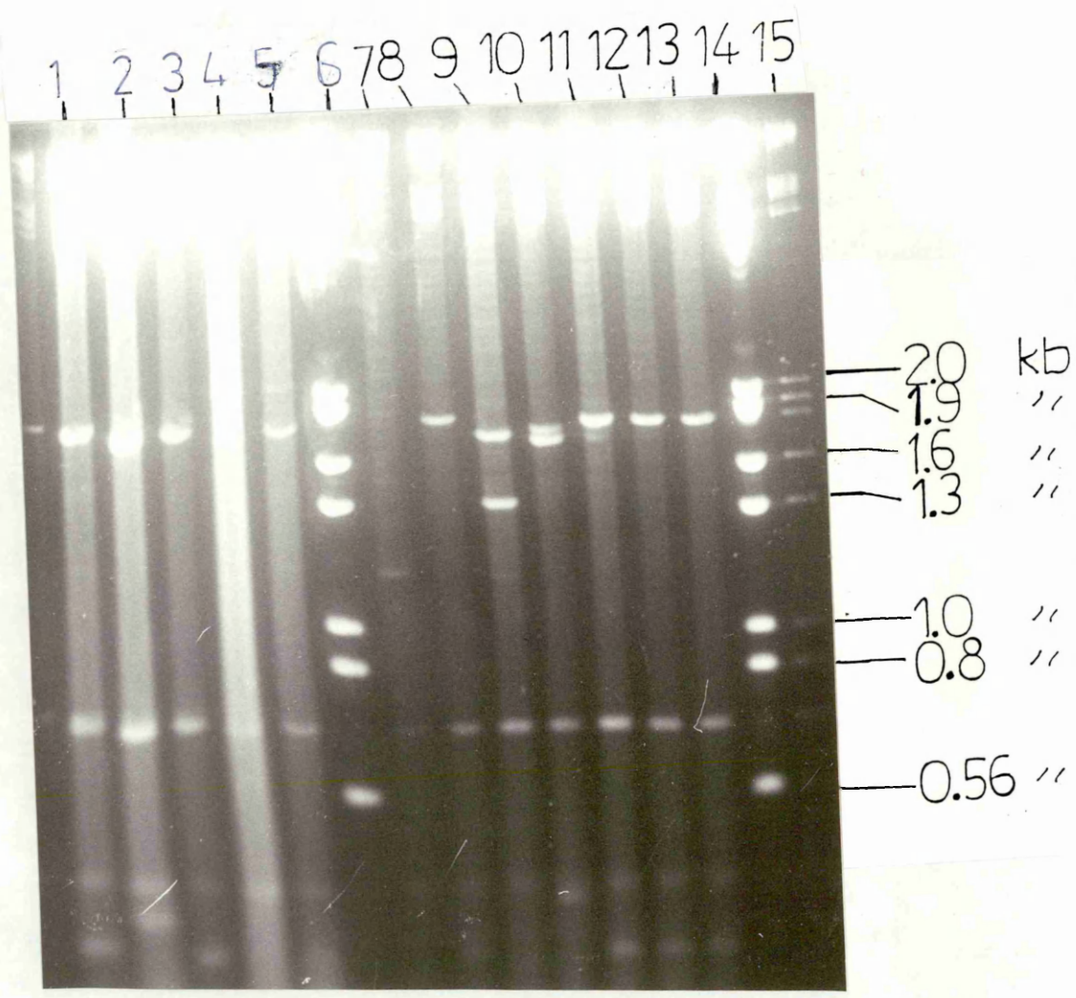


Figure 3.10 Fibronectin clones digestion. Various FN clones recovered from screening a placental library have been digested with BglII/HindIII restriction enzymes.

1-\HCF12
2-\HCF14
3-\HCF126
4-\HCF168
5-\HCF177
6-Lambda marker (EcoRI/HindIII)
7-Lambda gt10
8-\HCF11
9-\HCF17
11-\HCF24
12-\HCF126
13-\HCF168
14-\HCF177
15-Same as 6
16-Lambda gt10

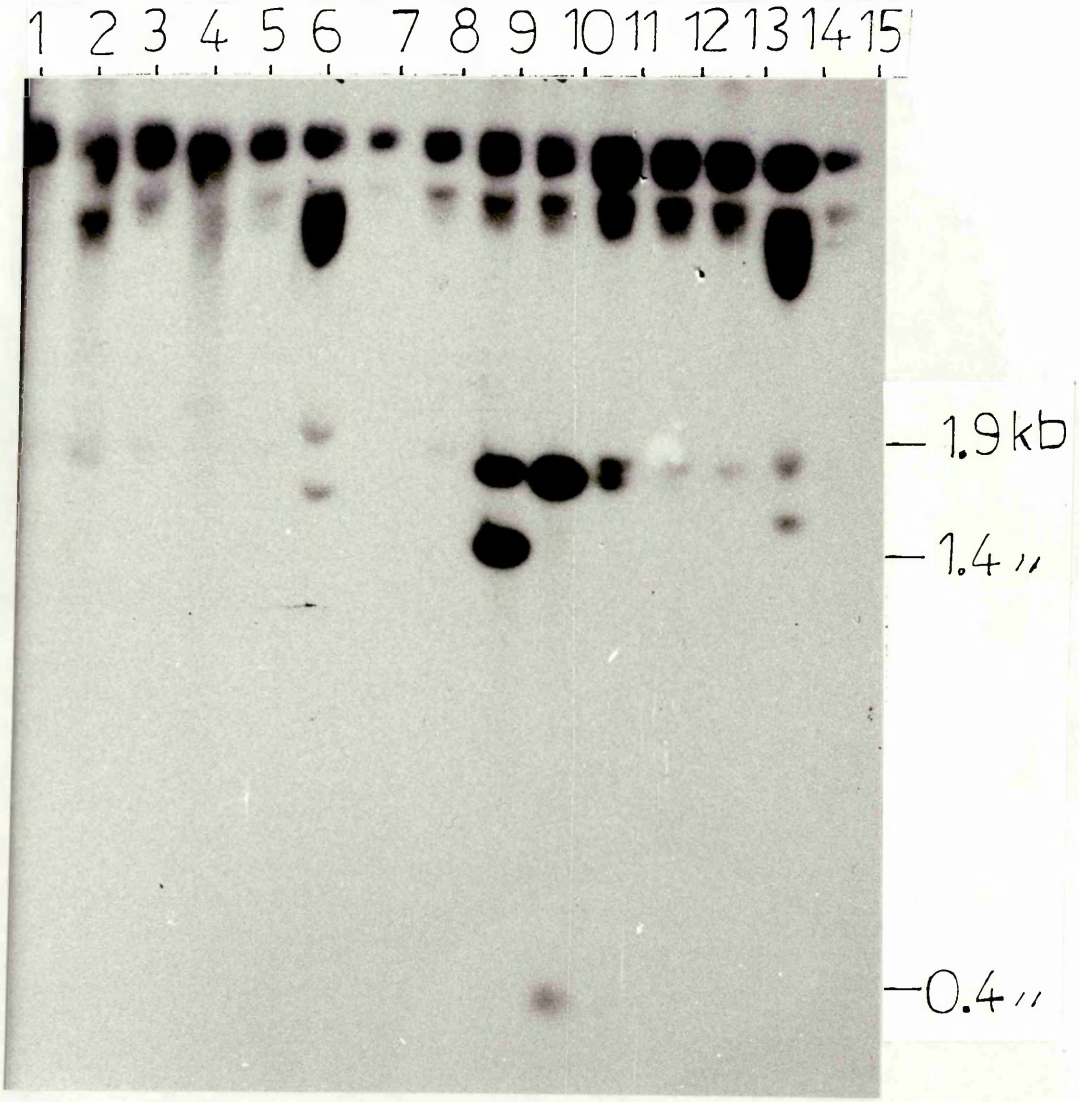


Figure 3.11 Placental FN clones hybridised with FN insert. Only inserts from two of the clones (\HCF17 and 24) have hybridised to the probe, and other clones either do not have inserts (not big enough) or the inserts are small (the figure is the hybridised filter of the gel in figure 3.10).

1-\HCF12
2-\HCF14
3-\HCF126
4-\HCF168
5-\HCF177
6-Lambda marker (EcoRI/HindIII)
7-Lambda gt10
8-\HCF11
9-\HCF17
11-\HCF24
12-\HCF126
13-\HCF168
14-\HCF177
15-Same as 6
16-Lambda gt10

IIICS specific fragments from pFH23 and pFH1 respectively, it became apparant that the recovery of such small fragments could not be easily achieved. It was then decided to cut the three FN clones (pFH1, pFH23 and pFH154) using various restriction enzymes, some of which would generate ED-I and IIICS fragments (Appendix 2). For this purpose pFH1 was cut with HaeIII, BstEII, EcoRI and BamHI restriction enzymes to generate a 204bp from the IIICS region (Fig. 3.12). The pFH23 was digested with HpaII, Sau3A, EcoRI and BamHI restriction enzymes to generate a 183bp fragment from the ED-I region (Fig. 3.12 and Appendix 2). These digestions, alongside some other digestions of these and other clones, were run on a 1.6% agarose gel (Fig. 3.12). The restriction enzymes for digesting the \HCF17 DNA were chosen as such that they would have at least one recognition site in the IIICS or ED-I regions. The information about the recognition sites had come from the available FN sequence (Kornblihtt *et al.*, 1985).

The above gel was Southern blotted and hybridised with the HCF17 insert. The autoradiograph of this blot showed that HCF17 hybridised to the 5' end of the pFH1 and to the 3' and 5' end of the pFH23 but not to the 204bp IIICS or 183bp ED-I fragemts (Fig. 3.13). The result of digesting the \HCF17 DNA when combined with the result of the hybridisation suggested that the IIICS and ED-I might have been spliced out (Figs. 3.12-13). These results combined with the results obtained above (Section 3.3, Fig 3.13) also indicated that the \HCF17 was most probably limited by the two EcoRI sites present in the FN sequence (Appendix 2).

3.3.2 Subcloning \HCF17 into pUC18

In order to determine the exact sizes of the spliced regions, more restriction analysis was needed. Large amount of HCF17 DNA was needed for such restriction analysis, but DNA preparation, from phage, usually does not yield the same amount DNA when compared

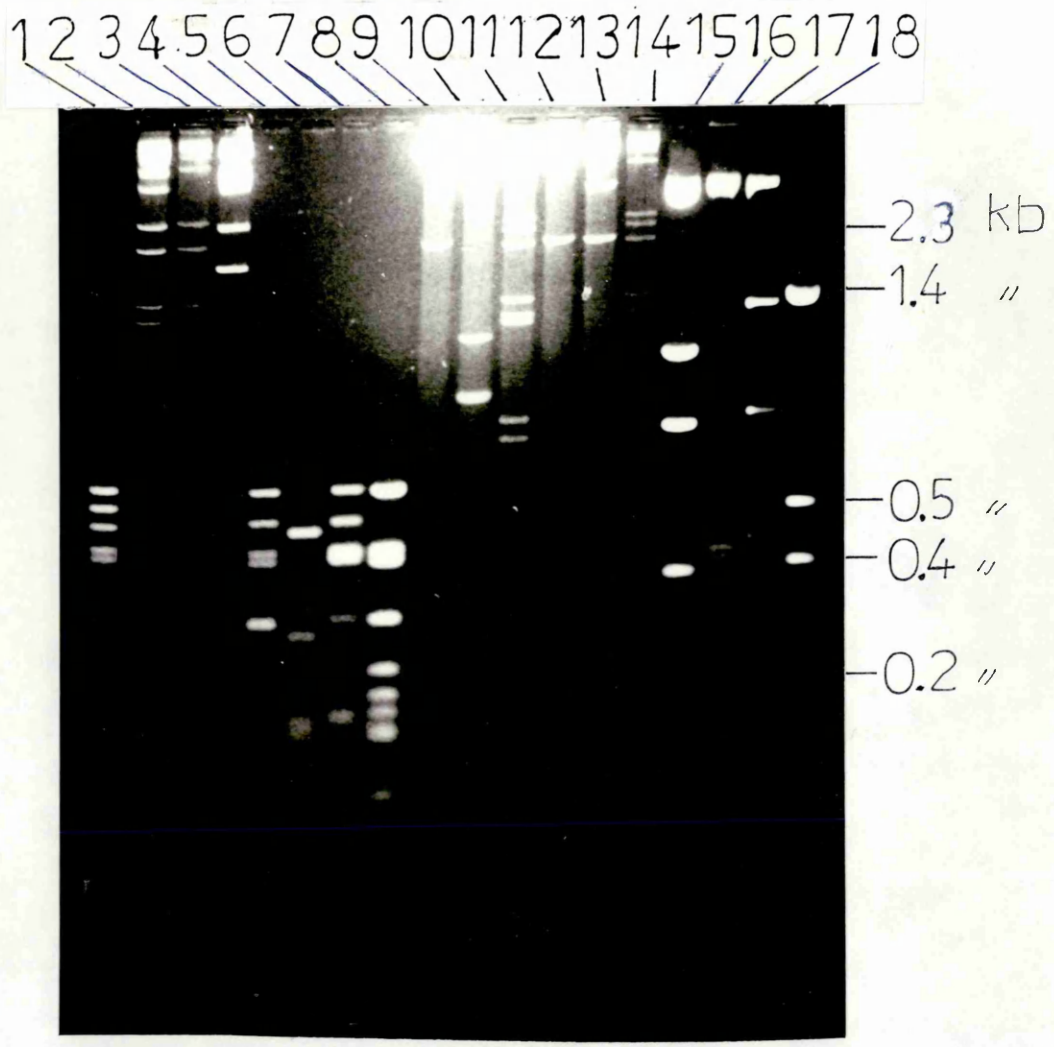


Figure 3.12 FN clones digested with various restriction enzymes. The digestion was mainly done to see the restriction pattern of \HCF17. It was hoped that these digestions would show whether ED-I and IIICS were present or absent. RI means EcoRI and * means digestion.

1- pBR322 Hae 3	digest	10-\HCF17 Bgl2/RI	digest
2- \HCF17 BstE 2	*	11-\HCF17 BstE2/RI	*
3- \HCF11 BstE 2	*	12-\HCF17 EcoRV/RI	*
4- Lambda marker Pvu 2	*	13-\HCF17 XhoI/RI	*
5-pFH1 Hae3/BstE2/RI/BamHI	*	14-\HCF11 BstE2/RI	*
6-pFH23 Hpa2/Sau3A/RI/BamHI	*	15-pFH1 Bgl2/BamHI/RI	*
7-pFH154 Hae3/BamHI/RI	*	16-pFH23 BstE2/BamHI/RI	*
8-Pat153 Hae3	*	17-pFH154 BstE2/BamHI/RI	*
9-\HCF17 RI	*	18-pUC18 HinFI	*

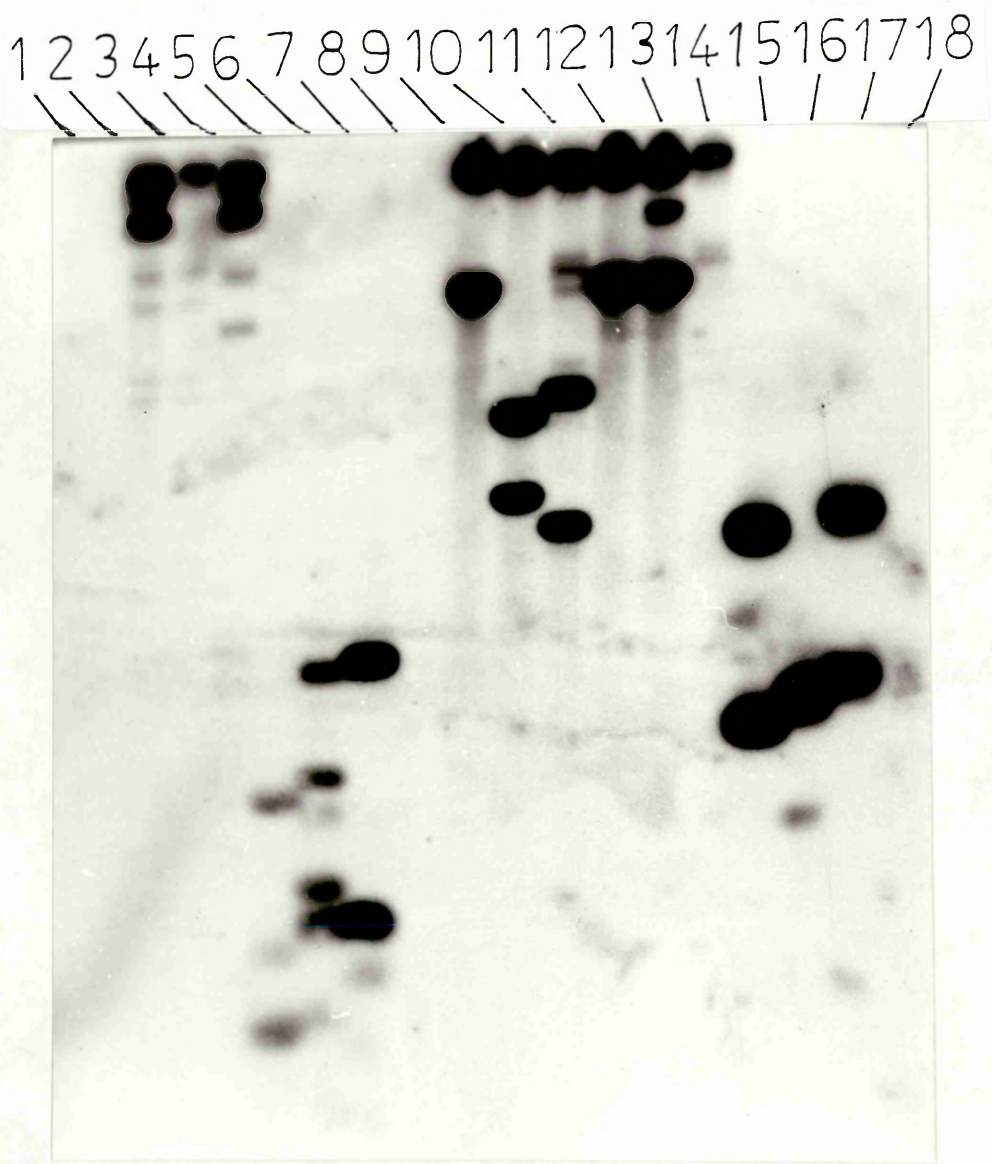


Figure 3.13 Southern blot hybridisation of the gel in figure 3.12. The gel was hybridised with the \HCF17 insert. RI menas EcoRI restrction enzyme and * means digestion.

1- pBR322 Hae 3	digest	10-\HCF17 Bgl2/RI	digest
2- \HCF17 BstE 2	*	11-\HCF17 BstE2/RI	*
3- \HCF11 BstE 2	*	12-\HCF17 EcoRV/RI	*
4- Lambda marker Pvu 2	*	13-\HCF17 XhoI/RI	*
5-pFH1 Hae3/BstE2/RI/BamHI	*	14-\HCF11 BstE2/RI	*
6-pFH23 Hpa2/Sau3A/RI/BamHI	*	15-pFH1 Bgl2/BamHI/RI	*
7-pFH154 Hae3/BamHI/RI	*	16-pFH23 BstE2/BamHI/RI	*
8-Pat153 Hae3	*	17-pFH154 BstE2/BamHI/RI	*
9-\HCF17 RI	*	18-pUC18 HinFI	*

with plasmid DNA preparation (Maniatis *et al.*, 1982 and personal experience). It was therefore decided to subclone the \HCF17 insert into a high copy number plasmid vector such as pUC18. There was another main advantage for such subcloning because pUC18 is, relatively speaking, much smaller size than \gt10 and therefore restriction manipulation of the pHCF17 would be much easier.

For the subcloning purpose, \HCF17 DNA was cut with EcoRI restriction enzyme to release the insert. The digested DNA was run on a 0.8% agarose gel. The insert DNA fragment was recovered from the gel using NAE-DEAE membrane (adapted from Young *et al.*, 1985; Chapter 2). The recovered DNA was phenol extracted and resuspended in TE buffer. On the other hand the pUC18 vector DNA was restricted with EcoRI and then dephosphorylated to reduce the recircularisation of the vector during ligation (Maniatis *et al.*, 1982; Chapter 2). After dephosphorylation, the DNA was then phenol extracted, ethanol precipitated and resuspended in TE.

The dephosphorylated vector and the insert DNA were mixed in different molar ratios and then prepared for ligation. The ligated DNA was transformed into DS941 cells and plated on selective media in presence of Xgal and IPTG. In these conditions the colonies containing the recombinant clones would be white and those containing the parental plasmids would be blue. After overnight incubation, several white colonies were picked and regrown on matrix plate. Colony lysate gel was prepared from these colonies and the positive clones were identified by having bigger sizes (Fig. 3.14). Alkaline lysate plasmid DNA was prepared from these clones and the DNA were tested, on agarose gel, for containg insert. The pHCF17 DNA was digested for further analysis.

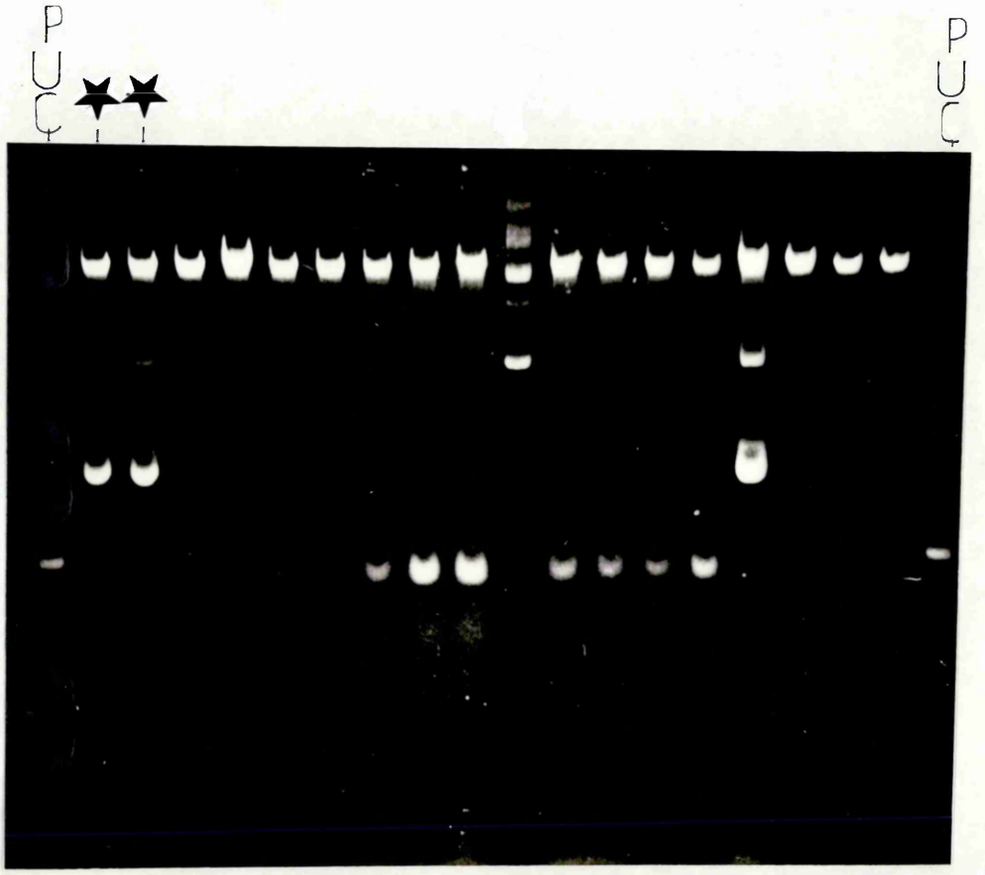


Figure 3.14 Single colony gel from subcloning HCF17 insert into pUC18. From those colonies chosen some show increase in size (*) which indicate that they contain an insert. Uncut pUC18 DNA was used as marker.

3.3.3 Restriction Analysis of the IIICS and ED-I Regions in pHCF17

The pHCF17 DNA was digested with the enzymes listed in the Table 3.1. The table lists two possible sets of restriction fragments which could be generated if the pHCF17 clone contains or lacks both of the two splicing regions (i.e. IIICS and ED-I). The predictions were based on the available DNA sequence (Kornblihtt *et al.*, 1985). These assumptions were based on the preliminary findings mentioned above in that the IIICS and ED-I might have been spliced out in HCF17. So if the digestions, with the enzymes listed, would generate either of the two sets then it could be concluded that the IIICS or ED-I or both are being spliced or are present, depending on the pattern seen.

The pHCF17 DNA was digested with the enzymes listed in the Table 3.1. Every digestion was combined with the EcoRI digestion (double digesion). The reason for the double digestions was that the EcoRI digestion would release the insert therefore eliminate any pUC18 sequence being carried over with the HCF17 fragments. Such a contamination, if not eliminated, would make the size estimation of the HCF17 fragments (from the Southern blot hybridisation) very difficult. The digested DNA were run on a 1.6% agarose gel and photographed (Fig. 3.15). The gel was then Southern blotted and probed with pHCF17 insert (Fig. 3.16).

Based on the results shown (Figs. 3.15-16) and also with the help of the size markers bands (lambda and pUC18, Fig. 3.15), a calibration curve was prepared and the size of the observed fragments for the pHCF17 were measured (Fig. 3.17). The values for the estimated and observed fragments are shown (Table 3.1 and Table 3.2 respectively). In most cases the total size of the observed fragments were similar (i.e. + or - 50bp) to the total size of the expected ones (e.g. 1990bp in total). There were, however, few exceptions such as lack of digestion with the EcoRV enzyme or in some cases the

Figure 3.15 Restriction analysis of pHCF17. The pHCF17 DNA was digested with various restriction enzymes. Some of these enzymes have also digested the pUC vector. RI means EcoRI and * means digestion.

1-pUC8 AccI/RI	digest	11-Lambda PvuII marker	
2-pHCF17 AccI/RI	*	12-pHCF17 RsaI/RI	digest
3-pHCF17 PstI/RI	*	13-pHCF17 TaqI/RI	*
4-pHCF17 SstI/RI	*	14-pHCF17 XhoII/RI	*
5-pHCF17 XbaI/RI	*	15-pHCF17 HaeII/RI	*
6-pHCF17 EcoRV/RI	*	16-pHCF17 HpaII/RI	*
7-pHCF17 BglII/RI	*	17-pHCF17 Sau3A/RI	*
8-pHCF17 BstEII/RI	*	18-pHCF17 HhaI/RI	*
9-pHCF17 BalI/RI	*	19-pUC18 Sau3A/RI	*
10-pHCF17 StuI/RI	*		

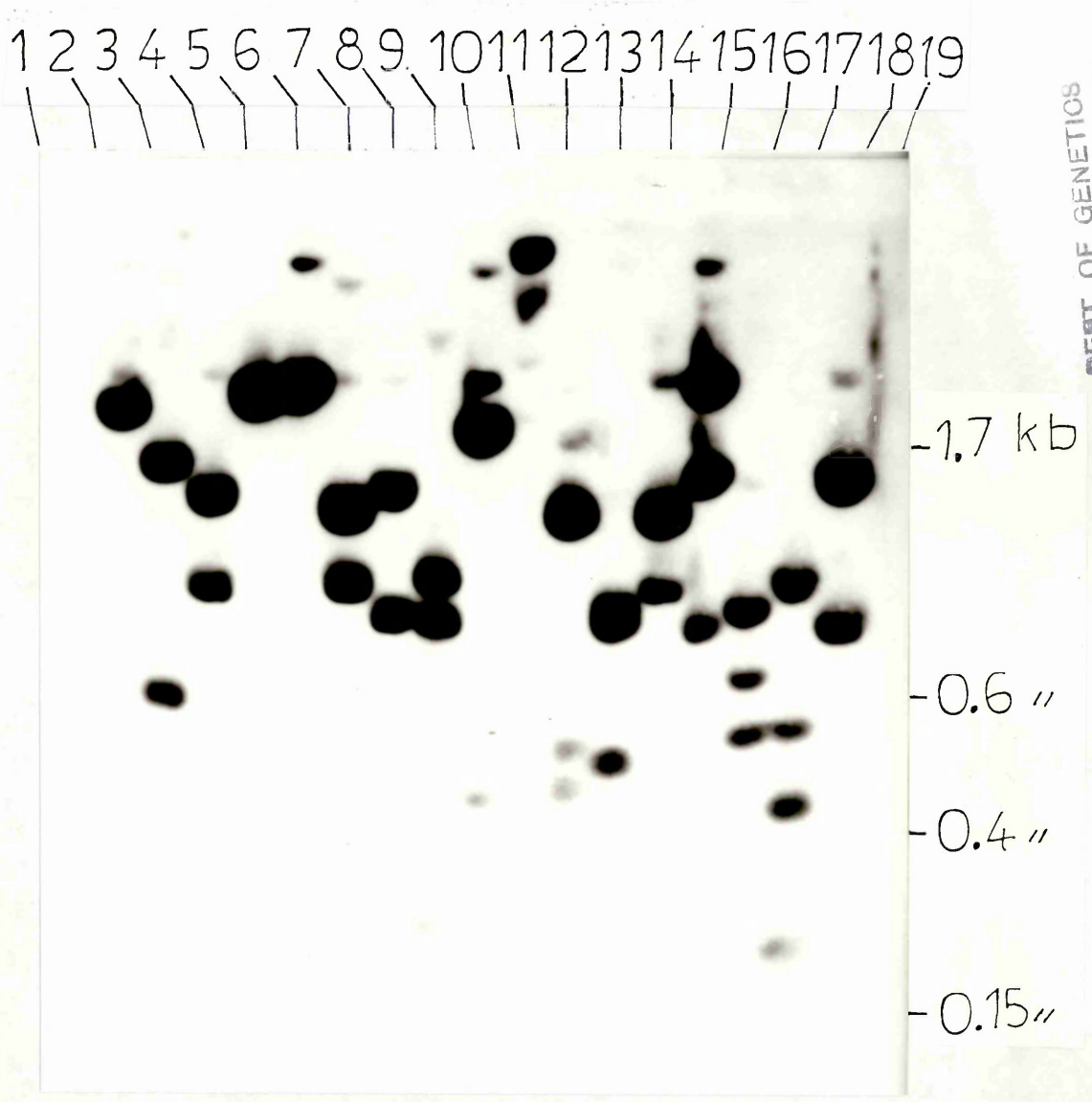


Figure 3.16 Southern blot hybridisation of pHCF17 insert with pHCF17 insert after various restriction digests. The gel in Figure 3.15 was Southern blotted and hybridised with the pHCF17 insert. RI means EcoRI restriction enzyme and * means digestion.

1-pUC8 AccI/RI	digest	11-Lambda PvuII marker	
2-pHCF17 AccI/RI	*	12-pHCF17 RsaI/RI	digest
3-pHCF17 PstI/RI	*	13-pHCF17 TaqI/RI	*
4-pHCF17 SstI/RI	*	14-pHCF17 XhoII/RI	*
5-pHCF17 XbaI/RI	*	15-pHCF17 HaeII/RI	*
6-pHCF17 EcoRV/RI	*	16-pHCF17 HpaII/RI	*
7-pHCF17 BglII/RI	*	17-pHCF17 Sau3A/RI	*
8-pHCF17 BstEII/RI	*	18-pHCF17 HhaI/RI	*
9-pHCF17 BalI/RI	*	19-pUC18 Sau3A/RI	*
10-pHCF17 StuI/RI	*		

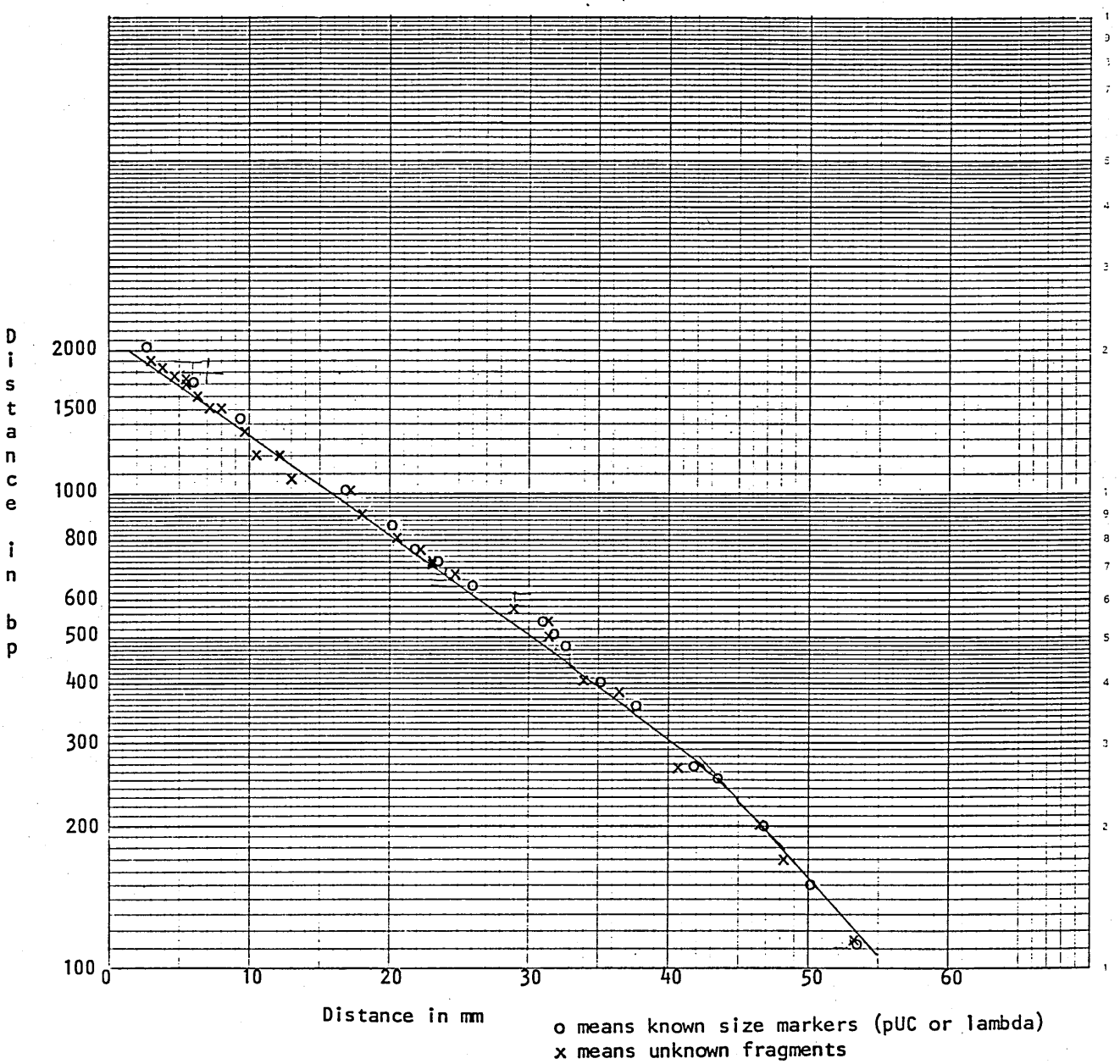


Figure 3.17 Calibration curve. The physical distance of each fragment is measured from the top of the gel (photo) and then after ploting every distance, the size of the unknown fragments are estimated compared with the size of the marker fragments.

Enzyme	Fragments expected to be seen in bp	
	With IIICS and ED-A	Without IIICS and ED-A
AccI	2353, 270	1720, 270
BalI	1075, 1028, 205, 179, 93, 43	895, 758, 205, 93, 43
BglII	1409, 827, 387	1139, 851
BstEII	862, 759, 726, 276	1225, 765
EcoRI	2623	1990
EcoRV	1601, 1022	1238, 752
HaeII	1638, 985	1275, 715
HhaI	1637, 986	1274, 716
HpaII	960, 850, 507, 205, 101	785, 597, 507, 101
PstI	1796, 536, 177, 114	1340, 536, 114
RsaI	753, 744, 558, 460, 108	1149, 460, 385
Sau3A	703, 623, 387, 340, 165, 123, 120, 120, 42	854, 473, 340, 165, 120, 42
SstI	1443, 1180	1173, 817
StuI	1410, 840, 373	1617, 373
TaqI	1163, 704, 436, 255, 65	800, 754, 436
XbaI	2512, 111	1879, 111
XhoII	1409, 927, 387	1139, 851

Table 3.1 Restriction fragments expected to be generated when HCF17 is cut with the restriction enzymes listed (the size of the expected fragments were deduced from the published FN sequence, Kornblihtt et al., 1985; Appendix 2). The expected sizes were based on the assumption that both of the spliced regions were either present or absent. These asumptions were based on the preliminary restriction map of HCF17 and HCF24. Whenever the assumption was that the IIICS was present, its size was assumed to be 360bp but if it was assumed to be absent, its size was assumed to be 267bp (see text for detail).

Enzyme	Estimated and observed fragments of pHCF 17 in bp		
	Obseved		Estimated
AccI	1750, 280	[2030]	1720, 270
BalI	890, 750, 200,,100	[1940]	895, 758, 205, 93, 43
BglII	1180, 870	[2050]	1139, 851
BstEII	1260, 760	[2020]	1225, 765
EcoRI	2000	[2000]	1990
EcoRV	2000	[2000]	1238, 752
HaeII	1300, 710	[2010]	1275, 715
HhaI	1300, 730	[2030]	1274, 716
HpaII	760, 590, 480, 110	[1940]	785, 597, 507, 101
PstI	1400, 540, 110	[2050]	1340, 536, 114
RsaI	1150, 450, 370	[1965]	1149, 460, 385
Sau3A	870, 470, 340, 170	[1850]	854, 473, 340, 165, 120, 42
SstI	1200, 840	[2040]	1173, 817
StuI	1580, 360	[1940]	1617, 373
TaqI	760, 730, 410	[1900]	800, 754, 436
XbaI	1850, 110	[2060]	1879, 111
XhoII	1120, 830	[1950]	1139, 851

Table 3.2 The size of the estimated and observed restriction fragments for the restriction enzymes listed are shown. In most cases the total sizes of the observed fragments (shown within brackets [ ]) were similar (i.e. + or - 50bp) to the total size of the expected ones (1990bp). There were few exceptions (e.g. EcoR V lack of digestion or fragments too small to be seen, Sau3A and BalI). Other differences (larger than 50bp) could have been caused by inaccuracy of readings from the calibration curve. For more information see Table 3.1 and the text.

generated fragments were too small to be easily detected on the gel (e.g. Sau3A and BalI). Also larger than 50bp differences could have been caused by inaccuracy of readings from the calibration curve due to the way calibration curve are made and the unknown sizes of the fragments are measured.

The above findings confirmed the absence of IIICS and ED-I sequences in the HCF17 clone. The absence of 267bp from the IIICS and 270bp from the ED-I sequence are obvious from the sizes of the fragments seen (Figs. 3.15-16 and Table 3.2). The presence of splicings and their sizes can be supported by the following arguments which is based on the above findings:

(a) If the total 360bp of IIICS was present in the HCF17 then the EcoRI/BglII digestions should have generated an 827bp and a 387bp fragments and if it was absent it should have generated only an 851bp fragment instead. The observed fragment was 870bp (close to the 851bp) which suggest the absence of all of the 360bp IIICS sequence.

(b) If the total of 360bp from the IIICS was present then the EcoRI/Sau3A digestions should have generated a 703bp, a 387bp and a 123bp fragments and if it was absent it should have generated only an 853bp fragment. The observed fragment was 870bp (close to 853bp) which again suggests the splicing of all of the 360bp from the IIICS sequence.

(c) If the total 360bp of IIICS was present in the HCF17 then the EcoRI/BstEII digestions should have generated an 862bp and a 726bp fragments and if it was absent it should have generated a 1225bp fragment instead. The observed fragment was 1260bp (close to 1225bp) which again suggests the absence of all of the 360bp IIICS sequence.

(d) If the 270bp of ED-I was present in the HCF17 then EcoRI/HaeII digestions should have generated a 985bp fragment and if it was absent it should have generated a 715bp fragment. The observed fragment was 720bp which suggests the absence of all of the 270bp of ED-I sequence.

(e) If the 270bp of ED-I was present in the HCF17 then EcoRI/BstEII digestions should have generated a 276bp and a 759bp (1035bp) fragments and if it was absent it should have generated a 765bp fragment. The observed fragment was 760bp again suggesting the absence of all of the 270bp from ED-I sequence.
(f) If the 270bp of ED-I sequence was present in the HCF17 then the EcoRI/SstI digestions should have generated a 1443bp fragment and if it was absent it should have generated a 1173bp fragment. The observed fragment was 1180bp which suggests the absence of all of the 270bp ED-I sequence.

The above findings indicated that the entire ED-I and IIICS regions (270bp and 360bp respectively) had been spliced out. Confirmation of the absence of the total sequence from the IIICS region was more difficult because the published DNA sequence by Kornblihtt *et al.* (1985) was lacking the extra 93bp and only had 267bp out of the 360bp. The knowledge about the rest of the IIICS sequence came from another work (Sekiguchi, 1986) and their sequence showed that another 93bp can be also spliced out. This extra 93bp increased the total size of the IIICS to 360bp.

The exact size of the IIICS, however, was not accurately measurable by the above types of experiments and it was necessary to resolve it by, for example, sequencing the pHCF17 clone (see section 3.6).

3.4 Restriction Analysis of the \HCF24

The preliminary restriction analysis done on \HCF24 DNA showed that the insert (HCF24) was about 2.6kb that is:

1700 + 1650 + 390 = 3740bp so 3740 - 1140 = 2600bp

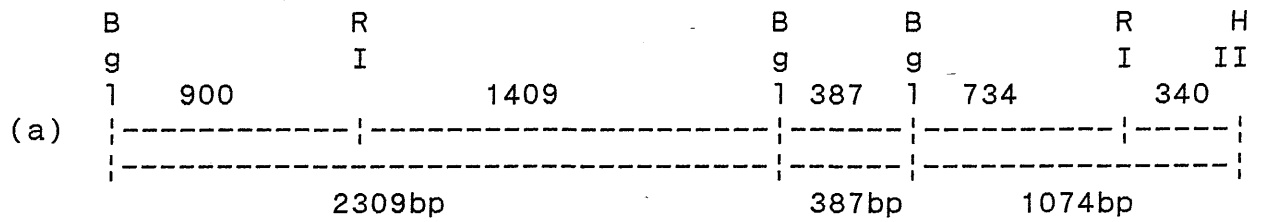
[The 1140bp is from \gt10 arms (Fig. 3.18)].

Generation of the 390bp fragment from the BglII/HindIII digests suggested the presence of some or all of the 360bp from the IIICS region. The digestion

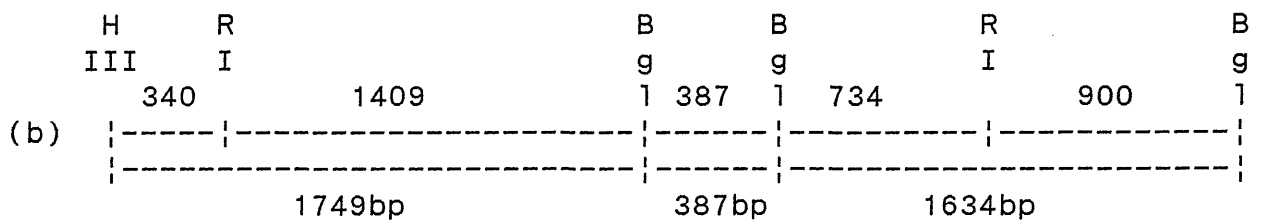
also showed the orientation of cloning into the \gt10 and it was deduced from one of the two possibilities shown in Fig. 3.18. The orientation map was deduced from the observed fragments on the gel and from the knowledge that within the limit of \HCF24 clone (i.e. hybridising to 5' end of the pFH 1 and pFH23 and not hybridising to the 3' end of the pFH1 or 5' end of the pFH154) there is no HindIII site and there are two BglII site, one of them within the IIICS region (Appendix 2). Finally when the \HCF24 was digested with BglII/HindIII, fragments of about 1700bp, 1650bp and 380bp were observed (Figs. 3.10-11). This finding suggested that the possibility shown in the prediction (b) in Fig. 3.18 may be correct.

The above finding, however, did not clearly show whether the \HCF24 clone contained the sequences corresponding to the two splicing regions or not. For this purpose more restriction digests were needed to find out the inclusion or splicing of IIICS and ED-I sequences. A set of restriction digests were, therefore, carried out with some of the enzymes listed in the Table 3.1. The pHCF17 DNA was also digested with the same enzymes to help the analysis as well as seeing whether the predicted fragments shown in Table 3.1 were in agreement with observed fragments for both of the clones. The digested DNA were run on 1.6% agarose gel and then photographed (Fig. 3.19). The gel was then Southern blotted and probed with pHCF17 insert (Fig. 3.20).

The results showed that the EcoRI digest generated a fragment similar in size with pUC18 (about 2.6kb, Fig. 3.19). This finding, when compared with the BglII/HindIII digests (Section 3.3), indicated that the \HCF24 clone had no internal EcoRI site which may suggest that the clone was flanked by two EcoRI sites present in the published sequence (i.e. 3' and 5' end of the \HCF17 and \HCF24 and the 5' end of the \HCF11). Restriction analysis showed that the position of these sites were the same as the position of the internal EcoRI sites present within the FN cDNA sequence. A



Expected BglII/HindIII fragments 2309bp, 387bp and 1074bp



Expected BglII/HindIII fragments 1749bp, 387bp and 1634bp

Figure 3.18 Possible orientations which /HCF24 might have been cloned into /gt10. The possibilities have come from the assumption that /HCF24 clone was contained within the two EcoRI sites (see text and Appendix 2). In each possibility the predicted fragments which might have been generated are shown (a and b; the lower numbers). Number in the upper lines are in base pairs (bp). Bgl means BglII, RI means EcoRI and HIII means HindIII restriction sites.

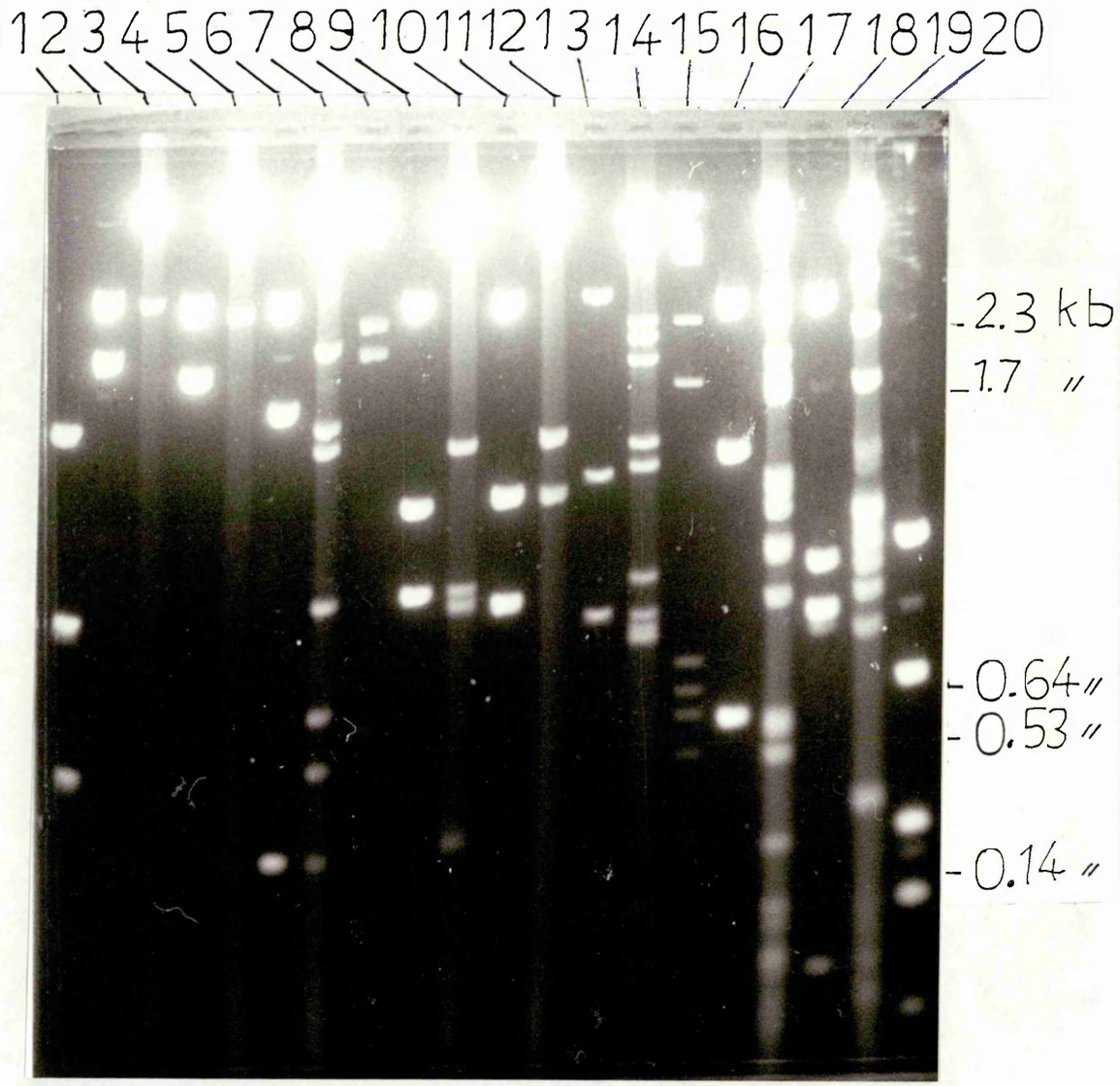


Figure 3.19 Restriction analysis of Fibronectin clones pFH17 and \HCF24. These two clones were restricted with various restriction enzymes to estimate the size of \HCF24 size and splicing pattern. RI means EcoRI and * means digestion.

