IMMUNOLOGICAL AND MOLECULAR GENETIC STUDIES ON TROPHOBLAST MEMBRANE PROTEINS

A thesis submitted for the Degree of

Doctor of Philosophy at the

University of Glasgow

by

Debra Ann Nickson

Institute of Genetics

Church Street

Glasgow

October 1985

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

This thesis is dedicated to my mother and father,

for all their love and help.

ACKNOWLEDGEMENTS

Firstly it is with much gratitude that I thank my supervisor, Dr. Roger Sutcliffe, for his help, advice and especially his enthusiastic encouragement throughout the course of this work.

I wish to thank Dr. P.M. Johnson for gifts of antisera and Dr. J. Scaife for his advice and gifts of E.coli host strains. For their time, assistance and gifts of material I am indebted to, the staff of the labour suite at the Queen Mother's Hospital, Glasgow, and the staff of the Royal Samaritans Hospital for Women, Glasgow.

I am grateful to all members of the department who have given assistance and advice. For their help, freindship and tolerance my special thanks go to the "placental group" Nael Abu-Hasan, Salwa Khalaf, Craig Livingstone and particularly Susan Henderson for her technical assistance. My studies were financed by a studentship from the Medical Research Council and I wish to thank Professor David Sherratt for allowing me to persue my studies in the Institute of Genetics.

Finally, my acknowledgement would not be complete without thanking Birkbeck for his skill at the drawing board.

CONTENTS

CHAPTER 4 : Preparation and screening of a syncytiotrophoblast cDNA library

SUMMARY

1. In recent years the plasma membrane components of the human trophoblast have attracted increasing interest. Many investigators have wondered about the extent to which trophoblast components may contribute to the outcome of pregnancy, in particular the extent to which genetic variation in trophoblast components contributed to diseases of pregnancy. The HLA transplantation antigens play a major role in the success of organ transplants, HLA determinants have been detected on the extravillous cytotrophoblast of the placenta. The role these determinants play in the outcome of pregnancy is unknown, as is the relationship of these placental HLA determinants to the classic HLA A and B allotypes. Classical HLA determinants are not detected on the placental syncytiotrophoblast microvilli. Placental alkaline phosphatase is the major external component of placental microvilli and is known to show genetic variation, having three common alleles and also 15 rare alleles which account for 2.5% of the PLAP phenotypes. The function of this genetic variation to date is unknown. Though trophoblast specific antigens have been identified xenogeneically no conclusive work has been done to detect antibodies in maternal sera to trophoblast surface components.

2. This thesis describes two lines of investigation. The first was a serological approach to find out whether placental surface determinants are recognised by the maternal immune system. For this, syncytiotrophoblast plasma membranes prepared from term placentae were tested in a passive haemagglutination

assay for their ability to function as antigenic determinants with term maternal sera. The second was a molecular genetic approach involving the creation of placental cDNA libraries. These libraries were screened for cDNA clones which would aid in the characterisation of (a) the histocompatibility antigens present in the cytotrophoblast cells of the term chorionic plate and, (b) the enzyme placental alkaline phosphatase (PLAP) which has a high level of genetic polymorphism and is quantitatively the major cell membrane protein of the syncytiotrophoblast.

3. Using the passive haemagglutinin assay, agglutinins against trophoblast components were detected in term maternal sera at titres above those of non-pregnant control sera. These agglutinin titres were independant of the family relationship between individual sera and trophoblast samples. They were uneffected by adsorption with excess packed fetal or paternal red blood cells or paternal lymphocytes. The agglutinins of both normal control and term maternal sera were found to elute in the macroglobulin fraction of serum having a mw of >350Kd and were shown to be a component of euglobulin precipitates. The agglutinin titres remained in sera and were unaffected by the adsorption of IgM and IgG from the sera. It was concluded that these agglutinins were not a maternal antibody response against trophoblast surface components.

4. mRNA prepared from syncytiotrophoblast cells, and from purified cytotrophoblast cells of the chorionic plate were analysed by <u>in vitro</u> translation and immunoprecipitation. The major polypeptide synthesised from syncytiotrophoblast mRNA was hPL, accounting for 15-20% of the translated polypeptides. In

cytotrophoblast mRNA preparations this polypeptide accounted for only 1-5% of the synthesised polypeptides. Immunoprecipitation of the *in vitro* translated polypeptides from syncytiotrophoblast mRNA using anti-PLAP specifically precipitated polypeptides of 56Kd and 58Kd. Both PLAP and hPL mRNA levels were shown to increase with gestational age.

5. cDNA was synthesised from syncytiotrophoblast mRNA, sized fractionated and the cDNA of 1Kb and greater (42% of the total cDNA synthesised) was cloned. The library constructed in the lambda vector \gt10 consisted of 7.8×10^4 recombinants. It was screened with PLAP oligonucleotides and 26 clones were isolated from the amplified library. These clones are in the process of being further characterised. The library was also screened with the HLA class I cDNA probe pDP001 and clones which hybridised to the probe were detected. This is of great interest since the avaliable serological evidence indicates that class I determinants are absent from the syncytiotrophoblast.

6. If the clones detected with the PLAP oligonucleotide probe are PLAP cDNA they will be used to obtain an independent DNA assessment of the number of PLAP alleles and characterisation at the molecular level of the three forms, A,B and M, of PLAP. Also the molecular relationship between placental alkaline phosphatase, intestinal alkaline phosphatase and tissue unspecific alkaline phosphatase can be assessed and compared with the PLAP-like molecules found in tumour cells. If the clones detected by the HLA probe pDP001 are homologous to known HLA sequences one can determine the relationship between these syncytiotrophoblast determinants, the cytotrophoblast HLA

determinants, once cloned, and those expressed on other tissues.

7. The usefulness of molecular genetics is the ability to study genes which cannot be studied easily by classical methods, but this requires the availability of probes to detect the genes. At present the syncytiotrophoblast cDNA library can only be screened with probes for genes which have already been discovered. The importance of independent serological methods is that they may provide evidence for new immunological systems. The ability to acquire human monoclonal antibodies to placental determinants by forming hybridomas from Epstein-Barr transformed maternal lymphocytes may enable one to screen for new placental systems and the use of an expression vector, such as \gt11 containing a placental cDNA library, might then provide a means of establishing the molecular genetics of such systems.

ABBREVIATIONS

a - alpha
B - beta
X - gamma
λ - lambda

Ac - acetate
AP - Alkaline phosphatase
ATP - adenosine triphosphate
BSA - Bovine serum albumin
dATP - deoxyadenosine triphosphate
dCTP - deoxycytidine triphosphate
dGTP - deoxyguanosine triphosphate
dTTP - deoxythymidine triphosphate
DNA - deoxyribonucleic acid
cDNA - Complementary DNA
ssDNA - single stranded DNA
dsDNA - double stranded DNA
DEPC - Diethylpyrocarbonate
DTT - Dithiotheitol
EDTA - ethylenediaminetetra-acetic acid (disodium salt)
EtBr - ethidium bromide
EtOH - ethanol
FCS - Fetal calf serum
FN - Fibronectin
FRBC - Formalized red blood cells
hCG - Human chorionic gonadotrophin
hPL - Human placental lactogen
Hu - Human
HLA - Human lymphocyte antigens
IAP - Intestinal alkaline phosphotase
IPTG - Isopropyl-B-D-thiogalactopyanoside
MHC - Major histocompatibility complex
NRS - Normal rabbit serum
PAGE - Polyacrylamide gel electrophoresis
PAPP - Pregnancy-associated plasma protein
PBS - Phosphate buffered saline
PDB - Phage dilution buffer
pfu - Plaque forming unit
PLAP - Placental alkaline phosphatase
PP - Placental protein
RBC - Red blood cells
RNA - ribonucleic acid
mRNA - Messenger RNA

RT - Room temperature
SDS - sodium dodecylsulphate
SSC - sodium cloride, sodium citrate
TA - Trophoblast antigen
TBE - Tris-borate, EDTA
TCA - Trichloracetic acid
T_D - Dissociation temperature
TE - Tris, EDTA
TLX - Trophoblast cross reactive antigen
Tris - tris (hydroxymethyl) amino ethane
VCS - Vector Cloning Systems
X-gal - 5-bromo-4-chloro-3-indolyl-B-galactoside

Measurements

mA - milliamps (10^{-3} A)
mV - millivolts (10^{-3} V)

Kd - kilo Dalton

bp - base pair
Kb - kilo base pair

^{o}C - degrees centigrade

g - centrifugal force equal to gravitational acceleration

g - gramme
mg - milligramme (10^{-3} g)
ug - microgramme (10^{-6} g)
ng - nanogramme (10^{-9} g)

l - litre
ml - millilitre (10^{-3} l)
ul - microlitre (10^{-6} l)

mw - Molecular weight
w/v - weight/volume

M - Molar (moles per litre)

pH - acidity [negative $\log_{10}$ (Molar concentration H^+ ions)]

log - logarithm

CHAPTER 1

I N T R O D U C T I O N

INTRODUCTION

1:1 The Placenta

The human placenta's role in its limited life span of forty weeks is to maintain the fetal environment, thus the structure and function of the placenta change during its period of growth as is required by the developing embryo and fetus. It acts as a physical barrier separating the maternal and fetal 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). One of the quantitatively major cell membrane proteins of these syncytiotrophoblast microvilli is placental alkaline phosphatase (PLAP). PLAP is a member of a well studied group of enzymes (McComb et al, 1979). Though having many common structural and functional features PLAP differs from the other alkaline phosphatases due to its polymorphism. Although not involved in maternal immunity PLAP is a highly heterogenous protein having three common alleles, which encode for 97.5% of PLAP phenotypes, and 15 rare alleles (Harris et al, 1974; Doellgest & Fishman, 1976). Abu-Hasan (1983) demonstrated the existance of a membrane bound form of PLAP (M-PLAP). The different properties expressed by M-PLAP when compared with the previously described A-PLAP and B-PLAP suggested that a membrane associated form of heterogeneity existed and raised the question as to whether this was due to

post-translational processing or differential splicing of primary RNA transcripts, as is the case for immunoglobulins.

It is not yet clear how the fetus is protected from the maternal immune system during pregnancy. Though the microvilli and all villus trophoblast seem to lack HLA class I determinants, HLA class I determinants of some sort are expressed on the extravillous trophoblast. The principal antigens recognised as foreign on an allograft are the major histocompatibility (HLA) antigens, and women have been shown to mount humoral immune responses against paternally-derived HLA antigens during pregnancy. Since the fetus expresses those histocompatibility antigens, its survival *in utero* suggests that either the fetal antigens are shielded from significant maternal attack by trophoblastic tissue, or that there is some local modification in the mother's immune response leading to a failure to recognise or adequately respond to fetal antigens. Such local modifications could involve the production of immunosuppressive molecules (Waltman *et al* 1971), or the elicitation of blocking factors (Hellstrom *et al* 1969). The success of the placenta as an allograft was at first thought to be a property of the uterus, but the successful development of fetuses in non-uterine ectopic sites, and the ability of the uterus to support the rejection of allografts (see Beer and Billingham, 1976) showed that the uterus *per se* is not an immunologically privileged site. Nevertheless, suppressor cell accumulation in the uterine decidua of murine pregnancies has been shown to be correlated with successful pregnancies (Slapsys & Clark, 1983 and Clark *et al* 1983). The supressor cells are thought to protect the fetal allograft from cell mediated immune rejection. In human pregnancies the lack of

blocking factor production is thought by some to be correlated with chronic aborters (Rocklin et al, 1979).

Trophoblastic tissue is a priori a good candidate for protecting the fetus. The trophoblast syncytium is known to be a highly specialised membrane which covers the chorionic villi of the placenta and its zone of contact between the maternal and fetal tissues. It is trophoblast cells which invade the uterus and, though in direct contact with the maternal immune system, survive without eliciting an immune response. For this reason alone the trophoblast tissue is worthy of study. Also, most placental proteins such as PLAP are localised in the syncytial trophoblast, thus for a greater understanding of this tissue it has become necessary to investigate the role these trophoblast components play.

1:1:1 Development of the placenta

The maternal uterus provides the implantation site and the blood supply necessary for placental development. Even before implantation the outer layer of the blastocyst proliferates to form the trophoblast cell mass. The trophoblastic cells which derive from the polar trophectoderm and give rise to the placenta are morphologically different from the inner cell mass of the blastocyst which forms the embryo proper (Edwards, 1977). Due to the trophoblast infiltration of the endometrial epithelium the blastocyst becomes completely embedded by the 11-12th day. After implantation the trophoblast cells begin to differentiate into the peripheral layer of primitive syncytiotrophoblast and an inner layer of cytotrophoblast which proliferates to form the precursors of the primary villi (Enders,

1965; 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, which contains fibroblasts, phagocytotic Hofbauer cells and collagen fibres, the villi are classed as secondary villi. The tertiary villi arise from the secondary villi with the development of fetal capillaries in the mesenchymal cores. 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 170um in the first trimester to 40um 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 syncitium join together to form the cytotrophoblastic shell are derived from the cytotrophoblast found in the Langhans layer of the placenta (Boyd and Hamilton 1970; Wynn, 1972).

1:1:2 **Syncytiotrophoblast and Cytotrophoblast**

There are considerable morphological differences between the syncytiotrophoblastic and cytotrophoblastic cells. The cytotrophoblast cells show considerable variation in shape, which is dependant on their positioning in the placental structure and

the gestation period. They have little endoplasmic reticulum and a scarce population of organelles (Boyd & Hamilton 1970). The uninucleated cytotrophoblast cells fuse to form the syncytiotrophoblast cells which have a high concentration of mitochondria, lysosomes, vesicles, vacuoles, microtubules, microfilaments and a high abundance of endoplasmic reticulum and ribonucleoprotein. Enders (1965) suggested that it was the syncytiotrophoblast which was the endocrinologically active tissue of the placenta (Wynn, 1975). 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 through gestation. The 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 (Truman & Ford, 1984 and Hamilton & Hamilton, 1977). In early pregnancy the microvilli are long and close together but as pregnancy progresses they become shorter, branched and more seperated. At term their length is only 0.8um compared to 1.5um at 10 weeks (Tighe et al 1967). The function of the microvilli is uncertain but as they efficently increase the surface area of the placenta in contact with the maternal blood and since it is known that if the maternal nutrient supply is restricted the microvilli proliferate, thus they may facilitate placental transfer and absorption (Jones and Fox, 1979).

1:1:3 **Placental proteins**

Studies of placental proteins were stimulated with the discovery by Smith , Brush and Lucket (1974) that microvillous preparations could be obtained by differential centrifugation of the saline extract from whole placental tissue. Besides their use as placental markers it was hoped that placental specific proteins would play a role in the monitoring of placental function and wellbeing during pregnancy. Using SDS gel electrophoresis, Carlson et al (1976) identified 16 major protein and 10 major glycoprotein subunits from trophoblast microvilli membrane preparations. By ^{32}P labelling experiments, a glycoprotein of 66 Kd was shown to be placental alkaline phosphatase. Smith et al (1977) observed trophoblast protein bands from 33-200 Kd and the prominant band of 40 Kd was thought to be actin. Kelley et al (1979) found 20 peptide bands. One of the principal bands seen was a glycoprotein of mw 69 Kd, placental alkaline phosphatase. Using 2-D SDS gel electrophoresis and isoelectric focusing Wada et al (1977) observed 17 major sialoglycoprotein subunits ranging in isoelectric points from pH 4.6- 7.2. Placental alkaline phosphatase was identified as a 66 Kd protein with an isoelectric point of 6.3, transferrin was identified as a 69 Kd protein. Many groups have analysed the constituents of placental membrane preparations and due to differing preparation protocols there are discrepancies in the actual number of protein components identified on SDS gel. electrophoresis. Though many of the proteins have not been positively identified, albumin, transferrin and placental alkaline phosphatase are found to be major components of all trophoblastic preparations (Ogbimi et al, 1979; Okamura et al,

1981; Kantor et al, 1981). PLAP is a structural component of the placenta whereas albumin and transferrin, though present in the placental membrane preparations, are not synthesised by the placenta. Albumin derives from the placenta's blood supply and transferrin is present due to its uptake by receptors on the surface of the trophoblast membranes (Faulk & Galbraith, 1979).

Hormonal function can be detected in the maternal sera during pregnancy. Human chorionic gonadotrophin (hCG) is a placental glycoprotein hormone found predominantly in the syncytiotrophoblast (Kurman et al, 1984). hCG reaches its peak of activity in maternal sera at 10-12 weeks of pregnancy. It is structurally and functionally related to luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) (Fiddes & Goodman, 1979). Other protein hormones secreted by the placenta which have biological properties similar to the FSH and TSH are human chorionic thyrotrophin (hCT) and human releasing hormone (hRH) (Pattillo et al, 1983). Human placental lactogen (hPL) is a placental hormone which cross reacts with antisera against human growth hormone (hGH). HPL's biosynthesis also occurs predominantly in the syncytiotrophoblast (Tabarelli et al 1983a&b) and its detectable level in the maternal serum during pregnancy parallels placental growth (Friesen, 1973; Handwerger et al, 1981). With the exception of hCG, the concentrations of all placental proteins in the maternal circulation substantially parallels the growth of the placenta (Hytten & Leitch, 1971).

The human placenta produces a wide range of proteins in addition to the trophoblast specific protein hormones and enzymes which have been shown to have analogs in non-pregnant adults. The

placenta synthesises a number of placental specific proteins that have no known function or analog. Using antisera directed against placental extracts in gel-diffusion experiments, Thornes (1958) demonstrated the presence of protein antigens in the sera of pregnant women which were absent from the sera of non-pregnant adults (Bohn et al 1983). From Thornes' findings of placental specific proteins in pregnancy sera many more placental specific proteins in placental extracts and pregnancy sera have been identified with antisera directed against different placental fractions. The placental proteins a_2-globulin and B_2-globulin were of the first to be detected in pregnancy sera by immunoelectrophoresis (for review see Bohn et al, 1983). Bohn (1979) described several different placental proteins, SP1 from pregnancy sera and PP_1-PP_5 from placental extracts, all but PP_5 being trophoblast specific. More recently Bohn and co-workers (1983) have identified specific soluble tissue proteins PP_{10}-PP_{12} as well as several other soluble antigens which though they were derived from placental membranes their tissue specificity has not been established. Using antisera to pregnancy plasma Lin et al (1974) detected four specific proteins PAPP-A,B,C and D. PAPP-C was found to be identical to SP1 and PAPP-D was later identified as the placental hormone hPL.

Not all the placental proteins detected by immunochemical methods were specific for trophoblast tissue or even pregnancy. The placental proteins having no identified comparable material in normal adults are characterized as being specific to placental trophoblast and classed as pregnancy proteins. The pregnancy proteins which are secreted into the maternal sera are SP1, PAPP-A and PAPP-B. SP1 and PAPP-A were

shown to be localised and most probably synthesised in the villous syncytiotrophoblast (Lin & Halbert, 1976; Bohn 1979). The pregnancy proteins concentrated in the placental tissue are PP_5, PP_{10}, PP_{11} and PP_{12}. The concentration of the placental proteins in the maternal circulation parallels the growth of the placenta for those proteins studied. Thus it would seem that the production of almost all placental proteins depends on the mass of the trophoblast. The concentration of placental proteins shows a slow rise in the first trimester, rapid in the second trimester and plateauing by 36 weeks (Hytten & Leitch, 1971). The placental proteins fall into three groups (1) proteins with hormonal functions, (2) proteins with enzymic 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, apparently normal pregnancies have come to term without the synthesis of one or more of the placental proteins (Chard,1982; Gordon & Chard, 1979). Though many of the proteins produced by the placenta have no proven function they are studied with the hope that the more that is known about the structure of the proteins the closer one will be in deducing their function.

1:1:4 **Monoclonal antibodies against placental determinants**

Since the advent of somatic cell hybridisation technology (Kohler & Milstein, 1975) a wide variety of monoclonal antibodies have been raised against human placental trophoblast preparations (Sunderland *et al*, 1981; Johnson *et al*,1981a&b & 1982; Johnson & Molloy, 1983; Brown *et al* 1983; Loke *et al*, 1984). Several of the trophoblast monoclonals are non-specific, being raised against surface membrane proteins common to both

trophoblast and other tissues. These non-specific monoclonals raise the possibility of shared membrane complexes between the placenta and other tissues. H310 and H316 are examples of non specific monoclonals showing shared membrane complexes. They both recognise an antigenic epitope specificity to trophoblast and lymphocytes (Brown et al, 1983). Among Johnson et al's(1981a&b & 1982) monoclonals which were found to be trophoblast specific are H315 which recognised PLAP and first trimester syncytiotrophoblast and H317 which is specific for PLAP. Sunderland et al (1981c) has also produced monoclonal antibodies which are specific for human syncytiotrophoblast, NDOG1 and NDOG2. Both NDOG1 and NDOG2 antigens were secreted placental proteins. NDOG2 was shown to be specific for PLAP. The NDOG1 antigen is present in pregnancy sera for only six hours after delivery thus it is neither SP1, PAPP-A or PLAP as these secreted proteins have longer half-lives.

Monoclonal antibodies have been used to show the distribution of different antigens on placental tissue. Loke & Day (1984) monoclonal 18B/A5 which is raised against first trimester trophoblast preparations reacts discretely with cytotrophoblast from the chorionic villi. H310 and H317 detect the cytotrophoblast layer on amniochorionic membranes and all the villous trophoblast in the placental bed tissue (Johnson & Molloy, 1983). Bulmer et al (1984) showed that H315 also reacts strongly with villous syncytiotrophoblast. H316 and NDOG1 can be used to stain for syncytial sprouts and cytotrophoblastic cell columns. As all the trophoblast monoclonals available react with villous trophoblast the monoclonals used as tissue markers for the non-villous trophoblast are TROMA1 and LE61. These are

specific for intermediate filaments. Using monoclonal antibodies raised against HLA determinants (W6/32, B_2M) Sunderland et al (1981b&c) showed that non-villous trophoblast expresses class I HLA antigens determinants but not class II HLA DR antigen determinants. The syncytial layer of trophoblast and the underlying zone of cytotrophoblast has not been shown to express any HLA antigens; for further discussion see pp 25-28.

1:1:5 **Trophoblast specific antigens**

The fetal/placental unit constitutes an allogeneic graft of which the trophoblast cells are a priori a good candidate for eliciting a maternal immune response. They are in direct physical contact with the cells of the maternal endometrium and are found in the maternal circulation (Goodfellow & Taylor, 1982). Whether mothers make antibodies to trophoblastic proteins is not known. Davies and Sutcliffe (1982) and Davies et al (1982a&b) have provided evidence for maternal recognition of trophoblast using isotope release in the presence of maternal lymphocytes by chromium-labelled syncytiotrophoblastic cells. Davies & Brown (1985) have detected anti-trophoblast antibodies in the sera of pregnant women though these results have yet to be confirmed by other groups. Faulk et al (1978) prepared rabbit anti-human sera from trophoblastic tissue. The antisera was made specific for the trophoblastic tissue by absorption with normal human adult tissue. Anti-trophoblast sera was separated into two catagories anti-trophoblast antigen 1 (anti-TA1) and anti-trophoblast antigen 2 (anti-TA2). Anti-TA1 recognised TA1 antigens present on the syncytiotrophoblast microvilli and on certain human cell lines whereas anti-TA2 recognised TA2 antigens found on

trophoblast, lymphocytes, villous fibroblasts and endothelium. The TA2 antibodies could be absorbed from anti-trophoblast sera to leave TA1 antisera. The anti-trophoblast sera were studied for the ability to interact or inhibit immunoassays using known placental proteins, the results indicated that the TA1 antigens were tissue specific for the trophoblastic derivatives of human amniochorion (McIntyre & Faulk, 1979). Whyte and Loke (1979) described another unique anti-trophoblast antigen TrPM which was raised against syncytiotrophoblast and found to react against surface membrane components of syncytiotrophoblast using immunohistology. The trophoblastic antigens specified by antisera which would seem to be structural components of the syncytiotrophoblast membrane have been described as placental proteins (Klopper & Chard, 1979).

Faulk et al (1978) noted that anti-trophoblast sera directed against different placentae trophoblast preparations were cytotoxic for some but not all donor lymphocytes and bore no correlation with the ABO or HLA types of the donors (McIntyre & Faulk, 1982a). Thus the presence of allotypic trophoblast-lymphocyte cross reactive antigens (TLX) was proposed. Though the TLX antigen system has not been adequately defined recognition of paternally derived TLX specificities on the placental surface has being suggested for the maintainance of pregnancy (Faulk, 1981). Recent results from a controlled trial of immunisation treatment of recurrent aborters showed that 78% of pregnancies were successful followed immunisation of women with their husband's lymphocytes compared with 37% for the women immunised with their own lymphocytes (Mobray et al, 1985).

1:2 Alkaline Phosphatase

Although placental alkaline phosphatase (PLAP) is not involved in maternal immunity, it is polymorphic, expressed in tumours and a member of the well studied group of Alkaline phosphatases [orthophosphosphoric-monoester phosphohydrolase (alkaline optimum) E C 3.1.3.1] (for review see McComb et al, 1979). AP's, which hydrolyse a wide range of monophosphates at alkaline pH (10-10.5), are present in most human tissues. The AP's are glycoproteins which are membrane associated and detected as cell surface antigens. The greatest AP activity is shown in liver, bone, kidney, intestine and placenta as the APs from these tissues show many common structural and functional features suggesting that they are involved in similar biological processes.

1:2:1 Different Human Alkaline Phosphatase Isoenzymes

The different molecular forms of the enzyme are characterised and distinguished from each other by their thermostability, differential inhibition, by antigenic specificity and electrophoretic properties (Fernley, 1971; Stinson & Seargeant, 1974; Goldstein et al,1980a, Fishman, 1974; Moss, 1982). The enzymes also show different peptide maps on 2-D gels and differ in their N-terminal amino acid sequences (Seargeant & Stinson, 1979; McKenna et al, 1979; Badger & Sussman, 1976; Komoda et al, 1981). Using these properties two distinct types of of human AP were distinguished;tissue un-specific AP and tissue specific AP. A comparison of properties suggests one locus encodes for the tissue un-specific AP of the liver, bone and kidney and two loci for the tissue specific

forms; one for the placental form and one for the intestinal form. The evidence for this is summarised in table 1.1. There is some evidence of a fourth locus in humans expressed in testis and thymus, these tissues express trace amounts of placental-like AP (Millan, Erikssan & Stigbrand 1982).

1:2:1:1 **Tissue un-specific Alkaline Phosphatase** is the major AP isoenzyme found in liver, kidney and bone. Though the isoenzymes from these tissues differ in their thermostability (Moss & Whitby, 1978), using polyclonal antisera the AP enzymes of these tissues have been shown to be immunochemically identical (Boyer, 1963). The inhibition of the isoenzyme's catalytic activities by L-homoarginine is also identical for the AP enzymes of these tissues (Fishman & Sie, 1970). They all contain terminal sialic acid and partially bind to Concanavalin A and lentil lectin Sepharose (Butterworth & Moss, 1966; Lehmann, 1980). The difference in the electrophoretic properties of these enzymes is thought to be due to their differing levels of sialic acid as the desialated forms have the same isoelectric point (Smith et al, 1973; Stinson & Seargeant, 1981).

1:2:1:2 **Tissue specific Alkaline Phosphatases** are distinguished from the tissue unspecific AP by thermostability, immunochemistry and by enzyme inhibition. L-homoarginine inhibits the catalytic activities of tissue unspecific AP whereas the tissue specific APs are not inhibited by L-homoarginine but by L-phenylalanine (Ghosh & Fishman, 1966). The tissue specific APs intestinal AP and placental AP (IAP & PLAP) were shown to be related to each other by their cross reactivity to polyclonal antisera raised against either IAP or PLAP neither reacted to polyclonal antisera raised against liver, bone or kidney AP's.

TABLE 1.1

Isoenzyme type	Tissue unspecific AP	Tissue specific IAP	PLAP
mw	136-170Kd	140-170Kd	116-125Kd
N-terminal sequence	Leu-Val-Phe	Phe-Ile-Pro	Ile-Ile-Pro
pH optima	10.1-10.2	10.1-10.2	10.7
Heat stability			
56°C 15 min	+/++	++	+++
65°C 5 min	-	-	+++
Amino acid sensitivity (5mM)			
L-phenylalanine	-	+++	+++
L-homoarginine	+++	-/+	-/+
L-leucine	+	++	+
Neuraminidase sensitivity	+	-	+
Lectin binding	+	-	+++
Immunoreactivity with antisera to:			
Liver AP	+++	-	-
Bone AP	+++	-	-
Kidney AP	+++	-	-
Intestinal AP	-	+++	+
Term placental AP	-	+	+++

Table 1.1: Properties of the major occuring human alkaline phosphatases; adapted from Stigbrand (1984).

a **Intestinal Alkaline Phosphatase** this AP isoenzyme is found in the small intestine, its catalytic activity is inhibited by L-phenylalanine (Ghosh & Fishman, 1966). Though a glycoprotein, IAP does not contain sialic acid and so it does not bind to ConA or lentil lectin Sepharose (Lehmann, 1980).

b **Placental Alkaline Phosphatase** PLAP is found in the term placenta. PLAP can be distinguished from the other AP's due to its heat stability; at 65°C the other human AP's are completely inactivated whereas PLAP resists denaturation (Moss et al, 1972; Berdalis & Hirschlorn, 1972). Like IAP the catalytic activity of PLAP is inhibited by L-phenylalinine (Ghosh & Fishman, 1966). In common with the tissue unspecific AP PLAP is a glycoprotein containing terminal sialic acid and binds completely to ConA and lentil lectin Sepharose (Lehmann, 1980). Trace amounts of heat stable AP besides PLAP are also present in human tissue. These are defined as PLAP-like as they differ from PLAP in their greater sensitivity to L-leucine. PLAP-like AP have been found in normal testis, endocervix, and in lung and thymus tissue (Chang et al, 1980; Goldstein et al, 1980b; Nozawa et al, 1980; Goldstein et al, 1982).

The human AP's are similar in that they all occur as dimers composed of monomeric units of near identical structure. From the different properties they display it would seem that they are a multilocus enzyme family, encoded for by at least 3 gene loci whose location in the genome is not known (Harris, Hopkinson & Robson, 1974). One loci encodes for the tissue unspecific AP, two seperate loci encode for the tissue specific AP, IAP and PLAP and a fourth loci may encode for the low levels of PLAP-like AP found in normal adult tissue (Seargeant et al,

1979; McKenna et al, 1979). An explanation for the occurence of the different human AP loci is that they have decended from a common ancestral gene which has duplicated during evolution to give rise to several different genes by continual divergence. PLAP would be assumed to be a late evolutionary gene product as it is only detected in orangutan, chimpanzee and human placentae (Goldstein et al, 1980a). The absence of the PLAP isoenzyme in lowland gorillas is unexpected as there is no evidence of divergence of lowland gorillas and man after their divergence from orangutans (Donald & Robson, 1974). The PLAP isoenzyme also displays a high degree of polymorphism. Using starch gel electrophoresis 18 different allelic variants are evident for PLAP which is in contrast to the tissue unspecific AP's and IAP which show no allelic variants (Harris et al, 1974). This suggests that the mutants which affect the PLAP loci are not present in the other AP loci indicating that PLAP has arisen later in the evolutionary trail than the other human AP's.

1:2:2 Placental Alkaline Phosphatase Polymorphism

Electrophoretic variation in PLAP extracted from different term placentae was first reported by Boyer (1961). Three common types of electrophoretic patterns were found as well as a rarer band termed the D-variant which migrated slower than the other bands. Based on the population frequency of the electrophoretic variants three common PLAP alleles were genetically determined. Boyer's findings were confirmed by Robson and Harris (1965 & 1967). Six common phenotypes and nine rare phenotypes of PLAP were identified using single and dizygotic twin placentae from different racial populations (Robson &

Harris, 1965 & 1967). The rare phenotypes were usually found in combination with one of the common alleles. The D-variant was reported as a rare allelic variant by Beckman and Beckman (1968 & 1969) and it was shown to be inhibited by L-leucine (Doellgest & Fishman 1976). Besides the 3 common alleles there is now evidence for 15 or more rare alleles. The incidence of these rare allele heterozygotes is 2-3%, whereas with 22 other enzyme loci tested only in 5 loci did the frequency of heterozygotes exceed 0.3% (Donald & Robson, 1973; Harris et al 1974). The 3 alleles which encode for the six common phenotypes are, PI^1 which shows a slow mobility on starch gels, PI^2 which has a fast mobility and PI^3 that has an intermediate mobility. These alleles make up 97-98% of the PLAP variants.

The nomenclature for the PLAP alleles was altered to a numerical system by Donald & Robson (1973). The common alleles are numbered 1-3 and the rare alleles 4-18. Thus the common homozygous phenotypes are 1-1, 2-2 and 3-3 and the heterozygotes 2-1, 3-1 and 3-2. The electrophoretic patterns of the six common phenotypes in starch gels shows that heterozygotes have 3 bands, compared with the homozygotes single band. As PLAP is a homodimer two of the bands seen for the heterozygotes are the same as those seen for the two homozygotes. The third band, which migrates in an intermediate position, is a hybrid molecule created from the monomeric subunits of the two alleles. The gene frequencies of the common alleles vary in different racial groups though in all groups PI^1 is the most common, being between 57-94%. In Caucasian populations the PI^2 allele has a frequency of 24-34% whereas in both Asiatic Mongoloid and African Negro populations the frequency of PI^2 is lower, 2-8% and 2-5% respectively. The PI^3

allele is present at a low frequency in both Caucasian and Negro populations (4-9%) but in the Chinese and Japanese populations the frequency is between 20-24% (Beckman & Beckman, 1969; Donald & Robson, 1973).

The variant allele phenotypes of PLAP were identified and can be distinguished using starch gel electrophoresis by the different mobilities of their bands (Boyer, 1963; Donald & Robson, 1973). The variants can also be distinguished by antigenic variation (Wei & Doellgast, 1980) and by variations in thermostability (Holmgren & Stigbrand, 1976). The variant phenotypes are identical with respect to their pH optima, Km values and inhibition by L-phenylalanine and L-leucine. The structural basis for the polymorphism of the PLAP alleles has not been elucidated (Jemmerson et al, 1984). Amino acid substitutions account for the allelic electromorphs Adh-f and Adh-s in Drosophila alcohol dehydrogenase (Krietman, 1983). Thus amino acid substitutions could also account for the different alleles of PLAP. Some amino acid substitutions, though altering the antigenic determinants, would not affect the electrophoretic mobility. This is seen with the PLAP variants since monoclonal antibodies recognise the different PLAP phenotypes seen on starch gels and other phenotypes which are not distinguished on starch gels (Slaughter et al, 1981; Millan et al, 1982b).

1:2:3 Synthesis of Alkaline Phosphatase during pregnancy

Three forms of AP are detected in the placenta and maternal sera during gestation (Sakiyama et al, 1979). (1) Heat labile AP, specific to the first trimester between 6-10 weeks. (2) Heat stable PLAP, found in the second and third trimesters.

(3) The third form of AP in the placenta is visualised between 11-13 weeks of pregnancy and is a hybrid of first trimester AP and PLAP (Fishman et al, 1976; Sakiyama et al, 1979).

The first trimester heat labile AP is inhibited by L-homoarginine and inactivated by antisera directed against human liver AP, though not by antisera directed against PLAP. Thus the first trimester AP is a tissue unspecific AP having properties similar to liver AP. It differs in its electrophoretic mobility in nondenaturing polyacryamide gels, two bands are seen the slower band corresponding to liver AP but the faster band lacks any known antigenic determinants (Sakyama et al, 1973). In early pregnancy the heat stable PLAP activity is low in concentration and it begins a progressive increase from 28 weeks to term, when over 90% of the alkaline phosphatase in term placenta is PLAP (Sussman & Bowman, 1968; Fishman et al, 1976; Sakiyama et al, 1979). PLAP is detectable in the serum of pregnant woman from about 16 weeks onwards with the highest levels occuring at term (200-300ng/ml). After delivery the level of PLAP detectable in the maternal sera decreases, until by the sixth day there is no detectable PLAP in the maternal serum (Holmgren et al, 1978; Fishman et al, 1976). Using a radioimmunoassay Holmgren et al (1978) showed that there was no correlation with placental weight, blood type, parity of the mother, sex of the baby and the levels of PLAP.

1:2:4 Molecular Heterogeneity of Placental Alkaline Phosphatase

Being a placental surface membrane enzyme PLAP shows up to 10-fold greater enzyme activity levels in syncytiotrophoblastic tissue compared with the enzyme activity

levels in the whole placenta. The expression of PLAP is limited to the syncytiotrophoblastic microvilli where it is exposed to the circulating maternal blood (Whitsett & Lessard, 1975; Jones & Fox, 1976). PLAP is a sialoglycoprotein composed of pairs of 69K mw disulphide bonded polypeptide chains and when extracted from homogenates of whole placentae it shows two forms of heterogeneity. On starch gels they resolve as two zones of enzyme activity, A and B which are detected in all human placentae (Ghosh et al, 1968; Beckman et al, 1966; Robson & Harris, 1965). The A form (A-PLAP) has a greater electrophoretic mobility than the B form (B-PLAP). Having three times the specific activity of B-PLAP A-PLAP constitutes the major zone of enzyme activity in both total placental extracts and maternal serum during pregnancy. The native mw of these forms is 140Kd for A-PLAP and 200Kd for B-PLAP (Doellgast et al, 1977). Both A-PLAP and B-PLAP share similar heat stability, express the same polymorphism and when treated with neuraminidase they both show retarded zones of enzyme activity (Robson & Harris, 1965). It has been suggested that B-PLAP is an aggregate of A-PLAP associated with a non-AP protein. As B-PLAP resists chromatographic resolution in several systems it may be a membrane-associated form of A-PLAP, poor chromatographic resolution seems to be a property of membrane bound proteins. (Wallach & Winzler, 1974; Contractor, 1983).

Placental microvillous plasma membrane fractions were shown biochemically to be enriched in PLAP when compared with total placental extracts Smith and Brush (1978), using a modification of Smith et al (1974) method for the preparation of placental microvillous membranes, showed a 14 fold enrichment for PLAP. Ogbimi et al (1979) reported a 3.8-7.6 fold enrichment

whereas Booth et al (1980) using a modified method from Smith et al (1974) observed PLAP enrichment of 24.6 fold. PLAP extracted just from the syncytiotrophoblastic microvilli shows three zonal bands when resolved on starch gels containing detergent (Abu-Hasan et al, 1984; Abu-Hasan & Sutcliffe, 1984a&b & 1985). The bands were the A and B forms of PLAP as seen in whole placental extracts and a third (M-PLAP) which ran between the A and B forms. Abu-Hasan (1984) showed the presence of small amounts of M-PLAP in whole placental extracts on starch gels containing 0.5% triton X-100. M-PLAP is the major form of PLAP found in the syncytiotrophoblastic microvilli. It has a native mw of 725Kd, expresses the same genetic polymorphism as the A and B forms and it gives reactions of identity with antibodies directed against A-PLAP. On non-reducing SDS gels the mobility of A-PLAP dimers is 140Kd and that of M-PLAP is 116-118Kd which suggests that M-PLAP is hydrodynamically a more compact molecule than A-PLAP (Abu-Hasan & Sutcliffe, 1984a, 1984b & 1985). The structural basis for the differences between the three isoenzymes of PLAP is not known but that they are polypeptide products of the same translational system is indicated by their identical genetic polymorphism.

The high mw M form of PLAP suggested that this was a molecular aggregate form of the other PLAP isoenzymes or an artifact aggregate arising from the insolubility of the extracted membrane proteins. There is evidence against M-PLAP being an aggregation artifact, the yield of M-PLAP is not affected by the use of detergents in the extraction of PLAP from the microvilli. M-PLAP and A-PLAP can be seperated using hydrophobic chromatography, M-PLAP bound to an alkyl-agarose column and was eluted with hydrophobic solvents whereas A-PLAP did not bind to

the column (Abu-Hasan & Sutcliffe, 1984a&b & 1985). M-PLAP can be cleaved from the surface of the syncytiotrophoblast microvilli using the protease subtilisin; the subtilisin cleaved M differing from native M-PLAP in being 2Kd smaller. The 2Kd region was shown to be cleaved from the carboxy-terminus of M-PLAP as both native and subtilisin cleaved M have a common N-termini (Abu-Hasan & Sutcliffe, 1984b & 1985). The N-terminal amino acid sequence for M-PLAP was in common with the N-terminal amino acid sequence of A-PLAP (extracted from the syncytiotrophoblast microvilli) and agreed with the Dansyl-Edman sequencing studies on PLAP extracted from total placental extracts (Green & Sussman 1973).

