MITOCHONDRIAL GENE EXPRESSION IN EARLY SEA URCHIN DEVELOPMENT

A thesis submitted for the Degree of
Doctor of philosophy at the
University of Glasgow

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June 1990

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This thesis is dedicated to my mum and dad.

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

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ABBREVIATIONS

12S rRNA	- large subunit mitochondrial rRNA
16S rRNA	- small subunit mitochondrial rRNA
Acetyl CoA	- acetyl coenzyme A
ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
ATPase 6 and 8	- ATP synthase subunits 6 and 8
bis-acrylamide	- N, N', methylene-bis-acrylamide
BSA	- bovine serum albumin
cDNA	- complementary DNA
COI, II and III	- cytochrome c oxidase subunit I, II and III
CoQ	- coenzyme Q
dH_2O	- distilled H_2O
D-loop	- displacement loop
DHU	- dihydro-uridine
DMF	- dimethylformamide
DNA	- deoxribonucleic acid
DNase	- deoxyribonuclease
dNTP	- deoxyribonucleotide triphosphate
ddNTP	- dideoxyribonucleotide
dsDNA	- double-stranded DNA
DTT	- dithiothreitol
EDTA	- ethylenediamine tetra-acetic acid
EtBr	- ethidium bromide
FAD	- flavin adenine dinucleotide (oxidised)
$FADH_2$	- flavin adenine dinucleotide (reduced)
Fig.	- figure
GTP	- guanosine 5'-triphosphate
GMP	- guanosine 5'-monophosphate
H-strand	- heavy strand of mitochondrial DNA

HEPES	- N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HSP	- heavy strand promoter
IPTG	- Isopropyl- -D-thiogalactopyranoside
L-strand	- light strand of mitochondrial DNA
LSP	- light strand promoter
MOPS	- 3-[N-morpholino]propane sulphonic acid
mRNA	- messenger RNA
mtTF1	- mitochondrial transcription factor 1
NAD^+	- nicotinamide adenine dinucleotide (oxidised)
$NADH/H^+$	- nicotinamide adenine dinucleotide (reduced)
ND1-ND6	- NADH dehydrogenase subunits 1-6.
PEG	- polyethylene glycol
RNA	- ribonucleic acid
rRNA	- ribosomal RNA
RNase	- ribonuclease
SDS	- sodium dodecylsulphate
ssDNA	- single-stranded DNA
TCA cycle	- tricarboxylic acid cycle
TMED	- N, N, N', N',-tetramethylenediamine
Tris	- tris(hydroxymethyl) amino ethane
tRNA	- transfer RNA
u	- units
URF	- unassigned reading frame
vol	- volume
X-gal	- 5-bromo-4-chloro-3-indolyl-B-galactopyranoside

Units

bp	- base pairs
Ci	- Curies
cm	- centimetres
cpm	- counts per minute
^{o}C	- degrees centigrade
g	- grammes
g	- centrifugal force equivalent to gravitational acceleration
hr	- hours
kb	- kilobases/kilobase pairs
l	- litres
mCi	- millicuries
M	- Molar
min	- minutes
ml	- millilitres
mm	- millimetre
mM	- milliMolar
mMol	- milliMoles
mV	- millivolts
ng	- nanogrammes
nt	- nucleotides
nMol	- nanomoles
µCi	- microcuries
OD	- optical density
pH	- acidity [$-\log_{10}$(molar concentration of H^{+} ions]
rpm	- revolutions per minute
sec	- seconds

μCi	- microCuries
μg	- microgrammes
uJ	- microJoules
$\mu J.mm^{-2}$	- microJoules per square millimetre
μm	- micrometres
μl	- microlitres
v	- volts

ACKNOWLEDGEMENTS

I would like to thank Howy Jacobs for excellent advice and suggestions over the course of this work, and also for his meticulous comments on this thesis. I am also very grateful to the other people who proofread this thesis, namely Andy Mayhook, Rick Sewards and Amy Bednarz.

Thanks are also due to the other members of the third floor for making it an enjoyable place to work in, providing diverse musical tastes, shoulders to cry on, etc. Special thanks are also due to Joyce Moore and Arvind Hundal for kindly providing several solutions and electric timers, when these had run out at (more or less) crucial moments, to Keif Matthews for advice on lambda preps.,to Mary Burke for her help, and to the media ladies. I would also like to thank Margaret White and Linda Kerr for typing my reference list out for me, and for instruction on the finer points of word processing.

I would like to thank my other friends in the Genetics Department, past and present, for their companionship at all times of the day and night, either in Reids or the department itself.

Last, but not least, I would like to thank all my flatmates over the last 3 and a bit years for making my time more enjoyable and interesting, particularly my present flatmates Kenny Bain and Julie Muir, and my ex-flatmates Mike Levitt and Eddy Clarke.

Summary

Mitochondrial transcripts are differentially expressed, particularly the rRNAs which are expressed at a higher level than the mRNAs and tRNAs. Most work on animal mitochondrial gene expression has been done in vertebrates, in which differential expression of the rRNA genes relative to the mRNA/tRNA genes seems to be achieved at the level of transcriptional initiation/termination. Transcription of the contiguous rRNA genes initiates in an intergenic sequence located immediately upstream of them, but is subsequently attenuated to limit RNA polymerase readthrough into the mRNA/tRNA genes. The mRNA and rRNA genes are thought to have distinct transcriptional initiation sites located immediately downstream of the rRNA initiation site, but RNA polymerase initiating at this downstream site reads through the attenuator into the mRNA/tRNA genes. Sequence analysis has shown that the organisation of the sea urchin mitochondrial genome differs from that found in vertebrates. Of particular importance for the control of gene expression is the fact that the rRNA genes are separated by the genes encoding 2 mRNAs and several tRNAs, and also an extended intergenic sequence. These differences mean that alternative mechanism from that in vertebrates must be operating to give differential expression of the rRNA and mRNA/tRNA genes.

There is also a further level of complexity in sea urchin mitochondrial gene expression, in that the ratio of rRNA:mRNA is developmentally modulated. Using RNA filter hybridisations, I have shown that the ratio of rRNA:mRNA is developmentally modulated. mRNA levels increase about ten-fold between the egg and blastula stages of development, whereas rRNA levels decrease slightly. The levels of high molecular weight transcripts containing the information for more than one gene change between different developmental stages, suggesting that pathways of RNA synthesis operate in a developmentally regulated manner.

In order to get an insight into how the rRNA:mRNA ratio

is established and developmentally modulated in sea urchins, I mapped mitochondrial transcripts at the egg and blastula stages of development. These experiments showed that the 5' ends of both rRNA genes are directly adjacent to their upstream genes, so neither rRNA gene has an intergenic sequence directly upstream. However, the 3' ends of both rRNAs overlap with the transcripts encoded by the adjacent downstream genes. These overlapping transcripts must be synthesised by mutually exclusive pathways, most likely at the level of RNA processing. Transcript termini seemed to be the same at both developmental stages.

I have used two complementary techniques in order to map transcriptional start sites, and transcription units, on the sea urchin mitochondrial genome. Firstly I have used *in vitro* capping with the enzyme guanylyl transferase, which specifically labels the 5' triphosphate groups on RNA (which result from transcriptional initiation rather than processing). This approach has not given entirely consistent results, but has identified two regions of the sea urchin mitochondrial genome which are complementary to transcripts with 5' triphosphate termini.

Secondly I have ^{3}H-labelled sea urchin RNA *in vivo*, in pronase-permeabilised sea urchin eggs, and have identified the major labelled mitochondrial transcripts by gel and hybridisational analyses. I have used this method along with ultra-violet (u.v.) mapping, in which transcription is terminated in a dose-dependent fashion depending on distance from the transcriptional initiation site. This can be used to order genes within transcription units, and has shown that whereas the synthesis of 16S rRNA is relatively u.v. resistant, the synthesis of both 12S rRNA and COI mRNA are sensitive to even the lowest doses of u.v. irradiation. This suggests that 16S rRNA is synthesised from a transcriptional initiation site located closely upstream, and that COI mRNA and 12S rRNA are both synthesised from transcriptional initiation sites located far upstream of their genes. The genes encoding COI and 16S rRNA are adjacent, demonstrating that at least in some cases adjacent genes are not

necessarily co-transcribed from the same promoter. 16S rRNA and COI mRNA may have distinct primary transcripts, and the choice of which mature transcript is selected by the mutually exclusive synthetic pathways may be determined by their repective sites of transcriptional initiation, perhaps by affecting secondary structure at the 16S rRNA/COI gene boundary.

The u.v.-mapping data is thus consistent with a transcriptional initiation site located just upstream of the 16S rRNA gene. Upstream of the 16S rRNA gene, within the gene encoding ND2, is a TTATATATAA motif. TTATATATAA-like motifs are also found at several other locations within the genome, between divergently transcribed genes, and within the single extended intergenic sequence (just upstream of the replication origin). The strategic locations of the TTATATATAA-like motifs, along with the u.v.-mapping data, make the TTATATATAA-like motifs strong candidates for a bidirectional promoter element.

Fine transcript mapping has shown that the 5' termini of some transcripts map to this TTATATATAA-like consensus, and the abundance of these termini are developmentally modulated. However, some of the high molecular weight transcripts detected on northern blots are larger than adjacent TTATATATAA motifs, which presumably do not simply demarcate transcription units. This is in agreement with the u.v.-mapping data and would suggest that this mitochondrial genome has multiple, overlapping transcription units.

On the basis of the work described in this thesis, I would like to propose that, despite differences imposed by genome organisation, there are also a number of underlying similaritites in the control of mitochondrial gene expression in sea urchins and vertebrates.

expected if they ended at

CHAPTER 1

INTRODUCTION

1.1 Mitochondrial structure and function

Eukaryotic cells contain a number of structurally and functionally distinct membrane-bound compartments called organelles. Some organelles have been shown to contain DNA-encoded genetic information. These are the nucleus (which contains the most information), mitochondria, chloroplasts (in plants), and recently the centrioles of Chlamydomonas (Hall et al, 1989).

There are essentially two theories about how this subcellular organisation has evolved from prokaryotic cells, which lack membrane-bound organelles (Margulis, 1972; Gray, 1982). The endosymbiotic theory suggests that the organellar genomes were originally located in different cells, which associated symbiotically, and that subsequently there was a transfer of information from other organelle genomes to the nucleus. This resulted in an increase in the information content of the nuclear genome, with a concomitant decrease in information content of the other organelle genomes. The alternative theory suggests that the organelle genomes originated, and then became physically compartmentalised, within a single cell. Evidence seems to support an endosymbiotic origin for mitochondria (Gray, 1982). This evidence includes sequence similarities between mitochondrial rRNA genes (particularly of plants) and prokaryotic, rather than nuclear, rRNA genes, and structural similarities between the tRNA molecules of mitochondria and prokaryotes (Gray, 1982). This evidence is consistent with a different cellular origin for mitochondria and nuclei, unless there are widely different selective pressures acting on nuclear and mitochondrial DNA sequences. The original bacterial endosymbiont may have been related to present day Paracoccus and certain Rhodospirillaceae, since these have a similar respiratory physiology and biochemistry to mitochondria (Gray, 1982).

Mitochondria are found in almost all eukaryotic cells. The number of mitochondria per cell, and their intracellular location and morphology are tissue-dependent in multicellular eukaryotes. For example, mammalian erythrocytes lack mitochondria, whereas in the human liver there are 500-1000

per cell (Nelson, 1987). In general, mitochondria are several μm long and about 0.5 μm in diameter. Mitochondria contain two membranes with distinct properties. The outer membrane contains only a few known enzymes and is permeable to molecules with molecular weights up to 5 kilodaltons. In contrast, the inner membrane contains a large number of enzymes and is impermeable to most ions and polar molecules. The membranes compartmentalise the interior of the mitochondrion into 2 distinct spaces, the internal or matrix space, and the inter-membrane space between the inner and outer membranes. New mitochondria are thought to arise from the division of pre-existing mitochondria, rather than de novo (Attardi and Schatz, 1988).

Mitochondria are of considerable interest because of their important role in metabolism. They are the cellular location of the central metabolic reactions of aerobic respiration, conserving most of the energy in the cell in the form of high energy phosphate bonds in ATP and GTP. Aerobic respiration can be divided up into 3 sets of reactions, which can be compartmentalised temporally and spatially within the mitochondrion (Fig. 1.1). (1) The citric acid (or TCA) cycle takes place in the mitochondrial matrix, and is catalysed by soluble and inner membrane proteins. This is a cyclical set of reactions, in which the oxidation of Acetyl CoA is coupled to the reduction of the coenzymes NAD^+ and FAD to $NADH/H^+$ and $FADH_2$ repectively. Acetyl CoA is synthesised in mitochondria from pyruvate (a product of glycolysis in the cytosol) or from fatty acids. (2) These reduced coenzymes are reoxidised in the electron transport chain by the stepwise transfer of electrons donated by the coenzymes along a series of 4 enzyme complexes to molecular oxygen. These enzyme complexes are (with the exception of cytochrome c oxidase) located in the inner membrane and have increasing redox potentials along the chain. The energy released is used to pump protons across the inner mitochondrial membrane creating a pH and electrochemical gradient. (3) Finally, ATP is generated using the energy stored in the transmembrane electrochemical proton gradient. This is catalysed by the F_1F_0 multiprotein complex (ATP synthase) which spans the inner membrane. The F_1 subcomplex catalyses the formation of ATP on the inner surface, while protons flow across the membrane through the integral membrane subcomplex F_0.