1-pUC18 TaqI digest	11-pHCF17 SstI/RI digest
2-pHCF17 RI *	12-\HCF24 SstI/RI *
3-\HCF24 RI *	13-pHCF17 BstEII/RI *
4-pHCF17 XbaI/RI *	14-\HCF24 BstEII/RI *
5-\HCF24 XbaI/RI *	15-Lambda PvuII marker
6-pHCF17 StuI/RI *	16-pHCF17 PstI/RI *
7-\HCF24 StuI/RI *	17-\HCF24 PstI/RI *
8-Lambda Hind3 marker	18-pHCF17 BalI/RI *
9-pHCF17 BglII/RI *	19-\HCF24 BalI/RI *
10-\HCF24 BglII/RI *	20-pUC18 Sau3A *

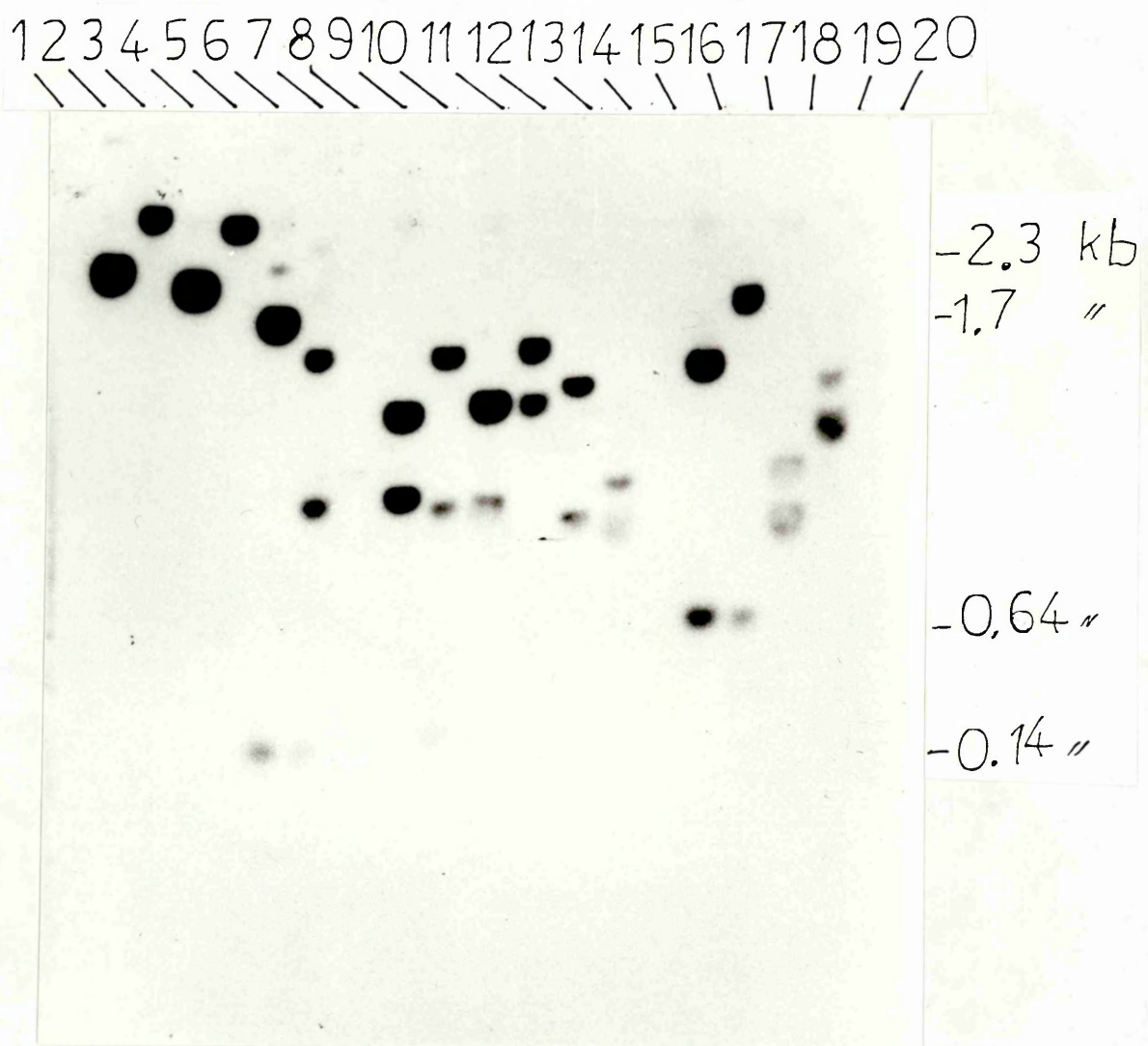


Figure 3.20 Southern hybridisation of \HCF24 clone. The clone was probed with the pHCF17 insert and the gel came from the digestion shown in Figure 3.19. RI means EcoRI restriction enzyme and * means digestion.b

1-pUC18 TaqI digest	11-pHCF17 SstI/RI digest
2-pHCF17 RI *	12-\HCF24 SstI/RI *
3-\HCF24 RI *	13-pHCF17 BstEII/RI *
4-pHCF17 XbaI/RI *	14-\HCF24 BstEII/RI *
5-\HCF24 XbaI/RI *	15-Lambda marker PvuII
6-pHCF17 StuI/RI *	16-pHCF17 PstI/RI *
7-\HCF24 StuI/RI *	17-\HCF24 PstI/RI *
8-Lambda marker HindII	18-pHCF17 BalI/RI *
9-pHCF17 BglII/RI *	19-\HCF24 BalI/RI *
10-\HCF24 BglII/RI *	20-pUC18 Sau3A *

possible reason for this observation might have been due to the lack of methylase protection of the EcoRI sites during the cDNA cloning. If this was the case then none of the above clones would have been long enough to contain the third splicing region, ED-II ED-B) (Gutman and Kornblihtt 1987).

In order to see whether the entire 360bp from the IIICS region, including the rarely observed 93bp at the 3' end of the IIICS region, was present in \HCF24 clone a similar approach to \HCF17 was taken (Table 3.3 and Fig. 3.19 respectively).

The observation indicated the presence of the total 360bp of the IIICS region. The presence of the 360bp can be argued as follow:

(a) The StuI digestion of \HCF24 would have generated a 1317bp if the 360bp was absent but a fragment of about 1400bp was generated. The size of the observed fragment is about 1400 rather than 1300 because the band is equal in size with the constant band of BglII digest of \HCF24 (1409bp); compare tracks 7 and 8 (Figs. 3.19-3.20).

(b) The presence of entire 360bp can also be suported by the observation that BglII digestion of \HCF24 had generated a fragment of about 820bp instead of 730bp. The size of the fragment is about 820bp rather than 730bp because the band is equal in size with the constant band of SstI digest of pHCF17 (821bp); see tracks 10 and 11 and also it is not equal to the 765bp band of BstEII digest of pHCF17, compare tracks 10, 11 and 13 (Figs. 3.19 and 3.20).

(c) The SstI digestion of \HCF24 would have generated a 1087bp instead of 1180bp if the 360bp was absent but the observed fragment was about 1180bp. This can be supported by the fact that the band is equal in size with the constant band of SstI digest of pHCF17 (1173bp) and it is bigger than the 1139bp fragment of BglII digest of pHCF17 suggesting it being bigger that 1087bp; see tracks 12, 11 and 10 respectively (Figs. 3.19 and 3.20).

(d) The presence of entire 360bp can be suported as well by the observation that BstEII digestion of \HCF24 had

generated an 860bp fragment instead of 769bp. The band is about 860bp instead of 769bp because it is bigger than the constant band of BstEII and SstI digests of pHCF17 (765bp and 821bp respectively); see tracks 14, 13 and 11 respectively (Figs. 3.19 and 3.20).

The presence of ED-I can be supported by the band shifts seen above (Figs. 3.19-20 and Table 3.3). More proofs can be given by fragments generated by the BstEII digestion. There is a BstEII site within the published sequence for the ED-I region (Appendix 2). If one assumes that ED-I has been spliced out (but IIICS being present) then BstEII digest should have generate three fragments, 1035bp, 864bp and 726bp and if ED-I was present it should have generated four fragments, 864bp, 759bp, 726bp and 276bp (Appendix 2). There were no 1035bp fragment present and the largest fragment observed was about 870bp which all suggested the presence of ED-I (Figs. 3.19 and 3.20; see also the argument which was presented to support of the presence of the IIICS sequence in \HCF24 above, d). Further proof was obtained when \HCF24 was restricted with XhoI. There is only one XhoI restriction site present in the FN sequence, between the two EcoRI sites. This site is located within the ED-I region (Appendix 2). If the XhoI digestion of \HCF24 could have generated two fragments (1857bp and 768bp), it would have meant that the ED-I was present and if it had generated only one fragment (2353bp) then it would have mean that the ED-I had been spliced out. The XhoI digestion of \HCF24 indeed generated only two fragments of about 1850bp and 800bp suggesting the presence of the ED-I (Figs. 3.21-22).

3.5 Restriction analysis of \HCF11

As it was mentioned earlier, the \HCF11 clone was also recovered from the cDNA library and the tertiary screening showed that it hybridised to the 3' end of the EcoRI digest of pFH1 (section 3.2.3). This finding

Enzyme	360bp present	267bp present	Observed
EcoRI	2623	2530	2600
XbaI	2512, 111	2419, 111	2500
StuI	1410, 840, 373	1317, 840, 373	1400a
BglII	1409, 827, 387	1409, 734, 387	820b
SstI	1443, 1180	1443, 1087	1180c
BstEII	862, 759, 726, 276	769, 759, 726, 276	860d
PstI	1796, 536,177,114	1703, 536, 177, 114	1800
BalI	1073, 1028, 205, 179 93, 43	980, 1028, 205, 179 93, 43	1070

Table 3.3 Restriction digest prediction of /HCF24. The fragments sizes show two assumptions: either the entire 360bp or only 267 bp of the IIICS sequences are present. The fragments which should contain the extra 93bp of the IIICS have been underlined. The right column shows the observed sizes of such fragments. The small letters (e.g. a,b,c and d) are the observed fragments which support the inclusion of the 93bp (for detail see the text).

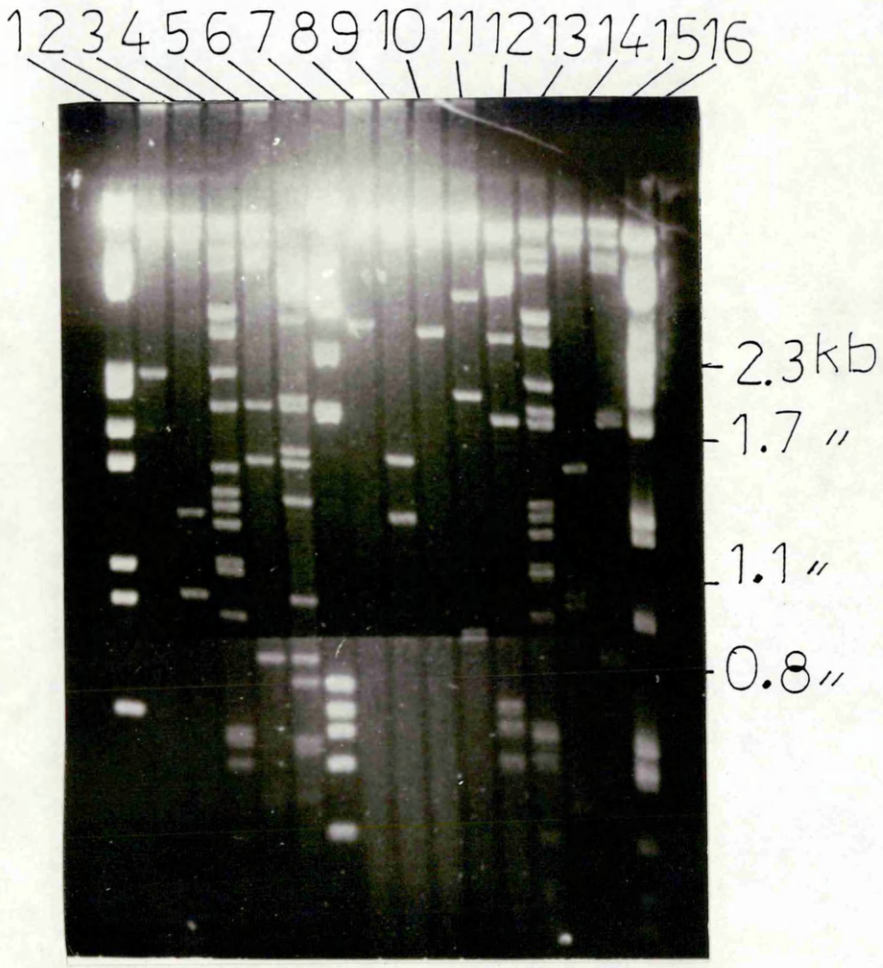


Figure 3.21 Restriction analysis of \HCF24. The clone was digested with XhoI/EcoRI to see whether it generates two fragments (1857 bp and 768 bp when ED-I was present) or only one fragment (2353 bp when ED-I if absent). Three fragments can be seen on the gel but Southern blotting will tell if there is two fragments or one. The XhoI/EcoRI digest is in track 11.

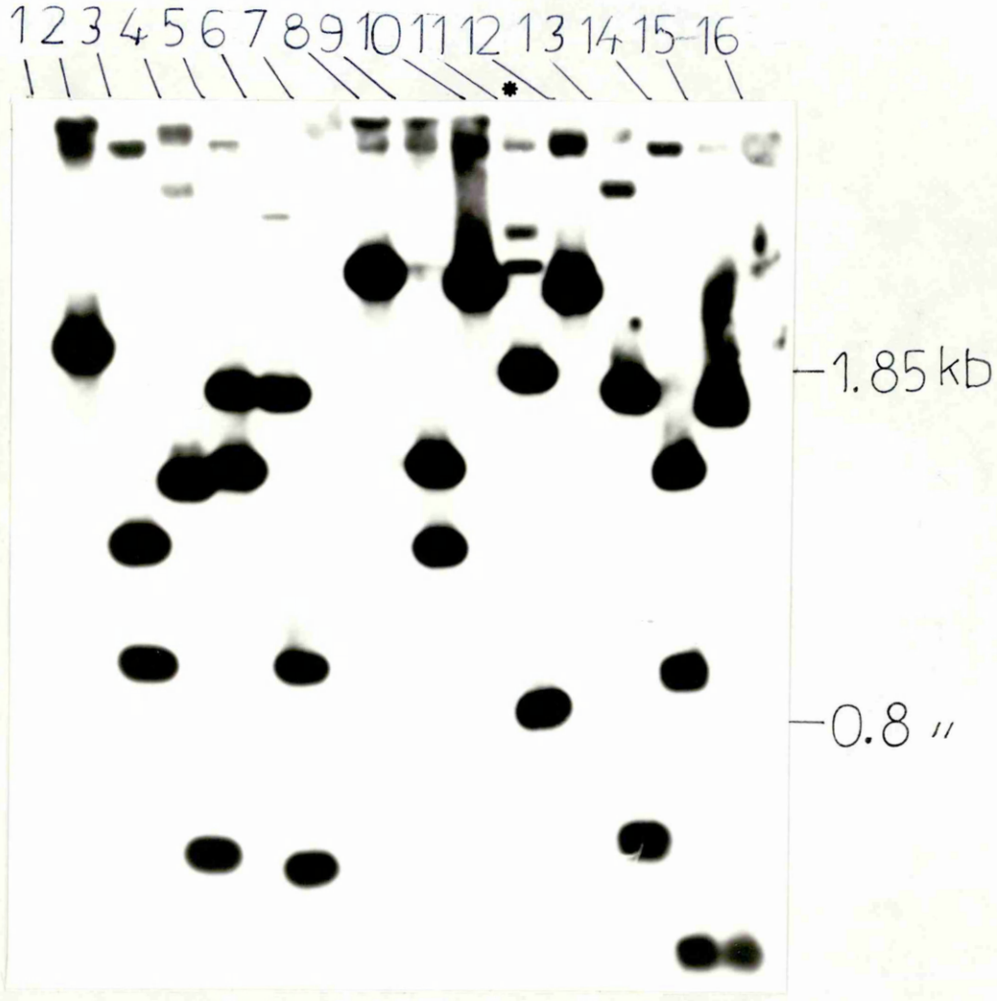


Figure 3.22 Southern analysis of \HCF24 clone. The clone was digested with XhoI/EcoRI to see whether it generates two fragments (1857 bp and 768 bp when ED-I was present) or only one fragment (2353 bp when ED-I if absent). Two fragments have hybridised to the probe (pHCF17 insert) which confirms the presence of the ED-I in the \HCF24 clone. The XhoI/EcoRI digest is shown by a (*).

suggested that this clone might not be long enough to extend toward the two splicing regions (i.e. IIICS and ED-I). The \HCF11 DNA was restricted with BglII/HindIII restriction enzymes to find out the size of this clone and to see whether it extended beyond the EcoRI site present at the 3' end of the cDNA (Appendix 2). The restriction digest analysis showed that the \HCF11 was about 620bp and hybridisation probing showed that this clone did not hybridised to the HCF17 clone indicating that it was extended toward the 3' end of the EcoRI site in the 3'end of the cDNA while \HCF17 was extended toward the 5' end of the EcoRI site (3.10-11). Because of this finding no further analysis on this clone was carried out.

3.6 Sequencing the HCF17 Clone for the Absence or Presence of IIICS

The restriction analysis of the \HCF17 which was mentioned earlier, suggested that the IIICS was spliced out but it was said that the exact size of spliced sequences could not be accurately measured. The accurate measurement of the splice fragment seemed important since several observations have indicated that the IIICS shows complex pattern of splicing which can generate up to 5 FN isoforms (Chapter 1). The best way to resolve this ambiguity was to determine the DNA sequence of the regions flanking the IIICS. At the time dideoxy chain termination method of Sanger *et al.* (1977), using M13 vectors, was the preferred method for sequencing therefore it was decided to suclone pHCF17 into M13.

3.6.1 Subcloning HCF17 into M13mp18

For the subcloning purpose the pHCF17 DNA was cut with EcoRI/StuI. The EcoRI digest would release the insert and the StuI digest cuts the clone only once, close but away from the IIICS regions. Such a digestion would generate two fragments, 1620bp and 373bp. The

bigger fragment would containe the IIICS splice site and the StuI would be only 147bp from the 5' end of the IIICS region (Appendix 2). Thus about 150bp sequence from the StuI site should be able to confirm the exact size of the spliced sequence in this region.

The EcoRI/StuI digests of pHCF17 was phenol extracted and after ethanol precipitation was resuspended in TE buffer. The M13 DNA was cut with EcoRI/SmaI enzymes and then the DNA was dephosphorylated to reduce the chance of recircularisation of the vector during the subcloning. The M13 DNA was also phenol extracted and ethanol precipitated and then resuspended in TE buffer. The two DNA were mixed and allowed to ligate at 7^{o}C overnight. The ligated DNA was transformed into competent JM101 *E. coli* cells. The cells were grown in the presence of Xgal and IPTG to allow the distinction between the recombinants and parental phages.

White positive plaques of M13 were picked and double stranded DNA from these clones were made by STET prep (Chapter 2). The DNA were dotted onto a nylon membrane and hybridised with 3' end of the pFH154 probe. The use of 3' pFH154 was to select only clones containing the IIICS spliced region (Fig. 3.23). Several positive clones were identified (Fig. 3.24) and single stranded DNA were made from some of them and used for determining the DNA sequences.

3.6.2 Sequencing the Flanking Regions of the Spliced IIICS in pHCF17

The DNA sequencing was performed following the instruction in the BRL "M13 Cloning/Dideoxy Sequencing Instruction Manual" supplied with the BRL Sequencing Kit with few minor changes reflected in the methods section (Chapter 2). After determining the right dideoxy nucleotides concentration (by performing a series of sequencing reactions and testing them by TCA precipitation, results not shown), the new sequencing

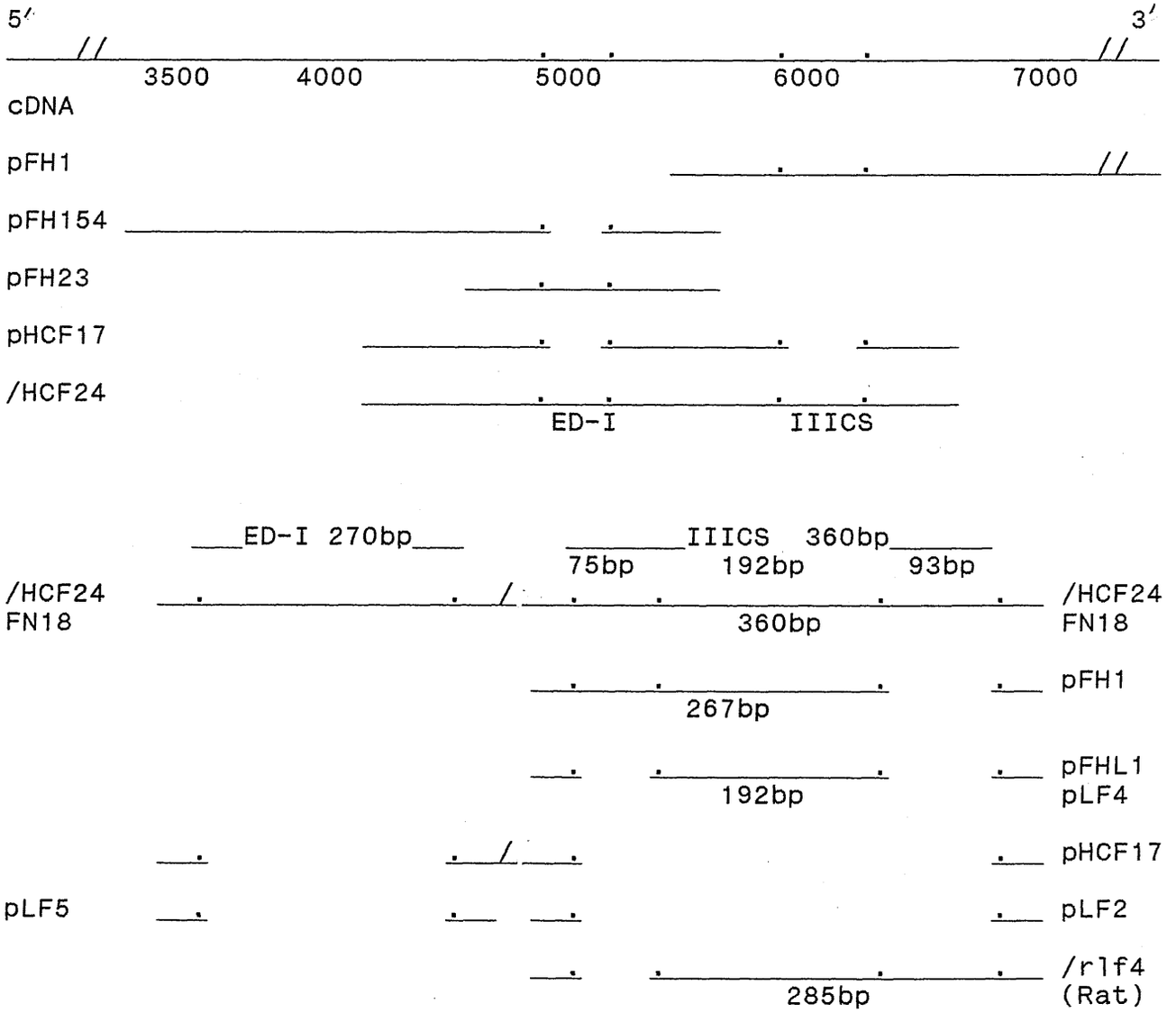


Figure 3.23 Schematic representation of human and rat liver FN cDNA variation at ED-A and IIICS regions. At the ED-A region there are two variants one with the ED-A [e.g., pFH23, Kornblihtt et al. (1984) and FN18 Bernard et al. (1985)]. The other one without the ED-A [e.g., pFH154, Kornblihtt et al. (1984) and pLF5 Sekiguchi et al. (1986)]. There have been four variations reported in the IIICS region in human: FN18 (Bernard et al. 1985) and /HCF24 both contain 360 bp, including both the 75bp and 93 bp extra segment flanking the central 192 bp; pFH1 (Kornblihtt et al. 1983) lacks the 93 bp flanking segment; pFHL1 (Umazawa et al. 1985) and pLF4 (Sekiguchi et al. 1986) lack both the 5′and 3′ flanking sequences; pLF2 (Sekiguchi et al 1986) and pHCF17 lack the entire 360 bases. The fifth variation in IIICS region have been reported to exist in rat liver [e.g. /rlf4 Schwarzbuer et al. (1983) which lacks the 5′ 75 bp flanking sequence. Dots locates the alternative splicing sites, -//- represent continuity and -/ - indicates contiguity.

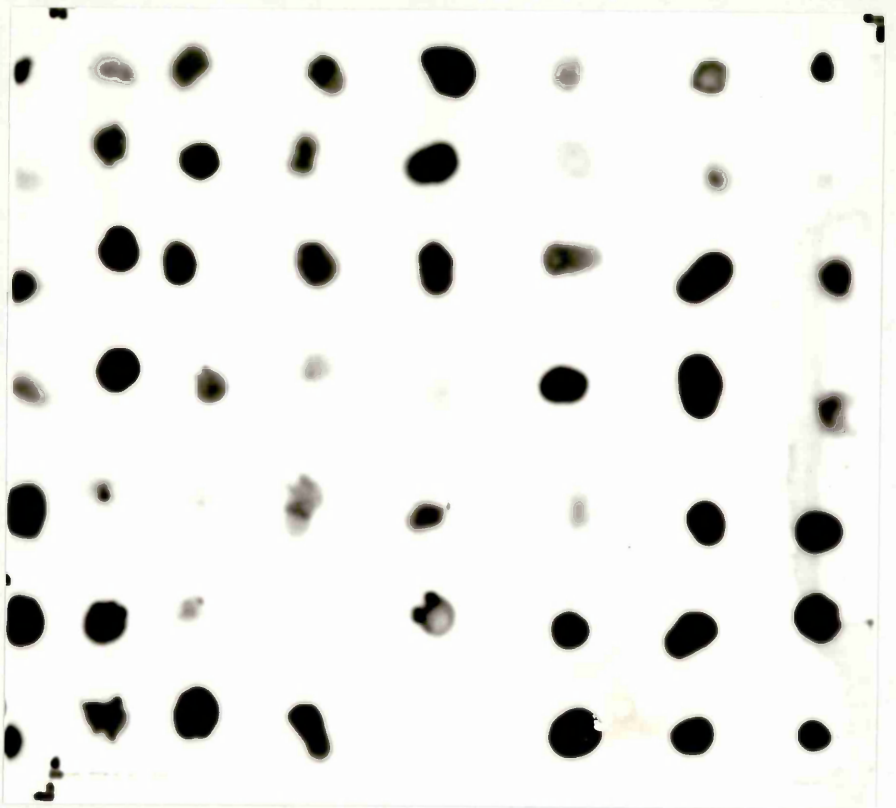


Figure 3.24 Subcloned HCF17 insert into M13. The pHCF17 clone was subcloned into the M13 and positive clones were easily identified by filter hybridisation with pHCF17 insert.

were performed and run on 8% polyacrylamide gel.

The autoradiography of the above gel helped to determine more than 200 bases of the HCF17 clone (Fig. 3.25). The determined sequences were compared with the published FN cDNA sequences (Kornblihtt *et al.*, 1985; Bernard *et al.*, 1985; Sekiguchi *et al.*, 1986). The comparisons confirmed the earlier prediction and showed that all of the 360bp of the IIICS had been spliced out. This meant that even the rarely seen 93bp at the 3' end of the IIICS was absent in the HCF17 clone (Figs. 3.23 and 3.26). The nucleotide sequence of the 200bp showed no other changes (e.g. deletion, addition or point substitution) when compared with the published FN sequence (Kornblihtt *et al.*, 1985).

Discussion

FN proteins are one of the major component of the extra cellular matrices and they are involved in a wide variety of biological activities (Chapter 1). These proteins have been intensely studied in recent years and their molecular and protein structures have been characterised. The difference between cellular and plasma FN is thought to have been mainly caused by the differences at the mRNA level. These differences have been cause by alternative splicing of the FN pre-RNA precursors (Kornblihtt et al., 1985; Dufour et al., 1988; see also Chapter 1). Despite these findings which have been mainly from human normal and transformed cell lines as well as plasma, little is known about the molecular structure of human placental FN.

A cDNA library, originated from chorionic plate of human term placenta, was screened for FN clones using human FN cDNA probes. The screening resulted in the

```
           10            20            30            40
TGT CTC CGA TGA TAA TGA CCG GAC CTT GGC CCT TGG CTT ATA TGT TAA

50            60            70            80            90
ATA CAG TAA CGG GAC TTC TTA TTA GTC TTC TCG CTC GGG GAC TAA CCT

  100             *           120           130           140
TCC TTT TTC TGT CCT GTT CTT CGA GAG AGA GTC TGT TGG TAG AGT ACC

    150           160           170           180           190
CGG GGT AAG GTC CTG TGA AGA CTC ATG TAG TAA AGT ACA GTA GGA CAA

       200           210           220           230           240
CCG TGA CTA CTT CTT GGG AAT GTC AAG TCC CAA GGA CCT TGA AGA TGG

          250
TCA CGG TGA GAC TGT CC
```

Figure 3.25 Nucleotide sequence of the 3' and 5' flanking regions of the spliced IIICS in human placental cDNA clone HCF17. Only the sequence of one of the strands (sense strand) is shown. The splice site is indicated by a star (*) between TGT C*CT GTT. The amino acid coding sequence for Gly (GGA) at the splice site has not been altered by splicing.

(a) [270bp spliced]

3' CCTTTTTCTGTCtgctcgaag.../ /...cttacatcCACTCCTTTAGG 5'

5' GGAAAAAGACAGacgagcttc.../ /...gaatgtagGTGAGGAAATCC 3'

(b) [360bp spliced]

3' CCTTTTTCTGTCtgctcgaag.../ /...ggagatgtcCTGTTCTTCGA 5'

5' GGAAAAAGACAGacgagcttc.../ /...cctctacagGACAAGAAGCT 3'

Figure 3.26 DNA sequence flanking the IIICS splice site. If the sequence shown in (a) were to be found (BIG letters) in the nucleotide sequence of the HCF17 clone then only 267bp would have been spliced out in IIICS region and if the sequence in (b) were found (BIG letters) then 360bp was missing. The actual finding was the sequence shown in (b), which means 360bp in the IIICS region had been spliced out. The small letters show some of the unspliced sequences which would have been present if there would have been no splicing. The sequence of small letters in (a) are from Kornblihtt et al. (1985) and in (b) are from Bernard et al. (1986). Underlined are the consensus sequences for the 5' splice sites (i.e. GTGAG) and for the 3' splice sites (i.e. YYYYYYNAG where Y represents pyrimidine and N any base; Sekiguchi et al., 1986).

identification of at least three clones (/HCF11, /HCF17 and /HCF24) of variable sizes. DNA from these clones were subjected to restriction analysis and it was found that two of them (/HCF17 and /HCF24) were from the same region of the FN transcripts. The differences in size, between these 2 clones, were thought to have been caused by alternative splicing of the primary mRNA transcripts.

Restriction mapping showed that indeed these differences had been caused by the alternative splicing of the two splicing regions (ED-I & IIICS). Detailed restriction analysis of one of these clones (pHCF17) showed that a 270bp sequence from the ED-I region and 360bp sequence from the IIICS region had been spliced out. The absence of all of the 360bp from the IIICS region was also confirmed by the DNA sequencing. The other clone, /HCF24, contained the whole of the sequences for both regions. The \HCF11 clone was not extended toward the two splicing regions to make it interesting for further analysis.

The role of FN proteins isoforms with or without ED-I or/and IIICS is not fully understood yet. The significance of ED-I being present or absent is discussed in Chapter 4 and would not appear here, but the possible significance of IIICS is given here.

Direct evidence has been obtained that alternative splicing in the IIICS region accounts for some of the fibronectin subunit variants. Antibodies have been raised against the 95 amino acid (285bp) sequence of the IIICS synthesized as a beta-galactosidase fusion protein in lambda gt11 (Schwarzbauer et al., 1985). In immunoblotting experiments, the antiserum reacted only with the larger subunit of rat and hamster plasma fibronectin (and not to the smaller subunit presumably IIICS^{-}). The serum also recognized all subunits of cellular fibronectin. The results thus indicate that some of the molecular weight difference between the

subunits of plasma fibronectin arises from alternative splicing of the IIICS. The antibody data also show that cellular fibronectin are characterised by the presence of part or all of the IIICS sequence. However, the functional implications of this and the more subtle variations in this region remain unclear.

One exception to the above statment is that the alternative splicing of FN mRNAs in the IIICS region means that the CS1 sequence, (Chapter 1) which promotes cell adhesion of melanoma or neural crest cells, is present in certain but not all FN variants. The CS1 binding site (presumably present in HCF24) may have similar role in placenta. Presence of two different isoforms of FN (with or without IIICS) may mean that one type specifically promotes the adhesion or migration of one cell type rather than another. It is conceivable that different kinds of FNs carrying some or all of total complement of adhesion sites are secreted at specific regions of the placenta and even embryo where they modulate cellular behavior according to the environment of the region or their degree of differentiation. This assumption may be supported by the findings that some areas of the embryo are never occupied by migrating neural crest cells, even though they contain high amounts of FN (Dufour et al., 1988).

CHAPTER 4

IMMUNOHISTOLOGICAL STUDIES OF PLACENTAL FIBRONECTIN

4.1 Introduction.

Previous studies in this laboratory, using anti-FN antibody (Ab), have shown that FN could be detected in the chorionic plate of human placenta (Khalaf et al., 1985). These findings were, in part, confirmed by the screening of a placental cDNA library, described in the previous chapter (Chapter 3). There was evidence for the expression of FN molecules with and without ED-I (ED-A) and IIICS sequences in the chorionic plate of human placenta. It was then decided to confirm these findings at the serological level. The aim was to extend the study for these two splicing patterns as well as the third region, ED-II (Chapter 1) in the chorionic plate and other placental tissues. At that time only monoclonal antibodies (McAbs) against ED-I and ED-II epitopes (IST-9 and IST-8 respectively) and another McAb against a common FN epitope, IST-2, were available (Zardi et al., 1984; 1987; Sekiguchi et al., 1985; Castellani et al., 1986; Carnemolla et al., 1989). It was therefore decided to explore the pattern of expression of FN and two of its isoforms, ED-I and ED-II, in placental tissues.

Anticipating a differential expression of FN isoforms in normal placentae, it was decided to compare this expression with that found in placentae from women who had experienced some types of complications during their pregnancies (these placentae will be refered to as "clinical placentae" hereafter, to differentiate them from normal ones). However, only a limited number of clinical placentae were available at that time, therefore it was not intended that this would be a large series to explore.

Normal and clinical placental sections were studied for the expression of the above epitopes using the three McAbs, IST-2, IST-8 and IST-9. The study was aimed at finding any possible differences between normal and clinical placentae as well as any possible

relationship between the clinical complications and the level of FN and its isoforms expression. This is because FN plays important role during embryogenesis and development (D'Ardenne and McGee, 1984). In fact FN, in the extracellular matrix, is not only involved in trophoblastic differentiation, but also it supports many other cellular migratory and developmental changes occurring during embryogenesis and provides support for the integrity of the placental membranes which is necessary for the maintenance of pregnancy (Alpin and Foden 1982). Any significant difference between normal and clinical placentae in their FN content may suggest that low level of FN might have been one of the causes for that particular abnormality (for review in the role of FN in disease see D'Ardenne and McGee (1984).

4.2 Staining for Fibronectin and Its Isoforms.

4.2.1 Optimisation of the Staining Procedures.

Several normal and clinical human placentae were collected, shortly after delivery, from the Queen Mother's Hospital, Glasgow. The tissues were cut from three different anatomical regions of placenta; chorionic plate (CP), utero-placental interface (N) and the outer margin of the placenta (CPN) (Fig. 4.1). The frozen sections from these tissues were prepared for staining as described in the methods section (Chapter 2).

The staining technique was first optimised on normal term placentae using an Ab which was known to stain some of the human placental tissues (i.e. W6/32, Barnstable et al., 1978; Khalaf et al., 1985, respectively). Several steps in the staining procedure were tested and optimised for the reduction of background and enhancement of signal. These procedures included blocking endogenous peroxidases, adsorption of second Ab, blocking the non-specific binding sites on the section (with blocking agent), optimising the duration of diaminobenzidine (DAB) treatment and working out the right concentration of primary and peroxidase-conjugated second antibodies (see also the methods section in Chapter 2).

Blocking the endogenous peroxidases present on the sections was found to be necessary to reduce some of the background associated with them. These endogenous peroxidases were efficiently blocked by treating the sections with 0.1% periodic acid as described in the methods section (Chapter 2).

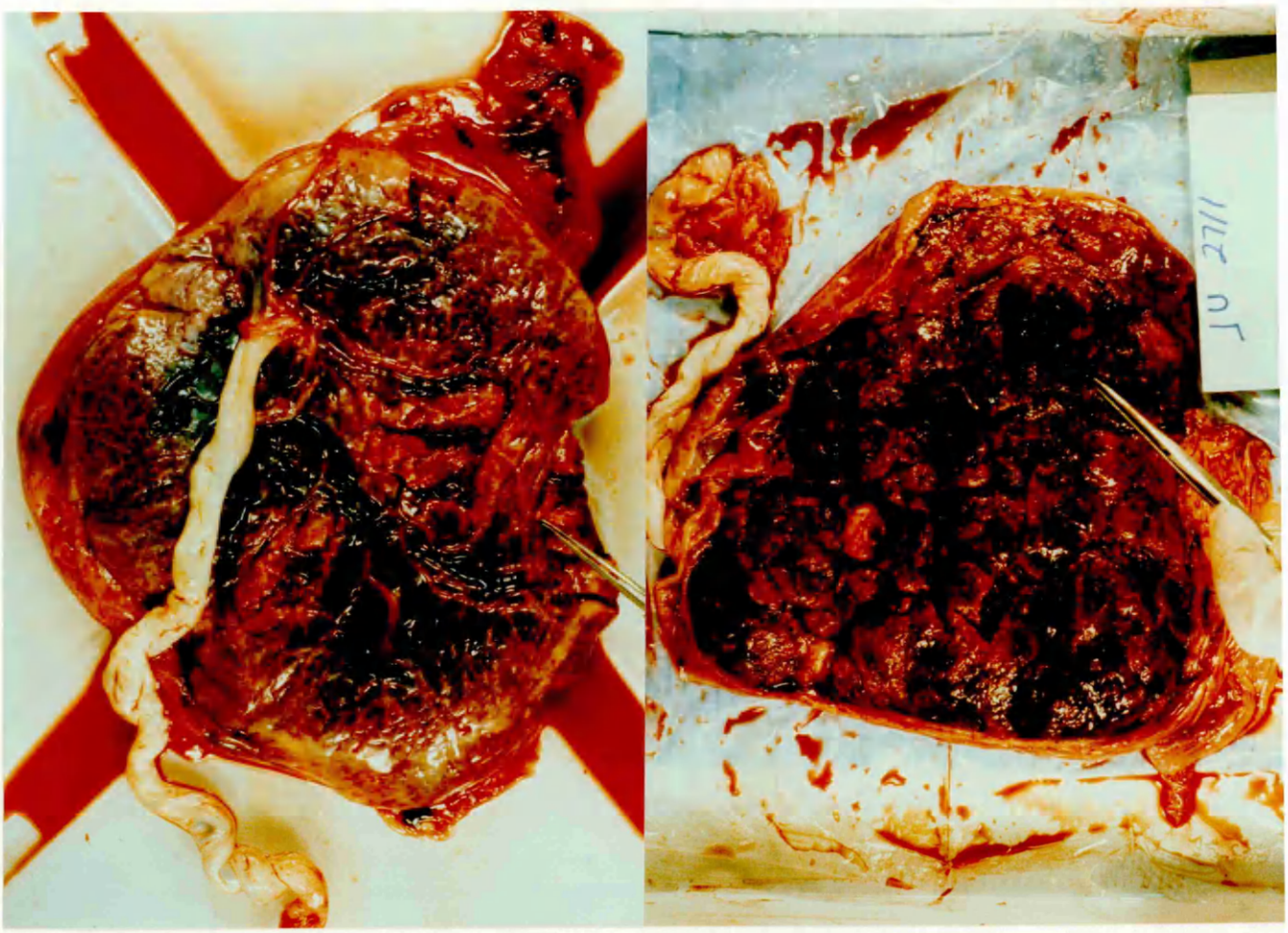


Figure 4.1 Two human placentae which were used for the study of FN expression are shown. (a) Placenta showing the fetal side after removal of amnion. Forceps show the position of placental margin. (b) Placenta from maternal side (N layer). The area where tissues were usually taken are shown (by forceps).

It was found that when the sections were stained using only untreated 2nd Ab, there was weak staining (Fig. 4.2). This was thought to have been caused by interactions between the Ab combining sites on the 2nd Ab and proteins on the placental section. This background staining was found to diminish by adsorbing the 2nd Ab with adsorbants such as human serum. The background was usually completely eliminated if the staining procedure included blocking of the section with BSA.

The adsorption of the 2nd Ab was carried out using four adsorbants (BSA, homogenized placenta, human serum and human serum from pregnant woman). On the contrary to the BSA effect for treating the sections, in here only human serum from pregnant woman completely eliminated the background staining. The reason that serum from pregnant woman was more effective than the other adsorbants in adsorbing the 2nd Ab, may be because of cross reactivity with the proteins which had been exchanged between placenta and maternal serum. It has been documented that some small proteins cross placental cells in either direction (Sutcliffe 1975). If the affinity of the second Ab was for these kinds of proteins then adsorbing the 2nd Ab with a material from the same origin (i.e. a pregnant woman) was more likely to adsorb those Ab combining sites and, as a consequence, reduced the associated background staining.

On the basis of the results described above, a routine procedure was adopted to eliminate or reduce the background when staining placental sections, which was to treat the sections with periodic acid and BSA, and to adsorb the 2nd Ab with human serum from pregnant woman.

It was found that DAB treatment caused some non-specific background, but the pattern of background staining was different from those mentioned above. The argument in support of non-specificity was that areas without any tissue section were also stained, giving rise to some brown patches (Fig. 4.3). This kind of

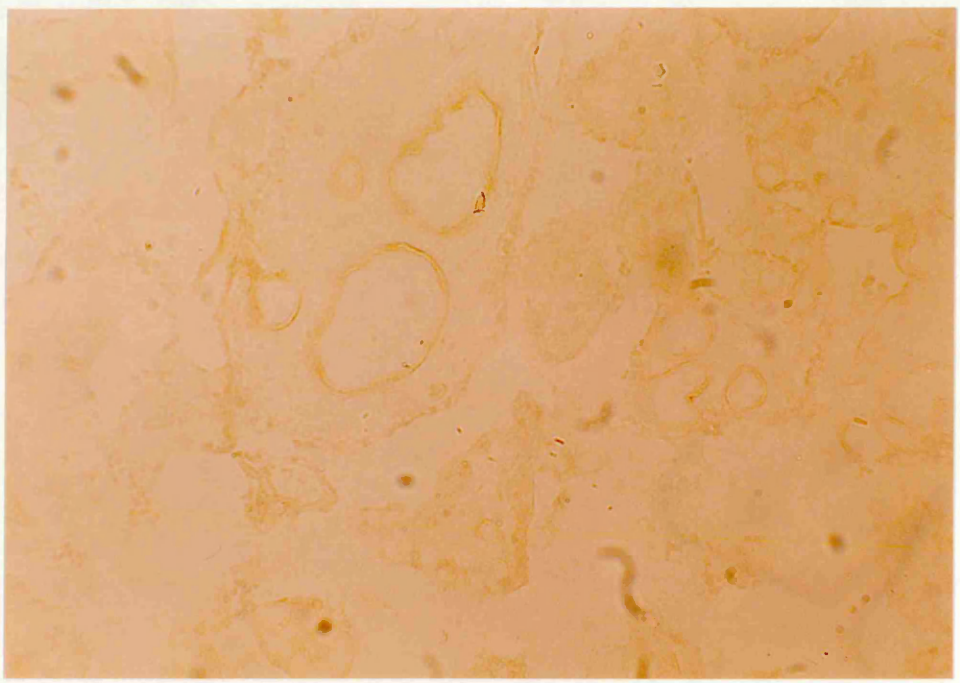


Figure 4.2 A section from placental margin. The section has been stained only with unadsorbed second Ab. A faint uniform staining is seen. Very faint haematoxyline staining is also present.

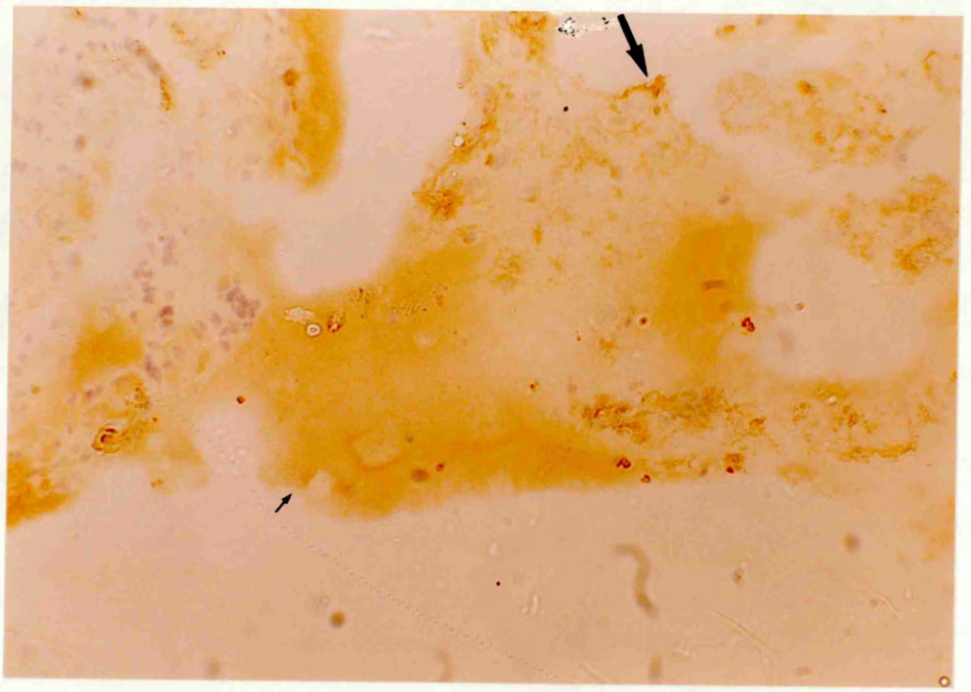


Figure 4.3 A section from placental margin stained with W6/32 mAb showing background staining caused by DAB treatment. DAB non-specific staining (small arrow) can be distinguished from the W6/32 specific staining (bigger arrow). DAB staining can be misleading and usually was seen as brown patches or zones of variable sizes even where no tissue was present.

background staining was controlled by reducing the DAB treatment to the minimum required, combined with washing the section extensively under running water.

The effect of counterstaining the nuclei with haemotoxylin, for better interpretation of the results, was also tested. Counterstaining identified the cell types as either single nucleated cells or multinucleated cells but it was found that too strong counterstaining tended to reduce the strength of the initial staining therefore the strength of counterstaining was kept to minimum necessary in most cases.