The majority of PLAP in the maternal serum is of the A form rather than the M form (Abu-Hasan unpublished). It has been recently found that a serum protein factor, which is not inhibited by a wide range of exogenous protease inhibitors, has the ability to convert M-PLAP to the A and B forms (Livingstone,J.C. personal communication). The factor behaves as a glycoprotein in binding to ConA sepharose. Though hepatic and endothelial forms of serum lipoprotein lipase bind to ConA sepharose they did not stimulate conversion. The factor was shown to elute as an unimodal peak on DEAE sepharose at the same point as Lecithin cholesterol acyl transferase (LCAT), which can act as a lipoprotein phospholipase (Doi & Nishida, 1981). The ability of LCAT to convert M to the A and B forms is being tested. The evidence indicates that the A and B forms of PLAP are the conversion products of the M form, but since the native sizes of A-PLAP and B-PLAP are a lot less than that of M-PLAP the M form is not just a simple aggregate of A and B. The A and B forms of PLAP would seem not to exist in their native forms until after

conversion of the M complex with the serum factor.

The expression of PLAP is limited to the syncytiotrophoblastic microvilli the enzyme being present on the plasma menbrane surface (Tokumitsu et al, 1979). With the active site of the enzyme located on the maternal facing side of the microvilli one would assume that its function involves its contact with maternal blood. It has been demonstrated that ADP-ase is a strong inhibitor of platlet aggregation and placental extracts have been shown to contain ADP-ase activity (Hutton et al, 1980). PLAP may be involved as an ADP-ase in the degradation of ADP in the placenta and its role may be to maintain the fluidity of the blood in the placental sinuses. It has also been suggested that PLAP may be associated with transport mechanisms across the placenta (Truman & Ford, 1984; Shirazi et al).

1:2:5 **Tumour Alkaline Phosphatases**

Fetal and embryonic proteins are expressed in tumours at high levels. The re-expression of genes normally active only in the early stages of development suggests that the tumour malignancy may involve regulation disturbances in the expression of these genes (Manes, 1974; Fishman & Sell, 1976). As detectable low levels of these proteins are found in some normal tissue it is not known whether the proteins are tumour specific or just the over production of existing fetal proteins (Ruoslahti & Seppala, 1972; Usategui-Gomez et al, 1974; Millan et al, 1982c). Some of the oncofetal antigens expressed in tumours are carcinoembryonic antigen (CEA), alpha fetoprotein (AFP) and PLAP (Abelev et al, 1963; Gold & Freeman, 1965; Fishman et al, 1968a&b; Wahren et al, 1979).

Tumour PLAP was first observed as an oncofetal antigen in a male patient (Regan) who had an oat cell carcinoma of the lung (Fishman et al, 1968abc). With regards to immuno cross reactivity and catalytic properties amino terminal sequence, peptide mapping, isoeletric point, subunit mw Regan and PLAP were found to be identical (Fishman et al, 1968a; Green & Sussman, 1973; Lehman, 1975). Another tumour related PLAP was found in a patient (Nagao) with pleural carcinomatosis: this Nagao isoenzyme was found to be distinct from the PLAP and Regan isoenzyme due to its increased sensitivity to L-leucine (Nakayama et al, 1970). This would suggest that the Nagao isoenzyme represents the expression of a gene related structurally but distinct from the PLAP gene whereas Regan would seem to be the re-expression of the PLAP gene. The re-examination of 39 cancer patient sera containing AP which had been classified as PLAP+ on electrophoretic and calalytic properties showed that many were inhibited by L-leucine and on starch gels they showed the phenotype of the very rare D-variant of PLAP (Wei & Doellgast, 1981; Millan et al, 1982c).

PLAP and PLAP-like isoenzymes are clinically useful tumour markers as elevated levels of PLAP and PLAP-like AP are found in several different malignancies; pancreas, lung, breast, colon, lymphnodes, kidney, stomach and bladder (Fishman & Stolbach, 1979; Stigbrand & Engvall, 1982; Lange et al, 1982; Jeppsson et al, 1983). The highly sensitive and specific, solid phase enzyme immunoassay (EIA) for the identification of PLAP with monoclonal antibodies such as H317 detected measurable levels of circulating PLAP-like isoenzyme in 34% of ovarian and 25% of cervical carcinoma patients. H317 has also been used

successfully in detection of tumour recurrences in ovarian cancer patients by antibody guided *in vivo* radionucleotide imaging techniques (McLaughlin *et al*, 1983). This and the sensitive sandwich enzyme-linked immunoassay reported by Millan and Stigbrand (1982) increase the diagnostic value of PLAP and PLAP-like isoenzymes. AP with PLAP and PLAP-like properties have been demonstrated in cell lines derived from carcinoma of the cervix (Hela) and other human tumours. Out of eight Hela cell lines studied by Benham *et al* (1978) six contained PLAP-like AP the others were liver-like AP and an unidentified AP isoenzyme, this was distinguishable from the known human AP's. Though cross reactive with PLAP, using polyclonal antibodies directed against PLAP, the cell line AP isoenzymes can be distinguished from PLAP by their sensitivity to inhibition using L-leucine and by the fact they fail to show the electrophoretic polymorphism of PLAP (Millan *et al*, 1982b&c).

1:3 Transplantation Antigens

The major reason for graft failure between genetically dissimilar individuals (allogenic grafts) is a host immune response to the cell surface antigens of the donor which differ from those of the host. These surface antigens are the histocompatibility antigens. The genes of which can be divided into two main categories, major histocompatibility complex (MHC) and the minor histocompatibility antigens, which represent allogeneic polymorphism to a much lesser extent. The MHC encodes for three classes of genes I, II and III. The MHC of humans (HLA) and mouse (H-2) appear extremely homologous with both encoding the same three classes of genes (Hood *et al*, 1984; Bodmer &

Bodmer, 1984). The three class I antigens which are responsible for graft rejection are designated A, B & C in humans and K, D & L in mice. The mouse hematolymphoid differentiation antigens Qa-1, Qa-2, Qa-3 and TL (Tla gene) are also being described in humans (Hood et al, 1984; Bodmer & Bodmer, 1984). The HLA class II antigens are called DR, DQ and DP which correspond to the H-2 class II antigens A alpha, A beta, E alpha and E beta. These are found on macrophages, T and B cells. Their function is to provide self recognition. This self recognition element allows the helper t cells to interact with foreign antigen to produce lymphokines which stimulate the proliferation of cytotoxic T cells or suppressor T cells (Hood et al, 1984; Steinmetz, 1984). The HLA complex class III genes code for the complement components C2, C4 and BF.

1:3:1 **HLA and the Placenta**

Matching donor and host HLA antigens and the suppression of any residual immune response is standard practice for organ graft success (Playfair, 1982; Hood et al 1984). The placenta, though fed by its own fetal vessels, is a semi-allogenic graft which survives in direct physical contact with the maternal immune system without rejection (Lala et al, 1983). Usually for maximum allograft rejection response the HLA antigens of both class I and II are needed to be recognised by the allo reactive T cells of the host. Precursors of the cytotoxic T cells (Tc) bearing class I receptors respond to the graft's class I antigens, whereas precursors of the helper cells (Th) recognise the class II antigens. After clonal expansion and maturation, the Tc, with the help of the Th in the form of interleukin 2,

destroys the graft cells by recognition of the class I antigens.

The distribution of HLA antigens on early and term placentae has been studied using immunohistology. Both class I and II antigens were shown to be absent from villous trophoblast (Faulk & Temple, 1976; Goodfellow et al, 1976; Sunderland et al, 1981a&b). More recent studies of extra-villous trophoblast locations has shown class I expression (Sunderland et al, 1981a; Redman et al, 1984). The cytotrophoblast of the uteroplacental interface which invades the uterine decidua, the cytotrophoblastic columns cell islands and the cytotrophoblasts of the chorionic plate all express HLA class I antigens, as detected by the monoclonal antibodies W6/32 and B_2M. However the syncytial layer of trophoblast and the underlying zone of cytotrophoblast lacks HLA class I antigens (Sunderland et al, 1981a; Redman et al, 1984). The extra-villous trophoblast which reacts with the monoclonal W6/32 did not express the expected fetal HLA A and B allotypes (Redman, 1983). Thus the class I determinants detected on the placenta are qualitatively different from those detected on other tissue. They either belong to HLA C or to a yet unknown HLA class I locus. The latter possibility would mirror murine H-2 where of 36 distinct class I genes from BALB/C genomic cosmid library 10 are uncharacterised (Goodenow et al, 1982; Steinmetz, 1984; Redman, 1983). Class II antigens were not expressed on the villous or non villous trophoblast using the monoclonal antibodies (Sunderland 1981a&b, Redman, 1983). In culture Brami and co-workers (1983) found cells of trophoblastic origin from 9-12 week placentae which expressed HLA-DR. Greater investigation of trophoblast cells in culture is needed to confirm this result. Khalaf (1985) isolated cells from the

chorionic plate and the uteroplacental interface (Nitabuch's layer). These placental cells were cultured for 2-4 months and their antigenic determinants studied using monoclonal antibodies. Both cell preparations showed positive staining for HLA class I antigens with only a few cells staining positively for HLA class II antigens (Khalaf et al, 1985a&b).

The antigenic status of the trophoblast is the key determinant for immune interactions between the mother and the fetus. The absence of HLA class II antigens could possibly make the trophoblast immune from graft rejection though class I antigens alone, in the mouse, are sufficient for tissue rejection (Lala et al, 1983; Billington & Bell, 1983; Klien, 1975). Also the expression of the class I or Class I-like determinants on the extra-villous invading trophoblast might be important in eliciting the maternal immune system to respond ineffectively (McIntyre & Faulk, 1982a&b).

1:4 Molecular analysis of the placenta

During gestation the human placenta synthesises and secretes into the maternal serum, many proteins and peptide hormones which are unique to pregnancy. The syncytiotrophoblastic and probably the cytotrophoblast cells are actively involved in this synthesis and secretion (Contractor et al, 1977). From this it is apparent that the transcription of mRNA and its translation into protein is occurring in the placenta (Sybulski & Tremblay, 1967; Dancis et al, 1976; Klopper & Chard, 1979). By the study of mRNA from different placental tissues one will be able to localise gene expression. The analysis of the placental genes at the mRNA and cDNA cloning level will give an insight to the

possible mechanisms involved in the regulation of gene expression; whether proteins are encoded by single genes or gene clusters. Also sequence data derived from cDNA clones will enable the similatities and differences between placental and other non-placental related genes to be specifically charecterised.

Analysis during placental development of nucleic acids showed that the RNA content per cell was 2.5 times higher in the first trimester placenta compared with term placenta. Total RNA and protein synthesis increased linearly with gestational age and DNA synthesis and cell division ceased after 36 weeks. The analysis of nuclease activity showed that deoxyribonuclease activity declined during gestation whilst the ribonuclease activity remained constant throughout (Brody, 1953; McKay *et al*, 1958; Winick *et al*, 1967). That the levels of specific mRNA present vary during placental development according to the placenta's requirements was shown by Chatterjee *et al* (1976). By extracting mRNA from polyribosomes in early and term placentae Chatterjee *et al* (1976) studied the synthesis of two placental peptide hormones, hCG and hPL. The mRNA was *in vitro* translated in a wheat germ cell free system. *In vitro* translated mRNA from early placenta synthesises eight fold more hCG alpha protein than mRNA from term placentae. This corresponds to the peak of hCG detected in maternal sera at 10-12 weeks of gestation and its decline in later pregnancy (Chatterjee & Monro, 1977; Daniels-McQueen *et al*, 1978). The hPL hormone reaches its maximal level in the maternal serum at term, which is reflected in the *in vitro* translated mRNA. Term placental mRNA synthesised 4-5 times more hPL when compared with mRNA from first trimester placentae (Boime *et al*, 1976). Synthesis of hPL using an equal amount of mRNA from

term and first trimester placentae showed that the enhanced synthesis of hPL at term is the result of an increased proportion of the mRNA coding for hPL. Thus the proportion of the total mRNA available which codes for a specific protein varies and this determines the amount of protein formed at different stages in pregnancy.

1:4:1 Placental mRNA

RNA can be extracted from the syncytiotrophoblastic tissue of placenta using a strong denaturant and separated from the placental DNA by centrifugation through a caesium chloride cushion (Boime et al, 1976; Seeburg et al, 1977; Fiddes & Goodman, 1979). To obtain intact RNA of high quality the rate of denaturation of the RNase present in tissue must exceed the rate of RNA degradation by RNase. Chirgwin et al (1979) reported that the rate of denaturation was maximized by the combined use of a strong denaturant (5M guanidinium thiocyanate) and reductant (0.1M B-mercaptoethanol). The denaturant eliminates nucleolytic degradation of the RNA by its denaturation of all the cellular proteins present, while the reductant breaks the protein disulphide bonds which are necessary for RNase activity. For eukaryotic cells only 1% to 5% of the total cellular RNA is mRNA. This mRNA is heterogeneous in size from a few hundred bases to several kilobases and encodes for the synthesis of all the proteins in the cell. Due to the poly(A) tail at the 3` end of nearly all mRNAs the mRNA can be purified from the rest of the cellular RNA by chromatography using oligo(dT) cellulose (Aviv & Leder, 1972).

1:4:1:1 *in vitro* **translation of mRNA** After enriching for polyadenylated RNA by repeated chromatography on oligo(dT) cellulose (Aviv & Leder, 1972) the placental mRNA was *in vitro* translated, in the presence of ^{35}S-methionine, into various polypeptides which were analysed using SDS-polyacrylamide gels (Boime *et al*, 1976; Seeburg *et al*, 1977; Fiddes & Goodman, 1979 & 1980). The reticulocyte cell free system was used in preference to other translational systems for the synthesis of the placental translation polypeptides as it has a very low background of endogenous protein synthesis and the capability to synthesis polypeptides of high molecular weights (Pelham & Jackson, 1976). The major proteins *in vitro* synthesised by mRNA extracted from first trimester placenta were of apparent mw 13Kd and 16Kd. These were shown to be the pre-forms of the alpha and beta subunits of hCG by immunoprecipitation, using antisera directed against the mature hCG. The 13Kd and 16Kd pre-forms of the alpha and beta subunits of hCG were visible in only trace amounts in *in vitro* synthesised term mRNA. The major *in vitro* synthesised protein at term was of apparent mw 24Kd. Up to 20% of the incorporated ^{35}S-methionine was present in this polypeptide whereas there was only low incorporation of ^{35}S-methionine in the rest of the synthesised term polypeptides. This 24Kd polypeptide was shown to be the preform of hPL by its specific immunoprecipitation with antisera directed against the mature hPL protein.

1:4:1:2 **Immunoprecipitation** It is possible to isolate by immunoprecipitation procedures nearly any cellular protein to which specific antibodies are available, as long as the extraction procedures retain the antigenicity of the protein (Kessler, 1975). Immunoprecipitation is usually performed in two

stages. The specific antibody is first added to the solution containing the antigen to be isolated and the antigen-antibody complex is then precipitated. Precipitation is facilitated using an agent that by binding to the antigen-antibody complex gives it sufficient mass to precipitate. Protein A, a protein component of certain strains of Staphlococcus aureus cell walls has a high affinity for the Fc region of IgG molecules, binding specifically and strongly (Kronvall et al 1970). Since formalin fixed S. aureus retain this cell wall protein, these cells precipitate antigen-antibody complexes and thus act as a solid phase immunoadsorbent (Kessler, 1975). The advantages of using Protein A compared with double antibody techniques are that the uptake of the antigen-antibody complex occurs very rapidly and there is no need for a second antibody. Once bound, the complex cannot be displaced by excess free antibody or normal IgG and the antigen can then be eluted from the cells using SDS and B-mercaptoethanol at high temperatures. Immunoprecipitation using protein A has been successfully used to to detect specific translational products of placental mRNA using antibodies directed against hPL and hCG (Seeburg, et al 1977, Fiddes & Goodman, 1979 & 1980). Therefore it was used to detect whether PLAP and other placental proteins were translated from cytotrophoblast and syncytiotrophoblast mRNA.

1:4:2 Cloning of placental genes

Of the in vitro synthesised polypeptides in placental mRNA it is the two major synthesised polypeptides, hCG and hPL, which have been studied in the most detail. Due to the differences in translational activities between first trimester

and term placental mRNA, cDNA synthesised from these two sources show differences in their restriction enzymes digestion pattern. By this approach discrete restriction fragments were generated for the predominant cDNA species. Comparison of the cDNA restriction enzymes fragment patterns from first trimester and term mRNA samples enabled the cDNA fragments corresponding to alpha hCG, beta hCG and hPL to be identified. The DNA fragments generated by HaeIII and HhaI digestion of single stranded cDNA from term mRNA enriched for hPL enabled sequence analysis and cloning of this cDNA species. From the hPL cDNA clones, four hPL-like genes have been isolated two of which have been shown to produce the same mature hormone (Barrera-Saldana et al, 1982). The analysis of cDNA clones bearing sequences corresponding to Beta hCG has shown that the this subunit is encoded for by at least three non allelic genes (Policastro et al, 1983).

As the other in vitro synthesised polypeptides are at low abundances they can not be studied by the elution of the predominant cDNA fragments from gels and the direct cloning of these DNA fragments. The analysis of placental genes which cannot be directly cloned involves the synthesis and cloning of total cDNA from placental mRNA. The identification and isolation of cDNA clones for genes which are expressed at low levels requires probes. A knowledge of the amino acid sequence of the gene product enables synthetic oligonucleotides to be used as hybridisation probes (Suggs et al, 1981). Whereas the expression of the gene in E.coli and the availability of antibodies to the gene product enable specific clones to be identified by immunoassays (Broome & Gilbert, 1978; Kemp & Cowman, 1981; Young & Davis, 1983).

1:4:2:1 **Synthetic Oligonucleotides** When the sequence of at least part of the polypeptide product for the gene of interest is known synthetic oligonucleotides can be designed. From the amino acid sequence alone a single oligonucleotide probe could not be synthesised which was 100% homologous to the DNA sequence as the genetic code is almost totally degenerate with 61 of the possible 64 triplets coding for 20 amino acids. Only two amino acids, methionine and tryptophan, are represented by a single codon. To minimize ambiguities in the synthetic oligonucleotide the amino acid sequences which have the fewest codon possibilities are chosen (Suggs et al, 1981; Anderson & Kingston, 1983; Jaye et al, 1983).

The use of oligonucleotides for probing or priming cDNA depends on the availability of amino acid sequence data. Up to 1982, the only data available for PLAP was for the residues 1-4 (Green & Sussman, 1973):

Ile-Ile-Pro-Val-

In 1983, N.S. Abu-Hasan in this laboratory sequenced the residues 1-13 of M-PLAP and A-PLAP (Abu-Hasan & Sutcliffe, 1984 & 1985):

5 10

Ile-Ile-Pro-Val-Glu-Glu-Glu-Asn-Pro-Asp-Phe-Trp-Asn-

Analysis of Abu-Hasan's data showed that short oligonucleotides with acceptable redundancies could be deduced (table 1.2). This sequence was confirmed when data appeared for residues 1-42 (Ezra et al, 1983):

5 10 15

Ile-Ile-Pro-Val-Glu-Glu-Glu-Asn-Pro-Asp-Phe-Trp-Asn-Arg-Glu-

20 25 30

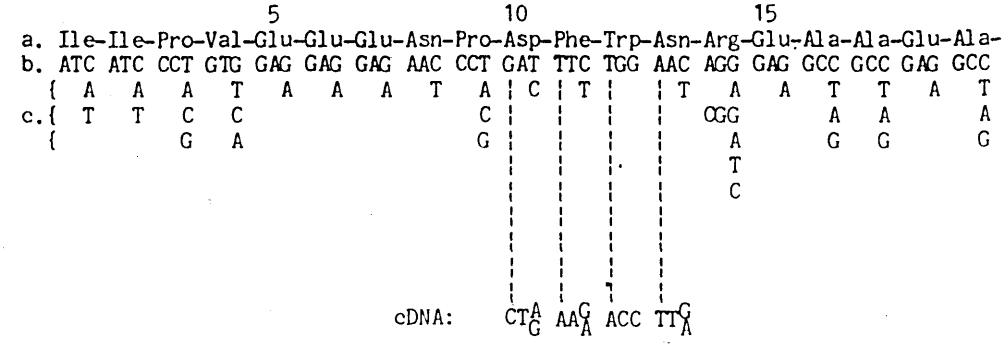
Ala-Ala-Glu-Ala-Leu-Gly-Ala-Ala-Lys-Lys-Leu-Gln-Pro-Ala-Gln-

35 40

Thr-Ala-Ala-Lys-Asn-Leu-(Ile)-Ile-Phe-Leu-Gln-Asp-

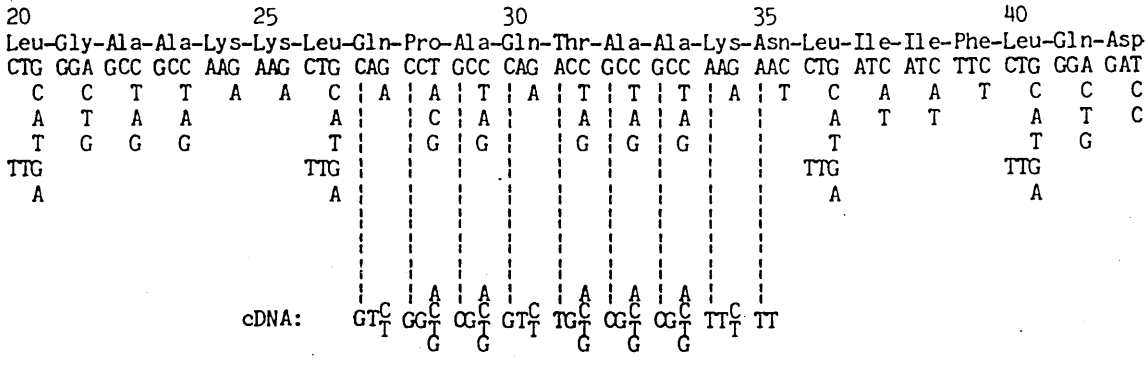
Consider in table 1.2 the amino acid sequence for the amino terminal end of the PLAP polypeptide. At position 12 there is a tryptophan residue coded for by an unique codon (UGG) the flanking amino acids at positions 10-13 are each coded by pairs of synonymous codons. Thus a synthetic oligonucleotide of 12 bases from the first base of codon 10 would contain 3 mixed sites each with a pair of alternative bases (table 1.2). Thus to obtain an oligomer which would be ≥ 100% complementary to PLAP mRNA, 8 different oligonucleotide sequences are needed. A larger perfectly matched oligonucleotide is highly desirable however, the cost of synthesising one is also high. Covering all the possible arrangments of codons for a amino acid sequence limits the size of oligonucleotides which can be practically synthesised (Sood et al, 1981; Suggs et al, 1981; Breslow et al, 1982; Stetler et al, 1982; Orkin et al, 1983). For example an oligonucleotide of 26 bases from the first base of codon 27 to the second base of codon 35 would contain 8 mixed sites, 3 with a pair of alternative bases and the others with a choice of four alternative bases (table 1.2). Thus 8192 unique oligonucleotide sequences are needed to obtain one which is 100% homologous.

Although 100% homologous oligonucleotides have been used successfully to probe and detect cDNAs it is possible and perhaps



I. Short Oligonucleotides covering all the possible codon sequences:-

(1) 3'-CTAAAAACCTTA-5'
(2) 3'-CTAAAAACCTTG-5'
(3) 3'-CTGAAGACCTTA-5'
(4) 3'-CTGAAGACCTTG-5'
(5) 3'-CTGAAAACCTTA-5'
(6) 3'-CTGAAAACCTTG-5'
(7) 3'-CTAAAGACCTTA-5'
(8) 3'-CTAAAGACCTTG-5'



II. Long Oligonucleotide based on:-

i codon usage : 3'-GTCGGACGGGTCTGGCGGCGGTTCTT-5'

ii codon usage at 3` end and covering all the codon possibilities at the 5` end

(1) 3'-GTCGGACGGGTCTGGCGGCGGTTCTT-5'
(2) 3'-GTCGGACGGGTCTGGCGGCGATTCTT-5'
(3) 3'-GTCGGACGGGTCTGGCGGCGTTTCTT-5'
(4) 3'-GTCGGACGGGTCTGGCGGCGCTTCTT-5'
(5) 3'-GTCGGACGGGTCTGGCGGCGGTTTTT-5'
(6) 3'-GTCGGACGGGTCTGGCGGCGATTTTT-5'
(7) 3'-GTCGGACGGGTCTGGCGGCGTTTTTT-5'
(8) 3'-GTCGGACGGGTCTGGCGGCGCTTTTT-5'

Table 1.2: Amino acid sequence for the N-terminal end of the PLAP polypeptide, showing all the possible codon sequences for these amino acids. The cDNA sequences used for the deduction of the PLAP oligonucleotides I and II are aligned with their codon sequences.

a. The amino acid sequence data for PLAP, Ezra et al, 1983 and Abu-Hasan & Sutcliffe, 1984 & 1985.

b. The statistically favoured codons, specified from the codon usage known for human fetal beta globins ^{C}X and ^{A}X, Smithies et al,1981 and Shen et al, 1981.

c. The other possible codons

more successful to use longer oligonucleotides and to tolerate a certain level of mismatch. When the codon uncertainty is ignored the increased length of the oligonucleotide is used to confer probe specificity (Agarwal et al; 1981; Jaye et al, 1983; Anderson & Kingston, 1983; Ullrich et al, 1984). The use of a longer unique oligonucleotide as a specific probe involves:

(1) The selection of the amino acid sequence which has the least codon redundancy. By avoiding regions containing amino acids encoded for by 6 codons the degeneracy of codons is limited to the third base position and thus a oligonucleotide probe would be at least 66% homologous.

(2) The selection of statistically favoured codons. Codon usage is determined from sequenced cDNAs corresponding to related proteins.

(3) The selection of nucleotides giving the greatest stability of mismatches during hybridisation where no preference is seen with codon usage. Jaye et al (1983) found that a G:T mismatch at the codon third position though less stable than the G:C base pair contributed to the stability of the oligonucleotide/mRNA duplex whereas an A:C mismatch would not. However, Lathe (1985) suggests that the base pairing energy associated with a G:T mismatch generates a less stable oligonucleotide/mRNA hybrid for human coding sequences. Also Smith (1983) noted that the stability of G:T pairs in DNA was substantially less than that in RNA.

(4) The selection of an oligonucleotide sequence with minimum secondary structure which would reduce hybridisation.

For a longer unique oligonucleotide for PLAP a region where the degeneracy of codons was limited to the third base

position was chosen and the statistically favoured third base was chosen. The degenerate third base positions for the PLAP amino acid codons was specified by the codon usage known for human fetal beta globins C and A (Smithies et al, 1981; Shen et al, 1981) and verified from the human codon usage table of Lathe (1985). Thus with codon usage the oligonucleotide of 26 bases from the first base of codon 27 to the second base of codon 35 would have 8 bases selected on the basis of statistically favoured codons and it would be at least 69% homologous (table 1.2).

A combination of the above two methods can also be used for the synthesis of oligonucleotides. At the 3`end of the oligonucleotide the third base of each codon is specified by codon usage whereas at the 5`end, where 100% homology is more important, especially when the oligonucleotide is being used to prime cDNA synthesis, each alternative arrangment of bases is specified (table 1.2).

As even a single base pair mismatch alters the thermal stability of the oligonucleotide/DNA duplex by altering the hybridisation conditions one can select for perfect homology or for specified levels of mismatch. The hybridisation and washing stringencies are based on the dissociation temperature of the duplex calculated from the proportion of G/C and A/T in the oligomer (Gillam & Smith, 1979; Suggs et al, 1982; Winter el al, 1982; Norrander et al, 1983). The dissociation temperatures for the different PLAP oligonucleotides, given as examples above, are shown in table 1.3.

TABLE 1.3

DISSOCIATION TEMPERATURE T_D = [dG.dC x 4°C] + [dA.dT x 2°C]

	PLAP oligonucleotides	TD
I. homologous bases	3'-CT_AA_AC_TT_-5'	24°C
I. (1)	3'-CTAAAAACCTTA-5'	30°C
(2)	3'-CTAAAAACCTTG-5'	32°C
(3)	3'-CTGAAGACCTTA-5'	34°C
(4)	3'-CTGAAGACCTTG-5'	36°C
(5)	3'-CTGAAAACCTTA-5'	32°C
(6)	3'-CTGAAAACCTTG-5'	34°C
(7)	3'-CTAAAGACCTTA-5'	32°C
(8)	3'-CTAAAGACCTTG-5'	34°C
II. homologous bases	3'-GT_GG_CG_GT_TG_CG_CG_TT_TT-5'	58°C
II.i	3'-GTCGGACGGGTCTGGCGGCGGTTCTT-5'	88°C
II.ii (1)	3'-GTCGGACGGGTCTGGCGGCGGTTCTT-5'	86°C
(2)	3'-GTCGGACGGGTCTGGCGGCGATTCTT-5'	86°C
(3)	3'-GTCGGACGGGTCTGGCGGCGTTTCTT-5'	88°C
(4)	3'-GTCGGACGGGTCTGGCGGCGCTTCTT-5'	86°C
(5)	3'-GTCGGACGGGTCTGGCGGCGGTTTTT-5'	84°C
(6)	3'-GTCGGACGGGTCTGGCGGCGATTTTT-5'	84°C
(7)	3'-GTCGGACGGGTCTGGCGGCGTTTTTT-5'	84°C
(8)	3'-GTCGGACGGGTCTGGCGGCGCTTTTT-5'	86°C

Table 1.3: The dissociation temperatures for the different PLAP oligonucleotides shown in table 1.2.

1:5 Cloning Vectors

The use of a λ vector for the preparation of cDNA libraries in preference to a plasmid vector makes use of the efficency and reproducibility of *in vitro* packaging of λ DNA as a method of introducing the cDNA sequences into *E.coli* compared with DNA transfection employed by plasmids (Young & Davis, 1984). *In vitro* packaging of λ DNA uses cell extracts derived from two induced λ lysogens whose prophage have complementary mutations in genes required for mature phage assembly, by mixing the two extracts with λ DNA the DNA is packaged into infectious "phage" particles (Becker & Gold, 1975). The frequency of which cDNA clones of specific mRNAs appear in a cDNA library is usually dependent on the abundance of that message in the mRNA population. To isolate cDNA clones of PLAP which is assumed to be a low abundence mRNA a much larger cDNA library needs to be constructed; compared with that needed for the isolation of cDNA from a high abundant mRNA. This is not just because of the minimum number of clones required to obtain a complete representation of the low abundance mRNAs but also due to the sampling variation and preferential cloning of certain mRNA sequences (Huynh, Young & Davis, 1984).

1:5:1 Lambda Vectors gt10 and gt11

The choice between the lytic cycle, the release of progeny phage, and the lysogenic pathway which leads to the intergration of the λDNA into the host genome is dependant on both 'phage and host factors. The phage cII protein is the primary control function in the establishment of lysogeny (Herskowitz & Hagen, 1980). To establish lysogeny the regulatory

activities carried out by cII and cIII are the negative regulation of lytic functions and the positive regulation of the cI gene repressor protein for the maintainance of lysogeny by the repression of early transcription and the consequent blocking of late gene expression (Echols & Green, 1971; Reichardt & Kaiser, 1971). The positive regulation of the int gene is also under the control of cII and cIII, the int protein recognizes sequences at the att sites of both host and 'phage genomes and calalyzes the prophage insertion into the host genome (Chung & Echols, 1977).

Thus bacteriophage which are mutant in genes cI, cII or cIII are unable to follow the lysogenic pathway and form clear plaques. Both \gt10 and \gt11 are suitable cloning vectors as both are mutant in cI \gt11 has a temperature sensitive cI repressor which is inactive at 42°C whereas \gt10 becomes cI^- by the insertion of foreign DNA at its EcoR1 site. \gt10 produces phage libraries which can be screened using nucleic acid probes; genomic DNA, cDNA, RNA, cloned DNA fragments or synthetic oligonucleotides. Whereas \gt11 libraries can be screened with antibody probes as the inserted cDNA fragments are expressed as polypeptides.

1:5:1:1 **\gt10:** The cloning vector \gt10 contains a unique EcoR1 site within the phage repressor gene (cI); figure 1.1 gives the structure of \gt10. Fragments of cDNA up to 7.6 Kb pairs in length can be inserted into this EcoR1 site to obtain packagable \gt10 DNA/cDNA molecules. As \gt10 is not a replacement vector an insert fragment is not required for packageable \gt10 DNA molecules (Murry et al, 1977). Therefore cDNA libraries cloned into \gt10 can consist of up to 99% parental phage (Huynh et al,

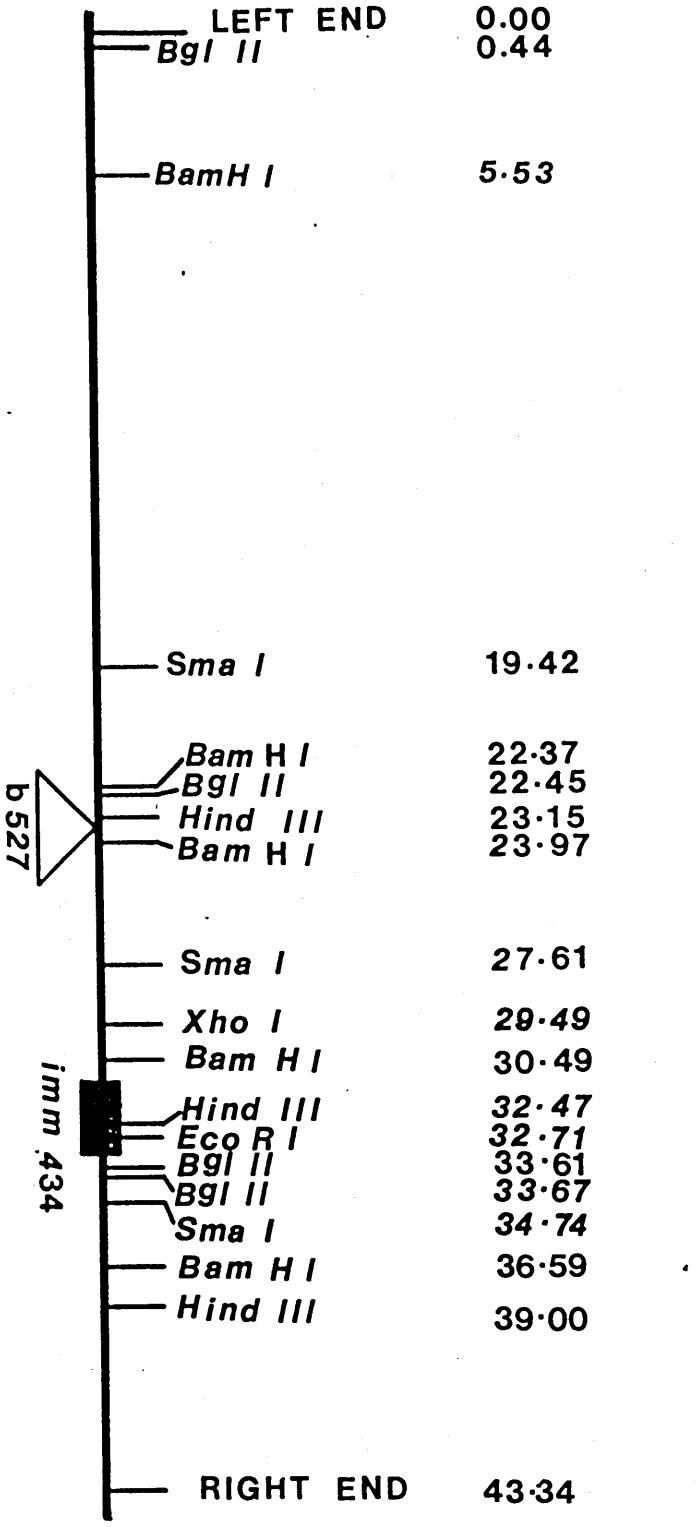


Figure 1.1: Map of λgt10 with restriction endonuclease cleavage sites in kilobase pairs. The b527 deletion represents 8.3% of the DNA sequences removed from the wild type lambda and the imm434 subsitution replaces 6.4% of the wild type lambda. The insertion of cDNA into the unique EcoR1 site inactivates the cI gene enabling recombinant clones to be identified by morphological differences. Reproduced from Huynh, Young & Davis, 1984.

1984). Parental \gt10 phage are cI$^+$ and form turbid plaques whereas the presence of inserted cDNA inactivates the repressor gene generating cI$^-$ phage which form clear plaques. Thus the recombinant phage are distinguished from parental, nonrecombinant, phage by their plaque morphology. By using an E.coli hflA host for the amplification of a \gt10 library one can select against the parental phage (Hoyt et al, 1982). On E.coli hflA hosts cI$^+$ phage lysogenize efficiently and plaque formation is supressed. However, cI$^-$ phage have the same plaque forming efficiency on E.coli hflA$^+$ or E.coli hflA$^-$ host strains. Thus the amplification of \gt10 cDNA libraries on an E.colihflA host eliminates the nonrecombinant phage from the library and produces large recombinant plaques of uniform size and shape for screening with nucleic acid probes.

1:5:1:2 **\gt11**: The expression vector \gt11 produces a temperature sensitive repressor (c1857), which is inactive at 42^{o}C, it also contains the amber mutation (S100) which renders it lysis defective in E.coli host cells which lack the amber supressor supF; the structure of this vector is shown in figure 1.2. When incubated at 42^{o}C using an E.coli supF host the \gt11 vector is capable of producing a polypeptide specified by the inserted cDNA therefore one can screen cDNA libraries with antibodies directed against the inserted DNA polypeptide sequences (Young & Davis, 1983) Up to 8.3 Kb pairs of cDNA can be ligated into the unique EcoR1 site of the \gt11 DNA within the structural gene for B-galactosidase. It is because of this insertion site, 53bp upstream from the B-galactosidase translation termination codon and within the Lac Z gene, that the vector is capable of expressing foreign DNA sequences as B-

Fig 1.2

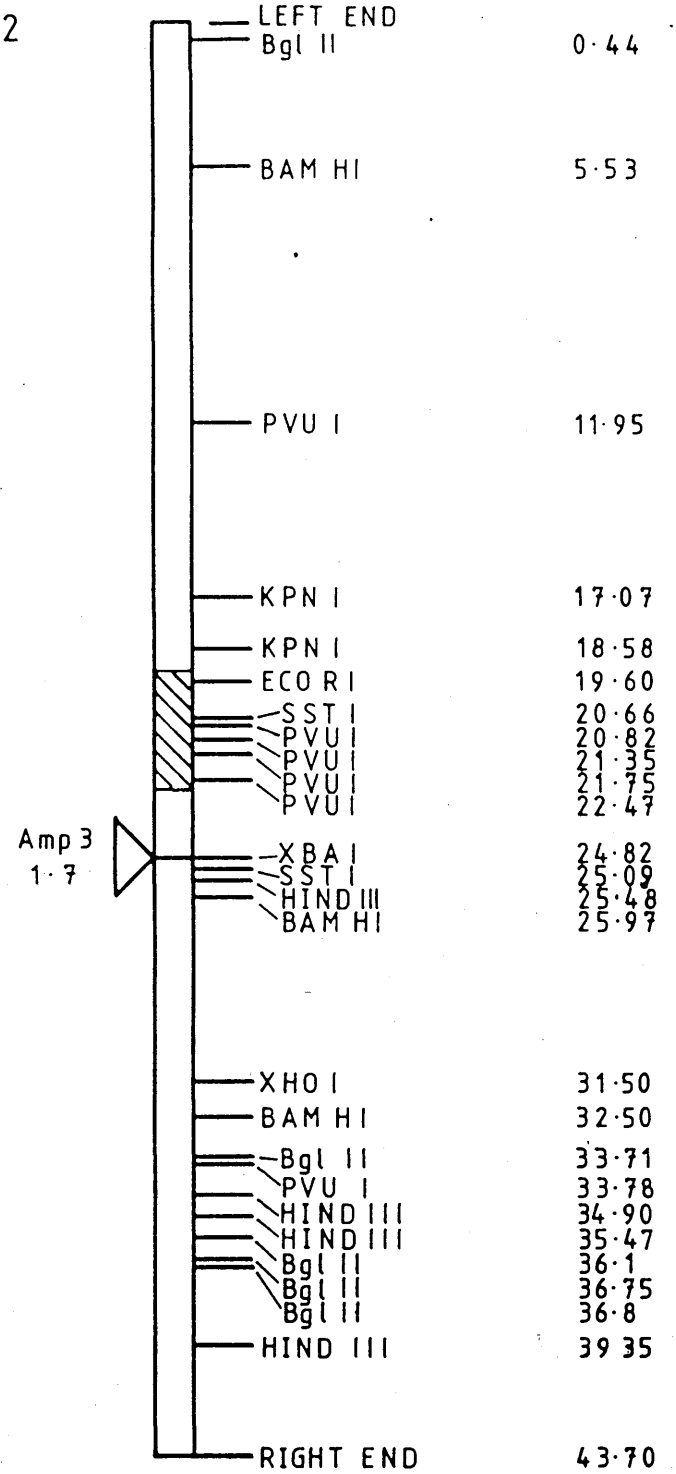


Figure 1.2: Map of \gt11 with restriction endonuclease cleavage sites in kilobase pairs. The transcriptional orientation of lacZ is indicated by the horizontal arrow. The unique EcoR1 site is situated within the structural gene for B-galactosidase. Foreign DNA inserted at this site is expressed as a B-galactosidase fusion protein. Reproduced from Huynh, Young & Davis, 1984.

galactosidase fusion proteins. Also the positioning of the insertion site within the LacZ gene enables recombinant phage to be distinguished from the non recombinant, parental, phage by their morphology. Using a E.coli SupFlacI^{+} host strain in the presence of the lac operon inducer, IPTG (Isopropyl B-D-thiogalactopyranoside), and the B-galactosidase substrate, X-gal, parental \gt11 phage produce blue plaques whereas recombinant phage plaques are colourless (Young & Davis, 1983). To overcome the problems associated with the production of foreign proteins that may be toxic to the host, E.coli which produce large amounts of lac operon repressor are used. The lac repressor prevents lac Z directed expression of the fusion product until the lac repessor is inactivated by the addition of IPTG. In this way detectable amounts of fusion protein can be made before the IPTG is added. Another problem associated with fusion proteins in E.coli is their stability, for this reason lon mutant hosts are used as a deficiency in lon protease increases the stability of the fusion protein and thus facilitates the antibody screening procedure.