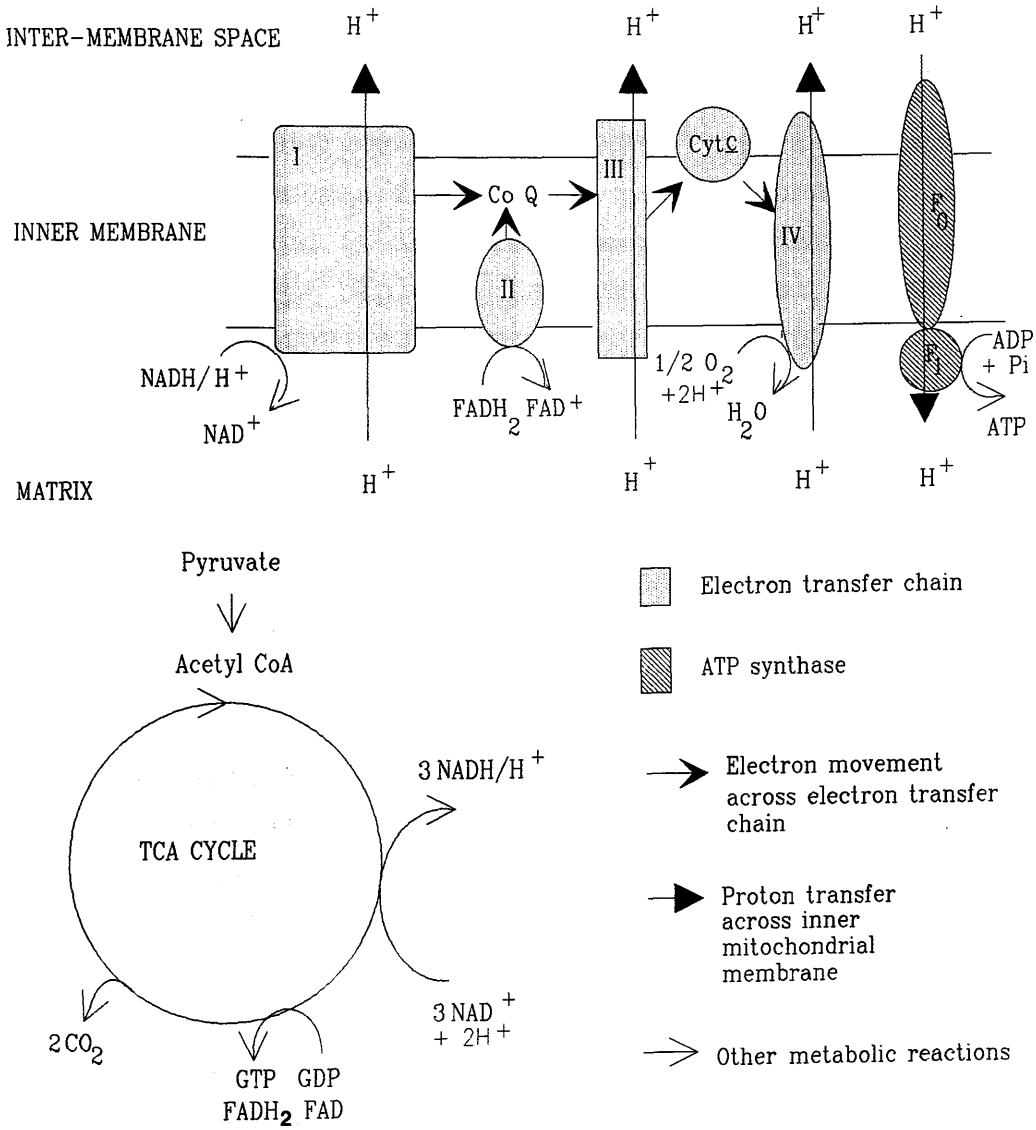


Figure 1.1 Summary of the reactions involved in aerobic metabolism. Components of the electron transfer chain are shown as I, NADH dehydrogenase (complex I); II, succinate dehydrogenase (complex II); III, cytochrome b–c$_1$ complex (complex III); IV, cyt c , cytochrome c oxidase (complex IV). Electrons donated by a single $NADH/H^+$ or $FADH_2$ give rise to 3 or 2 protons being transported across the inner mitochondrial membrane, respectively. One molecule of ATP is synthesised for each H^+ transported back into the mitochondrial matrix by the ATP synthase complex. Not to scale. Other abbreviations are used as described on page (x) of this thesis.

In addition, mitochondria contribute to the synthesis of several other cellular metabolites, such as pyrimidines, phospholipids, heme and urea (Attardi and Schatz, 1988).

1.1.2 Mitochondrial DNA

Mitochondrial DNA has been of considerable research interest. Differences in mitochondrial DNA have been used to estimate the evolutionary divergence time between animals, (particularly closely related animals) since mitochondrial DNA evolves 5-10 times faster than nuclear DNA (at least in some organisms), and it is free from recombination, inherited in an effectively haploid and uniparental manner, and contains no multicopy sequences (reviewed by Brown, 1985).

Mitochondrial DNA has also been of medical interest for two reasons. In mice, a minor transplantation antigen MTF has been shown to be encoded by the upstream portion of the gene for ND1 (Loveland *et al*, 1990). MTF is presented by a class I MHC molecule (encoded by the nuclear *hmt* gene) and beta-2-microglobulin (Richards *et al*, 1989; Fischer-Lindahl *et al*, 1989). Secondly, a number of neuromuscular diseases in humans are known to be caused by mutations in mitochondrial DNA (Grivell, 1989a). These vary from single base changes such as in Lebers Hereditary Optical Neuropathy (Wallace *et al*, 1989), to deletions in a proportion of mitochondrial DNA molecules such as in Chronic Progressive External Opthalmoplegia (Holt *et al*, 1988).

Studies on mitochondria have provided some interesting and novel examples of genome organisation and gene expression, which are discussed below, as well as information on how gene expression in two cellular compartments is coordinated.

1.2 Mitochondrial genomes

1.2.1 Overview

Sequence analysis has shown that mitochondrial genomes contain only a small amount of genetic information. This is limited to that for structural genes encoding some of the proteins involved in aerobic respiration, the RNA components of the translational apparatus, and, in some cases, a few of

introns. In human mitochondria, the reading frames for two proteins partially overlap, and they are both translated from the same mRNA (Fearnly and Walker, 1986). Those animal mitochondrial genomes, which have been characterised in this detail, have been shown to be covalently closed circular molecules. Some animal mitochondrial genomes have been shown to have D-loop structures, spatially localised in their genomes close to their leading strand replication origin. D-loops are triple-stranded structures in which one of the parental DNA strands has been displaced by a family of short DNA molecules, which are thought to arise as a result of a premature arrest of replication at defined sites (Doda et al, 1981; Cairns and Bogenhagen, 1986; Gillum and Clayton, 1978). Within these constraints, the physical organisation of the genome shows some variability between species (Table 1.1). The human mitochondrial genome is illustrated in Figure 1.2.

1.2.3 Plant mitochondrial genomes

Higher plant mitochondrial genomes are considerably larger and more complex than those of animals, varying from 200 kb in Brassica species to 2500 kb in watermelons (Levings and Brown, 1989). Several factors contribute to this size difference, including the presence of genes for the subunit of F_1 ATP synthase, several ribosomal proteins, and a 5S RNA which is found in plant mitochondrial ribosomes. The other two rRNAs, which are also mitochondrially encoded like in other organisms, are larger (26S and 18S) than those found in animal mitochondria, and the mitochondrial genomes of many species contain sequences of plastid and nuclear origin, as well as sequences which are similar to those which encode reverse transcriptase (Schuster and Brennicke, 1987). All higher plant mitochondrial genomes characterised to date, apart from that of Brassica hirta, are organised as a number of subgenomic circular molecules, which are potentially interconvertible by recombination between repeats (Lonsdale et al, 1984). The mitochondrial genome of Brassica hirta is a single circular molecule of 208 kb, and does not contain any recombinational repeats (Palmer and Herbon, 1987).

Chlamydomonas reinhardtii is the only lower plant mitochondrial DNA to have been reported in detail, and differs from higher plant mitochondrial DNA in being a linear

Organism	Molecular genome structure	Gene content	Size	Genome organisation	Reference
Sea Urchin	CCC, D-loop	Standard	15.6 kb	Single 121bp major intergenic region, rRNA genes on same strand, but separated by major intergenic region, 2 mRNA genes and tRNA gene cluster	Jacobs et al, 1988; 1989
Starfish	CCC	Standard	16 kb	Single major intergenic region, rRNA genes encoded by different strands of the genome. tRNA genes clustered.	Smith et al, 1989
Ascaris suum (nematode)	CCC	Lacks A8	14.2 kb	2 major intergenic regions, rRNA genes separated by 4 mRNA genes and 5 tRNA genes, unusual tRNA structures	Wolstenholme et al, 1987; 1989
Human	CCC, D-loop	Standard	16.6 kb	Single major intergenic region, rRNA genes closely linked, no extended (>4 gene) tRNA gene cluster	Anderson et al, 1981
Mouse	CCC, D-loop	Standard	16.3 kb	As for human	Bibb et al, 1981
Drosophila yakuba	CCC	Standard	19.5 kb	As for human, but major intergenic	Clary and Wolstenholme, 1985
Zea mays (higher plant)	Multipartite	Lacks ND2-ND6. Contains f1 ATPase subunit a.		rRNA genes separated. Large intergenic regions.	Attardi and Schatz, 1988
Chlamydomonas reinhardtii (lower plant)	Linear molecule	Lacks ND3, ND4, ND4L, COIII, COII, A6 and A8.	15.8 kb	rRNA genes organised in modules. Single major intergenic region of 25-200 bp in region sequenced to date.	Gray and Boer, 1988

Organism	Molecular genome structure	Gene content	Size	Genome organisation	References
Saccharomyces cerevisiae (yeast)		Lacks ND1-6. Contains A9, RNA maturases, optional intron transposition endonucleases, RNA component of RNAse P-like enzyme, 2 additional tRNAs, 3 URFs, ribosomal protein gene	70-82 kb	rRNA genes far apart on the genome large intergenic regions, tRNA gene cluster. Introns in 3 genes optional between strains.	Grivell, 1989b
Torulopsis glabrata (yeast)		Lacks ND1-6. May lack A8. Contains ribosomal protein gene.	19 kb	rRNA genes separated by ribosomal protein gene. Not completely sequenced. tRNA genes clustered.	Clark-Walker and Sriprikash, 1983 Clark-Walker et al, 1985
Neurospora crassa (filamentous fungus)	CCC	Lacks ND4 and ND4L Contains several URFs and introns	60 kb	rRNA genes separated by 2 mRNA genes and several tRNA genes, tRNAs clustered, introns in several genes.	Kennel and Lambowitz, 1989
Aspergillus nidulans (filamentous fungus)	CCC	Contains A9, an URF and introns	33.25 kb	rRNA genes separated by 2 mRNA genes and one of the 2 tRNA clusters. Several genes contain introns.	Dyson et al, 1989; Kochel et al, 1981.
Trypanosoma brucei (protozoan)	2 Unrelated classes of molecules	No ND4L, ND6, A6, or A8. Contains several URFs, one of which is now known to be encrypted COIII.	Maxicircles 22 kb Minicircles around 1 kb	Maxicircles resemble metazoan mitochondrial DNA, large major intergenic region (strain dependent in size),tRNAs possibly nuclear-encoded.Function of minicircles unknown.	Stuart, 1987.

Table 1.1: Mitochondrial genome organisation. All genomes contain genes for the large and small subunit rRNAs, tRNAs (as denoted by the standard 1 letter code: E, T, P, Q, N, L, A, W, C, V, M, D, Y, G, L_{UUR}, I, R, K, S_{UCN}, H, S_{AGN}, F) ND1, ND2, ND3, ND4, ND5, ND6, ND4L, COI, COII, COIIII, , A6, A8 and cyt b unless otherwise stated. Abbreviations are used as described on page, apart from CCC which stands for covalently closed circular. The molecular genome structure is only given where it is known, for example only those animal mitochondrial genomes which are known to contain D-loops are mentioned specifically, although other mitochondrial genomes mentioned may also in fact contain D-loops.

molecule, and lacking several genes found in higher plant mitochondria (Gray and Boer, 1988). Many of the tRNAs are thought to be transported from the cytosol, which might also be the case for a proportion of bean mitochondrial tRNAs (Green et al, 1987). The most bizarre feature of the Chlamydomonas mitochondrial genome is the rRNA genes, which are split up into several modules spread over 6kb. These may function in the Chlamydomonas mitochondrial ribosome as a non-covalent network of small RNAs (Boer and Gray, 1988).