4.2.2 Staining Placental Tissues for FN

After the optimisation of the staining, placental serial sections were stained for the expression of fibronectin and two of its isoforms, ED-I and ED-II. For each staining experiment, at least one set of normal placental sections was stained for comparison with the clinical ones. Each set of sections, from a particular placenta contained at least six slides. One slide was used for negative control (i.e. the section was treated according to the routine procedure for antibody staining without using any Ab). The second slide was stained to check if any possible background was associated with the second Ab (i.e. ngative control for the 2nd Ab). The third slide was stained with W6/32, an anti HLA class 1 McAb (Barnstable et al., 1978) as a positive control. W6/32 was chosen because it has been shown to stain the trophoblast cells strongly in the chorionic plate of human term placenta (Khalaf et al., 1985). The W6/32 staining was used for two purposes; firstly as an indicator of the success or failure of staining, and secondly to identify the origin of cells staining positively on serial sections, since HLA class 1 determinants are expressed differently in chorionic plate (CP) and chorionic villi (CV) (Khalaf et al., 1985; Sunderland, Redman and Stirrat, 1981; and this work, Fig. 4.4). At least three other slides were

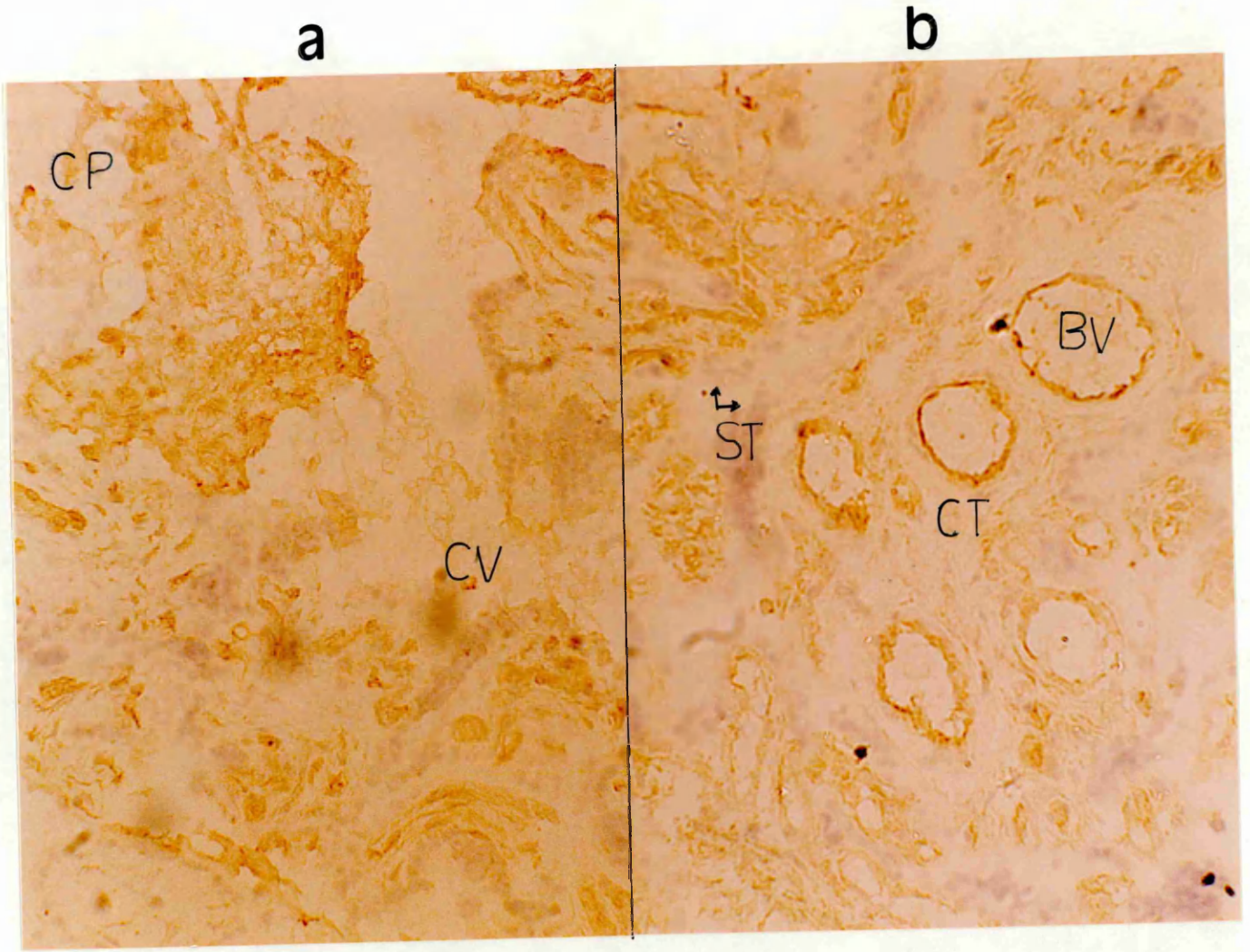


Figure 4.4 Two sections from placental margin have been stained with W6/32 (an anti HLA class 1 determinant Ab). (a) Chorionic plate cells (CP) and some areas in the chorionic villi (CV) are stained strongly but syncytitrophoblasts are either negative or very faintly stained. (b) Within the chorionic villi the blood vessels (BV) have stained strongly and the connective tissues (CT) have stained faintly and the syncytiotrophoblast (ST) were negative.

stained for FN and its isoforms (i.e. at least one with IST-2, one with IST-8 and one with IST-9). Usually at least two different types of placental sections (e.g. slides from either chorionic plate, CP and feto-maternal margin, CPN or CPN and maternal side of the placenta, N) were stained, but because of the similarities in the results, only the results of CPN stainings are given here (though a few CP and N slides are given as well).

The clinical placentae, with their apparant clinical condition, are listed in Table 4.1. The normal placenta at the end of the list represents several similar placentae used, and it refers to normal term placentae in which the delivery had been normal and there was no apparant clinical complications with the pregnancy.

4.2.2 Results of Staining Clinical and Normal Placentae for FN and Its Isoforms.

The placentae listed in Table 4.1 were stained with McAb against the epitopes of FN isoforms. The concentrations of the McAbs used for staining are listed in Table 4.2. In few cases more concentrated Abs were used to confirm that the negative staining was due to the very low level or possibly absence of FN isoform expression, and not due to the experimental variations or any other reasons. Higher concentrations of these McAbs also showed that there were inter-individual variations because some sections were still negative at these higher concentrations of Abs, This meant that there was very little or no expression of FN isoforms (Table 4.2).

Due to the wide variations in the pattern and intensity of staining, it is difficult to describe the result of every slide, so they are described using numbers to correspond to the intensity of staining (i.e. $<0.5^{+}-5^{+}$). The results are summarized in Table 4.2. The table shows the name of the placentae, the type of McAbs

No	Name	Weeks deliv.	Babies weight	Remarks on clinical conditions
1	GR	39	3.2 kg	mild pregnancy-induced hypertension
2	MK	28	1.21 kg	pre-term labour
3	JU	39	3.21 kg	pregnancy-induced hypertension
4	MC	31	0.95 kg	fetal growth retardation (infant death)
5	HA	35	2.37 kg	spontaneous rupture of membrane
6	OB1	38	2.84 kg	} twin pregnancy, pregnancy-induced hypertension
7	OB2	38	2.6 kg	
8	BE	38	3.46 kg	diabetes
9	FR	35	2.19 kg	renal hypertension
10	HAR	39	3.56 kg	diabetes
11	FL	31	1.28 kg	hypertension. oligohydramnios
12	N	term		normal

Table 4.1 Clinical complications associated with the pregnancies of those placentae used in this work. Several normal placentae were used but are not listed in the table.

No.	Group	Name	IST-2		IST-8		IST-9	
			diln.	result	diln.	result	diln.	result
1	I	FR	1/20	3+_4^{+}	1/10	<0.5^{+}	1/10	1^{+}
2		HAR	1/20	3^{+}-4^{+}	1/10	2^{+}	1/10	1^{+}-2^{+}
3		OB2	1/20	4^{+}-5^{+}	1/10	1^{+}	1/10	<0.5^{+}
4		MK	1/20	3^{+}-4^{+}	1/10	1^{+}	1/10	1^{+}
5		Normal	1/20	3^{+}	1/10	1^{+}-2^{+}	1/10	<0.5^{+}
6	II	MC	1/20	3^{+}	1/10	2^{+}	1/10	2^{+}-3^{+}
7		OB1	1/20	4^{+}-5^{+}	1/10	2.5-3^{+}	1/10	2-2.5^{+}
8		GR	1/20	4^{+}-5^{+}	1/10	2^{+}-3^{+}	1/10	1^{+}
9		Normal	1/20	3^{+}-4^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
10	III	JU	1/20	3^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
11		BE	1/20	3^{+}	1/10	1^{+}-2^{+}	1/10	0.5^{+}
12		FL	1/20	2^{+}-3^{+}	1/10	0.5-1^{+}	1/10	0.5^{+}
13		FR	1/20	2^{+}-3^{+}	1/10	1^{+}-2^{+}	1/10	0.5-1^{+}
14		Normal	1/20	1^{+}-2^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
15	IV	HA	1/20	1^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
16		MC	1/20	1^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
17		MK	1/20	2^{+}-3^{+}	1/10	<0.5^{+}	1/10	1^{+}
18		FL	1/20	2^{+}	1/10	0.5^{+}	1/10	0.5^{+}
19		Normal	1/20	2^{+}-3^{+}	1/10	<0.5^{+}	1/10	<0.5^{+}
20	V	Normal	1/10	3^{+}	1/2	3^{+}	1/2	1.5^{+}
21		Normal	1/10	3^{+}	1/5	<0.5^{+}	NT	NT
22		Normal	NT	NT	1/2	1^{+}	NT	NT
23		Normal	NT	NT	1/5	<0.5^{+}	NT	NT

Table 4.2 Slides which had been stained together are shown as groups. Each group contained some clinical and at least one normal slide. Group V contains only normal placental sections which had been stained with more concentrated Ab (NT means not tested).

used and their dilutions as well as the results of the stainings (shown by numbers). The arrangment of data has been based on the groups of slides which were stained together. This was chosen to show that there were minor inter-group variations in the intensity of staining, as well as within group variations. The inter-group variation is thought to have been caused by minor experimental differences, whereas the within-group variation was most probably caused by differences in the levels of expression. For example, it seemed that groups I and II generally had stronger staining than group IV. The within group variations were quite obvious in all 4 groups (Table 4.2). The results in group V are from normal placental sections, which had been stained with more concentrated Abs than other sections as indicated above. The results indicate that some of these sections were still almost negative ($<0.5^+$) at higher Ab concentration (nos. 21 and 23).

The interpretation of these numbers is that, for example 1^+ was fainter than 2^+ and 5^+ was stronger than 4^+ etc. Usually the numbers were based on the overall intensity of staining in the chorionic plate and chorionic villi and not on the intensity of staining on the blood vessels or some unusually strongly staining areas (e.g. fibrinoid staining, Fig. 4.5).

These numbers should be used to compare the results obtained with a particular McAb, and can not be compared with results for another McAb. For example, faint (1^+) staining with IST-2 is not the same as that with IST-8 or IST-9 (Figs. 4.6 and 4.7), since IST-2 staining was generally stronger than IST-8 or IST-9 staining (Figs. 4.8 and 4.9 respectively). Nonetheless, the numbers corresponding to the intensity of staining for IST-8 and IST-9 are similar.

A $<0.5^+$ result means that, with similar concentrations of Abs, overall very faint or negative staining was observed. On the other hand values greater than 0.5^+ mean positive staining. These values were,

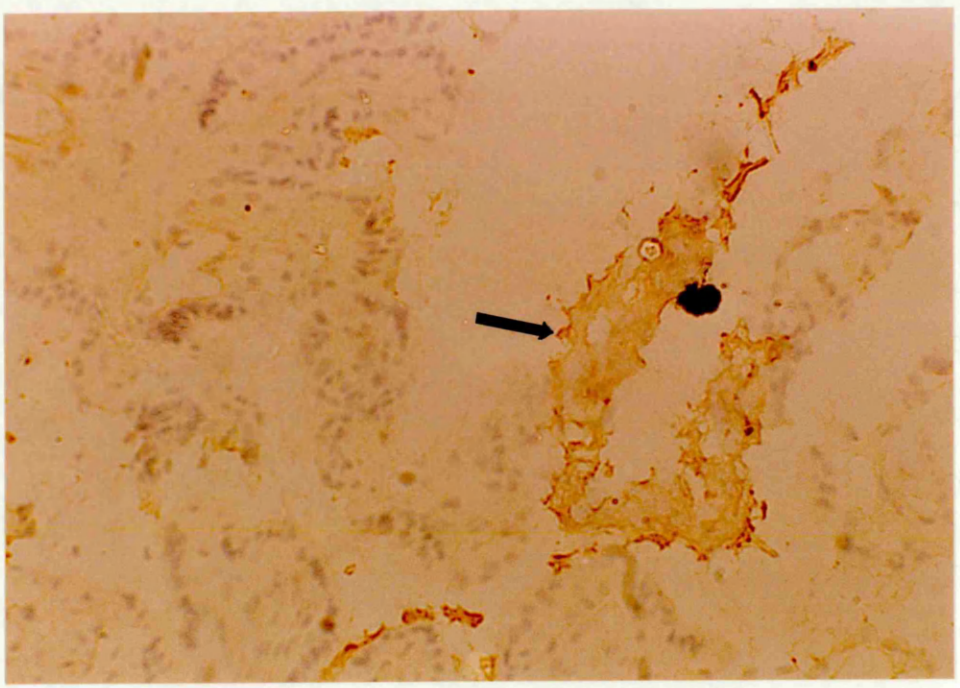


Figure 4.5 A section from placental margin stained with the IST-2 mAb. The figure shows strong fibrinoid (arrow) staining compared to the blood vessels in the chorionic villi.

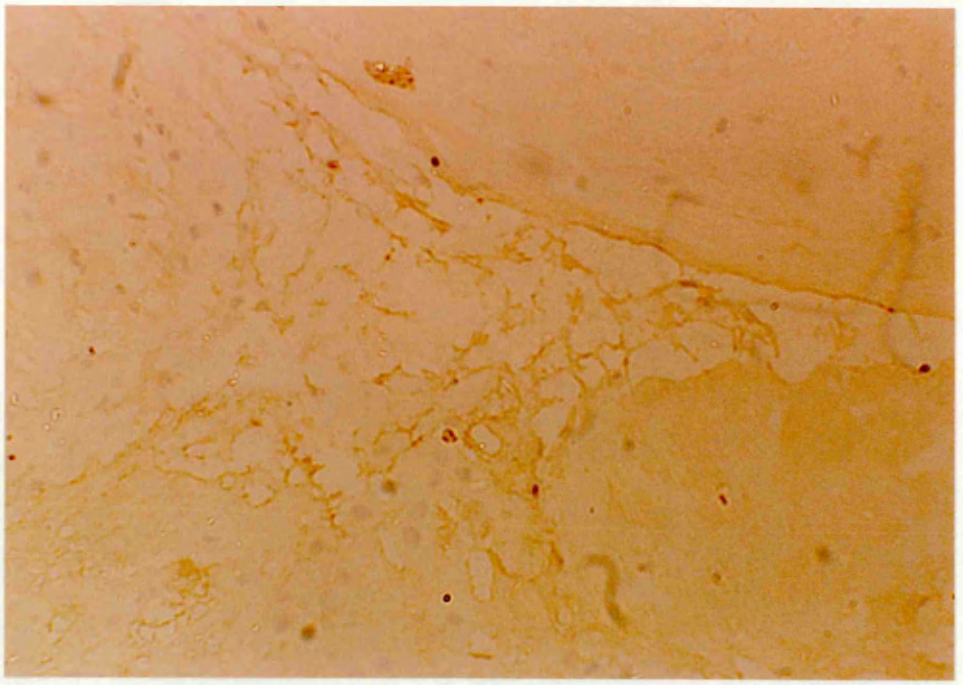


Figure 4.6 A section from placental margin showing a typical 1^+ staining with the IST-2 mAb. The staining is within the chorionic plate and some tissues are intact and some broken as a result of freezing.

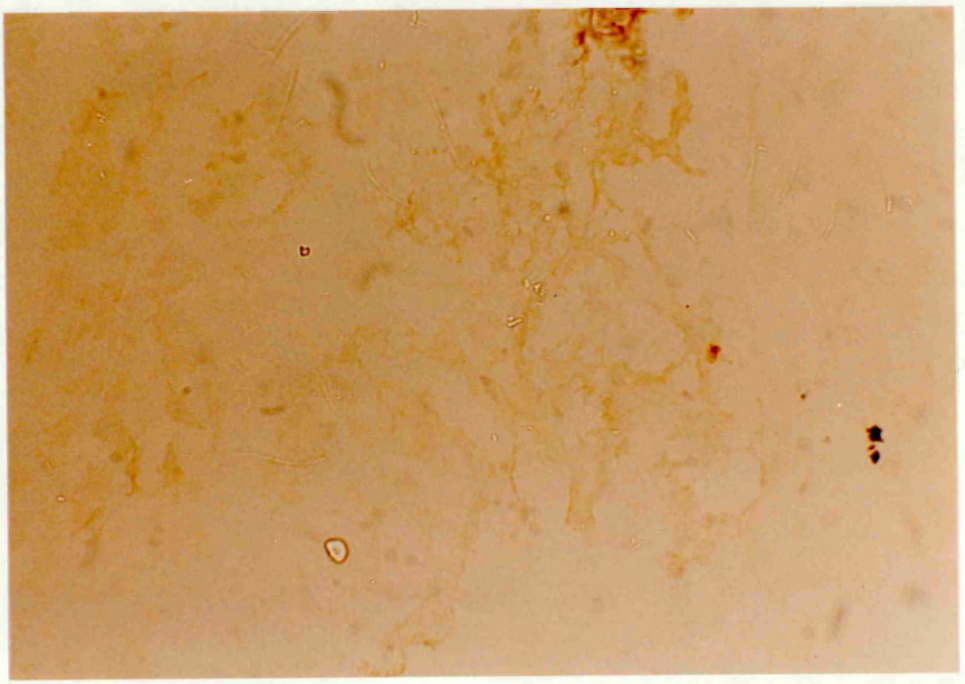


Figure 4.7 A section from placental margin showing a typical 1^+ staining with the IST-8 mAb. 1^+ for the IST-9 mAb staining would be similar. The staining is within the chorionic plate. Compare the intensity of staining with that in Figure 4.6.

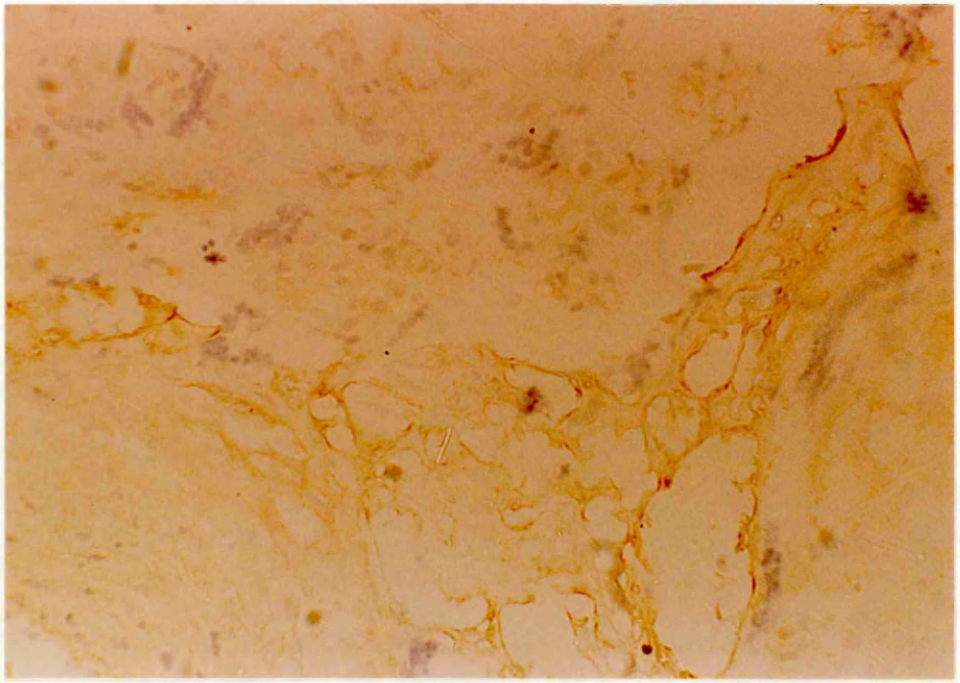


Figure 4.8 A section from placental margin showing a typical 2^{+}- 3^{+} staining with the IST-2 mAb. 2^{+}- 3^{+} staining is the intensity of the staining in the positive area.

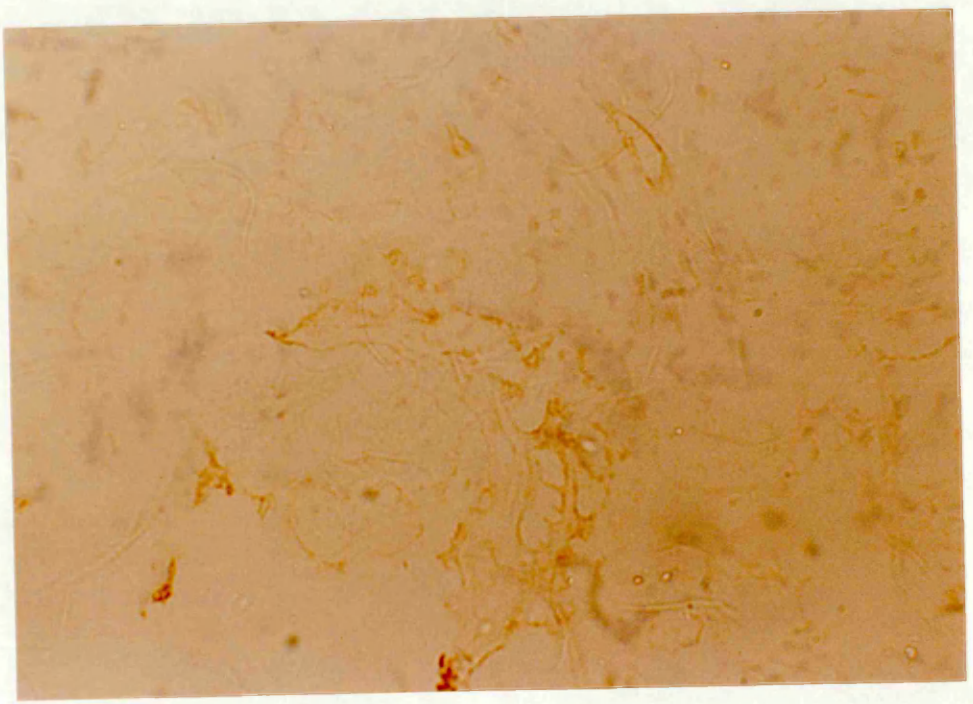


Figure 4.9 A section from placental margin showing a typical 2^{+}-3^{+} staining with the IST-8 mAb. 2^{+}-3^{+} intensity with the IST-9 would be similar. The staining intensity is the intensity of the positive area. Compare the intensity of staining with that in Figure 4.8.

however, judged from overall staining, seen after scanning the entire section, since no particular section was identified to stain uniformly. For example, in most cases CP had more staining than CV which may have been directly related to the level of expression of FN and its isoforms within the chorionic plate.

Staining showed that there were usually few things in common between the results obtained by all of the three McAbs, irrespective of their staining intensity. In general extracellular matrices in the chorionic plate had stained much more strongly than those in the chorionic villi (Fig. 4.10), and the matrices of the blood vessels stained more intensely than matrices in other cells either in the chorionic plate or chorionic villi (Fig. 4.11). Within the chorionic plate cytotrophoblasts were staining strongly, as were the blood vessels and chorionic epithelium (Fig. 4.12).

Within each villus the blood vessel(s) stained more intensely than the connective tissue around the blood vessels, and villus syncytiotrophoblast cells were usually negative for FN (Fig. 4.13). Sections from the utero-placental interface (N layer) stained much more faintly than those from the chorionic plate and were similar to those found for the chorionic villi (Fig. 4.14).

Sometimes staining with the Ist-2 showed that there was faint staining within large blood vessels even after peroxidase blocking. This could have been caused by fetal serum FN, which is abundant in the fetal circulation (Molnar et al., 1988). The pattern of staining was, however, different from those of endothelial cells forming the blood vessels (compare Fig. 4.15 with Figs. 4.12 and 4.13).

The staining results for IST-2/IST-8 and IST-2/IST-9 are compared in Table 4.3, and results are

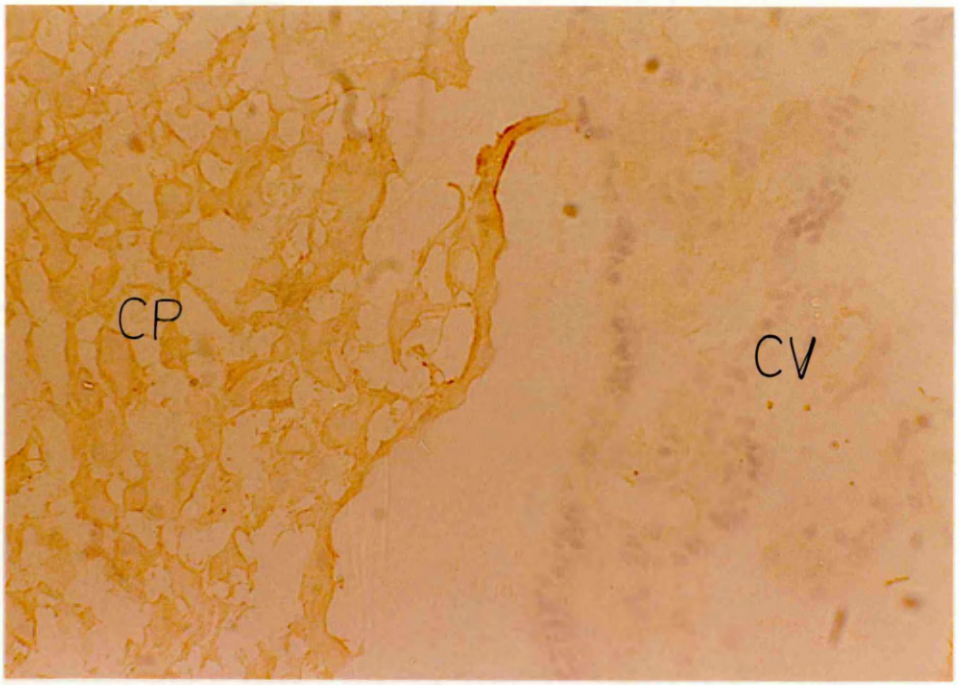


Figure 4.10 A section from placental margin stained with the IST-2 mAb. The figure shows that there is much more staining for FN in the chorionic plate (CP) than in the chorionic villi (CV). The tissues in the CP are broken, most probably due to the freezing of the placental tissue for sectioning.

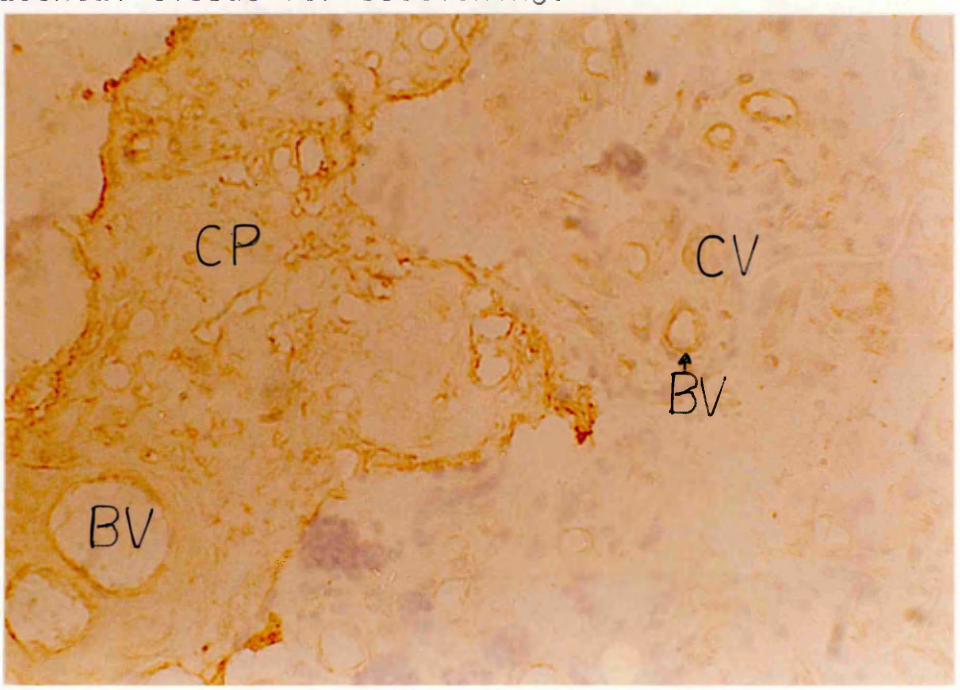


Figure 4.11 Blood vessels stained for FN. The figure shows that generally the blood vessels (BV) in the chorionic plate (CP) and chorionic villi (CV) staining more intensely than other tissues. This was however less obvious in the CP, maybe because of usual strong staining of the other cells in the CP. The section is from placental margin stained with IST-2 mAb.

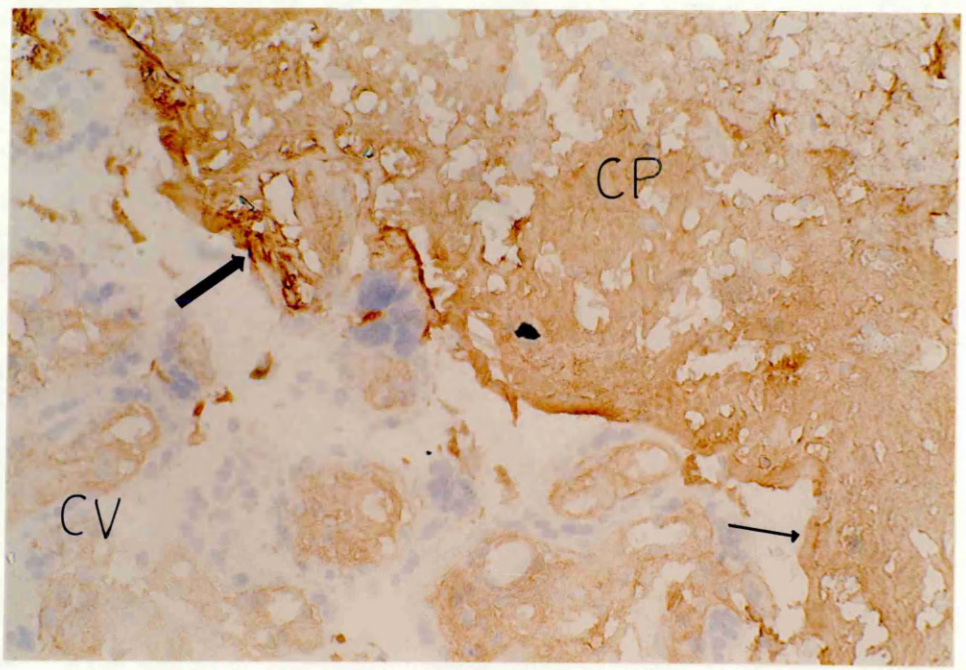


Figure 4.12 Chorionic plate tissues stained for FN. The figure shows that within the chorionic plate (CP) some cells were staining more intensely than others (the differentiation of these cells, without double staining, is however difficult). The chorionic epithelium (small arrow) was usually staining the same as blood vessels but most of the time they appeared to stain more, this was most probably due to the folding of these tissues during sectioning (bigger arrow). The section is from placental margin stained with the IST-2 mAb.

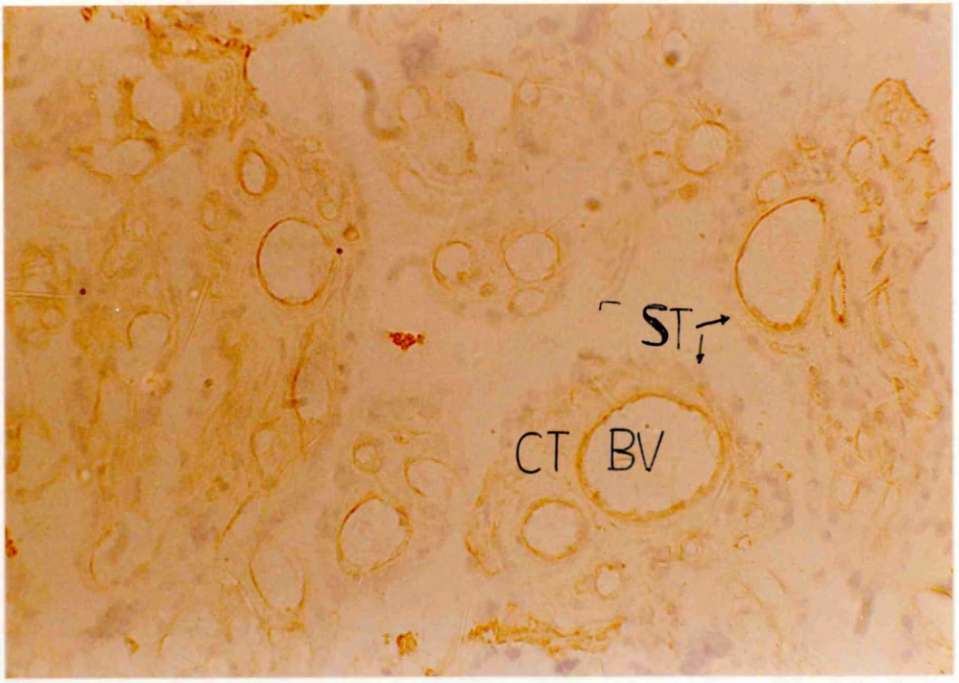


Figure 4.13 Chorionic villi tissues stained for FN. The figure shows a typical staining in the chorionic villi. The blood vessels (BV) are clearly stained more intensely than the connective tissues (CT) within each villus. Syncytiotrophoblast cells (arrow) were usually negative for FN. The section is from placental margin stained with the IST-2 mAb.

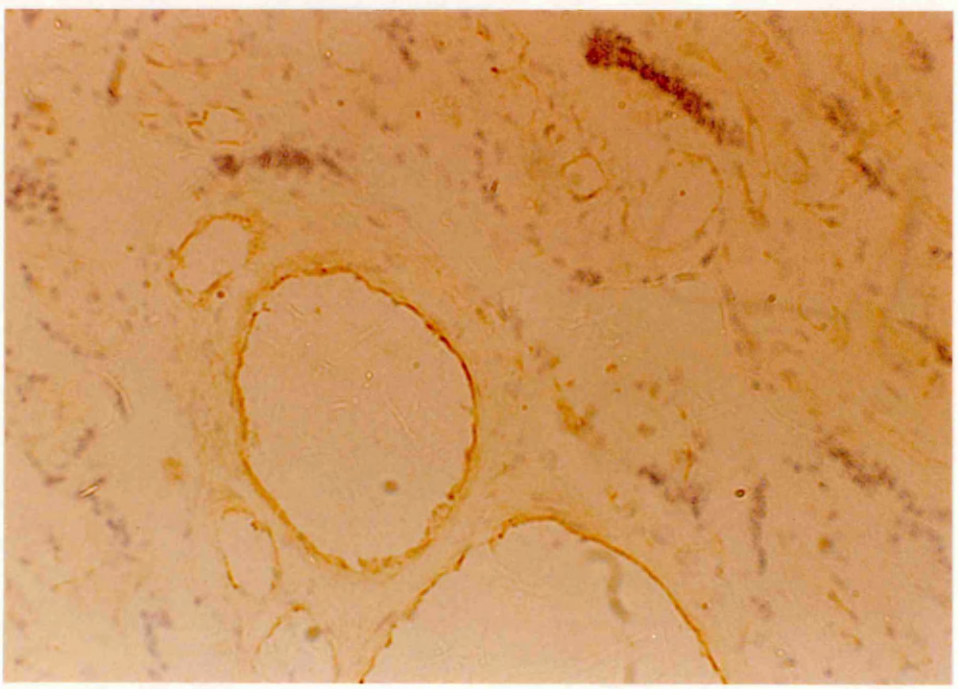


Figure 4.14 Tissues form the feto-maternal interface stained for FN. The figure shows that only blood vessels in the Nitabuch's layer have stained for FN. The section is from Nitabuch's layer of placenta stained with the IST-2 mAb.

Figure 4.15 Blood vessels stained for FN. The figure shows a large blood vessel staining intensely for FN. Within the blood vessel there are some stainings which may have come from plasma FN. The staining inside the blood vessel is different from those for extracellular matrices of endothelial cells of the blood vessel and the epithelial cells surounding the blood vessel. The section is from the chorionic plate of human placenta stained with the IST-2 mAb.

arranged into two groups of placentae. Group one is arranged by putting the strongest IST-8 or IST-9 stainings first and the faintest ones at the end, irrespective of intensity of IST-2 staining. In group two, the arrangement has been based on the intensity of IST-2 staining, because the staining with IST-8 and IST-9 had been negative.

In both cases (i.e. the IST-2/IST-8 and IST-2/IST-9 comparisons) there seemed to be no strong correlation in the intensity of staining between IST-2 and the other two McAb. For example, GR showed 4^+-5^+ staining for IST-2 and 2^+-3^+ for IST-8 (no. 2, IST-2/IST-8, Table 4.3), whereas OB2 showed 4^+-5^+ for IST- 2 but only 1^+ for IST-8 (no. 8, IST-2/IST-8, Table 4.3). IST-2 and IST-9 also showed the same kind of non-concordance as was seen between IST-2 and IST-8 (e.g. compare MC and OB2 for IST-2/IST-9 comparisons, Table 4.3).

One interesting point worth mentioning is the difference in the pattern of staining of the placental sections from twins (OB1 and OB2). These two placentae were still connected by their amnion after delivery, then they were separated and treated as two independent placentae. Blocks of placental tissue, from these two placentae, were kept separate from one another to avoid any possible mixup. Both of these placentae stained with equal intensity with IST-2, but the OB1 slides stained positively with IST-8 and IST-9 (2.5^+-3^+ and 2^+-2.5^+ respectively); whereas the OB2 slides stained weakly with IST-8 (1^+) and stained almost negative ($<0.5^+$) with IST-9 (Table 4.3). The significance of these findings is not apparent, especially as the two placentae and the related babies' weights did not appear to be significantly different (Table 4.1). The difference may have been due to inter-group variations in the pattern of staining, which have also been observed with normal placentae. The two groups containing these slides (groups I and II, Table 4.2) have, neverthless, shown similar intensities of staining which contradicts this assumption.

IST_2 and IST_8 Comparisons					IST_2 and IST_9 Comparisons			
No.	Name	IST_2	IST_8	Group	Name	IST_2	IST_9	Group
1	OB1	4^{+}-5^{+}	2.5^{+}-3^{+}	A	MC	3^{+},	2^{+}-3^{+}	A
2	GR	4^{+}-5^{+}	2^{+}-3^{+}	A	OB1	4^{+}-5^{+}	2^{+}-2.5^{+}	A
3	MC	3^{+},	2^{+}	A	HAR	3^{+}-4^{+}	1^{+}-2^{+}	A
4	HAR	3^{+}-4^{+}	2^{+}	A	GR	4^{+}-5^{+}	1^{+}	A
5	Norm.	3^{+},	1^{+}-2^{+}	A	MK	3^{+}-4^{+}	1^{+}	A
6	BE	3^{+},	1^{+}-2^{+}	A	FR	3^{+}-4^{+}	1^{+}	A
7	FR	2^{+}-3^{+}	1^{+}-2^{+}	A	MK	2^{+}-3^{+}	1^{+}	A
8	OB2	4^{+}-5^{+}	1^{+}	A	FR	2^{+}-3^{+}	0.5^{+}-1^{+}	A
9	MK	3^{+}-4^{+}	1^{+}	A	BE	3^{+},	0.5^{+}	A
10	FL	2^{+}-3^{+}	0.5_1^{+}	A	FL	2^{+}-3^{+}	0.5^{+}	A
11	FL	2^{+},	0.5^{+}	A	FL	2^{+},	0.5^{+}	A
12	FR	3^{+}-4^{+}	$<0.5^{+}$	B	OB2	4^{+}-5^{+}	$<0.5^{+}$	B
13	Norm.	3^{+}-4^{+}	$<0.5^{+}$	B	Norm.	3^{+}-4^{+}	$<0.5^{+}$	B
14	JU	3^{+},	$<0.5^{+}$	B	Norm.	3^{+},	$<0.5^{+}$	B
15	MK	2^{+}-3^{+}	$<0.5^{+}$	B	JU	3^{+},	$<0.5^{+}$	B
16	Norm.	2^{+}-3^{+}	$<0.5^{+}$	B	Norm.	2^{+}-3^{+}	$<0.5^{+}$	B
17	Norm.	1^{+}-2^{+}	$<0.5^{+}$	B	Norm.	1^{+}-2^{+}	$<0.5^{+}$	B
18	HA	1^{+},	$<0.5^{+}$	B	HA	1^{+},	$<0.5^{+}$	B
19	MC	1^{+},	$<0.5^{+}$	B	MC	1^{+},	$<0.5^{+}$	B

Table 4.3 Comparisons of results obtained for IST-2/IST-8 and IST-2/IST-9. For full discussion see the text.

The staining results obtained for IST-8 and IST-9 were compared to find out whether there was any correlation between the expression of ED-I and ED-II, and if so, whether they both had the same tissue specificity (i.e. chorionic plate vs chorionic villi, Table 4.4). The results have been arranged in to groups for easier comparisons, and IST-2 results are shown for cross reference. In the first group, the placentae had stained with both IST-8 and IST-9 with the same intensity. In group 2, both IST-8 and IST-9 staining was positive, but IST-8 staining was slightly stronger. Group 3 contains those which were positive for IST-8 but negative for IST-9, and group 4 shows slides which were negative for IST-8 but positive for IST-9. Finally group 5 shows slides which were negative for both Abs.

Comparisons of groups 1 to 5 showed that in 79% of the cases both IST-8 and IST-9 had either stained, or had failed to stain (Table 4.4). This concordance can be further subdivided into 47% of the slides being positive with both McAbs (nos. 1-7), and 31% being negative with both McAbs (nos. 14-19). On the other hand only 21% of slides were positive for one and negative for the other indicating no correlation for expression (nos. 10-13). The 79% concordance may suggest that ED-I and ED-II epitopes are, most of the time, expressed together (but with different intensities). At the same time, ED-I and ED-II were not expressed to the same levels as the common FN epitope recognised by IST-2.

Generally the expression of ED-I or ED-II was higher in the clinical cases than in normal placentae. In fact 3 out of 4 normal placentae had no or very faint staining for ED-I or ED-II, and the 4th one was negative for ED-I and showed faint to moderate staining for ED-II. In contrast only 2 out of 11 slides (18%) from clinical placentae failed to stain for ED-I and ED-II (nos. 15 and 18, JU and HA respectively, Table 4.4). The failure of the third clinical slide (no. 19, Table 4.4) to stain for ED-I or ED-II could have been due to the fact that the tissue was from the N layer and N

No.	Name	Sectn.	IST-2	IST-8	IST-9	Comments
1	OB1	Margin	4^+-5^+	2.5-3^+	2-2.5^+	+ve IST-8 and IST-9 (the same intensity of IST-8 and IST-9 but usually IST-9 a little less).
2	HAR	Margin	3^+-4^+	2^+	1^+-2^+	
3	MK	Margin	3^+-4^+	1^+	1^+	
4	MC	Margin	3^+	2^+	2^+-3^+	
5	FR	Margin	2^+-3^+	1^+-2^+	0.5_1^+	
6	FL	Margin	2^+-3^+	0.5_1^+	0.5^+	
7	FL	CP	2^+	0.5^+	0.5^+	
8	GR	Margin	4^+-5^+	2^+-3^+	1^+	+ve IST-8 and IST-9 but IST-8 > IST-9
9	BE	Margin	3^+	1^+-2^+	0.5^+	
10	OB2	CP	4^+-5^+	1^+	$<0.5^+$	+ve IST-8 but $<0.5^+$ IST-9
11	Norm.	Margin	3^+	1^+-2^+	$<0.5^+$	
12	FR	CP	3^+-4^+	$<0.5^+$	1^+	$<0.5^+$ IST-8 but +ve IST-9
13	MK	CP	2^+-3^+	$<0.5^+$	1^+	
14	Norm.	Margin	3^+-4^+	$<0.5^+$	$<0.5^+$	$<0.5^+$ IST-8 and IST-9
15	JU	Margin	3^+	$<0.5^+$	$<0.5^+$	
16	Norm.	Margin	2^+-3^+	$<0.5^+$	$<0.5^+$	
17	Norm.	Margin	1^+-2^+	$<0.5^+$	$<0.5^+$	
18	HA	Margin	1^+	$<0.5^+$	$<0.5^+$	
19	MC	N	1^+	$<0.5^+$	$<0.5^+$	

Table 4.4 Comparisons of results obtained for IST-8 and IST-9. The tissues stained were from CP, N and margin. CP = chorionic plate, N = Nitabuch's layer, Margin = chorionic plate-Nitabuch's margin and BV = blood vessels. For interpretation of numbers see the text.

layer has shown low expression for all three FN epitopes (no. 19, Table 4.4 and Fig. 4.14). In support of this argument, the other slides from the same person, but different areas of the placenta, stained positively for both ED-I and ED-II (no. 4, Table 4.4).

It may also be concluded that ED-I and ED-II expressed independently of each other, because even when in 47% of the cases they both were positive, the intensity of staining for a particular placenta was not the same (Table 4.4). Overall it could be said that ED-I and ED-II showed similar tissue specificity with each other themselves as well as with the common FN epitope.

The difference in the intensity of staining seen between IST-2 on the one hand and the other two McAbs on the other may have been caused by other factors apart from differences in the level of expression of FN isoforms. It may have been caused, for example, by the difference in the affinity of the three McAbs used. Reviewing the published works of the group (Zardi et al., 1984; 1987; Sekiguchi et al., 1985; Castellani et al., 1986; Carnemolla et al., 1989) from which these Abs had originated, did not help to compare the affinity of these Abs. Usually these Abs have been reported in different papers and even then they have not been quantitatively compared. When they have been compared then usually the comparisons have not been done serologically so as to be useful in this work. In fact it may not be correct to say that the affinity of McAbs can be compared, because even two different McAbs raised against the same molecule may differ in affinity. This is because each of them may recognise a different epitope of the same molecule so the intermolecular attractive forces which exist between the Ab combining sites and the antigenic determinant (Roitt, Brostoff and Male, 1985) will be different.

4.3 Discussion

Placental sections generally showed more staining in the chorionic plate than in the chorionic villi. Within the chorionic plate, the extracellular matrices of the chorionic epithelium, cytotrophoblast cells and endothelial cells of the blood vessels stained more heavily for FN and its isoforms than other cells present. The presence of more FN protein in the chorionic plate may be because of the role of the chorionic plate as a vital barrier between the amniotic cavity and the maternal blood circulation, as well as the existence of more compact extracellular matrices which are holding the cells together, since FN has been found to have a vital role in the maintenance of tissue and organ integrity (Akiyama and Yamada, 1987).

Normal and clinical placentae generally showed no difference in the intensity of staining with the IST-2 McAb. IST-2 stained the placental sections more strongly then the other two splice specific McAbs (IST-8 and IST-9) that is to say, comparatively, little ED-I and ED-II isoforms were seen to be expressed on these sections. The findings can be summarised as follows:

(1) There were inter-individual variations for the expression of FN (IST-2 positives). These variations might have been caused by either experimental differences or different level of expression in these placentae, as has been seen by other researcher (Zhu et al., 1984).

(2) Usually IST-2 staining was much stronger than staining with the other two Abs, IST-8 and IST-9.

(3) Usually whenever IST-2 staining was strong, the other McAbs (IST-8 and IST-9) tended to be strong as well, but rarely as strong as IST-2 staining.

(4) Usually IST-8 and IST-9 showed a similar level of staining whenever they were positive.

(5) The intensity of staining for IST-8 and IST-9 was independent of each other, therefore the expression of ED-A$^+$ and ED-II$^+$ may be controlled independently.

(6) The three McAbs usually showed similar pattern of

staining on a given part of the placental tissue. In other word, despite their differences in the level of staining intensity, all three Abs showed stronger staining for the chorionic plate than chorionic villi and so on.

(7) ED-I^+ and ED-II^+ FN molecules were usually expressed more in clinical placentae than in normal placentae.

(8) Due to the limits of detection of staining, imposed by the nature of the work and the means of detecting positive signals, a negative staining (i.e. $<0.5^+$) for any of the above Abs does not mean a total absence of signal. Even when a slide was judged negative, a few faint positive areas could be seen as well, negative staining on a particular slide means that the overall intensity of staining was judged negative in comparison with other slides.

Experiments done on separate occasions showed that there were inter-group as well as within group variations. These variations were most probably caused either by experimental variation or differences in the level of expression.

The staining for ED-I and ED-II showed that normal and clinical placentae differentially expressed these two epitopes, but not the common FN epitope recognised by IST-2. Clinical placentae usually had higher levels of expression of ED-I and ED-II than normal placentae, but there were inter-individual differences among the clinical placentae in the intensity of the staining, which could not easily be related to their medical complications. ED-I^- FN proteins have been known to be the plasma type and ED-I^+ are usually the cellular type. The reason behind the higher expression of ED-I^+ FN protein in the clinical placentae is not known but may be related to some of the observed complications, may be in that the pattern of alternative splicing is altered as is the case with the transformed cells (Kornblihtt et al., 1987; Zardi et al., 1987; Dufour et al., 1988). The significance of these findings however, is not clear

and it needs further investigation into the role of the ED-I and ED-II epitopes in FN protein activity.

The expression of FN isoforms in human placenta has not been reported so far, but there have been suggestions that while ED-II$^+$ FN is absent in normal tissue, they are generally expressed in tumour cells and fetal tissues (Zardi et al., 1987). It can therefore be suggested that because placental cells show similarities with fetal cells, espcially in the early stages of development, the same type of expressions may be anticipated in placental cells. At least judging from the results found in this study, none or very little of the ED-II$^+$ FN variant was seen to be expressed in normal placentae. Clinical placentae on the other hand, may express higher amounts of the ED-II$^+$ FN variant, as assessed by IST-8 staining.