1:5:1:3 **\Amp3**: The cI temperature sensitive 'phage \Amp3 is identical to \gt11 except for the insertion at the Xba1 site of \gt11 of a 1.7 Kb fragment from pBR322 (the B-lactamase gene) which codes for ampicillin resistance (Young & Davis, 1983a&b).

CHAPTER 2

SEROLOGICAL STUDIES OF TROPHOBLAST MEMBRANE COMPONENTS

2:1 INTRODUCTION

Trophoblast-specific antigens have been identified xenogeneically using heterologous antisera and monoclonal antibodies (Faulk et al 1978, McIntyre & Faulk 1979, Loke et al 1980, Sunderland et al 1981, Johnson et al 1981, Brown et al 1983). Also investigations of abortion prone couples have indicated that inadequate maternal recognition of allotypic fetal trophoblastic-lymphocyte cross reactive (TLX) antigens leads to pregnancy failure (McIntyre & Faulk 1982a). Allotypic TLX antigens would seem to be to a stimulus to signal the mother to mount a blocking response to inhibit allograft rejection reactions but this system has not been studied in detail and antibodies against TLX have not been detected in the maternal sera.

While studying trophoblast-lymphoblast interactions Dr M. Davies in this laboratory developed a passive haemagglutination assay, using preparations of human syncytiotrophoblast microvilli, to determine the titre of xenogeneic anti-syncytiotrophoblast sera. In pilot experiments he detected anti-trophoblast agglutinins in term maternal sera. The aim of the work in this chapter was to discover whether these agglutinins were maternal antibodies directed to alloantigenic or isoantigenic trophoblast antigens. Firstly I had to establish the haemagglutination assay techniques in my own hands. Several syncytiotrophoblast microvilli preparations were assayed against serial dilutions of different term maternal sera and control sera. Then an attempt was made to absorb the agglutinins from the sera with potential antigens. Finally, several different antibody

purification systems were used to determine whether the agglutinins co-purified with the maternal antibodies, either IgG or IgM.

The passive haemagglutination assay was used in preference to the more sensitive ELISA assay because there was concern that Fc receptors present on the syncytiotrophoblast plasma membrane might lead to false positive results in the ELISA assay.

2:2 MATERIALS and METHODS

2:2:1 Preparation of solubilised placental plasma microvillous membrane proteins

The microvillous membrane vesicles were prepared according to the method of Smith, Brush and Luckett (1974). Briefly, a fresh term placenta from which cord and membranes had been removed was washed rapidly in ice-cold 0.11M calcium chloride followed by ice cold saline 0.9% (w/v). The washed tissue was cut into small pieces, suspended in 0.9% (w/v) saline and agitated for 30 minutes at 4°C. The saline wash was removed and centrifuged at 80xg for 10 minutes. The supernatant was centrifuged first at 10,000g for 5 min then at 100,000g for 30 minutes. The resulting pellet of placental villous membrane vesicles was resuspended in phosphate buffered saline (PBS) pH 7.4 in a tight fitting glass homogenizer and washed by centrifugation at 100000g for 30 minutes. The pellet was solubilized by resuspending in 1% Nonidet NP40 (v/v) 10mM glycine in 3.8 mM Tris HCl (pH9.2) to a final protein concentration of 3mg/ml. This was stirred for 60 minutes at 4°C then centrifuged at 100000g for 60 minutes. The supernatant was dialysed against PBS (pH 7.4) for 18 hours at 4°C. The amount of solubilized membrane present was estimated by determination of the protein content using the Folin method, bovine serum albumin was used as the standard (Lowry et al 1951).

2:2:2 Preparation of protein-coated formalized red blood cells

Formalized red blood cells were prepared using tannic acid (Herbert, 1978). Briefly, washed and packed human red blood

cells (RBC) were prepared by centrifugation of whole blood at 3000g for 5 minutes. The RBC pellet formed was washed with saline (0.15M NaCl) three times then resuspended to 12.5% (v/v) in PBS. The RBCs were then formalized at room temperature by dialysing against formalin for three hours, then the dialysis sac was ruptured and the mixture stirred for a further 18 hours. The formalized RBC (FRBC) were washed and resuspended to 25%(v/v) in PBS and then incubated with an equal volume of tannic acid (0.1 mg/ml) for 15 minutes at 37°C. After washing with PBS the tanned FRBC were resuspended to 25%(v/v) in PBS and incubated for 30 minutes at 37°C with an equal volume of solubilized placental microvillous membrane protein (1 mg/ml). The protein coated FRBC (target cells) were washed three times in PBS containing stabilizing protein and resuspended in the same buffer to a final concentration of 1% (v/v) target cells. The pretreatment of FRBC with tannic acid can cause agglutination of FRBC in the absence of added agglutinin. This effect was prevented by the addition of a protein stabilizer to the final buffer. The optimal concentration of three different stabilizing proteins were determined by titration.

2:2:3 **Passive Haemagglutination Assay**

Sera from patients at term and non-pregnant females and male controls were adsorbed with equal volumes of 25% FRBC for 30 minutes at room temperature. The adsorbed sera were serially diluted in PBS containing the protein stabilizer. To 50ul aliquots of each dilution were added 20ul of 1% target cells and incubated overnight. The agglutinin titre of each serum was determined as the highest dilution that showed agglutination.

2:2:4 **Serum Fractionation**

A column (1.5x30 cm) of AcA34 Sepharose (LKB) was equilibrated in PBS. Samples (1ml) of maternal and control sera were adsorbed with FRBC and passed over the column at a flow rate of 5ml per hour; 1ml fractions were collected. For euglobulin precipitation (Schwick et al 1978), adsorbed sera were dialysed against water at 4°C overnight. The euglobulin precipitates which formed were precipitated by centrifugation and redissolved in PBS to the original volume of serum. The supernatants and euglobulin samples were assayed for agglutinins to placental microvillous membrane proteins and for IgM and IgG.

2:2:5 **Determination of IgM and IgG concentrations in serum fractions**

Radial immunodiffusion is one of the simplest gel precipitation methods for quantitative assessment of protein concentration is (see Thompson, 1977). The gels used were 1mm thick and contained a standard concentration of antibody (anti-IgM or anti-IgG) in 1% agarose buffered with barbitone buffer pH8.6 (1.1% barbituric acid, 7% Na barbitone, 1% calcium lactate). The serum fractions were placed in wells 2cm apart and allowed to diffuse into the gel. The radial diffusion of antigen will continue while the antigen is in excess since soluble immune complexes are formed. When the concentration of antigen is sufficiently reduced an antibody-antigen insoluble complex forms and precipitates. After washing the gels with PBS to remove soluble protein the ring of precipitate can be stained with protein stain (0.2% coomassie blue, 50% methanol, 7% glacial acetic acid) and its diameter measured. The size of the

immunoprecipitation ring increases in proportion to the quantity of antigen present (Mancini et al, 1965). Thus by measuring the precipitate rings for reference dilutions of a standard serum a calibration curve can be obtained.

2:2:6 **Cyanogen-Bromide activation of Sepharose**

Using the method of Porath et al (1967), 12g of sepharose 4B (Pharmacia) was washed with 2-3 volumes of distilled water, drained and suspended in 10ml of distilled water. This was added to 1.2g CNBr dissolved in 20ml distilled water. The pH of the suspension was brought to pH 11.3 with 2M NaOH and kept at this pH by the addition of 2M NaOH and continuous stirring until the mixture equilibrated. It was then poured into a column and washed several times with cold water and finally with 0.1M $NaHCO_3$. Aliquots of the drained and activated sepharose were added to the anti-human immunoglobulins (Seward) and the mixtures stirred for 16 hours at room temperature. Unbound sites were blocked with 1M ethanolamine pH8 (Sigma) for 1-2 hours.To remove non-covalently absorbed protein the antibody:sepharose was washed three times with 1M NaCl then finally washed with PBS until no more absorbance was detected in filtrates.

2:2:7 **Iodination of Antibody**

Purified immunoglobulins were iodinated using a modification of the method of Hunter & Greenwood (1962), in which the final concentration of chloramine-T and NaS_2O_4 were 5ug/10ul and 12ug/10ul respectively. To 5ul of ^{125}I (20MBq) was added 10ul of 0.5M phosphate buffer to bring the pH of the mixture to pH

7.5. To this was added 10ul of the immunoglobulin sample to be iodinated (1mg/ml) and 10ul (0.5mg/ml) of chloramine-T. The mixture was left for 20 seconds then 10ul solution of NaS_2O_4 (1.2mg/ml) and 40ul of 0.12M KI in PBS was added to saturate the solution with cold iodine. The solution was separated into immunoglobulin bound ^{125}I and unbound ^{125}I by passing over a Sephadex G-25 column (Pharmacia), equilibrated using 1mg/ml BSA in PBS. Two peaks of ^{125}I activity were seen, the first is bound ^{125}I and the second the free ^{125}I salt. The fractions of the first peak were pooled and again fractionated to remove any free ^{125}I remaining in the bound ^{125}I peak. The second fractionation column was either sepharose CL-6B or sephadex G-200 (Pharmacia) depending on whether ^{125}I-IgM or ^{125}I-IgG was to be fractionated. Fractions which gave the highest specific binding to their respective anti-HuIg sepharose were used in the radioimmunoassays.

2:2:8 **Radioimmunoassay**

Serum antibodies, IgM and IgG, were assayed indirectly by competition with ^{125}I-HuIg for binding to anti-HuIg sepharose conjugate in 1mg/ml BSA in PBS. Aliquots (20ul) of each dilution were added to 50ul of anti-HuIg sepharose. To this was added 20ul of ^{125}I-HuIg (5000 cpm). This was shaken overnight at room temperature and the unbound ^{125}I-HuIg supernatant was removed after centrifugation at 4000g for 15 minutes. The sepharose was washed 3 times with 2ml PBS and recentrifuged (4000g 15 minutes). The sepharose bound fraction of ^{125}I-HuIg was counted on an LKB minigamma counter.

2:2:9 **Removal of IgM and IgG from maternal and control sera**

After adsorption with FRBC several maternal and control sera were adsorbed overnight with an excess amount of anti-HuIg sepharose, specific for either human IgG or IgM. After centrifugation, at 4000g for 15 minutes, the supernatants were assayed for the presence of agglutinins with the passive haemagglutination assay. The supernatants were also assayed for the presence of residual IgM or IgG using radial immunodiffusion plates and radioimmunoassays.

2:3 RESULTS

2:3:1 Optimisation of the sensitivity of the passive haemagglutination assay

The sensitivity of targets is affected by the method of tanning the FRBC (Herbert 1978). Placental microvillous targets differing only in their tanning procedures were tested using antisera against the microvillous protein. The results (table 2.1) show that maximum sensitivity was achieved by tanning the cells in a 25% (v/v) solution of FRBC using 0.3-0.4 mg/ml tannic acid. Since the stabilising protein used may also affect the sensitivity of the assay, different proteins (FCS, BSA and NRS) were assayed to find optimal concentrations to stabilize target cells. The results (table 2.2) show that all three sources of proteins can stabilize targets. Although BSA increased the titre of the assay 10-fold compared with FCS, the increase in agglutinin titre for term maternal sera was accompanied by an increase in control sera agglutination. Thus the difference in titre between maternal and control sera was not greatly increased by the use of BSA for stabilisation in place of FCS. NRS was found to be unsatisfactory for use as a stabilizer as there was difficulty in determining the agglutin$\tilde{x}$ end point when titering maternal and control sera.

2:3:2 Titres in maternal sera

Maternal and control sera were assayed against four different placental microvillous targets (T10-T13). The results (table 2.3) show that the maternal sera have consistantly higher agglutinin titres than the control sera. Maternal and control

TABLE 2.1

Target number	% FRBC	Tannic acid mg/ml	Agglutinin titres* log_2
1	25%	0.1	19
2	25%	0.2	18
3	25%	0.3	20
4	25%	0.4	20
5	25%	0.5	16
6	5%	0.1	16

* The agglutinin titres are minus the background titre. The mean background titre was 8 with a range of 7-9

Table 2.1: Placental microvillous protein targets differing only in their tanning procedure were tested for their agglutibility using rabbit antisera against placental microvillous protein. The sensitivity of the targets as shown by the agglutinin titres is effected by the different tanning procedures.

TABLE 2.2

Stabilising protein	percentage protein to stabilize 1% targets	number of expts	Agglutinin titres log 2 maternal term sera mean	range	human male sera mean	range
FCS	1.5%	7	8	6-10	4	3-5
BSA	0.2%	7	10	8-13	5	4-6
NRS*	3.0%	-	-	-	-	-

Table 2.2: Different proteins were assayed to find the optimal percentage of each protein needed to stabilise the targets. The effect of the different protein stabilisers on the agglutinin titres for term maternal and male sera is shown. The mean and range of agglutinin titres for both term maternal and male sera differed depending on the stabilizer protein used.

* NRS this protein though capable of stabilizing the targets was found to be unsatisfactory as difficulty in determining agglutinin end-points was experienced.

TABLE 2.3

		Agglutinin titres Log_2			
	Targets	T10	T11	T12	T13
maternal serum	40136	6	6	7	7
"	40147	7	7	8	7
"	40754	8	8	8	7
"	36350	7	7	8	7
"	22525	7	7	7	7
"	28572	7	7	7	7
"	40250	8	8	7	6
male serum	A	4	5	3	3
"	B	3	3	3	3
"	C	4	4	4	3
"	D	4	4	4	4
"	E	4	4/5	3	3
"	F	3	3	3	3
"	G	4	6	5	3

Table 2.3: Agglutinin titres of maternal term and human male, control, sera assayed using four placental microvillous membrane protein targets; the stabilising protein being 1% FCS.

sera had similar background titres of agglutinins against control targets coated with human serum albumin. The agglutinin titre of each maternal serum was assayed against placental microvillous membrane samples from the same pregnancy and from different pregnancies. The results (table 2.4) showed that the agglutinin titres were independent of the relationship between individual sera and the samples of placental microvillous membrane.

Adsorption of maternal sera with an excess of packed paternal or fetal RBC and paternal white blood cells was ineffective in adsorbing out the agglutination activity of maternal sera tested (table 2.5).

2:3:3 Partial characterisation of the agglutinins

The passive haemagglutination assay was used to compare the agglutinin titres of maternal sera euglobulin precipitates and their supernatants (table 2.6). The majority of the agglutinins and the serum IgM were present in the euglobulin fraction. Three of the four sera tested showed no detectable agglutinins or IgM in the supernatant.

Gel filtration on AcA 34 showed that the maternal serum fractions with maximum agglutinin titre (15-18ml; figure 2.1) correlated with the peak of IgM concentration (12-20 ml; figure 1). The actual agglutinin peaks differed for individual maternal sera when assayed with different placental microvillous targets (T11-T13; figure 2.1). Comparing the fractionated term maternal and control sera showed that, though control agglutinins appeared in the same range of fractions, there was a considerably higher titre of agglutinins present in the term maternal sera (figure 2.1).

TABLE 2.4

		Agglutinin titres $\log_2$				
	Targets	T2	T3	T4	T6	T8
Maternal serum	2	10	9	9	8	8/9
"	3	9	10	9	8	9/10
"	4	9	10/11	8/9	8	10
"	6	9	9/10	7	7/8	8/9
"	8	9/10	10	9/10	8/9	10

control titre: mean=5, range=4-6

Table 2.4: The agglutinin titre of maternal sera assayed ,in duplicate, with targets made from placental samples from the same source (underlined) were compared with the agglutinin titres of the same sera assayed with targets made from placental samples from different sources. The protein stabiliser used was 1% FCS.

TABLE 2.5

		Blood groups			Adsorbtion:	AGGLUTININ TITRES LOG 2 None			Paternal RBC			Fetal RBC			Paternal WBC		
		mother	baby	father	Targets:	T11	T12	T15	T11	T12	T15	T11	T12	T15	T11	T12	T15
Maternal serum	28572	A+	A-	O+		7/8	8	8	7	6	6	4/5	5/6	4	7	8	8
"	40353	O+	A+	A+		7	6/7	8/9	5	4/5	5	5	5	5	7	6	8
"	40142	A+	O+	O-		8	6	7	6	6	6	5/6	4	6/7	7	5/6	7
"	40250	O+	O+	O-		8	8	9	7	7	7/8	7	6/7	7/8	8	8	8
"	40014	O+	O+	O+		7	6	7	6	6	6/7	6/7	5/6	6	7	6	7
"	40754	O+	O+	B+		9	8	8	8	7	7	6	6/7	6	8	8	8
"	40136	O+	O+	O+		8	7	8	7/8	7	7	NT	NT	NT	8	7	7
"	40147	B+	O+	A+		8	8	9	7	6	6	7	6	6	7/8	7	7/8
CONTROL	SERUM					3	4	4									
"	"					2/3	3	3									

Table 2.5: The agglutinin titres of term maternal sera were assayed in duplicate with three different targets after adsorbing the sera with paternal and fetal red blood cells. Details of ABO and Rh-D blood groups for the maternal, paternal and fetal groups are also shown.

TABLE 2.6

Maternal Sera	serum fractions	Agglutinin titres Log_2 using targets T12	T13	IgM mg/l
40250	Euglbn	6	4	1000
	Supnt	2	2	117
40136	Euglbn	6	4	880
	Supnt	<1	<1	78
40147	Euglbn	6	6	880
	Supnt	<1	<1	39
40148	Euglbn	4	6	660
	Supnt	<1	<1	23
CONTROL	Euglbn	4	3	ND
	Supnt	1	1	ND

Table 2.6: The agglutinin titres of term maternal sera were assayed using two different targets after the sera had been fractionated by euglobulin precipitation into supernatant and euglobulin fractions. Both fractions were assayed for IgM concentration using radial immunodiffusion. The assay was calibrated with standard human serum (Seward).

ND= Not determined

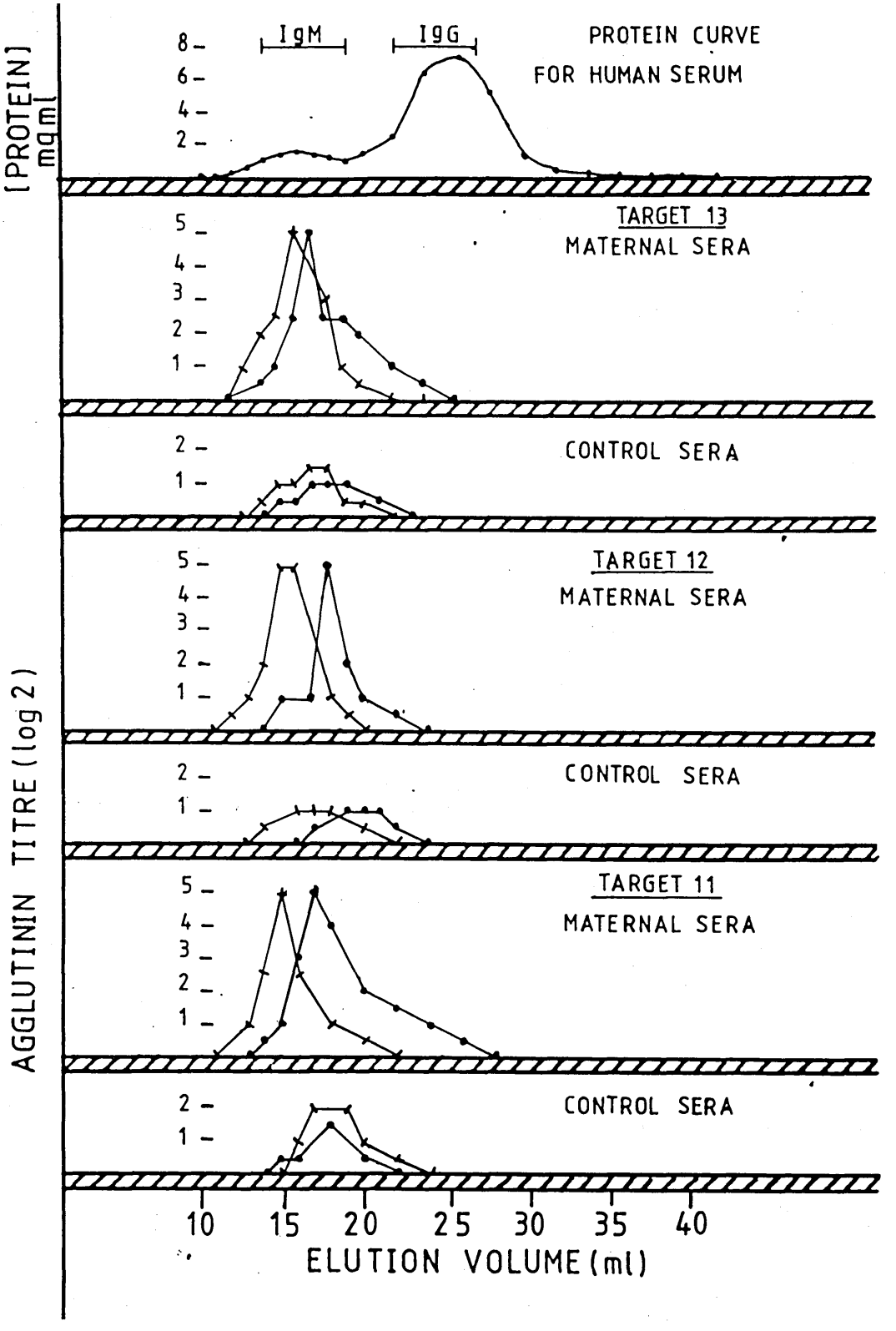


Figure 2.1: Gel fractionation on AcA 34 shows that the serum fractions with the maximum agglutinin titres for both term maternal and control sera appear in the first peak of the serum proteins (12-20 ml). The serum fractions showing maximum IgM activity are 14-19 ml whereas IgG appears in serum fractions 22-28 ml.

In a further experiment to characterize the agglutinins serum agglutinin titres were determined after IgM or IgG had been removed from the sera by adsorption with sepharose coupled with anti-human IgM or anti-human IgG. Maternal sera adsorbed with unconjugated sepharose was used as a absorption control. The efficiency of antiboby adsorption was determined indirectly by a radioimmuno-competition assay. The results of figures 2.2a & 2.2b show that after adsorption with anti-HuIg sepharose the levels of antibody remaining were below the detection level of the assay as no competition for the binding of ^{125}I-Ig was detected. Thus less than 0.5mg/l of IgM remained after adsorption with anti-IgM sepharose (figure 2.2a) and less than 2.5mg/l of IgG after adsorption with anti-IgG sepharose (figure 2.2b). Sera adsorbed with unconjugated sepharose displayed the same competition profile as unabsorbed sera (figures 2.2a&b). Quantitative results were obtained by radial immunodiffusion. When compared with sera adsorbed with unconjugated sepharose a 6000 fold reduction of both IgM and IgG concentrations was seen after adsorption with anti-IgHu sepharose (tables 2.7 & 2.8). The results tables 2.7 & 2.8 also show that no significant difference was seen between the agglutinin titres for maternal sera after adsorption with anti-IgM sepharose or anti-IgG sepharose when compared with the agglutintin titres of sera adsorbed with control sepharose. Thus as the maternal sera agglutinin titre remains after the successful removal of IgM or IgG from the maternal sera the agglutinin seen is not an antibody of the IgM or IgG class.

Fig 2.2

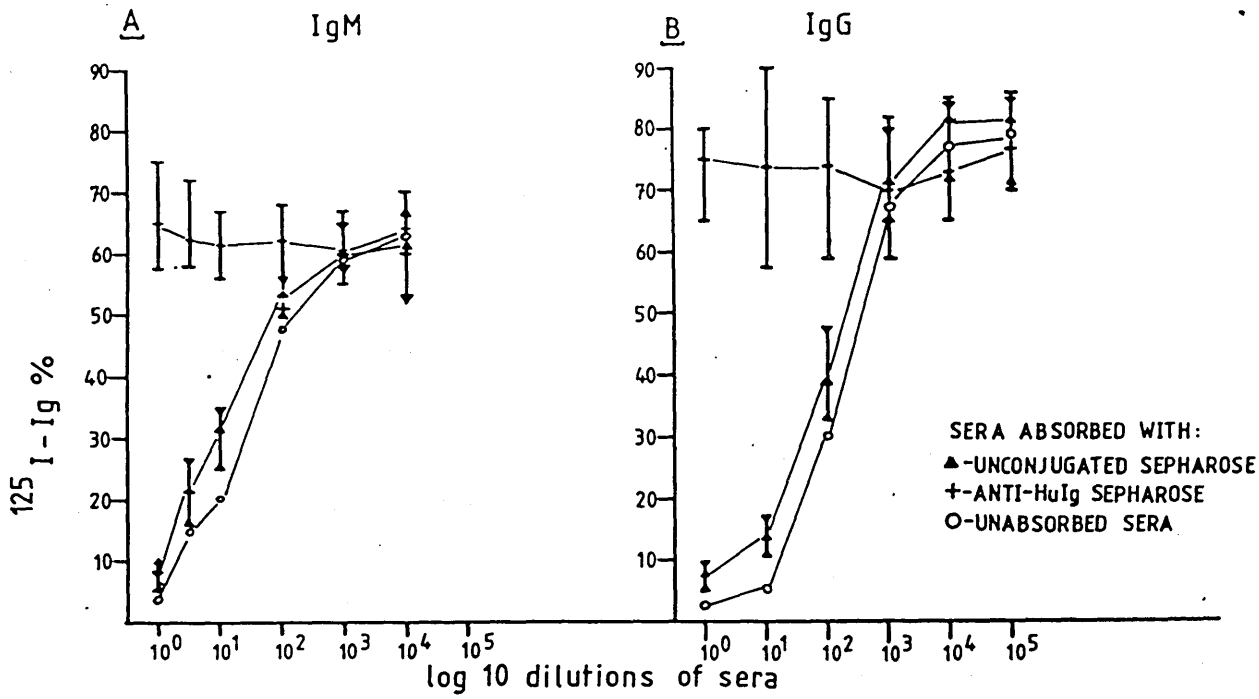


Figure 2.2: This shows the percentage bound ^{125}I-HuIg when in competition with maternal sera for binding to anti-HuIg sepharose.

a. Shows the mean and range percentage bound ^{125}I-IgM to anti-IgM sepharose (listed in table 6) before and after adsorption with either anti-IgM sepharose or unconjugated sepharose. The sensitivity of this competition assay, was 0.5mg/l of IgM.

b. Shows the mean and range percentage bound ^{125}I-IgG to anti-IgG sepharose (listed in table 7) before and after adsorption with either anti-IgG sepharose or unconjugated sepharose. The sensitivity of this competition assay was 2.5mg/l of IgG.

TABLE 2.7

Maternal serum	adsorption treatment	Agglutinin titres $\log_2$ TARGETS T5	T7	T9	IgM mg/l
40014	untreated	ND	ND	ND	
	control seph.	8	8	8	660
	anti-IgM seph.	10	10	11	0.07
40148	untreated	ND	ND	ND	
	control seph.	8	9	8	880
	anti-IgM seph.	8	9	8	0.18
40142	untreated	ND	ND	ND	
	control seph.	8	9	8	880
	anti-IgM seph.	8	9	8	0.09
40163	untreated	11	12	10	
	control seph.	11	12	11	1170
	anti-IgM seph.	11	11	12	0.23
22525	untreated	11	12	10	
	control seph.	10	11	12	1170
	anti-IgM seph.	11	12	12	0.12
28572	untreated	11	10	12	
	control seph.	11	12	11	880
	anti-IgM seph.	11	11	12	0.18
29605	untreated	12	12	13	
	control seph.	11	11	12	880
	anti-IgM seph.	11	11	13	0.18
control serum	untreated	6	6	5/6	
	control seph.	5	5	6	1170
	anti-IgM seph.	5	6	6	0.10

Table 2.7: The agglutinin titres and IgM concentration of maternal sera, after adsorption with either anti-IgM sepharose or control sepharose were assayed. The stabilising protein used in the agglutinin assays was 0.2% BSA.

ND= Not determined

TABLE 2.8

Maternal serum	adsorption treatment	Agglutinin titres $\log_2$ TARGETS T10	T12	T13	IgG mg/l
40100	untreated	12	11	10	
	control seph.	8	7	7	7120
	anti-IgG seph.	8	7	7	0.7
30686	untreated	10	9	9	
	control seph.	8	8	8	7120
	anti-IgG seph.	8	8	8	1.4
40142	untreated	9	9	9	
	control seph.	8	8	8	6000
	anti-IgG seph.	8	8	8	1.2
40014	untreated	7	7	7	
	control seph.	7	7	7	6000
	anti-IgG seph.	7	7	7	1.2
40148	untreated	ND	10	9	
	control seph.	8	9	8	10000
	anti-IgG seph	8	9	8	2.0
control serum	untreated	5	5	5	
	control seph.	4	4	4	8500
	anti-IgG seph.	4	4	4	1.6

Table 2.8: The agglutinin titres and IgG concentrations of maternal sera after adsorption with either anti-IgG sepharose or control sepharose. The stabilising protein used in the agglutination assay was 0.2% BSA.

ND= Not determined

2:4 DISCUSSION

Using the passive haemagglutination assay, agglutinins against plasma membrane proteins of the syncytiotrophoblast microvilli were detected in term maternal sera at agglutinin titres above those of non-pregnant control sera (table 2.3). In an attempt to characterise the agglutinins term maternal and control sera were fractionated on an AcA 34 column, the agglutinins of both the maternal and control sera were found to elute, together with IgM, in the macroglobulin fraction of serum having a molecular weight greater than 350 000 daltons (figure 2.1). The agglutinins were also found to precipitate in euglobulin precipitation experiments which precipitated IgM (table 2.6). As the agglutinins co-elute and precipitate in the IgM fraction of serum it was thought that they were IgM, though an IgG antibody:antigen complexes could also have mw of greater than 350Kd. Thus the agglutinins were tested to see whether they could be adsorbed with anti-IgM or anti-IgG (figures 2.2a&b). The term maternal sera showed the same agglutinin titres after either adsorption with anti-IgM sepharose, anti-IgG sepharose or unconjugated sepharose. Since the anti-globulin sepharoses were very effective at specificly adsorbing either IgM or IgG, these results indicate that the maternal agglutinins are not an antibody of either the IgM or IgG class.

This study indicates that the agglutinin response seen in term maternal sera is not an antibody response against plasma membrane proteins of human syncytiotrophoblast microvilli. It can be speculated that the agglutinins seen in term maternal sera may be increased levels, due to pregnancy, of agglutinins found in

all sera. The control sera agglutinins properties parallel those of maternal agglutinins, the agglutinins appearing in the same serum fractions, after gel fractionation and are shown to be components of euglobulin precipitates. The origin and nature of this agglutinin response in term maternal sera to the syncytiotrophoblast microvilli is unclear but the agglutinin has no specificity for individual placenta since sera show similar agglutinin titres against different preparations of placental microvillous membranes.

CHAPTER 3

PREPARATION AND ANALYSIS OF mRNA FROM SYNCYTIOTROPHOBLAST AND CYTOTROPHOBLAST TISSUE

3:1 INTRODUCTION

The aim of the work described in this chapter was to prepare and analyse placental mRNA populations. This was an essential step before cDNA could be made or cloned (chapter 4). The guanidinium thiocyanate method of Chirgwin *et al* (1979) was used to isolate RNA from the placental cytotrophoblast or syncytiotrophoblast tissue. The mRNA preparations were assayed for protein synthesis by *in vitro* translation using rabbit reticulocyte lysate and the translate visualised on SDS-PAGE gels.

As PLAP can be purified from placental microvilli in mg amounts it was hoped that its mRNA would be detectable in the placental syncytiotrophoblast mRNA. Thus the detection and specific immunoprecipitation of PLAP and other placental proteins from the *in vitro* translated polypeptides of syncytiotrophoblast mRNA was attemped with both polyclonal and monoclonal anti-PLAP sera. The *in vitro* translation of syncytiotrophoblast and cytotrophoblast mRNA populations enabled a comparison to be made between the placental polypeptides synthesised by both. This was of some significance since it has emerged that the monoclonal antibody H315 which stains both syncytiotrophoblast and cytotrophoblast tissue is directed against PLAP (P.M. Johnson personal communication). Thus PLAP may also be detected in cytotrophoblast mRNA *in vitro* translated polypeptides.

The cytotrophoblast mRNA was prepared from the chorionic plate of the placenta. This cytotrophoblast has been shown by immunohistology to express HLA class I determinants and it was hoped that mRNA for these determinants would be detected

As well as the *in vitro* translation of mRNA populations DNA probes were also used to screen the placental mRNA by dot hybridisation. As part of the amino acid sequence of PLAP is known, oligonucleotide sequences of PLAP could be deduced. The chemically synthesised PLAP oligonucleotides were used to screen placental syncytiotrophoblast and cytotrophoblast mRNA in search of PLAP mRNA. As a control, for the specificity of the PLAP oligonucleotide, an hPL oligonucleotide of the same length and a similar base composition was also used. The mRNA dot blots were also screened with a cDNA probe (pDP001) for the HLA class I sequences.

3:2 MATERIALS and METHODS

3:2:1 Preparation of placental RNA

To minimise ribonuclease activity during the preparation of placental poly(A^+) RNA, sterile disposable plastic-ware was used wherever possible as it is essentially free of ribonucleases (RNase). All solutions were prepared using chemicals reserved for work with RNA and were treated with 0.1% diethylpyrocarbonate (DEPC Sigma) overnight and autoclaved. As DEPC is unstable in the presence of Tris buffers these solutions were just autoclaved.

Syncytiotrophoblast and cytotrophoblast RNA were extracted from human term placentae. Syncytiotrophoblast RNA was also extracted from the placentae of premature births (30 weeks onwards) and from first trimester clinical abortions (10-14 weeks). To protect the RNA from degradation by ribonucleases a solution of guanidinium thiocyanate and 2-mercaptoethanol was used (Chirgwin, et al 1979). The protocol used for the extraction of the RNA was an adaptation of the method of Fiddes & Goodman (1979). Fresh human placentae were washed in saline to remove blood. The syncytiotrophoblast tissue obtained from the microvillous membranes was cut into small pieces and RNA was either extracted immediately or the tissue was snap frozen for later use. Cytotrophoblast tissue was obtained from the chorionic plate. This material was either snap frozen for later use or washed in trypsin for an hour to remove any contaminating material (Khalaf, 1985; Khalaf et al, 1985a&b). After the trypsin treatment the tissue was snap frozen. With the aid of a blender 10g of fresh or snap frozen tissue was dissolved in 200ml of 4M

guanidinium thiocyanate in 50mM Tris HCl pH 7.5, 10mM EDTA , 5% 2-mercaptoethanol. The solution was centrifuged at 10,000 rpm for 10 minutes in a Beckman JA-14 rotor. The supernatant was adjusted to 2% sarcosyl then heated at 65°C for 5 minutes. After adding 0.1g of caesium chloride per ml of the solution, it was layered over 5ml cushions of 5.7M caesium chloride in 10mM EDTA and centrifuged at 25 000 rpm for 16 hours at 20°C in a Beckman SW28 rotor. The supernatant was removed by aspiration to leave the RNA pellet which was transferred into sterile plastic tubes and dissolved in 10mM Tris HCl (pH 7.5), 1mM EDTA, 5% sarcosyl and 5% phenol. Then the RNA was extracted twice with an equal volume of phenol/chloroform and once with an equal volume of chloroform. The RNA was precipitated from the aqueous phase with two volumes of ethanol in the presence of 0.2M sodium acetate (pH 5.5) overnight at -20°C. After centrifugation the RNA pellet was washed in cold 95% ethanol and recentrifuged. The RNA pellet was then dried and dissolved in sterile DEPC-treated distilled water and stored at -70°C until needed.

3:2:2 Agarose Gel Electrophoresis

To determine the purity and quality of the placental RNA 0.1-1.0ug samples were diluted to 20ul in the final sample buffer, FSB (5xFSB=0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) and electrophoresed through a 1.2% agarose gel in TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA) at 50mA for 1 hour. After staining the gel for 30 minutes with the fluorescent intercalating dye ethidium bromide the gel could be examined in ultraviolet light. RNA of a high quality and purity shows three distinct ribosomal bands.

3:2:3 Quantitating the RNA

To determine the amount of RNA in a preparation which was without significant amounts of contaminants, as determined by agarose gel electrophoresis, spectrophotometric measurement of the amount of UV irradiation absorbed by the bases was quantified. Readings are taken at 260nm and 280nm. From the 260nm reading the amount of RNA in the sample can be calculated as an OD_{260} of 1 corresponds to 40ug/ml of RNA. Pure preparations of RNA have OD_{260}/OD_{280} of 2.0; protein or phenol contamination reduce this value.

3:2:4 Preparation of polyadenylated RNA from total placental RNA

Chromatography on oligo (dT) cellulose (Aviv & Leder, 1972) was the method used to separate the polyadenylated RNA [poly (A^+) RNA] from the total RNAs. A small volume of oligo (dT) cellulose (BRL) was equilibrated in sterile RNase-free high salt buffer (0.5mM KCl, 20mM Tris HCl pH 7.5, 1mM EDTA). A 1ml column was poured into a plastic pipette plugged with non-absorbent DEPC-treated cotton wool. The column was washed with 5 volumes of high salt buffer. After adjusting the RNA sample to 1mM EDTA it was heated to 65°C for 5 minutes, cooled, and adjusted to 0.5M KCl. The RNA sample was then diluted to at least 10 times the column volume with high salt buffer and applied to the column. The flow-through was collected and reapplied to the column. After binding the RNA the column was washed with several column volumes of high salt buffer to remove the bound poly(A^-) RNA. To elute the poly(A^+) RNA the column was washed with sterile DEPC-treated distilled water. The eluted poly(A^+) RNA was adjusted to 1mM EDTA, heated to 65°C for 5 minutes, cooled, adjusted to 0.5M

KCl, diluted with high salt buffer and reapplied to the column for one further cycle of chromatography. The peak poly (A^+) RNA fractions were precipitated in 2 volumes of ethanol in the presence of 0.2M sodium acetate at -20°C overnight. After centrifugation in a microfuge for 15 minutes the poly (A^+) RNA pellet was washed in 95% ethanol and recentrifuged. The resultant pellet was dried and then dissolved in sterile RNase-free water and stored at -70°C.

3:2:5 In vitro translation of placental RNA

Placental RNA samples were translated in the presence of ^{35}S-methionine (NEN) using a rabbit reticulocyte lysate cell free system (Amersham International plc.). RNA translation was carried out in a mixture containing 80% (v/v) lysate with ^{35}S-methionine to give a final concentration of 1uCi/ul. The samples were incubated at 30°C for 90 minutes. An equal volume of a RNase solution (RNase 2.5ug/ml, 40mM EDTA, 1% methionine) was then added and the samples incubated for a further 15 minutes. The samples were assayed for the incorporation of ^{35}S-methionine into synthesised protein products by TCA-precipitation and SDS-PAGE gels.