1.2.4 Fungal mitochondrial genomes

Yeasts have the most studied fungal mitochondrial genomes. Saccharomyces cerevisiae has one of the most complex of these, with a genome size intermediate between that of mtDNA in plants and animals (70-82 kb). This large genome size is due to the presence of apparently non-coding intergenic regions, consisting of (A+T)-rich regions interspersed with so called (GC) clusters (Bernardi, 1982). The latter may act as sites for intramolecular recombination (Zamaroczy and Bernardi, 1987). Introns are also present in most strains (Tzagaloff and Myers, 1986). Other species of yeast have smaller mitochondrial genomes (Clark-Walker and Sriprikash, 1983; Wilson et al, 1989), as a result of shorter intergenic regions and fewer introns. The S. cerevisiae mitochondrial genome is illustrated in Figure 1.2.

1.2.5 Protozoan mitochondrial genomes

The protozoan parasite Trypanosome brucei is unusual in having a mitochondrial genome composed of two unrelated classes of molecules: minicircles, which are around 1 kb in size, and maxicircles, which are around 22 kb in size (Stuart, 1987). The function of the minicircles is unknown, whereas the maxicircles seem to resemble conventional mitochondrial genomes.

1.3 Differential expression of mitochondrial genes at the RNA level

Efficient protein synthesis (in both mitochondria and the cytosol) requires a large excess of rRNA over mRNA. This

can be achieved either at the transcriptional level (with rRNAs being transcribed at a higher level than mRNAs) or the post-transcriptional level (with rRNAs being more stable than mRNAs). Additionally, different mitochondrial mRNAs and tRNAs are present in different steady-state concentrations (Attardi and Schatz, 1988). Regulation at both the transcriptional and post-transcriptional levels seem to contribute towards the differential expression of mitochondrial RNAs, although, as discussed in detail below, the relative contribution at these two levels differs between organisms.

Although it is clear that different mitochondrial transcripts are represented in different amounts, there is conflicting evidence on whether this is a constitutive effect, or whether the levels of mitochondrial transcripts are modulated to meet different cellular requirements. There is evidence that mitochondrial transcription, at least in mammalian tissue-culture cells, might be in excess of cellular demands and not rate limiting for protein synthesis. During experiments in which mitochondrial transcription in African green monkey cells was 85% inhibited by photodamage of 5'-bromodeoxyuridine-substituted DNA (Lansman and Clayton, 1975), mitochondrial protein sythesis was maintained for 48 hours. If the mitochondrial mRNA and rRNA half lives in these cells are similar to those in HeLa cells (50-80 minutes and 3-4 hours respectively), much of the RNA would be depleted after this length of time. This maintenance of protein synthesis could be explained by an increase in RNA stability, or, alternatively, the remaining 10-20% of RNA synthesis may be enough to support normal levels of mitochondrial translation. Similar results have been obtained by England et al (1978).

However, in sea urchin eggs (Rinaldi et al, 1977; 1979), and in Xenopus laevis (Rinaldi and Giudice, 1985), it has been shown that the nucleus exerts a negative control over RNA synthesis, suggesting that transcription of the mitochondrial genome may be modulated in some cases. In addition, in the parasite Trypanosome brucei, there are differences in expression of mitochondrial transcripts between the bloodstream and insect forms of the parasite, corresponding with a lack of a functional Krebs cycle and cytochromes in the bloodstream form (Feagin et al, 1985; Feagin and Stuart, 1985). These differences in mitochondrial

RNA synthesis might, however, be related to polyadenylation (Section 1.11.1), or editing (Section 1.11.4), of mitochondrial transcripts, rather than transcription.

1.4 Experimental approaches used to study mitochondrial gene expression

To a large extent, similar experimental approaches have been used to examine mitochondrial RNA synthesis in different organisms. The mapping of transcriptional initiation sites is of central importance to understanding how gene expression is controlled, since promoters and many other elements involved in the control of transcription are found at the 5' end of transcription units. However, mapping a 5' end by a nuclease protection or primer extension experiment is not necessarily equivalent to mapping a site of transcriptional initiation, since 5' ends can also be generated by RNA processing. Nevertheless, the 5' ends arising from transcriptional initiation differ chemically from those arising from RNA processing, in that they have 5' triphosphate rather than monophosphate groups. The enzyme guanylyl transferase specifically radiolabels 5' triphosphate groups which lack any cap structure, such as in mitochondrial RNA, by adding a ^{32}P GMP group (Levens *et al*, 1981a), which allows transcriptional initiation sites to be distinguished from RNA processing sites.

Mitochondrial RNA half-lives have been determined by two techniques. One of these, the pulse-chase technique, involves radio-labelling RNA *in vivo* with a labelled ribonucleotide, and then blocking transcription with an inhibitor and monitoring the decay of the labelled RNA in the absence of *de novo* synthesis. The other involves a labelling period, after which the label is removed from the medium but is still present in the cells. RNA half-lives can be calculated from the kinetics of incorporation of this label, which decreases in intracellular concentration over time. Of these two techniques, the kinetics of incorporation of label is thought to be more reliable, since transcriptional inhibitors may have general effects on the organism besides inhibiting mitochondrial RNA polymerase. For example, ethidium bromide has commonly been used as an inhibitor in experiments to measure mitochondrial RNA half-lives, since it inhibits

mitochondrial RNA synthesis more than nuclear RNA synthesis (Penman et al, 1970), perhaps as a result of the different physical properties of the two genomes. Ethidium bromide might have a greater effect on supercoiled mitochondrial DNA than on the linear nuclear genome. However there is evidence suggesting that ethidium bromide leads to degradation of the mitochondrial genome (Penman et al, 1970), and it might have indirect effects on mitochondrial RNA synthesis via an interaction with either nuclear DNA, or nuclear-encoded RNA.

1.5 Regulation of mitochondrial RNA levels in animals

1.5.1 Trancriptional regulation

1.5.1.1 HeLa cell mitochondrial transcription

Much of the detailed work on animal mitochondrial transcription has been done in HeLa cells, in which differential rates of transcription are thought to be more important than post-transcriptional regulation in determining the higher level of rRNA over mRNA. This argument is based on the fact that whereas rRNA half-lives are only 2-5 times longer than mRNA half lives, the steady-state level of mitochondrial rRNA is 30-60 times higher than that of mitochondrial mRNA (Gelfand and Attardi, 1981; Attardi et al, 1982). When calculated on the basis of their steady-state level and half-life, this corresponds to a 20-60 fold difference in synthetic rate between the two classes of RNA. From this it has been calculated that, in a single cell generation, the rRNA genes would be transcribed around 50 times, whereas on average the mRNA genes would only be transcribed once or twice.

In vitro capping experiments using guanylyl transferase, along with S1 nuclease mapping of RNA isolated from nascent transcription complexes, have shown that transcription of both strands initiates at either side of the major intergenic region of HeLa cell mitochondrial DNA (Fig. 1.2)(Ojala et al, 1981a; Montoya et al, 1982; Cantatore and Attardi, 1982; Yoza and Bogenhagen, 1984). This means that Hela cell mitochondrial transcription units are polycistronic. Paradoxically, pulse labelling and hydridisation experiments have shown that the entire L-strand seems to be transcribed

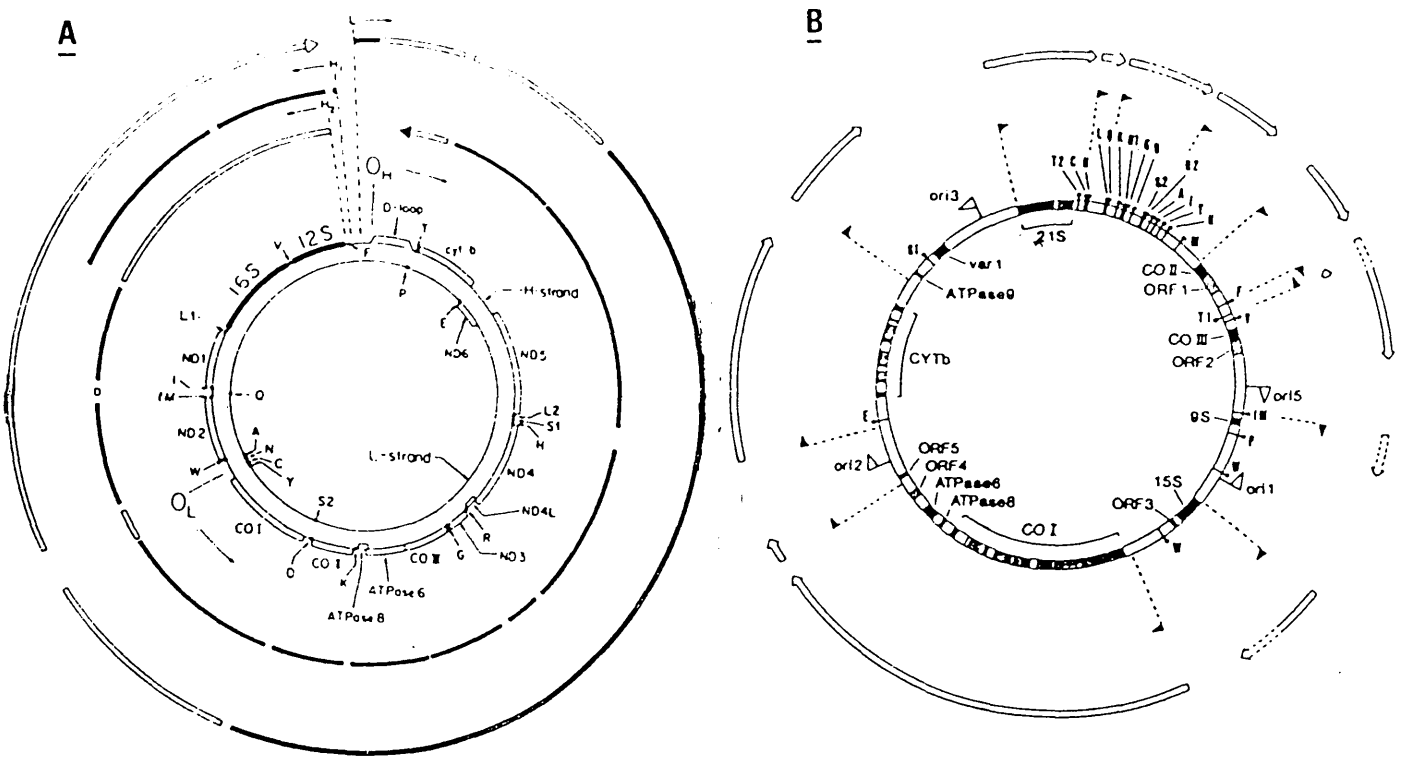


Figure 1.2: Transcriptional organisation of the human and yeast mitochondrial genomes. (A) The human mitochondrial genome. The inner two circles represent the two coding strands of the genome. In the outer portion of the diagram, the curved black bars represent identified functional transcripts other than tRNAs which result from processing of the two polycistronic transcripts of the H-strand starting at either H1 (rDNA initiation site) or H2 (total H-strand initiation site). Cross hatched bars represent stable RNA species resulting from transcription of the L-strand. The white bars represent unstable, and presumably nonfunctional, transcripts. (B) The S. cerevisiae mitochondrial genome, organised as for (A), with the genome as the inner circle and the transcripts as the outer circle. The black flags represent sites of transcriptional initiation, and point in the direction of transcription. The white flags represent origins of replication. The identified primary transcripts are represented as curved open bars with the arrow head indicating the 3' end of the transcripts. Transfer RNAs are indicated according to their decoding specificities by the universal 1 letter amino acid code. Other abbreviations are as described at the start of this thesis, apart from ORF (open reading frame), ori (replication origin) and ATPase, ATP synthase. From Attardi and Schatz (1988).

at a 3-fold higher rate than the H-strand, even though it only contains 7% of the genome's coding information (Aloni and Attardi, 1977; Cantatore and Attardi, 1982).

Most of the studies of human mitochondrial gene expression have concentrated on the H-strand, which contains the coding information for both of the rRNAs, most of the tRNAs and all but one of the mRNAs. Montoya et al (1983) have proposed a model for the differential expression of rRNAs and mRNAs based on the selective initiation and termination of transcription (Fig. 1.3). Their model suggests that transcription of the rRNAs and the mRNAs initiates independently at two separate sites. According to their model transcription initiates more frequently at the upstream site and is attenuated at the 3' end of the 16S rRNA gene to produce the precursor to the rRNA molecules. The model further proposes that transcription initiates less frequently at the downstream site, but in this case reads through the attenuator at the 3' end of the 16S rRNA gene, resulting in the production of mRNAs and the remaining tRNA molecules.

The Montoya et al (1983) model is based on three lines of evidence. Firstly, capping experiments identified two H-strand initiation sites, one of which mapped 20-40 nucleotides upstream of the gene encoding $tRNA^{phe}$, whereas the other mapped just upstream of the 12S rRNA gene. The upstream site (the proposed initiation site for rRNA synthesis) was a more abundant substrate for capping reactions, which might be consistent with it being a more frequently used initiation site (although this assertion might not be strictly valid since the level of capping is also dependent on RNA secondary structure, i.e. the accessibility of the 5' triphosphate to the enzyme, and on the stability of the 5' triphosphate-containing residue). There is, however, some controversy regarding the existence of two transcriptional initiation sites, which is discussed below.