At the same time the rather low expression of the ED-I$^+$ FN on most placental sections was unexpected, since most cellular forms of FN contain ED-I isoforms. This may have been caused either by the low affinity of the IST-9 McAb or predominance of cellular form of ED-I$^-$ FN molecules on these tissues. However, at the molecular level (at least in the chorionic plate) both types of FN isoforms (i.e. ED-I$^-$ and ED-I$^+$) were detected (Chapter 3). The detection was not however, a quantitative one but the discussion which follows might apply to the findings at the molecular level. Another possibility for the observed differences between IST-2 and IST-9 could have been caused by contribution of plasma type (ED-I$^-$) FN molecules in these tissues, since plasma FN is exclusively of ED-I$^-$ type (Dufour et al., 1988). If such an assumption is true then the ED-I$^-$ isoform could have come from either fetal serum or maternal serum. If this was true then the plasma type FN in either case, could have been incorporated into the extracellular matrices of the placental cells. Since plasma type FN lacks the ED-I sequence (ED-I$^-$) then they could not be detected by the McAb directed against the ED-I epitope (ED-I$^-$) but could be detected by IST-2.

The above possibility may not be correct, since there have been reports which do not support the notion of plasma FN incorporation into the placental tissues (Isemura et al., 1984; Zhu et al., 1984 and Munakata et al., 1988). One group have shown that FN found in the human placenta was different from adult or fetal plasma FN, in having twice the carbohydrate content (Zhu and Laine, 1987). These FN were called placental FN to differentiate them from plasma FN (Zhu et al., 1984).

Another report has shown that the FN molecules newly synthesised by the chorionic villi of human placenta resembled placental FN in carbohydrate content and their reduced binding ability to denatured collagen (Munakata et al., 1988). In that study the chorionic villi of human term placenta was incubated with labelled amino acid and carbohydrate ([^{14}C]leucine and [^{3}H]glucosamine), to see whether there were any newly synthesised FN molecules in those tissues. If there was any then the labelled amino acid and carbohydrate would be incorporated and could be measured. Munakata et al. showed that the chorionic tissues actively incorporated the labelled molecules and they also observed that the newly synthesised FN molecules were not secreted to the culture medium but were retained within those tissues. Based on their findings Munakata et al. have concluded that FN molecules in the placenta are synthesised locally and retained by tissues involved. In fact several FN synthesising cells, including fibroblastic stromal cells, and endothelial cells in the chorionic villi of human placenta, have been detected recently (Yamada et al., 1987). A similar thing may occur the chorionic plate, so similar investigations to those done by Munakata et al. (1988) need to be carried out.

Another point in support of non-plasma origin for these FN is the finding described in last chapter (Chapter 3) in that DE-I^{-} clone was isolated from placental library. Since plasma FN is made only in hepatocytes in the liver (Kornblihtt et al., 1984a,b;

Umazawa et al., 1985 Sekiguchi et al., 1986a) and then secreted into the blood stream, therefore no mRNA from hepatocytes would be found in placenta and any such mRNA has to have placental origin.

Based on the above findings and assumptions it may therefore be concluded that the strong staining of the chorionic plate sections with IST-2 and not with the other two McAbs, IST-8 and IST-9, was probably due to the predominant presence of ED-I$^-$ and ED-II$^-$ FN molecules in those tissues.

It was then decided to quantify the amount of FN mRNA isoforms produced in chorionic plate of the human term placenta, so a collaboration was set up with a group in Oxford, (Dr. F.E. Baralle, Sir William Dunn School of Pathology, University of Oxford) to do quantitative S1 analysis on these mRNAs. The quantitative S1 analysis of mRNA was attempted a few times using splice-specific cDNA probes, but the experiments did not produce any clear and quantitative results, primarily due to difficulties in obtaining intact RNA. The presence of good quality mRNA is one of the most important factors in any quantitative S1 analysis (Greene and Struhl 1987). It may be that a proportion of the chorionic plate cells were degenerating during and after delivery in which case the mRNA present in those cells would probably be rapidly degraded.

There are few other ways of investigating the pattern of expression of FN and its isoforms in the placental tissues. One is the isolation of placental cells from different anatomical regions and examination of them for the expression of FN and its isoforms using specific Abs. FN proteins could be purified from placental tissues such as chorionic plate or chorionic villi, and then be digested with proteolytic enzymes such as those which have already been used to study FN and its isoforms (e.g. thermalysin and Cathepsin-D; Zardi et al., 1987). The digested fragments could then be

tested with specific McAbs to see which tissue produces what kind of FN isoforms. This technique has already been used to show that tumour-derived and SV 40-transfected human cells release ED-I^+ FN molecules in about 10 times greater amounts than normal human fibroblast cells, by which the McAbs IST-9 and IST-4 (which recognises a determinant common to all FN types) in a competition radioimmunoassay system (for details see Borsi et al., 1987). Similar technique may be used to quantify the amount of FN and its isoforms produced by different placental tissues.

At the DNA and cellular level, ED-I, ED-II and IIICS DNA probes which already exist (two of these, IIICS and ED-I, were obtained from the work described in the previous chapter), could be used to do *in-situ* hybridisation on placental tissue sections in order to localise cells producing FN as well as ED-I^+ and ED-II^+ mRNA.

Any future research, besides investigating some of the unknowns discussed above, should be directed toward establishing the biological as well as developmental roles of these splicing isoforms.

CHAPTER 5

CDM8 CLONING VECTOR

5.1 Introduction

The aim of the work presented in this chapter was to utilize an already existing high efficiency cloning and expression vector, CDM8, (Aruffo and Seed, 1987a; Seed, 1987; Fig. 5.1) for cloning a placental cDNA library. The reason for using CDM8 vector is that cDNA cloned into CDM8 can be expressed in mammalian cell lines such as COS cells (Chapter 6) and the coded protein can be expressed in its native conformation coupled with the appropriate patterns of post-translational modifications. The CDM8 is a plasmid based vector and it can be easily made in large amounts and also it can be manipulated easier than for example lambda based expression vector (Maniatis *et al.*, 1982).

The work in this chapter, however, was focused on optimising the cloning efficiency of the CDM8 vector. The optimisation steps included preparation of the vector DNA, addition of adaptors to DNA fragments to be cloned into the CDM8 vector, cloning of the inserts into CDM8 and finally transformation of the recombinants into competent MC1061/p3 (MC) *E. coli* cells.

5.2 BstX1 Digestion of CDM8 Vector

In order to clone foreign DNA into CDM8 vector, the vector DNA had to be digested with BstX1 restriction enzyme. CDM8 DNA was restricted with the BstXI enzyme and a stuffer band of about 350 bp and a vector band of about 4.1 Kbp were seen on an agarose gel as expected (Simmons personal communication, Figure 5.2).

BstX1 digestion of the vector would usually release the stuffer. Presence of the stuffer in the ligation mix with the vector, in theory, would favour recircularisation of the vectors during cloning into the CDM8 vector. In fact it was observed that when CDM8 vector was cut with BstX1 enzyme and then religated

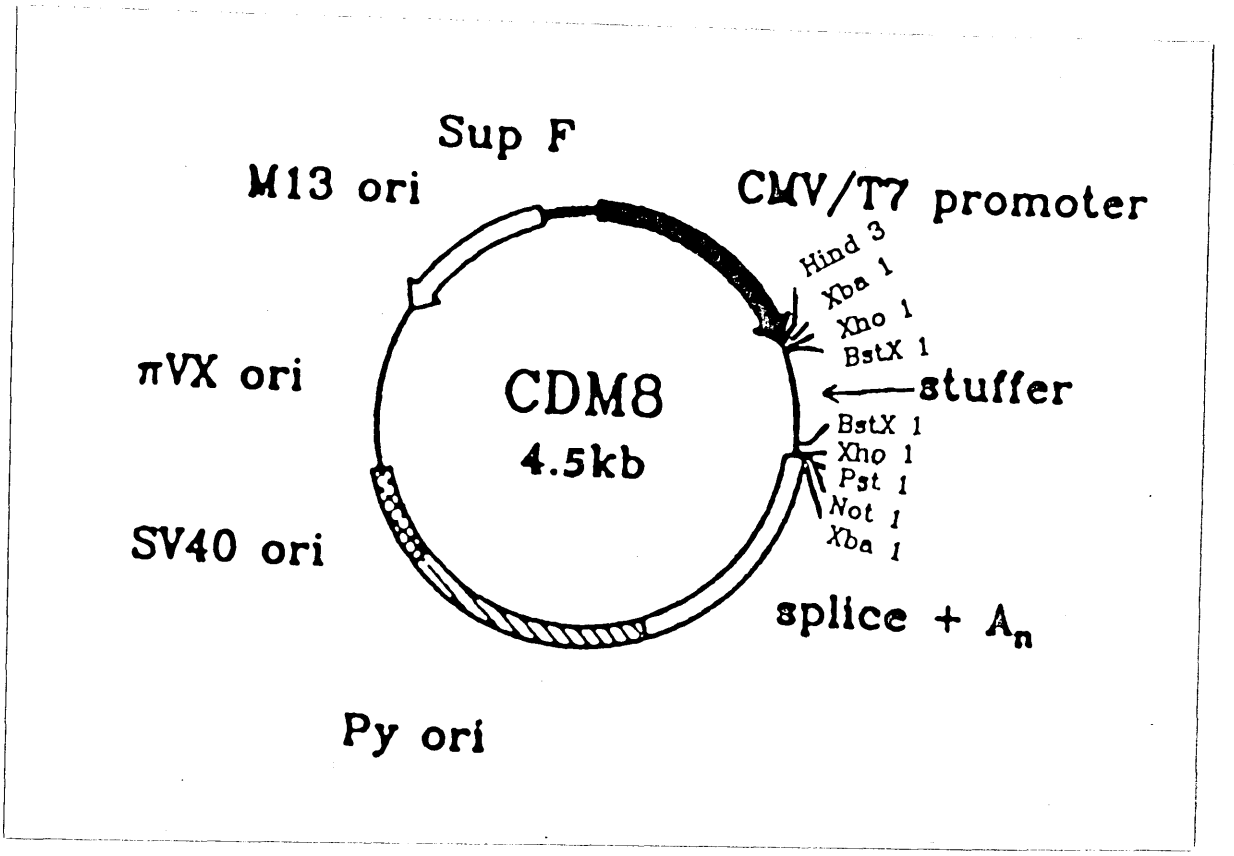


Figure 5.1 Map of CDM8 cloning vector. The 4512 bp vector is made of 8 segments. PiVX is from pBR322 origin. M13 ori is from M13 origin. Sup F is from synthetic tyrosine suppressor tRNA gene (sup F gene). CMV/T7 are from human cytomegalovirus and T7 RNA polymerase promoter. The two polylinker regions are separated by a replaceable fragment called stuffer. Splice $+A_n$ is from pVS2 and are splice and polyadenylation signals. Py ori is from polyoma origin. Finally SV40 ori is from simian virus 40 origin. Some of the restriction endonuclease sites flanking the BstX1 cloning sites are shown. The directions of transcription are indicated by the arrows.

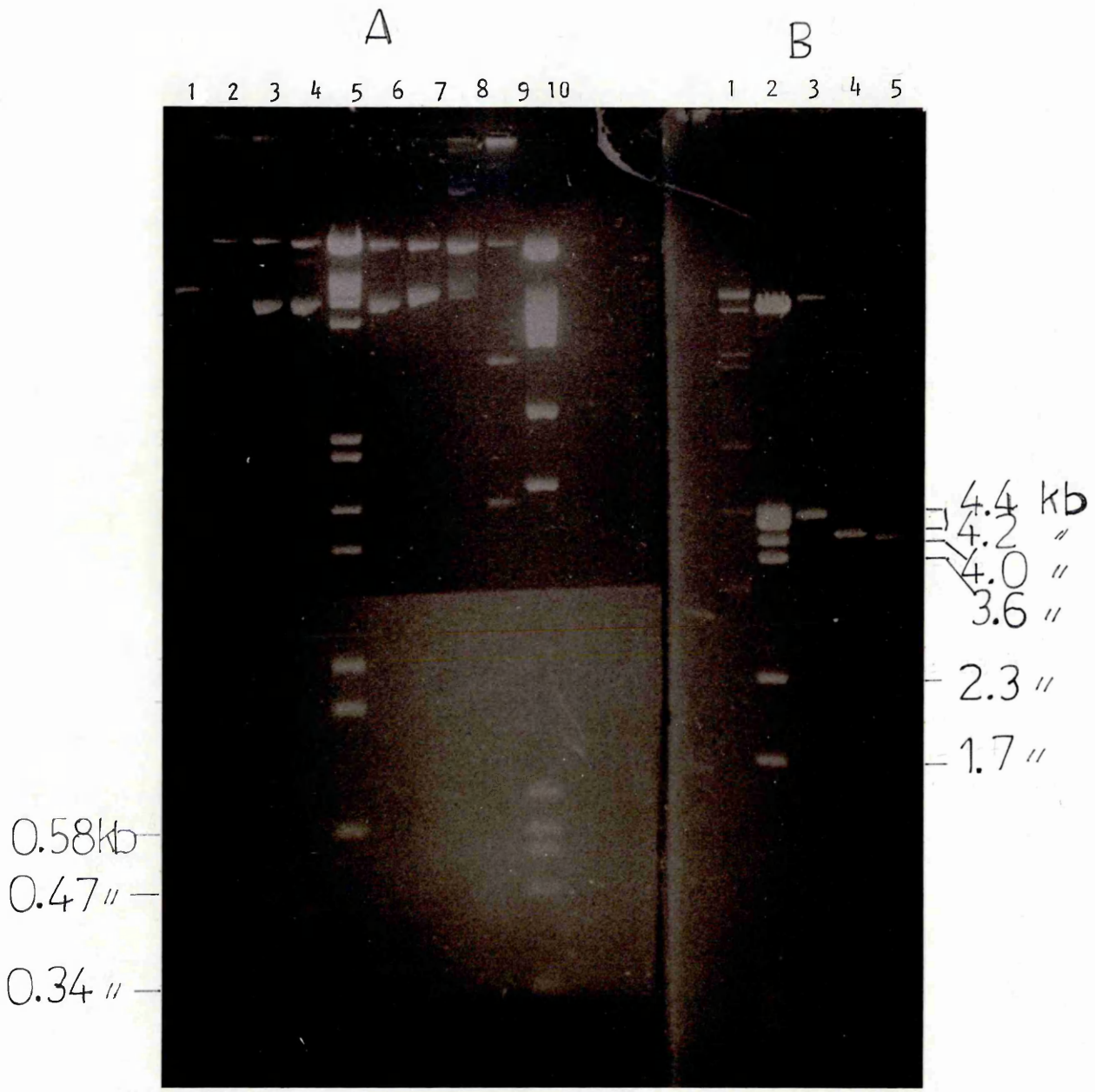


Figure 5.2 BstX1 digested CDM8 vector. CDM8 digested with BstX1 retriction enzyme to show the size of the insert (arrow) (a). The size of the vector is shown on the right (b).

(a)

1-pBR322 uncut
2-CDM8 uncut
3-CDM8 XbaI
4-CDM8 Hind3/PstI
5-Lambda RI/Hind3
6-CDM8 BstXI
7-CDM8 Hid3
8-CD2 BstX1
9-CD2 XbaI
10-Lambda PvuII

(b)

1-CD2 XbaI
2-Lambda PvuII
3-CDM8 Hind3
4-CDM8 BstXI
5-CDM8 PstI/Hind3

without removal of the stuffer, most of the vectors recircularised (data shown later in this chapter).

5.3 Separating the CDM8 Vector from the Stuffer

In order to make stuffer free CDM8 vector, the BstXI digested DNA was either "Gene Cleaned" (Chapter 2) or separated using KOAc gradient. To separate the vector from the stuffer by Gene Cleaning method, the digested DNA was electrophoresed on a 0.6% TAE gel and the vector band was cut out from the gel and purified (Fig. 5.3). The purified DNA was checked on an agarose gel to confirm the recovery of DNA at the end of the purification procedure (Fig. 5.4).

The other method of separating the vector from the stuffer involves restricting the CDM8 DNA and overlaying it on to a 5%-20% KOAc gradients (see Chapter 2). After spinning down the gradient, a thick band for CDM8 and a fainter band for stuffer were usually visible under UV light. These bands were either recovered directly, by syringing them out, or indirectly by fractionation (Chapter 2). The DNA recovered by fractionation were tested on an agarose gel to find out which fraction contained the vector or the stuffer DNA. This was also done to see how efficient the recovery of DNA from the fractions had been (Fig. 5.5). In both cases (direct recovery or fractionation), the efficiency of recovery was poor. The fractionation method of recovery was primarily done to test the fraction collection effeciency on a known amount of DNA for optimisation purpose.

5.4 Efficiency of Ligation of the Purified DNA

The "Gene Cleaned", stuffer free, CDM8 vector was test religated to see whether the BstXI digested vector would recicularise. The ligation mix was transformed into MC *E. coli* competent cells. The frequency of

Figure 5.3 The BstX1 digested CDM8 DNA on TAE agarose gel to be excised and Gene Cleaned. The vector band in track 2 was excised from the gel and purified.

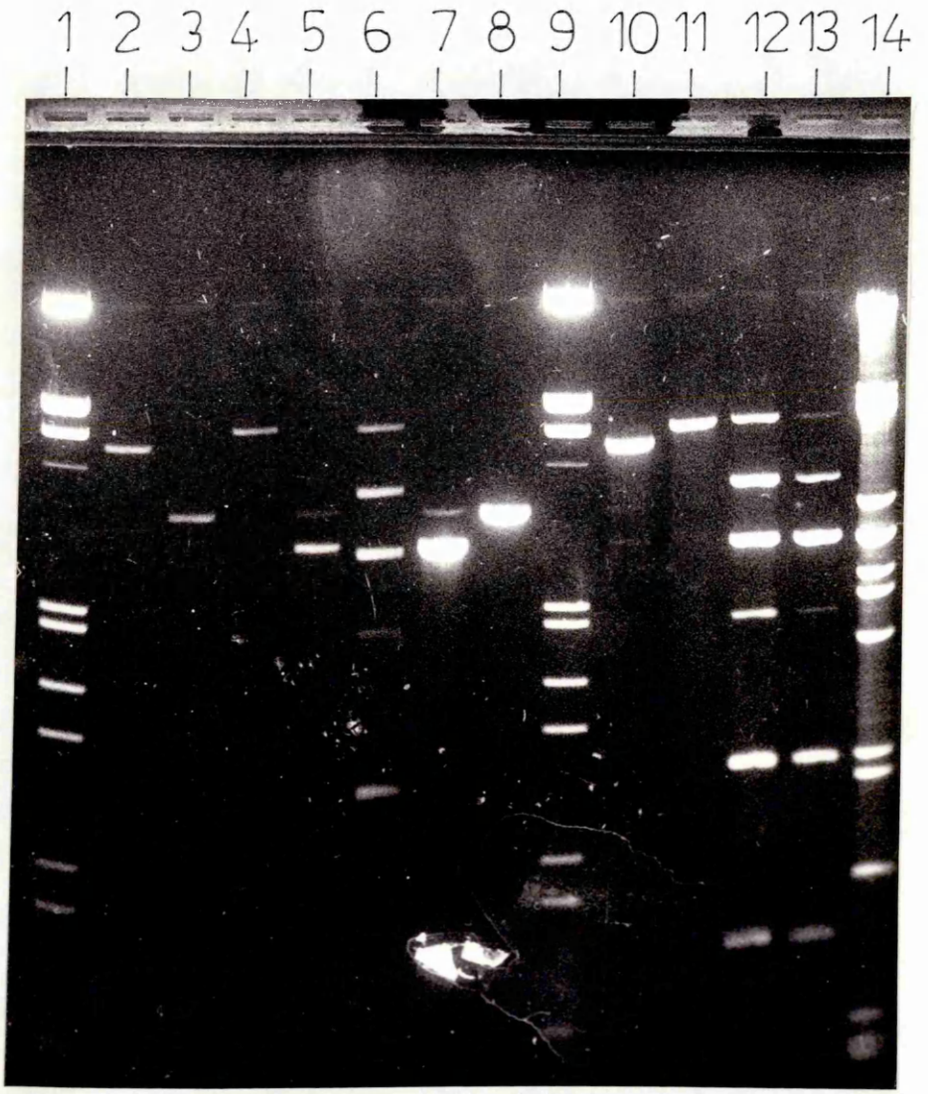


Figure 5.4 The Gene Cleaned DNA on agarose gel. The Gene Cleaned DNA from CDM8 BstXI digests are shown in tracks 2 and 10. Lambda HindIII EcoRI digested markers are shown in track 1 and 9.

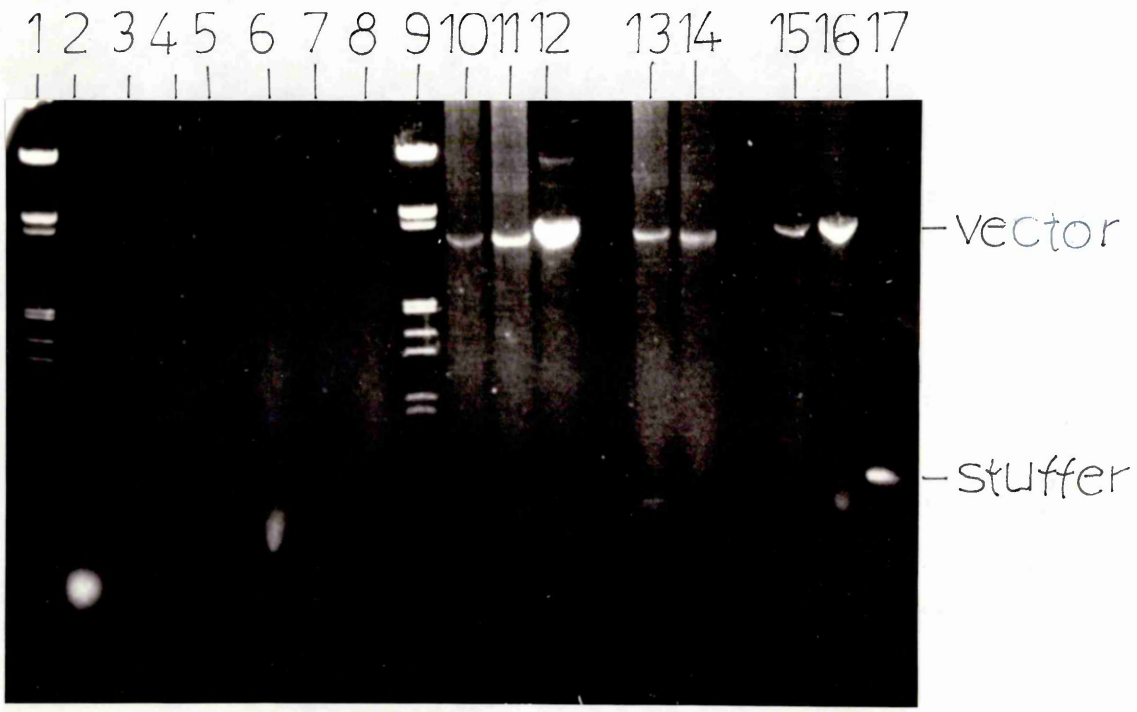


Figure 5.5 The DNA purified using a 5%-20% KOAc gradiant. The figure shows that the early fractions 2-8 do not contain much DNA but most vector DNA are in tracks 9-16. The stuffer DNA is seen in track 17. Tracks 1 and 9 contain lambda Hind3/EcoRI marker.

transformantion was similar to untransformed MC *E. coli* cells, confirming that vector DNA could not religate in the absence of the stuffer. This was expected because the band which was recovered by Gene Cleaning was free from stuffer and no undigested vector band was visible, on the gel, indicating that the digestion had been mostly complete (Fig. 5.4). Similar observation was seen when the fractionated vector DNA (from KOAc gradient) was test ligated and transformed into the MC *E. coli* cells.

Purified CDM8 vector and stuffer (by either the Gene Cleaning or KOAc gradient) were ligated back to each other to check whether purified vectors can religate to purified stuffers as expected. This was done to confirm that the cohesive ends in the vector (or even the stuffer) were intact and had not been nibbled by over digestion. The ligated mix was transformed in to the MC *E. coli* cells and the frequency of transformation was $2\text{-}7\text{x}10^4$ colonies/ug of DNA. With the same experiment $7\text{x}10^6$ colonies were obtained for each ug of uncut CDM8 DNA. The rather high frequency of transformation from the purified DNA showed that the stuffer was ligating back to the vector. This indicated that probably most of the cohesive ends were left intact.

5.5 Preparing DNA Fragments for Cloning into CDM8

It was anticipated that optimising the steps involved in the CDM8 cloning system, including the addition of the adaptors to cDNA, the cloning of cDNA into CDM8 and most importantly fractionating adaptor added cDNA through KOAc gradient would be difficult and risky because the cDNA might be lost during the cloning procedures. It was therefore decided that before cloning any placental cDNA library into CDM8 it would be better if the cloning procedures were tested using less complex series of clonable fragments. For this reason pBR322 DNA (Sutcliffe, 1978) was chosen to provide the required

fragments. It was thought that monitoring the experimental steps involving the cloning of pBR fragments would be easier than those of cDNA, since only a few fragments of known sizes were involved, observation of the purified DNA on the gel would be easier. Another reason was that the preparation of the pBR fragments was much cheaper and quicker than cDNA. Therefore, it was decided that pBR DraI/PvuII double digests of the pBR plasmid would give rise to the desired fragments (Fig. 5.6). For this purpose pBR DNA was cut with DraI/PvuII restriction enzymes and 3 major fragments were obtained (2.4 Kbp, 1.16 Kbp, and 0.7 Kbp; Fig. 5.7). The DraI/PvuII digested DNA fragments were usually extracted with phenol/chloroform and ethanol precipitated, or Gene Cleaned to obtain pure DNA fragments. These fragments were used for addition of BstX1 specific adaptors (see below).

5.6 BstX1 Specific Adaptors

Cloning cDNA into CDM8 usually involves the addition of two BstX1 specific adaptors to either ends of the cDNA (blunt ligations). The "adaptored" cDNA is then ligated to the BstX1 digested CDM8 vector. The same type of procedures should be followed for cloning pBR fragments into the CDM8. For this purpose two oligonucleotides were obtained which shared 8 complementary bases which could form a duplex at the right temperature (22^{o}C, Fig. 5.8). The duplex would give one blunt end and one cohesive end (typical nature of every adaptor). The sequences of the two oligonucleotides, as well as their complementary BstX1 digested ends of CDM8 after removal of the stuffer, are shown (Fig. 5.9).

The sizes of the two oligonucleotides for making adaptors were confirmed by end labelling some with gamma-^{32}P-ATP and running them on a 20% polyacrylamide gel (Fig 5.10). Since duplex formation is essential to make adaptors, the two oligonucleotides were tested for

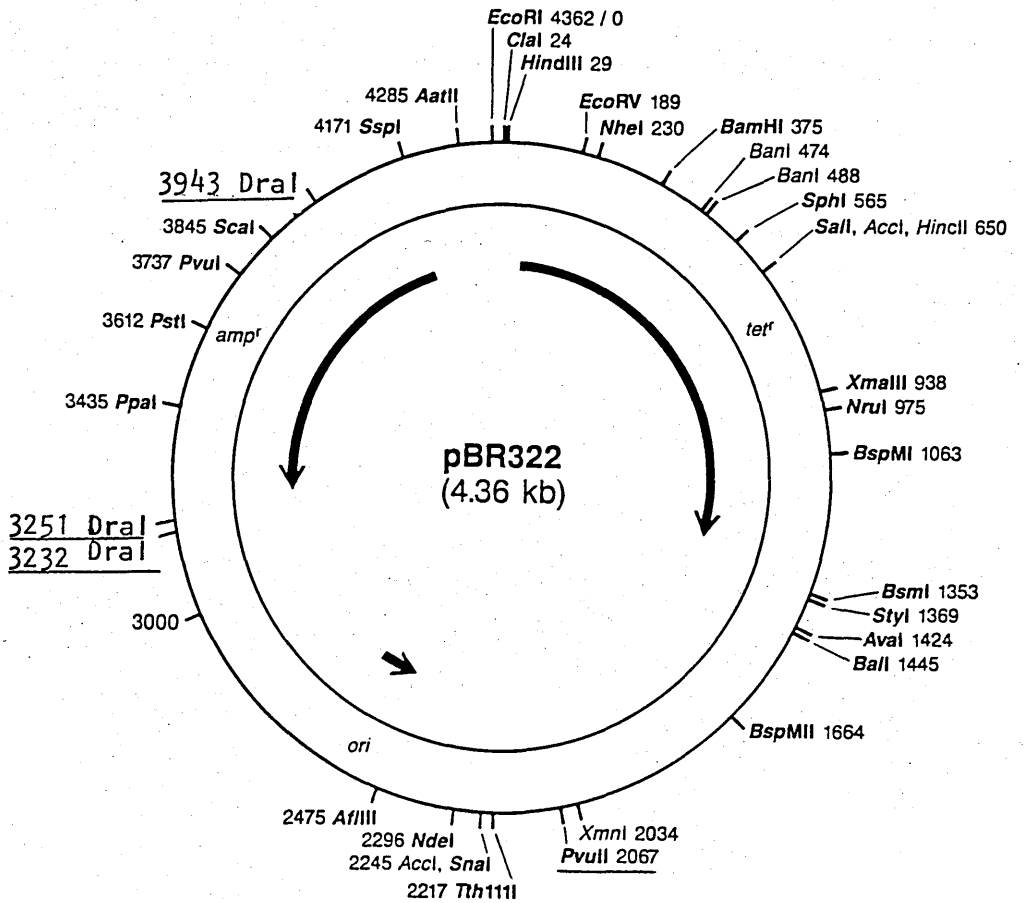


Figure 5.6 The pBR322 plasmid with some of the restriction sites. The two restriction enzyme sites (DraI and PvuII) are underlined.

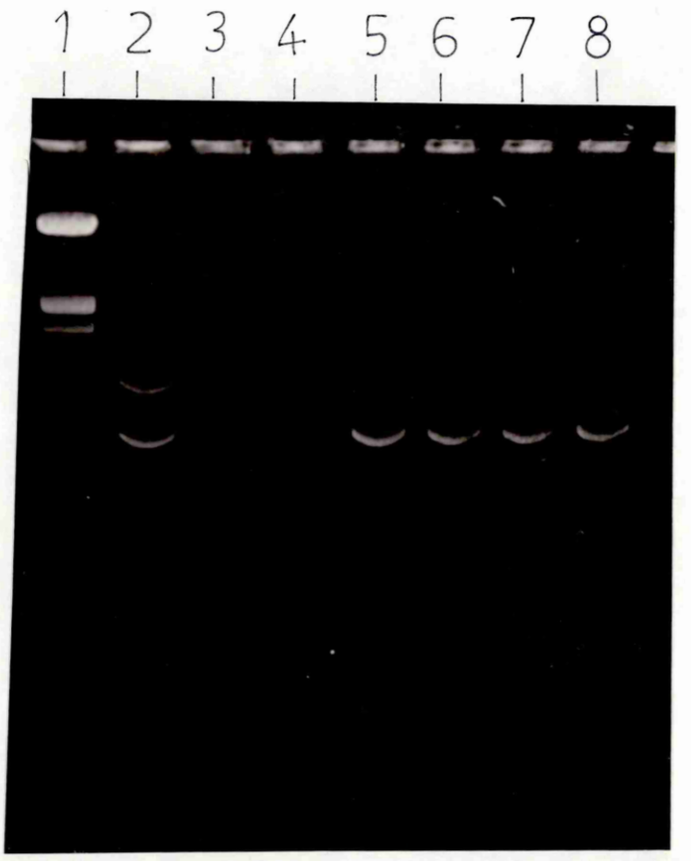


Figure 5.7 The pBR DNA cut with DraI and PvuII restriction enzymes. pBR DNA was cut in separate occasions and some are shown here (tracks 5-8).

			TM
5' C T T T A G A G C A C A	3'	3 (G/C) X 4	= 12
3' G A A A T C T C	5'	5 (A/T) X 2	= 10
			—
	Total TM		22°C

Fig. 5.8 The melting temperature of the BstX1 adaptor (duplex form). The (TM) is calculated from the base pairs formed and is 22°C.

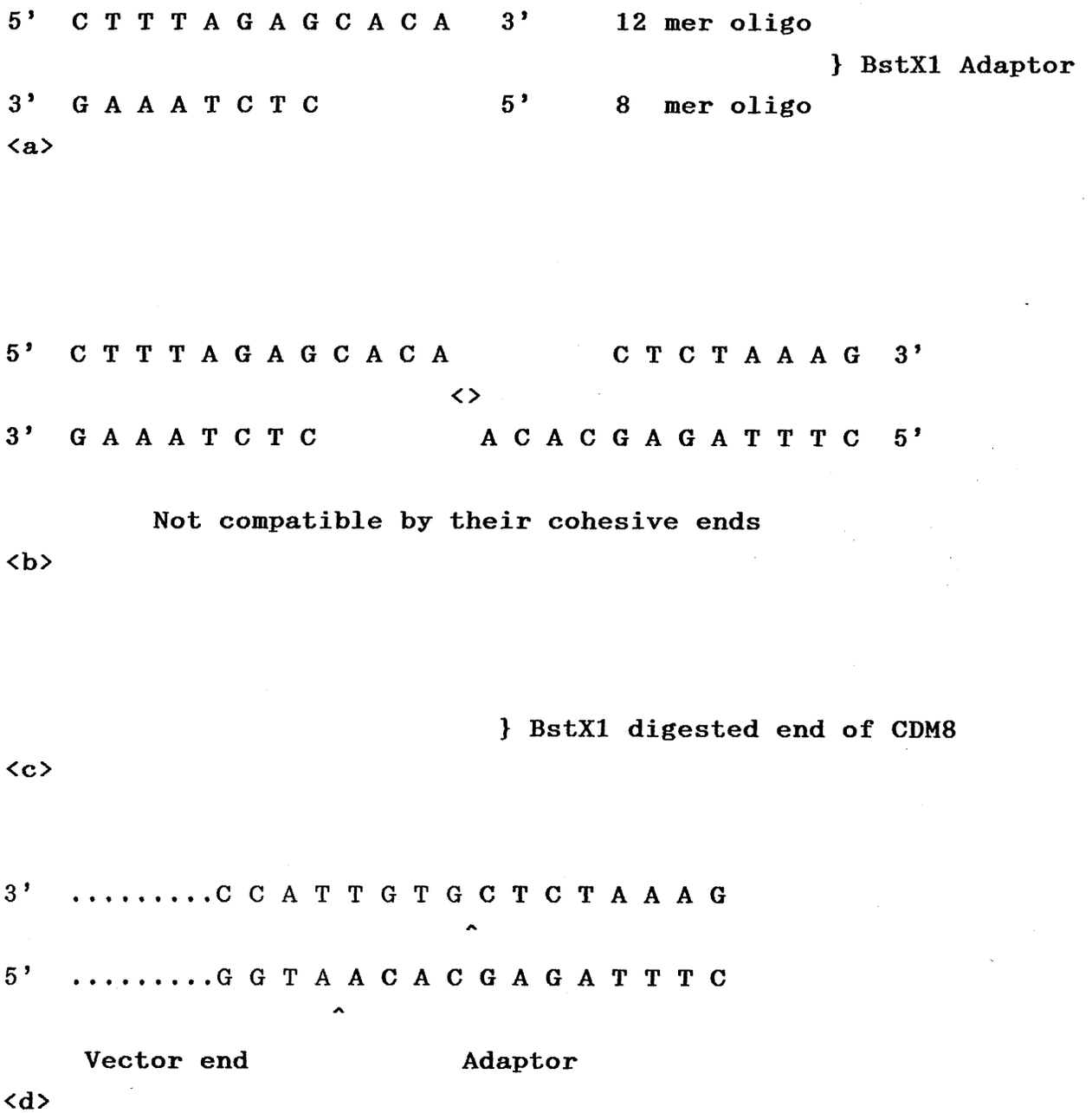


Fig. 5.9 BstX1 specific adaptors. <a> is formed by duplex of 12 mer and 8 mer oligonucleotides. Two adaptors can not ligate at their cohesive ends <b>. BstX1 digested of CDM8 vector <c>. The BstX1 specific adaptor is compatible to the BstX1 digested ends of the vector <d>. <> means not compatible and ^ means site of joining the two ends.

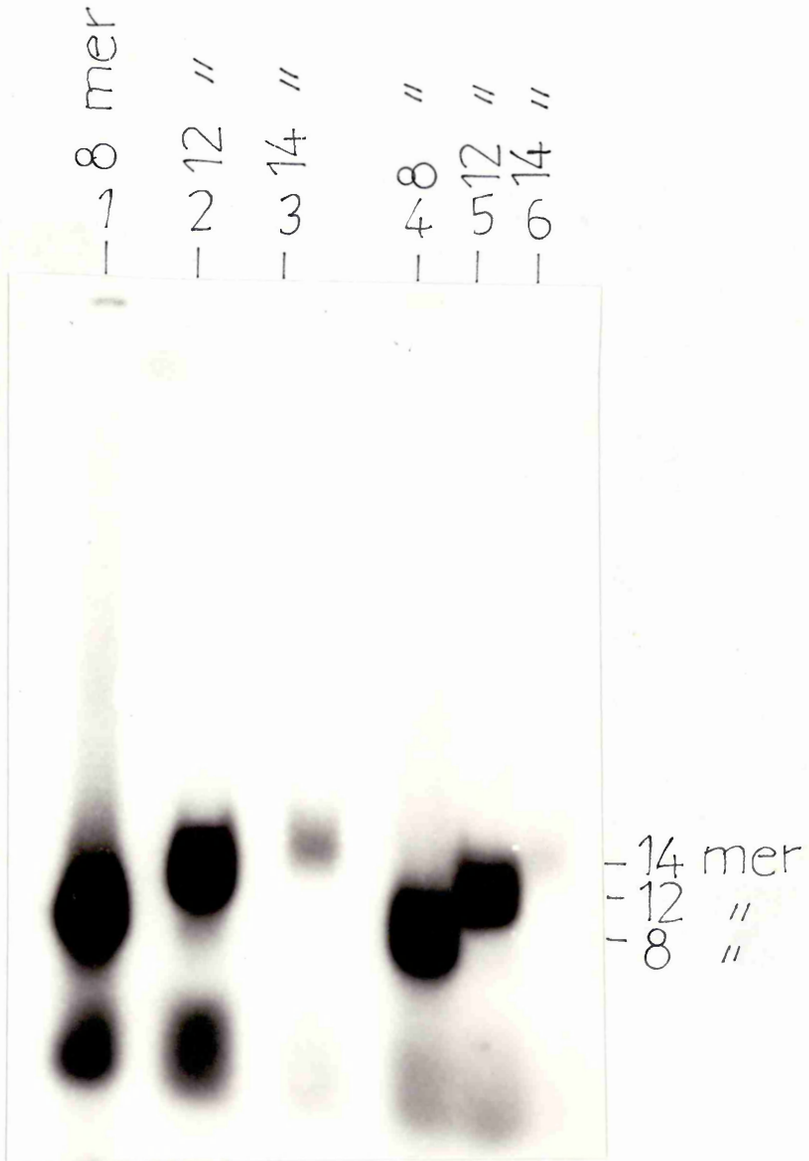


Figure 5.10 The end labelled oligonucleotides on polyacrylamide gel. The size of oligonucleotide primers are compared with another oligonucleotide of 14 mer.

duplex formation by mixing the two end labelled oligonucleotides at 4°C. This method of testing duplex formation was thought to be a quick though indirect way of confirming that the correct sequences were present for the adaptor formation.

An aliquot from the oligonucleotide mixture was also ligated to see whether the duplex forms were actually adaptors. The ligation reaction was carried out at 12°C for only 3 hours. It was hoped that by limiting the time of ligation some of the adaptors would stay as single adaptors and others would ligate to each other. This way both duplex formation and blunt end ligation of the adaptors could be shown to occur. An aliquot from the oligonucleotide mix and another aliquot from the ligation reaction were run on a non-denaturing polyacrylamide gel. The gel was run at 4°C to avoid melting or dissociating the adaptor into the two oligos. The gel was set for autoradiography and it was observed that indeed the adaptor had one blunt end and one sticky end, i.e. the banding pattern showed the presence of only one duplex band and one double adaptor band as was predicted (Fig. 5.11). These adaptors had only one compatible end, and according to the sequence, this must be the blunt end.

5.7 Addition of Adaptors to pBR322 Fragments.

In order to clone pBR322 fragments into BstX1 digested CDM8 vector, adaptors formed from end labelled oligonucleotides were ligated to the blunt ended pBR fragments. The oligonucleotides were present in an excess molar ratio (>125:1) to ensure that pBR fragments would not ligate each to other. Ligation of adaptors to the pBR fragments was also observed by autoradiography (Fig. 5.12). Comparisons of digested pBR fragments before and after adaptor additions showed (on gel and autoradiograph respectively) that the adaptors had been added efficiently (compare Figs. 5.7 and 5.12).

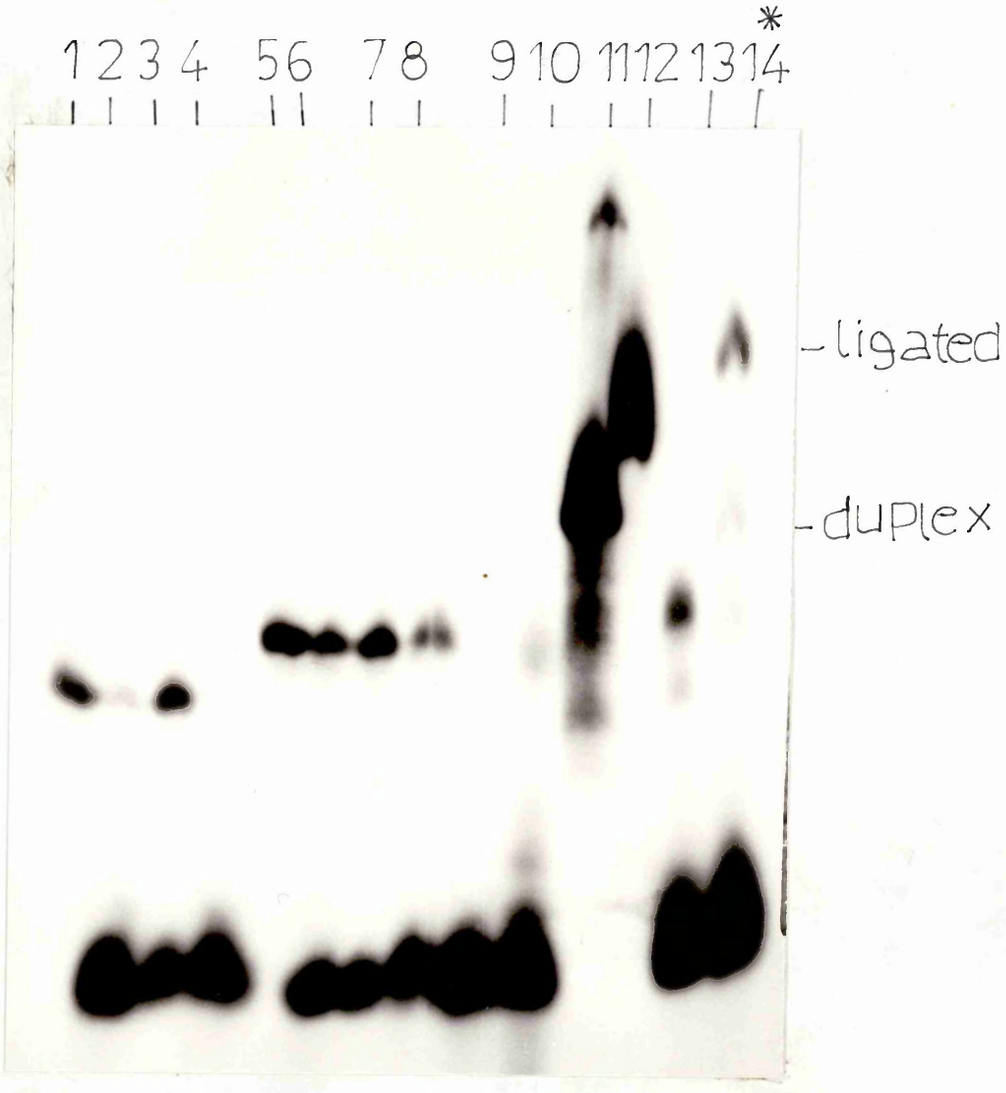


Fig. 5.11 End labelled oligonucleotides on a non-denaturing 20% polyacrylamide gel. The Oligonucleotides specific for forming BstX1 adaptors, duplex (adaptor) formed and blunt end ligated adaptors are shown.

1-4 8 mer
4-8 12 mer
9-12 markers
9 15 mer
10 15 mer
11 21 mer
12 26 mer
13 duplex (adaptor)
14 two adaptors ligated

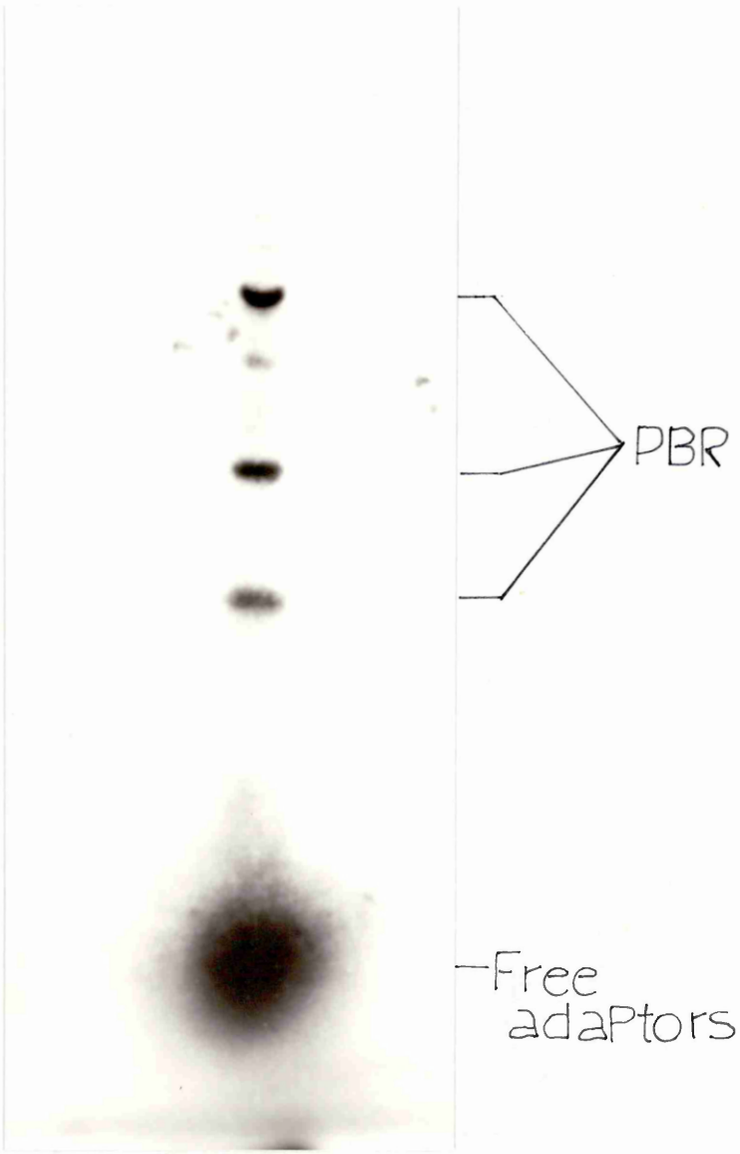


Figure 5.12 The autoradiograph of adaptored pBR on agarose gel. By endlabelling the primers which make up the adaptor it is clear that they have ligated to PBR fragments.

5.8 Need to Remove Free Adaptors

If the high number of free adaptors which are present in the adaptor added pBR ("adaptored pBR") ligation mix are not removed then they could block the BstX1 sites of the vector. This would drastically reduce the efficiency of the cloning of pBR fragments into CDM8 vector (or if double stranded cDNAs are involved). In theory the adaptors could block the two ends of the vector by at least 4 different ways (Fig. 5.13). Removal of free adaptors from the adaptored pBR was therefore absolutely necessary. The free adaptors usually were separated from the "adaptored" pBR fragments by fractionation through a 5-20% KOAc gradient (Aruffo and Seed 1987a). For this purpose usually a small amount of the oligonucleotides were end labelled prior to ligation to pBR. The labelling was primarily done to monitor the recovery of the adaptored pBR fragments more effectively. This was achieved by monitoring the presence of radioactivity in the collected fractions.

In order to recover the DNA, the fractions were ethanol precipitated using linear polyacrylamide as carrier of DNA according to the recommended procedure (Aruffo and Seed, 1987a and personal communication). It was usually observed that very little DNA was recovered each time. That is to say the total amount of DNA recovered was not comparable to the amount which had been layered on to the gradient. The efficiency of recovering the DNA was usually tested by, in most cases, running a sample from each fraction on an agarose gel. On other occasions the recovery was also monitored by measuring the amount of radioactivity recovered as mentioned above.