3:2:6 Analysis of translational protein products using TCA precipitation

The percentage incorporation of ^{35}S-methionine into the translational products was assayed by TCA-precipitation. Samples of the cell free translation mix were spotted onto filter discs and dried. The discs were washed 3 times with ice cold 10% TCA to precipitate protein and hence the incorporated ^{35}S-methionine.

The discs were then washed in methanol, dried and counted using liquid scintillation. Duplicate sample discs, which had not been TCA precipitated, and blank discs were counted to get the total reaction counts (^{35}S-methionine present in the translation mix) and the radioactive background counts.

3:2:7 Analysing translational protein products using Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The ^{35}S labelled translational protein products were resolved on either 9% or 5-15% SDS-PAGE gels, table 3.1 (Laemmli, 1970). To the translation samples was added 25ul SDS-PAGE sample buffer (30% glycerol, 6% SDS, 15% 2-mercaptoethanol, 150mM Tris HCl pH 6.7, 0.05% bromophenol blue). The samples were boiled for 2 minutes and loaded on SDS-PAGE gels. The gels (15x20x0.2cm) were electrophoresed at 40mA for 4 hours then soaked for 1 hour in 10% acetic acid/50% methanol to precipitate and fix the proteins in the gel. After fixing, gels were washed in 7% acetic acid then enhanced for autoradiography by soaking for 1 hour in EN^{3}HANCE (NEN). Following impregnation of the enhancing solution, the fluorescent material in the gel was precipitated by washing the gel in tap water for 1 hour. The gel was then dried, under vacuum, on Whatman 3MM paper and exposed on X-ray film (Kodak X-Omat S) and developed, using Kodak developing fluids.

3:2:8 Immunoprecipitation Assay

The immunoprecipitation of lysate translated proteins was carried out using the procedure of Kraus et al (1981 & 1982). Firstly, the translated protein sample was diluted to 200ul with buffer A (150mM NaCl, 10mM EDTA, 0.5% Triton X-100, 2% methionine

RESOLVING GEL	a 9%	b 5%	15%
Acrylamide:bis (30:0.8)	10.5ml	3.0ml	9.0ml
Resolving buffer 18.15g Tris] to 100ml 0.4g SDS] H_2O pH 8.9	9.0ml	4.5ml	4.5ml
Distilled H_2O	16.2ml	10.5ml	-
50% Glycerol	-	_	4.35ml
10% Ammonium Persulphate	225ul	150ul	75ul
TEMED	15ul	15ul	15ul

STACKING GEL	4.5%
Acrylamide:bis (30:0.8)	1.8ml
stacking buffer 5.9g Tris] to 100ml 0.4g SDS] H_2O pH 6.7	3.0ml
Distilled H_2O	7.2ml
10% Ammonium Persulphate	100ul
TEMED	10ul

Running buffer/litre |- 6.3g Tris
4.0g Glycine
1.0g SDS

Table 3.1: Solutions used in the preparation of (a) 9% and (b) 5-15% sodium dodecylsulphate polyacylamide gels.

pH7) and incubated at RT for 30 minutes with 3-5ul of normal rabbit serum. This step removed non-specific antigen complexes. After incubation 30ul of 10% (w/v) formalin fixed Staph A cells (BRL) washed and resuspended in buffer A were added and incubated for a further 10 minutes. The sample was centrifuged and the supernatant added to 5-10ul of rabbit antisera which were specific for human placental proteins. (DAKO & Seward). After incubating at RT for 30 minutes the sample was diluted to 1ml with buffer A, adjusted to 0.1% SDS and incubated at 4°C overnight.

To precipitate the antibody-antigen complex 50ul of 10% (w/v) formalin fixed Staph A cells washed and resuspended in buffer A were added and the mix incubated at 4°C for 15 minutes, then another 50ul of 10% (w/v) formalin fixed Staph A cells were added and the mix incubated for a further 15 minutes. After centrifuging the cell pellet was washed 3 times with 500ul of buffer B (150mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 10mM Tris HCl pH 7.2).The final pellet was resuspended in 50ul of SDS-PAGE gel sample buffer (6% SDS, 15% B-mercaptoethanol, 30% glycerol, 150mM Tris HCl pH 6.7), and heated at 100°C for 2 minutes. After centrifugation the supernatant was analysed on 9% SDS-PAGE gels for the immunoprecipitated proteins.

3:2:9 Competition assay to test specificity of precipitated polypeptides.

The specificity of the polypeptides precipitated by the anti-PLAP sera was demonstrated by competition for immunoprecipitation using PLAP protein extracted from term placenta (prepared in this laboratory) and commercially obtained

PLAP (Sigma). To the lysate translated protein samples were added log_{10} dilutions of PLAP protein in Buffer A. The samples were then diluted to 200ul with Buffer A and the immunoprecipitation assay continued as normal.

3:2:10 Synthetic oligonucliotides

Three synthetic oligonucleotides each 14 bases long were obtained from Pfizer Central Research:

hPL 5`-TTTCCTCCATGTTG-3`

PLAP-1 5`-TC$^{A}_{G}$TTCCA$^{A}_{G}$AAATC-3`

PLAP-2 5`-TC$^{A}_{G}$TTCCA$^{A}_{G}$AAGTC-3`

The purity of these olignucleotides was assessed by gel electrophoresis. The oligonucleotide for hPL was also sequenced at Pfizer to verify its base composition. For the mixed PLAP probes the percentage of each probe was assessed by using different butyl groups for each base:

PLAP-1a	5`-TCATTCCAAAAATC-3`	22%
PLAP-1b	5`-TCGTTCCAGAAATC-3`	28%
PLAP-1c	5`-TCGTTCCAAAAATC-3`	25%
PLAP-1d	5`-TCATTCCAGAAATC-3`	25%
PLAP-2a	5`-TCATTCCAAAAGTC-3`	22%
PLAP-2b	5`-TCGTTCCAGAAGTC-3`	28%
PLAP-2c	5`-TCGTTCCAAAAGTC-3`	25%
PLAP-2d	5`-TCATTCCAGAAGTC-3`	25%

3:2:11 Labelling Oligonucleotides

The oligonucleotides were labelled with T4 polynucleotide kinase (Maniatis et al, 1982). In a total volume of 20ul 1x kinase buffer (5x kinase buffer 0.5M Tris HCl pH 7.6,

0.1M $MgCl_2$, 1mM EDTA, 50mM dithiothreitol, 1mM spermidine) 40ng of the oligonucleotide were phosphorylated with 5 units of T4 kinase (BRL) and 50uCi of [χ^{32}P]ATP (specific activity= 7000Ci/mMole). This mix was incubated at 37°C for 30 minutes then the reaction stopped using 2ul of 0.5mM EDTA. After adjusting the volume to 100ul with TE the labelled DNA was separated from the unincorporated [χ^{32}P]ATP by chromatography on a Sephadex G25 column (8cmx0.5cm).

3:2:12 **Northern blotting using 14mer oligonucleotides as probes**

Under high salt conditions denatured poly (A^+) and ribosomal RNA transfer efficiently to nitrocellulose filters. To remove salt and other contaminants from the RNA (10ug/track) it was extracted with phenol/$CHCl_3$. The aqueous phase was precipitated in two volumes of ethanol at -20°C. After centrifugation in a microfuge for 15 minutes the RNA precipitate was washed with ice-cold 95% ethanol and recentrifuged. The RNA was then denatured with 20ul freshly made glyoxal mix (15% deionised glyoxal [pH>6], 50% dimethylsulphoxide, 10mM sodium phosphate buffer, pH 7) at 50°C for 1 hour. The mix was cooled on ice and 5ul of sample buffer (0.02% bromophenol blue in 50% [v/v] glycerol) was added. The samples were electrophoresed overnight at 30V on a 1.5% agarose horizontal gel (size) using 10 mM phosphate buffer (pH 6.8-7.0) as the running buffer, the buffer recirculating to maintain the pH. Two extra lanes were run on the gel over and above those needed for the blot; one of non-denatured RNA and the other of glyoxal denatured RNA. These two lanes were stained with acridine orange (30ug/ml) to check that the RNA had been denatured and not degraded. The rest of the gel

was blotted by placing on a sheet of Whatman 3MM paper saturated with 20xSSC (1xSSC: 0.15NaCl,15mM Na citrate) from a reservoir. A nitrocellulose filter (Schleicher & Schuell) cut to the same size as the gel and with the positions of the the gel lanes marked with biro was soaked in distilled H_2O, then in 20xSSC and laid over the gel. Sheets of Whatman 3MM paper followed by paper towels were laid over the filter ensuring that the only contact between the 20xSSC reservoir and the paper towels above the gel was through the gel. The transfer of RNA from the gel to the nitrocellulose was complete after blotting overnight (Thomas, 1980). The nitrocellulose was then removed from the gel and baked at 80°C for 3 hours. The filter can now be probed using specific DNA sequences (McMasters & Carmichael, 1977 and Thomas, 1980). The filter was soaked in 6xSSC, then sealed in a bag with 0.5ml/cm^2 of prehybridisation solution (6xSSC,10x Denhardt's buffer, 0.2% SDS, 0.25mg/ml sperm DNA) at 65°C for 1hour and 20°C overnight. The filter was washed in 6xSSC for 2 minutes then sealed in a bag containing the hybridisation solution (6xSSC, 10x Denhardt's buffer, [α^{32}P]-labelled probe $5x10^6$ cpm (Cherenkov) per filter) and hybridized overnight at 20°C. After hybridization the filter was washed in 6xSSC at RT for 5 minutes then at several intermediate temperatures until the dissociation temperature (T_D) of the probe was reached. To estimate T_D for the oligonucleotide probe the equation T_D= 2°C per dA.dT and 4°C per dG.dC nucleotide pair was used, shown in table 1.3. (Suggs et al, 1981). The filter was then autoradiographed using Kodak X-Omat S film.

3:2:13 **Dot hybridisation**

Instead of running the RNA on denaturing gels then transferring to nitrocellulose filters by northern blotting the denatured RNA samples were diluted in 15xSSC and spotted onto dry filters. Both denatured cytotrophoblast RNA samples from term placentae and syncytiotrophoblast RNA samples from various stages of gestation were diluted and spotted onto dry filters. After air drying for 30 minutes the filters were baked at 80°C for 3 hours. They were probed with PLAP or hPL ^{32}P 5` end labelled 14 base oligonucleotides using the prehybridisation, hybridisation and washing conditions described for the northern blots. The MHC class I DNA probe, pDP001, was also used to probe the dot filters. Here the filters were hybridised overnight at 65°C with $5x10^6$ cpm (Cherenkov) per filter. The filters were washed at 65°C under various salt concentrations 5xSSC to 0.1xSSC.

3:3 RESULTS

3:3:1 Agarose Gel Electrophoresis of RNA

1.2% agarose gels were run to determine the purity of the placental RNA. Distinct ribosomal RNA bands (28S, 18S & 5S), with a background smear of heterogeneous mRNA, suggested that the mRNA sample was undegraded. Comparison of "good" and "poor" RNA preparations as determined by RNA agarose gels with the *in vitro* translation of polypeptides showed that no high molecular weight polypeptides were synthesised using RNA shown to be degraded on agarose gels (figure 3.3).

3:3:2 Optimisation of in vitro translation of placental RNA

Several concentrations of total RNA and poly(A^+) RNA, purified from the syncytiotrophoblast, were used to optimise the translation of mRNA. The translation was assessed, using TCA precipitation, by the incorporation of ^{35}S-methionine into translated polypeptides. Figure 3.1 shows that the addition of increasing amounts of both total and poly(A^+) RNA to the *in vitro* translation mix resulted in a biphasic rise of ^{35}S-methionine incorporation. For poly(A^+) RNA this terminated in a plateau with the maximal incorporation at RNA levels greater than 25ng. The maximal incorporation for total RNA was observed with 900ng of RNA. The translation of greater concentrations of total RNA resulted in a decrease in polypeptide synthesis. Thus 36 times less poly(A^+) RNA than total RNA is needed to saturate the system (figure 3.1). This is as expected since only 1-5% of total cellular RNA from eukaryotic cells is message.

Fig 3.1

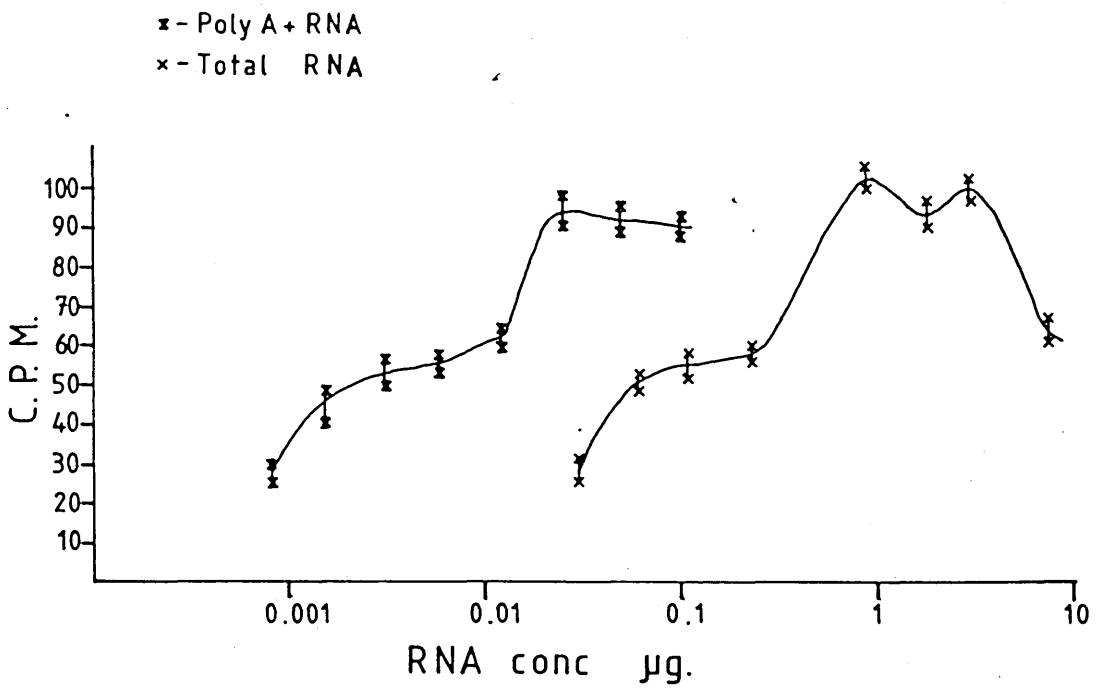


Figure 3.1: The optimum RNA concentrations necessary for <u>in vitro</u> translations was assayed by the TCA precipitable incorporation of ^{35}S-methionine into the synthesised polypeptides.

The RNA optima were also seen with gel electrophoresis of the translated polypeptides. Figures 3.2a & 3.2b show that that the number of polypeptide bands visible increased with increased RNA concentration until saturation of the system with RNA. In figure 3.2a track 4 (30ng total RNA) and figure 3.2b track 3 (0.78ng poly(A^+)) the only placental translated polypeptide visible was of mw 24Kd. This polypeptide was shown by immunoprecipitation to be pre-hPL and represents 15-20% of the translated polypeptides of syncytiotrophoblastic RNA (results shown in the next section). The band of mw 48Kd was endogenous to the lysate system and seen in the absence of RNA. A gradual increase of protein synthesis which duplicates the TCA precipitation results (figure 3.1) is seen in figure 3.2a tracks 5-8 (60-450ng total RNA) and figure 3.2b tracks 4-7 (1.5-12.5ng poly(A^+)).

The maximal protein synthesis using poly(A^+) RNA was seen in figure 3.2b, tracks 8-10 (25-100ng). There was no significant difference in the intensity of the polypeptide bands in these tracks indicating a plateau of ^{35}S-methionine incorporation due to the saturation of polypeptide production at concentrations of poly(A^+) RNA greater than 25ng. For total RNA the greatest protein synthesis was seen with 0.9ug of RNA (figure 3.2a, track 9). Figure 3.2a tracks 10-12 (3.6-7.2ug) show a decrease in translation though more was visible in track 11 (3.6ug) compared with tracks 10 & 12 (1.8-7.2ug). Even with this anomaly, a trend of decreased ^{35}S methionine incorporation was evident when more than 1ug of total cellular RNA was *in vitro* translated. This decrease may be due to the accumulation of translational inhibitors or just due the actual concentration of

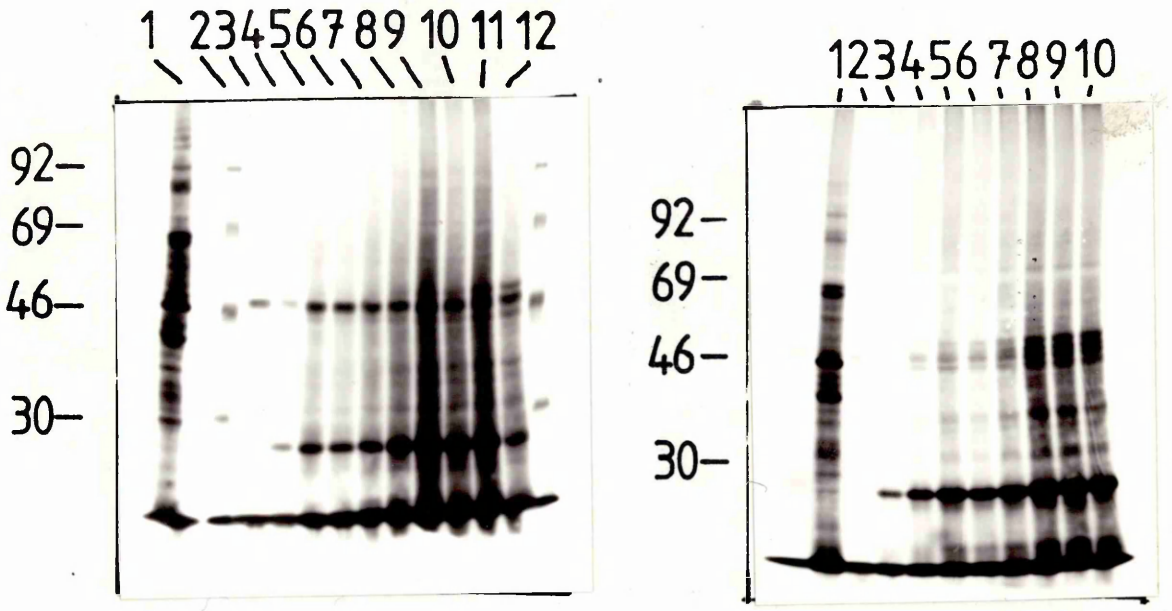


Figure 3.2: Autoradiograph of the ^{35}S-labelled *in vitro* translated products of human placental RNA separated on 9% SDS-PAGE gels. (a) Several concentrations of total (unfractionated) syncytiotrophoblast RNA samples. (b) Several concentrations of poly (A^+) syncytiotrophoblast RNA samples.

(a) 1. Translation positive control, using HSV-1 mRNA
2. mw markers
3. Translation negative control, using H_2O in place of RNA
4. Translation products obtained using 30ng total RNA
5. " " " " 60ng " "
6. " " " " 120ng " "
7. " " " " 240ng " "
8. " " " " 480ng " "
9. " " " " 900ng " "
10. " " " " 1.8ug " "
11. " " " " 3.6ug " "
12. " " " " 7.2ug " "

(b) 1. Translation positive control, using HSV-1 mRNA
2. Translation negative control, using H_2O in place of RNA
3. Translation products obtained using 0.78ng total RNA
4. " " " " 1.5ng " "
5. " " " " 3.0ng " "
6. " " " " 6.0ng " "
7. " " " " 12.5ng " "
8. " " " " 25.0ng " "
9. " " " " 50.0ng " "
10. " " " " 100.0ng " "

ribosomal RNA present.

From these results the placental RNA concentration optima can be determined for the reticulocyte cell free translation system. The optimal concentration of poly (A^+) RNA and total RNA was 2ng/1ul and 75ng/1ul of translation mix.

3:3:3 Analysis of placental syncytiotrophoblast RNA translational products

Before enrichment for poly(A^+) RNA the quality, purity and ability to synthesise polypeptides was determined for total cellular RNA by RNA agarose gels, OD_{260}/OD_{280} ratios and *in vitro* translation. Figure 3.3 shows that RNA preparations which have degraded ribosomal bands have degraded mRNA which is only able to synthesise low molecular weight polypeptides. Thus RNA preparations where ribosomal RNA was shown to be very degraded were not enriched for poly(A^+) RNA.

Figure 3.4 shows a comparison of polypeptides translated before (track 1) and after (track 2) enrichment for poly (A^+) RNA. A more distinct protein profile was seen after poly (A^+) enrichment. The protein profiles of tracks 1 and 2 (figure 3.4) indicate no loss of translated polypeptides after poly(A^+) RNA selection. The RNA which did not bind to the oligo (dT) cellulose but eluted from the column in the high salt wash was able to synthesise translation proteins (track 5), which were comparable to those synthesised by the unfractionated RNA (track 1). After the second cycle of chromatograpy the material eluted, from the oligo (dT) cellulose column, in the high salt wash was unable to synthesise polypeptides (track 6).

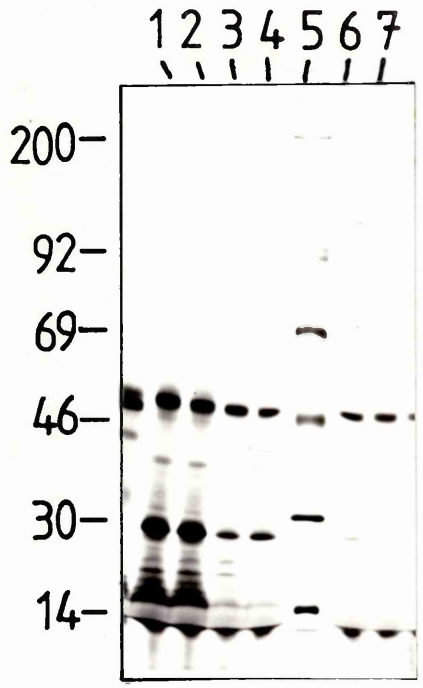


Figure 3.3: Autoradiograph of the ^{35}S-labelled *in vitro* translated products of human placental RNA which were shown to have degraded ribosomal bands, using 1.2% agarose gels. The translational products were separated on a 5-15% SDS-PAGE gel.

1. Slightly degraded ribosomal RNA bands
2. " " " " "
3. Degraded ribosomal RNA bands
4. " " " "
5. mw markers
6. Very degraded ribosomal RNA bands
7. " " " " "

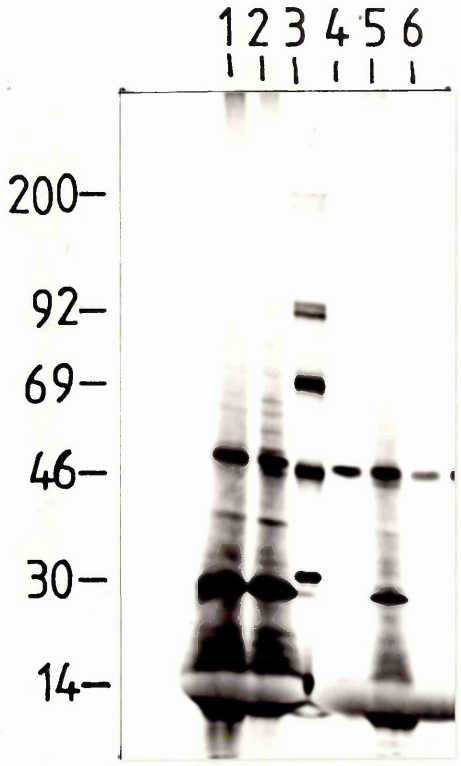


Figure 3.4: Autoradiograph of ^{35}S-labelled *in vitro* translated products of human placental RNA, separated on 5-15% SDS-PAGE gel. 1ug of total RNA and 50ng of (polyA$^+$) RNA were translated in a cell-free translation system in the presence of ^{35}S-methionine. From 50g of placental syncytiotrophoblastic tissue 10ug of (polyA$^+$) RNA could be extracted.
1. Total RNA translation products
2. Poly(A$^+$) RNA translational products
3. mw markers
4. Translation system blank
5. Poly(A$^-$) RNA translational products
6. Final high salt wash material

The ^{35}S label was incorporated into polypeptides which had molecular weights of up to 100Kd (figure 3.5a). The greatest proportion, 15-20%, was present in a 24kD band which was shown to be pre-hPL (results shown in the next section). This profile of the synthesised polypeptides was seen using 12 different full-term and 5 different premature placental mRNA preparations. The translation of RNA from three first trimester placentae gave polypeptide profiles similar to those of term placentae, though the intensity of some of the bands differed (figure 3.5b). This may be due to actual differences between first trimester and term placentae or due to differences in the RNA extraction protocol. RNA from term was extracted either from syncytiotrophoblast or cytotrophoblast whereas due to the small size of first trimester placentae RNA was extracted from the total placentae.

3:3:4 Analysis of placental cytotrophoblast RNA translational products

Figure 3.6 shows that cytotrophoblast RNA and syncytiotrophoblast RNA have very similar polypeptide profiles. The major difference being the intensity of the 24kd, pre-hPL, band. The intensity of this band is greatly reduced in cytotrophoblast RNA preparations especially when the cytotrophoblast cells had been treated with trypsin to remove contaminating cells before the RNA was extracted.

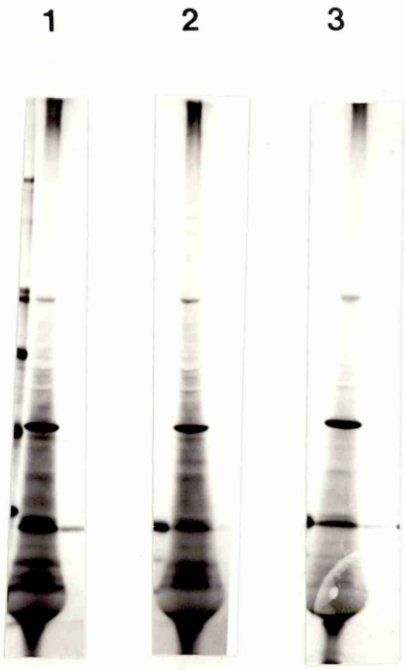


Figure 3.5: Autoradiograph showing the polypeptide profile obtained after the <u>in vitro</u> translation of:

1. Full-term syncytotrophoblast mRNA sample
2. Premature syncytotrophoblast mRNA sample
3. First trimester mRNA sample

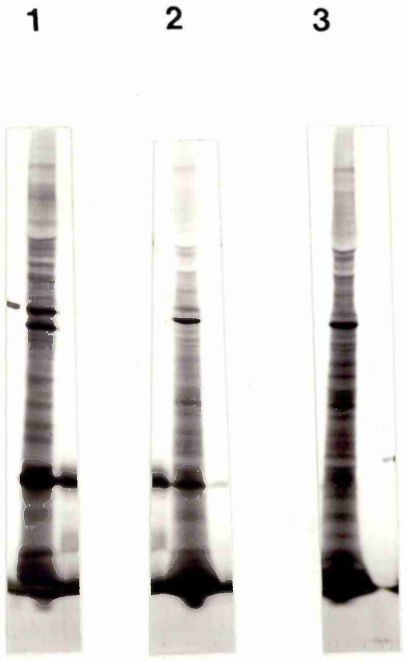


Figure 3.6: Autoradiograph comparing the ^{35}S-labelled *in vitro* synthesised polypeptide profiles of:

1. Full-term syncytotrophoblast mRNA sample
2. " cytotrophoblast mRNA sample
3. " trypsinised cytotrophoblast mRNA sample

3:3:5 **Analysis of immunprecipitated proteins**

The proteins synthesised in the presence of placental RNA, using the lysate cell free system, were immunoprecipitated with both polyclonal and monoclonal rabbit antisera specific for the placental proteins listed in table 3.1. Normal rabbit sera was used as the control negative serum.

The incorporation of ^{35}S-methionine during the *in vitro* translation of both syncytiotrophoblast and cytotrophoblast mRNA was assayed by the measurement of the TCA precipitable counts in the immunoprecipitated proteins (figure 3.7). The results show that the two RNA samples contain different levels of placental proteins. The major polypeptide of syncytiotrophoblast was hPL; 15-20% of the incorporated ^{35}S-methionine was precipitated with anti-hPL. Less than 1% of the synthesised polypeptides from trypsinised cytotrophoblast and 4.5-6% from non-trypsinised cytotrophoblast were precipitated with anti-hPL. Anti-FN and anti-B_2M precipitated 5% of cytotrophoblast RNA translated polypeptides but less than 0.1% of the syncytiotrophoblast RNA translated product. With anti-PLAP this was reversed. An average of 7% of the syncytiotrophoblast polypeptides were precipitated with polyclonal antisera for PLAP but with PLAP monoclonal antisera this percentage dropped (figure 3.7). From the cytotrophoblast RNA translation <0.1% of the polypeptides were precipitated with either monoclonal or polyclonal PLAP antisera.

The immunoprecipitated polypeptides were also analysed on SDS-PAGE gels. No polypeptide bands were visualised from immunoprecipitates which were less than 5% of the total synthesised polypeptides. Antisera specific for hPL precipitated a single protein band 24Kd, from syncytiotrophoblast RNA

ANTISERA	SPECIFICITY
anti-hPL	hPL
anti-PLAP(DAKO)	PLAP
anti-PLAP	"
TA 76	"
TA 78	"
PIG 17	"
H17E2	"
anti-SP_1	SP_1
anti-hCG	hCG
anti-PAPP-A	PAPP-A
anti-cytker	cytokeratin
ker/λ8	"
7A11	"
Anti-FN	fibronectin
W6/32	HLA class I
B_2M	"

table 3.1: List of the polyclonal and monoclonal antisera used in the analysis of the <u>in vitro</u> translated placental RNA samples.

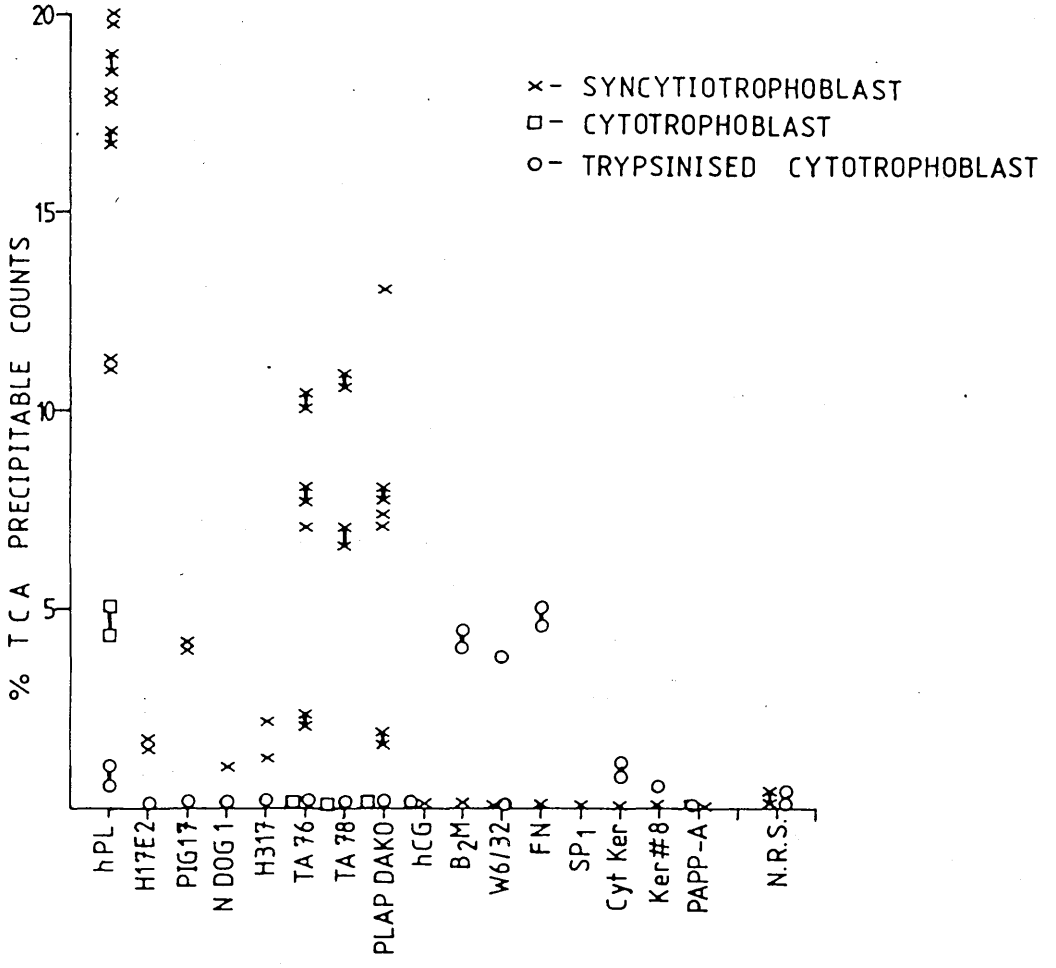


Figure 3.7: The incorporation of ^{35}S-methionine into specific placental mRNA polypeptides, as assayed by immunoprecipitation and TCA precipitation.

synthesised polypeptides (figure 3.8), which corresponded in size to that reported for pre-hPL (Fiddes & Goodman 1979, Seeburg et al 1977). This band of 24Kd co-precipitated, non-specifically, when antisera other than anti-hPL were used. It also precipitated in the presence of the formalin fixed Staph A cells and normal rabbit serum. To reduce the amounts of hPL co-precipitated the samples were pre-precipitated using normal rabbit serum and formalin fixed Staph A cells as this precipitation in the absence of antisera is due to a specific reaction of the hPL with Staph A cells (Fiddes & Goodman, 1979). Though pre-hPL could be immunoprecipitated from non-trypsinised cytotrophoblast RNA translations only 4-5% of the incorporated ^{35}S-methionine was precipitated with anti-hPL compared with 15-20% from syncytiotrophoblast RNA translations (figure 3.7). As this pre-hPL band was not detected by immunoprecipitation from trypsinised cytotrophoblast RNA translations (figure 3.9) it was assumed that unless the cytotrophoblast tissue was treated with trypsin before RNA extraction the cytotrophoblast RNA preparation would be contaminated with syncytiotrophoblast RNA.

Several antisera to PLAP were used for the immunoprecipitation of both cytotrophoblast and syncytiotrophoblast RNA synthesised polypeptides. Figure 3.10a shows that though polypeptides were precipitated from syncytiotrophoblast translations with anti-PLAP none were detectable from comparable cytotrophoblast translations. From syncytiotrophoblast RNA translations the apparent mw of the bands immunoprecipitated using anti-PLAP (DAKO) were a doublet of 58Kd and 56Kd, a triplet at 48Kd, 46Kd and 44Kd and a band at 35Kd (figure 3.10b). Rabbit antisera raised against placental villous

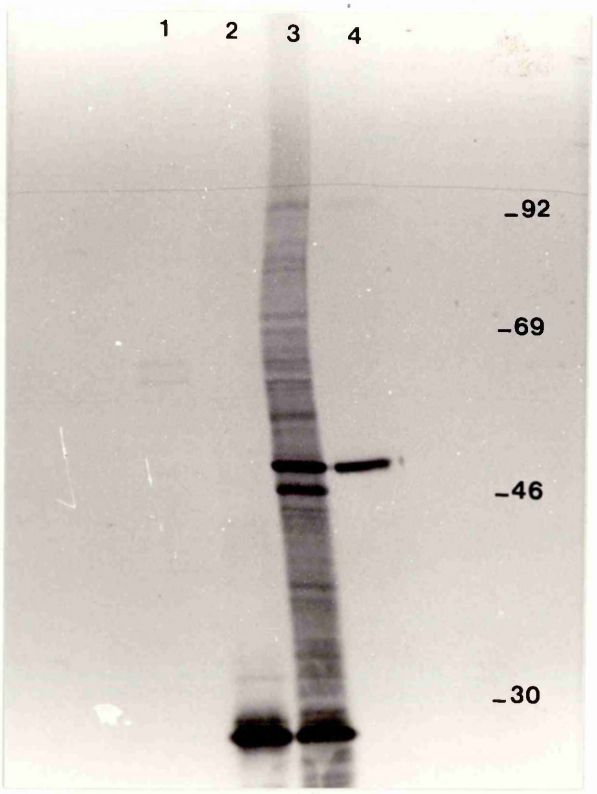


Figure 3.8: Autoradiograph of the ^{35}S-labelled *in vitro* translated products of syncytiotrophoblast mRNA, immunoprecipitated with rabbit antiserum against hPL(DAKO) or PLAP(DAKO), separated on a 9% SDS-PAGE gel. The precipitated band of 24KD corresponded in size to pre-hPL.

1. Poly(A^+) RNA translation products precipitated with antiserum against PLAP (DAKO)
2. Poly(A^+) RNA translation products precipitated with antiserum against hPL (DAKO)
3. Poly(A^+) RNA translation products
4. Translation system blank

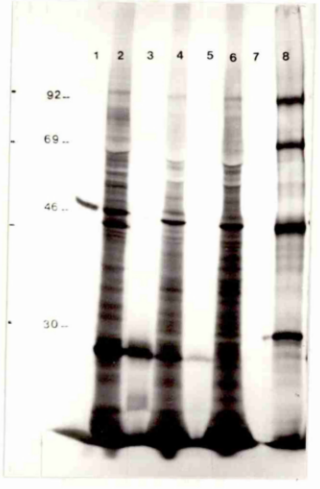


Figure 3.9: Autoradiograph of the ^{35}S-labelled *in vitro* translated products of syncytiotrophoblast, cytotrophoblast and trypsinised cytotrophoblast mRNA; immunoprecipitated with rabbit antiserum against hPL(DAKO) and separated on a 9% SDS-PAGE gel.

1. Translation system blank
2. Syncytiotrophoblast mRNA translation products
3. Syncytiotrophoblast mRNA translation products precipitated with antiserum against hPL
4. Cytotrophoblast mRNA translation products
5. Cytotrophoblast mRNA translation products precipitated with antiserum against hPL
6. Trypsinised cytotrophoblast mRNA translation products
7. Trypsinised cytotrophoblast mRNA translation products precipitated with antiserum against hPL
8. mw markers

Figure 3.10: Autoradiograph of ^{35}S-labelled *in vitro* translated products of term placental mRNA samples immunoprecipitated with rabbit antiserum against PLAP(DAKO) and separated on a 9% SDS-PAGE gel band at 35KD.