Secondly, two physiologically distinct types of transcript traversing the rRNA genes could be distinguished on the basis of their kinetic properties and termini. The Montoya model suggests that one of these transcript types is destined to become rRNA, whereas the other is there to link the mRNA promoter with the downstream mRNA genes (the linker transcript in Fig. 1.3). Kinetic experiments to map the

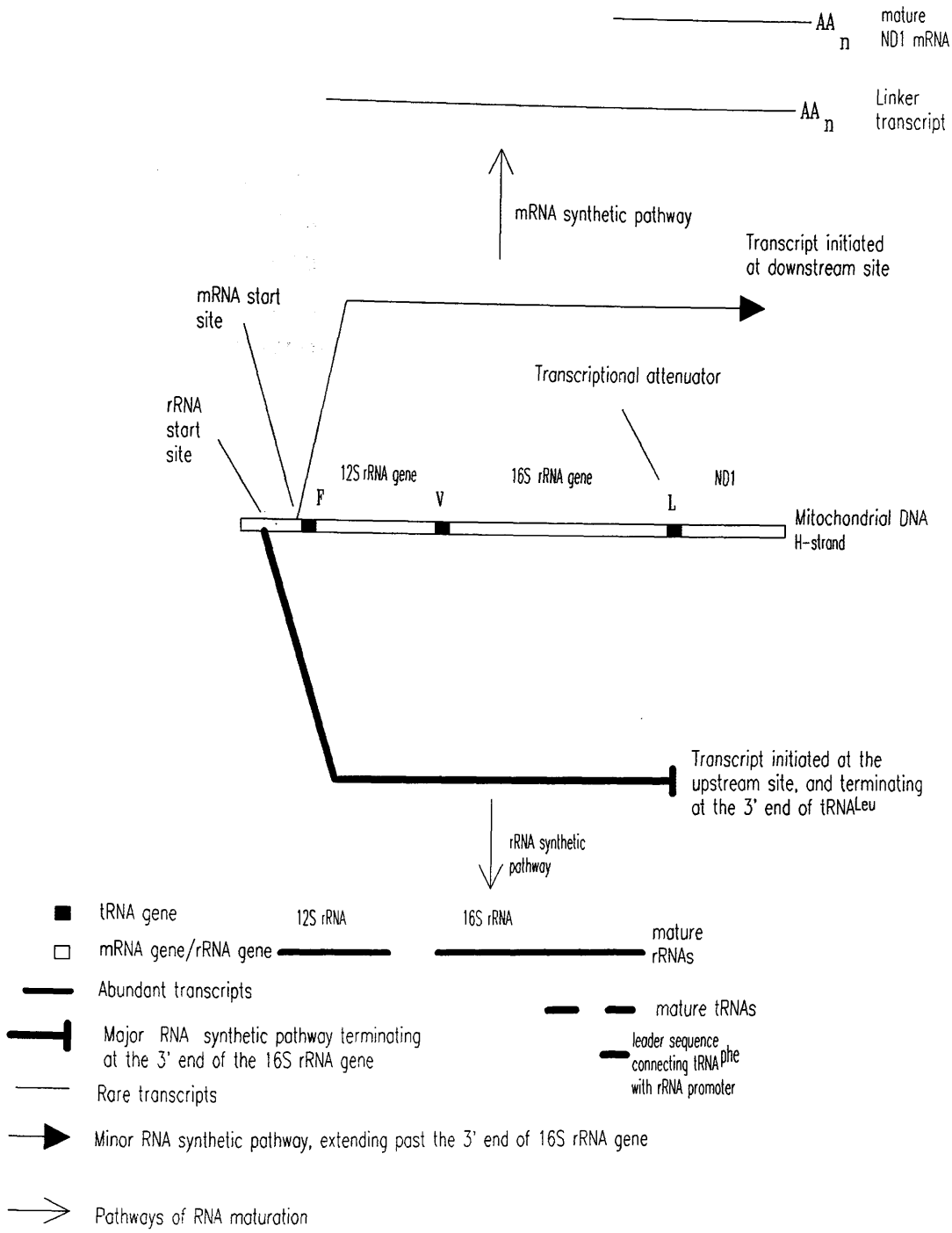


Figure 1.3: Transcriptional attenuation/overlapping transcript model of HeLa cell mitochondrial transcription. Full explanation in text. Transfer RNAs are abbreviated according to the universal code: F (phenylalanine), V (valine) and L (leucine). The linker transcript results from the RNA polymerase moving from the mRNA start site to the mRNA genes. The leader sequence results from the movement of RNA polymerase from the rRNA start site to the beginning of the gene for tRNA(F).

turnover of transcripts were carried out by labelling cells for 4 hours with ^{32}P-inorganic phosphate, and then pulsing them with ^{3}H-uridine for 10 minutes. When analysed by gel electrophoresis, the ratio between the amount of incorporated ^{3}H and the amount of labelled phosphate in an RNA species was taken as an indication of the ratio of newly synthesised to steady-state RNA. On the basis of this ratio there were two types of RNA species complementary to the rRNA region of the H-strand. One type showed evidence of a rapid turnover which would be expected of a rRNA precursor (given the accumulation of label in the mature rRNA), and the other a much slower turnover, which would be expected of a mRNA precursor.

At their 3' termini the putative mRNA precursors were polyadenylated like mature mRNAs, whereas the putative rRNA precursors were not (mature rRNAs are only oligoadenylated). At their 5' termini a proportion of the putative rRNA precursors had a leader extending 25 nucleotides upstream of the tRNA^{phe} gene to the putative rRNA initiation site.

Thirdly, there is some firm evidence that some form of transcriptional attenuation takes place at the 3' end of the 16S rRNA gene. Pulse labelling experiments showed that ^{3}H-uridine labelling of RNA species encoded downstream of the 16S rRNA gene occurs at a lower level than for those encoded upstream. Also, two tRNAs, tRNA^{val} and tRNA^{leu}, which are encoded by the rDNA region of the genome, are expressed at a higher steady-state level than other mitochondrial tRNAs, and which would not be predicted from the codon usage in HeLa cell mitochondrial DNA. Both these lines of evidence are consistent with a transcriptional termination event occuring at the 3' end of the 16S rRNA gene (Montoya et al, 1983). Unlike other HeLa cell mitochondrial transcripts, which have precise termini thought to arise from endonucleolytic processing, the 3' end of 16S rRNA is ragged, suggesting that there is some imprecision in the event leading to its formation. This could be characteristic of a transcriptional attenuation event, although it could also be explained as 3' clipping by ribonucleases. This latter explanation is unlikely, since if ribonucleases were responsible, then they would have to be specific for the 3' end of 16S rRNA. Most importantly, an activity promoting transcriptional termination in vitro has been isolated from HeLa cells

(Kruse et al, 1989). Proteins present in the active fractions bound to a DNA sequence within the gene for $tRNA^{leu}$. These proteins had no detectable RNA binding or processing activity. The HeLa cell mitochondrial termination sequence functions bidirectionally in vitro, and so may also prevent active L-strand transcription into the rRNA genes in vivo.

The 3' end of 16S rRNA can be folded up into a hairpin structure (Dubin et al, 1982), which resembles a bacterial rho-independent transcriptional terminator sequence (Rosenberg and Court, 1979). Despite this structural similarity, the importance of this hairpin is debateable since it is outside the region of DNA shown by deletion analysis to be important for termination in vitro, and whereas the HeLa cell mitochondrial transcriptional termination factor interacts with DNA to arrest transcription, the bacterial rho factor interacts with RNA.

The molecular basis of the differential transcription of mitochondrial rRNA and mRNA is not known. However, it has been shown that the synthesis of mitochondrial rRNA and mRNA can be dissociated experimentally, which is consistent with different enzyme complexes being responsible. Analysis of RNA labelled by incorporation of radiolabelled precursors in isolated mitochondria showed a decrease in the ratio of rRNA synthesised relative to mRNA, as compared with the relative levels synthesised in vivo in intact cells (Gaines and Attardi, 1984a and 1984b). This difference could be further accentuated by the presence of intercalating drugs such as actinomycin D and ethidium bromide, low temperature, the presence of Calcium and Manganese ions, and a low exogenous ATP concentration (Gaines and Attardi, 1984a, 1984b; Attardi et al 1985; Gaines et al, 1987a). The requirement for a high level of ATP for rRNA synthesis in organello is consistent with the results of in vitro experiments using purified mitochondrial RNA polymerase, where transcriptional initiation from the upstream H-strand promoter (which has been proposed to be responsible for the transcription of rRNA) was dependent on a high level of ATP (Narasimhan and Attardi, 1987).

The most controversial feature of the Montoya model for HeLa cell mitochondrial gene expression is the existence of two physiologically distinct transcriptional initiation sites. Transcription from the proposed downstream site cannot

be reproduced *in vitro*, although a number of minor initiation events occur in its region (Walberg and Clayton,1983; Chang and Clayton, 1984; Bogenhagen *et al* 1984; Shuey and Attardi, 1985). A possible explanation for this discrepancy might be that the enzymatic machinery initiating transcription at the downstream site is different, and so requires either different *in vitro* conditions for optimal transcription, or a different purification protocol. However, initiation at the downstream site would be predicted to be a less frequent event *in vivo* by the model of Montoya *et al* (1983). There is a precedent for a site of transcriptional initiation influencing the position of transcriptional termination. In prokaryotes, the first 30 nucleotides transcribed from a promoter can affect the efficiency of termination at a *rho*-dependent site, perhaps by interacting with the polymerase to convert it from a termination-proficient to a termination-resistant status (Goliger *et al*, 1989; Telesnitsky and Chamberlin, 1989). Also in prokaryotes, there are anti-termination sites, located far upstream of the termination sites which they govern, at which the RNA polymerase might become modified, so as to allow readthrough at the termination site (Ptashne, 1986). It has been shown that at one of these antitermination sites, *nut* of phage lambda, a protein specifically becomes associated with the elongating RNA polymerase, and subsequently prevents transcription being terminated at several sites located downstream in the transcription unit (Barik *et al*, 1987).

1.5.1.2 Generality of the HeLa cell mitochondrial transcription model to other animal mitochondria

The transcriptional control mechanisms operating to establish differential levels of rRNA relative to mRNA/tRNA in HeLa cells may be provide a model system for the study of mechanisms operating in other mitochondria. This might be the case particularly for vertebrate mitochondrial gene expression, and also some invertebrates like *Drosophila*. These have a similar gene organisation to human mitochondrial DNA, with closely spaced rRNA genes situated immediately downstream of the major intergenic region of their genomes (Section 1.2, Table 1.1). As in HeLa cells, the rRNA genes could be contained within a single transcription unit

initiating within the major intergenic region, and distinct from that responsible for the transcription of the mRNA genes.

Experimental evidence is consistent with this idea for mouse Ehrlich Ascite cell mitochondria, in which differential expression of the rRNA and mRNA genes seems to occur predominantly at the transcriptional level, like in HeLa cells. During short labelling periods in isolated, digitonin-treated mouse mitochondria, during which RNA turnover would be minimal, the rRNAs accumulated to a 20-40 fold higher level than the transcripts encoded by the rest of the genome (Kantheraj et al, 1983). Additionally, like in HeLa cells, the mouse mitochondrial L-strand seems to be transcribed at a higher level than the H-strand (Bhat et al, 1984). Two initiation sites for mouse H-strand transcription have been mapped in mouse L-cell mitochondrial DNA (Fig. 1.3), but whether these are physiologically distinct from each other is not known. The same enzyme seems to initiate transcription at both these sites in vitro (Chang et al, 1986c). A termination activity which specifically terminates transcription at the 3' end of the 16S rRNA gene has been isolated from mouse L cells (Christianson and Clayton, 1986, 1988), and is presumably the functional equivalent of the terminator purified from HeLa cells.

In vitro capping experiments have mapped the transcriptional initiation sites of Xenopus mitochondrial DNA to its single large intergenic sequence, immediately upstream of the adjacent rRNA genes (Bogenhagen et al, 1985, 1986b; Bogenhagen and Romanelli, 1988). Within this intergenic region there are multiple transcriptional initiation sites for both strands of the genome, all of which support transcription in vitro (see Section 1.9). Whether physiologically distinct transcripts initiate at these sites is not known. In addition, elsewhere on the genome there are numerous promoter motifs (see section 1.9), but transcriptional initiation has not been detected from these sites. A block of sequence similarity to the sequences involved in transcriptional termination in HeLa cells occurs at the 3' end of the 16S rRNA gene in Xenopus mitochondrial DNA (El Meziane et al, 1989).

Further tentative evidence that the model proposed for HeLa cell mitochondrial transcription might be a paradigm to

explain the control of mitochondrial transcription in some other animals has come from sequence comparisons, which have shown that there are hairpin structures similar to that found in the HeLa cell mitochondrial genome downstream of the mouse (Van Etten et al , 1980), bovine, hamster and mosquito (Dubin et al, 1982) 16S rRNA genes. However, as described above, the physiological importance of these hairpin structures is not clear. The 3' end of hamster mitochondrial 16S rRNA has been shown to be ragged, which as described above might be indicative of formation by a transcriptional attenuation event (Dubin et al, 1981).