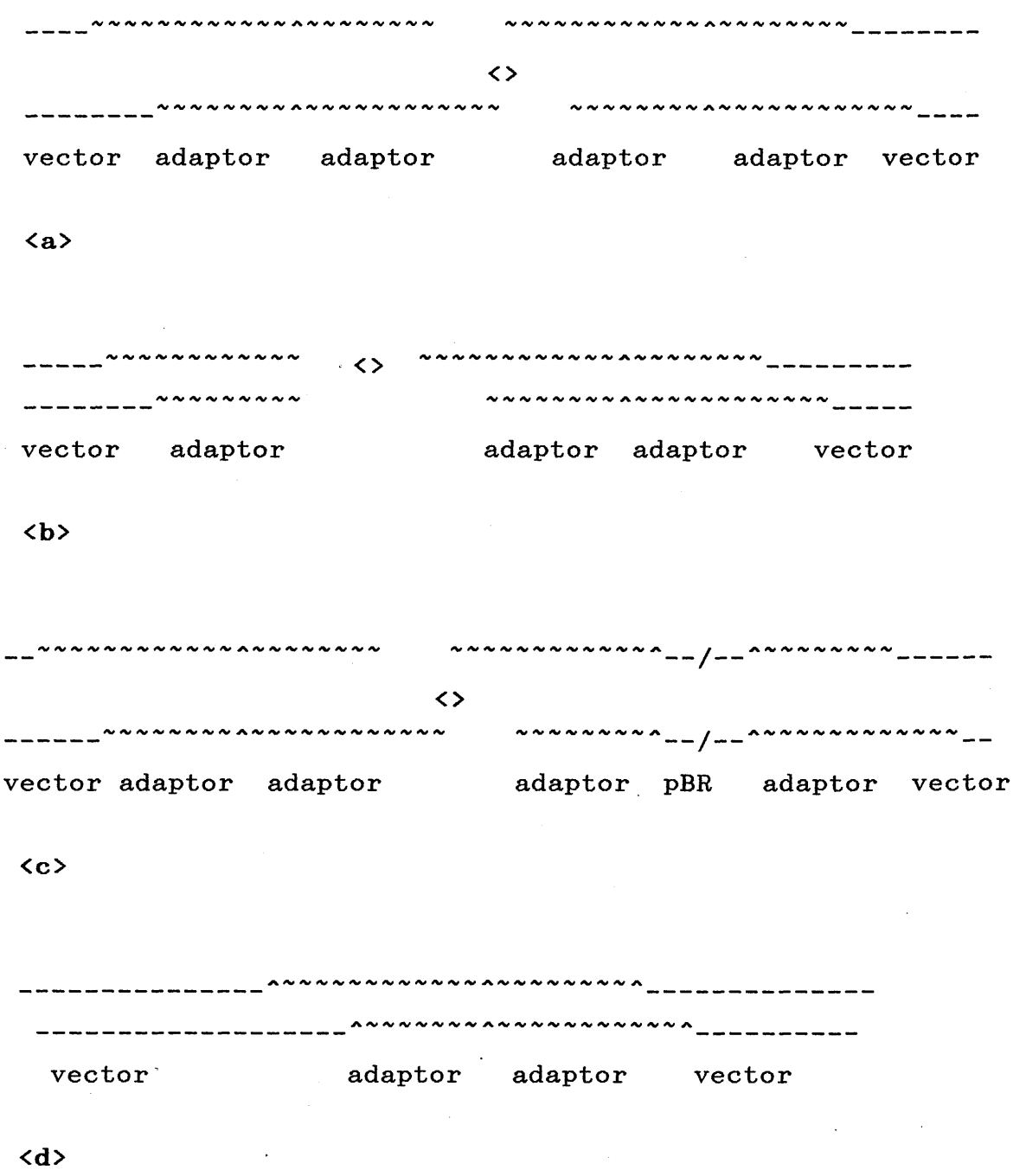


Fig 5.13 Free adaptors interference with the ligation of pBR fragments to vector DNA. There are at least four po bilities that free adaptors may block the vector sites threrefore may reduce the formation of pBR/CDM8 recombinants. In <a> both ends are blocked by double adaptors. In <b> one end is blocked by a single adaptor and the other one by a double adaptors. In <c> one end is blocked by adaptors but the other end is ligated by the pBR fragment but can not join to the other end because of the blockage. In <d> adaptors have joined the two ends of the vector and have created false recombinant. Sites of ligation are shown (^) and <> means the ends are nor compatible.

5.9 Optimising the Ethanol Precipitation of the Fractionated DNA

To test the efficiency of precipitating the fractionated DNA, BstX1 digested CDM8 DNA was spun through a 5-20% KOAc gradient. The collected fractions were precipitated as above, using linear polyacrylamide. Some of the resuspended materials were run on an agarose gel to check the efficiency of the DNA recovery. This was also done to find out which fraction contained the vector or the stuffer DNA. The gel showed that the amount of DNA recovered was much less than the amount of the DNA which had been layered on the gradient (Fig. 5.14). This experiment also suggested that this method of recovery of DNA was not so efficient one and an alternative method should be employed.

In order to improve the ethanol precipitation of DNA recovered from KOAc gradient, it was decided to test several different salts and carriers besides linear polyacrylamide. This was done to find out which of these would aid the precipitation of the DNA more effectively. An experiment was therefore set up to test the above hypothesis, that is the linear polyacrylamide does not recover DNA efficiently. In this experiment variable amounts of uncut pUC18 or CDM8 DNA (50-200 ng) were precipitated using salts or carriers listed in Table 5.1. Glycogen was included in this experiment because it is recomended that collagen would efficiently precipitate nucleic acids (Tracy, 1981; Helms et. al., 1985). It has also been shown that as little as 5 pg DNA could be precipitated if glycogen was used to precipitate DNA (Boehringer Mannheim UK supplied information leaflet). The DNA recovered above, by ethanol precipitation, were electrophoresed on agarose gels (Figs. 5.15 and 5.16).

It was shown that fractionated DNA from KOAc gradients were not recovered efficiently using linear polyacrylamide or the salts listed in Table 5.1. The

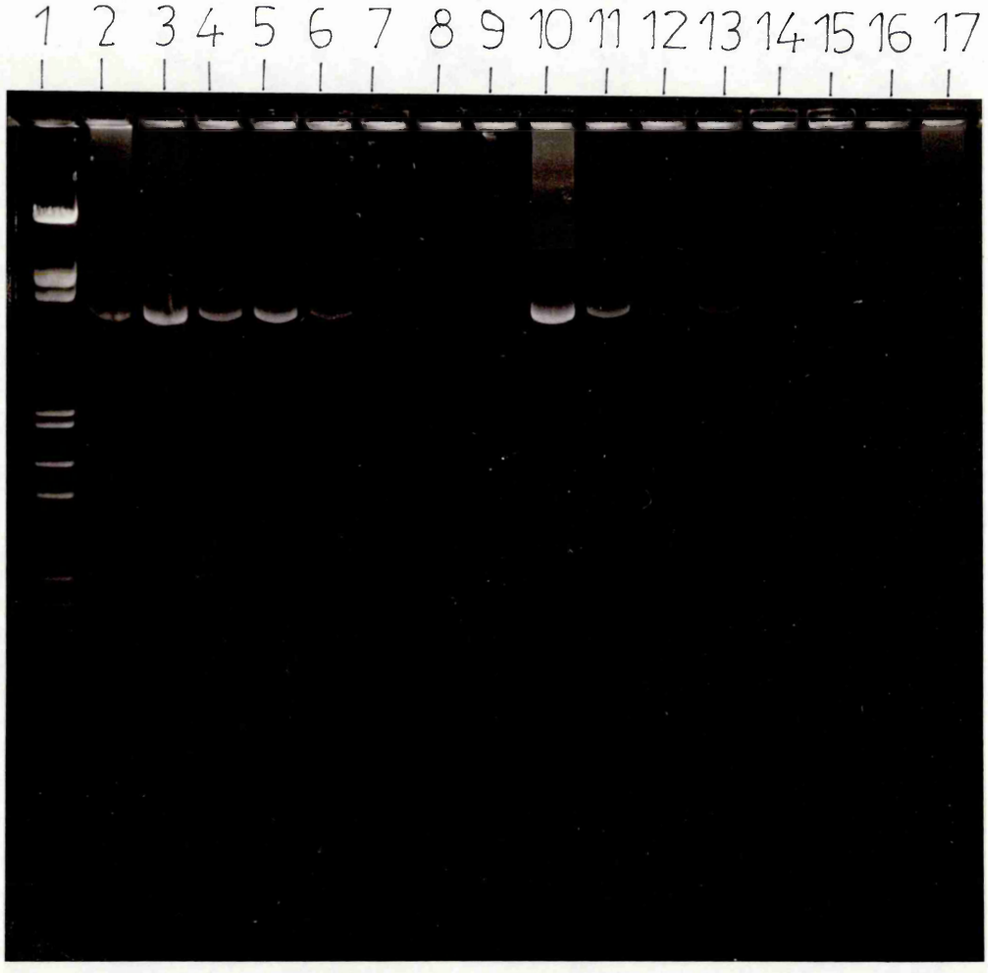


Fig. 5.14 Ethanol precipitated DNA on an agarose gel. DNA samples which had been recovered from fractions by using linear polyacrylamide as carrier, were run on a 0.7% agarose gel. Two sets of experiments are run in this gel (tracks 4-9 and 11-16). DNA in tracks 2, 9, 17 are cut but unfractionated CDM8 DNA as marker. Some stuffer DNA have also been recovered (faint bands in tracks 7, 14 and 15, nevertheless the recovery was poor.

Table 5.1 Ethanol precipitation of pUC18 or CDM8 DNA using different salts or carriers. Variable amounts of DNA were precipitated using either a single carrier/salt or combination of salts/carriers to find out which combination gives the best DNA recovery. The pellete formation was also recorded to compare them with the intensity of the DNA bands on the gel. l. acrylamide = linear acrylamide. l. polyacryl = linear polyacrylamide. NS = not sure. The * indicates that the DNA in these two tubes were in dH_2O rather than KOAc.

No.	DNA	Con. ng	Carriers/Salts	Con. ug	Pellete
1	pUC18	200	l. polyacryl.	20 ug	NS
2	pUC18	100	l. polyacryl.	20 ug	2+
3	pUC18	50	l. polyacryl.	20 ug	3+
4	CDM8	200	l. polyacryl.	20 ug	2+
5	CDM8	100	l. polyacryl.	20 ug	2+
6	CDM8	50	l. polyacryl.	20 ug	2+
7	pUC18	100	l. acrylamide	20 ug	2+
8	pUC18	50	l. acrylamide	20 ug	3+
9	pUC18	100	tRNA	10 ug	2+
10	pUC18	50	tRNA	10 ug	2+
11	pUC18	100	NaCl	0.1 M	1+
12	pUC18	50	NaCl	0.1 M	1+
13	pUC18	100	NaoAc	0.25 M	-
14	pUC18	50	NaoAc	0.25 M	-
15	pUC18	100	AmmoAc	2 M	1+ NS
16	pUC18	50	AmmoAc	2 M	1+ NS
17	pUC18	100	glycogen	50 ug	4+
18	pUC18	50	glycogen	50 ug	4+
19	pUC18	100	glycogen	200 ug	4+
20	pUC18	50	glycogen	200 ug	4+
21	pUC18	100	l. polyac/NaoAc	20/0.25	-
22	pUC18	50	l. polyac/NaoAc	20/0.25	-
23	pUC18	100	l. polyac/NaoAc	20/0.25	- *
24	pUC18	50	l.polyac./NaoAc	20/0.25	- *

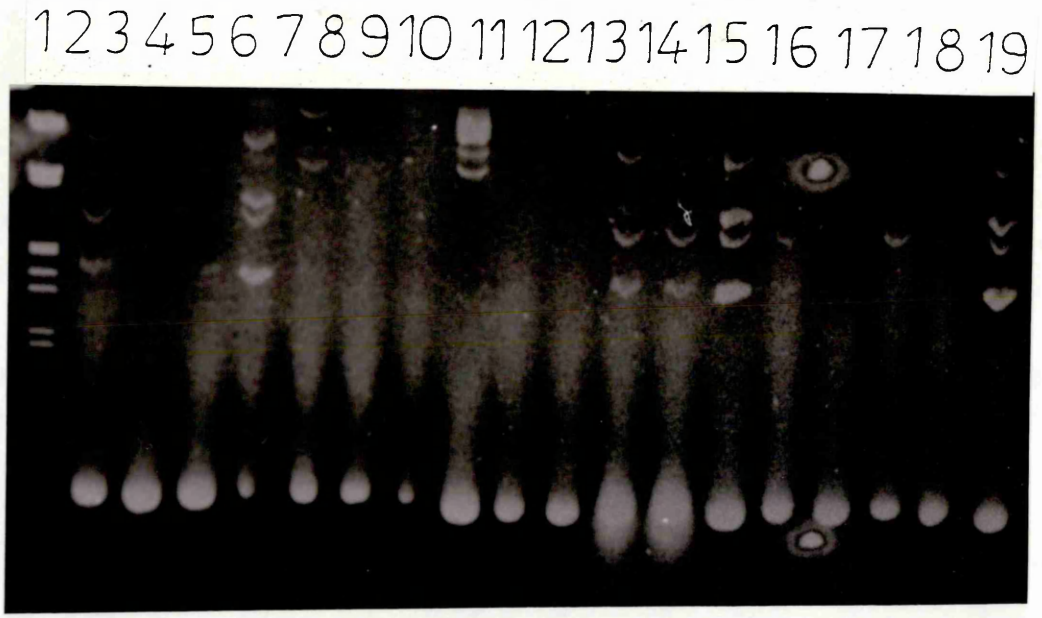


Figure 5.15 Ethanol precipitated DNA using various salts or other carriers were run on an agarose gel. The DNA samples which were precipitated as outlined in the Table 5.1 were run on a 0.8% gel. Samples from tube 1-14 were run on this gel and from 15-24 were run on gel seen in Fig. 5.16.

1. \ Hind III/EcoRI marker
2. DNA from tube 1
3. DNA from tube 2
4. DNA from tube 3
5. Uncut pUC18 (200 ng)
6. DNA from tube 4
7. DNA from tube 5
8. DNA from tube 6
9. Uncut CDM8 (200 ng)
10. DNA from tube 7
11. DNA from tube 8
12. DNA from tube 9
13. DNA from tube 10
14. Uncut pUC18 (200 ng)
15. DNA from tube 11
16. DNA from tube 12
17. DNA from tube 13
18. DNA from tube 14
19. Uncut pUC18 (200 ng)

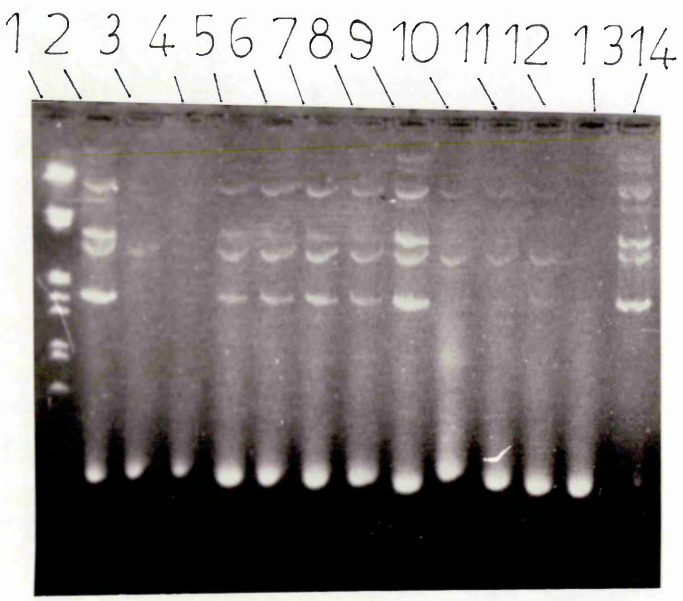


Figure 5.16 The remaining samples from ethanol precipitated DNA using various salts or other carriers. The DNA samples which were precipitated as outlined in the Table 5.1 were run on a 0.8% gel. Samples 1-14 were run on gel seen in Fig 5.15 and samples 15-24 are seen here.

1. \ Hind III/EcoRI marker
2. Uncut pUC18 (200 ng)
3. DNA from tube 15
4. DNA from tube 16
5. DNA from tube 17
6. DNA from tube 18
7. DNA from tube 19
8. DNA from tube 20
9. Uncut pUC18 (200 ng)
10. DNA from tube 21
11. DNA from tube 22
12. DNA from tube 23
13. DNA from tube 24
14. Uncut pUC18 (200 ng)

recovery was, however, more efficient if either tRNA or glycogen was used. It was therefore decided to use either tRNA or glycogen to recover DNA from fractionation.

5.10 Removing Free Adaptors After the Addition of Adaptors to pBR

Based on the results of the above precipitation experiments it was decided to remove free adaptors from the "adaptored" pBR complex using 5-20% KOAc gradients. The fractions were also precipitated using glycogen (as a carrier of DNA) instead of linear polyacrylamide. Material for this experiment was prepared by first ligating adaptors to pBR and fractionating the ligation mix. The fractions were then collected (as described in the method section) and glycogen was added to each fraction to a final concentration of 20 ug/ml. The DNA in the fractions were then ethanol precipitated and then resuspended in TE. A sample from each fraction (fractions 1-11 only) was loaded on to an agarose gel to check the efficiency of the recovery of the DNA this time (Fig. 5.17). pBR DNA (270 ng/track, tracks 1 and 13) was run as marker, as well as to indicate the amount of DNA recovered from the fractions. The result showed that the DNA, in each fraction, had been recovered efficiently and also it showed that the DNAs were concentrated in the early fractions (fractions 1-6; tracks 2-7 respectively). By calculating the amount of DNA layered on to the gradient and the estimated amount recovered from precipitating the fractions (as seen on the gel, Fig. 5.17), it was assumed that most of the DNA had been recovered. The adaptored pBR DNA from fractions 2 to 6 were used for the following experiment.

5.11 Cloning pBR into CDM8 Vector

Adaptored pBR from the above fractions (1/3 of the total amount recovered from each fraction) were ligated

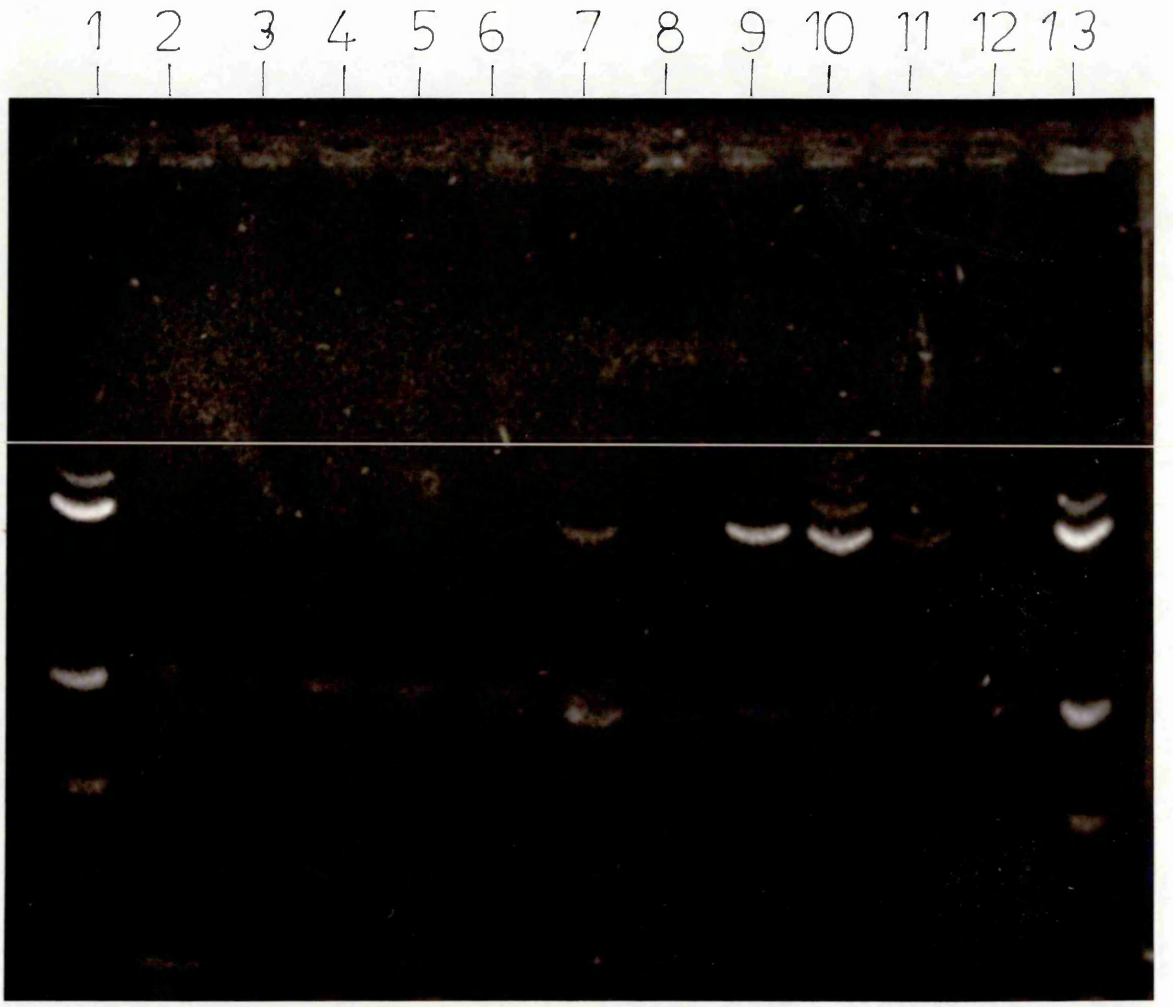


Fig. 5.17 The adaptor added pBR DNA recovered from KOAc gradient. These fragments were ethanol precipitated using glycogen as carrier. The figure shows that first of all DNA were concentrated mainly in fractions (tracks 9 and 10) and the recovery had been efficient.

1. pBR Dra1/PvuII marker (270 ng)
2. Fraction 1
3. Fraction 2
4. Fraction 3
5. Fraction 4
6. Fraction 5
7. Fraction 6
8. Fraction 7
9. Fraction 8
10. Fraction 9
11. Fraction 10
12. Fraction 11
13. pBR Dra1/PvuII marker (270 ng)

to the CDM8 vector. Some of the ligated materials were transformed into the competent MC *E. coli* cells and grown on the selective media. The frequency of transformation was calculated based on the concentration of CDM8 DNA and not on the concentration of the "adaptored" pBR DNA (Table 5.2) because, as mentioned above, the concentration of the "adaptored" pBR was not accurately measurable from the gel (Table 5.2). The actual transformation frequency may have been even higher if the calculation was based on the estimated concentration of pBR. The uncut CDM8 plasmid was also transformed as control for the efficiency of transformation. Untransformed competent MC *E. coli* cells were grown on Amp. and Tet. agar plates to measure the frequency of revertants (Table 5.2).

Comparing the frequencies of transformation for the uncut CDM8 and pBR recombinants (3×10^6 and 3.7×10^3 colonies per ug of DNA respectively), showed that both frequencies (i.e. uncut and pBR recombinants) were rather low. In fact the frequency of transformation for the uncut CDM8 was about 100 times lower than what had been reported (i.e. $3\text{-}5 \times 10^8$, Aruffo and Seed 1987a). This low frequency of transformation indicated that the ligation, fractionation and transformation in the CDM8 system need to be optimised even further if cloning of rare messeges was the objective.

In the following experiments, tests were made to check:

(a) The optimum concentration of uncut CDM8 vector, or the cut and religated CDM8 vector DNA to give high frequency of transformation.

(b) If there was any batch variation in competent MC *E. coli* cells.

(c) If there was any difference in the frequency of transformation caused by different length of ligation.

No.	DNA Sample	Track Fig. 5.	Con. of Vector	Freq. of Transform.
1	pBR/CDM8	2	24 ng	$4x10^2$
2	pBR/CDM8	3	24 ng	$1.6x10^3$
3	pBR/CDM8	4	24 ng	$2x10^3$
4	pBR/CDM8	5	24 ng	$3.7x10^3$
5	pBR/CDM8	6	24 ng	$1.2x10^3$
6	CDM8 uncut	-	10 ng	$3.1x10^6$
7	MC1061/p3	-	-	2 colonies

Table 5.2 Transformation frequencies for pBR-CDM8 recombinants. A sample of fractionated DNA seen in the Fig. 5.19 were transformed into MC *E. coli* cells. pBR in numbers 1-5 correspond to the DNA from tracks 2-6 in Fig 5.19 respectively. Frequencies have been calculated for one ug DNA. Uncut CDM8 transformation was done to show the transformation efficiency with supercoiled DNA. Untransformed MC *E. coli* were plated to show the frequency of revertants.

5.12 DNA Concentration Effect on the Frequency of Transformation:

Experiments were done to find the optimum concentration of uncut DNA to give the optimum frequency of transformants (Tables 5.3). The results showed that increasing the amount of DNA beyond a certain concentration did not produce proportionally higher frequencies of transformation (Fig. 5.18).

Highest frequency was usually found when 1-10 ng uncut DNA was transformed (8 X 10^7, Table 5.3 and Fig. 5.18). This frequency ($8x10^7$) was only 5 times less than that reported by Seed and collegues (i.e. $3-5x10^8$, Aruffo and Seed, 1987a). Similar effect was also observed if CDM8 DNA was cut and religated and then transformed (data not shown).

5.13 Effect of Batch Variations of Competent Cells on the Frequency of Transformation.

Several batches of competent MC *E. coli* cells were made according to the recomended procedure (Aruffo and Seed, 1987a). In preparing these competent cells several other factors such as the stage of cell growth, time and speed of pelleting the cells, the effect of detergent free glassware and duration of cell storage at $-70^{o}C$ were also tested.

In these experiments several batches of competent cells were tested for the frequency of transformation using 10 ng uncut CDM8 DNA. Untransformed MC *E. coli* cells from each batch was grown on Amp. and Tet. plates as control for the frequency of revertants.

The findings could be summarised as follow:

(a) Cells had to be in log phase growth (OD_{600}= 0.6) to give highest frequencies (results not shown).

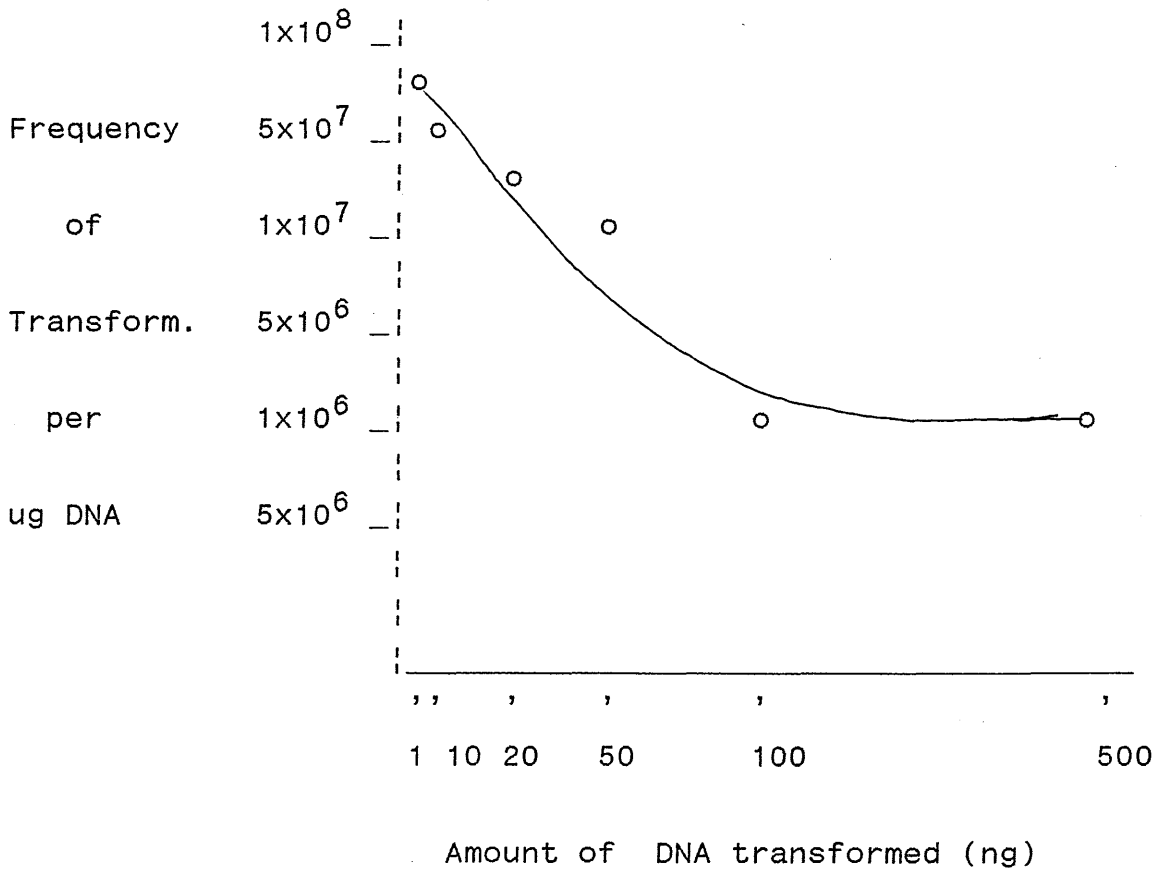


Fig. 5.18 DNA concentration effect on the frequency of transformation. The figure shows that frequency of transformation decreases as DNA concentration increases, starts to level off and then drops as the DNA concentration increases.

DNA	DNA con.	Frequency
Uncut CDM8	1 ng	$8x10^7$
Uncut CDM8	10 ng	$6.5x10^7$
Uncut CDM8	20 ng	$4x10^7$
Uncut CDM8	50 ng	$1.8x10^7$
Uncut CDM8	100 ng	$2x10^6$
Uncut CDM8	500 ng	$2.6x10^6$

Table 5.3 DNA concentration effect on the frequency of transformation. The frequencies are for one ug uncut CDM8 DNA.

(b) The cells had to be spun down at lower speed than recomended (i.e. 2-2.5 krpm instead of the recomended 4.2 krpm, Aruffo and Seed, 1987a). In fact the higher speed of spinning (more than 2.5 krpm) gave a lower frequency of transformation (batches 5-9, Table 5.4 and Fig. 5.19).,

(c) There was no need for glassware to be washed to a greater extent than was already practiced in the Department (results not shown).

(d) Duration of cells storage at -70°C (for one week to 6 months) had no significant effect on the frequency of transformations, and frequencies tended to reflect the differences observed in the batch variation seen in Table 5.4 (results not shown).

There were large variations in the frequency of transformation between different batches of competent cells prepared in the same way (from $2x10^6$ to $8x10^7$ colonies/ug DNA, batches 1-4, Table 5.4).

Other experiments were also done to see whether altering the time of ligation would make any difference in the frequency of transformation. In order to do this, a ligation reaction was set up using Hind III cut CDM8 DNA. Aliquots were removed after 3 and 5 hours of incubation and the rest of the material was allowed to incubate ovenight. The transformation of the ligated CDM8 showed that the overnight incubation gave slightly but not significantly higher transformation frequency (results not shown).

5.14 CDM8 BstX1 Digestion and Transformation

Ligatability of purified vector DNA (prepared as described above) was tested by ligating some of it with purified stuffer DNA (Figs. 5.4 and 5.5). The frequency of transformation in these cases was between 100-1000 times lower than for uncut DNA ($7x10^4$ vs $7x10^6$ for KOAc purified/uncut DNA and $6.9x10^4$ vs $8.1x10^7$ for "Gene Cleaned"/uncut DNA). These findings indicated that

Batch No.	krpm	con.	Frequency
1	2.5	10ng	$8x10^7$
2	2.5	10ng	$3.5x10^7$
3	2.5	10ng	$9.5x10^6$
4	2.5	10ng	$2x10^6$
5	3.0	10ng	$3.8x10^6$
6	3.5	10ng	$1.8x10^5$
7	3.7	10ng	$1.1x10^5$
8	4.0	10ng	$9x10^4$
9	4.2	10ng	$3x10^4$

Table 5.4 Effect of batch variations of competent cells on the frequency of transformation. There were inter-batch variation and different batches of competent cells gave different frequencies (batches 1-5). Nevetheless it is obvious that spinning the cells at higher speed, had dramatically reduced the frequency of transformation (batches 5-9). krpm means the speed at which the cells were pelleted, and con. means the concentration of DNA which were transformed.

digested and purified vector DNA would religate to the stuffer but the ligation is not particularly efficient, perhaps due to the damaged sticky ends, caused during the purification procedures. Another reason for having low frequency of transformation may be that the calculation of the frequencies of transformation was based on inaccurate estimation of the amount of DNA involved. The frequency of transformation for KOAc purified uncut vector was usually 10 times less than that of uncut and unpurified vector. This may suggest that either the purification steps had some negative effect on the transformability of the vector or that the amount of DNA recovered was overestimated.

Tests were also made to find out by how much frequencies varied when CDM8 DNA was ligated and transformed after a single digestion (HindIII), BstX1 double digestion without any separation of the stuffer (no purification), BstX1 double digestion with separation of stuffer from the vector (BstX1 digested and purified the vector and the stuffer either by "Gene Clean" method or by KOAc gradient) and cloned adaptored pBR fragments (into CDM8). Results showed a systematic reduction in the frequency of transformation for every step involved (Table 5.5). Some of the reasons for such behaviour will be given later (Discussion section). It should be added that these findings were consistent but were affected by the transformability of the competent cells and the amount of DNA being transformed as mentioned above. For example, HindIII digestion usually gave 10 fold fewer transformants than uncut DNA irrespective of the variation in the competent cells. Double digestion without any separation of the vector from the stuffer (no KOAc purification) gave 100 times fewer transformants than uncut vector. Finally religation of purified vector and stuffer gave roughly the same frequency as cloned pBR, which was about 10^3 times less than uncut DNA. Improving the efficiency of transformation may still increase these frequencies to the acceptable level.

CDM8 DNA	Ligation	Frequency
Uncut CDM8	-	7.6×10^7
HindIII cut	+	2×10^6
BstX1 cut	+	3.8×10^5
CDM8/stuffer KOAc purified	+	7×10^4
CDM8/pBR KOAc purified	+	6.5×10^4

Table 5.5 Effect of digestion and religation on the frequency of transformation. Generally every time CDM8 DNA was cut and religated before or after purification, there was a 10 fold drop in the frequency of transformation and the more the manipulation the bigger the drop was. There was about 10^3 drop between uncut DNA and cloning pBR into CDM8 vector.

5.15 Discussion

Several factors were tested in order to increase the frequency of transformation. The findings are summarised below:

It was necessary to recover DNA efficiently from a KOAc gradient. The recommended procedure, in which polyacrylamide was used as a carrier in the ethanol precipitation of the fractionated DNA, (Aruffo and Seed, 1987a) was not as efficient as using glycogen. Another factor that needed to be considered was the variation between batches of competent cells. Separating free adaptors from "adaptored" DNA was absolutely necessary otherwise free adaptors would block the cloning sites.

The best frequency of transformation which was obtained for pBR fragments (used as cDNA substitute), using similar procedures to those used by Seed and colleagues (Aruffo and Seed, 1987a), gave $6.5x10^4$ recombinants per ug of pBR DNA. At the same time the best frequency for uncut (supercoiled) CDM8 vector was $8x10^7$ per ug of DNA. The library construction efficiencies usually observed by Seed and Aruffo group per ug of mRNA have been between 0.5 to $2x10^6$ recombinants (Aruffo and Seed 1987a) and the transformation efficiency for supercoiled (uncut) vector has been $3-5x10^8$ colonies per ug of DNA. The best frequency of transformation with uncut DNA obtained in this work was only 10 times less than that found by Seed group (Aruffo and Seed, 1987a).

There could have been several reasons for not obtaining a high frequency of transformation. In summary, they could have been caused by:

Inaccurate estimation of the amount of DNA transformed, especially in cases where purified DNA had to be ligated, purified and then transformed.

The probable damage to the sticky ends of the vector and even the adaptors during the digestions of the vector or purification steps.

The lower frequencies of ligation which is usually associated with the blunt end ligations (Struhl, 1987) and this may have been one of the contributary causes where addition of adaptors to the pBR was involved.

The presence of a small amount of adaptors which have been left over from the purification steps blocked some of the vector ends and therefore reduced the number of ends available for ligation to the "adaptored pBR".

CDM8 cloning system, despite the low efficiency of transformation seen above and the difficulties involved, still looks attractive because cDNA cloned into CDM8 can be transfected into COS cells for immunoselection and immunostaining to see whether the cloned cDNA can be expressed on the surface of the COS (see Chapter 6). The special features of the CDM8 (Chapter 1) allow the recombinant clones to be amplified in MC *E. coli* cells, and if needed, the recovered clones may be sequenced without any need for further subclonings.

As mentioned, it was observed that the CDM8 cloning system was not producing high frequency of transformation. One of the main reasons was that several steps were involved which were directly or indirectly contributing to the low frequency of transformation. Therefore the CDM8 cloning system may not be suitable for cloning rare messages but may be used for cloning and subsequently recovering moderate to abundant messages from a cDNA library by using the COS expression system. These kind of difficulties may have been the reason behind the fact that in most of the reported cases, which CDM8 or CDM8 like cloning stratgies have been used, only abundant messages have been the target. For example CD2, CD7 and CD28, of which all three are T-cell antigens, have been isolated from a library made from human T-cell line HPB-ALL abundant for such

messages (Seed and Aruffo, 1987; Aruffo and Seed, 1987b; 1987a respectively). Even when a placental cDNA library was screened for monocytes receptor (Simmons and Seed, 1988) it was chosen because of placental richness of monocyte transcripts.

Inefficiency of CDM8 system to clone rare messages may be overcome by using other types of expression system (e.g. lambda gt11). It is known that the cloning efficiency of lambda vectors is much greater than plasmid base vectors (Maniatis *et al.*, 1982; Lech and Brent, 1987). Although the efficiency of packaging only approaches 10%, phages, once packaged, form plaques on *E. coli* with an efficiency of 1. On the other hand with the best transformation efficiency, i.e. 10^8/ug pBR322 DNA, less than 1 in 1000 plasmids become transformed into cells (Lech and Brent, 1987). The only main disadvantage of using lambda gt11 as cloning vector is that the fusion polypeptides produced are not in their native conformation and Abs may not be able to recognize them. This problem may be overcome by using polyclonal Abs which have been raised against purified proteins. The CDM8 cloning vector may, in here, be useful in that any isolated clone (e.g. isolated by using lambda gt11 and subcloned into the CDM8) can be tested to see whether it is expressed on the surface of COS cells or be amplified in the MC *E, coli* cells for making DNA for further analysis or sequencing.

Lastly while the above work was approaching its final stages, our group was given a placental library cloned into CDM7, a vector similar to CDM8, by Dr. David Simmons (University of Oxford). Due to the importance of screening the placental library and the work involved, the above work which was originally aimed at preparing CDM8 vector for cloning the cDNA library, was left at the stage reported above.

CHAPTER 6

OPTIMISATION OF DEAE-DEXTRAN TRANSFECTION

AND

IMMUNOSCREENING OF PLACENTAL cDNA LIBRARY

6.1 INTRODUCTION

The aim of the work described in this chapter was to screen a human placental cDNA library for isolating placental specific clones. The placental cDNA library was expressed in the COS expression system (Seed and Aruffo 1987a and Chapter 1) and immunoscreened with the monoclonal antibodies (McAbs) which had been raised and tested against human placental determinant (Chapter 1).

The COS expression system protocol contains several important steps (e.g. DNA transfection, panning, harvesting, Hirt DNA prep, amplification of DNA etc., Chapter 1). The first aim was to optimaise some of the steps involved. These included DEAE-dextran transfection, harvesting the cells and immunostaining. The optimisations of the steps involved were tested by using CD2 DNA (Seed and Aruffo, 1987).

6.2 Optimisation of DEAE-Dextran Transfection

6.2.1 Transfecting COS Cells with CD2 DNA

Several different protocols are available for transfecting DNA into mammalian cells (e.g. DEAE-dextran or $CaPO_4$ transfection and electroporation), of the three gene transfer methods only the DEAE-dextran transfection was mainly used for transfecting DNA into the COS cells. These transfections were mainly for the primary screening and immunofluorescence (FITC) staining purposes.

Several important factors in the DEAE-dextran transfection steps were tested and optimised to give higher frequencies of transfectants. These factors and steps included the concentrations of the COS cells, the DEAE-dextran and the DNA, harvesting the cells,

preparing the cells for staining and finally the FITC staining.

In order to optimise the DEAE-dextran transfection, using the COS expression system, several transfection experiments were carried out. In these experiments CD2 DNA (cloned into CDM8, Seed and Aruffo, 1987 and Chapter 1) was used to monitor the efficiency of transfection. The reasons for using CD2 instead of the placental cDNA library, for optimising the system, was that the CD2 clone has been isolated using the COS expression system (Seed and Aruffo 1987a). Furthermore the CD2 antigen (Ag) can be expressed on the surface of the COS cells and these Ags have been shown to bind to the anti-CD2 antibody (Ab) during the panning and staining (Seed and Aruffo 1987; Seed 1987; Peterson and Seed 1987; Bierer *et al.*, 1988). Also good quality anti-CD2 McAbs are commercially available. Finally the CD2 DNA unlike cDNA is obtainable in large quantities which make the optimisation of the steps easier.

In order to transfect the CD2 DNA into COS cells a DEAE-dextran/DNA mix was prepared then added to the appropriate culture dish and the cells were incubated (Chapter 2). Every 30 minutes the dishes were observed under the microscope for the formation of vacuoles which indicated that the uptake of DNA was occuring. Usually after 3 hours most cells had formed vacuoles and longer incubation gradually would have a detrimental effect (cells would become bursted because of over-vacuolisation). The lysis of the cells was, however, mostly occuring 4-5 hours after the DEAE-dextran treatment, which by this time the DEAE-dextran/DNA mix had already been removed (usually after 3 hours) to minimize the damage to the cells.

After removal of the DEAE-dextran/DNA the cells were shocked by addition of 10% DMSO to enhance DNA uptake (Lopata *et al.*, 1984). The cells were then washed and incubated for 24 hours. After that the cells were trypsinized in order to detach the cells from the

dishes. The detached cells were transferred into fresh culture dish and incubated for 24 hours to allow the expression of the coded proteins. It was observed that the transfer of cells, after the initial 24 hour incubation, was absolutely necessary. Probably the reason was that if the cells were not transferred as above they could not be detached easily with PEA (PEA = PBS, EDTA and azide; See Page 80) treatment to harvest the cells. The only other way to detach the cells was to trypsinize them. Trypsinization on the other hand was not favoured at this stage, because trypsin being a proteolytic enzyme might digest some of the Ags expressed on the cell surface. This in turn would cause reduction in the number of cells being panned (during the cDNA screening) and also reduction in the intensity of staining. Therefore after the final 24 hours of incubation the cells were lifted by PEA treatment and resuspended in PBS/FCS. The resuspended cells were transferred on to slides by cytospinning.

The cytospun cells were stained with anti-CD2 McAb by indirect immunofluorescence (FITC) staining in order to find out the efficiency of the DEAE-dextran transfection. The stained cells were counterstained with the nuclear stainer, propidium iodide. In these experiments untransfected COS cells were used as a negative control and a McAb against HLA class 1 determinant (W6/32, Barnstable *et al.*, 1978), was used as a positive control since untransfected COS cells were shown to express the HLA class 1 Ags (Fig 6.1).

In order to determine the efficiency of transfection, the percentages of the cells expressing the CD2 Ag was calculated by counting positive cells from at least five different, randomly chosen, fields of vision in a microscope. Usually each field had between 50 to 200 cells. The same procedure was followed for counting the total number of cells stained with W6/32 or propidium iodide. The percentage of W6/32 was calculated from the total W6/32 positive cells divided by the total nuclei present in the same fields since nuclei negative

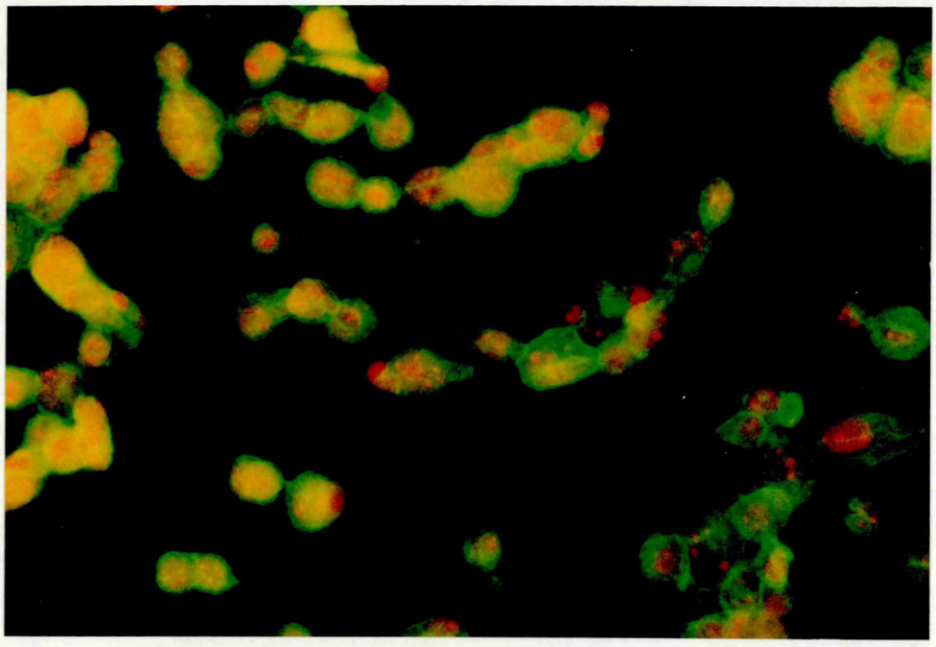


Figure 6.1. Untransfected COS cells stained with W6/32 Ab. COS cells clearly have stained with this Ab and therefore W6/32 staining of COS cells was used as positive control.

for W6/32 are thought to be lysed cells (no cell membrane or cytoplasm, Fig. 6.2).

The effects of various concentrations of DEAE-dextran, cell and the CD2 DNA were tested on the efficiency of transfection. In every case the CD2 DNA was transfected and the percentages of positive cells expressing the CD2 Ag were adjusted to take into account the proportion of lysed to intact cells.

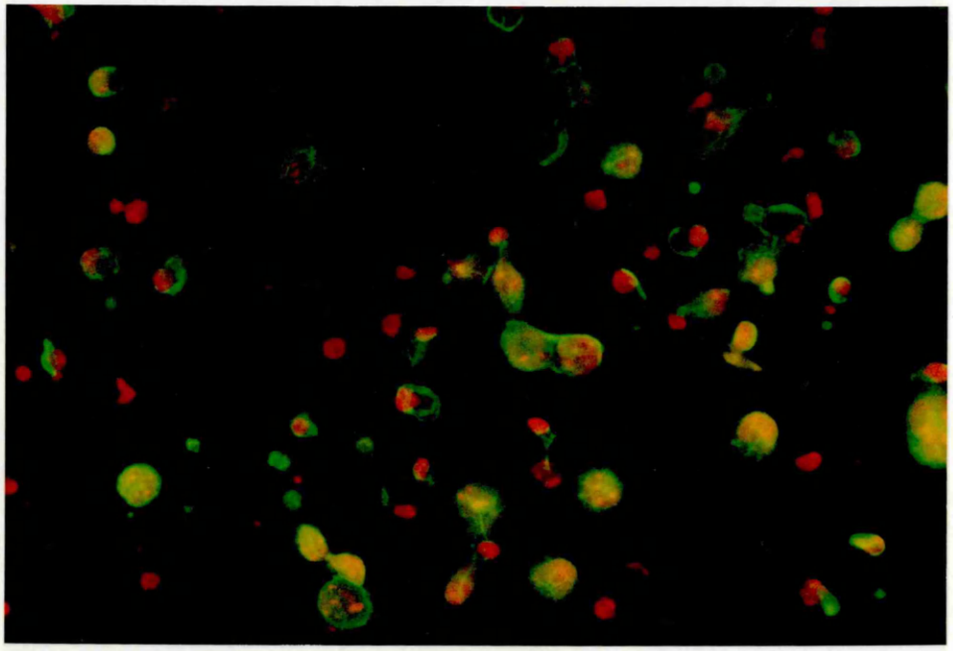
6.2.2 Effect of DEAE-dextran and Cell Concentration on the Efficiency of Transfection

The effect of DEAE-dextran concentration on the frequency of transfection was tested using constant amount of CD2 DNA. The concentration of DEAE-dextran was found to be very crucial in increasing the efficiency of transfection. It was found that experiments carried out with the same number of cells and lower concentration of DEAE-dextran gave significantly higher number of CD2 positives than those with higher concentration of DEAE-dextran (Fig. 6.3).