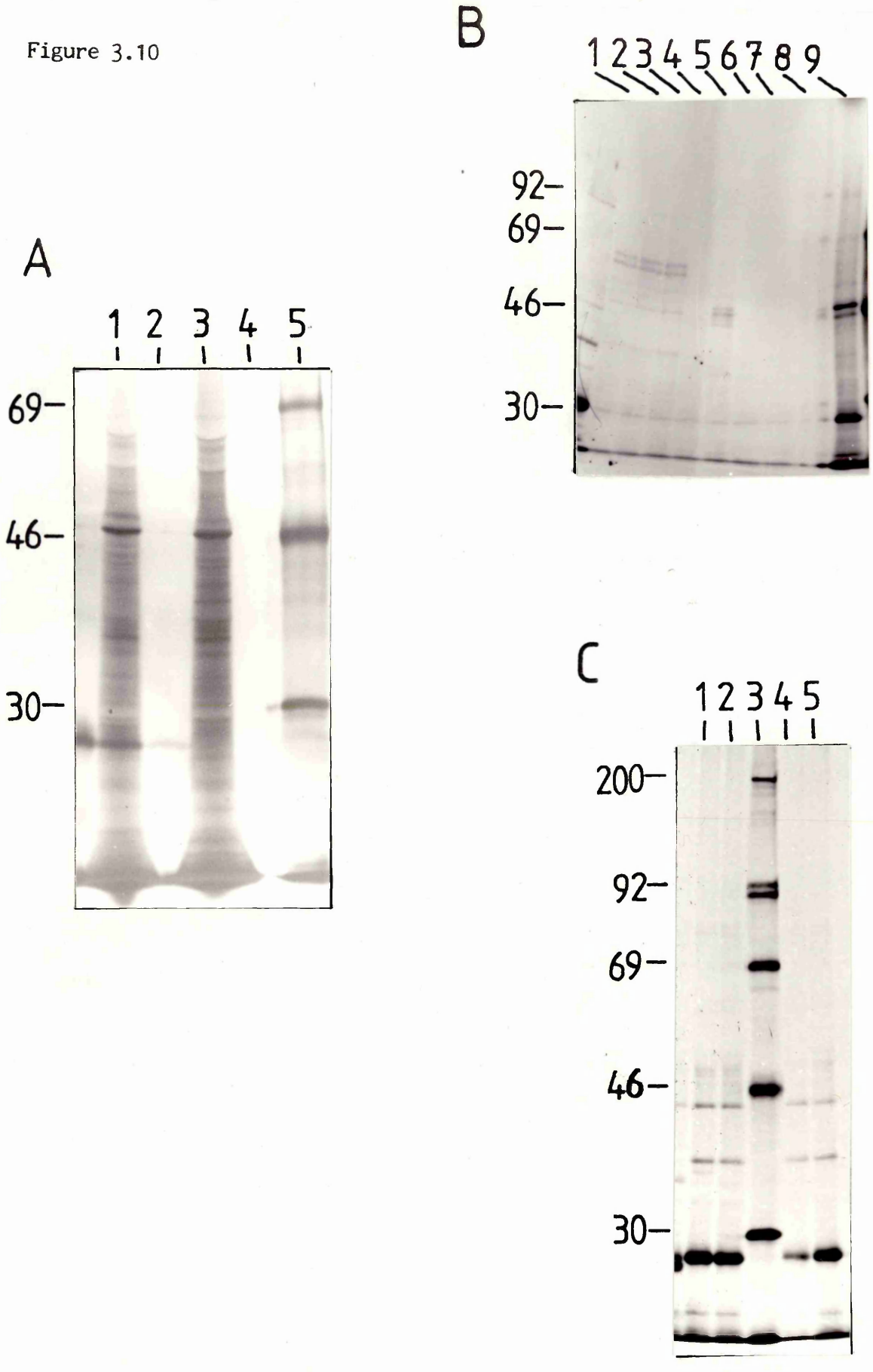
(a) 1. Cytotrophoblast mRNA translation products
2. " translation precipitated with anti-PLAP
3. Trypsinised cytotrophoblast mRNA translation products
4. " " translation precipitated with anti-PLAP
5. mw markers

(b) 1. Poly(A^+) RNA (p1) translation products precipitated with anti-PLAP (DAKO)
2. Poly(A^+) RNA (p2) translation products immunoprecipitated with anti-PLAP (DAKO)
3. Poly(A^+) RNA (p3) translation products immunoprecipitated with anti-PLAP (DAKO)
4.Poly(A^+) RNA (p1) translation products precipitated in the absence of anti-PLAP (DAKO)
5. Poly(A^+) RNA (p1) translation products immunoprecipitated with anti-PLAP (TA76/TA78)
6. Poly(A^+) RNA (p1) translation products plus 150ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
7. Poly(A^+) RNA (p2) translation products plus 150ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
8. Poly(A^+) RNA (p3) translation products plus 150ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
9. Poly(A^+) RNA (p1) translation products

(c) 1. Poly(A^+) RNA (p1) translation products immunoprecipitated with TA78
2. Poly(A^+) RNA (p2) translation products immunoprecipitated with TA78
3. MW markers
4. Poly(A^+) RNA (p1) translation products immunoprecipitated with TA76
5. Poly(A^+) RNA (p2) translation products immunoprecipitated with TA76

facing page

Figure 3.10



membranes which were found to have anti-PLAP activity (obtained from Dr. P.M. Johnson) immunoprecipitate bands at 44Kd and 35Kd. A very faint band of 77kD was also visible in some translations (figure 3.10c). Since there may be a decline in PLAP mRNA production at term, translational proteins obtained from placental poly(A^+) RNA at different stages of gestation (30 weeks and 12 weeks) were immunoprecipitated using either anti-hPL or anti-PLAP sera. Anti-hPL precipitated the 24Kd protein in both samples though there was less 24Kd protein in the first trimester sample (figure 3.11a). Anti-PLAP (obtained from Dr. P.M. Johnson) precipitated bands of 44Kd, 35Kd and 24Kd for the 30 week placental sample, the 24Kd pre-hPL was precipitated in the 12 week placental sample (figure 3.11a). With anti-PLAP (DAKO) a precipitable band of 44Kd was detected in premature mRNA samples and no bands were precipitated from first trimester mRNA samples (figure 3.11b).

Syncytiotrophoblast mRNA translates were also immunoprecipitated with the PLAP monoclonal antisera H317, PIG17 and NDOG2. Though immunoprecipitated polypeptides were detected by TCA precipitation less than 5% of the polypeptides were precipitated (figure 3.7) and when analysised on SDS-PAGE gels no precipitable bands were visable.

3:3:6 Competition assay to test specificity of polypeptides precipitated with antisera directed against PLAP

The specificity of the polypeptides precipitated by the anti-PLAP sera was demonstrated by competition with pure PLAP-A protein extracted from the term placenta or commercially obtained PLAP (Sigma). Figure 3.12 shows that the addition of excess PLAP

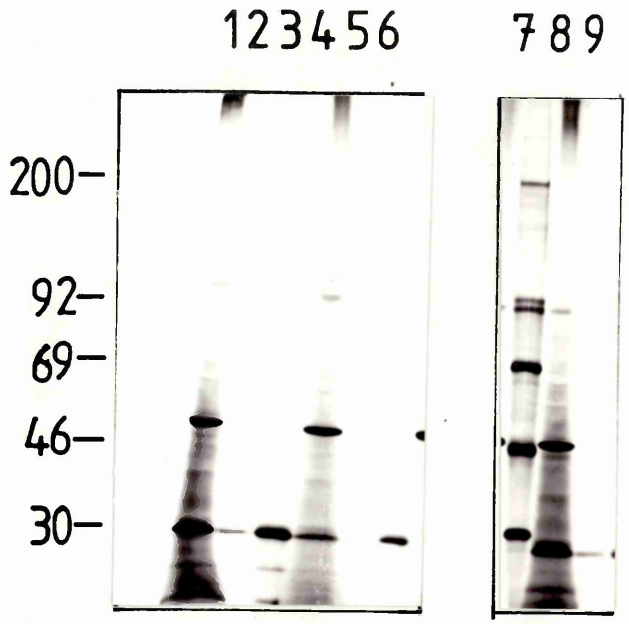


Figure 3.11: Translation products of placental poly(A^+) RNA obtained at different stages of gestation (30 weeks and 12 weeks) separated on a 5-15% SDS-PAGE gel. Anti-hPL (DAKO) precipitated the 24KD pre-hPL protein in both the samples. Anti-PLAP (TA76) precipitated bands of 44KD, 35KD and 24KD for the 30 week placental sample. Anti-PLAP (DAKO) precipitated a band of 44KD from the 30 week sample. The 24KD pre-hPL was precipitated from the 12 week placental sample with anti-PLAP (TA76) serum.

(a) 1. Poly(A^+) RNA translation products from 30 week placenta
2. Poly(A^+) RNA translation products from 30 week placenta precipitated with antisera against PLAP (TA76)
3. Poly(A^+) RNA translation products from 30 week placenta precipitated with antisera against hPL (DAKO)
4. Poly(A^+) RNA translation products from 12 week placenta
5. Poly(A^+) RNA translation products from 12 week placenta precipitated with antiserum against PLAP (TA76)
6. Poly(A^+) RNA translation products from 12 week placenta precipitated with antiserum against hPL (DAKO)

(b) 7. mw markers
8. Poly(A^+) RNA translation products from 30 week placenta
9. Poly(A^+) RNA translation products from 30 week placenta precipitated with antisera against PLAP (DAKO)

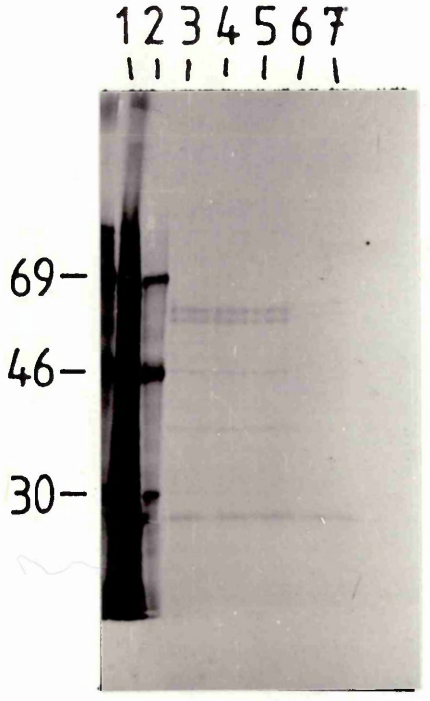


Figure 3.12: Competition Assay; ^{35}S-labelled *in vitro* translated products immunoprecipitated with rabbit antiserum against PLAP (DAKO) in the presence of excess unlabelled PLAP. The addition of excess PLAP prevented the immunoprecipitation by anti-PLAP(DAKO) of the 58KD and 56KD polypeptide bands, whereas the 24KD polypeptide band remained.

1. Poly(A^+) RNA translation products
2. mw markers
3. Poly(A^+) RNA translation products immunoprecipitated with anti-PLAP (DAKO)
4. Poly(A^+) RNA translation products plus 1ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
5. Poly(A^+) RNA translation products plus 10ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
6. Poly(A^+) RNA translation products plus 100ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)
7. Poly(A^+) RNA translation products plus 150ug of unlabelled PLAP immunoprecipitated with anti-PLAP (DAKO)

prevented the immunoprecipitation by anti-PLAP(DAKO) of the 58Kd and 56Kd polypeptide bands, whereas the 24Kd polypeptide band remained after the addition of excess PLAP. (figure 3.12).

3:3:7 Labelling of Oligonucleotides using T4 kinase

After ^{32}P labeling the oligonucleotides using T4 kinase the labelled DNA was separated from the unincorporated [$\chi^{32}P$]ATP by chromatography on a Sephadex G25 column (8cmx0.5cm). Two peaks of ^{32}P activity were seen. That the first peak contained the labelled DNA and the second peak the unincorporated [$\chi^{32}P$]ATP was tested by TCA precipitation. Only the first peak contained precipitable counts and thus the labelled oligonucleotides. The percentage yield of incorporated ^{32}P for the kinasing reaction ranged from 55-76%.

3:3:8 Northern Blots

Placental RNA denatured using glyoxal and DMSO was fractionated on a 1.5% agarose gel and transferred to a nitrocellulose filter as described. After hybridising overnight at 20°C with either PLAP or hPL ^{32}P 5` end labelled 14 base oligonucleotides each filter was washed at 20°C. A band of homology was seen with the 14 base hPL oligonucleotide probe (figure 3.13). No bands were seen when the two 14 base PLAP oligonucleotide probes were used. Washing the filter at intermediate temperatures until the dissociation temperature of the hPL oligonucleotide probe was reached, showed a band of 1Kb to be present in 2 term placental RNA samples and a 33 week placental RNA sample. There was no band seen in the 11 week placental RNA sample; not enough hPL message may be present in

Figure 3.13: Northern blot of placental syncytiotrophoblast RNA samples probed using a hPL oligonucleotide probe

1. Full-term RNA sample
2. Premature RNA sample
3. First trimester RNA sample
4. First trimester RNA sample
5. Premature RNA sample
6. Full-term RNA sample

this mRNA sample for hybridisation.

From these results one can see that the hPL oligonucleotide was able to hybridise to the RNA samples to a band of sufficient coding capacity for the pre-hPL message. The pre-hPL message has been shown by Shine et al (1977) and Goodman et al (1980) to be 815 residues long. The PLAP oligonucleotides did not show any homology with the RNA samples. This could be due to the fact that the PLAP message is of too low an abundance to be detected by a short oligonucleotide probe.

3:3:9 Dot Hybridisation

Denatured cytotrophoblast RNA samples from term placentae and syncytiotrophoblast RNA samples from various stages of gestation, were diluted and spotted onto dry filters. The dot blots were probed with PLAP or hPL 14 base oligonucleotides. After hybridisation with the oligonucleotides probes, the filters were washed under varying stringency conditions. The dissociation temperature T_D is the highest stringency wash possible without dissociation of the homologous probe.

Washing at 30°C with 5xSSC the T_D for the 11 homologous bases in the PLAP 14mer oligonucleotide mixtures showed that the binding intensity of the PLAP probes was proportional to the syncytiotrophoblast RNA samples gestation stage (figure 3.14a). No binding of the PLAP oligonucleotide was seen with the 9 week RNA sample (figure 3.14a, track 1). Very little PLAP binding to the cytotrophoblast RNA samples was seen. Under higher stringency washes the PLAP probe dissociated and hybridisation was not detected at any stage of gestation. The T_D for the hPL 14mer oligonucleotide probe is 40_oC. This probe hybridised to all the

RNA samples at the low stringency wash, 30_oC with 5xSSC, was used (figure 3.14b). The intensity of probe binding to the RNA samples was a lot stronger with syncytiotrophoblast RNA compared with cytotrophoblast RNA. The hPL probe remained hybridised under the high stringency wash at 40_oC and 5xSSC (figure 3.14c). At this temperature the intensity of hPL binding is proportional with the syncytiotrophoblast RNA samples gestation stage. The results also showed that decreasingly lower salt washes at 40_oC causes the hybridised hPL probe to dissociate as does stringency washes at temperatures above 40_oC.

Under the low stringency wash of 5xSSC at 65_oC the pDP001 probe hybridised with all the RNA samples though the intensity of hybridisation was greatest for the trypsinised cytotrophoblast RNA samples (figure 3.14d) At the high stringency wash of 0.1xSSC at 65_oC faint hybridisation to both syncytiotrophoblast and cytotrophoblast RNA samples could still be detected (figure 3.14e). This result indicates that there may be class I homologous sequences in both syncytiotrophoblast and cytotrophoblast placental RNA samples.

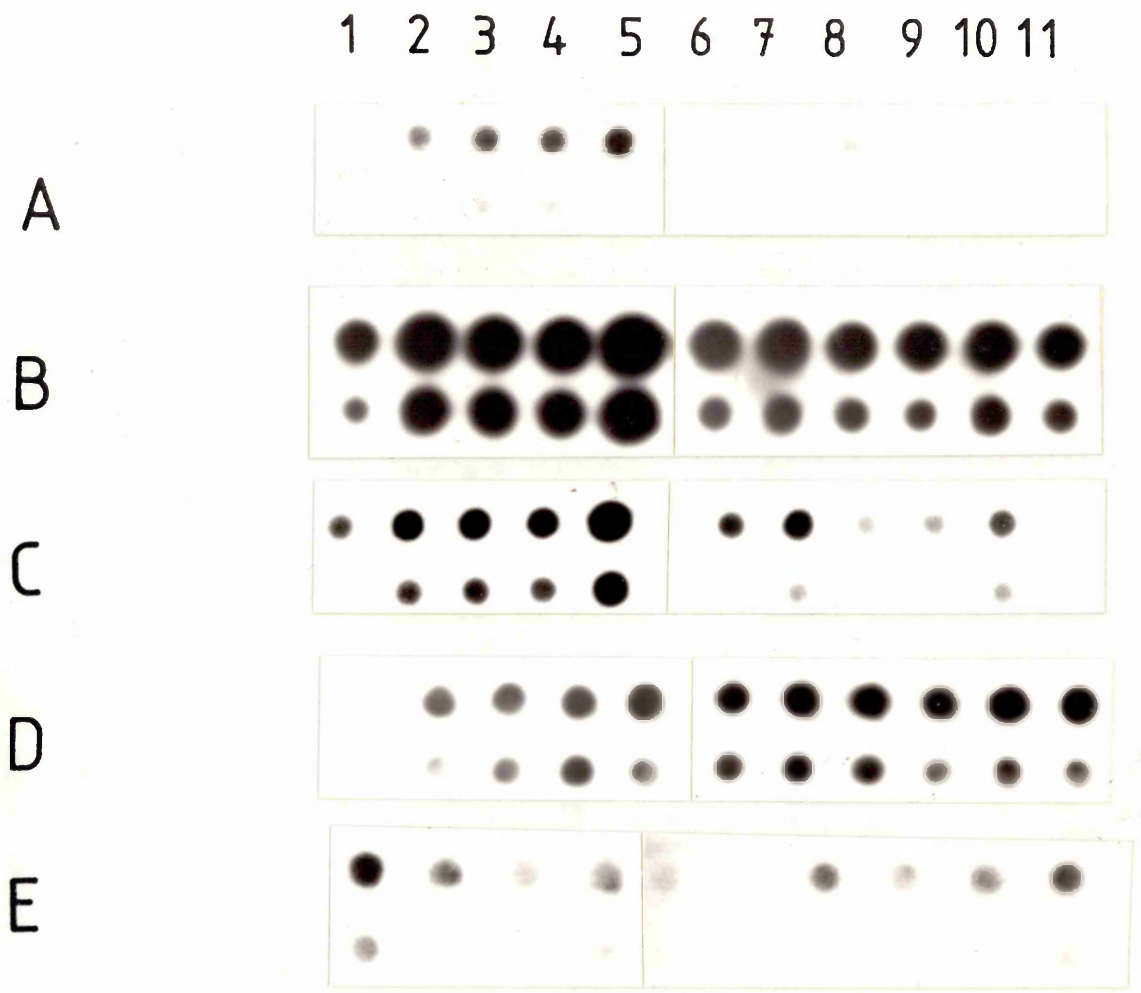


Figure 3.14: Dot hybridsisation of samples of syncytiotrophoblast RNA from various stages of gestation, term cytotrophoblast and trypsinised cytotrophoblast RNA. Probed using (a) PLAP 14mer oligonucleotide probe, washing conditions 30°C with 5xSSC (b) hPL 14mer oligonucleotide probe, washing conditions 40°C with 5xSSC (c) hPL 14mer oligonucleotide probe, washing conditions 40°C with 1xSSC (d) pDP001, washing conditions 65°C with 1xSSC (e) pDP001, washing conditions 65°C with 0.1xSSC

1. 9 week syncytiotrophoblast RNA sample
2. 11 week syncytiotrophoblast RNA sample
3. 30 week syncytiotrophoblast RNA sample
4. 33 week syncytiotrophoblast RNA sample
5. 40 week syncytiotrophoblast RNA sample
6. Term cytotrophoblast RNA sample (8)
7. Term syncytiotrophoblast RNA sample (8)
8. Term trypsinised cytotrophoblast RNA sample (8)
9. Term cytotrophoblast RNA sample (7)
10. Term syncytiotrophoblast RNA sample (7)
11. Term trypsinised cytotrophoblast RNA sample (7)

3:4 DISCUSSION

The mRNA populations extracted from syncytiotrophoblast and cytotrophoblast were analysed by *in vitro* translation. Both unfractionated placental RNA samples and poly (A_+) enriched samples synthesised the same polypeptides. The polypeptide bands were more distinct for the poly (A_+) enriched samples (figure 3.4). Whereas increased concentrations of unfractionated RNA caused a decrease in translation efficiency, no decrease in the translation efficiency was noticed with the addition of excess poly (A_+) RNA (figures 3.1, 3.2a & 3.2b). It was assumed that the decreased efficency was due to an accumulation of translational inhibitors present in the untranslated RNA sample. The presence of inhibitors was shown by the fact that the translational efficiency of high quality control mRNA was reduced by the addition of unfractionated placental RNA.

The major difference between the synthesised polypeptide profiles of syncytiotrophoblast and cytotrophoblast mRNA was the intensity of the 24kd, pre-hPL, band (figure 3.6). The intensity of this band is greatly reduced in cytotrophoblast RNA preparations especially when the cytotrophoblast cells had been treated with trypsin to remove contaminating cells before the RNA was extracted. This pre-hPL polypeptide was the predominant translational product for syncytiotrophoblast mRNA (figure 3.5a & 3.8). The other predominant polypeptides ranged in size from 86-20Kd with fainter polypeptide bands visible beyond 100Kd. Several of these proteins have been immunoprecipitated. The *in vitro* translation of human placental mRNA by Seeberg *et al* (1977) and Fiddes & Goodman (1979) centred on the low mw polypeptides

hPL and HCG. In term placental *in vitro* translated mRNA they precipitated the predominant polypeptide of 24Kd with anti-hPL; this polypeptide represented 20% of the translated polypeptides. Polypeptide bands of mw 35Kd and 22Kd were also present. These polypeptide bands were seen in my *in vitro* translated syncytiotrophoblast mRNA samples and by immunoprecipitation the 24Kd pre-hPL band was shown to represent 15-20% of the translated polypeptides (figures 3.7 & 3.8). The band of mw 48 Kd which was present in the translational products even in the absence of mRNA was due to the labelling of proteins or macromolecules present in the lysate mix by mRNA independant processes.

Ito and Chou (1983) showed that the immunoprecipitation using anti-PLAP of the cell free *in vitro* translation products of choriocarcinoma poly (A_+) RNA precipitated a protein of apparent mw 60Kd. The specificity of this precipitated polypeptide was shown by a competition assay using PLAP from term placentae; excess PLAP prevented the immunoprecipitation of the 60Kd band. As PLAP and choriocarcinoma AP have been shown to have the same immunological reactivity it has been postulated that the placental form of AP and the tumouragenic forms of AP are encoded by the same gene (Rosen *et al*, 1975). Thus if anti-PLAP precipitates a 60Kd protein from *in vitro* translated choriocarcinoma poly (A_+) RNA one would expect a band of similar mw from *in vitro* translated placental syncytiotrophoblast poly(A_+) RNA to be immunoprecipitated using anti-PLAP. The mw of the polypeptides specifically immunoprecipitated by anti-PLAP were 58Kd and 56Kd. Other proteins were also precipitated using anti-PLAP sera but they were not shown to be specific for PLAP by competition assays (figure 3.12). The weak intensity of the PLAP

bands compared with the 24Kd pre-hPL band may be due to the antisera used. These were directed against the glycosylated form of PLAP and not against the non-glycosylated form produced by the cell free translation systems. The messenger RNA for PLAP is also of low abundance and thus it is not translated as efficiently as hPL in the cell free translation system. The hPL protein has a half life of 15 minutes and thus needs to have an abundant mRNA to keep the levels of hPL high. Whereas PLAP is a stable protein it was assumed that its messenger RNA was of low abundance. Though the data is based on a relatively small number of placenta (20), the evidence from immunoprecipitation and dot hybridisation (figures 3.11 & 3.14) suggest that the levels of PLAP mRNA in the syncytiotrophoblast mRNA samples increases with the gestational age.

CHAPTER 4

PREPARATION AND SCREENING OF A SYNCYTIOTROPHOBLAST cDNA LIBRARY

4:1 INTRODUCTION

The aim of work described in this chapter was to construct cDNA libraries from the mRNA populations of cytotrophoblast and syncytiotrophoblast whose preparation and analysis was presented in chapter 3. These libraries were to be to be screened for sequences coding for PLAP and subsequently for HLA class I antigens. The construction of a cDNA library involves several steps:

(1) The preparation of the vector DNA.

(2) Reverse transcription of mRNA into cDNA.

(3) The ligation of the cDNA into the vector DNA.

(4) The introduction of the ligated material into E.coli.

(5) Screening of the resultant library for cDNA clones.

(6) Amplification of the recombinant cDNA library.

Preparations of placental cDNA were ligated into the lambda vectors gt10 and gt11. For ease of presentation, the results for cloning placental cDNA into \gt10 and the screening of the resultant placental cDNA library will be presented before the work on \gt11. However, at the start of the work it was necessary to choose whether to clone PLAP cDNA into \gt10 or the related expression vector \gt11. The only means of screening \gt10 clones was a short oligonucleotide sequence determined from the 5` amino acid sequence of PLAP. Considering that the cDNA is synthesised from the 3` end poly A tail and S1 digestion of the hairpin structure removes 5`end sequences it seemed more attractive to use the \gt11 expression vector. Here cloned cDNA is expressed as a fusion protein which can be screened with antibodies. As some of the antigenic determinants on PLAP might

be specified by through codons further towards the 3` end of the mRNA the cloning of placental syncytiotrophoblastic cDNA into \gt11 was attempted first. In retrospect this was probably not the best choice as there is no selection for recombinants in \gt11 and a library cloned into \gt10 can be transferred to \gt11 for antibody screening. Further encouragement to clone into \gt10 came with the arrival of long oligonucleotides to PLAP as well as cDNA probes for HLA class I antigens.

4:2:1 \gt10

The genotype for \gt10 is \srI\1° b527 srI\3° imm^{434} (srI434^{+}) srI\4° srI\5° it was constructed by in vitro techniques using the right arm of \607 and left arm of \518 (Huynh et al, 1984).

a. **E.coli strains used:**

(1) LE392 ¦- hsdR^{-} hsdM^{+} supE supF thr met lacY1

(2) BNN102 ¦- hsdR^{-} hsdM^{+} supE thr leu thi lacY1 tonA21 hflA150 [chr::Tn10]

The construction of BNN102 is described by Young & Davis (1983).

b. **Media used**

LB broth: 10g Bacto-tryptone(Difco), 5g Bacto-yeast extract, 10g NaCl, H_2O to a litre, adjusted to pH7.5 with sodium hydroxide

LB agar: same as for LB with the addition of 15g Bacto-agar (Difco).

LB soft agar: same as for LB with the addition of 6.5g Bacto-agar (Difco) and $MgSO_4$ to 10mM.

4:2:2 \gt11

The genotype of \gt11 is lac5 /\(shindIII\2-3) sri\3° cI857 srI\4° nin5 srI\5° Sam100 the construction of \gt11 is described by Young & Davis (1983). The \gt11[amp3] is identical to \gt11 except for a 1.7Kb amp3 fragment from pBR322 inserted into its Xba1 site.

a **E.coli strains used:**

(1) Y1088 ¦- /\ lacU169 supE supF hsdR^{-} hsdM^{+} metB trpR tonA21 proC::Tn5 (pMC9)

(2) Y1090 ¦- Δ *lacU169* *proA*$^+$ Δ*lon* *araD139* *strA* *supF* *hsdR*$^+$ *hsdM*$^+$ [*trpC22*::*Tn10*] (pMC9)

The construction of Y1088 and Y1090 is described by Young & Davis (1983).

b. **Media used**

BBL broth: 10g BBL-tripticase, 5g NaCl, H_2O to a litre, adjusted to pH7.5 with sodium hydroxide.

BBL agar: same as for BBL-broth with the addition of 15g Bacto-agar (Difco).

BBL soft agar: same as for BBL-broth with the addition of 6.5g bacto-agar (Difco) and $MgSO_4$ to 10mM. Just before use this was adjusted to a concentration of 5mM IPTG (Sigma) and 0.1mg/ml of Xgal (Sigma). IPTG (isopropyl B-D-thiogalactopyranoside) was prepared as a 1M stock solution in distilled H_2O and stored at -20°C. Xgal (5-bromo-4-chloro-3-indolyl-B-D-thiogalactopyranoside) was prepared as a 40mg/ml solution in dimethylformamide and stored at -20°C.

4:2:3 **Preparation of plating cells**

Host cells Y1088, Y1090, LE392 and BNN102 were used as plating cells. An overnight culture from a single colony was grown in 20ml LB medium containing 0.2% maltose to induce phage receptors on the *E.coli* cell surface (Enquist & Sternberg, 1979). 1ml of the overnight culture was added to 50ml of LB medium containing 0.2% maltose and the culture grown with aeration to log phase. The cells were collected by centrifugation at 2K for 5 minutes, resuspended in 10ml sterile 10mM $MgSO_4$ and stored at 4°C. The plating cells were viable for 2 weeks.

4:2:4 **Preparation of λ vector gt10**

Single plaque stocks which contained the lowest percentage of spontaneous clear-plaque phage were used for large scale preparation of λgt10 'phage. From these stocks ten plate stocks were prepared using an adaptation of the protocol of Davis *et al* (1980), as follows. $5x10^5$pfu of phage stock were added to 100-150ul of plating cells (LE392), incubated at 37°C for 15 minutes and then plated on LB plates (90mm) supplemented with 0.2% glucose in 3mls of molten (45°C) soft LB agar. After 5-6 hours incubation at 37°C the plates were chilled and overlaid with phage dilution buffer, PDB, (10mM Tris HCl pH7.4, 10mM $MgSO_4$, 0.01% gelatin). The plates were left at 4°C overnight to allow the 'phage to diffuse into the buffer. The overlay was removed and any bacterial or agar comtamination was removed from the phage solution by centrifugation at 6K in a JA-10 Beckman rotor for 10 minutes at 4°C.

4:2:5 **Preparation of λ vector gt11**

The bacteriophage λgt11[amp3]DNA was prepared by inducing the λgt11[amp3] lysogen c600 at 43°C using an adaptation of the procedure of Huynh, Young & Davis (1984). After testing that the c600 λgt11[amp3] lysogen is induced at 43°C 20ml of LB medium was inoculated with a single colony of the lysogen and incubated overnight at 32°C. The overnight culture was used to inoculate a litre of LB medium. This was incubated at 32°C with aeration until an OD_{600} of 0.6 was reached. To induce lytic growth the temperature of the culture was increased rapidly (by using pre-warmed water bath) to 43°C for 15 minutes then dropped to 37°C and incubated for a futher 3 hours with aeration.

To the culture was added 10ml $CHCl_3$ and incubation at 37°C with aeration was continued for a futher 15 minutes. The bacterial debris was removed from the culture by centrifugation at 6K in a JA-10 Beckman rotor for 10 minutes at 4°C.

4:2:6 **Preparation of λ vector DNA**

The gt10 or gt11 'phage were pelleted from suspension by centrifugation at 23K in a SW-27 Beckman rotor for 90minutes at 4°C and the pellet resuspended in 5ml of λ buffer (10mM Tris HCl pH7.5, 10mM $MgCl_2$, 1mM EDTA) at 4°C. To this was added an equal volume of CsCl in λ buffer (0.71g/ml). The phage was then banded by centrifuging at 55K in a VTi 55 Beckman rotor for 8 hours at 20°C. The phage band was removed in 0.5ml volume through the side of the tube with a syringe fitted with a 25 gauge needle. The phage was further purified by stepping the phage up through CsCl (0.71g/ml of λ buffer), the phage solution was over laid with CsCl (0.71g/ml of λ buffer) and centrifuged at 55K in a VTi 65 Beckman rotor for 4 hours at 20°C. The phage band was removed in 0.5ml volume as above diluted with an equal volume of λ buffer and dialysed overnight at 4°C against TE to remove the CsCl. To obtain λ DNA the phage solution was extracted 3x with equal volumes of phenol. The final aqueous phase was dialysed against TE to remove any residual phenol and the λ DNA stored at 4°C.

4:2:7 **EcoR1 digestion of λ vector DNA**

To 5ug of the λvector DNA in TE was added 5ul 10x HSB and 20U of EcoR1 in a total volume of 50ul. This was incubated at 37°C for 1 hour and the reaction stopped by the addition of 2ul (0.5M) EDTA and heating at 70°C for 10 minutes. To check that the

digestion had gone to completion samples of EcoR1 digested and undigested gt10 or gt11 were run on a 0.8% agarose gel in TBE buffer. EcoR1 digestion of \gt11(amp3) (45.4Kb) of 19.6Kb and 25.8Kb. EcoR1 digestion \gt10 (43.34 Kb) yeilds bands of 32.71Kb and 10.63Kb.

4:2:8 **Commercially obtained \vector DNA**

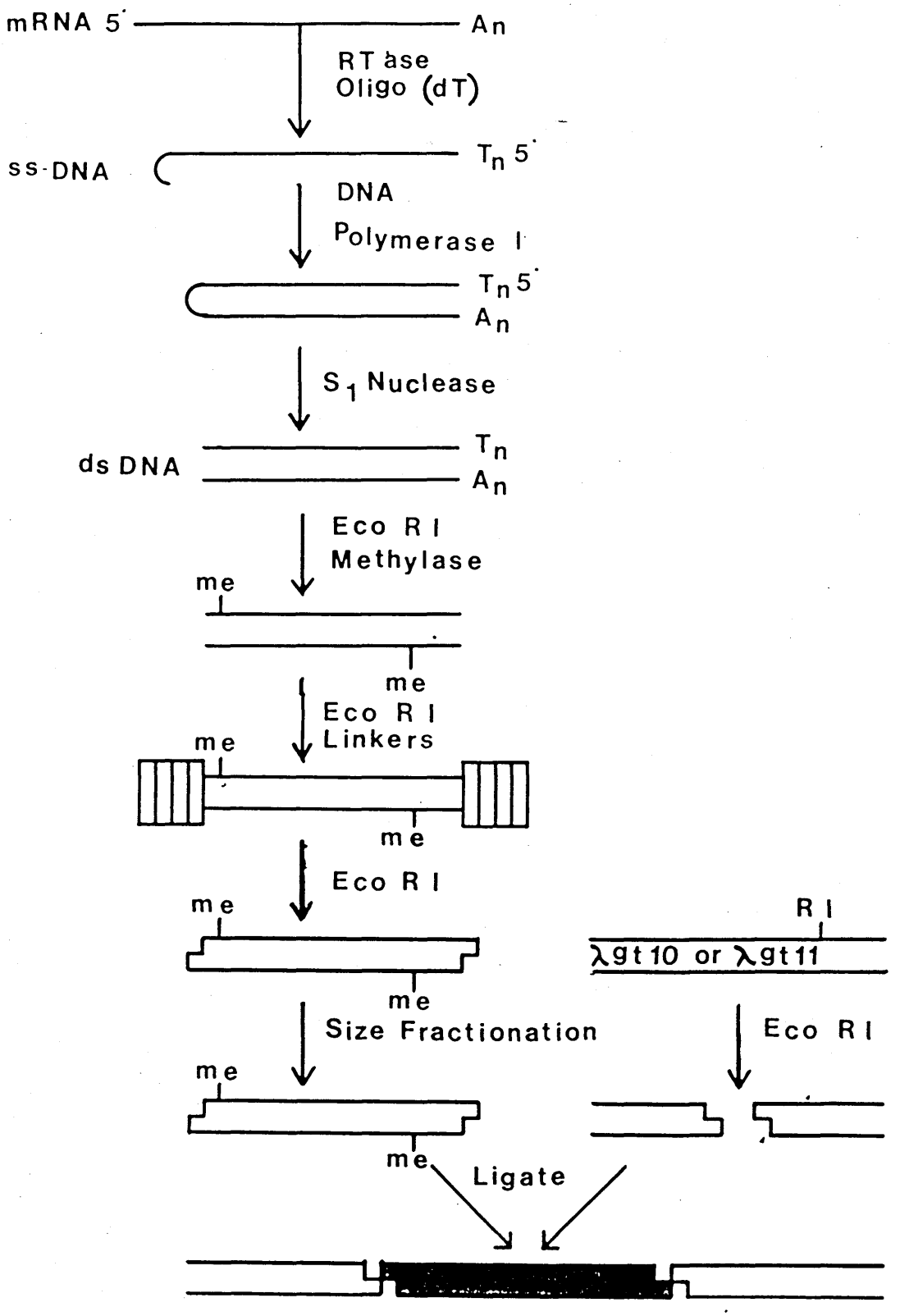
Dephosphorylated lambda gt11 arms were obtained from Vector Cloning Systems (3770 Tansy Street, San Diego, CA 921211).

4:2:9 **Preparation of cDNA**

cDNA was synthesised using placental poly (A^+) RNA and oligo(dT) as a primer (Wickens et al 1978). In a sterile eppendorf was added 30ul of dH_2O containing 2ug of poly(A^+) RNA, and 10U RNasin (BRL). The sample was heated at 70°C for 3 minutes, cooled on ice then to it was added 10U RNasin, 5ul 10x 1st strand buffer (0.4M KCl, 0.08M $MgCl_2$, 4mM DTT, 0.5M Tris HCl pH8.5) 10ul of 5mM d(A,G,C,T)TP, 1ul (0.5ug) oligo (dT), 10uCi [α^{32}P] dATP (specific activity= 800Ci/mMole), 20U reverse transcriptase (BRL). This was incubated at 42°C for 2 hours. The cDNA product was seperated from the the mRNA template by heat denaturation, 1.5 minutes at 100°C, then cooled immediately on ice [1% of the reaction product was kept to run on an alkaline agarose gel].

The second strand of the cDNA was synthesised using the 3' terminus of the first strand as a the primer. To the tube containing the first strand reaction mix was added 20ul of 5x 2nd strand buffer (250mM Hepes pH6.9, 250mMKCl, 50mM$MgCl_2$S) 2ul of 5mM d(A,G,C,T)TP and 60U of DNA polymerase to catalyse the

Diagrammatic scheme for double-stranded cDNA synthesis
and cloning into lambda vector gt10 or gt11



Recombinant λgt 10 or λgt 11

reaction (Efstratiadis et al, 1976 & Wickens et al, 1978). The volume was adjusted to 100ul using dH_2O and the mix incubated at 15°C for 3 hours [1% of the reaction product was kept to run on an alkaline agarose gel]

The single stranded hair pin loop connecting the two strands of the DNA was digested away with S1 nuclease. The S1 nuclease (BRL) was titrated to determine the optimal amount. Two-fold dilutions of the enzyme from 300u-4800u/ml in 4ul of S1 buffer were incubated with 1ul of the second strand synthesis at 37°C for 30 minutes. The digestion products were analysed on an alkaline agarose gel and the optimum S1 concentration was determined as that which reduced the size distribution of the cDNA smear to that seen after first strand synthesis. To the tube containing the second strand synthesis reaction product was added 400ul of S1 buffer (30mM NaAc pH 4.4, 0.25M NaCl, 1mM $ZnSO_4$) and S1 nuclease. This was incubated at 37°C for 30 minutes then the reaction stopped by adding 10ul of 0.5M EDTA (pH8.0), [1% of the reaction product was kept to run on an alkaline agarose gel]. Protein contamination was removed from the cDNA mix by extracted twice with an equal volume of phenol:$CHCl_3$ saturated with 10mM Tris HCl (pH7.5) then with $CHCl_3$. The aqueous phase was precipitated at -20°C using 2 volumes of EtOH. The precipitate was pelleted by centrifuging for 15 minutes in a microfuge at 4°C.

To increase the number of flush ended double stranded cDNA molecules the cDNA pellet was treated with DNA polymerase 1 (Seeburg et al, 1977). After drying the pellet was resuspended in 10ul containing , 6U T4 DNA polymerase (BRL), 0.1mM d(A,G,C,T)TP, 50mM Tris HCl (pH8), 6mM $MgCl_2$, 25mM KCl and 1ug

BSA. The mix was incubated at 37°C for 30 minutes then extracted twice with an equal volume of phenol:$CHCL_3$ saturated with 10mM Tris HCl pH7.5), then with $CHCl_3$. The aqueous phase was precipitated at -20°C using 2 volumes of EtOH and 1/10 volume of sodium acetate.

4:2:10 **Size distribution of the cDNA**

The 1% reaction volume reserved from the first and second strand synthesis of the cDNA and after S1 treatment were run on an agarose gel (1.2% agarose, 50mM NaCl, 1mM EDTA) in alkaline buffer (30mM NaOH, 1mM EDTA) to analyse the size distribution of the reaction product. The samples were loaded on the gel in 20ul of sample buffer (50mM NaOH, 1mM EDTA, 2.5% Ficoll, 0.025% Bromocresol Green) and run at 30V until the front had moved 8cm, after which the gel was soaked in 7% TCA at RT for 30 minutes, dried and autoradiographed using X-Omat S film.

The incorporation of [^{32}P]_CTP into the cDNA during the first and second strand synthesis reaction was tested by TCA precipitation. Knowing the amount of RNA one started with and the specific activity of the label an estimate of the amount of cDNA synthesised was calculated.

4:2:11 **EcoR1 Methylation of cDNA**

To protect the cDNA from digestion by EcoR1 the EcoR1 cleavage sites were methylated using S-adenosyl-L-methionine (Sigma) and EcoR1 methylase (BioLabs). The cDNA pellet obtained after flush-end treatment using T4 polymerase was dried and resuspended in 20ul EcoR1 methylase buffer (50mM Tris HCl pH7.5, 1mM EDTA, 5mM DTT, 400ug/ml BSA) plus 2ul of freshly made 100uM

S-adenosyl-L-methionine and 0.5ul EcoR1 methylase. This was incubated at 37°C for 30 minutes followed by 70°C for 10 minutes. The solution was extracted with an equal volume of phenol:$CHCL_3$ saturated with 10mM Tris HCl (pH7.5), the organic phase was re-extracted twice with 50ul TE. The aqueous phases were pooled and extracted twice with $CHCl_3$ and precipitated at -20°C using 2 volumes of EtOH and 1/10 volume of 3M sodium acetate.

To test that the methylation reaction did indeed protect the cDNA, plasmid DNA was also methylated as described and then both EcoR1 methylated and non-methylated plasmid DNA were tested for their restriction pattern using EcoR1. To 18ul of either untreated plasmid DNA or methylated plasmid DNA were added 2ul of 10x high salt buffer (100mM NaCl, 50mM Tris HCl pH7.5, 10mM $MgCl_2$, 1mM DDT) and 0.2ul(2U) EcoR1. This was incubated at 37°C for 1 hour and the reaction stopped by the addition of 0.2ul(0.5M) EDTA.