In contrast, a number of animal mitochondrial genomes have been shown to have a different physical organisation from that of human mitochondrial DNA (Table 1.1), in that their rRNA genes are separated by large regions of DNA containing other genes, which is the case in sea urchins (Jacobs et al, 1988; Cantatore et al, 1987) and the nematode Ascaris suum (Wolstenholme et al, 1987; 1989); or are on different strands of the genome as is the case in the starfish Asterias forbesii (Smith et al, 1990). An understanding of how transcription is controlled in one of these organisms, the sea urchin, is one of the aims of this project (Section 1.12 and 1.13). There is no reported work on how mitochondrial transcription is controlled in either starfish or nematodes.

1.5.2 Post-transcriptional regulation

There is evidence that the steady-state RNA levels in animal mitochondria are determined at least in part by differential RNA decay, with RNAs of differing stabilities accumulating to different extents.

The half-lives of H-strand-encoded RNAs in mammalian mitochondria differ most between different classes of RNA. RNA stability measurements in HeLa cells showed that mitochondrial transcripts were relatively metabolically unstable, with mRNAs having a half-life of around 1 hour, and rRNAs having half-lives 2-5 times longer than this (Gelfand and Attardi, 1981; Attardi et al, 1982; Zylber and Penman, 1969, 1971). As described in Section 1.5.1, this difference in half-life makes a quantitatively smaller contribution to the differential expression of rRNAs and mRNAs than their

relative rates of transcription.

In contrast, differences in RNA turnover are responsible for the 20-150 fold higher expression of individual tRNAs relative to individual mRNAs derived from the H-strand (see Attardi and Schatz, 1988). Differences in RNA stability are also thought to be the major contributory factor leading to differences in the steady-state levels of different mRNAs in HeLa cell mitochondria (Gelfand and Attardi, 1981; Attardi et al, 1982). This may well be the case in mouse mitochondria, where an analysis of in organello pulse-labelled RNA showed that all mRNAs were labelled (and so presumably synthesised) at approximately the same rate (Bhat et al, 1984). Although steady-state levels of mitochondrial mRNAs were not measured in these experiments, differences in steady-state level were inferred from differences in the steady-state levels of the mouse mitochondrially-encoded proteins (Bhat et al, 1982). However, inferring steady-state RNA levels from the levels of proteins synthesised is not totally justifiable, since other factors as well as RNA levels (for example translational efficiencies) would affect protein labelling in these experiments.

Differences in RNA stability are thought to make the major contribution to establishing the different steady-states of HeLa cell mitochondrial L-strand encoded transcripts. Although there is some evidence to suggest that transcriptional attenuation may take place at the 16S rRNA/ $tRNA^{leu}$ boundary (discussed above), it has been reported that most of the L-strand is transcribed as giant polycistronic precursor transcript, with all but one of the regions (7S RNA) being rapidly turned over (Aloni and Attardi, 1977; Cantatore and Attardi, 1982). 7S RNA has a half-life comparable with an H-strand encoded mRNA (Gelfand and Attardi, 1981), which together with the higher rate of transcription of the L-strand compared to the H-strand (Fisher and Clayton, 1988), make 7S RNA the most abundant polyadenylated transcript in HeLa cell mitochondria.

There may be some form of coupling between RNA degradation and RNA synthesis or processing in HeLa cells, since there is a disparity in the observed RNA half-lives when measured by incorporation kinetics or decay of labelled RNA after blocking of transcription: in experiments in which RNA synthesis is inhibited, the RNA is more stable (Gelfand

and Attardi, 1981).

Differences in RNA half-lives may be the result either of transcript structure (for example hairpin loops or other secondary structures which may protect against nucleases, or alternatively act as target sites for nuclease action), or of sequence-specific trans-acting factors protecting RNAs from degradation by nucleases. It has been suggested that the protection from ribonucleases of mitochondrial rRNA transcripts by ribosomal proteins, and mRNA transcripts by ribosomes, may be important in determining the differential levels of rRNA and mRNA (Jacobs, 1989). This view is supported by the observation that puromycin (which disrupts ribosomes) increases the rate of mitochondrial mRNA degradation, unlike chloramphenicol (which does not disrupt ribosomes) (Avadhani, 1979); and also by the observation that an increased rate of rRNA turnover was associated with a failure to form mitochondrial polyribosomes in ionophore activated sea urchin eggs (Craig and Innes, 1978). In contrast Gelfand and Attardi (1981) found that the half-lives of partially purified HeLa cell mitochondrial polysomal mRNAs were similar to those of total mitochondrial mRNA, although the half-life of mitochondrial polyribosomal rRNA (8-9 hours) was longer than the half-life of total mitochondrial rRNA (2.5-3.5 hours).

1.6 Regulation of mitochondrial RNA levels in fungi

1.6.1 Transcriptional regulation

In contrast to those animal systems described above, the mitochondrial genome of *Saccharomyces cerevisiae* has been shown to have at least 20 sites of transcriptional initiation (Fig. 1.2), both by *in vitro* capping experiments (Edwards *et al*, 1983; Levens *et al*, 1981a, 1982; Christianson and Rabinowitz, 1983), and by transcriptional studies in *rho*$^-$ mutants. *Rho*$^-$ mutants are respiratory deficient petite mutants with very large deletions in mitochondrial DNA. Only those mutants which contained a promoter mapped by capping experiments supported mitochondrial transcription (Christianson *et al*, 1983; Thalenfield *et al*, 1983; Locker and Rabinowitz, 1981).

All of the *S. cerevisiae* mitochondrial transcription

units are polycistronic. These transcription units contain different functional classes of RNA, and the small and large rRNAs are in different transcription units (Fig. 1.2). The frequency of transcriptional initiation plays a major role in regulating mitochondrial gene expression in S. cerevisiae, with the relative strength of promoters varying up to 20-fold depending on the sequences in and around the promoter (Mueller and Getz, 1986).

Like in HeLa cell mitochondria, transcriptional attenuation may be used as a means of regulating gene expression in S. cerevisiae, since pulse-labelling experiments have shown a dramatic decrease (up to 17-fold) in the labelling of promoter distal transcripts, in some of the transcription units (Mueller and Getz, 1986). The combination of different promoter strengths and transcriptional attenuation produces differences in the rate of transcription which vary over a 50-fold range (Attardi and Schatz, 1988).

Similarly there seem to be multiple transcription units, containing several genes, in filamentous fungi such as Aspergillus nidulans (Dyson et al, 1989) and Neurospora crassa (Kennell and Lambowitz, 1989). In Neurospora crassa, the small and large rRNAs have been shown to be in different transcription units.

1.6.2. Post-transcriptional regulation

Differential RNA stability may be a further important determinant of steady-state RNA levels in yeast. For various transcripts, the rates of synthesis predicted from pulse-labelling experiments should lead to a 5-10 fold higher steady-state level of RNA than is actually found (Mueller and Getz, 1986).

50% of the ribonuclease activity found in yeast cells is in the mitochondria (Hoffman et al, 1986). In yeast mitochondria at least some proteins have been identified which protect specific messages from degradation. The best characterised of these is CPB1. Yeast strains with mutations in the gene encoding this protein lack a transcript for cyt b (Dieckman et al, 1984). CPB1 interacts with the 5' end of the cyt b message and may either be an endonuclease responsible for 5' processing of cyt b from its polycistronic precursor

(Fig. 1.2); and/or protect cyt b mRNA, or its precursors, from degradation (Grivell, 1989b). Of these two possibilities the latter is more likely, since the CPB1 protein interacts with the leader of the cyt b message far downstream from its 5' processing site (Dobres et al, 1985; Dieckmann and Mittelmeier, 1987).

1.7. Regulation of mitochondrial RNA levels in higher plants

Very little is known about how plant mitochondrial RNA levels are regulated (Levings and Brown, 1989), in comparison with knowledge of animal and fungal systems.

Many plant mitochondrial genes have multiple transcripts of varying sizes. In vitro capping experiments have shown that these mainly arise as a result of multiple sites of transcriptional initiation, rather than multiple processing pathways. For example, in maize there are 3 initiation sites upstream both of the cyt b and COIII genes, and as many as 6 upstream of the ATPase subunit 9 gene (Mulligan et al, 1988a, 1988b). In contrast, the maize 26S rRNA is transcribed from a single promoter, and the 18S and 5S rRNA genes (which are adjacent) are co-transcribed from a single promoter.

The fact that there are no apparent consensus sequences involved in the promoting plant mitochondrial transcriptional initiation, even within species, may indicate either multiple mitochondrial RNA polymerase molecules with different specificities, or that mitochondrial RNA polymerase initiation sites may be distant from their promoters. Alternatively, it has been suggested that plant mitochondrial RNA polymerase may not require a specific consensus sequence in order to initiate transcription (Mulligan et al, 1988b; Isaac et al, 1985; Young et al, 1985).

In contrast to the 5' termini of plant mitochondrial transcripts, there are usually single 3' termini for transcripts containing each gene. There are some primary and secondary structure motifs at the 3' ends of genes, but whether these correspond to sites of transcriptional termination, RNA processing, or some other function is not clear (Schuster et al, 1986).

1.8 The enzymology of mitochondrial transcription

Mitochondrial RNA polymerases have been purified and characterised from human (Chang and Clayton, 1984; Fisher and Clayton, 1985; Fisher *et al*, 1987), *Xenopus* (Bogenhagen and Insdorf, 1988) and yeast mitochondria (Levens and Howley, 1985; Winkley *et al*, 1985; Schinkel *et al*, 1986). These mitochondrial RNA polymerases appear to be complexes of two polypeptides: a core polymerase, which is the enzymatic moiety but is either weakly selective or nonselective for its promoter, and initiates randomly on DNA; and a specificity subunit without any polymerase activity, but required for specific initiation of RNA synthesis.

The above mentioned mitochondrial RNA polymerases transcribe random DNA sequences at a high level. This has led to the suggestion that mitochondrial RNA polymerases might require less inherent specificity compared with nuclear RNA polymerases, because of the low sequence complexity of mitochondrial DNA.

There is evidence, at least in yeast, for the existence of more than one kind of specifity subunit, since factors which have been biochemically purified by different groups have different properties. In one case a 70 kD polypeptide was purified, which binds specifically to the mitochondrial promoter sequence in the absence of polymerase (Ticho and Getz, 1988), whereas in the other a specifity factor of 43 kD was isolated, which did not interact with DNA in the absence of polymerase (Schinkel *et al*, 1987, 1989).

The specificity subunit of *Xenopus* mitochondrial RNA polymerase holoenzyme (Bogenhagen and Insdorf, 1988) resembles the 43 kD yeast specifity subunit (Schinkel *et al* 1987, 1989), in that it interacts directly with the polymerase, rather than with DNA. These two specificity subunits might interact with their respective core polymerases either prior to, or at the same time as interacting with DNA. There is some evidence for the latter possibility in the case of the yeast 43 kD specificity subunit (Schinkel *et al*, 1987),

In contrast, there is evidence that the specificity subunits of human and mouse mitochondrial RNA polymerase, which have been termed human and murine mtTF1 respectively, are similar to each other but differ from the yeast and

Xenopus specificity subunits. Both human and murine mtTF1 have been biochemically purified and characterised (Fisher and Clayton, 1985, 1988; Fisher et al, 1987, 1989). They have been shown to interact with specific DNA binding sites independently of the core polymerase (see section 1.9), and subsequently interact with the core polymerase to give the mitochondrial RNA polymerase holoenzyme. mtTF1 is probably responsible for promoter recognition and selection to provide pre-initiation complexes for the relatively nonselective RNA polymerase core enzyme to bind (Fisher and Clayton, 1988).

The gene encoding the yeast mitochondrial RNA polymerase has been cloned and sequenced (Kelly et al, 1986; Masters et al, 1987). It shows blocks of sequence similarity with the phage T3, T7 and SP6 RNA polymerases, particularly at the carboxyl terminus of the protein, which in the phage polymerases is involved in promoter recognition and catalysis. This suggests that functions related to promoter recognition and catalysis may be conserved between the phage and mitochondrial RNA polymerases, whereas the stability of the promoter complex (which involves the amino terminus in the phage enzymes) may be achieved by a different mechanism (Masters et al, 1987). A common feature of both the mitochondrial polymerases and these phage polymerases is that they provide primers for replication of their template genomes as well as transcripts (Schinkel and Tabak, 1989). The ancestral form of both these enzymes may have provided such a dual function before the evolution of specific primase enzymes.

1.9 Template requirements for mitochondrial transcription

Mitochondrial DNA transformation systems have recently been developed (Johnston et al, 1988), along with techniques to re-introduce isolated mitochondria into tissue-culture cells (King and Attardi, 1988), which should allow experiments to examine the template requirements for mitochondrial transcription in vivo. No data from these sorts of experiment have been published yet. For this reason, most of the detailed information on the template requirements for mitochondrial transcription has come from in vitro transcription systems.

Characterised mitochondrial promoter sequences are species-specific, and do not generally support high levels of transcription *in vitro* with a heterologous polymerase (Chang *et al*, 1985). For this reason, it has been necessary to develop homologous transcription systems in order to study the template requirements for mitochondrial transcription in different organisms. These studies have revealed both similarities and differences in the template requirements for mitochondrial transcription between species.