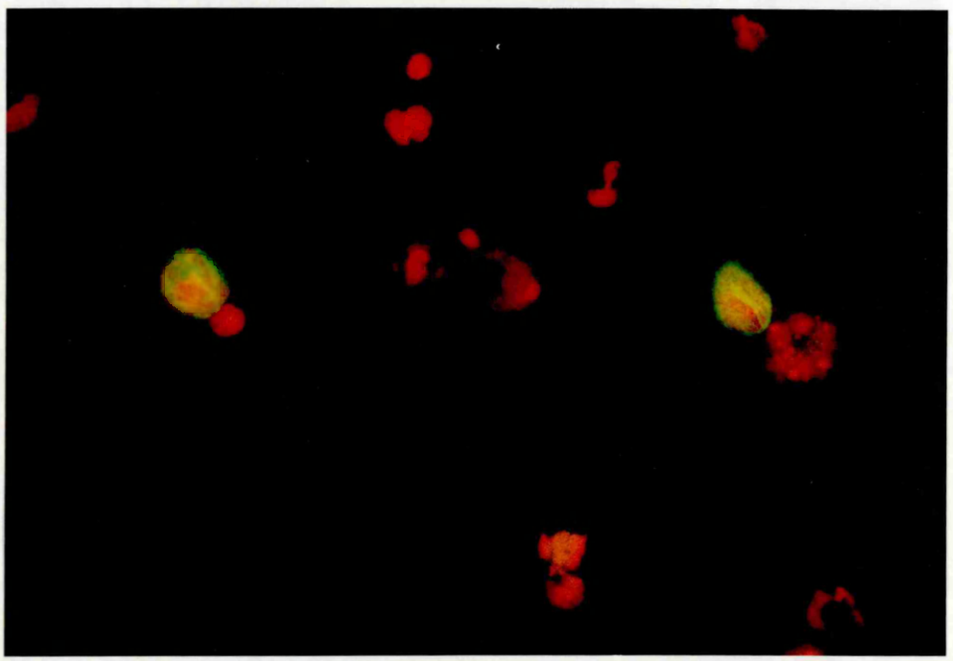
The effect of cell concentration was also tested and it was found to be as important as the concentration of DEAE-dextran in increasing the efficency of transfection. Generally dishes with 50% confluency (i.e. about $1x10^5$ cells per dish) produced the highest number of CD2 positives when compared with less or more than 50% confluency (i.e. $0.5x10^5$ and $2x10^5$ cells per dish respectively; Fig. 6.3).

6.2.3 DNA Concentration Effect on Transfection

The concentration of DNA was also found to be important in increasing the transfection efficiency (Fig. 6.4). It was also found that the positive effect of DNA concentration was limited and after certain concentration, addition of more DNA did not produce any



A



B

Figure 6.2. Lysed or damaged COS cells do not stain with W6/32 (A), CD2 COS cells transfected with CD2 DNA were used for the optimisation experiments (B). The COS cells which are damaged during cell harvest or cytospinning lack most or part of the cytoplasm and therefore, do not stain the same way as intact cells do. The remaining nuclei have stained only with propidium iodide.

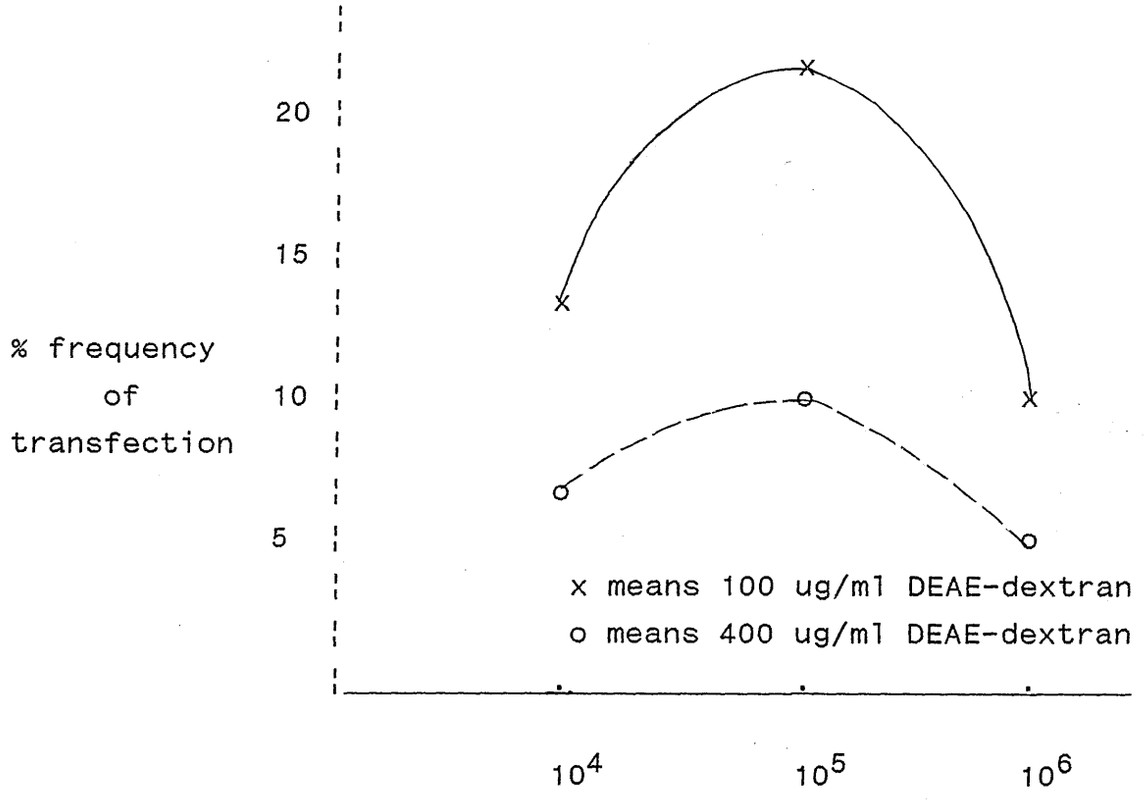


Figure 6.3 Effect of cell and DEAE-dextran concentration on the efficiency of transfection. Generally dishes with about 50% confluency (about $1x10^5$ cells per a 10 ml culture) with either of the two DEAE-dextran concentrations, gave more transfectants than dishes with less or more confluency (i.e. $1x10^4$ and $1x10^6$). When two dishes had roughly the same number of cells the one with less DEAE-dextran concentration gave higher frequency of transfection. The DEAE-dextran concentrations are shown for 100 ug/ml (solid lines) and for 400 ug/ml (broken lines).

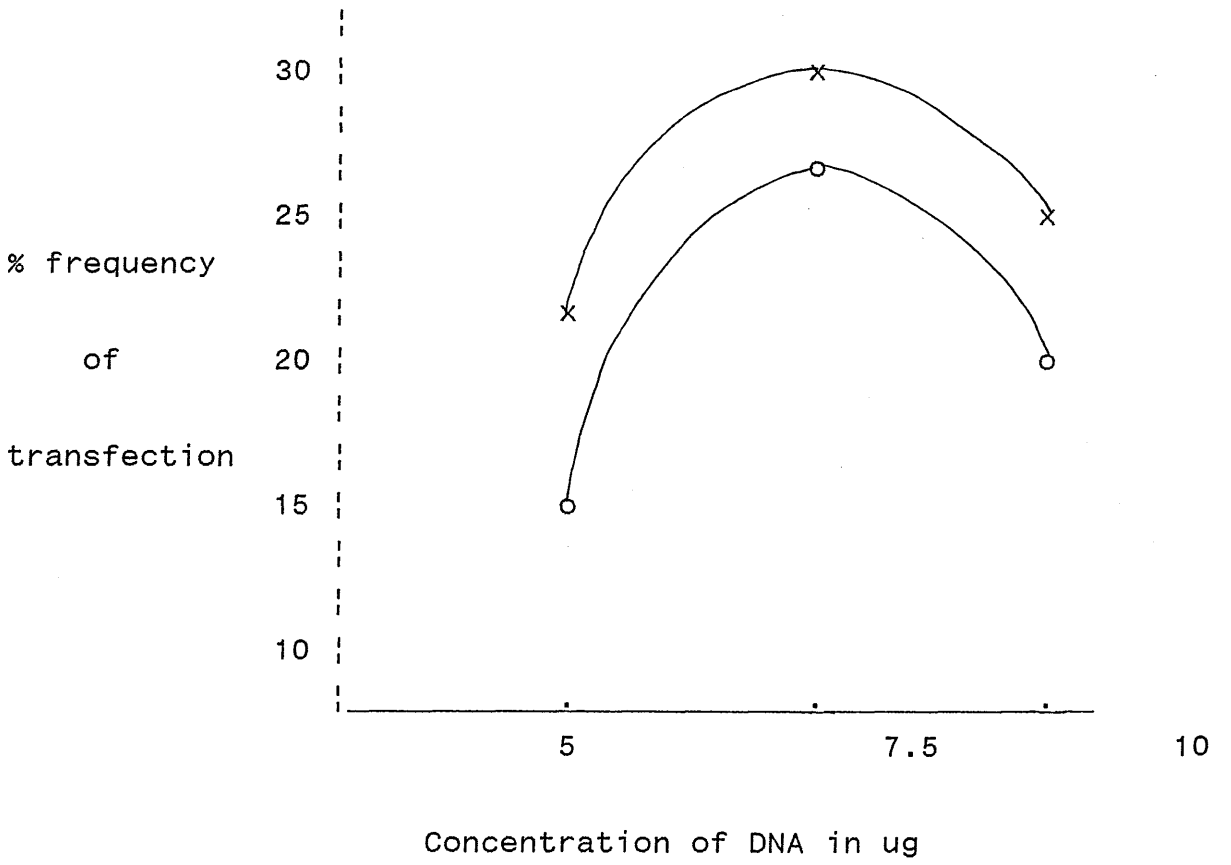


Figure 6.4 Effect of DNA concentration on the efficiency of DNA concentration. The DNA concentration effect was tested using two different cell concentration. Generally the DNA concentration of 7.5 ug per 10 ml culture produced the highest frequencies of transfection in either of the cell concentration. Using more DNA (10 ug vs 5 ug) did not produce proportional increase in the frequency of transfection. The DNA concentrations on $1x10^4$ cells is shown as solid line (x) and on $1x10^5$ is shown as broken lines (o).

proportional increase in the number of positive cells. The highest frequencies were usually observed when 7.5 ug DNA was used but the highest number of transfectants, per microgram DNA, was achieved when only 5 ug DNA was used for each dish. The frequency of transfection, before optimisation, was ranging between 5 to 10% and after optimisation was ranging between 15 to 30%. Close analysis of the results and the conditions would suggest that the transfection efficiency could have been optimised even further but the obtained values were sufficient for this work.

6.2.4 Cytospinning and Immunofluorescence Staining

In order to examine the efficiency of transfection, the transfected cells expressing the CD2 Ags had to be stained. Staining and counterstaining proved to be very valuable for determining the efficiency of transfection as well as showing any possible damage to the cells. In order to stain the cells they had to be transferred on to slides. Two methods of cell preparation and staining were tested for comparing the pattern and the intensity of staining in each methods.

In one of these method the cells were grown on coverslips laid down inside a tissue culture dish and after overnight incubation the cells were transfected with DNA. After the transfection and incubation, the coverslips were washed with PBSA and fixed in acetone. The coverslips were then glued on to slides and the cells were stained. The staining result indicated that several disadvantages were found to be associated with this method of cell preparation. These could be summarized as:
(a) The cells were usually in clumps. (b) The background staining was too high. (c) Even the W6/32 staining quality was poor. (d) This method was too laborious with no real advantage.

The other method of cell preparation was by

cytospinning (Chapter 2). Compared with the above method, cytospinning was found to be more convenient and the staining was clear with low or no background. The results of optimisation given in this chapter are, therefore, from the cytospinning.

Cytospun cells were stained indirectly with either immunoperoxidase or immunofluorescence (FITC) stainings. The FITC staining was chosen over immunoperoxidase staining for its clarity and simplicity. The FITC staining usually took less time than immunoperoxidase, the stained cells were in better shape at the end and the staining results were less ambiguous to interpret. The main disadvantage of FITC staining was that the intensity of staining could not be kept for a long time (maximum one week at $4^{o}C$), however, immunoperoxidase staining could be kept for much longer without any major fading of the staining signals as was seen in the work described in chapter 4. To overcome this problem with the FITC staining, the cells were observed using a Lites microscope equipped for epi-fluorescence microscopy and if necessary pictures were taken for record and later comparisons of results.

It has to be said that not all cytospun cells were found to stain with the same quality and clarity. Indeed some cytospun cells failed to stain well, some others had high background and sometimes these background staining were associated with lots of damaged or lysed cells. Several factors in the process of preparing cells and staining, were thought to be contributing to the above problems. These factors were associated with several steps in the process of cell culture, cell harvest and staining. These steps have been divided, for convenience, into pre-cytospinning, cytospinning and staining.

6.2.5 Pre-Cytospinning

Several steps prior to the cytospinning were

tested for their effect on the quality of the cells and subsequent staining. These steps were the DEAE-dextran transfection, the cell concentration, the DMSO treatment, the presence or absence of DNA in the transfection, the time of incubation in PEA and the recovery of the cells by trypsin instead of PEA. Slides from these tests were stained with W6/32 McAb and counterstained with propidium iodide. The staining together with counterstaining could show whether the cells were lysed/damaged or not, and if so to what extent. The result of pre-cytospinning factors affecting the outcome of the staining may be summarised as follows:

The staining showed that the DEAE-dextran transfection was not a contributory factor in cells lysis.

The concentration of the cells being transfected also showed no direct effect on the cells lysis.

Long DMSO treatment (>5 minutes) was found to be one of the causes of damage to the cells but the damage was particularly acute if the DMSO had been oxidised.

The transfection of the cells with or without DNA (mock transfection) had no adverse effects on the cells.

These observations were also true for cells being incubated in PEA for different length of the time.

6.2.6 Cytospinning (Cytocentrifugation)

In order to cytospin the transfected cells they were detached from the culture dished using PEA, concentrated first by spinning them down and resuspending in a small volume of PBS/FCS. Experience showed that these cells, especially if very concentrated, had to be declumped prior to cytospining, in order to achieve clear staining signals as well as to reduce the level of nonspecific background. The

declumping involved syringing the cells up and down through a 21G gauge needle. It was, however, found that usually the declumping process was one of the causes of damage to the cells and only gentle declumping had to be adapted to achieve both goals, less damage and better staining.

Besides declumping other steps which are involved were found to be important in obtaining good cytospun slides. These steps can be summarized as follow:

Cells had to be resuspended in a buffer containing serum (e.g. FCS) because it was found that serum was necessary to aid the cells attachment to the slide during cytospinning and also without serum clumping would occur.

It was also found that the cells were in better condition if they were kept at their concentrated form, on ice, during cytospinning. In this case an aliquot of cell suspension was diluted for every few rounds of spinning.

The best cell concentration, to give good staining, was about $1\text{-}5x10^3$ cells per slide. More concentrated cells usually would form clumps and usually would cause poor staining. On the other hand less concentrated cells usually did not spread well and tended to be found on the edges of the cytospun area.

The speed and the duration in which the cells were cytospun were also important, especially when the cells had become fragile (may be during harvesting or declumping process). The range of speeds tested were from 300 to 2000 rpm and the range of spinning times were from 3 to 10 minutes. The speed and the time of cytospinning which gave even spread of the cells on the slides without causing major damage to the cells were found to be around 500 rpm for 5 minutes.

Unused or poorly cleaned slides or coverslips

usually gave rise to a particular type of background staining (Fig. 6.5). The background staining on the poorly cleaned slides was not usually removable by washing the cells during staining therefore very clean slides and coverslips had to be used (see Chapter 2).

Cytospinning chambers also needed attention. The chambers had to be washed thoroughly with water and mild detergent (activated Cidex) at the end of cytospinning every day as well as during the cytospinning if some of the liquid from the previous run was left behind in the chamber.

Cells had to be fixed in acetone immediately after cytospinning to avoid exposing the cells to air, any delay in doing so usually caused the cells to burst. On the other hand no change or damage was observed if cells were overfixed (10 seconds up to 15 minutes).

The cytospun cells were either stained on the same day as they were prepared or they were stored at room temperature in a slide box. There was no apparant differences in the intensity or pattern of staining between the two slide types (i.e. fresh or stored slides).

Some times the FITC staining showed a small number of cells being damaged or lysed. At times the extent of the damage was too high to make the staining results reliable. For the purpose of distingushing slides at the pre-staining level, with high number of damaged cells the cells from randomly chosen slides were either stained with leishman or trypan blue. The unstained cells were also looked at under microscope for the same purpose. The idea was that if the damage to the cells could be detected at pre-staining level then the staining of slides with high number of damaged cells could be avoided. It was found that the damage to the cells could not be accurately measured on unstained slides under microscope. The leishman staining was not helpful either due to the fact that there was no

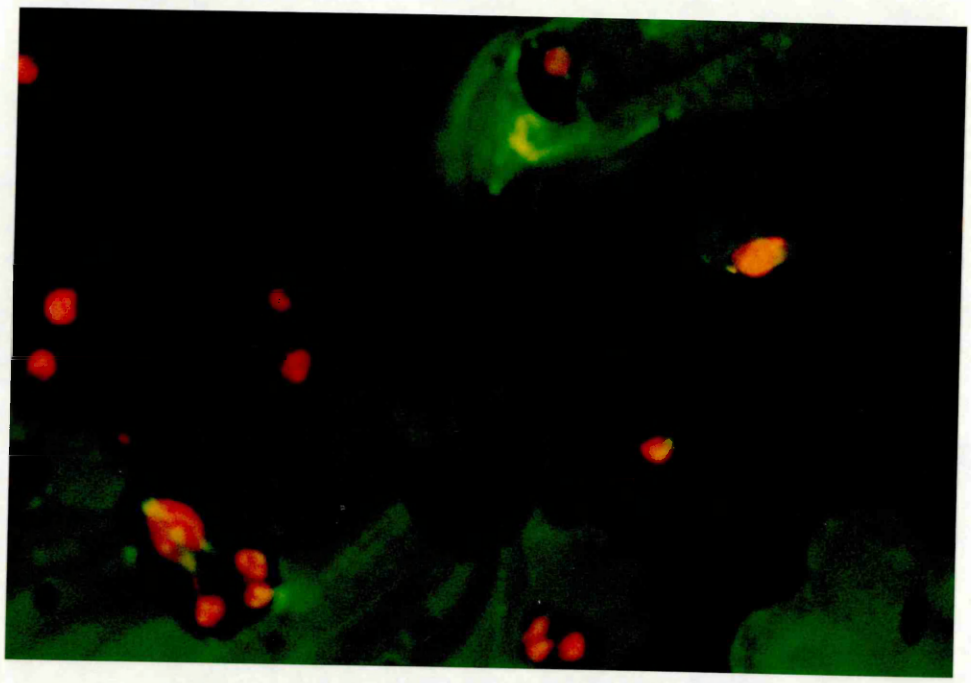


Figure 6.5 Background staining caused by free Ab on either coverslips or slides. The pattern of staining was easily distinguishable from other types.

correlation between the number of cells seen damaged after the Leishman staining and after the FITC staining using W6/32 McAb. When cells were stained with trypan blue the number of damaged cells seemed to be greater than seen with the W6/32 staining. The reason may be that trypan blue only stains the dead cells (Fig. 6.6) but W6/32 stains semi-damaged cells as well as healthy cells (Fig. 6.7). Trypan blue staining of uncytospun cells was therefore, unhelpful for the same reason. The estimation of the damage to the cells was only achieved by staining with W6/32 acompanied by counterstaining with propidium iodide.

6.2.7 Immunofluorescent Staining

Cytospun transfected cells were stained with several McAbs in order to identify the cells expressing novel determinants as target Ags (see section 6.3). The staining was also useful for measuring the efficiency of the transfection. Some transfected and untransfected COS cells were stained with only the second Ab (i.e. FITC conjugated) to work out the right dilution of second Ab and also to find out if there were any background on these slides caused by the second Ab.

After establishing the correct dilution of the second Ab, several different transfected cell preparations were stained. It was observed that in some cases there were high levels of background staining on the cells, on the coverslips or the slides. The staining on the cells was most probably caused by cross reactivity between Abs and some of the determinants on the COS cells. The nonspecific background staining on the coverslips or the slides was thought to have been caused by free Abs and improperly cleaned slides. Several different adsorbants (e.g. human and rabbit serum, BSA, intact and sonicated COS cells) or detergents (triton and NP40) as well as washing conditions were used in order to reduce the nonspecific stainings on these cells, slides or coverslips. It was

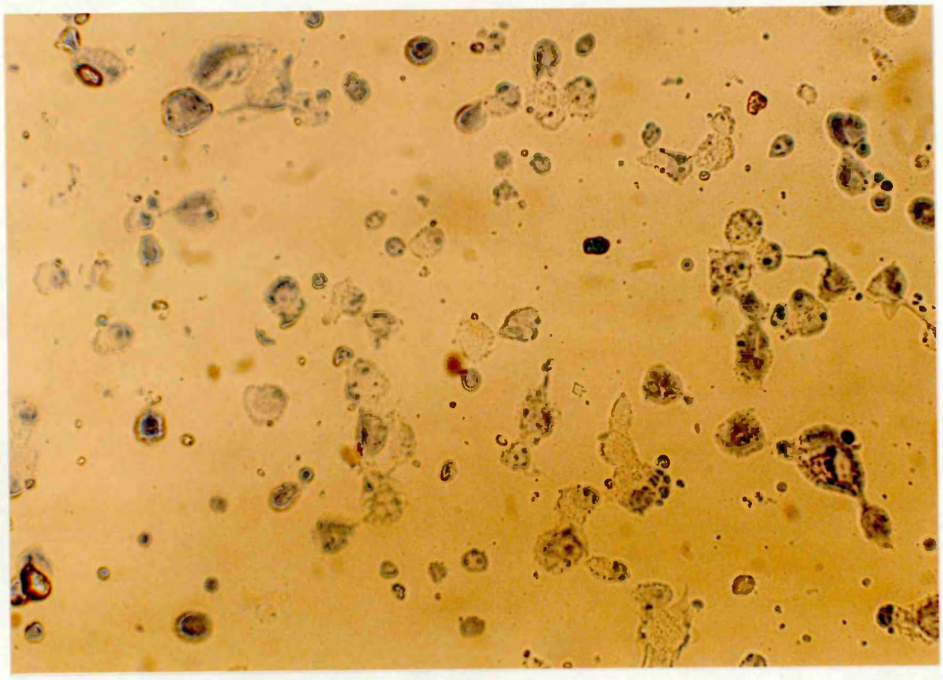


Figure 6.6 COS cells stained with trypan blue. The trypan blue staining usually over-represented the extent of damage to the cells and could not be used to assess the extent of damage at the pre-cytospining of pre-staining level.

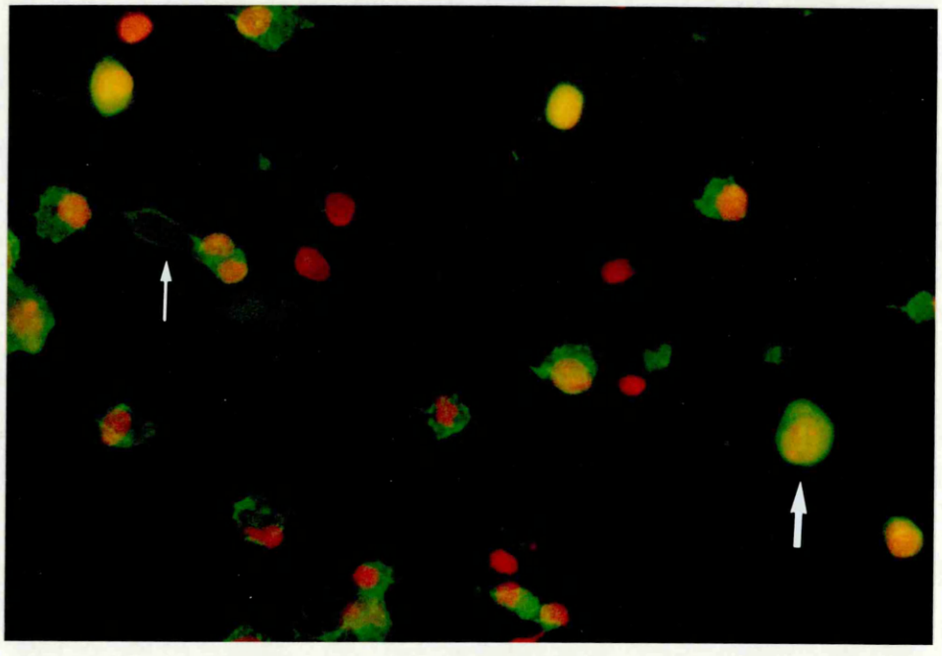


Figure 6.7 COS cells stained with W6/32 and counterstained with propidium iodide. The staining shows that both semi-damaged (small arrow) and intact (bigger arrow) cells had stained with the W6/32 McAb.

found that none of the adsorbant or detergents could eliminate the background staining caused in these ways. The only effective method of removing the nonspecific staining was excessive washing of the cells in a circulating bath of TBS for 15 minutes with three changes of the buffer (Chapter 2). The background staining caused by cross reactivity mentioned above was only associated with some of the McAbs used to screen the cDNA library (e.g. GB17 and GB25) and could not be controlled unless these Abs were diluted much further (see sections below).

The overall remarks about staining and backgrounds could be summerized as follow:

(a): In most cases the background was directly related to the intactness of the cells that is the more damaged the cells were the more background there was.

(b): Transfected cells generally gave higher background than untransfected cells when stained with irrelevant Ab. The reason was thought not to be associated with the changes which the transfection may have caused but may have been associated with the fact that the transfected cells had gone through more stages of handling before being cytospun (probably have become more prone to damage).

(c): Most recomended washing procedures (e.g. washing with a jet of washing buffer from washing bottle) failed to wash off the free Abs after the end of the Ab incubation. The best washing procedure, however, was circulating washing buffer.

(d): Prolonged incubation of first or second Ab had no apparant negative or positive effect on staining intensity but a minimum of 15 minutes incubation was used routinely.

(e): Some of the first Abs were giving high background even at very diluted forms (GB17 and GB25, Figs. 6.8-9, see also section 6.3).

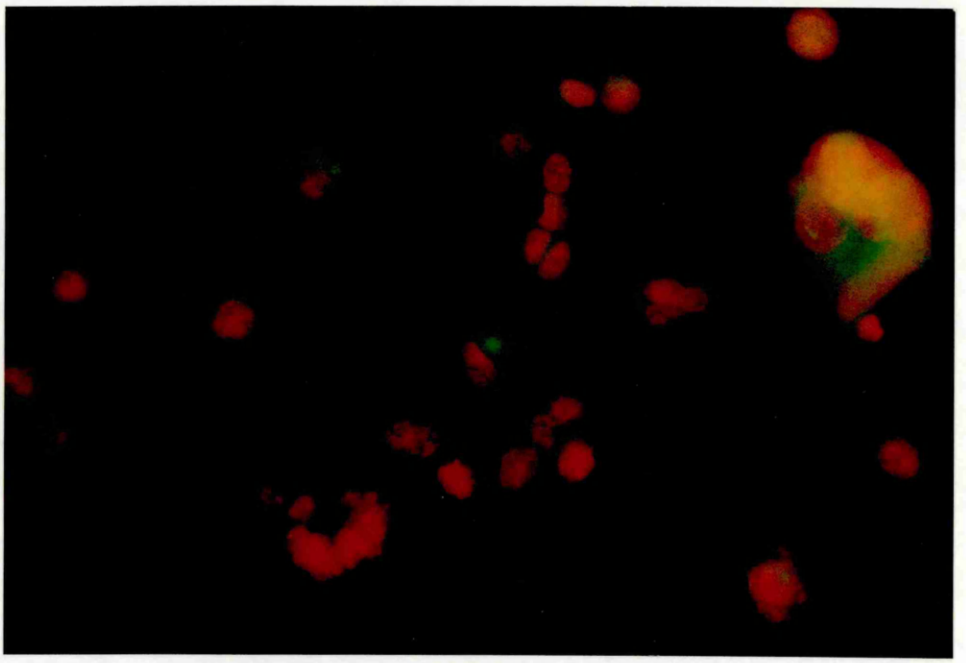


Figure 6.8 COS cells transfected with the DNA recovered by the GB24 McAb and stained with GB17 McAb. The DNA had been recovered from cells panned by the GB24 Ab and then back-transfected into the COS cells. The cells were stained with the GB17 McAb (1/50 dilution). There can be seen faint staining on most of the cells.

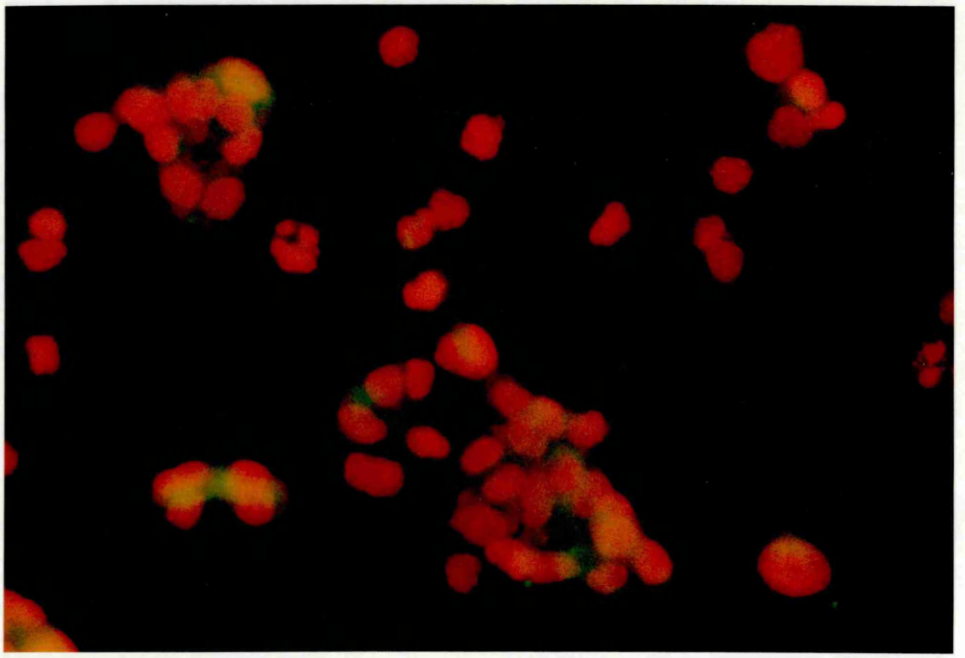


Figure 6.9 COS cells transfected with the DNA recovered by the GB24 McAb and stained with GB25 McAb. The DNA had been recovered from cells panned by the GB24 Ab and then back-transfected into the COS cells. The cells were stained with the GB25 McAb (1/100 dilution). There can be seen faint staining on most of the cells.

(f): Several FITC conjugated second Abs (sheep or goat anti-mouse Ab) were tested for enhancing the positive signals without causing background staining on negative cells. Those from Dako and Sigma usually gave better staining than those from SAPU (Scotish Antibody Production Unit) or Sera Lab. The second Ab from Dako was the one mainly used in this work.

6.2.8 Counterstaining

The FITC staining was usually followed by addition of the counterstainer, propidium iodide (Chapter 2). The result of FITC staining without counterstaining was difficult to interpret, especially if the staining signal was weak. The counterstaining also proved to be absolutely necessary to estimate the extent of damage to the cells, because burst cells would have only nuclei left which only stain with propidium iodide (Fig. 6.10). Overall the FITC staining combined with the counterstaining would usually produce much clear and interpretable staining.

It was found that the propidium iodide prepared as recommended in the method section (Yeh, Hsi and Faulk 1981) was stable for longer than suggested period of 6 months. In fact no apparent loss of activity was observed even after two years if the propidium iodide was kept in a dark bottle at 4°C. The recommended washing method was found to reduce the intensity of propidium iodide counterstaining (Yeh, Hsi and Faulk 1981), therefore, the propidium iodide was usually added directly to the cells at the final stage of staining, at $^{1}/_{10}$-$^{1}/_{20}$ dilution of the stock solution (5-10 ul of stock solution of propidium iodide diluted in 100 ul of glycerol/TBS, and 20-40 ul was used per slide, Chapter 2). This method of adding propidium iodide had at least three advantages.

(a) No further washing was necessary after addition of propidium iodide.

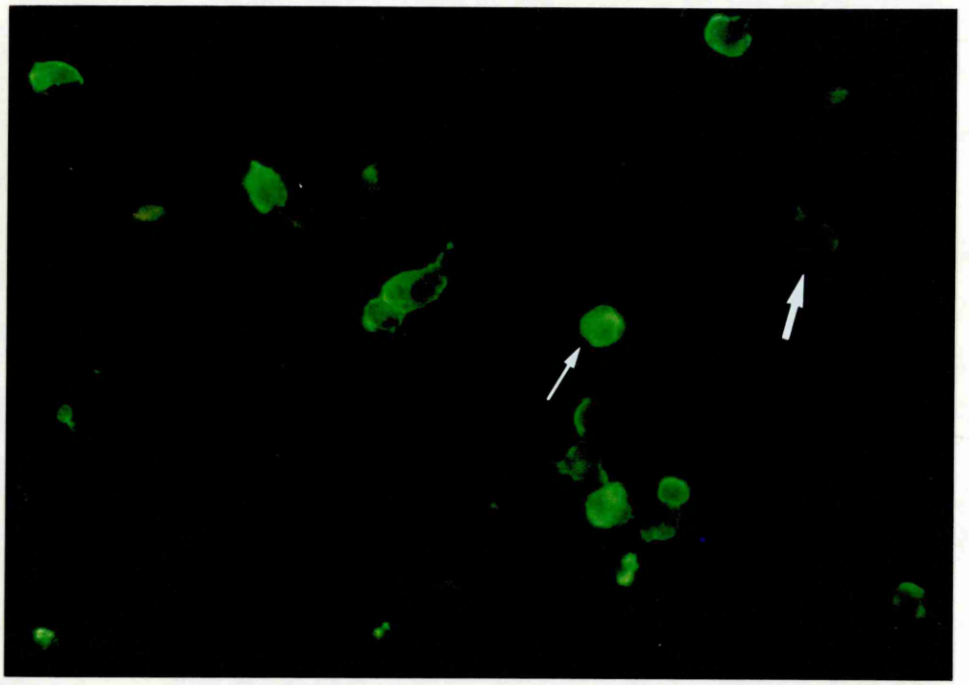


Figure 6.10 COS cells stained with W6/32 and counterstained with propidium iodide. The cells which had been mostly damaged show variable staining with W6/32. Some cells are strong (small arrow) others faint (bigger arrow). The counterstained nuclei are very faint and can not accurately show the extent of damage to the cells.

(b) Less propidium iodide was used in this way than adding to the second Ab.

(c) If more propidium iodide was needed it could be easily added to the cells.

6.3 Screening Placental cDNA Library

6.3.1 Immunoscreening

After the optimisation of the DEAE-dextran transfection and the handling of the COS cells from harvesting to staining, a human placental cDNA library, cloned into CDM7 (CDM7 is similar to the CDM8 introduced in Chapter 5), was transfected into COS cells by the DEAE-dextran transfection method. The advantage of using the DEAE-dextran transfection in the primary screening is given in Chapter 1. Each of the Abs listed in Table 6.1 was tested for panning untransfected COS cells. Among these Abs, GB17 and GB25, even at very dilute form, were usually panning the COS cells probably nonspecifically. Other Abs usually had little or negligible nonspecific binding to the untransfected COS cells. These Abs were therefore, diluted to give the lowest nonspecific panning of the untransfected COS cells. The diluted Abs were then used for panning the COS cells transfected with the library DNA. The transfected COS cells presumably expressing the novel determinants were selected by panning using a pool of Abs. Hirt DNA was made from these cells and transformed by electroporation into MC 1061/P3 (MC) *E. coli* in order to amplify the DNA.

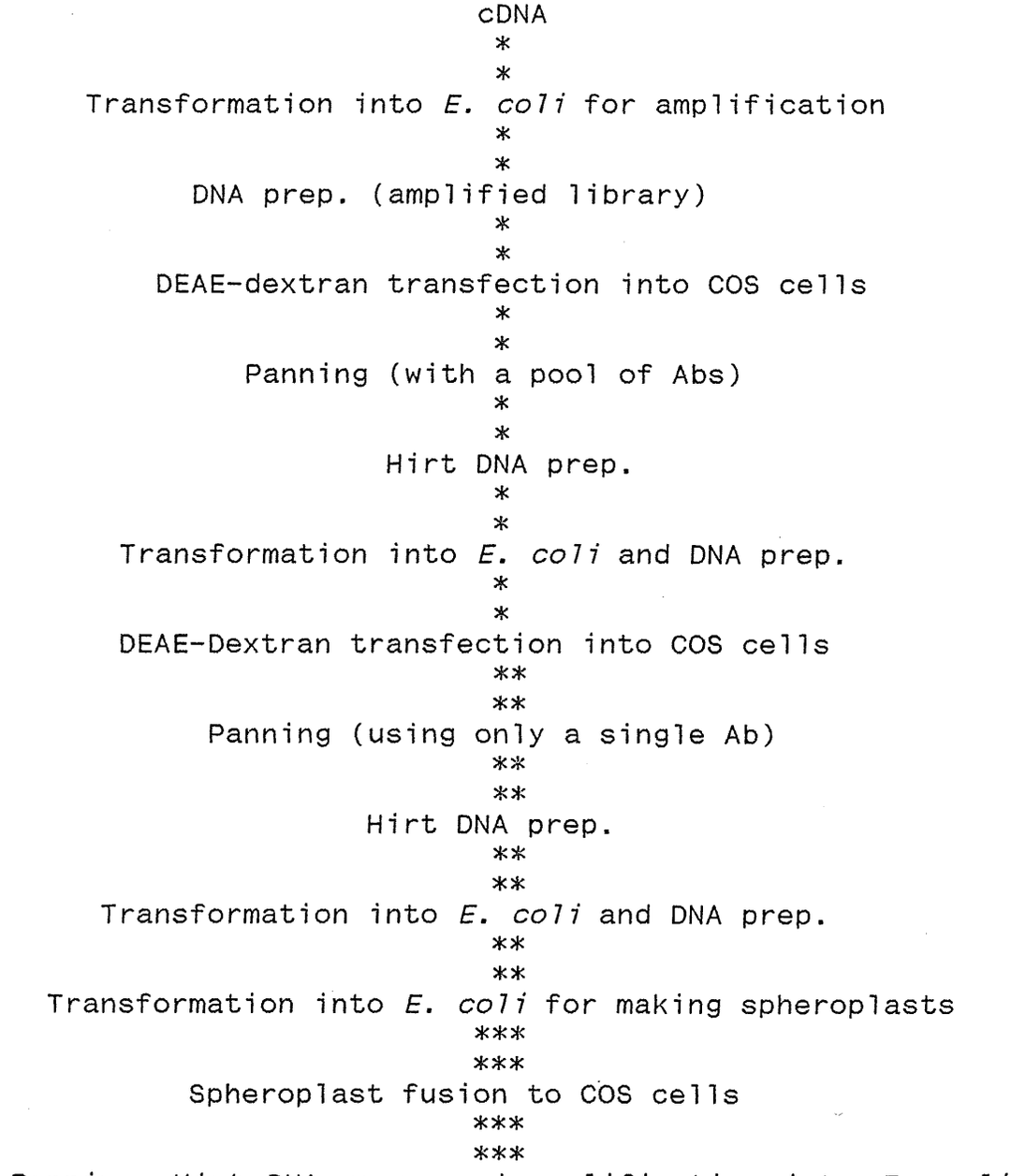
The colonies from electroporated cells, in the primary selections, were scraped from the agar plates, pooled and grown in L-broth. These *E. coli* cells were prepared for spheroplast fusion and fused into COS cells. The spheroplast fusion and electroporation were mainly carried out by Dr. Nickson in our laboratory.

Usually $6x10^9$ spheroplast *E. coli* cells were fused to $5x10^5$ COS cells. COS cells were incubated and panned (usually with a single Ab to select single population of cell surface determinants). This selection procedures are used for the secondary and tertiary screenings. The Hirt DNA was then prepared from COS cells which had bound to the panning dish and then transformed into MC *E. coli* cells for the amplification of selected clones. Several colonies were generated from the transformation which indicated that several cDNA clones might have been selected. Plasmid DNA were made from these cells and usually a pool of these clones were screened further (tertiary screening) by spheroplast fusion as described above. On the other hand some of the amplified DNA were either used for transfecting the COS cells for staining purpose or for restriction analysis (See flow diagram next page).

6.3.2 DEAE-dextran Transfection and Staining of the Immunoselected Clones

DNA from some of the tertiary screened clones were back-transfected into the COS cells for staining purposes. Staining was one of the most revealing way of allowing one to see whether the isolated clones were the target clones. The cytospun cells were then stained with the appropriate Ab to confirm the isolation of the target clone. The dilution of the primary Ab had already been worked out to give the lowest possible background on untransfected or mock-transfected COS cells (DEAE-dextran transfection without using any DNA). None of the selected clones stained positively with the Ab which had been used to select that particular clone during panning or subsequent screenings. Despite this fact, there were usually some background staining associated with some of these Abs (Fig. 6.8 and 6.9 and Table 6.1).

Results obtained above indicated that staining could not confirm that any of the immunoselected clones were the target clone(s). The failure was not due to the inefficiency of staining because CD2 transfection either



Flow diagram showing procedures for immunoscreening.
In this diagram a single * means primary, double * means secondary and triple * means tertiary screenings. Transformation into *E. coli* was either by $CaCl_2$ or by electroporation. Antibody (Ab) selection of transfected COS cells was either by panning or Dynabeads (see section 2.2.22).

No	Cells	1^ Ab	Dilut	Result	Comments
1	COS	-	-	-VE	Only second Ab was used
2	COS	W6/32	1/500	+VE	-ve W6 meant cells damaged (non-Transfectants)
3	COS	CD2	1/100	-VE	No background
4	COS	GB17	1/200	-VE	Usually faint background
5	COS	GB24	1/50	-VE	Usually faint background
6	COS	GB25	1/1000	-VE	Some background
7	COS	161G	1/50	-VE	No background
8	COS	FT	1/50	-VE	No background
9	CD2	CD2	1/10	+VE	Strong staining
10	GB17	GB17	1/200	-VE	Some background
11	GB24	GB24	1/50	-VE	As on GB24 above (same as 5)
12	CD2/ GB24	CD2	1/10	+VE	As on CD2 (same as 9)
13	CD2/ GB24	GB24	1/50	-VE	As on GB24 (same as 5)
14	GB25	GB25	1/1000	-VE	High background
15	161G	161G	1/50	-VE	No background
16	FT	FT	1/50	-VE	No background

Table 6.1 Summary of the results from staining the immunoselected clones with the appropriate and the control Abs. None of the selected clones stained with the appropriate Ab and in few cases the background was high. In these cases majority of the cells were positives indicating high degree of cross reactivity. The GB25 Ab usually gave the strongest of the backgrounds, even on untransfected cells and only 1/2000 dilution of this Ab gave very low background on untransfected cells(intact COS cells usually stain with W6/32).

by itself or mixed with other immunoselected clones (e.g. a pool of GB24 immunoselected clones) to act as a positive control stained positively with the anti-CD2 Ab which meant that the DEAE-dextran transfection, cytospinning and the staining were experimentally feasible (Fig. 6.11).

Several aliquots of the cDNA library were screened using the screening protocol described above. Every selected clone (by the Abs listed in Table 6.1) was usually analysed further by restriction analysis and staining. Based on several staining results similar to those listed in the Table 6.1, it was concluded that none of the immunoselected clones were the target clones.

The staining results seemed to be contradictory to the results obtained from the panning results seen in the primary and secondary screenings. In there the results indicated that the Abs were actually panning some transfected cells, presumably they had plasmids in them since it was shown that these selected clones have plasmid in them because the Hirt DNA from these cells, amplified in MC *E. coli*, produced colonies on selective agar plates. The panned cells should have therefore been mainly the transfected COS cells (having plasmid in them). It can then be argued that the binding of Abs to the transfected COS cells might have been due to the interactions between these Abs and some types of antigenic determinant on COS cells. At the same time it should be pointed out that all the Abs used in this work had been tested against untransfected COS cells and no significant panning was observed (except with GB17 and GB25 at higher concentrations). If one agrees with both arguments then it may be concluded that the Abs were panning transfected cells nonspecifically. The nature of the observed cross reactivity is not yet known.

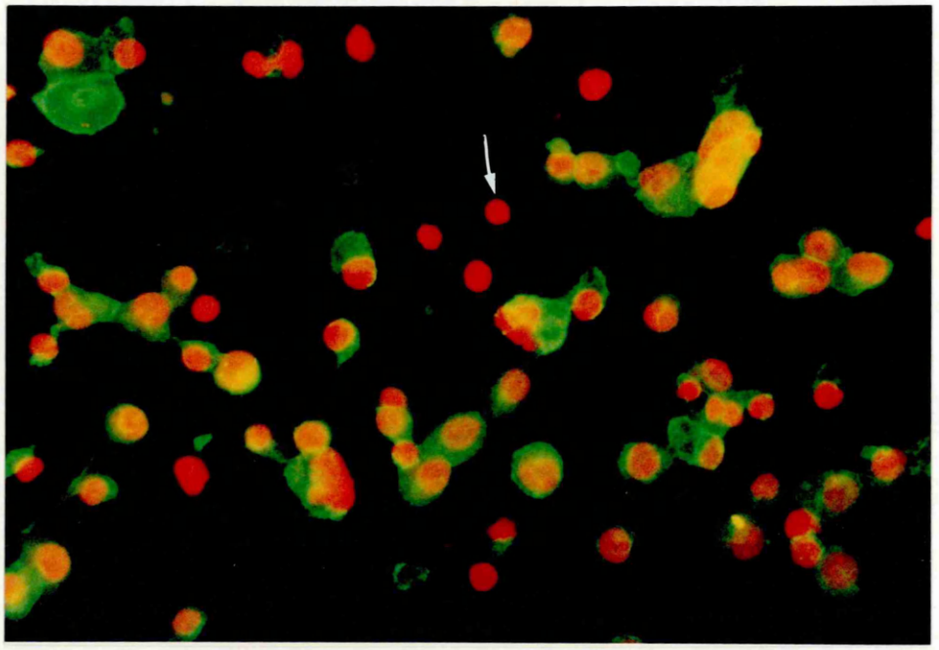


Figure 6.11 COS cells stained with W6/32 and counterstained with propidium iodide. Lack of W6/32 staining on some counterstained nuclei shows that the cells had been lysed and only the nuclei had remained.

6.3.3 Restriction Enzyme Analysis of Immunoselected Clones

The restriction analysis was carried out to confirm the presence of insert in the isolated clones and also to see if there were any similarities between the restriction patterns of the inserts. Usually after each round of selection, in order to find out the size of the inserts of the immunoselected clones, mini DNA preps were made from MC *E. coli* cells. The DNA were analysed by restriction digest analysis. The restriction analysis was carried out using several restriction enzymes (e.g. Pst I and Hind III) recognizing the sites flanking the insert in the polylinker region of the CDM7 (which has similar polylinker sites to the CDM8 vector described in Chapter 5). Those clones which had large inserts were pooled and back-transfected into COS cells for further enrichment (selection).

The immunoselected clones showed unpredictable restriction patterns. Some of the clones were not restricting with the enzymes which were known to cut the vector DNA flanking the cloning site (e.g. Pst I/Hind III). In some other cases the restricted DNA showed no insert, however, there were cases where plasmids with insert were seen (Fig. 6.12).

These restriction patterns could have arisen in several ways. One possibility is that the COS cells have been transfected with only the vector and the Abs have panned these cells probably randomly. Clones selected from these cells would obviously lack any insert, though the reason for such a selection is not obvious (may be due to the reasons discussed for deletion, see below). Some of the clones isolated in this way were actually linearised by cutting the plasmid with Pst I/Hind III instead of giving two bands, one for the vector and one for the insert. These plasmids could have come from re-circularisation of vectors during cDNA cloning. This possibility was not, however, expected because the library had been cloned into the BstX1 cloning sites in

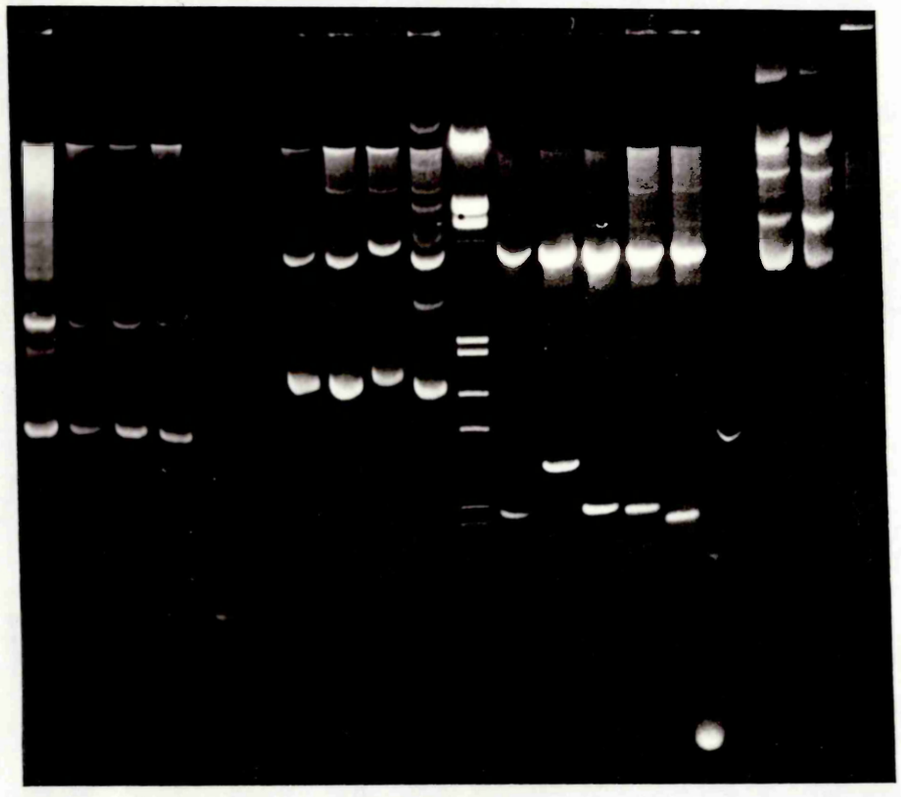


Figure 6.12 Restriction digest analysis of the immunonoselected clones. The clones showed unpredictable restriction patterns. Some were restricting and showing to have insert and others had no insert or were not restricting.