4:2:12 **EcoR1 Linker addition to the double-stranded cDNA**

The EcoR1 methylated cDNA pellet was resuspended to a total volume of 20ul in 5ul (1ug) kinased EcoR1 linkers. 1.5ul 10x ligation mix (250mM Tris HCl pH7.4, 100mM $MgCl_2$, 100mM DTT, 2mM spermidine, 10mM ATP), 5U T4 DNA ligase (BRL), 1U T4 RNA ligase (BRL) and incubated at 15°C overnight. The ligation reaction was stopped by heating at 65°C for 10 minutes. [1% of the reaction product was kept to run on a polyacrylamide gel].

4:2:13 **Removal of excess EcoR1 linkers**

After digestion with EcoR1 the excess linkers were removed by either isopropanol precipitation or size

fractionation:

a. **Isopropanol precipitation** To the tube containing the ligation mix was added 0.2 volumes of 3M NaAc (pH6) and 0.6 volumes of isopropanol. The solution was mixed and incubated at RT for 5 minutes. This was then centrifuged in a microfuge for 5 minutes at RT, the supernatant removed and the pellet washed with 70% EtOH then resuspended in 40ul TE [1% of both the supernatant and the resuspended pellet was kept to run on a gel]. To the pellet resuspended in 40ul TE were added 4.2ul of 10x high salt buffer (100mM NaCl, 50mM Tris HCl pH7.5, 10mM $MgCl_2$, 1mM DDT) and 2ul(20U) EcoR1. This was incubated at 37°C for 3 hours and the reaction stopped by the addition of 0.4ul(0.5M) EDTA. A second isopropanol precipitation was performed again, 0.2 volumes of 3M NaAc (pH6) and 0.6 volumes of isopropanol were added. The solution was incubated at RT for 5 minutes and then centrifuged in a microfuge for 5 minutes at RT. The supernatant was removed and after washing the pellet with 70% EtOH it was dried, resuspended in TE and stored at -20°C. [1% of both the supernatant and the resuspended pellet was kept to run on a gel].

The 1% volume samples of supernatant and pellet solution, reserved after the isopropanol precipitation steps, were loaded on a 10% polyacrylamide gel in 20ul of sample buffer (10%glycerol, 5% mercaptoethanol, 3% SDS, 0.0625M Tris HCl pH6.8, 0.01% bromophenol blue). The samples were electrophoresed at 20mA, using TBE running buffer (10.8g/l Tris, 55g/l boric acid, 9.3g/l EDTA) until the bromophenol blue marker is halfway down the gel. The gel was then dried and autoradiographed. From this gel one can check whether the ligation of the EcoR1 linkers to the cDNA had been sucessful and that all the excess EcoR1

linkers were removed, after digestion with EcoR1.

b. **Size fractionation** The cDNA can be size fractionated and the excess linkers removed by running the reaction mixture over a bio-Gel 50 column (0.2x25cm) at room temperature. The column was washed with 50ml of column buffer (10mM Tris HCl pH 7.5, 100mM NaCl, 1mM Na_3 EDTA). Excess λ DNA was used to pre-soak the column before it was calibrated. To calibrate, 5'^{32}P-end labeled Hinf I pBR322 fragments in loading buffer (1ul 0.25% bromphenol blue-50% glycerol/10ul TE pH7.5) were run through the column at a flow rate of 20ul per minute and 40ul fractions collected. The fractions were assayed using gel electrophoresis on a 1.2% agarose gel. After washing the column thoroughly with column buffer it was used to fractionate the cDNA. To the cDNA was added loading buffer (1ul 0.25% bromphenol blue-50% glycerol/10ul TE pH7.5) and the sample was run under identical conditions as used to calibrate the column. Thus the actual size of the cDNA in each fraction collected could be determined. The amount of radioactivity in each fraction was determined by counting the whole fraction on the ^{3}H channel of the scintillation counter. The column fractions containing cDNA of the desired size range were pooled and stored at -20°C.

4:2:14 Ligation of cDNA:(EcoR1)linker with EcoR1 digested λ vector DNA

For good ligation of the cDNA to the phage arms a molar ratio of 1:1 (cDNA:gt10 or gt11) in a total DNA concentration of 2ug was aimed at (Maniatis, Fritsch & Sambrook, 1982). Thus 2ug of EcoR1 digested λ vector DNA was co-precipitated with 40ng of cDNA at -20°C with 2 volumes of EtOH. The precipitate was

pelleted by centrifugation in a microfuge at 4°C for 15 minutes. The pellet was washed once with 70% EtOH, dried and resuspended with 2U T4 DNA ligase in 10ul ligation buffer (25mM Tris HCl pH7.4, 10mM $MgCl_2$, 10mM DDT, 1mM ATP, 0.2mM spermidine). This was incubated at 15°C overnight. The success of the ligation reaction was tested by running samples of the ligated cDNA:gt10 or cDNA:gt11 next to gt10 or gt11 and EcoR1 digested gt10 or gt11 on a 0.8% agarose gel in TBE buffer. One would expect to see one band for the ligated material.

4:2:15 **In Vitro packaging**

The product of the ligation reaction was packaged using λ DNA *in vitro* packaging kit and protocol as of Amersham International plc.

4:2:16 **Plating of packaged phage**

By plating out a sample of the "phage stock" obtained from packaging one can determine the packaging efficiency of the cDNA:λgt hybrids and the percentage of recombinant phage present. Dilutions of the "phage stock" were made in phage dilution buffer, PDB, (10mM Tris HCl pH7.4, 10mM $MgSO_4$, 0.01% gelatin). From the expected packaging efficiency $1x10^6$-$1x10^7$ pfu/ug of DNA packaged, dilutions of the "phage stock" were calculated to give approximately 200 plaques when 50ul of the dilution was plated. Dilutions 10-fold higher and lower also were prepared. To 100ul of the plating cells was added 50ul of the phage dilutions. This was incubated at 37°C for 15 minutes to allow the phage to adsorb to the *E.coli* cells. Then 3ml of molten (45°C) soft agar (BBL or LB) was added and the mix poured onto BBL or LB plates. After the

overlay had set the plates were incubated overnight at 42°C for gt11 or 37°C for gt10. By counting the plaques the phage titre of the "phage stock" could be calculated and thus the efficiency of the packaging reaction, derived in pfu (plaque forming units) per mg of DNA packaged, could be calculated.

For a λgt11 library the percentage of recombinant phage in the "phage stock" was determined by the numbers of colourless and blue plaques. Plaques produced by parental λgt11 phage are blue on Xgal plates whereas recombinant phage produce colourless plaques. The percentage of recombinant phage in λgt10 libraries were recognised by their clear plaque morphology, the parental λgt10 phage form turbid plaques.

4:2:17 **Screening the cDNA library with nucleic acid probes**

The cDNA library in λgt10 was screened by hybridisation of nucleic acid probes to DNA from the plaques transferred to nylon filters (PALL). The method used was an adaptation of the Benton & Davis (1977) protocol. Aliquots of the "phage stock" containing 2.5×10^4 pfu in 50ul were incubated with 150ul plating cells at 37°C for 15 minutes. After the adsorption of the phage to the cells 5mls of molten (45°C) soft LB agar was added and the mixes were poured onto dry LB plates which were incubated at 37°C, until the plaques were just beginning to touch. After chilling at 4°C for 1 hour the plates were returned to room temperature and a dry nylon filter was placed on the surface of each plate. Both the filter and the plate were marked for later orientation of plaques. As transfer of the phage DNA to the filter occurs rapidly the filter was removed from the plate after 1 minute and placed DNA side up on Whatmans 3MM paper

soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes, then transferred to neutralizing solution (3M NaAcetate pH5.5) for 5 minutes. The filter was blotted on Whatmans 3MM paper and air dried for 30 minutes. After drying the DNA was fixed to the filter by baking the filter at 80°C for 1 hour. Duplicate filters were made by placing a second filter on the plate and leaving it in contact with the plaques for 2 minutes before removing the filter and proceding as above.

As hybridisation times and temperatures are a function of the probe concentration and sequence the procedure differed depending on the probe being used. The hybridisation temperature and time for each probe used is shown in table 4.1. All the filters were prehybridised, in sealed bags, for 1 hour with 4ml/100cm^2 of hybridisation buffer (5x denhardts buffer, 5xSSPE, 0.2%SDS, 0.1mg/ml denatured nonhomologous DNA). After prehybridisation the labelled probe (2x10^{6}cpm/filter) was added to the bag and the filter hybridised. After hybridisation the filters were washed at the temperatures and salt concentrations shown in table.. After washing, the filters were autoradiographed using Kodak X-Omat S film.

4:2:18 Preparation of DNA probes

a. **Placental cDNA Probe** The single stranded cDNA probe was prepared by reverse transcribing human placental poly (A^+) RNA. In a sterile eppendorf tube was added 10U RNasin, 1ug poly (A^+) RNA, 10ul Random primer (1ug/ml) and 1ul of 10x 1st stand buffer to a final volume of 10ul. The sample was heated at 70°C for 5 minutes and quenched on ice. To it was added 4ul 10x 1st strand buffer, 7.5ul of 5mM d(G,C,T)TP, 2.5ul 20uM dATP, 100uCi

TABLE 4.1

Probe	Hybridisation temperature	WASHING CONDITIONS low stringency	high stringency	very high stringency
placental cDNA	65°C	2xSSPE RT	0.2xSSPE + 0.2% SDS, 65°C	0.1xSSPE + 0.2% SDS, 70°C
PLAPoligonucleotide(14)	RT	5xSSPE RT	1xSSPE + 0.2%SDS, 30°C	1xSSPE + 0.2% SDS, 32-40°C
PLAP oligonucleotide (26)	35°C	5xSSPE RT	1xSSPE + 0.2% SDS, 55°C	1xSSPE + 0.2% SDS, 65-85°C
hPL oligonucleotide (14)	RT	5xSSPE RT	1xSSPE + 0.2% SDS, 30°C	1xSSPE + 0.2% SDS, 40°C
pHPL815	65°C	2xSSPE RT	0.2xSSPE + 0.2% SDS, 65°C	0.1xSSPE + 0.2% SDS, 70°C
pDP001	65°C	2xSSPE RT	0.2xSSPE + 0.2% SDS, 65°C	0.1xSSPE + 0.2% SDS, 70°C

Table 4.1: The syncytiotrophoblast cDNA library in \gt10 was screened by hybridisation of nucleic acid probes to DNA from the plaques transferred to nylon filters. The hybridisation temperatures depended on the probe employed and are shown in the table. After hybridisation the filters were washed 3x at each of the stringency stages shown in the table. If the filter was kept moist, by sealing in a plastic bag, it was possible to autoradiograph after any of the washing stages and then resuming washing afterwards.

[$^{a-32}$P]dATP, 20U reverse transcriptase and dH_2O to a final volume of 50ul. This was incubated at 42°C for 2 hours then the reaction stopped by the addition of EDTA to 20mM. To separate cDNA from the unincorporated nucleotides the sample was run on a small G-50 column.

b. **Oligonucleotide Probes** These probes were prepared as shown in chapter 3.

c. **pDP001** This HLA class I insert in pBR322 (Snood et al, 1981; Devilee et al, 1984) and cultures of plasmid were kindly provided by Drs J.J. van Rood, M.J. Giphart and M. Tilanus. The plasmid DNA was nick translated in a total volume of 20ul. To 2ul (200ng) of plasmid DNA, containing the probe, was added 2ul of 10x nick translation buffer (0.5M Tris HCl (pH7.8), 50mM $MgCl_2$, 100mM B-mercaptoethanol) 1ul (50pmol) each of dATP and dCTP, 1ul (100pmol) each of dGTP and dTTP, 5ul (50uCi, 600Ci/mmol) each of [a-^{32}P] dATP and [a-^{32}P] dCTP, 1ul (0.04ng) DN-ase and 1ul (5u) DNA polymerase. This was incubated at 15°C for 1 hour. The labelled probe was isolated from the unincorporated nucleotides by sephadex G-50 chromatograpy (Devilee et al 1984).

d. **pHPL815** This full length cDNA insert for human placental lactogen in pBR322 (Barrera-Saldana, Robberson & Saunder, 1982) was a kind gift of Dr G Saunders. It was nick translated using the same protocol as shown for pDP001.

4:2:19 Screening the cDNA library with antibody probes

\gt11 libraries can be screened with antibody probes as plaques on a lawn of E.coli Y1090 (11cmx 11cm). 100ul of Y1088 plating cells were incubated with $2.5x10^4$ pfu of the library for each plate at 37°C for 15 minutes, then 300ul Y1090 plating cells

are added. After the adsorption of the phage to the cells 5mls of molten (45°C) soft BBL agar was added and the mix was poured onto dry BBL plates which were incubated at 42°C for three hours. Then the plates were removed to a 37°C incubator and overlaid with dry nitrocellulose filters which had been soaked in 10mM IPTG. The plates were incubated at 37°C for 5 hours then removed to RT. The position of the filter on each plate was marked and the filters removed. The filters were washed 5x in TS buffer (50mM Tris HCl pH8, 150mM NaCl) for 30 minutes then incubated at RT in 5% BSA in TS buffer (10-15ml/filter) for 1 hour. The filters were then incubated overnight, at RT, with antibody (rabbit antisera directed against PLAP or hPL) diluted 1:100 in 5% BSA in TS buffer. The filters were then washed again in TS buffer for 30 minutes before incubating in 1:1000 dilution of peroxidase-conjugated goat anti-rabbit IgG in 5% BSA in TS buffer for 4 hours. After a further 5 washes in TS buffer the reaction was developed by soaking the filters in either 3,3'diaminobenzidine (30mg/ml) in 50mM Tris HCl pH7.4 or o-phenylenediamine in phosphate-citrate buffer pH 5.0, 0.05% H_2O_2 was added to both solutions just before use. The reaction was terminated by washing the filters with water and drying.

As the antibodies used contained components which bind to antigens produced by E.coli the antibodies used were preadsorbed three times with E.coli lysate bound to nitrocellulose filters at a 1:10 dilution for 30 minutes.

4:2:20 Amplification of the cDNA library

The cDNA library was amplified by plating out the library at a density of 2.5×10^4 pfu per 90mm petri dish using

100ul of plating cells per plate and freshly made plates. After incubation for 6 hours the plate stock was prepared by overlaying the plates with cold PDB then leaving at 4°C overnight to allow the phage to diffuse into the PDB. The overlay solution was removed from the plates and to it was added a few drops of $CHCl_3$. This amplified phage stock was stored at 4°C (Davis, Botstein & Roth, 1980).

4:2:21 **Phage mini preps**

Individual colourless plaques for \gt11 and clear plaques for \gt10 were picked, using a pasteur pipette, from "phage stock" dilutions which give well seperated plaques. The plaques and put into 1ml of PDB containing 20ul of chloroform and these phage suspensions were stored at 4°C, to allow the phage to diffuse into the buffer. Several different procedures were used for the isolation of DNA from the phage suspensions.

a. **Liquid culture method**:- This method is an adaptation of the procedure of Leder et al (1977). To 4.5ml of LB plus was added 100ul of fresh bacterial cells from an overnight culture and 0.5ml of a `phage suspension. The mixture was incubated with aeration overnight (at 37°C for gt10 or 42°C for gt11), until lysis occured. Then 100ul of chloroform was added to the culture and it was incubated for a further 15 minutes before centrifuging at 8000g for 10 minutes at 4°C to remove the bacterial debris and the supernatant treated with RNase A (1mg/ml) and DNase I (1mg/ml) at 37°C for 30 minutes.

b. **Plate lysate method**:- This is an adaptation of the procedure described by Maniatis et al (1982). To 100ul of plating cells was added 50ul of the `phage suspension this was incubated at 37°C

for 15 minutes. Molton BBL 0.65% agarose was added and the mixes were poured onto freshly made BBL plates which were incubated at 42°C, until the plaques were beginning to touch. The phage were eluted from the agarose by adding 5ml of PDB to each plate and storing for 2-4 hours at RT with constant gentle shaking or at 4°C overnight. The bacterial debris was removed from the solution by centrifuging at 8000g for 10 minutes at 4°C. The supernatant was also treated with RNase A (1mg/ml) and DNase I (1mg/ml) at 37°C for 30 minutes.

The `phage particles were recovered from the supernatants by centrifugation at 48000g for 30 minutes at 4°C, the pellet was resuspended in 0.5ml of PDB. To isolate the DNA the resuspended phage particles were adjusted to 0.1% SDS and incubated at 65°C for 10 minutes with either 5ul of 0.5M EDTA (pH 8.0) or with 75ul of 2M NaCl/0.1M EDTA. The mix was extracted twice with phenol/chloroform, once with chloroform and once with ether. The final aqueous phase was precipitated using either an equal volume of isopropanol at -70°C or 2 volumes of ethanol. The pellet was washed with 70% ethanol, dried and resuspended in TE.

The DNA was restricted in a total volume of 20ul using 2ul of 10xHSB or 10x core buffer and 2 units of EcoR1. The DNA fragments of the restriction digest were analysed by gel electrophoresis.

4:3:1 Estimate of the amount of cDNA synthesised

From the incorporation of ^{32}P into the DNA during the first strand synthesis reaction, assayed by TCA precipitation (table 4.2), it is possible to estimate the amount of cDNA synthesised. Between 5-50% of the concentration of poly(A^+) RNA used should be reverse transcribed into cDNA. Using 2ug of placental poly(A^+) RNA the mass of cDNA synthesised was between 0.41-0.58ug (21-29%).

4:3:2 Analysis of the size distribution of the cDNA

The reserved 1% sample reaction volumes from the first and second strand synthesis of the cDNA and after S1 nuclease treatment were used to analyse the size distribution of the reaction products by running the three samples in adjacent tracks in an 1.2% alkaline agarose gel. Figure 4.1 (tracks 1 & 2) shows that the average size of the cDNA after first strand synthesis 1kb. After second strand synthesis the size of the cDNA doubled, due to the presence of the "hair pin" structure (figure 4.1, tracks 3 & 4). The "hair pin" was digested with S1 nuclease (figure 4.1, tracks 5-9). The optimal amount of S1 needed to reduce the size of the cDNA to that of the first strand reaction products was shown by the titration of S1 nuclease to be 24000u/ml (figure 4.1, track 6).

4:3:3 Analysis of EcoR1 Linker addition to the double-stranded cDNA

A 10% polyacrylamide gel in TBE buffer was run to check

TABLE 4.2

% TCA precipitable counts, after first strand synthesis : (a) 0.62%
(b) 0.65%
(c) 0.89%
(d) 0.85%
(e) 0.79%

$$\text{cDNA synthesised} = 4 \times \frac{\%\ \text{incorporation of } [^{32}P]\text{dATP} \times \text{reaction volume (ul)} \times \text{moles/ul} \times \text{mw of dNTP}}{100}$$

$$= 4 \times \frac{\%\ \text{incorporation of } [^{32}P]\text{dATP} \times 50 \times 10^{-3} \times 330}{100 \qquad\qquad 10^{6}}$$

$$= \%\ \text{incorporation of } [^{32}P]\text{dATP} \times 6.6 \times 10^{-7}\ \text{g}$$

cDNA synthesised using 2ug of poly (A^+) RNA: (a) 0.41ug
(b) 0.43ug
(c) 0.59ug
(d) 0.56ug
(e) 0.52ug

Table 4.2: Estimation of the amount of cDNA synthesised by assaying the incorporation of ^{32}P into the cDNA during the first strand synthesis by TCA precipitation.

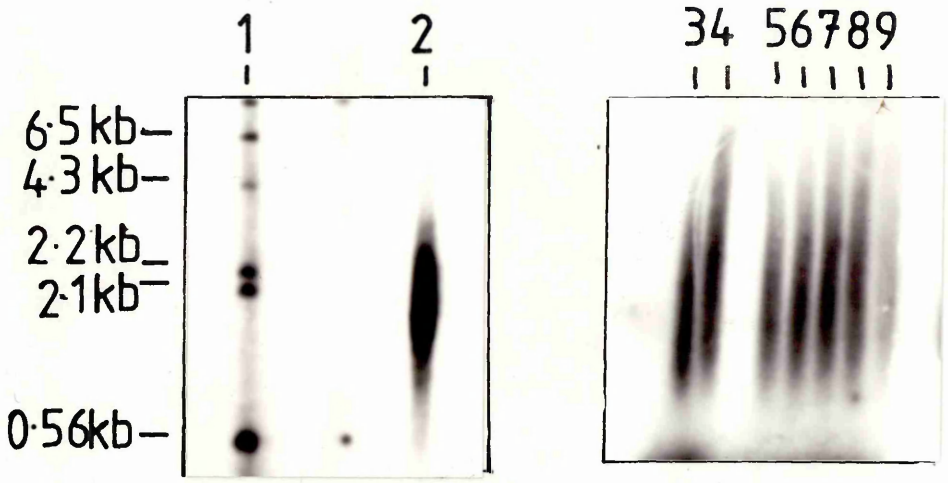


Figure 4.1: 1% samples of ^{32}P-labelled syncytiotrophoblast cDNA were run on 1.2% alkaline agarose gels, the gel was dried and autoradiographed.

1: Size standards, λ Hind III
2: First strand cDNA product
3: First strand cDNA product
4: Second strand cDNA product
5: cDNA after digestion with 4800u/ml S1 nuclease
6: cDNA after digestion with 2400u/ml S1 nuclease
7: cDNA after digestion with 1200u/ml S1 nuclease
8: cDNA after digestion with 600u/ml S1 nuclease
9: cDNA after digestion with 300u/ml S1 nuclease

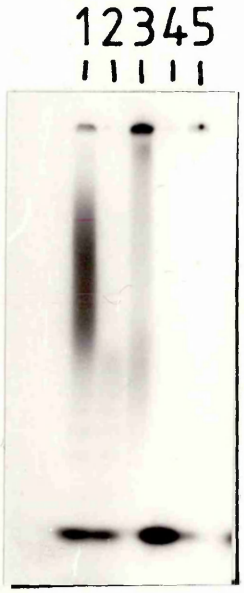


Figure 4.2: 10% TBE polyacrylamide gel, showing the removal of excess EcoR1 linkers with isopropanol precipitations and EcoR1 digestions.

1: cDNA, EcoR1 linker ligation mix
2: supernatant from the 1st isopropanol precipitation
3: precipitate " " " " "
4: supernatant from the 2nd isopropanol precipitation
5: precipitate " " " " "

that the excess EcoR1 linkers were removed from the cDNA sample by the isopropanol precipitations and EcoR1 digestion (figure 4.2). In track 1, a ladder of linker oligomers can be seen, due to the 100:1 molar excess of linkers present in the cDNA:(EcoR1)linker ligation. The first isopropanol precipitation has the effect of removing the linker ladder below 100bps (track 2 &3), as only DNA of >100bps was precipitated. The remaining linker oligomers were shown to have been digested by EcoR1 using a second isopropanol precipitation. Track 4, the non-precipitated material, shows that the linkers have been digested by the EcoR1 to unligated linkers and track 5, the precipitated material, shows the linker:cDNA free from excess linkers.

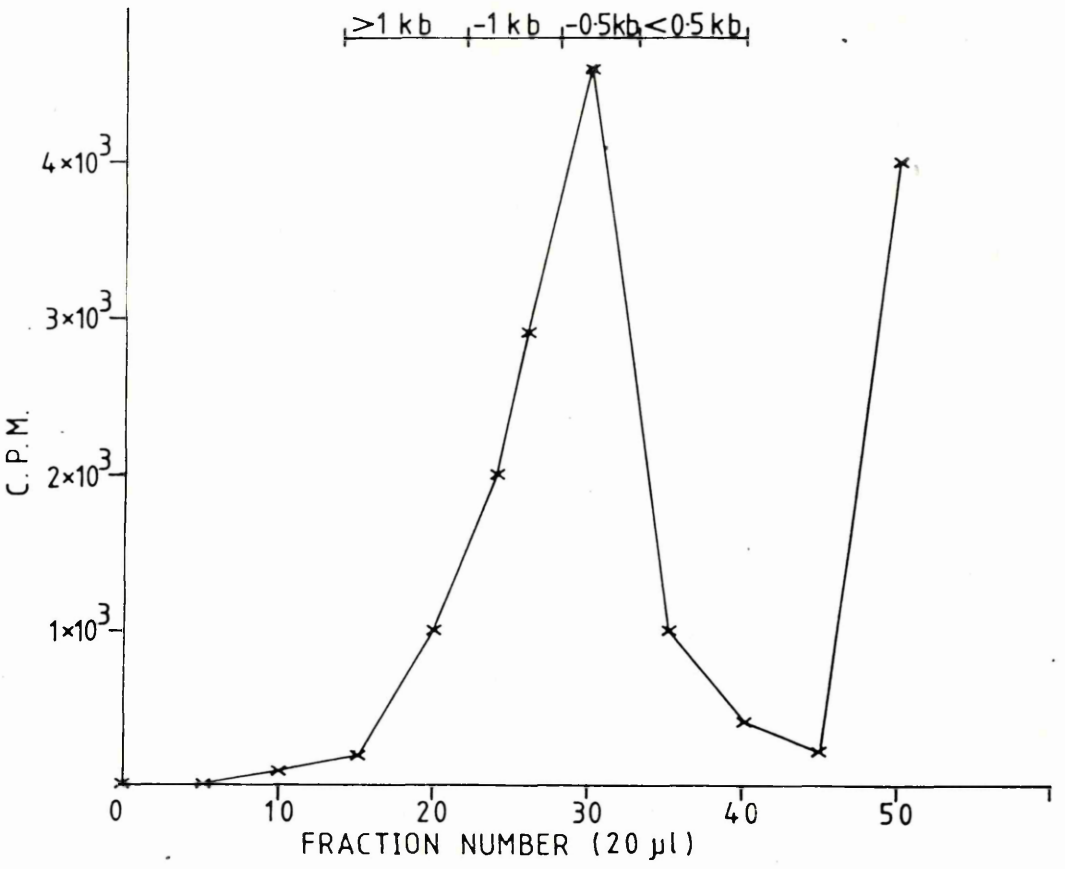
4:3:4 Size fractionation of cDNA

The size fractionation of ds cDNA using a bio-Gel 50 column gives an elution profile as shown in figure 4.3. By comparing this profile with that obtained with Hinf I pBR322 fragments the actual size of the cDNA in each fraction was determined and is shown in figure 4.3. From this it was possible to determine the actual size of cDNA used in the cDNA cloning procedure. Only cDNA fractions with a size of 1Kb and greater were used in the cloning of cDNA into \gt10.

4:3:5 Screening the cDNA library with nucleic acid probes

The cDNA library cloned into \gt10 contained 7.8×10^4 recombinants (table 4.3). To ascertain that these recombinants were of a placental origin they were screened using a placental cDNA probe. The results (figure 4.4) show that 10% of the recombinants hybridised to this probe.

Fig 4.3



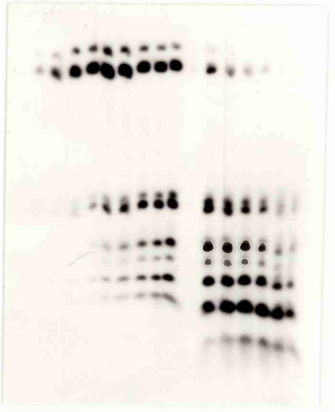


Figure 4.3: Size fractionation of ^{32}P-labelled syncytiotrophoblast cDNA. (a) The elution profile of the cDNA from a bio-gel 50 column. (b) The column was calibrated with Hinf I pBR322 fragments.

TABLE 4.3

\gt10:cDNA	Total pfu/ug	clear pfu/ug	% Recombinants
1 : 0	2.3×10^5	5.0×10^2	0.22
a 1 : 1	2.4×10^5	3.0×10^4	12.50
b 1 : 1	2.5×10^5	2.0×10^4	8.00
c 1 : 1	2.2×10^5	1.6×10^4	7.27
d 1 : 1	2.2×10^5	1.2×10^4	5.45

Table 4.3: Efficiency of syncytiotrophoblast cDNA cloning into \gt10. The total number of recombinant clones obtained was 7.8×10^4.

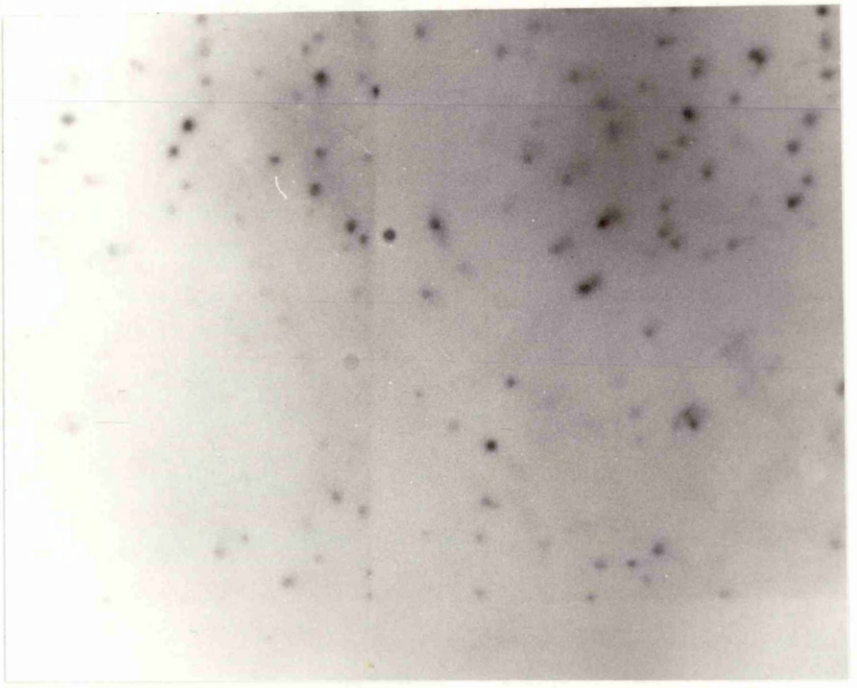


Figure 4.4: Screening of the recombinant \gt10 library with placental cDNA probe. Approximately 10% of the recombinants hybridized to the cDNA probe under stringent hybridization conditions.

The 14 base PLAP oligonucleotide probe did not hybridise to any of the recombinants in this λgt10:cDNA library, at low stringency washes. However, the 26 base PLAP oligonucleotide detected 24 recombinants in the amplified library; 55°C at high stringency washes. As the library was screened at a density of 250pfu/cm^2 the PLAP recombinants could not be picked without also picking some of the surrounding non-PLAP recombinants. These recombinants, after amplification, are in the process of being rescreened. Figure 4.5 shows that PLAP clones compose approximately a third of the recombinant clones present in the PLAP amplified stock.

The library was also screened with the 14 base oligonucleotide for hPL. Figure 4.6a shows that 0.01% of the λlibary screened remained hybridised to the hPL oligonucleotide probe after stringent washing conditions. This result was also seen with the full length hPL probe pHPL815 (figure 4.6b). The hPL mRNA accounts for 15-20% of the total mRNA present in the syncytiotrophoblast mRNA and thus one would expect a higher percentage of the recombinants to be hPL clones (Barrera-Saldana et al, 1982). However, the hPL gene has a known mRNA sequence of 815 bases and only cDNA fragments of 1Kb and greater were cloned. Thus hPL cDNA sequences may be under-represented in this library.

The library has also been preliminarily probed using the class I HLA cDNA probe pDP001. Under high stringency washing conditions this probe hybridised to 0.1% of the amplified library screened, figure 4.7.

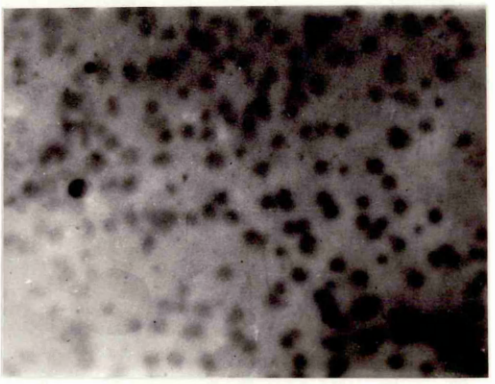


Figure 4.5: Rescreening of the `PLAP' recombinant,amplified, \gt10 clones with 26mer PLAP oligonucleotide. Approximately a third of the recombinants hybridized to the PLAP probe under stringent hybridization conditions.

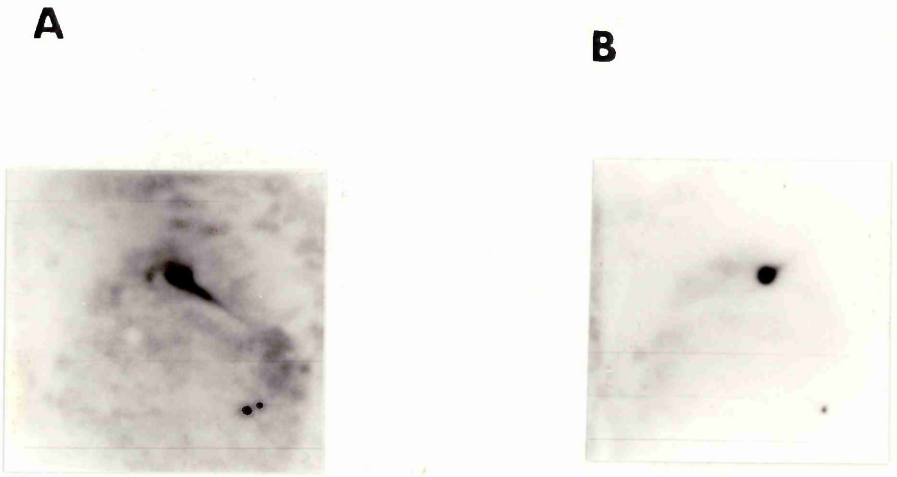


Figure 4.6: Screening the amplified recombinant \gt10 library with hPL probes. (a) hPL, 14mer, oligonucleotide (b) pHPL815 hPL cDNA probe. From a total of 25,000pfu plated only three of the recombinants hybridized to the probes under stringent hybridization conditions.

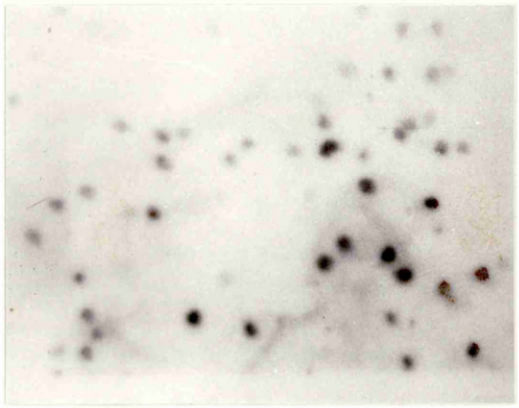


Figure 4.7: Screening the recombinant, amplified, \gt10 library with the class I HLA probe pDP001. Using stringent hybridization conditions. 30 clones hybridized to the HLA probe from a total of 25,000pfu plated

4:3:6 **Screening \ gt11 clones**

With the protocol outlined in the methods one expects up to 30% recombinants in a \gt11 library. Even with the DNA in great molar excess (table 4.4A) the maximum percentage of recombinants obtained was 3.4%. These recombinants were screened with antibody probes for hPL and PLAP on a lawn of Y1090. As no hybridisation was detected DNA was prepared from 10 randomly chosen clear plaques and digested with Eco RI. None of the DNA from these or other plaques tested showed insert fragments. In most of the cases this was due to the insensitivity of the DNA to digestion with EcoR1.

To obtain a higher percentage of recombinant phage, commercially obtained dephosphorylated \gt11 arms were used. The advantage of this is that with the removal of the 5` terminal phosphate the vector arms cannot be ligated together in the absence of insert DNA. Using VCS \gt11 arms 91-98% of the plaques obtained were colourless and thus recombinants. However, the total number of recombinants obtained was only 120, table 4.4B, the expected number was between $5x10^5$ and $1x10^6$. Several control ligations (table 4.4C) using the test insert, 4Kb EcoR1 Rheo fragment, provided with dephosphorylated gt11 arms gave $3.3x10^3$ recombinants. This was a thousand fold lower than quoted in the data sheet. The 120 placental cDNA recombinants were screened using antibody probes without sucess. A second batch of dephosphorylated \gt11 arms were obtained from VCS. The results obtained from test ligations was indicitive of poor dephosphorylation (table 4.4D).

TABLE 4.4

	Molar ratio	Total pfu/ug	Recombinant pfu/ug	% Recombinants
A. λgt11:cDNA				
a	1 : 0	5.9×10^7	-	-
	1 : 1	5.0×10^7	-	-
	2 : 1	2.3×10^7	-	-
b	1 : 0	1.5×10^6	-	-
	1 : 1	1.4×10^7	5.1×10^5	3.6
	2 : 1	3.0×10^6	-	-
c	1 : 0	1.1×10^7	-	-
	1 : 1	1.8×10^7	2.7×10^5	1.5
d	1 : 0	1.6×10^7	1.6×10^5	0.1
	1 : 1	7.0×10^6	3.5×10^4	0.5
	1 : 2	1.0×10^7	3.4×10^5	3.4
	1 : 4	7.1×10^6	1.33×10^5	1.9
	2 : 1	1.0×10^6	2.6×10^4	2.6
	4 : 1	2.3×10^7	2.0×10^4	0.08
B. VCS_1 λgt11:cDNA				
a	1 : 1	120	120	100
b	1 : 1	104	103	99
C. VCS_1 λgt11:Rheo				
a	1 : 1	3.9×10^3	3.85×10^3	99
b	1 : 1	3.2×10^3	3.2×10^3	100
c	1 : 1	2.9×10^3	2.85×10^3	98
d	2 : 1	5.44×10^3	5.38×10^3	99
D. VCS_2 λgt11:Rheo				
a	1 : 1	9.7×10^4	4.0×10^3	4
b	1 : 1	1.5×10^5	5.0×10^3	3.3

Table 4.4: Efficiency of cDNA cloning into λgt11:
A: Using different molar ratios of λgt11 arms and syncytiotrophoblast cDNA.
B: Using VCS dephosphorylated λgt11 arms (1st batch) and syncytiotrophoblast cDNA.
C: Using VCS dephosphorylated λgt11 arms (1st batch) and the 4Kb EcoR1 Rheo fragment as a test of the efficiency of cDNA cloning.
D: Using VCS dephosphorylated λgt11 arms (2nd batch) and the 4Kb EcoR1 Rheo fragment as a test of the efficiency of cDNA cloning.

4:4 DISCUSSION

A syncytiotrophoblast cDNA library has been cloned into \gt10. This is not a total library as only cDNA fragments of 1Kb and greater were cloned. The reason for this was primarily to ensure the presence of PLAP sequences capable of hybridising to the 5' oligonucleotides, it also seemed likely to exclude hPL clones from the library. As the hPL message accounts for 15-20% of the mRNA present it was hoped that its removal would enhance the chance of detecting PLAP clones. The short 14 base oligonucleotide for PLAP did not detect any clones whereas the longer PLAP oligonucleotide did. This is probably due to its increased size, though it was also slightly further (36 bases) from the 5`end.

The clones isolated for PLAP need to be further characterised, to ascertain the size of the cDNA inserts and to determine their homology to each other and to the PLAP probe used in their isolation. Once characterised, these clones can be used to screen the library for longer cDNA clones and in the stepwise method it may be possible to isolate cDNA clones covering the whole coding region for PLAP.

cDNA clones showing homology to the HLA class I DNA pDP001 were also detected in this library. This is interesting and these clones definitely need characterising since serological evidence to date indicates that classical class I determinants are thought to be absent from the syncytiotrophoblast (chapter 1). The main avenues for the investigation of these these clones would be restriction mapping, sequencing and the comparison of hybridisation patterns of these clones and other HLA cDNA probes

(see chapter 5).

Polyvalent antibodies have been used sucessfully to isolate genes from λgt11 libraries but this is very dependent on the titre of the antibody and whether it can bind to antigenic determinants on the fusion protein expressed in the gt11 vector. Colourless, recombinant, plaques were isolated from the gt11 library. However, no plaques were isolated by screening with hPL or PLAP antibody probes and no DNA insert fragments were detected. As the excess linkers were removed from the cDNA used for this library by isopropanol precipitation and not size fractionation, it was assumed that linker contamination had lead to the appearance of false recombinants. Unfortunately the commercially obtained VCS λgt11 dephosphorylated arms did not live up to expectations and only 120 recombinant plaques were obtained. At least 10^5 recombinants are needed to have a library containing clones of rare mRNAs.

CHAPTER 5

D I S C U S S I O N

DISCUSSION

In the last decade since the first reports of molecular cloning (Efstratiadis et al 1976), this technology has evolved into a invaluable tool in the molecular analysis of eukaryotic genes. The use of λ cloning vectors now make it possible to clone and express mRNAs which are of low abundance in the mRNA population (Huynh, Young & Davis, 1984).