1.9.1 Animal mitochondrial promoters

Accurate transcription from the upstream H-strand initiation site (Section 1.5.1) and from the L-strand initiation site can be reproduced *in vitro*, using purified HeLa cell mitochondrial RNA polymerase in a run-off transcription assay on a cloned mitochondrial DNA template (Walberg and Clayton, 1983; Chang and Clayton, 1984; Bogenhagen *et al*, 1984; Shuey and Attardi, 1985). Both of these initiation sites are weakly bidirectional (Chang *et al*, 1986). Combined with *in vitro* mutagenesis and footprinting experiments, these *in vitro* run-off transcription experiments have shown that both the light strand promoter (LSP) and the heavy strand promoter (HSP, which directs transcriptional initiation at the upstream H-strand initiation site), can be organised into 2 functional domains, each contained within about 50 nucleotides (Topper and Clayton, 1989; Fig.1.4). Firstly an 8 nucleotide sequence, present at each of the initiation sites, is responsible for interacting with the polymerase and specifying the correct site for transcriptional initiation. This is referred to as the core promoter since it can support accurate transcriptional initiation *in vitro* alone in the case of the HSP (but not the LSP). Secondly, sequences from 10 to 40 nucleotides upstream of the transcriptional initiation sites are required for efficient transcription (Chang and Clayton, 1984; Bogenhagen *et al*, 1984; Hixson and Clayton, 1985; Topper and Clayton, 1989). These upstream sequences contain the binding sites for the human mitochondrial RNA polymerase specificity subunit (mtTF1) described above (Section 1.8).

The mtTF1 binding sites are arranged in direct repeat,

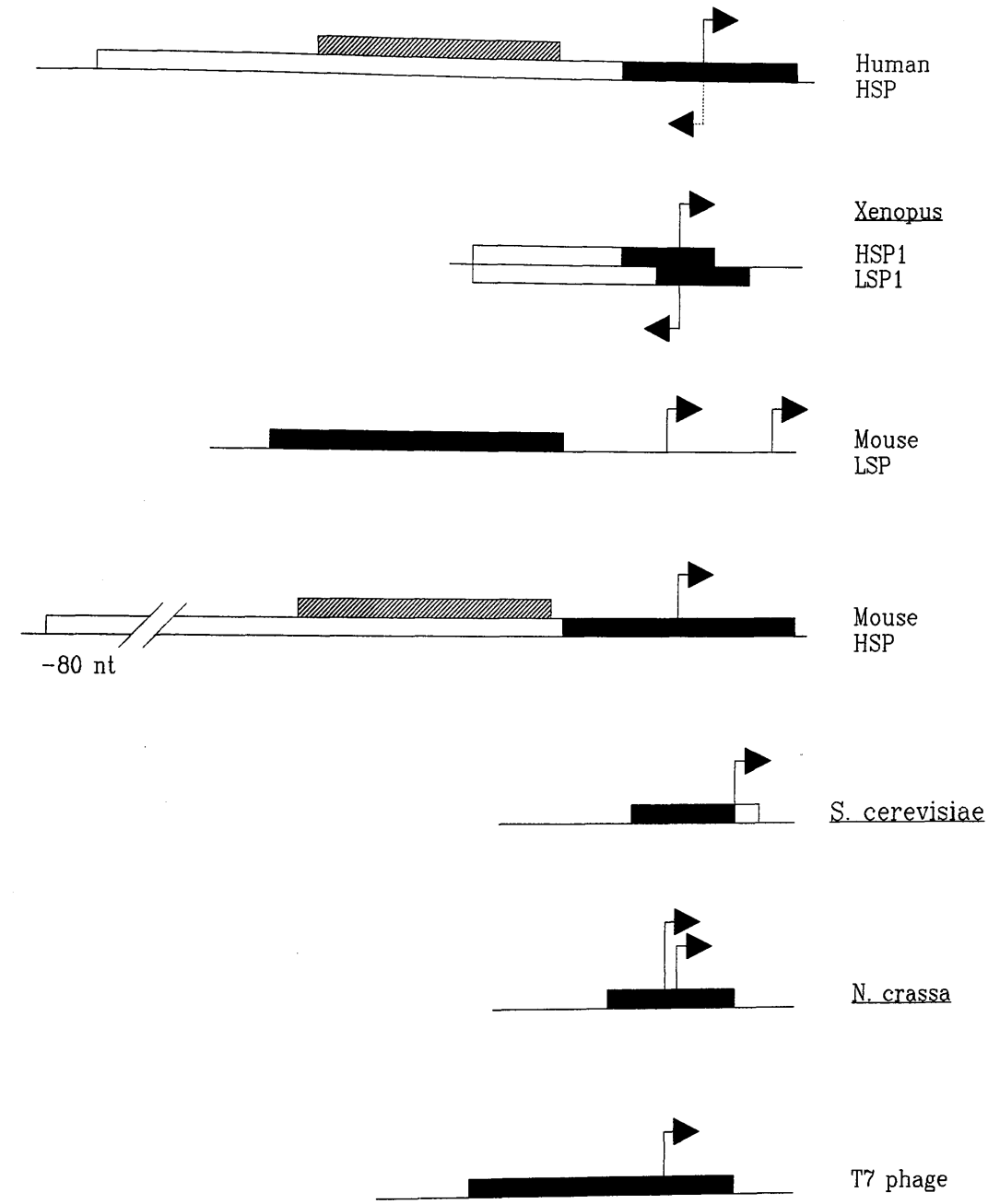


Figure 1.4: Organisation of mitochondrial promoters.

and so oppositely orientated with regard to the direction of transciption from the HSP and LSP. The HSP binds mtTF1 less strongly than the LSP *in vitro*, which may explain why it is a less active promoter *in vivo* (Fisher and Clayton, 1988). Several lines of evidence suggest that the existence of separate promoters for each strand of HeLa cell mitochondrial DNA may have occurred as a result of the duplication of a single bidirectional promoter which was responsible for the transcription of both strands. These are the observation that mtTF1 binding sites are in direct repeat, that a single mtTF1 binding site can activate two bidirectional promoters (Topper and Clayton, 1989), that both of the existing human mitochondrial promoters are weakly bidirectional (Chang *et al*, 1986), and that there are some non-functional elements with limited homology to the HSP and the LSP within the D-loop. The evolution of two promoters may have been appropriate, since the LSP and the HSP have distinct physiological roles- the HSP is responsible for transcript production for protein synthesis, whereas the LSP, in addition to transcript production, promotes transcription of the primer for leading-strand DNA replication (Chang and Clayton, 1985; Chang *et al*, 1987).

The apparent similarity in the architecture of the human HSP and LSP may be deceptive, since alterations at apparently equivalent positions in their core promoter, and in the upstream regions, have different effects on the rate of mitochondrial transcription *in vitro*. A basal level of transcription occurs from the core promoter alone in the case of the HSP, but not the LSP, and whereas linker-scanning mutations of the upstream region of the HSP all result in the same level of basal transcription *in vitro*, similar mutations in the LSP support a range of *in vitro* transcriptional levels (Topper and Clayton, 1989). Transcription from the LSP and HSP show different responses to changes in ATP concentration and the ratio of template to polymerase preparation *in vitro* (Narasimhan and Attardi, 1987). These observations suggest that the promoters interact differently with the identified transcription machinery; or that they interact with each other; or that there are other, promoter-specific, transcription factors involved which remain to be identified at the molecular level.

The mouse mitochondrial promoters are unusual in that

there is no homology between the HSP and the LSP, other than that they have a TA dinucleotide at the initiation site (Chang and Clayton, 1986a). The light strand promoter is similar in organisation to the HeLa cell mitochondrial promoters, with a 19 bp core promoter, and an upstream region required for efficient transcription (Fig. 1.4; Chang and Clayton, 1986b). The latter contains the binding site for murine mtTF (Section 1.8; Fisher et al, 1989). The mouse mitochondrial HSP is unusual in that it promotes transcriptional initiation at sites 9 and 18 nucleotides downstream of itself (1 and 2 turns of the helix) (Chang and Clayton, 1986c). Deletion analyses have shown no apparent requirement for flanking sequences for mouse HSP function in vitro. It is not known whether the same protein complex is responsible for initiating transcription at both the mouse LSP and HSP. Since the initiation events at the two sites showed different efficiencies in vitro different proteins may be involved.

The mitochondrial genome of Xenopus laevis contains two fully bidirectional promoters, and one unidirectional promoter which is responsible for initiating transcription of the light strand only (Fig. 1.4; Bogenhagen and Yoza, 1986; Bogenhagen et al, 1986a; Bogenhagen and Insdorf, 1988). Like the human mitochondrial promoters and the mouse LSP, the Xenopus mitochondrial promoters can be dissected into two functional regions: a core octanucleotide sequence necessary for a basal level of transcription, and regions of flanking DNA required for efficient transciption. Two of the core octanucleotide sequences are repeated in opposite orientations in the bidirectional promoters and partially overlap (Bogenhagen and Yoza, 1986; Figure 1.4). Flanking sequences seem to be less important for Xenopus mitochondrial transcription in vitro than for the human mitochondrial promoters or the mouse LSP, perhaps since its specificity factor binds the polymerase directly, like a bacterial sigma subunit, rather than binding to upstream elements (Bogenhagen and Romanelli, 1988; Section 1.8).

1.9.2 Fungal mitochondrial promoters

In yeast mitochondria, deletion analyses in conjunction with in vitro transcription reactions have identified a

nonanucleotide sequence, ATATAAGTA, which is required for transcription (Osinga et al, 1981; Christianson and Rabinowitz, 1983; Winkley et al, 1985; Schinkel et al, 1986; Fig. 1.4). Unlike most animal mitochondrial promoters, yeast mitochondrial promoters are not bidirectional. Sequences outside this nonanucleotide consensus probably contribute to promoter activity since there are several copies of this nonanucleotide sequence in the yeast genome for which no sites of transcriptional initiation can be detected by in vitro capping reactions. However, the failure to detect substrates for capping reactions at these sites could result from rapid RNA processing (Grivell, 1989b). Transcriptional efficiency in vitro is affected by the nucleotide 2 bases downstream of the initiation site, where strong and weak promoters can be differentiated by their ability to initiate transcription with limiting concentrations of RNA polymerase (Marczynski et al, 1989). This also seems to be the case in vivo. For example, the gene for ATPase 9 has 2 promoters, a weak one with a pyrimidine in this position, and a stronger one, located 78 nucleotides upstream, with a purine at this position (Biswas et al, 1985; Biswas and Getz, 1986a, 1986b, 1987). The role of other flanking nucleotides remains uncharacterised.

Recently, an in vitro transcription system has been set up for Neurospora crassa mitochondria (Kennel and Lambowitz, 1989). The template requirements have not been well characterised, but transcription initiates within a 15 nucleotide consensus sequence (Fig. 1.4). As in yeast, transcription of a single gene may initiate from several alternative sites, giving rise to multiple transcripts for a single gene (Kennel and Lambowitz, 1989).

Yeast mitochondrial RNA polymerase shows some similarity to those of T3, T7 and SP6 bacteriophages (section 1.8). The promoters of these bacteriophages consist only of an essential core region, which contains the initiation site, and there is no requirement for flanking sequences (Fig. 1.4) (Schinkel and Tabak, 1989). These phage promoters show no apparent nucleotide sequence similarity with the yeast mitochondrial promoter.

1.10. Mitochondrial DNA/protein interactions

Mitochondrial DNA protein interactions have been most thoroughly studied in S. cerevisiae. DNA bending is thought to occur when RNA polymerase binds to the promoter, since there is a correlation between the yield from run-off in vitro transcription reactions, the level of RNA polymerase binding (as measured by footprinting) and the level of DNA bending (measured by gel retardation) (Schinkel et al, 1988). In one case, transcriptional initiation in vitro can take place downstream of the large rRNA promoter independently of the mitochondrial polymerase specificity subunit, at an $(A)_5$ tract. (A)-rich tracts have been associated with DNA bending. The specificity subunit may act at other sites to produce, or stabilise, a bend in DNA.

This requirement for DNA bending may be general for mitochondrial promoters: runs of adenine residues follow the Xenopus mitochondrial promoters (Bogenhagen and Romanelli, 1988), and an in vitro transcription system for Neurospora crassa mitochondrial DNA has shown that the consensus sites for transcriptional initiation are preceded by a 15-28 nucleotide (A+T)-rich region, and then a (G+C)-rich hairpin structure (Kennel and Lambowitz, 1989). However, deletion of the (G+C)-rich region had no affect on the level of initiation in vitro, although the role of the (A+T)-rich region has not been examined.

Limited elongation of the nascent RNA chain in vitro, achieved by carrying out transcriptional initiation in a reaction with a limiting concentration of a nucleotide, in conjunction with DNase mapping, has shown that the polymerase-induced footprint becomes extended 3' from the transcriptional initiation site for about 8 nucleotides whilst its 5' border remains fixed. This suggests that some change in DNA structure or polymerase conformation on initiation of RNA chain elongation (Schinkel et al, 1988a; 1988b). Similar changes in footprint are seen for T7 RNA polymerase, but not that of E. coli (Schinkel and Tabak, 1989).

1.11 RNA processing

A number of processing events are required to generate

mature transcripts from nascent mitochondrial RNA. There are 6 documented types of RNA processing: endonucleolytic cleavage, polyadenylation, CCA addition to tRNA molecules, base modification, RNA editing and splicing.