CDM7 vector (Simmons personal communication) and it was documented that the Bstx1 sites in CDM8 (CDM7 likewise) do not have compatible ends to be able to religate (for full detail see Chapter 5).

Another possibility might have been that the inserts were very small and not detectable on the gel (e.g. >200 bp). This can be excluded because the library had been size fractionated and fragments smaller than 500 bp had been discarded (Simmons personal communication; Simmons and Seed, 1988). Inability to restrict some of the clones may have been caused by DNA impurities. To exclude this possibility, some of the DNA were cleaned further and restricted again. The same results were obtained suggesting that other causes such as loss of one of these two restriction sites might have been involved. It was also observed that in some cases the vector band was smaller than expected.

The above observations (i.e. lack of digestion, lack of insert and reduction in the vector size, etc.) seemed accelerated as the screening advanced from primary to tertiary. This effect was particularly investigated using GB24 Ab (Figs. 6.13- 14). These figures show that most of the clones have different sizes of vector band, suggesting that some kinds of deletion of the vector or cDNA insert might have occured during the screening process. This was also observed when CD2 was serially propagated through COS cells without Ag selection (panning).

The original cDNA library was transformed into MC cells and several colonies were randomly picked and DNA from these clones were restricted to ensure a similar size vector was present. This was tested by cutting the library DNA with Pst I/Hind III enzymes. Most clone showed only a single vector band probably suggesting that deleted vectors were not present in the library. Therefore reduction in the vector size may have occured during the screening process (Fig 6.15). The deletions were probably occuring after the DNA had been

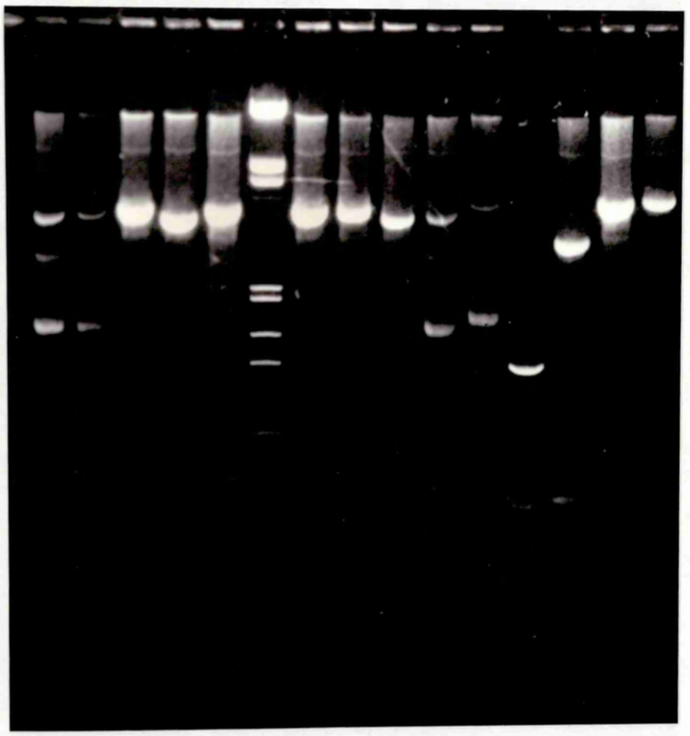


figure 6.13 Restriction pattern of immunoselected clones after the primary screening. Most clones have similar size vector band.

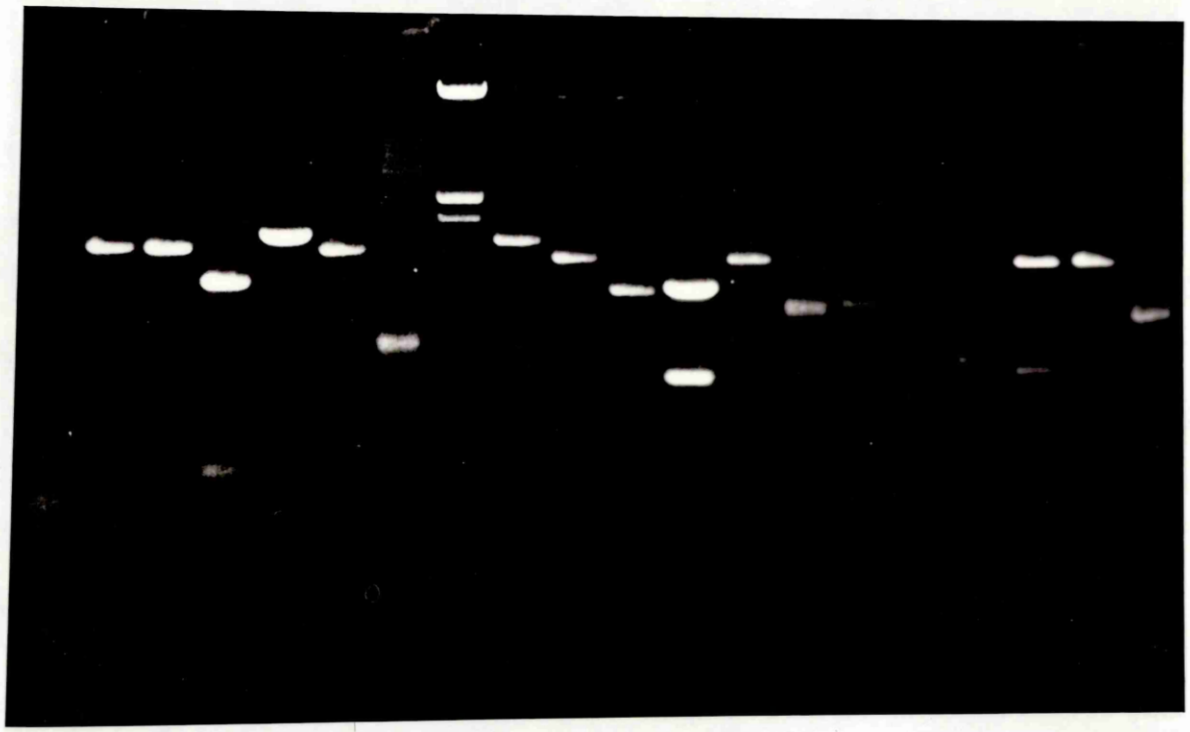


Figure 6.14 Restriction pattern of immunoselected clones after progressive selection. Compared with the primary selected clones (Fig. 6.13), most clones have different vector size bands suggesting they might have been deleted.

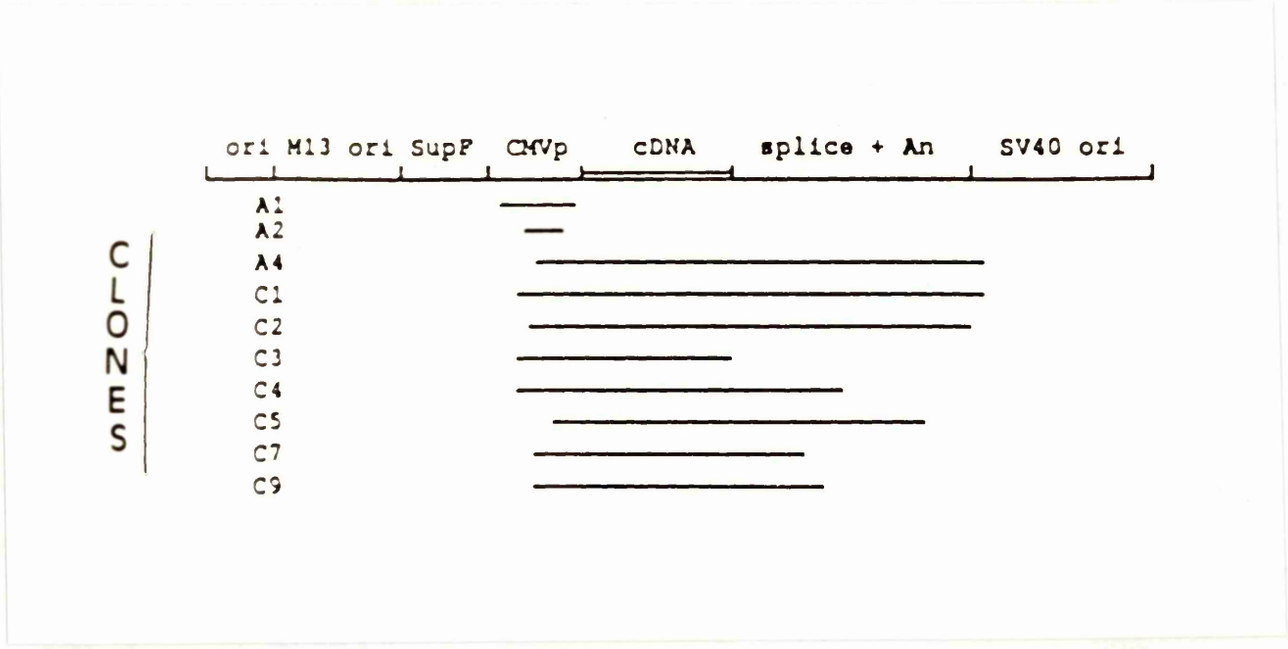


Figure 6.15 Deletion map of CDM8. The figure shows that 10 independent clones have been deleted in the COS cells. In almost every clone the non-essential sequences including CMV promoter have been deleted. lines cover deleted areas.

transfected into the COS cells or transformed into the MC *E. coli* cells.

Only the deletions which have occured in the nonessential regions of the vector (Polyoma ori and CMVp, the RNA promoter) as well as the insert could be detected (Fig. 6.16). Any possible deletion in the essential regions of the vector (SupF, piXV ori and SV40 ori) would lead to the loss of that particular plasmid during subsequent cell divisions. This kind of deletion though possible in theory, could not be shown on a gel. On the other hand deletions in the nonessential regions usually would not lead to the loss of that particular plasmid. It even sometimes, might be advantageous and lead to faster replication of that particular plasmid. If this happens then that particular plasmid would over grow and would be over-represented. For example deletion including the promoter region (i.e. CMVp) would lead to absence of mRNA transcription and the plasmid would therefore most of its activity to replication. This in turn causes over growth of promoter free plasmids.

In support of the above argument it was observed that progressive screening increased the frequency of deleted plasmids (Fig. 6.13-14). This may even suggest that the selection was favourable toward selecting cells containing the deleted plasmids. If this was the case then Abs should have panned the transfected COS cells randomly to allow the deleted plasmids to be selected as well. Eventhough panning of the deleted plasmids may not be favoured but because of over-representation they might be present among the selected clones as well (which was actually the case).

Detailed restriction analysis were later done in our laboratory to map the deleted regions. The analysis of deletions which had been recovered by back-transformation into MC *E. coli* cells have shown that deletions were mainly clustered around the flanking regions of the insert (i.e. CMV promoter, cDNA insert and splice + A_n) and it almost always included the

promoter (Fig. 6.15; Sutcliffe *et al.*, 1990). The results have indicated that all fragments essential to the survival of the plasmid in the COS cell or in the MC *E. coli* have remained intact. Finally the above findings may suggest that deletion could have been independent of the type of of McAb used to screen the library because all the immunoselected clones showed this phenomenon.

6.4 Discussion

The main focus of this chapter was on optimisation of the COS expression system at the level of DEAE-dextran transfection and staining. The data presented showed that several factors were important in optimising the efficiency of the DEAE-dextran transfection. The three main factors were shown to be the concentration of DEAE-dextran, cells and DNA. It was found that lower concentration of DEAE-dextran (for example 100 ug/ml) than recommended (i.e. 400 ug/ml, Seed and Aruffo, 1987) gave better transfection efficiency.

The cell concentration was also found to be important in increasing the efficiency of transfection. This effect was not linear and the efficiency decreased after certain cell concentrations. The percentage of cell confluency to give the best effect was in agreement with what has been reported (Seed and Aruffo, 1987) with an important difference in that the data presented here showed actual numerical concentration of the cells rather than percentage of confluency.

The DNA concentration showed similar effects as to the concentration of cells, that is the increase in efficiency of transfection was not proportional to the concentration of DNA. The data presented suggested that if the maximum number of transfectants per microgram of DNA was the objective (for example for screening a cDNA library), then DNA concentration should be optimised to

give the maximum number of transfectants per microgram of DNA.

Staining results showed that several factors may effect the intensity of the staining. These factors involved the cell culture, the cell harvesting, preparing the cells for cytospinning and the cytospinning itself.It was shown that none of the immunoselected clones proved to be the target clones. The Abs used were panning cells, probably randomly, due to Ab-protein cross reactivity. At the same time some Abs were panning more cells than the other Abs, may be because of lower specificity.

It was also observed that some of the immunoselected clones became deleted during transfection and enrichment steps. The deletions were, most probably, random within a clone but they were observed in the nonessential regions of the vector. Some of the failures in identifying the target clone may have been caused by the deletion phenomenon (deletions affecting the insert and also the promotor).

There may be other reasons for failure of Abs to select the right clone. One difficulty in selecting the right clone would be the presence of large amount of secondary structure in the mRNA, which does not permit the reverse transcriptase to make complementary strand during cDNA synthesis (Old and Primrose 1985). In this case the library would be deficient of that particular clone.

Another difficulty could be the absence of mRNA from the target tissue and consequently the corresponding clone in the cDNA library to code for the target polypeptide. It has to be emphasised that these Abs (except CD2) have been shown to stain placental trophoblast cells (Chapter 1). A cDNA from a human placental library is, therefore, expected to contain the corresponding clones.

These McAbs have been raised against various forms of trophoblast surface extracts (Chapter 1). Nothing is known about the abundance of corresponding messages. If these messages are low abundance then the COS experssion system may not be sensetive enough to select low abundance clones. In most of the reported cases the COS expression system have been used to screen cDNA libraries abundant for the target clone (Seed and Aruffo 1987; Aruffo and Seed 1987a, b; Simmons and Seed 1988 and also see Chapter 1).

The other reason for not selecting any specific clone could be the low affinity of Abs for the target Ag or high cross reactivity of some of these Abs with the non-target antigenic determinants on the COS cells which were observed during panning and staining.

One of the main advantages of the COS cell system is that Ags can be expressed in their native state as full-length polypeptides on the cell surface, but it is usually difficult to clone full length cDNA for long polypeptides. For example the polypeptide recognized by GB17 Ag is about 175 KDa and the message coding for such a big polypeptide may be too big to be present as full length cDNA clone in the library.

It would also be difficult to select clone whose antigenicity requires the presence of two different polypeptide chains (e.g. each HLA class 1 determinant consists of one polypeptide encoded by MHC gene and one smaller polypeptide, beta-2 microglobulin encoded by different gene, Roitt, Brostoff and Male 1985) unless one of the chains is normally expressed in COS cells (it should be noted that MHC, in here, is given as an example because as far as MHC is concerned COS cells express monkey's MHC which is very similar to human MHC).

Yet another possibility may be the problem of accessibility, that is to say whether the epitope

recognizable by a particular Ab is expressed on the surface, because Abs raised against purified non-membrane (not expressed on the surface) polypeptide would be able to recognise the same polypeptide on intact (viable) cells. This would be a serious problem if the Abs epecificity had been determined based on their reaction to the purified proteins (e.g. Western blotting) or on fixed cells are used for identifying surface determinants on intact cells. The reason is that fixed cells usually show different pattern of staining than viable cells. This difference may be because, in fixed cells. acetone fixation may partially delipidate the cell membrane which may make some proteins more exposed. The fixation may also cause other changes to the 3D structure of the Ags and consequently the McAb which had been tested on fixed cells may not be able to recognize the same cells in culture. This possibility might have been the case with the Abs used in this work, because most of these Abs had been tested and chosen for their activity on fixed cells (Chapter 1).

Different methods of screening should therefore be utilized which can complement each other. For example a lambda gt11 library can be screened with these Abs. The positive clones should be used for hybridisation probing for further screening the lambda gt11 and also to screen a placental cDNA library by plaque and colony lifts using the methods outlined in Chapters 2 and 3. Colonies which give positive signals can be transfected into COS cells for staining purpose. The problem associated with the affinity of these Abs for the target polypeptides would still be present for screening the lambda gt11 library but the deletion problem may be avoided (for more discussion on lambda gt11 see Chapter 7).

CHAPTER 7

CLONING FDO161G cDNA

7.1 Introduction

In the previous chapter (Chapter 6) the COS expression system was introduced and attempts were made to isolate novel determinants by immunoscreening. Due to the failure of the methods used, some other methods of investigation were suggested. Two of the suggested methods were employed and the results are described in this chapter. These methods of investigation included screening of the cDNA library by oligonucleotide probing and polymerase chain reaction (PCR) and screening of the lambda gt11 placental cDNA library.

Molecular approaches such as PCR or colony hybridisation (homology probing) techniques may be employed for the isolation of the target cDNA clone if some amino acid sequence of the target protein is known (Suggs et al., 1981; Hanks 1987; Cardin and Tavitian 1986; Gould et al., 1989). The PCR method, in theory, requires only knowledge of the amino acid sequence of the two short regions (approximately 7-10 amino acids in length) of the encoded protein (Gould et al., 1989 ; also see chapter 1).

Lambda gt11 expression vector which was developed by Young and Davis (1983a,b) can accomodate the cDNA library and allow the expression of the protein as a fusion protein (Chapter 1). Any clone unable to be isolated using the COS expression system, may be isolated using the lambda gt11 expression vector (chapter 1). Antibodies such as FDO161G can be tested against the fusion proteins and the DNA of the positive clones can be amplified rapidly by PCR.

The work in this chapter focuses on the amplification of DNA by PCR, colony hybridisation by oligonucleotide probing, screening of the lambda gt11 placental cDNA library and expression of FDO161G cDNA clone in COS cells.

7.2 PCR amplification of DNA

As mentioned above, PCR can be generated if some amino acid sequence of the target protein is known. Such information was available on the FDO161G glycopeptide as some of the amino acid sequence of the protein recognised by FDO161G Ab had already been partialy characterised (Muller 1989; Fig. 7.1).

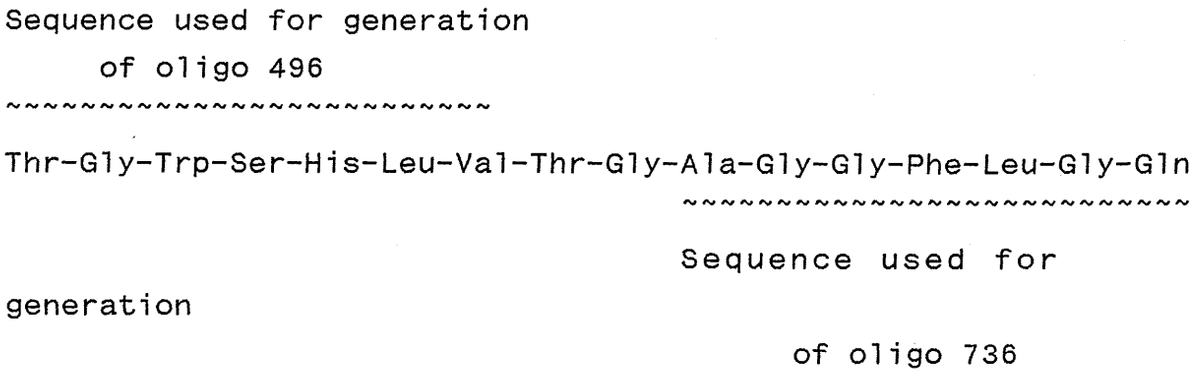


Figure 7.1 The N-terminus amino acid sequence of FDO161G glycopeptide and the position of the two oligos for generating 59 bases fragment.

PCR was therefore used to screen a placental cDNA library as well as genomic DNA. In these experiments two degenerate oligonucleotide mixtures, corresponding to each known peptide sequence of the FDO161G glycopeptide, had to be made to act as primers in the reaction. The primary aim of these experiments was to generate a small DNA fragment complementary to the 5' end of the gene coding for the FDO161G glycopeptide. Generation of such a fragment was hoped to enable us to isolate the complete cDNA clone (or the gene) coding for the FDO161G glycopeptide.

The two degenerate oligonucleotides (called 496 and 736; Fig. 7.2) were designed to coresponded to the amino acid sequence of the N-terminus of the FDO161G glycoprotein. They were chosen to contain all possible codon combinations that could encode the known amino acid sequences (in total 2^{12} degeneracies for each oligonucleotide). From the amino acids sequences chosen for the generation of the oligonucleotides, 4 had least degenerate codons (i.e. Trp, His, Phe and Gln), 2 had most degenerate codons (i.e. Ser and Leu), and others had their third nucleotide of the codon degenerate (Figs. 7.2-3).

The two oligonucleotides were designed to have complementary sequences to each of the opposite strands and were apart by 7 bp (Fig. 7.3). The amplification of the target sequence should generate a 59 bp fragment (26 bases for each of the oligonucleotides and 7 bases in between these two).

Anticipating difficulties in generating specific PCR product with these highly degenerate oligonucleotides, it was decided to optimise the amplification of the specific sequences by PCR using single population (non degenerate) primers specific to the CDM8 vector DNA. Oligonucleotides were, therefore, designed to have similar sizes to 496 and 736 (26 mer).

```
5' CTG CAG ACA GGA TGG AGC CAC CTA GT 3'
             C   C       T   T   C
             G   G     TCA       G
             T   T       C       T
                         G     T A
                         T       G
   ~~~~~~~~
   Pst site
```

(a) oligo primer 496

```
5' GCA GGA GGA TTC CTA GGA CA 3'
     C   C   C   T   C   C
     G   G   G       G   G
     T   T   T       T   T
               T A
                 G
```

(b) oligo 497