A syncytiotrophoblast cDNA library was constructed (chapter 4) in the lambda vector λgt10 and consisted of 7.8×10^4 recombinants. This was not a complete library of all the synthesised cDNA sequences, as the cDNA has been size fractionated. Only cDNAs of 1Kb and greater were cloned representing 42% of the total cDNA synthesised. The evidence that the recombinant 'phage did contain placental cDNA inserts was obtained by screening both the syncytiotrophoblast cDNA library and a control, Rheo, library with a random primed syncytiotrophoblast cDNA probe. Approximately 10% of the syncytiotrophoblast cDNA recombinants hybridised to this probe but none of the rheo recombinants.

Khalaf (1985) has done an extensive immunohistological study to measure the level of contamination in cytotrophoblast preparations from the chorionic plate. In the placental preparations she studied between 65-85% of the cells were cytotrophoblast, the main contaminants being endothelial cells and lymphocytes. No immunohistological work has been done to determine the level of contaminating cells in the syncytiotrophoblast preparations used in the creation of the cDNA library. The analysis of the mRNA in vitro translations shows

the major synthesised polypeptide to be hPL, a trophoblast tissue specific protein with an abundant message. By screening the syncytiotrophoblast library with DNA probes for genes known to be absent from the syncytiotrophoblast and present in the possible contaminants the percentage contamination could be assessed. Also the tissue specificity of interesting clones isolated can determined by dot hybridisations using RNA from placental and other tissue.

This placental syncytiotrophoblast cDNA library contains clones which hybridise to the PLAP oligonucleotide probes; if these clones are found to be of PLAP cDNA, they will be among the first human enzyme polymorphisms to be cloned. First one needs to determining the homology of the PLAP clones to each other, this can be done by restriction mapping of the clones. Southern blots of ^{32}P-labelled PLAP clones to restriction enzyme digested human DNA, and northern blots of placental RNA will enable the hybridisation patterns of the different PLAP clones to be compared. To ascertain without doubt that these clones do indeed contain PLAP sequences would involve comparison of the known amino acid sequence of PLAP with the di-deoxy sequenced PLAP cDNA inserts. These PLAP clones can be used to screen the library for longer cDNA clones, as these clones were detected by an oligonucleotide from the amino-terminal end of the PLAP amino acid sequence one would expect them to be near full-length. Because S1 nuclease is used to digest the hairpin structure the cDNA clones will lack some sequences corresponding to the 5` untranslated region of the mRNAs.

PLAP polymorphisms have been investigated at the protein level through monozygotic and dizygotic twin studies and by

population studies in different racial groups (Robson & Harris, 1965 & 1967; Beckman & Beckman, 1968 & 1969). At the protein level three common alleles and 15 rare alleles have been identified by starch gel electophoresis, though the structural basis for this polymorphism has not been determined (Donald & Robson, 1973; Harris et al, 1974). Clearly they must involve amino acid substitutions, these substitutions may be extensive since some monoclonal antibodies are known to recognise different PLAP phenotypes seen on starch gels as well as other phenotypes not detected by starch gels (Slaughter et al, 1981; Millan et al, 1982b). Sequencing of cDNA clones will reveal the extent of these amino acid substitutions.

As some of the amino acid substitutions alter restriction enzyme cleavage sites they can be detected in total genomic DNA by the use of cocktails of restriction enzymes and specific probes, also any restriction sites which map outside the gene but are in linkage disequilibrium will aid the detection of the different alleles. By using the cloned PLAP gene it may be possible to distinguish the PLAP genotypes at the DNA level. This has the advantage that one can phenotype any individual in the population rather than the placentae from a small subset of the population. At the moment the maternal genotype is deduced from placental phenotypes. The aim is to use the cloned PLAP gene to determine whether there is any correlation between PLAP genotype and pregnancy diseases. By determining the genotype of PLAP for mother, father and placenta in normal pregnancies, spontaneous abortions and complications of pregnancy, one may be able to detect a relationship between PLAP genotype and the success of pregnancy.

The evolution of the different forms of AP is of great interest, the structural basis of the unusual polymorphism of PLAP and the differences between the three human isoenzymes is not known. Post-translational modifications may account for the different catalytic and electrophoretic properties shown by these enzymes and the altered glycosylation could change the antigenic sites of the different enzymes. To determine the relationship between PLAP, PLAP-variants and the other human AP's requires structural studies. At the protein level Ezra et al (1983) have reported, to date, the 40 N-terminal amino acids for PLAP and PLAP-like variants to be identical. By studying PLAP at the molecular level one may be able to detect the reason for PLAP's high level of genetic polymorphism, which may be due to gene conversion. Also as PLAP or PLAP-like proteins are expressed in ovarian and some other tumours we can compare the nature of the villous PLAP gene with the PLAP-like gene expressed in tumours. The use of PLAP clones will enable us to determine how closely related the placental PLAP and tumour PLAP genes are. The PLAP clones can also be used to screen human genomic libraries for other alkaline phosphatase genes.

Immunohistology has indicated that HLA class I genes are only expressed on non-villous trophoblast (Sunderland et al, 1981a&b; Redman et al, 1984). Screening my syncytiotrophoblast cDNA library with the full length class I probe pDP001 has detected cDNA clones which hybridise to this probe. As no HLA class I determinants have been detected on the villous trophoblast by immunohistology the study of these HPL-like syncytiotrophoblast cDNA clones will determine the relationship between these syncytiotrophoblast HLA determinants and other

tissue HLA determinants. It is already known that the HLA determinants expressed on the non-villous cytotrophoblast are not the fetal or maternal HLA A and B allotypes (Redman, 1983). The further characterization of the human cytotrophoblast class I determinants depended on a supply of purified cytotrophoblast cells. Khalaf (1985) purified cytotrophoblast cells from the chorionic plate of term placenta. The resultant mRNA was analysed together with mRNA from the syncytiotrophoblast and is being used as a source of mRNA for the creation of a cytotrophoblast cDNA library. By probing future cytotrophoblast cDNA libraries with established clones of HLA class I genes, we will be able to detect cytotrophoblast HLA clones and establish the nature of the HLA genes expressed in this type of human cytotrophoblast. The available evidence suggests that they will either belong to HLA C or to a yet unknown HLA class I locus. The latter possibility would mirror murine H-2 where 36 distinct class I genes are found in the BALB/C genomic cosmid library, of which 10 are, as yet, uncharacterised (Goodenow *et al*, 1982; Steinmetz, 1984; Redman, 1983).

The placental cDNA libraries, both syncytiotrophoblastic and cytotrophoblastic, can also be screened for HLA class II antigens, though most of the available immunohistological results indicate that no class II determinants are detectable on placental tissue *in situ* (Faulk & Temple, 1976; Goodfellow *et al*, 1976; Sunderland *et al*, 1981a&b). Whether this is the case *in vitro* is not yet clear. HLA-DR antigens have been reported to be present in first trimester placental cultures, this work has yet to be confirmed by other groups (Brami *et al*, 1983). Khalaf (1985) failed to find class II expression in cultured

cytotrophoblast at term.

Allotypic expression of some paternal HLA antigens and ABO blood group antigens are known to be absent from the placenta (Faulk & Temple, 1976; Redman, 1983). Other minor histocompatibility antigens are very probably present in man since in the mouse is known to have more than 30 minor histocompatibility antigen loci. However, in neither mouse nor man have the expression of such systems been explored in detail in the placenta. The evidence for trophoblast components acting as antigenic determinants which elicit a maternal immune response is varied. The results presented in chapter 2 show that though agglutinins were present in term maternal sera they were not an antibody response against trophoblast components. Davies & Browne (1985a&b) using an ELIZA assay have detected anti-trophoblast antibodies in maternal sera during pregnancy, whereas N. Hole and P.M. Johnson (Liverpool group, unpublished observations) using a similar ELIZA assay detected no maternal antibody reactivity against trophoblast surface determinants. The cDNA libraries which are originally cloned into \gt10 can be easily transferred to \gt11 by digesting the \gt10 cDNA recombinant DNA with EcoR1, recovering the cDNA inserts from low melting point agarose and ligating this with EcoR1 cut \gt11 DNA. If maternally-recognised trophoblast antigens are detected and purified, polyclonal antibodies could be raised. Though it is almost certain that these antibodies would be of a low affinity they could be used to screen placental cDNA libraries constructed in the lambda expression vector \gt11. The \gt11 placental cDNA library could be screened with high affinity monoclonal antibodies from hybridomas formed from Epstein-Barr transformed maternal

lymphocytes as this method produces antibodies to antigens present in normal lymphocytes the antibody specificity needs to be confirmed before the antibodies are used. Several trophoblast antigens and antigen systems have been identified by xenogeneic antisera or monoclonal antibodies (Faulk et al, 1978; Johnson et al, 1981; Brown et al, 1983). Again by using a λ expression vector such as λ gt11 it may be possible to probe a placental cDNA library with these antibodies and characterise the genes involved.

B I B L I O G R A P H Y

Abu Hasan, N.S. (1983). Studies on human placental alkaline phosphatase Ph.D. Thesis.

Abu Hasan, N.S., Davies, M. and Sutcliffe, R.G. (1984). A high molecular weight species of placental alkaline phosphatase in human syncytiotrophoblast microvilli. Placenta 5, 159-174.

Abu Hasan, N.S. and Sutcliffe, R.G. (1984a). Purification and analysis of the microvillous (M) and A forms of placental alkaline phosphatase. Placenta 5, 71-82.

Abu Hasan, N.S. and Sutcliffe, R.G. (1984b). Molecular heterogeneity of placental alkaline phosphatase associated with microvillous membranes. In: Human Alkaline Phosphatases. Stigbrand, T. and Fishman, W.H. (eds). Alan R. Liss Inc., N.Y. pp 117-126.

Abu Hasan, N.S. and Sutcliffe, R.G. (1985). Placental alkaline phosphatase integrates via its carboxy-terminus into the microvillus membrane; its allotypes differ in conformation. Placenta 6 (in press).

Abelev, G.I., Perova, S.D., Khramkova, N.I., Postnikova, Z.A. and Irlin, I.S. (1963). Production of embryonal X-globulin by transplantable mouse hepatomas. Transplantation 1, 174-180.

Agarwal, K.L., Brunstedt, J. and Noyes, B.E. (1981). A general method for detection and characterization of an mRNA using an oligonucleotide probe. J. Biol. Chem. 25, 1023-1028.

Anderson, S. and Kingston, I. B. (1983). Isolation of a genomic clone for bovine pancreatic trypsin inhibitor by using a unique-sequence synthetic DNA probe. Proc. Natl. Acad. Sci. USA 80, 6838-6842.

Aviv, H. and Leder, R. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69, 1408-1412.

Badger, K.S. and Sussman, H.H. (1976). Structural evidence that human liver and placental alkaline phosphatase isoenzymes are coded by different genes. Proc. Natl. Acad. Sci. USA 73, 2201-2205.

Bagshawe, K.D. (1969). Choriocarcinoma: The clinical biology of the trophoblast and its tumours. Williams and Wilkins Co., Baltimore, pp 72-74.

Barrera-Saldana, H. A., Robberson, D. L. and Saunders, G. F. (1982). Transcriptional products of the human placental lactogen gene. J. Biol. Chem. 257, 12399-12404.

Becker, A. and Gold, M. (1975). Isolation of the bacteriophage lambda. A-gene protein. Proc. Natl. Acad. Sci. USA 72, 581.

Beckman, G. and Beckman, L. (1968). A genetic variant of placental alkaline phosphatase with unusual electrophoretic properties. Acta Genet (Basel) 18, 543-552.

Beckman, G. and Beckman, L. (1969). The placental alkaline phosphatase polymorphism; variations in Hawaiian populations. Hum. Hered. 19, 524-529

Beer, A. E. and Billingham, W. D. (1976). The immunobiology of mammalian reproduction. Prentice Hall, New York.

Benham, F.J., Povey, M.S. and Harris, H. (1978). Heterogeneity of alkaline phosphatase in different Hela lines. Somatic Cell Genet. 4, 13-25.

Benton, W. D. and Davis, R. W. (1977). Screening \gt recombinant clones by hybridization to single plaques in situ. Science 196, 180.

Berdalis, N.G. and Hirschhorn, U. (1972). Properties of placental alkaline phosphatase III. Thermo-stability and urea inhibition of isolated components of the three phenotypes. Biochem. Genet. 6, 1-8.

Billington, W. D. and Bell, S. C. (1983). Immunobiology of mouse trophoblast. In: Biology of the Trophoblast. Loke, Y. W. and Whyte, A. (eds). Elsevier North Holland, Amsterdam. pp 571-595.

Bodmer, J. and Bodmer, W. (1984). Histocompatibility 1984. Immunology Today 5, 251-254.

Bohn, H., Dali, F. and Luben, G. (1983). Human trophoblast specific products other than hormones. Biology of the Trophoblast. Loke, Y.W. and Whyte, A. (eds). Elsevier Science Publishers BU, pp 317-352.

Bohn, H. (1979). Placental and pregnancy proteins. In: Carcino - Embryonic Proteins - Chemistry, Biology, Chemical Applications, Lehman, F.G. (ed), Elsevier/Worth-Holland Biomedical Press, Amsterdam, pp 289-299.

Boime, I., McWilliams, D., Szezesna, E. and Camel, M. (1976). Synthesis of human placental lactogen messenger RNA as a function of gestation. J. of Biol. Chem. 251, 820-825.

Booth, A.G., Olaniyan, R.O. and Vanderpuye, O.A. (1980). An improved method for the preparation of placental syncytiotrophoblast microvilli. Placenta 1, 327-336.

Boyd, S.D. and Hamilton, W.J. (1970). The human placenta, W. Heffer & Sons, Cambridge.

Boyer, S.H. (1963). Human alkaline phosphatases. Discrimination by several means including starch gel electrophoresis of anti-enzyme-enzyme supernatant fluids. Ann. NY Acad. Sci. 103, 928-950.

Brami, C. J., Sanyal, M. K., Dwyer, J. M., Johnson, C. C., Kohorn, E. I. and Naftolin, F. (1983). HLA-DR antigen on human trophoblast. Am. J. Reprod. Immunol. 3, 165-174.

Breslow, J. L., Ross, D., McPherson, J., Williams, H., Kurnit, D., Nussbaum, A.L., Karathanous, S. K. and Zonnis, V.I. (1982). Isolation and characterization of cDNA clones for human apolipoprotein A-I. Proc. Natl. Acad. Sci. USA 79, 6861-6865.

Brody, S. (1953). Quantitative studies on nucleic acids and the nuclease activities during the development of the human placenta. Acta. Obstet. Gynaecol, Scand. 32, Suppl. 6, 1-32.

Broome, S. and Gilbert, W. (1978). Immunological screening method to detect specific translation products. Proc. Natl. Acad. Sci. USA 75, 2746-2749.

Brown, P. J., Molloy, C. M. and Johnson, P. M. (1983). Immunochemical identification of human trophoblast membrane antigens using monoclonal antibodies. J. Reprod. Immunol. 5, 351-361.

Bulmer, J. N., Billingham, W. D. and Johnson, P. M. (1984). Immunohistologic identification of trophoblast populations in early human pregnancy with the use of monoclonal antibodies. Am. J. Obstet. Gynaecol. 148, 19-26.

Butterworth, P.J. and Moss, D.W. (1966). Action of neuromusidase on human kidney alkaline phosphatase. Nature (London) 209, 805-806.

Carlson, R.W., Wada, H.G. and Sussman, H.H. (1976). The plasma membrane of human placenta. Isolation of microvillus membrane and characterization of protein and glycoprotein subunits. J. Biol. Chem. 251, 4139-4146.

Chang, C.H., Angellis, D. and Fishman, W.H. (1980). Presence of the rare D-variant heat-stable placental type alkaline phosphatase in normal human testis. Cancer Res. 40, 1506-1510.

Chard, T. (1982). Proteins of the human placenta: some general concepts. In: Pregnancy proteins. Grudzunskas Tevsner, Seppala (ed),Academic Press, Sydney, pp 3-21.

Chatterjee, M., Baliga, B.S. and Monro, H.N. (1976). Synthesis of human placental lactogen and human chorionic gonadotrophin by polyribosomes and messenger RNA from early and full term placentae. J. Biol. Chem. 251, 2945-2951.

Chatterjee, M. and Monro, H.N. (1977). Changing ratio of human chorionic gonadotrophin subunits synthesized by early and full-term placental polyribosomes. Biochem. Biophys. Res. Commun. 77, 426-433.

Chatterjie-Hasrouni, S., Montgomery, B. and Lala, P. K. (1983). Allo-antigenicity of trophoblast cells. Am. J. Reprod. Immunol. 3, 127-131.

Chirgwin, J.M., Przybla, A.E., MacDonald, J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. J. Biochemistry 18, 5294-5299.

Chou, J.Y. and Ito, F. (1984). Regulation of alkaline phosphatase gene expression in cultured placental cells. In: Human alkaline phosphatase. Stigbrand, T. and Fishman, W. H. (Eds). Alan R Liss Inc. New York, pp 167-186.

Chung, S. and Echols, H. (1977). Positive regulation of intergrative recombination by the cII and cIII genes of bacteriophage λ. Virology 79, 312-319.

Clark, D. A., Slapsys, R. M., Croy, B. A. and Rossant, J. (1983). Suppressor cell activity in uterine decidual correlates with success or failure of murine pregnancies. J. Immunol. 131, 540-542.

Clint, J.M., Wakely, J. and Ockleford, C.D. (1979). Differentiated regions of the human placental cell surface associated with the attachment of chorionic villi, phagocylosis of maternal erythrocytes and syncytiotrophoblast repair. Proc. R. Soc. London B 204, 345-353.

Contractor, S.F. (1983). Metabolic and enzymatic activity of human trophoblast. In: Biology of the trophoblast. Loke, Y.W. and Whyte, A. (eds), Elsevier Science Publishers BU, pp 236-281.

Contractor, S.F., Banks, R.W., Jones, C.J.P. and Fox, M. (1977). A possible role for placental lysosomes in the formation of villous syncytiotrophoplast. Cell Tissue Res. 178, 411-419.

Daniels-McQueen, S., McWilliams, D., Birken, S., Canfield, R. and Landerfeld, T. (1978). Identification of mRNAs encoding the and subunits of human chorionic gonadotrophin. J. Biol. Chem. 253, 7109-7114.

Dancis, J., Jansen, V. and Levitz, M. (1976). Transfer across perfused human placentae IV. Effect of protein binding on free fatty acids. Paediatr. Res. 10, 5-10.

Davies, M., McLaughlin, M.E.E. and Sutcliffe, R.G. (1982a). Immune responsiveness against the human placenta I. Generation of cellular and humanal activity in experimental animals. Immunology 47, 459-468.

Davies, M., McLaughlin, M.E.E. and Sutcliffe, R.G. (1982b). Immune responsiveness against the human placenta II. Response with xenogenetic lymphocytes in culture. Immunology 47, 469-477.

Davies, M. and Sutcliffe, R.G. (1982). A quantitative assay for the measurement of immune responses directed against the human placenta. J. Immunol. Methods 48, 373-383.

Davies, M. and Browne, C.M. (1985a). Anti-trophoblast antibody responses in normal human pregnancy. J. Reprod. Immunol. (In press).

Davies, M. and Brown, C.M. (1985b). Identification of selectively solubilised syncytiotrophoblast plasma membrane proteins as potential antigenic targets during normal human pregnancy. J. Reprod. Immunol. (In press).

Davis, R. W., Botstein, D. and Roth, J. R. (1980). A manual for genetic engineering. Advanced Bacterial Genetics.

Devilee, P., Warnaar, J. N. and Giphart, M. J. (1984). MHC homology between various mammalian species at the DNA level its relevance to MHC evolution. Expl. Clin. Immunogenet. 1, 90-98.

Doellgast, G.J. and Fishman, W.H. (1974). Purification of human placental alkaline phosphatase. Biochem. J. 141, 103-112.

Doellgast, G.J. and Fishman, W.H. (1976). L-leucine, a specific inhibitor of a rare human placental alkaline phosphatase phenotype. Nature 259, 49-51.

Doellgast, G.J., Spiegel, J., Guenther, R.A. and Fishman, W.H. (1977). Studies on human placental alkaline phosphatase purification by immunoabsorption and comparison of the A and B forms of the enzyme. Biochemica et Biophysica Acta 484, 59-78.

Doi, Y. and Nishida, T. (1981). Lecithin-cholesterol acyltransferase from human plasma. Meths. Enzymol. 71, 753-767.

Donald, L.J. and Robson, E.B. (1973). Rare variants of placental alkaline phosphatase. Ann. Hum. Genet. 37, 303-313.

Echols, H. and Green, L. (1971). Establishment and maintenance of repression by bacteriophage lambda: the role of the cI, cII and cIII proteins. Proc. Natl. Acad. Sci. USA 68, 2190-2194.

Edwards, R.G. (1977). Early human development: From oocyte to implantation. In: Scientific foundations of obstetric and gynaecology, 2nd Edition. Philip, E.A., Barnes, J. and Newton, M. (eds). Heineman, London. pp 175-252.

Efstratiadis, A., Kafabos, F.C., Maxam, A.M. and Moniatis, T. (1976). Enzymatic in vitro synthesis of globulin genes. Cell 7, 279.

Elston (1981). Gestational tumours of trophoblast. In: Recent advances in Histopathology, Anthony, P.P. and MacSween, R.N.M. (eds), Churchill-Livingstone, Edinburgh, Vol II, pp 149-161.

Enders, A.C. (1965). Formation of syncitium from cytotrophoblast in the human placenta. Obstet. Gynecol. 25, 378-386.

Ezra, E., Blacher, R. and Udenfriend, S. (1983). Purification and partial sequencing of human placental alkaline phosphatase. Biochem. Biophys. Res. Commun. 116, 1076-1083.

Faulk, W.P., Temple, A., Lovins, R.E. and Smith, N.C. (1978). Antigens of human trophoblasts - a working hypothesis for their role in normal and abnormal pregnancies. Proc. Natl. Acad. Sci. USA 75, 1947-1951.

Faulk, W.P. and Temple, A. (1976). Distribution of B_2-microglobulin and HLA in chorionic villi of human placentae. Nature 262, 799-802.

Faulk, W.P. and Galbraith, G.M.P. (1979). Trophoblast transferrin and transferric receptors in the host-parasite relationship of human pregnancy. Pro. Royal Soc. London B 206, 83-97.

Faulk, W.P. (1981). Human trophoblast antigens: role in normal and abnormal pregnancies. Acad. Med. Bel. 136, 379-388.

Fernley, H.N. (1971). Mammalian alkaline phosphatases. In: The Enzymes, Vol IV, Academic Press (NY) Boyer, P.D. (ed). pp 414-447.

Fiddes, J.C. and Goodman, H.M. (1979). Isolation, cloning and sequence analysis of the cDNA for the subunit of human chorionic gonadotropin. Nature 281, 351-356.

Fiddes, J.C. and Goodman, H.M. (1980). The cDNA for the B-subunit of human chorionic gonadotropin suggest evolution of a gene by readthrough into the 3'-untranslated region. Nature 286, 684-687.

Fishman, W.H., Inglis, N.R., Stolbach, L.L. and Krant, M.J. (1968a). Serum alkaline phosphatase isoenzymes of human neoplastic cell origin. Cancer Res. 28, 150-154.

Fishman, W.H., Inglis, N.R., Green, S., Anstiss, L.L., Ghosh, N.K., Reif, A.E., Rustigan, R., Krant, M.J. and Stolbach, L.L. (1968b). Immunology and biochemistry of Regar isoenzyme of alkaline phosphatase in human cancer. Nature 219, 697-699.

Fishman, W.H. and Sie, H.G. (1970). L-homoarginine an inhibitor of serum "bone and liver" alkaline phosphatase. Clin. Chim. Acta. 29, 339-341.

Fishman, W.H. (1974). Perspectives on alkaline phosphatase isoenzymes. Am. J. Med. 56, 617-650.

Fishman, W.H. and Sell, S. (1976). Onco-developmental gene expression, Academic Press, New York.

Fishman, W.H. and Stolbach, L.L. (1979). Placental alkaline phosphatase. In: Immunodiagnosis of cancer. Herberman, R.B., McIntyre, K.R. (eds), Marcel Dekker, New York, pp 442-449.

Fishman, L., Miyayama, H., Driscoll, S.G. and Fishman, W.H. (1976). Developmental-phase specific alkaline phosphatase isoenzymes of human placenta and their occurrence in human cancer. Cancer Res. 36, 2268-2273.

Fox, H. (1978). Placental structure. In: Scientific basis of obstetrics and gynaecology, MacDonald, R.R. (ed), Churchill Livingstone, Edinburgh, 2nd Edition, pp 27-59.

Friesen, H.G. (1973). Placental protein and polypeptide hormones. In: Handbook of physiology, Section 7, Vol II part 2. Greep, R.O. and Astwood, E.B. (eds). American Physiological Soc. Washington DC. pp 295-309.

Ghosh, N.K. and Fishman, W.H. (1966). On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. J. Biol. Chem. 241, 2516-2522.

Gillam, S. and Smith, M. (1979). Site-specific mutagenesis using synthetic oligodeoxyribonucleotide primers. 1 Optimum conditions and minimum oligodeoxyribonucleotide length. Gene 8, 81-97.

Gold, P. and Freeman, S.O. (1965). Specific carcinoembryonic antigen of the human digestive system. J. Exp. Med. 122, 467-481.

Goldstein, D.J., Rogers, C.E. and Harris, H. (1980a). Expression of alkaline phosphatase loci in mammalian tissues. Proc. Natl. Acad. Sci. USA 77, 2857-2860.

Goldstein, D.J., Blasco, L. and Harris, H. (1980b). Placental alkaline phosphatase in non-malignant human cervix. Proc. Natl. Acad. Sci. USA 77, 4226-4228.

Goldstein, D.J., Rogers, C.H. and Harris, H. (1982). A search for trace expression of placental-like alkaline phosphatase in non-malignant human tissues - demonstration of its occurrence in lung, cervix, testis and thymus. Clin. Chem. Acta 125, 63-75.

Goldstein, D.J. and Berkowitz, R.S. (1982). Major problems in obstetrics and gynaecology, gestational trophoblastic neoplasms, clinical principles of diagnosis and management. W.B. Saunders Co., Philadelphia, pp 11-63.

Goodfellow, P.N., Barnstable, C.J., Bodmer, W.F., Snary, D. and Crumpton, M.J. (1976). Expression of HLA ststem antigens on placenta. Transplant 22, 595.

Goodfellow, C.F. and Taylor, P.V. (1982). Extraction and identification of trophoblast cells circulating in peripheral blood during pregnancy. Br. J. Obstet. Cynaecol. 89, 65-

Goodenow, R.S., McMillan, M., Nicholson, M., Sher, B.T., Eakle, K., Davidson, N. and Hood, L. (1982). Identification of the class 1 genes of the mouse major histocompatibility complex by DNA-mediated gene transfer. Nature 300, 231.

Gordon, Y.B. and Chard, T. (1979). Specific proteins of the human placenta. In: Placental proteins. Klopper, Chard (eds). Springer, Berlin. pp 1-17.

Green, P.J. and Sussman, H.H. (1973). Structural comparison of ectopic and normal placental alkaline phosphatase. Pro. Natl. Acad. Sci. USA 70, 2936-2940.

Hamilton, W.J., Boyd, J.D. and Mossman, H.W. (1972). Human Embryology 4th Edition. Hefter, Cambridge.

Hamilton, W.J. and Hamilton, D.V. (1977). Development of the human placenta. In: Scientific foundations of obstetrics and gynaecology. 2nd Edition. Philip, E. A., Barnes, J. and Newton, M. (eds). Heinemann, London. pp 292-358.

Handwerger, S., Hurley, T.W. and Golander, A. (1981). Placental and decidual polypeptide hormones. In: Principles and practice of obstetrics and perinatology, Iffy, L. and Kamunetzky, M.A. (eds), John Wiley and Sons Inc., New York, pp 243-260.

Harris, H., Hopkinson, D.A. and Robson, E.B. (1974). The incidence of rare alleles determining electrophoretic varients: data on 43 enzyme loci in man. Ann. Hum. Genetics 37, 237-253.

Hellstrom, K.E., Hellstrom, I. and Brown, J. (1969). Abrogation of cellular immunity to antigenically foreign mouse embryonic cells by a serum factor. Nature 224, 914..

Herbert, W.J. (1978). In: Handbook of experimental immunology. Weir, D.M. (ed). Blackwell Scientific Publications.

Herskowitz, I. and Hagen, D. (1980). The lysis-lysogeny decision of phage λ: explicit programming and responsiveness. Ann. Rev. Genet. 14, 399-445.

Hertig, A.T. and Rock, J. (1945). Two human ova of the pre-villous stage, having a developmental age of about seven and nine days respectively. Contrib. Embryol. 31, 65-84.

Hertz, R. (1978). Choriocarcinoma and related gestational trophoblastic tumours in women. Raven Press, New York, pp 23-44.

Holmgren, P.A. and Stigbrand, T. (1976). Purification and partial characterization of two genetic variants of placental alkaline phosphatase. Biochem. Genet. 14, 777-789.

Holmgren, P.A., Stigbrand, T. Damber, M. and Schoultz, B.V. (1978). Serum levels of placental alkaline phosphatase in high risk pregnancies. J. Obstet. Gynecol. 54, 631-634.

Hood, L.E., Weissman, I.L., Wood, W.B. and Wilson, J.H. (1984). Immunology 2nd Edition. The Benjamin/Cummings Publishing Company Inc.

Hoshina, M., Hussa, R., Pattillo, R. and Boime, I. (1983). Cytological distribution of chorionic gonadotropin subunit and placental lactogen messenger RNA in neoplasms derived from human placenta. J. of Cell Biol. 97, 1200-1206.

Hoyt, M.A., Knight, D.M, Das, A., Miller, H.I. and Echols, H. (1982). Control of phage λ development by stability and synthesis of cII protein: role of the viral cIII and host hflA, humA and humD genes. Cell 31, 565.

Huang, K.P., Robinson, J.C. and Chou, J.Y. (1976). Phosphoprotein phosphotase activity associated with human placental alkaline phosphatase. Biochem. Biophys. Res. Commun. 70, 186-192.

Hunter, W.H. and Greenwood, F.C. (1962). Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature, 194, 495-496.

Hutton, R.A., Chow, F.P.R., Craft, I.L. and Dandona, P. (1980). Inhibitors of platelet aggregation in the fetoplacental unit and myometruum with particular reference to the SDP degrading property of placenta. Placenta 1, 125-130.

Huynh, T.V., Young, R.A. and Davis, R.W. (1984). Constructing and screening cDNA libraries in \gt10 and \gt11. In: DNA cloning; a practical approach. Glover, D. (ed). IRL Press, Oxford.

Hytten, F.E. and Leitch, I. (eds.) (1971). Physiology of human pregnancy. Blackwell Scientific, Oxford. pp 326-329.

Ito, F. and Chou, Y. (1983). Biosynthesis and processing of placental AP using AP synthesized by choriocarcinoma cells in vivo and in vitro. Biochem. and Biophys, Res. Com. Vol. III, 611.

Jaye, M., de la Salle, H., Schamber, F., Balland, A., Kohli, V., Findeli, A., Tolstoshev, P. and Lecocq, J-P. (1983). Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe deduced from the amino acid sequence of bovine factor IX. Nucleic Acids Research 11, 2325-2335.

Jemmerson, R., Shar, N., Takeya, M. and Fishman, W.H. (1984). Functional organization of the placental alkaline phosphatase polypeptide chain. In: Human Alkaline Phosphatases, Stigbrand, T. and Fishman, W.H. (eds). Alan R. Liss Ltd. Inc. New York. pp 105-115.

Jeppsson, A., Wahnen, B., Stigbrand, T., Edsmyr, F. and Andersson, L. (1983). A clinical evaluation of serum placental alkaline phosphatase (PLAP) in seminoma patients. Brit. J. Urol. 55, 73-78.

Johnson, P.M., Cheng, H.M., Molloy, C.M, Stern, C.M.M. and Slade, M.B. (1981a). Human trophoblast-specific surface antigens identified using monoclonal antibodies. Am. J. Reprod. Immunol. 1, 246-254.

Johnson, P.M., Ogbimi, A.O., Brown, P.J. and Shah, L.C. (1981b). Antigens of the human syncytiotrophoblast microvillous plasma membrane. Am. J. Reprod. Immunol. 1, 83-87.

Johnson, P.M., McLaughlin, P.J., Cheng, H.M., Molloy, C.M. and Brown, P.J. (1982). Cellular reactivity of monoclonal antibodies to human placental trophoblast surface antigens. Immunol. Reprod. 5, 520-521.

Johnson, P.M. and Molloy, C.M. (1983). Localization in human term placental bed and amniochorion of cells bearing trophoblast antigens identified by monoclonal antibodies. Am. J. Reprod. Immunol. 4, 33-37.

Jones, C.P.J. and Fox, H. (1976). An ultrahistochemical study of the distribution of acid and alkaline phosphatase in placentae from normal and complicated pregnancies. Journal of Pathology 118, 143-151.

Jones, C.P.J. and Fox, H. (1979). Ultrastructure of the placenta in prolonged pregnancy. J. Pathol. 126, 173-180.

Jones, W.B. (1975). Treatment of chorionic tumours. Clin. Obstet. Gynecol. 18, 247-265.

Jones, W.B. (1981). Trophoblastic tumours: prognostic factors. Cancer 48, 602-607.

Kantor, R.R.S., Galbraith, R.M, Emerson, D.L. and Galbraith, G.M.P. (1981). Placental alkaline phosphatase is a major specificity in antisera raised to human trophoblast membranes. Am. J. Reprod. Immunol. 1, 336-339.

Kawagoe, K., Svejare, M. and Machunami, R. (1978). Ultrastructural evidence of human chorionic gonadotrophin on trophoblastic surface. Acta Histochem. Cytochem. 11, 187-194.

Kellen, J.A. and Lustig, V. (1971). Patterns on amino acid inhibition of alkaline phosphatases as an aid in cancer diagnosis. Oncology 25, 239-246.

Kelley, L.K., King, B.F., Johnson, L.W. and Smith, C.H. (1979). Protein composition and structure of human placental microvillous membrane. External surface, sialic acid containing extrinsic and intrinsic components. Exp. Cell Res. 123, 167-176.

Kemp, D.J. and Cowman, A.F. (1981). Direct immunoassay for detecting Escherichia coli colonies that contain polypeptides encoded by cloned DNA segments. Proc. Natl. Acad. Sci. USA 78, 4520-4525.

Kessler, S.W. (1975). Rapid isolation of antigens from cells with a straphylococcal protein A-antibody adsorbent:parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115, 1617-1624.

Kessler, S.W. (1981). Use of protein-A-bearing staphylococci for the immune precipitation and isolation of antigens from cells. Methods Enzymol, 73, 442.

Khalaf, S.A. (1985). Studies on the cells from the basal and chorionic plates of human placenta. Ph.D. Thesis.

Khalaf, S.A., Henderson, S.J. and Sutcliffe, R.G. (1985). Purification and analysis of trophoblast cells from human chorionic plate. (In preparation).

Khalaf, S.A., Livingston, J.C., Nickson, D.A, Henderson, S.J. and Sutcliffe, R.G. (1985). Trophoblast cells and markers. (In press).

Klien, J. (1975). Biology of the H-2 complex. Springer Verlag, New York.

Klopper, A. and Chard, T. (Eds.) (1979). Placental Proteins. Springer-Verlag, Berlin, Heidelberg, New York.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cell secreting antibody of predefined specificity. Nature 256, 495-497.

Komoda, T., Sakagishi, Y. and Sekune, T. (1981). Multiple forms of human intestinal alkaline phosphatase: chemical and enzymic properties, and circulating clearances of the fast - and slow - moving enzymes. Clin. Chim. Acta 117, 167-187.

Kraus, J.P., Conboy, J.G. and Rosenberg, L.E. (1981). Pre-ornithine transcarbonylase. J. Biol. Chem. 256, 10739-10742.

Kraus, J.P. and Rosenberg, L.E. (1982). Purification of low-abundance messnger RNAs from rat liver by polysome immunoadsorption. Proc. Natl. Acad. Sci. USA 79, 4015-4019.

Kreitman, M. (1983). Nucleotide polymorphism at the alcohol dehydrogenase loci of Drosophila menogaster. Nature 304, 412-417.

Kronwall, G., Seal, U.S., Funstad, J. and Williams, R.C. (1970). Phylogenetic insight into evolution of mammalian F- fragment of G globulin using staphylococcal protein A. J. Immunol. 104, 140-147.

Kurman, R.J., Main, S.C. and Chan, H-C (1984). Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta 5, 349-370.

Laemmli, U.K. (1970). Cleavage structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lala, P.K., Chatterjie-Hasrouni, S., Kearns, M., Montgomery, B. and Calavincenzo, V. (1983). Immunobiology of the fetal-maternal interface. Immuno. Rev. 75, 87-116.

Lange, P.M., Millar, J.L., Stigbrand, T., Venella, R.L., Ruoslahti, E. and Fishman, W.H. (1982). Placental alkaline phosphatase as a tumour marker for seminoma. Cancer Res. 42, 3244-3247.

Lathe, R. (1985). Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. J. Mol. Biol. 183, 1-12.

Leder, P., Tiemeier, D. Enquist, L. (1977). EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: The \gt Wes system. Science 196, 175.

Lehman, F.G. (1975a). Immunological methods for human placental alkaline phosphatase (Regan isoenzyme). Clin. Chim. Acta. 65, 271-282.

Lehman, F.G. (1975b). Immunological relationship between human placental and intestinal alkaline phosphatase. Clin. Chim. Acta. 65, 257-269.

Lehman, F.G. (1980). Differentiation of human alkaline phosphatases by lectin binding affinity. Klin Wochenschr 58, 947-951.

Lin, T.M., Halbert, S.P., Kiefer, D., Spellag, W.N. and Gall, S. (1974). Characterization of four human pregnancy-associated plasma proteins. Am. J. Obstet. Gynecol. 118, 223-236.

Lin, T.M. and Halbert, S.P. (1976). Placental localization of human pregnancy associated plasma proteins. Science 193, 1249-1252.

Loke, Y.W. and Day, S. (1984). Monoclonal antibodies to human cytotrophoblast. Am. J. Reprod. Immunol. 5, 106-108.

Loke, Y.W., Day, S., Butterworth, B. and Potter, B. (1984). Monoclonal antibody to human cytotrophoblast cross-reacts with basal layers of some fetal epithelial surfaces. Placenta 5, 199-204.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951). Protein measurement with the fotin phenol reagent. J. Biol. Chem. 193, 265-275.

McComb, R.B., Bowers, G.N. and Posen, S. (1979). Alkaline phosphates. Plenum New York.

McIntyre, J.A. and Faulk, W.P. (1979). Antigens of human trophoblast: effects of heterologous antitrophoblast sera on lymphocyte responses in vitro. J. Exp. Med. 149, 824-836.

McIntyre, J.A. and Faulk, W.P. (1982a). Allotypic trophoblast-lymphocyte cross-reactive (TLX) cell surface antigens. Human Immunol. 4, 27-35.

McIntyre, J.A. and Faulk, W.P. (1982b). HLA and the generation of diversity in human pregnancy. In: Immunological Aspects of Placental Proteins. Klopper A. (Ed.). Placental Suppl.4, W. Saunders Eastbourne, 1-11.

McKay, D.G., Hertig, A.T., Adams, E.C. and Richardson, M.U. (1958). Histochemical observations on the human placenta. Obstet Gynecol 12, 1-36.

McKenna, M.J., Hamilton, T.A. and Sussman, H.H. (1979). Comparison of human alkaline phosphatase isoenzymes. Structural evidence for three protein classes. Biochem. J. 181, 67-73.

McLaughlin, P.J., Gee, H. and Johnson, P.M. (1983). Placental type alkaline phosphatase in pregnancy and malignancy plasma: specific estimation using monoclonal antibody in solid phase enzyme immunoassay. Clin. Chem. Acta. 130, 199-209.

McMasters, G.K. and Carmichael, G.G. (1977). Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acidine orange. Proc. Natl. Acad. Sci. 74, 4835.

Manes, C. (1974). Phasing of gene products during development. Cancer Res. 34, 2044-2052.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (eds)(1982). Molecular cloning: a laboratory manual. Cold Spring Harbor.

Millan, J.L., Beckman, G., Jeppsson, A. and Stigbrand, T. (1982). Genetic variants of placental alkaline phosphatase as determined by monoclonal antibodies. Hum. Genet. 60, 145-149.

Millan, J.L., Eriksson, A., and Stigbrand, T. (1982). A possible new locus of alkaline phosphatase expressed in human testis. Hum. Genet. 62, 293-295.