1.11.1 Endonucleolytic cutting

Mitochondrial transcription units are often bicistronic or polycistronic, which means that the primary transcript has to be endonucleolytically cleaved to generate mature transcripts (where these contain the coding information from single genes). The stage at which cleavage occurs in the sequence of processing events is not known. In human cells, the processing of mitochondrial H-strand encoded transcripts is likely to occur concomitantly with transcription, since genome sized H-strand transcripts cannot be visualised either in steady-state RNA (Ojala et al, 1980; Amalric et al, 1978), or in nascent transcription complexes (Cantatore and Attardi, 1982). On the other hand, genome-sized L-strand transcripts can be visualised. Other processing events may occur prior to endonucleolytic cleavage, since endonucleolytic processing of the hamster rRNA precursor is dependent on base modification (methylation) of the RNA (Dubin et al, 1985; Prince et al, 1986).

Transfer RNA sequences within primary transcripts are thought to be of central importance in acting as recognition signals for the processing machinery, presumably on the basis of their secondary structure. Transfer RNA genes are dispersed around the vertebrate genome in strategic locations between other genes (Anderson et al, 1981), and fine transcript mapping has shown that the 5' and 3' termini of several mRNAs as well as those of both rRNAs directly abut tRNA genes (Montoya et al, 1981; Battey and Clayton, 1980; Eperon et al, 1980; Ojala et al, 1980; Crews and Attardi, 1980). This may also be the case in Neurospora crassa, since the endpoints of the most abundant transcripts coincide with those of tRNA sequences (de Vries et al, 1986; Burger et al, 1985; Breitenburger et al, 1985). In contrast, tRNA excision in yeast does not concomitantly generate mature mRNA ends, and so additional cleavage events are nearly always required (Tzagaloff and Myers, 1986).

Transfer RNA genes are not found at the junctions

between all mitochondrial mRNA/rRNA genes, and therefore other processing activities are required besides those which recognise tRNA structure. For example, only 6 out of 12 mammalian mitochondrial mRNA sequences can be completely excised by tRNA excision . It has been suggested that the termini of these mRNAs may fold into tRNA-like structures, which can be recognised by the same processing machinery that excises tRNAs (Attardi et al, 1982; Ojala et al, 1981b). However, there is evidence that different enzymatic activities are responsible. In a comparison of steady-state and newly synthesised RNA in digitonin-treated mouse mitochondria, the processing of RNA precursors in which tRNAs demarcate mature transcripts was shown to be more rapid than for those which lack juxtaposed tRNAs, suggesting that there were at least two distinct types of processing activity present (Bhat et al, 1984).

In Neurospora crassa mitochondrial DNA, tRNA genes are more clustered than in mammalian mitochondrial DNA, so in many cases are not present between other genes. At these junctions there are complex hypothetical RNA secondary structures, which appear to correspond with sites of endonucleolytic cleavage. These sites are located near the beginning of base-paired palindromic sequences, and, to a lesser extent, near bulges in base paired regions (Agsteribbe and Hartog, 1987). Both these types of site are associated with RNA processing in E. coli, where different types of RNase are responsible. However, in contrast, in Aspergillus nidulans there is no evidence that transcript junctions not demarcated by tRNAs have any tRNA-like secondary structure (Dyson et al, 1989). At the 3' terminus of many mature yeast mRNAs is the sequence AUUAUUAUUCUU, which may act as a general signal for a 3' processing endonuclease (Zhu et al, 1987).

Experiments have been carried out to analyse endonucleolytic processing in vitro. An endoribonuclease activity has been isolated from HeLa cells, which can cleave the end of a bacterial tRNA precursor to give its mature 5' end (Doersen et al, 1985; Attardi et al, 1985). Like other RNase P enzymes (Altman and Smith, 1971) it has an RNA component. In vitro this mitochondrial RNAse P-like enzyme does not cleave a mitochondrial tRNA precursor, so it is possible that some other factor may be required for

mitochondrial tRNA excision, which is supplied by the bacterial tRNA precursor. Mitochondrial tRNAs lack some conserved features of their cytosolic counterparts, which may be important for processing. An RNase P-like enzyme, which cuts at the 5' termini of mitochondrial tRNA precursors, has also been isolated from yeast (Martin and Underbrink-Lyon, 1981). Like the animal mitochondrial RNase P, this is a ribonucleoprotein, but its RNA component is mitochondrially encoded (Miller and Martin, 1983). A processing activity which cuts 3' to tRNA sequences has been partially purified from yeast mitochondria; this might also be the enzyme responsible for CCA addition to tRNAs (Martin et al, 1989).

An RNA processing activity (RNase MRP) has been purified from mouse L-cell mitochondria. This cleaves the RNA which is thought to act as a primer for H-strand DNA synthesis. The enzyme introduces an endonucleolytic cleavage near the transition site for RNA to DNA synthesis (Chang and Clayton, 1987a; 1987b; Chang et al, 1987). Like RNase P, RNase MRP (Mitochondrial RNA Processing) contains an RNA component, which is encoded by the nucleus (Chang and Clayton, 1989).

1.11.2 Polyadenylation

Animal mitochondrial mRNAs are not capped (Grohman et al, 1978), but have poly(A) tails reported as being 55-60 nucleotides long (Battey and Clayton, 1980; Ojala et al, 1980, 1981b; Hirsch and Penman 1973; Ojala and Attardi, 1974). In mouse L-cell (Van Etten et al, 1983) and HeLa cell (Dubin et al, 1982) mitochondria 16S rRNA is oligoadenylated with 1-8 (A) residues added, and 12S rRNA is monoadenylated. In contrast, yeast mitochondrial RNAs are not polyadenylated (Groot et al, 1974).

Polyadenylation has two known functional roles in mitochondria. Firstly, it creates several translational stop codons, which in many animal mitochondrial messages are not genomically-encoded. In these cases, the mitochondrial gene ends in a U or UA, so that polyadenylation creates a UAA stop codon (Anderson et al, 1981, 1982).

Secondly, polyadenylation may also play a role in stabilising mitochondrial transcripts from exonucleolytic degradation in vivo. Measurements of the changes in size of

RNase A-resistant mRNA (i.e. the poly(A) tracts), in a pulse-chase experiment in Ehrlich ascites mitochondria, showed that poly(A) tails shortened with age. Ethidium bromide and puromycin, which accelerated the decay of mRNA, also enhanced the decay of poly(A) tails (Avadhani, 1979). Trypanosomes, which only have functional mitochondria for part of their life cycle, show differential poladenylation of mitochondrial transcripts over the course of development. A larger fraction of the transcripts are polyadenylated in the procyclic form of trypanosomes, present in the Tsetse fly, than in the forms of trypanosomes found in the mammalian bloodsteam form, which lack functional mitochondria (Stuart, 1987).

Poly(A) shortening also seems to be associated with nuclear RNA decay. For example, poly(A) tail shortening seems to be the first step in the decay of c-myc mRNA (Brewer and Ross, 1988), and globin mRNAs with poly(A) tails of less than 30 nucleotides were unstable when injected into Xenopus oocytes (Nudel et al, 1976). Poly(A) binding protein seems to be the important factor in blocking ribonuclease action: short poly(A) tails may not be stabilised by this protein, since it requires around 25 nucleotides for binding, and the level of protection from ribonuclease action may depend on the number of poly(A) binding proteins bound by a message (Bernstein et al, 1989).

However, the mechanism of mitochondrial polyadenylation is probably distinct from that in the nucleus, since mitochondrial poly(A) polymerases are different from nuclear poly(A) polymerases (Rose et al 1980), and there seems to be no polyadenylation sequence similar to that found in nuclear transcripts (Montell et al, 1983). There is the possibility that all mitochondrial RNA 3' ends are substrates for polyadenylation. For example, in addition to the mature messages of HeLa cell mitochondria, the excised 5' leader sequence of COI mRNA, which contains the antisense of 4 tRNAs, is polyadenylated. Conversely, it is also possible that the site of transcriptional initiation determines whether or not a transcript is polyadenylated, since in HeLa cells the putative rRNA precursors are not polyadenylated whereas the transcripts which have been proposed to arise as a result of linking the promoter with the mRNA genes are polyadenylated (Montoya et al, 1983; Attardi et al, 1985)

(section 1.4.1). Polyadenylation may be distinct from the endonucleolytic cleavage machinery since in HeLa cells polyadenylation, but not cleavage, is inefficient in isolated mitochondria as compared with the levels observed in intact cells, and can be independently stimulated by low temperatures or the addition of ATP (Gaines et al, 1987a).

1.11.3 CCA addition and base modification

Transfer RNAs are modified by CCA addition at their 3' end, and also by base modification to give methylated residues, pseudouridines, etc. (Clayton, 1984). Mammalian mitochondria have only one $tRNA^{met}$ which may be differentially modified in order to give a $tRNA^{met}_f$ for the initiation of translation, and a $tRNA^{met}_m$ to decode internal AUG codons. As discussed above (section 1.11), RNA is modified by methylation prior to endonucleolytic cleavage.

1.11.4 RNA editing

RNA editing may be described as any process, other than classical splicing, which results in an RNA molecule which has a different nucleotide sequence from its template DNA (Simpson and Shaw, 1989). RNA editing was first described in trypanosome mitochondria, where it involved the non-templated addition and/or removal of uridine residues from a variety of transcripts (Benne et al, 1986). RNA editing may have a functional role in Trypanosome mitochondrial translation, the existence of which is supported circumstantially (Shaw et al, 1989). RNA editing may act as a translational control mechanism to create AUG initiation codons (Feagin et al, 1987; Shaw et al, 1988); to create open reading frames either from frameshifts (Benne et al, 1986) or from cryptic, genomically encoded, copies of the gene (Feagin et al, 1988); or to create coding information for new amino acids, which may be important for protein stability and function. RNA editing may also be involved in controlling the developmental specificity of mitochondrial gene expression: respiratory activity is only found in the procyclic, but not the bloodstream form, of trypanosomes (Stuart, 1987).

RNA editing by (C) insertion has been described for mitochondrial RNA of the slime mould Physarum polycephalum

(see Simpson and Shaw, 1989). RNA editing has also been described in plant mitochondria, involving the conversion of cytidine to uridine residues in wheat (Covello and Gray, 1989; Gualberto et al, 1989), and in Oenothera (Schuster et al, 1990) leading to a change in the amino acid encoded. This type of editing does not involve nucleotide addition, and may result from base modification of existing nucleotide residues, in this case a deamination reaction. A similar phenomenon has been reported in a nuclear-encoded RNA, alipoprotein B mRNA, where there is a tissue specific cytidine to uridine conversion resulting in the formation of an in-frame stop codon and a truncated (Powell et al, 1987) protein containing only the N-terminal.

To date, there are no reports of RNA editing in animal mitochondria, although it has been suggested that the creation of stop codons by polyadenylation represents RNA editing in that it creates functional messages (Attardi and Schatz, 1988). The post-transcriptional nucleotide modifications of tRNA molecules might also be regarded as tenuous examples of RNA editing.

1.11.5 RNA Splicing

There is no evidence that splicing takes place in animal mitochondria. However, three S. cerevisiae mitochondrial genes contain multiple introns. Although many of these introns are capable of self-splicing in vitro, splicing in the cell seems to be a complex process, involving both proteins encoded by at least some of the introns (known as maturases), and a number of nuclear-encoded proteins (Grivell et al, 1989b). The fact that introns can self-splice in vitro suggests that the RNA itself is functionally important for the reaction. The role of proteins in these reactions is not well characterised. A yeast nuclear gene product involved in mitochondrial RNA splicing has been shown to be aminoacyl tRNA synthetase (Labouesse et al, 1987). This is also true of a Neurospora gene involved in mitochondrial RNA splicing (Akins and Lambowitz, 1987). In another case, a yeast nuclear gene involved in mitochondrial RNA splicing has been shown to have homology to an RNA unwindase (Seraphin et al, 1989).