```
3' CGT CCT CCT AAG GAT CCT GT TTC GAA 5'
     G   G   G   A   G   G
     C   C   C       C   C
     A   A   A       A   A
               A T
                 C
                           ~~~~~~~~
                           Hind3 site
```

(c) oligo primer 736

Figure 7.2 Oligos derived from the amino acid sequence of the FDO161G polypeptide. The sequences also show all possible degeneracies for each oligonucleotide. Each of the two oligonucleotides, 496 and 736 (a and c), has 10^{12} degeneracies. The sequence for 736 (c) is the complementary strand of the 497 (b). The restriction sites at the 5' end of the two oligonucleotides (496 and 736) were placed to facilitate later subcloning of the PCR fragments generated.

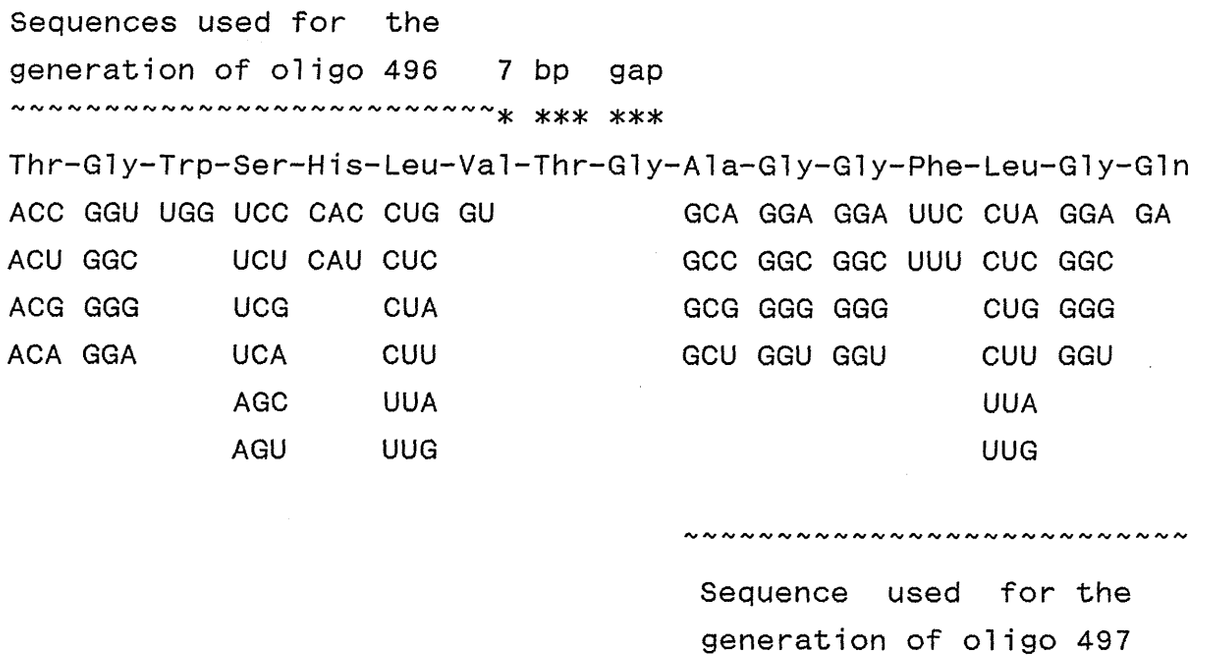


Figure 7.3 The amino acid derived sequences used for generation of the two oligonucleotides. The two oligonucleotides were made by considering all of the degeneracies written under each amio acid sequence. The DNA sequence for oligonucleotide 736 was the complementary strand of 497. The 7 bp distance between the two primers are shown (*).

These oligonucleotides were specific for the CDM8 plasmid, flanking the stuffer (Fig. 7.4). The aim was that by using these oligonucleotides any DNA insert in the stuffer region of the CDM8 would be amplified (e.g. CD2 or the stuffer itself).

PCR was performed on CDM8 and CD2 (cloned into CDM8) using oligonucleotides 380 and 382 (Fig. 7.4). The reactions were performed on uncut and cut and linearised (Not 1 cut) CD2 and CDM8 plasmids. The amplified fragments were run on an agarose gel (Fig. 7.5) and it was seen that more than one fragment has been generated for each plasmid (multiple CD2 fragments can not be seen in this figure but was seen on the autoradiograph of the Southern blot of this gel, see below). It also can be seen that Not 1 digested CDM8 has generated less fragments than uncut CDM8 (tracks 2, 3, 5 and 6). The gel was Southern blotted and hybridised with the CD2 probe to find out which one of the PCR generated fragments were CD2 specific. The reason for doing this was that only a 1.5 kb fragment was expected from the amplification of CD2 DNA but instead several fragments were generated (Fig 7.6). Computer analysis of the CD2 DNA sequences compare with the sequence of these two oligonucleotides (380, 382) showed that there were two other possible location in the CD2 sequence which these two oligonucleotides might have annealed which resulted in the amplification of the two smaller fragments.

Autoradiography of the hybridised Southern blot showed that the CDM8 fragments had not hybridised to the CD2 probe as predicted. On the contrary most of the fragments in the CD2 containing tracks had hybridised to the probe. One of the biggest of these fragments seemed to be the same size as the CD2 insert marker (1.5 kb, compare tracks 7, 8 and 10 with 9; Fig 7.6). The largest fragment of about 6 kb may have been generated by amplification of the circular plasmid. The two smaller fragments (i.e. 1.3 kb and 0.8 kb) had also hybridised to the CD2 probe.

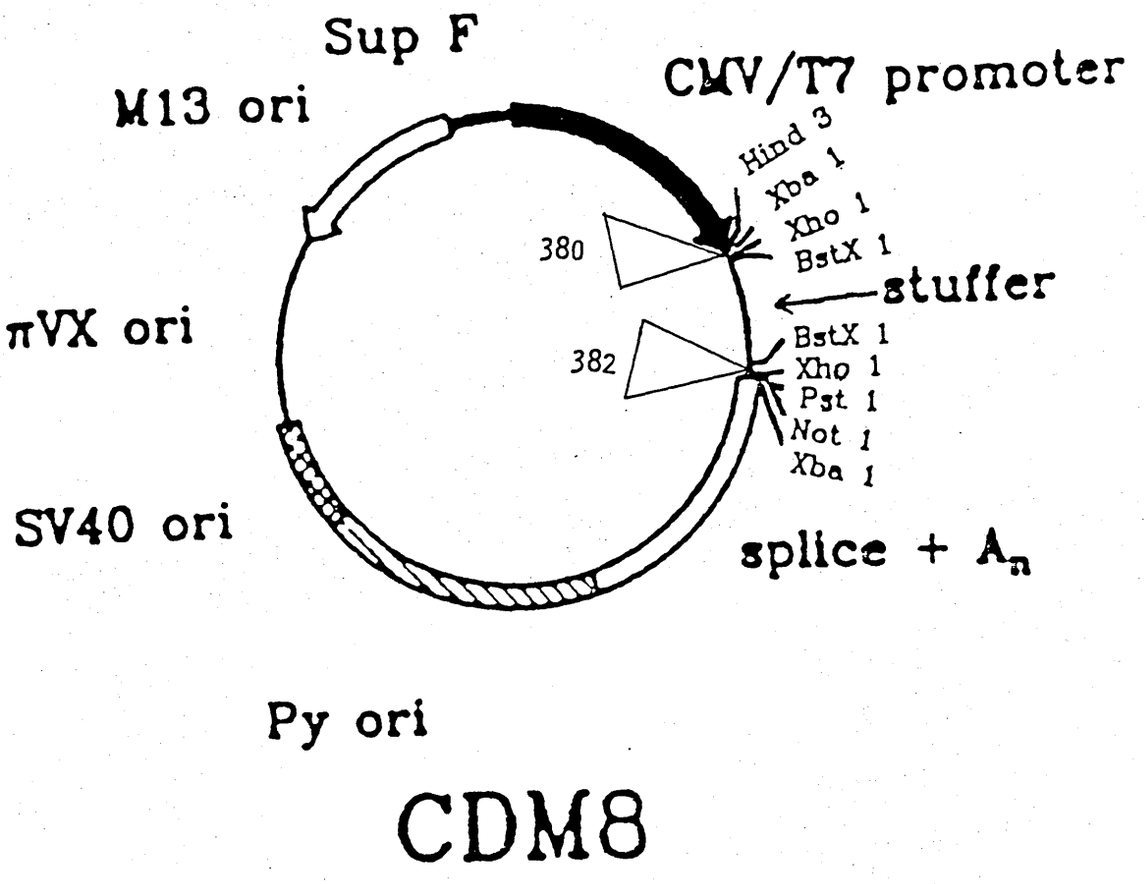


Figure 7.4 CDM8 specific oligonucleotides. These two oligonucleotides would hybridise to two specific target sequences flanking the stuffer. Oligo 380 would hybridise to the 5' end of the stuffer and 382 to the 3' end and any PCR, using these two primers, should amplify anything inserted into the stuffer region.

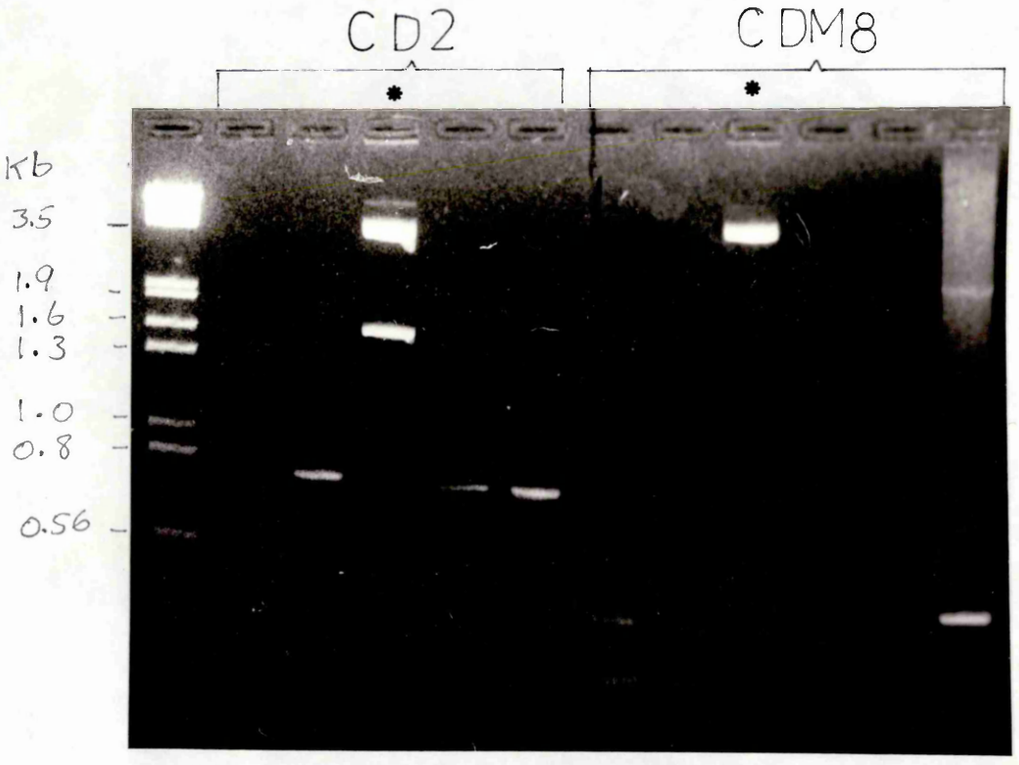


Figure 7.5 PCR amplified fragments of CD2 and CDM8 on a 1.5% agarose gel. Amplifications can be seen with both DNA. The CD2 amplified fragments are about 0.7 kb and CDM8 fragments are less than 0.5 kb. Digested CD2 and CDM8 DNA were used as markers (*).

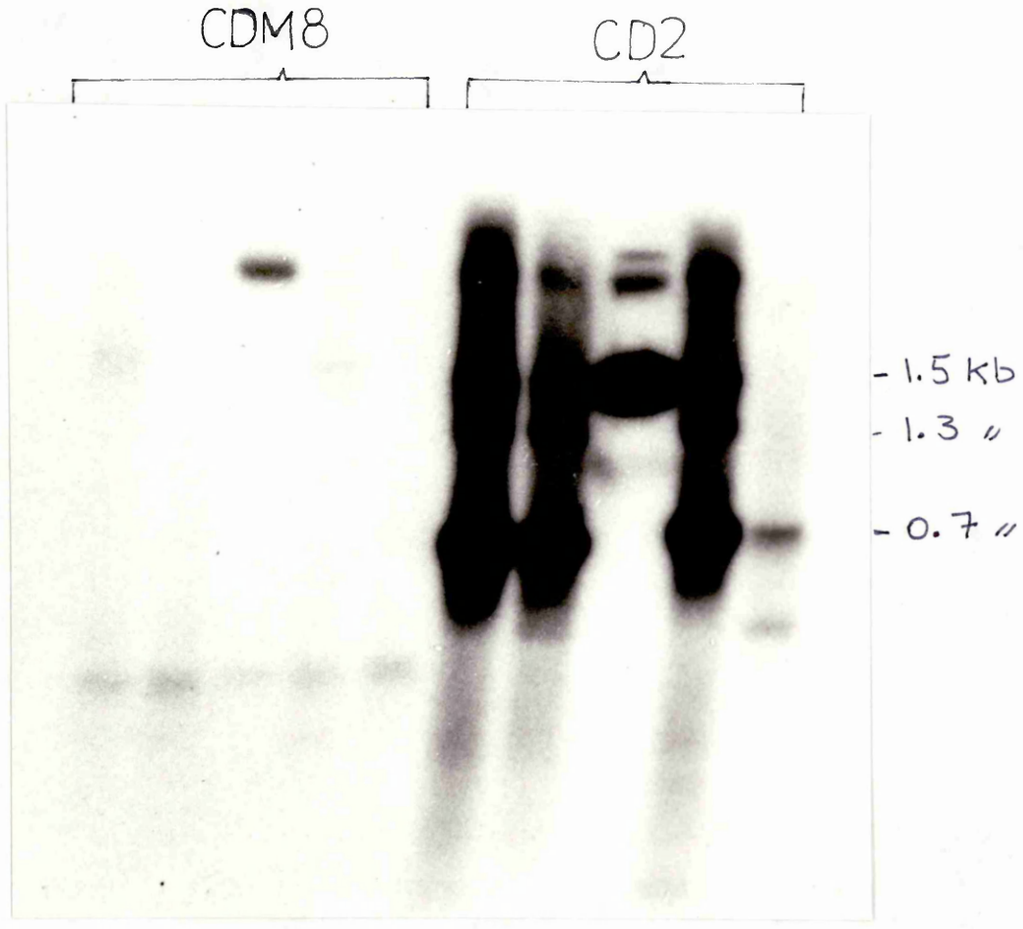


Figure 7.6 Southern blot of the amplified CD2 and CDM8 fragments hybridised with CD2 1.2 kb insert. The figure shows that the amplified DNA in CD2 tracks have hybridised to the probe and several different size DNA have been amplified, some smaller than the CD2 insert (i.e. smaller than 1.5 kb). None of the amplified DNA fragments in the CDM8 tracks have hybridised specifically to the CD2 probe.

The presence of more than one fragment among the amplified CDM8 stuffer was unexpected because in theory only one fragment of about 450 bp should have been generated. The sequences for these two oligonucleotides had been chosen, by the aid of computer, to have the least homology with the non target sequences in CDM8. The reason for seeing these fragments might be that the oligonucleotide primers have annealed weakly to the CDM8 DNA on non-target sites.

The above experiment showed that non-specific annealing is a major problem in amplifiying DNA fragments using PCR as has been suggested by Frohman and Martin (1990). Adjusting the length and temperature of the annealing and amplification seemed to improve the quantity of the 1.5 kb CD2 fragments. Also apparent was the observation that linearised DNA tended to generate less nonspecific fragments (Fig. 7.5).

7.3 PCR Amplification of the Placental cDNA Library

Attempts were made to amplify the placental library, described in the previous chapter (Chapter 6), using PCR. For this purpose the library DNA was first digested with Not 1 restriction enzyme and PCR was performed using the twosets of FDO161G specific primers (496 and 736). CD2 and CDM8 amplification as described above were used as positive controls. The amplified DNA were run on a 1.5% agarose gel. The gel showed that fragments generated from the CD2 and CDM8 were as above, but fragments generated from the library using FDO161G specific primers were mainly less than 500 bp (Fig. 7.7). It was thought that these fragments were of small size and that FDO161G specific fragments could be among them. Another PCR experiment was therefore set up using a combination of different annealing and extension times. The possibility of generating non-target fragments was tested by running PCR using either a single population or two different populations of primers (using either primer number 496 or 736 vs using

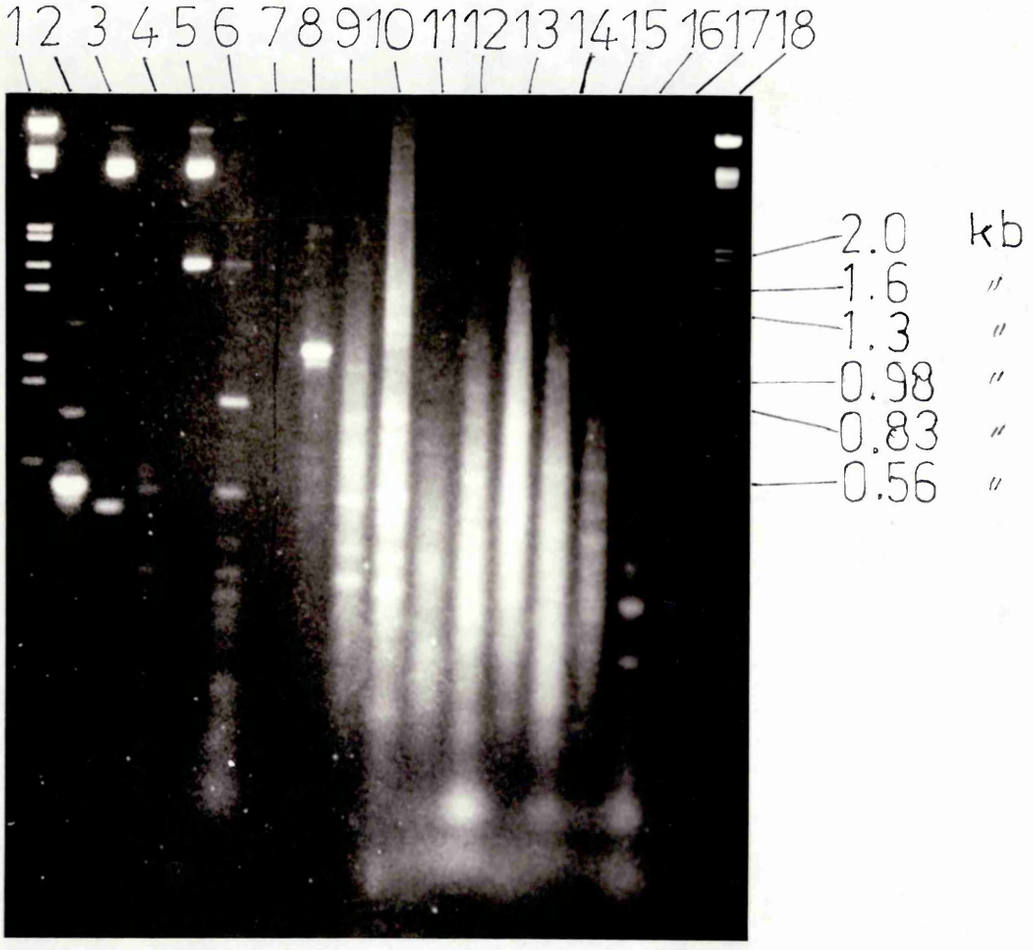


Figure 7.7 Amplified cDNA library on a 1.5% agarose gel. The two oligonucleotide 496 and 736 have generated fragments less than 500 bases long (tracks 16 and 17).

both 496 and 736 together respectively). In this experiment the primers were annealed at 45°C, extended at 72°C and denatured at 90°C. The size of the expected FDO161G specific fragment was too small to be seen in an ordinary agarose gel and had to be visualised in a polyacrylamide gel. Visualising the amplified fragments in the polyacrylamide gel was facilitated by the addition of a small amount of ^{32}P-gamma-dATP during the last 5 cycles of the PCR. The PCR products were run on a 12% polyacrylamide gel and after drying, the gel was autoradiographed and it showed that both single and double primers had generated fragments (Fig 7.8).

It was then decided to do a PCR amplification using a combination of single or double primers, with or without template. The templates were the cDNA, genomic DNA and some random DNA as a negative control. To avoid any possibility of DNA contamination of the primers or the reagents from the previous experiments, fresh aliquots of Taq polymerase, oligonucleotide primers, and buffers were used. The PCR was carried out for 30 cycles programmed to run 30 seconds for annealing at 45°C, 30 seconds for the extension at 72°C and one minute for denaturation at 90°C. The experimental set up is shown in Table 7.1. Addition of radiolabelled nucleotide and visualisation of the DNA fragments were as above.

The autoradiography of the resulting PCR product showed that most of the double primers, including template minus reaction (track 4, Fig. 7.9), have generated similar fragments. The similarities of the banding pattern and also the generation of fragments in the tube without any template may suggest that these fragments could have been caused by either self priming or by priming each other. Another possibility is that reagents in all of the tubes might have been contaminated with the same kind of DNA resulting in the generation of these fragments.

Figure 7.8 PCR amplified library DNA on a 12% polyacrylamide gel. In this gel it is obvious that reaction mixes with only single primers have also amplified some DNA; therefore it can not be said that the amplified DNA are specific amplification of target sequences. The size of amplified fragments can not be accurately measured from the markers used because the markers are single stranded and the amplified DNA most probably double stranded.

	1	2	3	4	5	6	7	8	9	10	11	12
Templ.	_	_	_	library			genomicT45			genomic SiHa		
496	+	_	+	+	_	+	+	_	+	+	_	+
736	_	+	+	_	+	+	_	+	+	_	+	+

Table 7.1 Experimental set up to exclude the possibility of contamination. No DNA, cDNA library DNA or genomic DNA were amplified by PCR. In each DNA sample there was two single primers and one double primers.

Figure 7.9 PCR amplified DNA from placental cDNA or genomic DNA on a 12% polyacrylamide gel. This gel contains the products of the amplification experiment described in Table 7.1. In this gel tracks 1-3 contain DNA from No. 1-3 in Table 7.1, tracks 6-8 from No. 4-6, tracks 9-11 from No. 7-9 and tracks 13-15 from No. 10-12 respectively. In most tracks some amplified DNA can be seen despite the fact that some did not have any template (i.e. tracks 1-3).

Several similar experiments, with proper controls, failed to generate any FDO161G specific fragments. Improving the sensitivity of the PCR by altering the length and the temperature of the steps involved, using fresh reagents etc. also failed to identify the FDO161G specific DNA. Some of the reasons for this failure will be discussed later in this chapter.

7.4 Colony Hybridisation of the Placental Library

The above placental library was also screened by the colony lift method of *in situ* hybridisation of bacterial colonies (Chapter 2). In these experiments the three FDO161G specific oligonucleotides (496, 497 and 736) were used. Several colonies which were picked as positives in the primary screening proved to be false positives in the subsequent screenings. This method of screening the library with highly degenerate oligonucleotides could not isolate any positive clone in several attempts which were made to optimise the system and try to reduce the noise signals. The main problem was that the background (false positives) signals were very strong and none of the treatments applied could improve the background without removing any possible positive signal. Nevertheless it has to be emphasised that only a limited number of attempts were made and it was then decided to abandon this method of approach.

7.5 Screening a Lambda gt11 cDNA Library

7.5 .1 Cloning FDO161G cDNA Clone

As shown above and in the previous chapter (Chapter 6) several methods were employed for identifying the FDO161G specific clone. All of these approaches, so far, had failed to identify the target clones. Another approach, in parallel, was carried out (in our lab by Dr. Nickson and Mr McBride, Nickson et al., 1990) using

a lambda gt11 placental cDNA library (containing $1.5x10^6$ independent clones). Their findings, which were carried out in parallel with my work, are given here, with their permission, to complement my own findings. Their work cover screening the lambda gt11 library, cloning the FDO161G cDNA clone, subcloning it into pUC18 and pCDNA1 and sequencing.

The placental library was immunoscreened with affinity purified polyclonal Abs to the FDO161G protein. Positive plaques were picked, replicated and re-screened twice. Consistently positive plaques were observed at a frequency of $2.5x10^{-5}$ plaques. After some preliminary restriction analysis, a bigger clone (DN1/6, 1.2 kb) was analysed further and several restriction sites were identified (Fig. 7.10). 1/6 clone was sequenced on both strands (using Sequenase kit from USB) and found to contain an open reading frame of 742bp and a 410bp untranslated region, terminating in a poly (A) site and poly (A) tail (Appendix 3). This clone was then used for isolating more clones by rescreening the same library by the plaque hybridisation method described in other chapters (Chapters 2 and 3). The idea was that those clones not being able to be expressed as a fusion protein (e.g. not being in frame or in right orientation) would be identified by the DNA hybridisation method of plaque lifts (e.g. isolation of FN clones in Chapter 3).

The rescreening of the library produced more than 48 positive clones (frequency of $3x10^{-3}$), and these clones were assessed for insert size by PCR using two oligonucleotide primers flanking the insert in lambda gt11. One of these clones (B3) was found to be about 1.6kb and it was subcloned into pUC18 for further analysis. Restriction mapping showed that it included the sequence of clone 1/6 (Fig. 7.10)

In order to find out whether the B3 clone was a full length cDNA, and also whether it was coding the same N-terminus amino acid sequence as the FDO161G

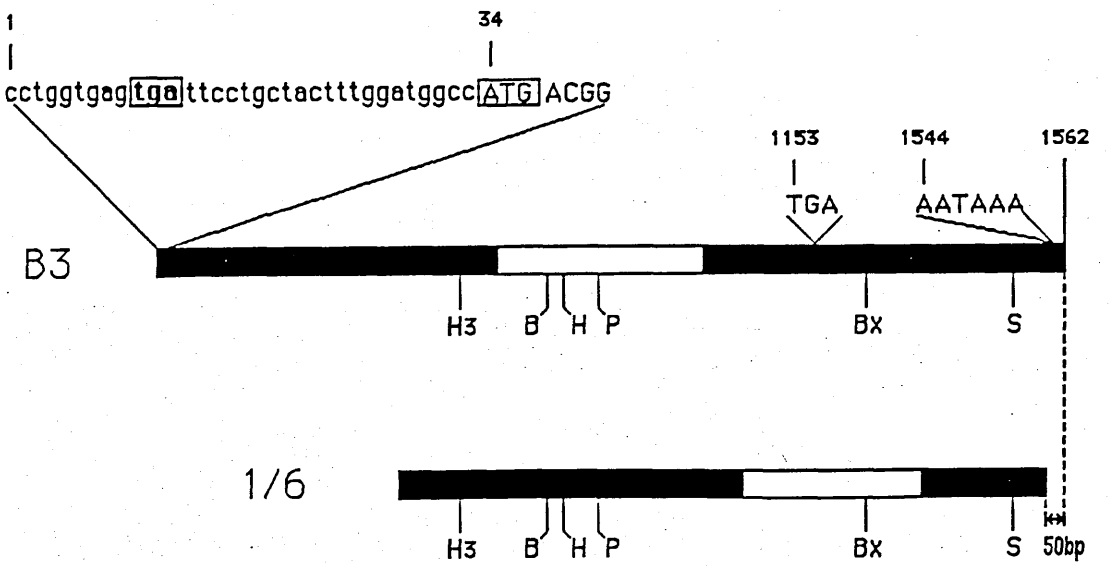


Figure 7.10 Diagram of the structure of the cDNA clones B3 and 1/6. The dark bars represent DNA sequence obtained off both strands of the respective cDNA. The translational stop codon is shown at position 1153. The 33 base pairs upstream of the initiation codon are shown including the inframe stop codon. Sizes are in base pairs; B- BamHI, Bx- BstxI, H- HincII, H3- HindIII, P- PstI, S- SacI.

glycopeptide, it had to be sequenced. The B3 clone (in pUC18) was therefore sequenced in both strands as well. B3 shared the same sequence for the 1150bp with clone 1/6. The additional 413bp of B3 was sequenced from both strands.

B3 sequence contained a complete open reading frame beginning with an AUG codon (Appendix 3) in a good consensus sequence for translational initiation (Kozac 1983). This is preceded by 33bp of 5' untranslated sequence containing a stop codon and an in-frame ATG at position 28, which is a weaker consensus sequence for translational initiation (Kozac 1983).

In situ hybridisation using clone DN1/6 as probe located the corresponding gene to the chromosome location 1p13.3 (Morrison et al., 1990). This lead us to become aware of the positioning of 3 beta-hydroxy-5-ene steroid dehydrogenase (HSD) at chromosome location 1p13 by Berube et al. (1989). Subsequently clones DN1/6 and B3 were shown (Nickson et al., 1990) to have nucleotide sequences identical to the open reading frame and 3' untranslated sequences of HSD (The et al., 1989).

The deduced amino acid sequence of the N-terminus from nucleotide 34 is in agreement with most of the N-terminus amino acid sequence of the FDO161G glycopeptide (Fig 7.11). This also shows that the AUG starting at position 34 is the initiator of translation as was suggested above. Among the first 24 amino acids deduced from the B3 sequence only one, at position 6 (His), does not agree with the amino acid sequence of FDO161G glycopeptide (cysteine, Cys, in HSD instead of histidine, His, in FDO161G, Fig 7.11).

The N-terminus amino acid sequence agreement between the FDO161G glycopeptide and HSD strongly suggests that these two proteins are the same. If that is the case then one expects that the HSD, being a dehydrogenase, is to be found in the cytosol and not on the surface of the cell (Alberts et al., 1989) as the

a

Thr [1]-Gly-Trp-Ser-<u>His</u> [5]-Leu-Val-Thr-Gly-Ala [10]-Gly

Thr [1]-Gly-Trp-Ser-<u>Cys</u> [5]-Leu-Val-Thr-Gly-Ala [10]-Gly

b

a

Gly-Phe-Leu-Gly-Gln-Arg-Ileu-Ileu-Arg [20]-Leu-Leu-Val-Lys

Gly-Phe-Leu-Gly-Gln-Arg-Ileu-Ileu-Arg [20]-Leu-Leu-Val-Lys

b

Figure 7.11 Amino acid sequences of the N-terminus of the FDO161G glycopeptide (a) and HSD (b). The only difference between these two sequences is in position [5] and is underlined.

data from the FDO161G experiments indicated (i.e. lack of immunoscreening). However, it has been shown that the FDO161G glycopeptide is expressed on the surface of human granulosa cells (Muller unpublished work but reported to the WHO Task Force).

In order to find out whether B3 can be expressed on the surface of the COS cells, the full length clone (B3) was subcloned into pCDNA1, (Invitrogen), a plasmid vector derived from CDM8. Recombinants were amplified in *E. coli* MC1061/p3 cells (Chapter 6) and purified plasmids were transfected into COS cells by DEAE-dextran transfection method (Chapter 1 and 6). The transfected cells were cytospun and stained with FDO161G McAb. The cells were counterstained with propidium iodide. W6/32 staining was used as a positive control and CD2 staining was used to show the frequency of transfection (see also Chapter 6).

The FITC staining showed that cells transfected with B3 DNA stained with the FDO161G McAb (Fig. 7.12). The frequency of transfection was similar to CD2 transfection (Fig. 7.13). These staining results were, however, obtained from acetone fixed cells. It was therefore decided to stain viable COS cells which had been transfected with the B3 DNA. For this purpose the DEAE-dextran transfected COS cells were treated as described in Chapter 6 (i.e. transfection, harvesting, ect.) short of cytospinning. The cells were then spun down and resuspended in PBS A. McAbs (FDO161G, CD2 or W6/32) were added to the cell suspension and then incubated at room temperature. The cells were then spun down and resuspended as above, then washed two times in PBS A. FITC conjugated anti-mouse second Ab was added to the cell suspension and after incubation they were washed as above. The resuspended cells were examined under microscope and the result indicated that the staining had not produced any clear result. Thisindicates that the FDO161G McAb binds to a polypeptide which is not on the surface of the COS cells. This FITC staining of the transfected COS cells

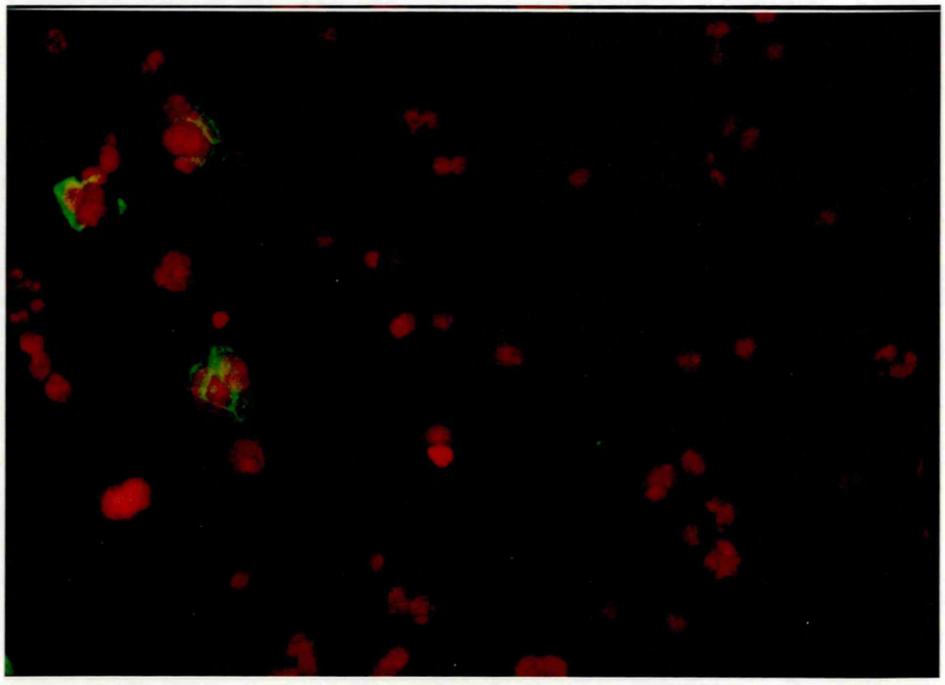


Figure 7.12 The COS cells transfected with the B3 DNA (cloned into pCDNA1) and stained with FDO161G McAb. The cells were also counterstained using propidium iodide (red).

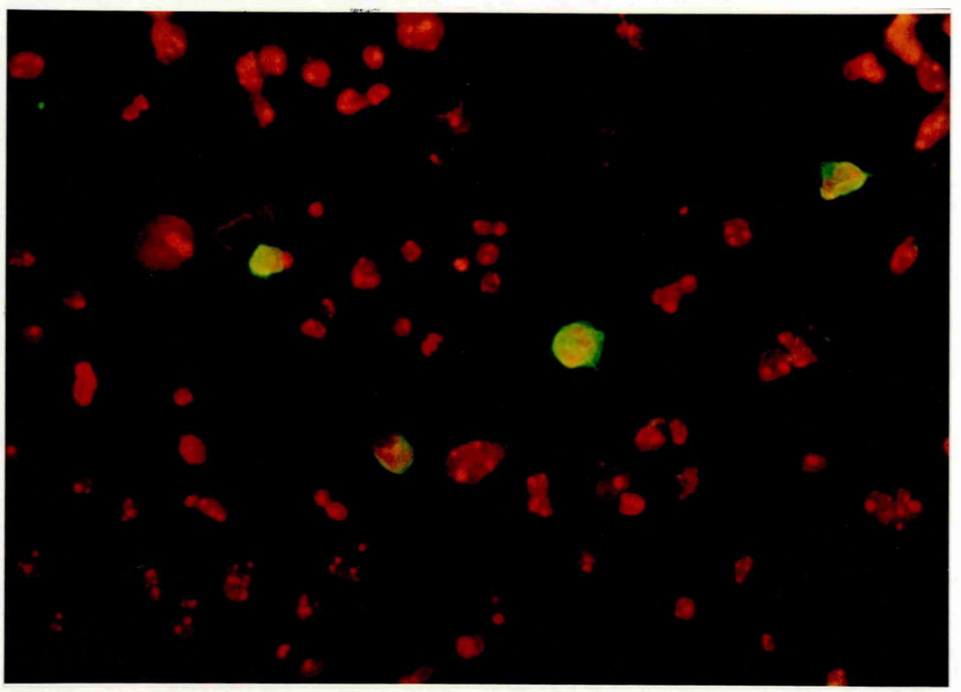


Figure 7.13 **The COS cells transfected with the CD2 DNA and stained with the anti-CD2 Ab.** The staining was done to access the eficiency of transfection. The cells were also counterstained using propidium iodide.

in solution and its subsequent findings were suported by the panning experiments, with immobilised FDO161G Ab, which did not show binding beyond the background level seen with mock transfected COS cells (results not shown). These findings together strongly indicate that, in the transfected COS cells, the FDO161G epitope is not accessible to the FDO161G McAbs.

7.6 Discussion

The results which were given in the previous chapter (Chapter 6) and earlier in this chapter showed that the methods applied in order to clone the FDO161G cDNA were not able to isolate any positive clone. Immunoscreening using the COS expression system, despite the fact that FDO161G Ab gave the least nonspecific panning of untransfected COS cells, did not isolate the target clone (Chapter 6). If we accept the observation that cytospun COS cells, transfected with B3 DNA, expressed the polypeptide recognised by the FDO161G McAb on fixed cells but not on the cells in solution (based on the results of panning or FITC staining of cells in solution) then the failure of panning or FITC staining of the viable cells may have been caused, most probably, by lack of FDO161G expression on the surface of the transfected cells. The positive staining of the fixed cells may be explained in that acetone fixing may have an effect on the cells (such as delipidation discussed in Chapter 6) which may result in the FDO161G polypeptide becoming accessible to the FDO161G Ab. On the other hand the finding that FDO161G glycopeptide is expressed on the surface of granulosa cells (shown by FACS method, Muller unpublished) may either be due to cross reactivity of the FDO161G McAb with a similar but different Ag on the surface of the granulosa cells or caused by technical artifacts (for example some granulosa cells have become bursted prior to the FACS and the HSD proteins somehow have bound to the surface of

intact cells and bound to the McAbs giving positive signals).

Hybridisation probing (using DN1/6 clone amplified by PCR) showed that 3 beta-HSD is not probably low abundance in the placental cDNA library used in this work (1/3000 or $3x^{-3}$). Nevertheless the immunoscreening of the lambda gt11 indicated that the expression of the HSD clones were greatly reduced (frequency of $2.5x10^{-5}$). The reduction in the level of expression could have been caused by several factors such as the protein not being expressable (see below), not being in-frame, etc. Failure of the COS system to isolate the FDO161G clone may have been due to the panning procedure as panning may be less sensitive than Ab screening of lambda gt11 clones. The reason for panning being less sensitive is that, besides panning itself, there are several steps involved (in the COS system) which may limit the isolation of the target clone. Some of these limiting steps can be summarised as follows:

Inefficient cloning of cDNA into cDM8 (probably less efficient than cloning into lamba gt11).

Variable efficiencies of transfection as well as being less efficient than plaque forming in the lamda gt11 system (even though the efficiency of packaging only approaches 10%, phage, once packaged, form plaques on *E. coli* with the efficiency of almost 100%, Lech and Brent, 1987). With 10-20% DEAE-dextran transfection efficiency only $1x10^4$-$2x10^4$ transfectants per ug of CDM8 DNA is produced (out of $1x10^5$ cells only 10-20% receive plasmid). This may be less efficient than plaque forming because less than 1 in 10^7 plasmids became transfected into COS cells (this is assumed if only one copy of plasmid enters each cell).

Panning may be inefficient because several steps are involved which may reduce its efficiency (such as several washing steps, Ab binding steps, centrifugations and etc., Chapter 2).

The screening of colony lifts and also amplifying cDNA or genomic DNA with the oligonucleotide primers, (derived from the partial amino acid sequence of the FDO161G glycopeptide), also failed to identify any clone. The reason in these two cases may be more clear, as the amino acid sequence derived from the DNA sequence of B3 (cloned from a lambda gt11 library as well as the amino acid sequence of the HSD) showed one very important variation from the amino acid sequence determined by the Muller group for the FDO161G epitope (Nickson et al., 1990 and Muller personal communication) . The amino acid comparisons showed that the sixth residue should have been cysteine rather than histidine, and that the codon sequence for His was one of the least degenerate. By using DNA sequences corresponding to histidine, instead of cysteine, to generate oligonucleotides (i.e. oligo 496) to run PCR, it is clear that such an oligonucleotide would not be able to anneal to the target site in the HSD gene or cDNA (codons for His are CAC or CAU but codons for Cys are UGU and UGC). Lack of having perfectly matched oligonucleotide among the 496 primers, to the 5' end of the gene, could have been the main reason for not being able to amplify the HSD sequence from the genomic DNA by PCR.

The above suggestions may be true for the cDNA, as well as the fact that not all cDNA clones would represent the full length message, and in most of times the 5' end of the sequence, in most of the times, would not be present among the target clones (Greene and Struhl, 1987). If this is the case, then using oligonucleotides complementary to the 5' end of the message would not identify those clones missing some sequences from their 5' end. In fact, the largest clone which was isolated from lambda gt11 by Ab screening was about 400 bp shorter than the full length cDNA and most if not all of the missing sequence was from the 5' end of the clone which supports the above prediction.

The 83 fold difference between the frequencies of clones being identified by Ab screening ($1/2.5x10^{-5}$) and the DNA hybridisation method of screening ($1/3x10^{-3}$) could have been caused by some of the following factors. Any cDNA to be expressed as an identifiable protein has to be in frame (3 possibilities) and in the right transcriptional orientation (two possibilities). The cDNA should contain the sequences coding for the epitope recognised by a given Ab (this does not seem to be the major problem for the FDO161G polyclonal Ab, because recognition of a clone lacking about 400 bp from the 5' end of the message showed that the epitopes recognised by the polyclonal Ab are not just coded for by the 5' end of the sequence). For the expression of the full length cDNA as a fusion protein (i.e. in lambda gt11) there should be no stop codon between the end of the beta-galactosidase transcription unit (lac Z gene) and the initiation codon in the cDNA. The cDNA sequence of B3 showed that it contained a stop signal at the 5' untranslated region, therefore such a signal would not allow expression of full length cDNA (containing the 5' UT region) as a fusion protein. The DNA hybridisation method of screening is not limited by any of the above factors and will therefore be more sensitive.

A question then arises in that would a protein derived from such a DNA sequence (i.e. HSD) be able to be expressed on the surface (as the results found by doing FACS analysis on the granulosa cells, performed by Muller, suggests)? The reason for proposing such a question is that it is now known that for a protein to be expressed on the surface of a eukaryotic cell, there should exist a stretch of usually more than 9 amino acids, having an uninterupted, uncharged, mainly hydrophobic property, to act as signal sequence (Heijne 1981; Pugsley 1989 and Chapter 1). Looking at the amino acid sequence of the HSD (Appendix 3) it shows little sign of containing a typical hydrophobic transmembrane sequence. Two hydrophobic regions between residues 76-92 and 284-305 have mean hydrophobicities (mean Gly residue; von Heijne 1981, 1985) of -3.0 and -4.9,

respectively. Whilst the latter one may be sufficiently long and hydrophobic enough to act as a transmembrane region, there is no hydrophobic leader-like sequence close to the N-terminus end.

It is known that the HSD is a microsomal enzyme (Koide and Torres 1965; Ferre et al., 1975) and it is commonly purified from microsomal extracts of cells (Ishi-Ohba et al., 1986a, b; Ishimura et al., 1988; Thomas et al., 1989). It is not known whether the placental HSD is in the outer side of the microsome, facing the lumen (which is topographically the outside of the cell), or whether it is in the inner side of the microsome, facing the cytoplasm (which is topographically inside the cell). More likely, is the possibility that the HSD is hydrophobic enough to be adsorbed to the cytoplasmic face of the endoplasmic reticulum, or that some form of glycolipid modification anchors it there.

HSD is also a dehydrogenase (Thomas et al., 1989) which uses NAD as cofactor and the sequence most similar to an NAD-binding beta-alpha-beta motif (Wierenga et al., 1986) lies at the amino-terminus, from Ser^{5} to Asp^{36}.. The question is where does the NAD-HSD interactions occur ; is it in the lumen of the microsomes or it is in the cytosol? In fact, most of the NAD-HSD interactions may occur in the cytosol around the mitochondria, (Alberts et al., 1987) and this may be why HSD can be co-purified with mitochondria (Ishimura et al., 1988; Thomas et al., 1989). If this is the case, then at least the epitope responsible for the dehydrogenase activity of the HSD would not be facing the lumen of the microsomes.

One may then wonder, how could FDO161G Ab identify this protein or at least part of it on the surface of the granulosa cells. Several possibilities may be relevant in dealing with this question. One possibility is that a small part or tail of this protein is exposed to the lumen of the microsome and is therefore

recognised by the FDO161G Ab. The second possibility is that some of the protein is carried out to the lumen of the endoplasmic reticulum (ER) during lipid transport across the ER membrane as in 5' nucleotidase. A third possibility is that FDO161G recognises two isoforms of the same protein which have arisen by differential splicing. The splice sequences would code for the leader sequence. The unspliced sequences would then have to code for the longer protein containing the signal peptide. Such a protein would be expressed. If this was the case then the spliced isoform has to code for the smaller protein (HSD). This possibility may be acceptable as the protein on the granulosa which was recognised by the FDO161G Ab has different size and protease V8 digest characteristics than the one recognised from human placenta (Muller 1989). Nonetheless it has been said that these findings are not strong enough to support the splicing hypothesis. If the mRNA population contains different isoforms, then the amount and size of each mRNA can be determined, for example, by primer extension technique (Ausubel et al. 1987).

Experiments are under way in our lab to use electron microscopy to find out whether the HSD is a membrane protein or not, because immuno-electron microscopy has failed to detect HSD in bovine adrenal mitochondria (Ishimura et al., 1988), and also to find out how much of this protein may be exposed to the outside of the cell. Another approach, in order to show whether viable transfected cells can express the HSD (coded by B3) protein on their surface, could be to analyse the B3 transfected COS cells with a fluorescence activated cell sorter (FACS) in a similar way as that done by Muller (personall communication and the project progress report to the WHO in 1989).

CHAPTER 8

GENERAL DISCUSSION

8.1 Placental Fibronectin

Fibronectin (FN) protein isoforms have several functions in the body and they play vital role for the integrity of human placenta. The multifunctional properties of FN may have been mediated by the ability of FN isoforms to bind to cell and other extracellular matrix components such as collagens and fibrin (Chapter 1). These and other functional properties which have been assigned to FN (Chapter 1) may have been made possible by the existence of multiple FN isoforms. For example the presence of CS1 cell binding site is within one of the alternative splice region (IIICS) (Humphries et al., 1987).

In order to find out whether more than one type of FN isoforms exist in human placenta and if so what are their tissue specificity, human placental FN was investigated at molecular and protein level. At the molecular level a placental cDNA library was screened for FN isoforms (Chapter 3). At the protein level three monoclonal antibodies (McAbs) were used in immunohistological studies (Chapter 4).

Screening a cDNA library from chorionic plate of human placenta resulted in the isolation of several clones which two were analysed further. Both clones extended toward the regions known to be alternatively spliced, ED-I and IIICS (Kornblihtt et al., 1984a,b). In one of these clones (HCF24) both regions were present and in another one both were absent (HCF17, Chapter 3).

The difference between the two clones isolated regarding the presence or absence of ED-I, may be functionally significant, because the presence of ED-I increases the distance between the cell-binding and heparin-binding sites, resulting in an enhanced binding activity of the cellular fibronectin molecule.

It is also known that the IIICS region can be alternatively spliced in 5 different ways (Kornblihtt et al., 1984a,b; Gutman and Kornblihtt 1987; Schwarzbauer et al., 1987; Dufour et al., 1988), two of which were observed in this work (Chapters 1 and 3).

The presence or absence of the entire IIICS sequences among the cDNA clones from the chorionic plate may be significant (if we asume that the HCF17 was from placental and not plasma) because it has been shown that some of the IIICS sequences code for a cell binding domain called connecting segment 1, CS1 (Humphries et al., 1986, 1987). The CS1 has been shown to promotes cell adhesion of melanoma or neural crest cells (McCarthy et al., 1986; Dufour et al., 1988). FN isoformed (for example coded by HCF24 clone) may have similar function in the placenta. Presence of both types may be significant in that one type ($IIICS^+$) specifically promote the adhesion or migration of some cells and other type does not. This suggestion may be supported by the finding that some areas of the embryo are never occupied by migrating neural crest cells, even though they have been shown to contain high amount of FN (Dufour et al., 1988).

The presence of the third alternative splicing region known as ED-II was not possible at the molecular level because none of the isolated clones were long enough to cover that area. However, some placental tissues stained positively with anti-ED-II Ab suggesting the presence of ED-II isoform in some but not all tissues from some but not all placentae. The pattern of regulation for such differences between different placentae is not known.

Many questions remain to be answered. In particular the functional significance of 20 possible FN subunits is not clearly understood. The alternative mRNA splicing which generates the FN variants is cell type specific

indicating that it is a regulated process. Little is known about the nature of this control mechanism. However, the availability of complete cDNA and genomic clones for FN offers an opportunity to investigate these problems experimentally. Thus continued progress may be anticipated in the study of this highly complex and multifunctional molecule. Any such findings may also help us to understand the precise role of each FN isoforms in placenta.

It has been reported that the pattern of glycosylation in placenta changes during placental development (Zhu and Laine, 1987). Questions which remain to be answered in this regard are: (i) How and why are the glycosylation patterns for these FNs change during development of the placenta? Is genetic programming of glycosyl transferase expression involved, or are different mRNA-splicing forms of FN being synthesized during stages of gestation which are glycosylated differently and take a different route through the Golgi? (ii) What effect, if any, does this change in carbohydrate have on the function of FN in the placental tissues? Some of these could have been addressed in this work but for the reasons discussed in Chapters 3 and 4, such as lack of anti-IIICS Ab, lack of good quality mRNA for S1 mapping and most importantly joining the WHO sponsored project limited the progress toward resolving some of these questions.

8.2 Placental Cell Surface Determinant

As a part of a team organized by the WHO Task force on Vaccines for Fertility Regulation our group was asked to join the investigation for determining the trophoblast surface markers. Our aim was to identify placental specific cDNA clone(s) corresponding to placental-specific polypeptide defined by McAbs (Chapter 6).

In order to clone cell surface determinants few criteria should be met. These include availability of an expression vector, of a mammalian or other type expression system, of good quality antibodies and a workable strategy. There are also other less frequently used methods such as oligonucleotide probing, PCR, etc. Nevertheless oligonucleotide probing and PCR are, in most of the times, dependent on the availability of partial amino acid sequences from the target determinant for generating degenerate oligonucleotides. The methods of approach which were employed during the course of work included oligonucleotide probing, PCR and immunoscreening.

Mixed-sequence oligonucleotide probes had been first used successfully for the isolation of a cloned cDNA encoding human beta 2-microglobulin (Suggs et al., 1981). For the fact that various problems are usually associated with use of degenerate oligonucleotides, various methods for oligonucleotide synthesis and hybridisation conditions have been suggested (Duby, 1987). Even computer programmes are available to determine the optimal structure of oligonucleotide probes from amino acid sequence (Yang et al., 1984). Formulas have also been devised to take into account the choice of codon for each amino acid; these are based on codon utilisation data, intercodon dinucleotide frequencies and other rules (Lathe , 1985). The more degenerate the oligonucleotides are the more probable that they contain the perfect match sequence, at the same time highly degenerate ones are usually associated with higher background.

The other approach for isolating cDNA sequences encoding a surface protein involves the use of PCR technique. This approach becomes possible either using known DNA sequences for generating primers or whenever

partial amino acids sequences are available to generate oligonucleotides. If DNA sequences are not available then degenerates oligonucleotides can be designed based on the information present in the amino acids sequences. Degenerate oligonucleotides have already been used for cloning cDNA clones (Lee et al., 1988; Girgis et al., 1988; Griffin et al., 1988). The problem with the PCR is that it is very sensitive and using highly degenerate oligonucleotides often leads to the amplification of un-specific sequences (Lee and Casky 1990). Conditions should therefore be optimised in order to reduce amplification of non-target sequences.

The third approach may be the use of immunoscreening methods. This can involve fusion protein expressed, for example, in *E. coli* through the use of lambda gt11 expression system or as native protein expressed in a mammalian cell system. The detection of fusion polypeptide is more governed by the availability of good quality Abs (polyclonal or monoclonal Abs) that recognise the linear structure of the protein. That is to say if an Ab can identify the target polypeptide on a Western-blot it is then expected to identify the same polypeptide from a fusion product. For this purpose polyclonal Abs are more preferred than McAbs (St. Jhon, 1987) since they recognise several different epitopes of the same protein.

Proteins which are expressed on a mammalian cell system can be detected by either monoclonal or polyclonal Abs. In such a system Abs which recognise a linear structure of a protein are useful, as are those Abs which recognise the native structure of the same protein. Limitations of such system are that the Ags should be expressed on the surface of the cell and full length cDNA encoding for such a protein may be needed for protein to pass through the membrane, to be properly processed, etc.

In both systems (i.e. lambda or/and mammalian) polyclonals are much superior than McAbs when they are used for the initial screening. This is because polyclonal Abs have multi recognition domains but McAbs recognise only a single epitope. McAb which can not recognise the target polypeptide in a Western blot may even be less useful , since very few of the expressed polypeptides would be in their native form.

As it was shown in the previous chapters (Chapters 5-7) that we started the work which was aimed at isolating trophoblast cell surface determinants. At that time only few poorly defined McAbs were available. None of these Abs were shown to recognise the target polypeptides on Western blots. In fact only for one of these Abs (i.e. FDO161) enough Ag could be purified to do Western-blot analysis or to raise polyclonal Abs.

Lack of McAb (or even polyclonal Abs) in one hand and the desire to isolate cell surface determinants limited our choice of screening systems to a mammalian expression system (Chapter 6). For reasons given in Chapter 6 (and above), none of the McAbs identified any target polypeptides. Polyclonal Abs against FDO161G Ag only became available toward the end of this work and were used for screening either COS or lambda expression systems. No clone was recovered from the COS system but the lambda library produced a clone (DN 1/6) which its DNA sequences suggested that it might be the HSD. Identification of this clone as HDS may explain why FDO161G polyclonal Ab did not produce positive clones from the COS system since HSD, most probably, is not a surface membrane protein.

In summary, due to several limitations and difficulties, such as lack of polyclonal Abs, lack of amino acid sequence from the target Ags, poor quality and poorly defined McAbs, inaccurate amino acids

sequences for generating oligonucleotides for oligonucleotide probing (colony hybridisation) or amplifying the target sequences by PCR and other reasons given in Chapters 5-7 no surface determinant was identified.

In any future investigation the above problems showd be avoided and a larger pool of Abs, possibly polyclonal Abs, be used. If polyclonal Abs are available it is best to used them against COS system since it is less likely that the identified polypeptide would be a non-surface protein. This will reduce the amount of work which is put into screening a lambda library and the disappointment in finding that the identified protein is not a cell surface determinant. If and when a cDNA clone is identified as encoding a cell surface protein it can then be expressed, on a mammalian system, for various reasons such as raising more Ab against the polypeptide for cross reactivity tests, doing Western-blot analysis and identification of domain specific McAbs which only recognises non-shared epitopes (placental specific epitope). For example the immunological contraception against hCG is directed only to the C-terminal amino acids 109-145 sequences of the beta-subunit, this is because other domains of hCG polypeptides are shared by other proteins in the body (Diczfalusy, 1986).

Whenever a trophoblast specific domain of a cell surface protein is identified, synthetic or bioengineered polypeptides can be used for production of vaccine and the ultimate tests on human-like primates. If successful you know what is next provided
cetain criteria are met:

1. The Ags used for immunization must be specific to the placenta and not cross-react with proteins of other tissues or body fluids.

2. The Ags must be available in pure form in sufficient large amounts.

3. Immunization against such Ags must prevent or disrupt implantation at a very early stage of pregnancy and without undesirable side effects.

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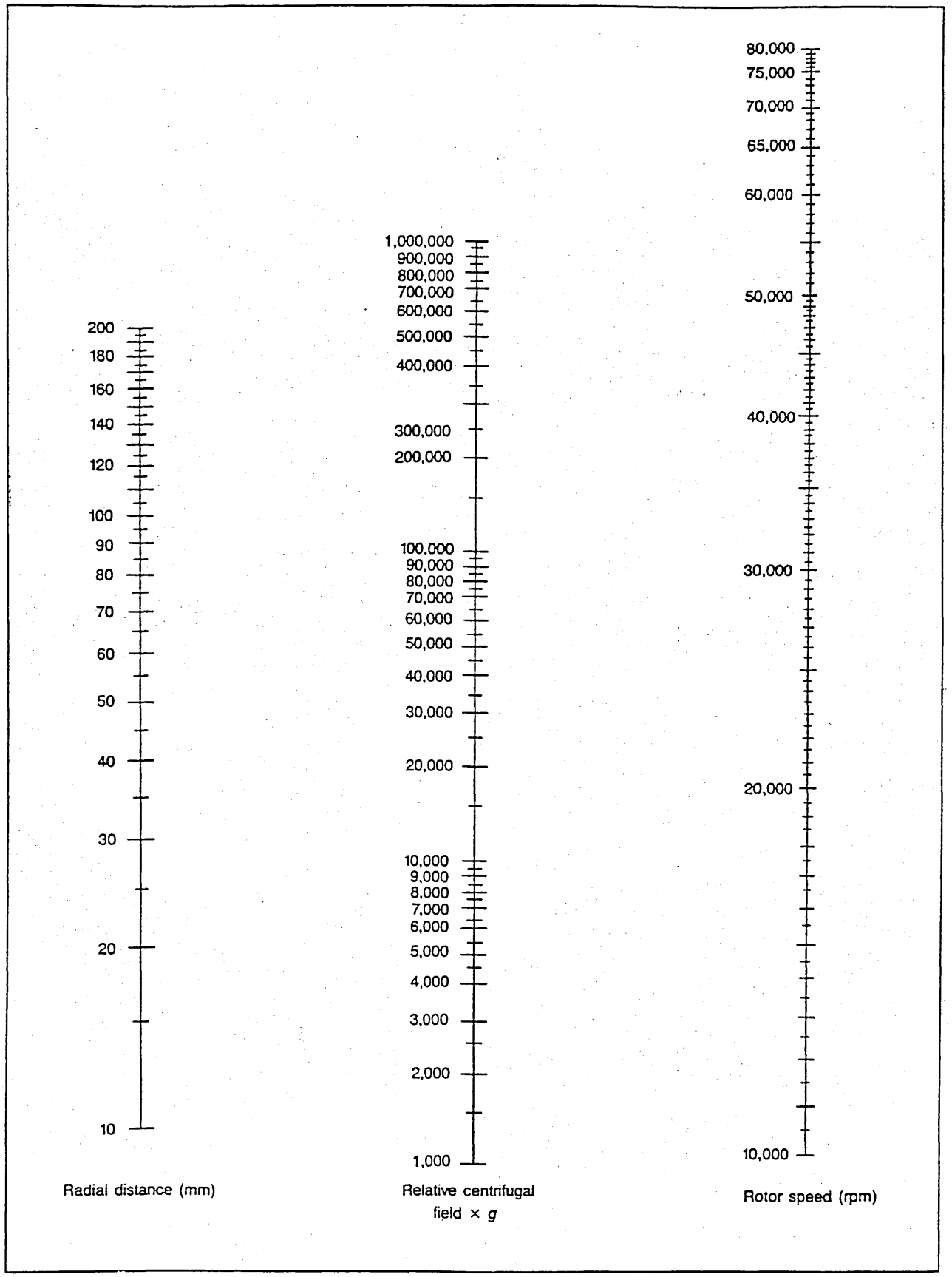
APPENDICES

Appendix 1

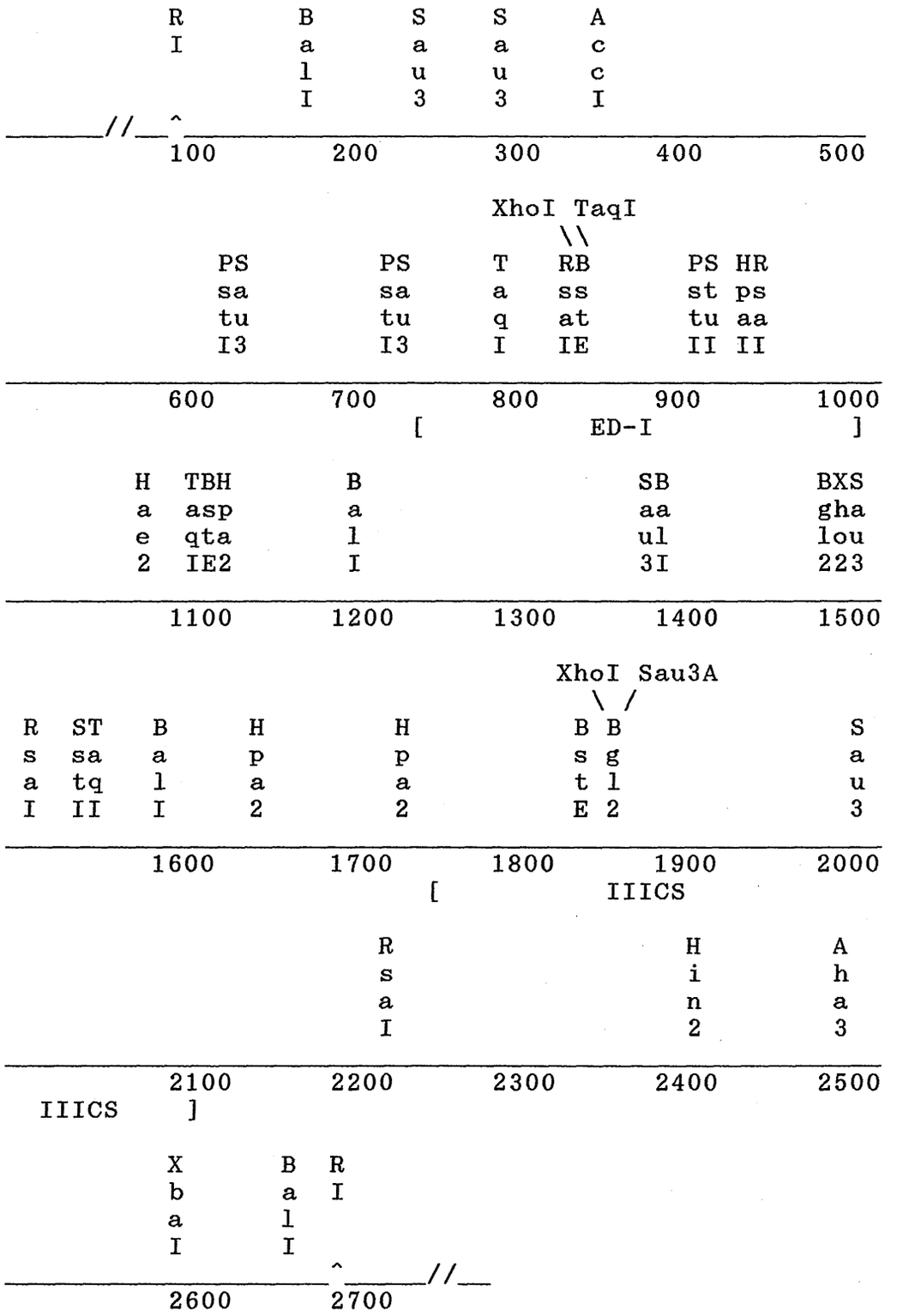
Relative Centrifugal Field Nanogram



Relative centrifugal field (RCF) nomogram. This nomogram is designed for Beckman centrifuges since most of the major spinning were done on the Beckman centrifuges (Chapter2). To determine an unknown value in a column, align ruler through known values in the other two columns. Desired value is found at the intersection of the ruler with the column of interest. The RCF can be determined for any rotor provided the radius (r) of such rotor is known by this formula $RCF=1.12r(rpm/1000)^2$.

Appendix 2

Restriction Map of Fibronectin cDNA



Restriction map of fibronectin between the two internal EcoRI (RI) sites. The position of the two alternative splicing regions are shown. Only those restriction sites are shown which were used in this work.

APPENDIX 3

Nucleotide and amino acid sequences of FDO161G (HSD) cDNA clone

```
  cctggtgagtgattcctgctactttggatggccATGACGGGCTGGAGCTGCCTTGTGACA
1 ---------+---------+---------+---------+---------+---------+ 60
  ggaccactcactaaggacgatgaaacctaccggTACTGCCCGACCTCGACGGAACACTGT

                                    MetThrGlyTrpSerCysLeuValThr
                                    1

  GGAGCAGGAGGGTTTCTGGGACAGAGGATCATCCGCCTCTTGGTGAAGGAGAAGGAGCTG
 61 ---------+---------+---------+---------+---------+---------+ 120
  CCTCGTCCTCCCAAAGACCCTGTCTCCTAGTAGGCGGAGAACCACTTCCTCTTCCTCGAC

  GlyAlaGlyGlyPheLeuGlyGlnArgIleIleArgLeuLeuValLysGluLysGluLeu
  10                                20

  AAGGAGATCAGGGTCTTGGACAAGGCCTTCGGACCAGAATTGAGAGAGGAATTTTCTAAA
121 ---------+---------+---------+---------+---------+---------+ 180
  TTCCTCTAGTCCCAGAACCTGTTCCGGAAGCCTGGTCTTAACTCTCTCCTTAAAAGATTT

  LysGluIleArgValLeuAspLysAlaPheGlyProGluLeuArgGluGluPheSerLys
  30                                40

  CTCCAGAACAAGACCAAGCTGACAGTGCTGGAAGGAGACATTCTGGATGAGCCATTCCTG
181 ---------+---------+---------+---------+---------+---------+ 240
  GAGGTCTTGTTCTGGTTCGACTGTCACGACCTTCCTCTGTAAGACCTACTCGGTAAGGAC

  LeuGlnAsnLysThrLysLeuThrValLeuGluGlyAspIleLeuAspGluProPheLeu
  50                                60

  AAGAGAGCCTGCCAGGACGTCTCGGTCATCATCCACACCGcctGTATCATTGATGTCTTC
241 ---------+---------+---------+---------+---------+---------+ 300
  TTCTCTCGGACGGTCCTGCAGAGCCAGTAGTAGGTGTGGCggaCATAGTAACTACAGAAG

  LysArgAlaCysGlnAspValSerValIleIleHisThrAlaCysIleIleAspValPhe
  70                                80
```

```
    GGTGTCACTCAcagAGAGTCTATCATGAATGTCAATGTGAAAGGTACCCAGCTCCTGTTA
301 ---------+---------+---------+---------+---------+---------+ 360
    CCACAGTGAGTgtcTCTCAGATAGTACTTACAGTTACACTTTCCATGGGTCGAGGACAAT

    GlyValThrHisArgGluSerIleMetAsnValAsnValLysGlyThrGlnLeuLeuLeu
    90                              100

    GAGGCCTGTGTCCAAGCTAGTGTGCCAGTCTTCATCTACACCAGTAGCATAGAGGTAGCC
361 ---------+---------+---------+---------+---------+---------+ 420
    CTCCGGACACAGGTTCGATCACACGGTCAGAAGTAGATGTGGTCATCGTATCTCCATCGG

    GluAlaCysValGlnAlaSerValProValPheIleTyrThrSerSerIleGluValAla
    110                             120

    GGGCCCAACTCCTACAAGGAAATCATCCAGAATGGCCATGAAGAAGAGCCTCTGGAAAAC
421 ---------+---------+---------+---------+---------+---------+ 480
    CCCGGGTTGAGGATGTTCCTTTAGTAGGTCTTACCGGTACTTCTTCTCGGAGACCTTTTG

    GlyProAsnSerTyrLysGluIleIleGlnAsnGlyHisGluGluGluProLeuGluAsn
    130                             140

    ACATGGCCCGCTCCATACCCACACAGCAAAAAGCTTGCTGAGAAGGCTGTACTGGCGGCT
481 ---------+---------+---------+---------+---------+---------+ 540
    TGTACCGGGCGAGGTATGGGTGTGTCGTTTTTCGAACGACTCTTCCGACATGACCGCCGA

    ThrTrpProAlaProTyrProHisSerLysLysLeuAlaGluLysAlaValLeuAlaAla
    150                             160

    AACGGGTGGAATCTGAAAAACGGCGGCACCCTGTACACTTGTGCCTTACGACCCATGTAT
541 ---------+---------+---------+---------+---------+---------+ 600
    TTGCCCACCTTAGACTTTTTGCCGCCGTGGGACATGTGAACACGGAATGCTGGGTACATA

    AsnGlyTrpAsnLeuLysAsnGlyGlyThrLeuTyrThrCysAlaLeuArgProMetTyr
    170                             180

    ATCTATGGGGAAGGAAGCCGATTCCTTTCTGCTAGTATAAACGAGGCCCTGAACAACAAT
601 ---------+---------+---------+---------+---------+---------+ 660
    TAGATACCCCTTCCTTCGGCTAAGGAAAGACGATCATATTTGCTCCGGGACTTGTTGTTA

    IleTyrGlyGluGlySerArgPheLeuSerAlaSerIleAsnGluAlaLeuAsnAsnAsn
    190                             200

    GGGATCCTGTCAAGTGTTGGAAAGTTCTCCACTGTTAACCCAGTCTATGTTGGCAATGTG
661 ---------+---------+---------+---------+---------+---------+ 720
    CCCTAGGACAGTTCACAACCTTTCAAGAGGTGACAATTGGGTCAGATACAACCGTTACAC

    GlyIleLeuSerSerValGlyLysPheSerThrValAsnProValTyrValGlyAsnVal
    210                             220
```

```
    GCCTGGGCCCACATTCTGGCCTTGAGGGCCCTGCAGGACCCCAAGAAGGCCCCAAGCATC
721 ---------+---------+---------+---------+---------+---------+ 780
    CGGACCCGGGTGTAAGACCGGAACTCCCGGGACGTCCTGGGGTTCTTCCGGGGTTCGTAG

    AlaTrpAlaHisIleLeuAlaLeuArgAlaLeuGlnAspProLysLysAlaProSerIle
    230                                 240

    CGAGGACAGTTCTACTATATCTCAGATGACACGCCTCACCAAAGCTATGATAACCTTAAT
781 ---------+---------+---------+---------+---------+---------+ 840
    GCTCCTGTCAAGATGATATAGAGTCTACTGTGCGGAGTGGTTTCGATACTATTGGAATTA

    ArgGlyGlnPheTyrTyrIleSerAspAspThrProHisGlnSerTyrAspAsnLeuAsn
    250                                 260

    TACACCCTGAGCAAAGAGTTCGGCCTCCGCCTTGATTCCAGATGGAGCTTTCCTTTATCC
841 ---------+---------+---------+---------+---------+---------+ 900
    ATGTGGGACTCGTTTCTCAAGCCGGAGGCGGAACTAAGGTCTACCTCGAAAGGAAATAGG

    TyrThrLeuSerLysGluPheGlyLeuArgLeuAspSerArgTrpSerPheProLeuSer
    270                                 280

    CTGATGTATTGGATTGGCTTCCTGCTGGAAATAGTGAGCTTCCTACTCAGGCCAATTTAC
901 ---------+---------+---------+---------+---------+---------+ 960
    GACTACATAACCTAACCGAAGGACGACCTTTATCACTCGAAGGATGAGTCCGGTTAAATG

    LeuMetTyrTrpIleGlyPheLeuLeuGluIleValSerPheLeuLeuArgProIleTyr
    290                                 300

    ACCTATCGACCGCCCTTCAACCGCCACATAGTCACATTGTCAAATAGCGTATTCACCTTC
961 ---------+---------+---------+---------+---------+---------+ 1020
    TGGATAGCTGGCGGGAAGTTGGCGGTGTATCAGTGTAACAGTTTATCGCATAAGTGGAAG

    ThrTyrArgProProPheAsnArgHisIleValThrLeuSerAsnSerValPheThrPhe
    310                                 320

     TCTTATAAGAAGGCTCAGCGAGATtTGGCGTATAAGCCACTCTACAGCTGGGAGGAAGCC
1021 ---------+---------+---------+---------+---------+---------+ 1080
     AGAATATTCTTCCGAGTCGCTCTAaACCGCATATTCGGTGAGATGTCGACCCTCCTTCGG

     SerTyrLysLysAlaGlnArgAspLeuAlaTyrLysProLeuTyrSerTrpGluGluAla
     330                                 340

     AAGCAGAAAACGGTGGAGTGGGTTGGTTCCCTTGTGGACCGGCACAAGGAGACCCTGAAG
1081 ---------+---------+---------+---------+---------+---------+ 1140
     TTCGTCTTTTGCCACCTCACCCAACCAAGGGAACACCTGGCCGTGTTCCTCTGGGACTTC

     LysGlnLysThrValGluTrpValGlySerLeuValAspArgHisLysGluThrLeuLys
     350                                 360
```

```
     TCCAAGACTCAGTGATTTAAGGATGACAGAGATGTGCATGTGGGTATTGTTAGGAGATGT
1141 ---------+---------+---------+---------+---------+---------+ 1200
     AGGTTCTGAGTCACTAAATTCCTACTGTCTCTACACGTACACCCATAACAATCCTCTACA

     SerLysThrGlnEnd
     370

     CATCAAGCTCCACCCTCCTGGCCTCATACAGAAAGTGACAAGGGCACAAGCTCAGGTCCT
1201 ---------+---------+---------+---------+---------+---------+ 1260
     GTAGTTCGAGGTGGGAGGACCGGAGTATGTCTTTCACTGTTCCCGTGTTCGAGTCCAGGA

     GCTGCCTCCCTTTCATACAATGGCCAACTTATTGTATTCCTCATGTCATCAAAACCTGCG
1261 ---------+---------+---------+---------+---------+---------+ 1320
     CGACGGAGGGAAAGTATGTTACCGGTTGAATAACATAAGGAGTACAGTAGTTTTGGACGC

     CAGTCATTGGCCCAACAAGAAGGTTTCTGTCCTAATCATATaCCAGAGGAAAGACCATGT
1321 ---------+---------+---------+---------+---------+---------+ 1380
     GTCAGTAACCGGGTTGTTCTTCCAAAGACAGGATTAGTATAtGGTCTCCTTTCTGGTACA

     GGTTTGCTGTTACCAAATCTCAGTAGCTGATTCTGAACAATTTAGGGACTCTTTTAACTT
1381 ---------+---------+---------+---------+---------+---------+ 1440
     CCAAACGACAATGGTTTAGAGTCATCGACTAAGACTTGTTAAATCCCTGAGAAAATTGAA

     GAGGGTCGTTTTGACTACTAGAGCTCCATTTCTACTCTTAAATGAGAAAGGATTTCCTTT
1441 ---------+---------+---------+---------+---------+---------+ 1500
     CTCCCAGCAAAACTGATGATCTCGAGGTAAAGATGAGAATTTACTCTTTCCTAAAGGAAA

     CTTTTTAATCTTCCATTCCTTCACATAGTTTGATAAAAAGATCAATAAATGTTTGAATGT
1501 ---------+---------+---------+---------+---------+---------+ 1560
     GAAAAATTAGAAGGTAAGGAAGTGTATCAAACTATTTTTCTAGTTATTTACAAACTTACA

     Ttaaa
1561 ----- 1565
     Aattt
```

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