Millan, J.L., Stigbrand, T., Ruoslahti, E. and Fishman, W.H. (1982). An allotype specific monoclonal antibody to placental alkaline phosphatase: Its characterization and use in the study of cancer-related phosphatase polymorphism. Cancer Res. 42, 2444.

Mochizuti, M., Morikawa, H., Kawaguchi, K. and Tojo, S. (1976). Growth hormone production and chorionic somatomammotropin in normal and molar pregnancy. J. Clin. Endocinal Metab. 43, 514-521.

Moss, D.W., Shakespeare, M.J. and Thomas, D.M. (1972). Observation on the heat-stability of alkaline phosphatase isoenzyme in serum. Clin. Chim. Acta. 40, 35-41.

Moss, D.W. and Whitby, L.G. (1975). A simplified heat-inactivation method for investigating alkaline phosphatase isoenzymes in serum. Clin. Chem. Acta. 61, 62-71.

Moss, D.W. (1982). Alkaline phosphates isoenzymes. Clin. Chem. 28, 2007-2016.

Mowbray, J.F., Liddle, H. and Underwood J. (1985). Controlled trial of treatment of recurrent spontaneous abortion by immunisation with paternal cells. Lancet, April. 941-943.

Murray, N.E., Brammar, W.J. and Murray, K. (1977). Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. 150 53.

Nakayama, T., Yoshida, H. and Kilamura, M. (1970). L-leuctine sensitive heat-stable alkaline phosphatase isoenzyme detected in a patent with pleuritis carcinomatosa. Clin. Chim. Acta 30, 545-548.

Norrander, J., Kemp, T. and Messing, J. (1983). Construction of improved M13 vectors using oligooxynucleotide-directed mutagenesis. Gene 26, 101-106.

Nozawa, S., Ohta, M., Izumi, S., Kayashi, S., Tsutsni, F., Kurihara, S. and Watanabe, K. (1980). Heat-stable alkaline phosphatase in normal female genital organ - with special reference to the histochemical heat-stability test and L-phenylalanine inhibition test. Acta Histochem. Cytochem. 13, 521-530.

Ogbimi, A.O., Johnson, P.M., Brown, P.J. and Fox, M. (1979). Characterisation of the soluble fraction of human syncytiotrophoblast microvillous plasma membrane-associated components. J. Reprod. Immunol. 1, 127-140.

Ogbimi, A.O. and Johnson, P.M. (1980). Further immunological and chemical characterisation of human syncytiotrophoblast microvillous plasma membrane-associated components. J. Reprod. Immunol. 2, 99-108.

Okamura, K., Powell, J.E., Lee, A.C. and Stevens, V.C. (1981). Characterisation of solubilised microvillous membrane proteins and glycoproteins from human placental syncytiotrophoblast. Placenta 2, 117-128.

Orkin, S.H., Daddona, P.E., Sherwach, D.S., Markham, A.F., Bruns, G.A., Goff, S.C. and Kelley, W.N. (1983). Molecular cloning of human adenosine deaminase gene sequences. J. Biol. Chem. 258, 12753-12756.

Pattillo, R.A., Hussu, R.O., Yorde, D.E. and Cole, L.A. (1983). Hormone synthesis by normal and neoplastic human trophoblast. In: Biology of trophoblast. Loke, Y.W. and Whyte, A. (Eds.). Elsevier Science Publishers BV, pp 283-316.

Pelham, H.R.B. and Jackson, R.J. (1976). An efficient mRNA-dependent transplation system from reticulocyte lysates. Eur. J. of Biochem. 67, 247-256.

Pierce, G.B. and Midgley, A.R. (1963). The origin and function of human syncytiotrophoblast giant cell. Am. J. Path. 43, 153-173.

Pijnenborg, R., Robertson, W.B., Brosens, I. and Dixon, G. (1981). Trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. Placenta 2, 71-91.

Playfair, J.H.L. (1982). Immunology at a glance. 2nd Ed. Blackwell Scientific Publications.

Policastro, P., Ovitt, C.E., Hoshuna, M., Fukuoka, H., Boothby, M.R. and Boime, I. (1983). The subunit of human chorionic gonadotrophin is encoded by multiple genes. J. Biol. Chem. 258, 11492-11499.

Porath, J., Axen, R. and Ernback, S. (1967). Chemical coupling of proteins to agarose gels. Nature 194, 1491-1492.

Redman, C.W.G. (1983). HLA-DR antigen on human trophoblast: A review. Am. J. Reprod. Immunol. 3, 175-177.

Redman, C.W.G., McMichael, A.J., Stirrat, G.M., Sunderland, C.A. and Tung, A. (1984). Class-1 major histocompatibility complex antigens on human extra-villous trophoblast. Immunol. 52, 457-468.

Reichardt, L. and Kaiser, A. (1971). Control of λ repressor synthesis. Proc. Natl. Acad. Sci. USA 68, 2185-2189.

Robson, E.B. and Harris, H. (1965). Genetics of the alkaline phosphatase polymorphism of the human placenta. Nature 18, 1257-1259.

Robson, E.B. and Harris, H. (1967). Further studies on the genetics of placental alkaline phosphatase. Ann. Hum. Genet. 30, 219-232.

Rocklin, R.E., Kilmiller, J.L. and Kaye, M.D. (1979). Immunobiology of the maternal-foetal relationship. Ann. Rev. Med. 30, 375.

Rosen, S.W., Weintraub, B.D., Vaitukaitis, J.L., Sussman, N.H., Hershman, J.M. and Muggia, F.M. (1975). Placental proteins and their subunits as tumour markers. Ann. Intern. Med. 82 71.

Rothman, P. and Saxena (1975). Primary amino acid sequence of follicle-stimulating hormone from human pituitory glands. J. Biol. Chem. 250, 6735-6746.

Ruoslahti, E. and Seppala, M. (1972). Studies of carcinoma-fetal proteins - III. Development of a radioimmunoassay for alpha-fetoprotein in serum of healthy human adults. Int. J. Cancer 8, 374-383.

Sakiyama, T., Robinson, J.C. and Chou, J.Y. (1979). Characterization of alkaline phosphatase from human first trimester placentae. J. Biol. Chem. 254, 935-938.

Schwick, H.G. (1978). In: Handbook of experimental immunology. Weir, D.M. (ed), Blackwell Scientific Publications.

Seargeant, L.E. and Stinson, R.A. (1979). Evidence that three structural genes code for human alkaline phosphatases. Nature 281, 152-154.

Seeberg, P.H., Shine, J., Martial, J.A., Ulrich, A., Baxter, J.D. and Howard, M. (1977). Nucleotide sequence of part of the gene for chorionic somatomammotropin: purification of DNA complementary to predominant mRNA species. Cell 12, 157-165.

Shen, S-H., Slighton, J.L. and Smithers, O. (1981). A history of the human fetal globin gene duplication. Cell 26, 191-203.

Shirazi, S., Colston, K.W. and Butterworth, P. (1978). Alkaline phosphatase: a possible transport protein for inorganic phosphate. Biochem. Soc. Trans. 6, 933.

Slaughter, C.A., Coseo, M.C., Canco, M.P. and Harris, M. (1981). Detection of enzyme polymorphism by using monoclonal antibodies. Proc. Nat. Acad. Sci. USA 78, 1124.

Smith, C.H., Nelson, D.N., King, B.F., Donohue, T.M., Ruzycki, S.M. and Kelley, L.K. (1977). Characterization of microvillus membrane preparation from human placental syncytiotrophoblast: A morphologic, biochemical and physiological study. Am. J. Obstet. Gynecol. 128, 190-196.

Smith, I., Perry, J.D. and Lightstone, P.J. (1973). Disc electrophoresis of alkaline phosphatase: mobility changes caused by neurominidose. Clin. Chem. Acta 45, 219-223.

Smith, M. (1983). In: Methods of DNA and RNA sequencing. Weissman, S.M. (ed). Praeger Press, New York. 23-68.

Smith, N.C., Brush, M.G. and Luckett, S. (1974). Preparation of human placental villous surface membrane. Nature 232, 302-303.

Smith, N.C. and Brush, M.G. (1978). Preparation and characterization of human syncytiotrophoblast plasma membrane. Med. Biol. 56.

Smithies, O., Engels, W.R., Devereux, J.R., Slighton, J.L. and Shen, S-H. (1981). Base substitutions, length differences and DNA strand asymmetries in the human $^{G}\gamma$ and $^{A}\gamma$ fetal globin gene region. Cell 26, 345-353.

Sood, A.K., Pereira, D. and Weissman, S.M. (1981). Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B by use of a oligonucleotide primer. Proc. Natl. Acad. Sci. USA 78, 616-620.

Steinmetz, M. (1984). Structure, function and evolution of the major histocompatibility complex of the mouse. TIBS 9, 224-226.

Stetler, D., Das, H., Nunberg, J.H., Saiki, R., Sheng-Dong, R., Mullis, K.B., Weissman, S.M. and Erlich, H.A. (1982). Isolation of a cDNA clone for the human HLA-DR antigen and chain by using a synthetic oligonucleotide as a hybridization probe. Proc. Natl. Acad. Sci. USA 79, 5966-5970.

Stigbrand, T. and Engwall, E. (1982). Placental proteins as tumour markers. In: Tumour markers. Sell, S. and Wahren, B. (Eds.). Human Press New Jersey.

Stigbrand, T. (1984). Present status and future trends of human alkaline phosphatases. In: Human Alkaline Phosphatases. Stigbrand, T. and Fishman, W.H. (eds). Alan R. Liss Inc. New York. 3-14.

Stinson, R.A. and Seargeant, L.E. (1981). Comparative studies of pure alkaline phosphatase from live human tissues. Clin. Chim. Acta 110, 261-272.

Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.H. and Itakura, K. (1981). Use of synthetic oligonucleotides as hybridization probes: Isolation of cloned cDNA sequences for human B_2-microglobulin. Proc. Natl. Acad. Sci. USA 78, 6613-6617,

Sunderland, C.A., Naiem, M., Mason, D.X., Redman, C.W.G. and Stirrat, G.M. (1981a). The expression of major histocompatibility antigens by human chorionic villi. J. Reprod. Immunol. 3, 323-331.

Sunderland, C.A., Redman, C.W.G. and Stirrat, G.M. (1981b). HLA, A, B, C, antigens are expressed on non villous trophoblast of the early human placenta. J. Immunol. 127, 2614-2615.

Sunderland, C.A., Redman, C.W.G. and Stirrat, G. (1981c). Monoclonal antibodies to human syncytiotrophoblast. Immunol. 43, 541-546.

Sussman, H.H. and Bowman, M. (1968). Placental alkaline phosphatase in maternal serum during normal and abnormal pregnancy. Nature 218, 359-360.

Sybulski, S. and Tremblay, P.C. (1967). Uptake and incorporation into protein of radioactive glycine by human placentae in vitro. Am. J. Obstet. Gynecol. 97, 1111-1118.

Tabarelli, M., Kofler, R. and Wick, G. (1983). Placental hormones: I : immunofluorescence studies of the localization of chorionic gonadotrophin, placental lactogen and prolactin in human and rat placenta and in the endometrium of pregnant rats. Placenta 4, 379-388.

Tabarelli, M., Kofler, R., Berger, P. and Wick, G. (1983). Placental hormones: II - Immunofluorescence studies of the localization of prolactin, placental lactogen and human chorionic gonadotrophin receptors in human and rat placenta. Placenta 4, 389-396

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragment transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5-201.

Thompson, R.A. (1977). Techniques in clinical immunology. Blackwell Scientific Publications.

Tighe, J.R., Garrod, P.R. and Curran, R.C. (1967). The trophoblast of human chorionic villus. J. Pathol. Bacteriol. 93, 559-567.

Tokimitsu, S., Kohnoe, K., Takeya, M. and Takeuchi, T. (1979). Localization of alkaline phosphatase isoenzymes in human cancer cells in vitro. Acta Histochem. Cytochem. 12, 631.

Truman, P. and Ford, H.C. (1984). The brush border of the human term placenta. Biochimca et Biophysica Acta 779, 139-160.

Ullrich, A., Berman, C.H., Dull, T.J., Gray, A. and Lee, J.M. (1984). Isolation of the human insulin-like growth factor I gene using a single synthetic DNA probe. EMBO J. 3, 361-364.

Usateiga-Gomez, M, Yeager, F.M. and Fernandez de Castro, A. (1974). Regan isoenzymes in normal human sera. Cancer Res. 34, 2544-2545.

Wada, H.G., Gornichi, S.Z. and Sussman, H.H. (1977). The sialoglycoprotein subunits of human placental brush border membranes characterized by two dimensional electrophoresis. J. Supramol. Struct. 6, 473-484.

Wahren, B., Holmgren, R.A. and Stigbrand, T. (1979). Placental alkaline phosphatase, alphafetoprotein and carcino-embryonic antigen in testicular tumors. Tissues typed by means of cytological smears. Int. J. Cancer 24, 749-753.

Wallach, D.F.H. and Winzler, R.J. (1974). Evolving strategies and tactics in membrane research. Springer-Verlag, Berlin, pp 94-122.

Waltman, S.R., Borde, R.M. and Borrios, J. (1971). Prevention of corneal homograft rejection by estrogens. Transplantation 11, 194.

Wei, S.C. and Doellgast, G.J. (1980). Specificity of alloenzyme absorbed antisera to human placental alkaline phosphatases for individual phenotypes. Biochem. Genet. 18, 1097-1107.

Wei, S.C. and Doellgast, G.J. (1981). Immunochemical studies of human placental-type variants of alkaline phosphatase. Structural differences between the Nagao isoenzyme and the placental D varient. Eur. J. Biochem. 118, 39-45.

Whitsett, J.A. and Lessard, J.L. (1978). Characteristics of the microvillus brush border of human placenta: insulin receptor localization in brush border membranes. Endocrinology 103, 1458-1468.

Whyte, A. and Loke, Y.W. (1979). Antigens of the human trophoblast plasma membrane. Clin. Exp. Immunol. 37, 359-366.

Wickens, M.P., Buell, G.N. and Schimke, R.T. (1978). Synthesis of double-stranded DNA complementary to lysozyme, ovomuconal and ovalbumin mRNAs. J. Biol. Chem. 253, 2483.

Winick, M., Coscia, A. and Noble, A. (1967). Cellular growth in human placenta. I - Normal placental growth. Pediatrics 39, 248-251.

Winter, G., Fersht, A.R., Wilkinson, A.J., Zoller, M. and Smith, M. (1982). Redesigning enzyme structure by site-directed mutagenesis : tyrosil tRNA synthetase and ATP binding. Nature 299, 756-758.

Wynn, R.M. (1972). Cytotrophoblastic specialization an ultrastructural study of the human placenta. Am. J. Obstet. Gynecol. 114. 339-355.

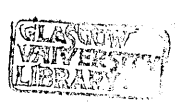
Wynn, R.M. (1975). Development and ultrastructural adaptations of the human placental. In: Human placentation. Brosews, I.A., Dixon, G. and Robertson, W.B. (Eds.). Excerpa Midica Amsterdam, pp3-21.

Young, R.A. and Davis, R.W. (1983). Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA 80, 1194-1198.

Young, R.A. and Davis, R.W. (1983). Yeast RNA polymerase 11 genes: isolation with antibody probes. Science 222, 778-782.

APPENDIX : PUBLISHED PAPERS

Khalaf, S.A., Livingstone, J.C., Nickson, D.A., Henderson, S.J. and Sutcliffe R.G. (1985). Trophoblastic cells and markers. In "Early Pregnancy factors". Ellendorff, F. and Koch, E. (Eds.). Perinatology Press.



EARLY PREGNANCY FACTORS
F. Ellendorff and E. Koch, eds.

TROPHOBLASTIC CELLS AND MARKERS

S.A. Khalaf, J.C. Livingstone, D.A. Nickson, S.J. Henderson and R.G. Sutcliffe*
Institute of Genetics, University of Glasgow, Church Street, Glasgow G11 5JS, Scotland, UK.

INTRODUCTION

In recent years the plasma membrane components of the human trophoblast have attracted increasing interest. This paper describes progress in two directions, the first being to isolate normal human placental cytotrophoblast cells in order to characterise their histocompatibility antigens. The second relates to the enzyme placental alkaline phosphatase (PLAP), which is the quantitatively major cell membrane protein of the syncytiotrophoblastic microvilli.

ISOLATION AND ANALYSIS OF CYTOTROPHOBLAST

The trophoblast of the placental villi lack β_2-microglobulin (β_2M) and the monomorphic determinants of the HLA class I heavy chain, defined by monoclonal antibodies such as W6/32 and PA 2.6 (6,21). This seems to apply to both the syncytial layer of trophoblast and the underlying zone of cytotrophoblast. Conversely, the cytotrophoblast of the uteroplacental interface, which invades the uterine decidua expresses HLA class I determinants and β_2M, as also does the cytotrophoblast of the reflected chorion (18,22). A similar pattern of MHC expression exists in the mouse (12). The further characterisation of human cytotrophoblastic class I determinants depends in part on the supply of purified cytotrophoblast cells. Ideally these should come from normal placentae rather than choriocarcinomas, especially since some cells change their pattern of MHC antigen expression when transformed. The chorionic plate of the placenta at term contains a substantial population of cytotrophoblast (Figure 1a). These cells stain readily for cytokeratins (figure 1c) which are epithelial markers which can be used to differentiate the cells from mesenchymal and endothelial cells (16,24). The cells are embedded in dense regions of fibronectin (figure 1b), covered by amniotic low columnar epithelium on its fetal aspect. After removal of the amnion serial frozen sections (figure 2) of the chorionic plate showed cells

*To whom correspondence should be addressed.

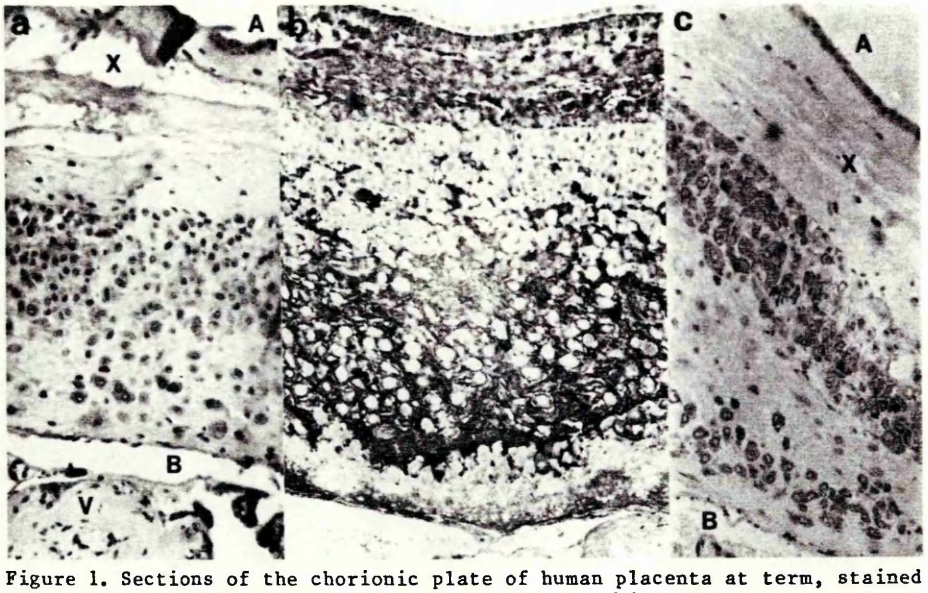


Figure 1. Sections of the chorionic plate of human placenta at term, stained (a) with H & E; (b) with rabbit anti fibronectin; (c) with mouse monoclonal PKK1 antibody to cytokeratin. In (c), the amniotic epithelium does not stain with PKK1. A = amnion; X = separation zone between amnion and chorionic plate; B = intervillus space; V = placental villus. Magnified x33.

staining for cytokeratins, β_2M and the class I HLA determinant W6/32.

We wished to characterise these HLA determinants for several reasons. Firstly, it is clear that in the mouse there are multiple class I genes in addition to the classic serologically detectable K and D loci; most of these genes map in the Qa/Tla region of mouse MHC (20). Since there is remarkable homology between many of the genes of human and mouse MHC, these genes may exist and be expressed in the human MHC. A recent report (18) on pregnancies in which the maternal and fetal HLA types were known to be different, showed that the expected fetal allotype could not be detected on cytotrophoblasts that expressed the monomorphic determinant.

We therefore attempted to purify the cytotrophoblast of the chorionic plate. Briefly, the amnion was removed, together with the main blood vessels and the underlying villus material. The dissected chorionic plate was trypsinised with 0.25% trypsin in culture medium containing antibiotics. After 1h the supernatant was discarded and four further aliquots of fresh trypsin were added in succession at 15 min intervals at 37C. The supernatants of these digestions were retained and the cells were recovered by filtration through muslin, followed by centrifugation to the interface of

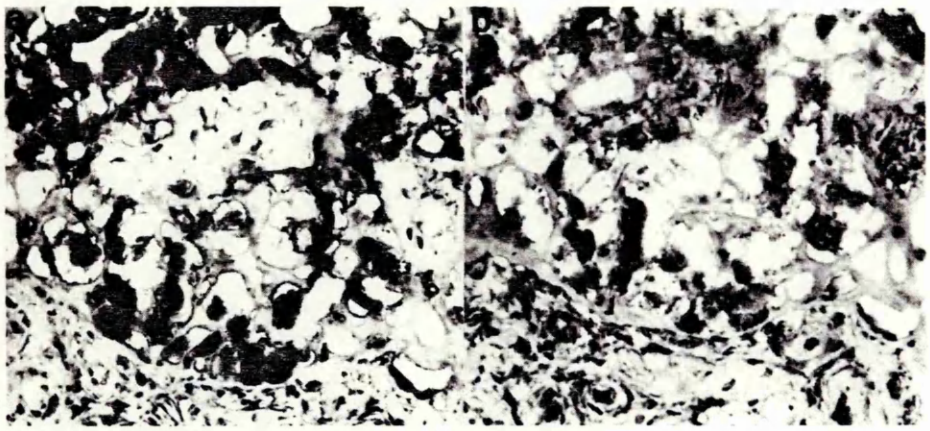


Figure 2. Serial frozen sections of chorionic plate stained (a) with monoclonal PKK1 anti-cytokeratin and (b) W6/32. Magnified x56.

TABLE 1. PERCENTAGES OF ISOLATED CHORIONIC PLATE CELLS STAINING FOR VARIOUS DETERMINANTS

PLACENTA:	A	B	C	D	E	F	G
SPECIFICITIES							
W6/32 (Class I)	50	62	79	58	64	89	76
β_2M (Class I)	59	70	75	53	60	75	70
DA6.231 (Class II)	15	21	6	-	-	-	-
Troma 1 (Int. filament)	57	59	80	-	-	-	-
Cytokeratin	68	69	86	61	65	83	70
Vimentin	4	6	11	4	9	10	8
Leucocyte common ag.	1	3	4	4	7	5	7
Blood group substance	10	8	12	2	10	11	6
Fibronectin	50	69	78	-	-	-	-
PAPP-A	60	70	73	-	-	-	-
SP1	42	51	58	-	-	-	-
hPL	39	52	56	-	-	-	-
α-hCG	19	25	23	13	10	22	23
β-hCG	12	13	9	2	9	10	11
H315	17	19	25	-	-	-	-
H316	36	49	58	-	-	-	-
7A11	-	-	-	55	60	62	73

15% and 52% percoll, in a discontinuous gradient. Where necessary, residual red blood cells were removed with a single treatment with Gray's solution. The viability of the final cell preparations ranged from 40-98% . Fuller details of the method are to be found elsewhere (14).

Cytospin preparations of the isolated cells were stained for a variety of histocompatibility and placental specificities, as shown in table 1. The majority of the cells stained for cytokeratins, as expected, and substantial numbers of cells expressed determinants for W6/32, β_2M, fibronectin, PAPP-A, SP1 and hPL. 10-25% expressed the alpha chain of hCG, and 2-13% the beta chain. It is probable that these cells are similar to intermediate cytotrophoblastic cells, which express hPL and are somewhat variable in morphology and in their expression of SP1 and beta hCG (15). Leucocyte common antigen and vimentin stained a small fraction of the isolated chorionic plate cells, indicating that there was little contamination with non-epithelial cells or leucocytes. The presence of endothelial cells was assayed through the affinity of the lectin Ulex europaeus I for the fucose residues of the ABH blood group substance (9). On this basis endothelial contamination was 2-11%. There was no evidence of fibroblast contamination in these preparations; no fibroblast outgrowths were ever observed in culture. However, despite the high viability of the cells, there was no sustained division in the cultures and we were unable to maintain the cells in vitro for more than 3-4 months. The provision of plasma clot as a culture substrate seemed to be beneficial and the cells digested the clot through the activation of plasminogen. Since the cells can often be found in association with fibrinoid (which is largely plasma clot; 23), it was hoped that this would help to establish long term cultures; but the cells failed to become established beyond one or two passages.

An attempt was made to type the cells for HLA antigens using the standard Terasaki microcytotoxicity test. Cytotoxicity to a range of antibodies was assessed by eosin dye exclusion, with the reaction of human lymphocytes as a positive control. The results (Table 2) show that, under the prevailing culture conditions, the chorionic plate cells are resistant to complement-mediated attack. Immunofluorescence staining showed that the antibodies were indeed binding to the cells in the microcytotoxicity assay, and also that complement component C3 was fixing to the cell surface. The failure to lyse the cells was not overcome by the use of sodium azideto inhibit antigenic modulation, or by the addition of a second antibody to increase complement fixation. We speculate that the cells are able either to

TABLE 2. PERCENTAGES OF VIABLE CHORIONIC PLATE CELLS AFTER TREATMENT WITH ANTIBODY AND RABBIT COMPLEMENT

	CHORIONIC PLATE CELLS			LYMPHOCYTES
	Ab + C′	C′	Ab	Ab + C′
FIRST ANTIBODY				
Normal Human Serum	69	65	68	98
	56	55	50	90
	80	77	75	85
W6/32	67	63	65	0.09
(anti-class I)	53	50	50	0
	73	76	70	0.05
anti- $_2$M	65	60	65	0
	51	50	50	0.02
	75	70	67	0.06
DA6.231	63	65	59	0.10
(anti-class II)	52	50	50	0.05
	75	69	72	0.09
Mouse anti-	60	61	59	0.09
trophoblast	54	50	52	0
	71	72	70	0.05

Lymphocyte viabilities in controls (C′;Ab) were 85-98%.

inhibit the effect of complement, or that plasma membrane repair proceeds at a rate which can overcome the effect of complement attack.

The cells are now being used as a source of mRNA for the creation of a cDNA library. This library will be probed with established clones of human class I genes to establish the nature of the MHC genes expressed in this type of human cytotrophoblast.

PLACENTAL ALKALINE PHOSPHATASE

The syncytiotrophoblastic microvilli of the human placenta are the major source of placental alkaline phosphatase (PLAP) during the second and third trimesters of pregnancy. PLAP is a tissue-specific polymorphic sialoglycoprotein, composed of pairs of disulphide-bonded polypeptide chains each of 69K molecular weight. When extracted from homogenates of whole placentae (7,19) PLAP can be resolved on starch gels into two bands (A-PLAP and B-

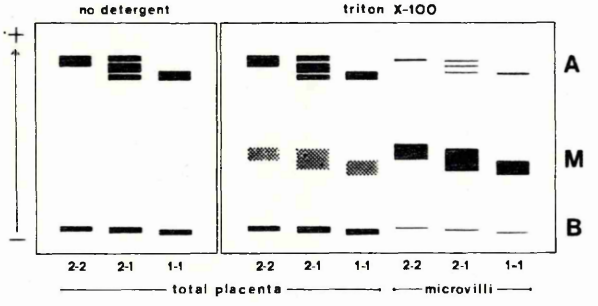


Figure 3. Diagram of the electrophoretic mobility of PLAP isoenzymes in starch gels, in the presence or absence of 0.5% triton X-100 in the gel. The phenotypes of each sample (2-2, 2-1, 1-1) are shown under each track.

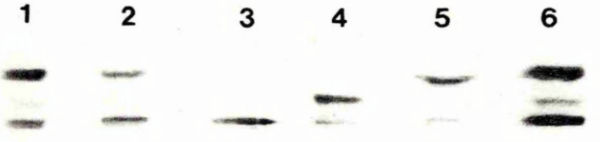


Figure 4. Triton-starch gel to show the effect of serum and of phospholipase C on M-PLAP. Tracks 1,6 = markers of A, M & B-PLAP; track 5 = A-PLAP; track 4 = M-PLAP; track 3 = M-PLAP treated with phospholipase C; track 2 = M-PLAP treated with human serum.

PLAP); A-PLAP has a native molecular weight of 140K and B-PLAP is about 200K. Recent studies from this laboratory (1-4) have shown that PLAP extracted from syncytiotrophoblastic microvilli has a native molecular weight of 725K and can be resolved in starch gels containing detergent as a band running between the A and B forms. We named this form M-PLAP (Figure 3). M-PLAP has a similar Km, pH optimum and heat stability to A-PLAP and gives reactions of identity with antibodies against A-PLAP. Notably, M-PLAP expresses the same genetic polymorphism as A-PLAP, showing that both isoenzymes are the product of the same transcriptional system. Initially, the finding of a high molecular weight form of PLAP suggested that this was an aggregation artifact due to the inherent insolubility of extracted membrane proteins. However, several pieces of evidence argued against this view. Use of detergents during the extraction of microvilli did not affect the yield of M-PLAP. On hydrophobic chromatography, the A form did not bind to alkyl-

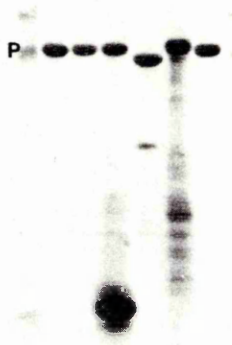


Figure 5. Reducing SDS polyacrylamide gel to show the effect of phospholipases on M-PLAP (the mobility of which is marked P on the gel). Tracks 1,8 = molecular weight markers; tracks 2,7 = M-PLAP; track 3 = M-PLAP + protease inhibitors (PI); track 4 = M-PLAP + phospholipase-A_2 + PI; track 5 = M-PLAP + phospholipase-C + PI; track 6 = M-PLAP + phospholipase-D + PI.

agarose, whereas the M form bound to the column and was eluted with hydrophobic solvents. In non-reducing SDS gels, the mobility of A-PLAP dimers corresponded to 140K, as expected. The mobility of M-PLAP dimers corresponded to 116-118K, suggesting a hydrodynamically more compact molecule (2-4).

The structural relationships between the PLAP isoenzymes might be better understood if we had some knowledge of the association of the M form with the plasma membrane. Cleavage of external membrane proteins with the protease subtilisin showed that M-PLAP can be cleaved off the microvilli as a virtually pure protein species, differing from uncleaved M-PLAP by lacking about 2K from its carboxy-terminus (3,4). We do not yet know whether the M form is anchored in the membrane by a sequence of hydrophobic amino acids, but suspect that covalently-bound lipid may provide an additional or alternative means of attachment to the plasma membrane. Figures 4 & 5 show the effect of various phospholipases on the mobility of M-PLAP in starch and SDS gels. As shown in figure 5, track 5, phospholipase C increases the mobility of the M polypeptide under reducing conditions (17). This suggests that there is a glyceryl moiety in covalent linkage with the protein and invites speculation that the hydrophobicity of M-PLAP is due to fatty acyl chains. To determine whether PLAP has a C-terminal hydrophobic sequence of amino acids requires amino acid sequencing or an analysis of cDNA clones. Poly(A)$^+$ RNA can be extracted from the placenta and can be translated in vitro into various polypeptides, including hPL (Figure 6). Our current work involves the cloning of placental cDNA into lambda gt10 and gt11 and the screening of

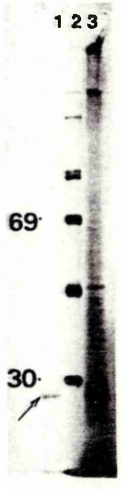


Figure 6. Translation of term placental poly $(A)^+$ RNA in a rabbit reticulocyte lysate cell-free system (Amersham International PLC). The polypeptides were resolved in a reducing 9% polyacrylamide gel and the incorporated ^{35}S-methionine was detected by autoradiography. Track 3 = total translated polypeptides; track 2 = molecular weight markers (see kdal values at left-hand side); track 1 = immunoprecipitation of translated polypeptides with rabbit anti-hPL and formalin-fixed staphylococci. The arrow in track 1 shows the immunoprecipitated hPL polypeptide.

such clones with synthetic oligonucleotide probes and anti-PLAP antibodies, respectively.

Although we have never been able to interconvert purified samples of the A and M forms by simple physical means, such as freezing or changes of solvent, we have recently found (17) that there is an apparently distinct protein factor in serum which can convert M-PLAP to the A and B forms (Figure 4). This conversion could not be inhibited by a wide range of exogenous protease inhibitors, suggesting that the factor is not proteolytic. The conversion activity does not fractionate with the serum lipoprotein fractions. Treatment of a volunteer (RGS) with intravenous heparin did not lead to a stimulation of conversion activity, indicating that neither the hepatic nor the endothelial forms of serum lipoprotein lipase (LPL) were the causative factors. On AcA 34 gel filtration chromatography, the factor elutes as a unimodal peak of about 60K molecular weight. It behaves as a glycoprotein in binding to Con-A sepharose. It elutes as a unimodal peak on DEAE sepharose at the same point as has been reported (5) for lecithin cholesterol acyl transferase (LCAT), which is capable of acting as a lipoprotein phospholipase. The ability of LCAT to convert M-PLAP to the A and B forms is currently being tested.

The foregoing results strongly indicate to us that the A and B forms of placental alkaline phosphatase are a conversion product of M-PLAP. It also explains why the majority of PLAP in maternal serum is of the A form rather than the M form (N.S. Abu-Hasan, unpublished). The conversion must involve some distinct chemical process, since the native sizes of the A and B forms

are less than M-PLAP. Further, since the dimers of M-PLAP are quite distinct in size from those of the A form, the A and B forms probably do not actually exist in the M complex until the latter is converted. However, there is some evidence (2, and J.C.L. unpublished) that M-PLAP exhibits molecular heterogeneity in discontinuous polyacrylamide gels. This may perhaps account for the generation of two conversion products from M-PLAP.

The function of PLAP remains enigmatic. Its expression is limited to the syncytiotrophoblastic microvilli and our biochemical data (3,4) support the electron microscopic evidence (13) that the enzyme is exposed to the circulating maternal blood. This tends to argue against the possibility that PLAP acts as a protein phosphatase which might antagonise or down-regulate the effects of membrane receptor phosphorylation (for example, of the EGF or insulin receptor). Such receptor phosphorylation occurs on the cytoplasmic domain of the receptor protein, and it is difficult to understand how an external phosphatase like PLAP could act on such a substrate. However, PLAP has been found to be active on artificially constructed phosphoproteins (10), so the possibility cannot be ruled out at this time.

Since the active site of the enzyme is located on the maternal side of the microvillus plasma membrane, our thoughts turn toward a function for the enzyme in contact with maternal blood, which might be of significance in the second half of pregnancy, when levels of the enzyme are maximal. The formal alternatives are that PLAP functions either as a soluble or as a membrane-bound enzyme. Because it is membrane-bound and not simply secreted, we believe it has a role on the plasma membrane, though it may also be present on exfoliated syncytial fragments circulating in maternal blood and its heat stability and long half-life mean that there may be some lesser effect of soluble enzyme in the systemic circulation. There is some evidence that PLAP helps to prevent inappropriate activation of blood platelets by dephosphorylating ADP (11). When stimulated by a variety of agents, including collagen III and thrombin, platelets become activated and secrete ADP, which triggers the activation of further platelets and so helps to establish blood coagulation. The ability of PLAP to use ADP as a substrate encourages us to pursue the hypothesis that PLAP functions to prevent platelet activation on the surface of placental microvilli.

ACKNOWLEDGEMENTS This work was supported by grants from the Medical Research Council.

REFERENCES

1. Abu-Hasan, N.S., Davies, M. and Sutcliffe, R.G. (1984) A high molecular weight species of placental alkaline phosphatase in human syncytiotrophoblast microvilli. Placenta 5, 159-174.
2. Abu-Hasan, N.S. and Sutcliffe, R.G. (1984) Purification and analysis of the microvillous (M) and A forms of placental alkaline phosphatase. Placenta 5, 71-82.
3. Abu-Hasan, N.S. and Sutcliffe, R.G. (1984) Molecular heterogeneity of placental alkaline phosphatase associated with microvillous membranes. In: "Human Alkaline Phosphatases", Eds: T. Stigbrand and W.H. Fishman. Alan R Liss inc., New York, 117-126.
4. Abu-Hasan, N.S. and Sutcliffe, R.G. (1985) Placental alkaline phosphatase integrates via its carboxy-terminus into the microvillus membrane; its allotypes differ in conformation. Placenta (in press).
5. Doi, Y. and Nishida, T. (1981) Lecithin-cholesterol acyltransferase from human plasma. Meths. Enzymol. 71, 753-767.
6. Faulk, W.P. and Temple, A. (1976) Distribution of beta 2 microglobulin and HLA in chorionic villi of human placentae. Nature 262, 299-802.
7. Ghosh, N.K. and Fishman, W.H. (1968) Purification and properties of molecular weight variants of placental alkaline phosphatase. Biochem. J. 103, 779-792.
8. Harris, H. (1982) Multilocus enzyme systems and the evolution of gene expression: The alkaline phosphatases as a model example. In: Harvey Lectures, series 76, London: Academic Press, 95.
9. Holthofer, H., Virtanen, I., Kariniemi, A.-L., Hormia, M. Linder, E. and Miettinen, A. (1982) Ulex europaeus I lectin as a marker forvascular endothelium in human tissue. Lab. Invest. 47, 60-66.
10. Huang, K.-P., Robinson, J.C. and Chou, J.Y. (1976) Phosphoprotein-phosphatase activity associated with human placental alkaline phosphatase. Biochem. Biophys. Res. Comm. 70, 186-192.
11. Hutton, R.A., Chow, F.P.R., Craft, I.L. and Dandona, P. (1980) Inhibitors of platelet aggregation in the fetoplacental unit and myometrium with particular reference to the ADP-degrading property of placenta. Placenta 1, 125-130.
12. Jenkinson, E.J. and Owen, V. (1980) Ontogeny and distribution of major histocompatibility (MHC) antigens on mouse placental trophoblast. J. Reprod. Immunol. 2, 173-181.
13. Jones, C.J.P. and Fox, H. (1976) An ultrahistochemical study of the distribution of acid and alkaline phosphatase in placentae from normal and complicated pregnancies. J. Path. 118, 143-151.
14. Khalaf, S.A., Henderson, S.J. and Sutcliffe, R.G. (1985) Purification and analysis of trophoblastic cells from the human placental chorionic plate. (In preparation.)
15. Kurman, R.J., Main, C.S. and Chen, H.-C. (1984) Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta 5, 349-370.
16. Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. Nature 283, 249-256.
17. Livingstone, J.C., Abu-Hasan, N.S., Clegg, R.J., Henderson, S.J., Marsden, H.S. and Sutcliffe, R.G. (1985) Structural relationships between the isoenzymes of human placental alkaline phosphatase; a serum factor converts the membrane form. (In preparation).
18. Redman, C.W.G., McMichael, A.J., Stirrat, G.M., Sunderland, C.A. and Ting, A. (1984) ClassI major histocompatibility complex antigens on human extra-villous trophoblast. Immunology 52, 457-468.
19. Robson, E.B. and Harris, H. (1965). Genetics of the alkaline phosphatase polymorphism of the human placenta. Nature 207, 1257-1259.
20. Steinmetz, M. (1984) Structure, function and evolution of the major

histocompatibility of the mouse. Trends Biochem. Sci. 9, 224-226.
21. Sunderland, C.A., Naiem, M., Mason, D.Y., Redman, C.W.G., and Stirrat, G.M. (1981) The expression of major histocompatibility antigens by human chorionic villi. J. Reprod. Immunol. 3, 323-331.
22. Sunderland, C.A., Redman, C.W.G. and Stirrat, G.M. (1981) HLA-A,B,C antigens are expressed in non-villous trophoblast of the early human placenta. J. Immunol. 127, 2614-2618.
23. Sutcliffe, R.G., Davies, M., Hunter, J.B., Waters, J.J. and Parry, J.E. (1982) The protein composition of the fibrinoid material at the human uteroplacental interface. Placenta 3, 297-308.
24. Wu, Y.-J., Parker, L.M., Binder, N.E., Beckett, M.A., Sinard, J.H., Griffiths, C.T. and Rheinwald, J.G. (1982) The mesothelial keratins: A new family of cytoskeletal proteins identified in cultured mesothelial cells and non-keratinising epithelia. Cell 31, 693-703.