Introns have also been found in the mitochondrial genomes of Aspergillus nidulans and Neurospora crassa, and some

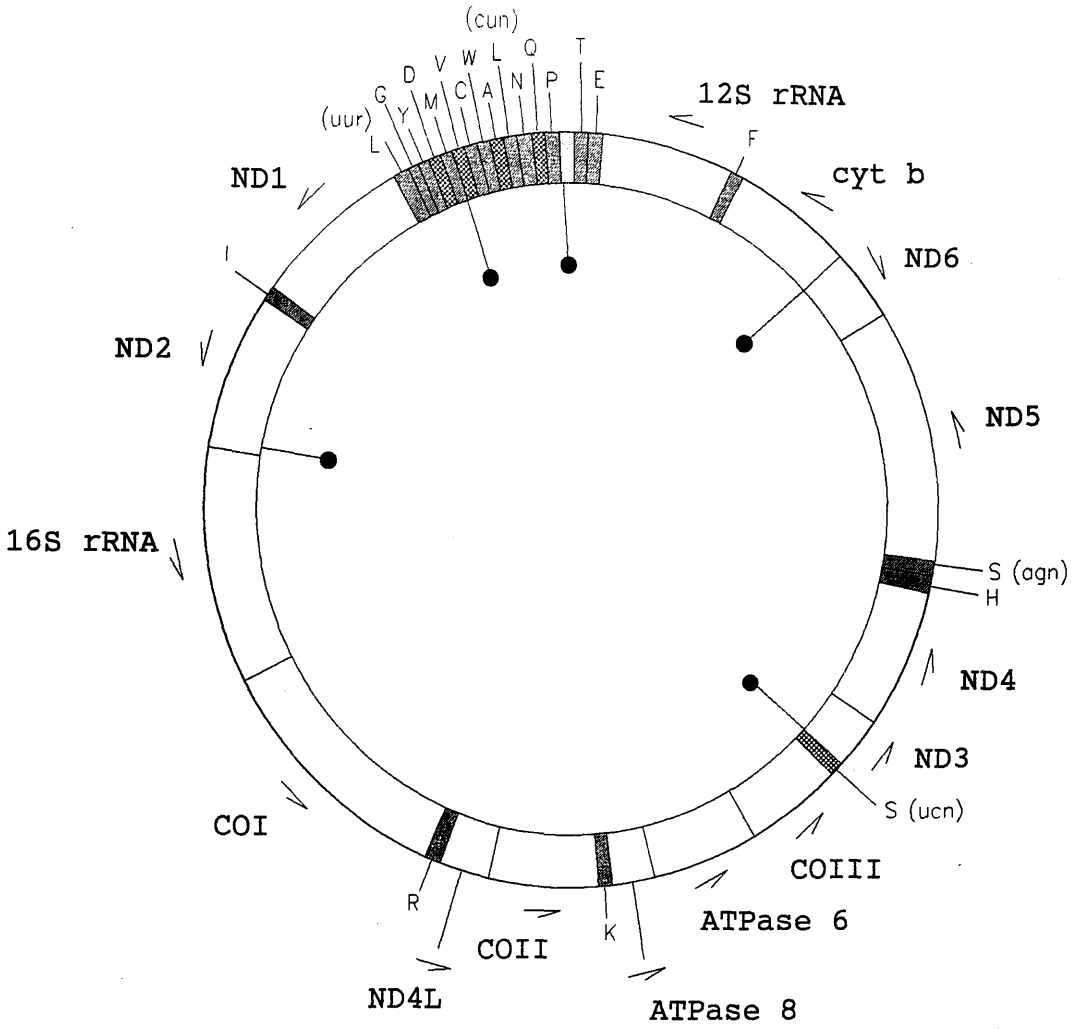
plant mitochondrial genomes (Levings and Brown, 1988; Fox and Leaver, 1981).

1.12 Sea urchin mitochondrial gene expression

The sea urchin mitochondrial genome contains the coding information for the same structural genes found in other characterised metazoan mitochondrial genomes, and a single major intergenic region containing a short D-loop (see Table 1.1).

The sea urchin mitochondrial genome shows two organisational differences from vertebrate mitochondrial genomes, which have important implications for mitochondrial gene expression, and thus make the sea urchin an interesting organism for studies of this kind (Fig. 1.5). Firstly, the genes for the rRNAs are separated by the genes encoding ND1 and ND2, 15 tRNA genes and the unassigned sequence region. This organisation precludes a simple transcriptional attenuation model for differential expression of the rRNAs and the mRNAs/tRNAs, as has been proposed for vertebrate mitochondria (section 1.4.1). However, there is evidence that in sea urchin mitochondria, as in other organisms, the rRNAs are expressed at a higher level than the mRNAs (Ruderman and Schmidt, 1981). If the single major intergenic region in sea urchin mitochondrial DNA contains the promoter region for the whole genome, as in vertebrate mitochondrial DNA, then 12S rRNA would be the last gene in the H-strand transcription unit to be transcribed, and differences in RNA levels would have to be determined post-transcriptionally. Alternatively, there might be multiple transcription units, perhaps combined with transcriptional attenuation. In this case it would be possible for a higher level of synthesis of rRNAs relative to mRNAs to be achieved at the transcriptional level, e.g. with promoter elements upstream of the rRNA genes, and transcriptional attenuation limiting readthrough of RNA polymerase into the downstream genes.

Secondly, the tRNA genes of the sea urchin are more clustered than in vertebrate mitochondrial DNA, with 15 out of 22 of them being located in a cluster around the major intergenic region. For this reason, tRNAs probably make a proportionally smaller contribution as signals for endonucleolytic processing than those in vertebrate



TTATATATAA–like sequences

tRNA genes (major coding strand)

tRNA genes (minor coding strand)

Major intergenic sequence

rRNA/protein–coding genes

transcriptional direction

Figure 1.5: Organisation of the mitochondrial genome of Strongylocentrotus purpuratus.

mitochondria (section 1.11.1).

An understanding of the strategies used to control mitochondrial gene expression in a number of different organisms might give an indication about the selective pressures leading to their evolution.

In addition, the sea urchin is a model system for studying early development. This is because it is possible to obtain large amounts of biological material, and synchronous cultures of developing embryos are easy to set up, in which embryogenesis proceeds at a fixed rate, can be monitored microscopically, and is subject to experimental manipulation by a wide variety of procedures (Czihak, 1984). It is of interest to see how mitochondrial gene expression is regulated in early development, since work on mitochondrial gene expression in animals has largely been confined to tissue-culture cells, comprising of only a single cell-type, which is not necessarily maintained in physiologically relevant conditions. Some studies of the developmental control of mitochondrial gene expression have recently been done on Xenopus (El Meziane et al, 1989), mice (Piko and Taylor, 1987), and rats (Gadaleta et al, 1985), and some work has been done on the response of mitochondrial gene expression to different respiratory demands in mammalian muscles and yeast (Williams, 1986; Williams et al, 1986; Mueller and Getz, 1986). Preliminary work has suggested that mitochondrial gene expression may be differentially regulated early in sea urchin development (Wells et al, 1982; Cabrera et al, 1983,).

A further advantage of the sea urchin as a model system is that the large quantities of biological material which can be obtained, make feasible the development of in vitro systems to study the biochemistry of the processes involved in mitochondrial gene expression.

1.13. Aims of the project

This project had four main aims:

(1) To help complete the sequencing of the genome, particularly regions of it which might be important in the control of gene expression, as part of a larger project which was going on in the laboratory (Jacobs et al, 1988).

(2) To examine the transcription pattern of the genome at different developmental time points, using RNA blot analyses, in order to get an insight into the mechanisms controlling developmental mitochondrial gene expression, and to quantitate differences in expression of rRNAs and mRNAs.

(3) To analyse transcript junctions, especially those of the rRNAs and their adjacent genes, in order to locate intergenic regions which might contain elements involved in mitochondrial gene expression. These experiments would also enable me to examine whether developmental changes in RNA levels could be associated with altered transcript termini, and, by inference, different synthetic pathways, at different developmental stages.

(4) To map transcriptional initiation sites and transcription units on the sea urchin mitochondrial genome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

The *E. coli* strains used were all derivatives of *E. coli* K-12, and are described in Table 2.1.

Table 2.1

Strain name	Genotype	Source
JM101	*sup*E, *thi*(*lac pro* A,B)/F' *Tra*D36, *pro*AB, *lac*I^{q}Z Δ M15	Yannisch-Perron *et al*, 1985
JM109	*rec*A1, *end* A1, *gyr*A96, *thi*, *hsd* R17, *Sup*E44, *rel*A1, λ^-, Δ(*lac pro*AB)[F'*Tra*D36*pro*AB *lac*I^{q}Z ΔM15]	Yannisch-Perron *et al* 1988
NM621	*hsd*R, *mcr*A, *mcr*b, *Sup*E44, *rec*D1009	Whittaker *et al*, 1988
JM83	*ara*, Δ(*lac pro*A,B), *rps*L, *hsd*R, ϕ80, *lac*Z ΔM15.	Vieira and Messing, 1982
DS941	*rec*F143, *thr*1, *leu*6, *thi*1, *lac*Y1, bX33, *sup*E44, *gal*K2, *ana*14, *xyl*5, mH1, *pro*A2, *his* 4, *argt*3, *str*31 F'(lacI^q)Z ΔM15	D.J. Sherratt (pers.comm.)

2.2 Plasmids and Bacteriophages.

Cloning vectors

pUC18 and pUC19: Both these plasmid vectors were used for cloning dsDNA (Norrander *et al*, 1983).

M13mp18 and mp19: Both these bacteriophage vectors were used for propagating ssDNA (Norrander *et al*, 1983).

Clone	Vector	Construction	Insert size (kb)	Type of clone	Gene content and orientation (strand synthesised by primer extension on M13 ssDNA template)	Reference
K34	M13mp8	EcoRI-SalI	0.6	Genomic subclone	Coding for cyt b	Elliott and Jacobs, 1989
Q4	M13mp19	SstI-XbaI	1.6	Genomic subclone	Coding for 12S rRNA, ND1,and tRNAs (E, T, P, N, L_{CUN}, L_{UUR}, W, C, M, Y, G). Noncoding for tRNAs (Q, A, V, D).	Elliott and Jacobs, 1989
T1	M13mp18	HindIII-XbaI	0.7	Genomic subclone	Coding for ND1 and tRNAs (M, Y, L_{UUR}, I). Noncoding for tRNA (D)	Elliott and Jacobs, 1989
T3	M13mp18	HindIII-XbaI	0.8	Genomic subclone	Coding for tRNA (I), ND1 and ND2	Elliott and Jacobs, 1989
ZH3A	M13mp19	SstI	2.4	Genomic subclone	Coding for COII, A8, A6, COIII. Noncoding for tRNA (K)	H.T. Jacobs, pers. comm.
N15	M13mp19	SstI-EcoRI	0.4	Genomic subclone	Noncoding for ND3, tRNA (S_{UCN}), COIII.	H.T. Jacobs, pers. comm.
J1	M13mp8	XhoI-EcoRI	0.6	Genomic subclone	Noncoding for ND6. Coding for cyt b	Elliott and Jacobs, 1989
N18	M13mp9	EcoRI-XhoI	0.6	Genomic subclone	Coding for ND6. Noncoding for cyt b	Elliott and Jacobs, 1989
R8	M13mp19	XbaI-HindIII	0.8	Genomic subclone	Coding for ND1 and ND2	Elliott and Jacobs, 1989
R6	M13mp19	XbaI-HindIII	0.7	Genomic subclone	Noncoding for ND1 and tRNAs (M, Y, G, L_{UUR}, I. Coding for tRNA (D).	Elliott and Jacobs, 1989
HP1	M13mp18	HindIII-PstI	0.7	Genomic subclone	Coding for 16S rRNA and COI	Chapter 4
HP2	M13mp19	HindIII-PstI	0.7	Genomic subclone	Noncoding for 16S rRNA and COI	Chapter 4
CD5	M13mp18	HindIII-SstI	0.8	Genomic subclone	Noncoding for 12S rRNA and tRNAs (E, T, P, N, L_{CUN}, L_{UUR}, W, C, M, Y and G). Coding for tRNAs (Q, A, V, D)	Elliott and Jacobs, 1989
H3	M13mp19	HindIII	1.3	Genomic subclone	Noncoding for ND2 and 16S rRNA	Chapter 3
H4	M13mp18	HindIII	1.3	Genomic subclone	Coding for ND2 and 16S rRNA	Chapter 3
A45	M13mp19	SstI-HindIII	1.4	Genomic subclone	Coding for ND3 and ND4	H.T. Jacobs, pers. comm.
SpG30	pBR322	BamHI-HindIII	0.6	cDNA	COI	Jacobs et al, 1983
SpP144	pBR322	BamHI-HindIII	1	cDNA	16S rRNA	Jacobs and Grimes, 1986
SpG410	pBR322	BamHI-HindIII	0.5	cDNA	12S rRNA	Elliott and Jacobs, 1989

pZH0.8	pUC19	SstI-HindIII	0.8	Genomic subclone	3' end of 12S rRNA, tRNAs (E, T, P, Q, N, L, A, W)	H.T. Jacobs, pers. comm.
pP12	pUC19	SalI-SstI	8.5	Genomic subclone	cyt b, 12S rRNA, tRNAs (E, T, P, Q, N, L_{CUN}, A, V, M, D, Y, G, L_{UUR}, I, R, W, C, F), ND1, ND2, 16S rRNA, COI, COII,ND4L	Jacobs et al, 1988
pZ1A	pUC19	SstI	7.0	Genomic subclone	As for pP12, but lacks cyt b and tRNA (F)	Jacobs et al, 1988
pZ1B	pUC19	SstI	7.0	Genomic subclone	As for pZ1A, but insert in opposite orientation	Jacobs et al, 1988
pZ2	pUC19	SstI	2.5	Genomic subclone	COII, A8, A6, COIII, ND3, tRNAs (K, S_{UCN})	Jacobs et al, 1988
pZ3	pUC19	SstI	2.4	Genomic subclone	ND3, ND4, tRNAs (H, S_{AGN}), ND5	Jacobs et al, 1988
pZH1.4	pUC19	SstI-HindIII	1.4	Genomic subclone	ND3, ND4	Jacobs et al, 1988
pH1.1	pUC19	HindIII	1.1	Genomic subclone	ND4, ND5, tRNAs (H, S_{AGN})	Jacobs et al, 1988
pH1.6	pUC19	HindIII	1.6	Genomic subclone	ND1 and ND2, tRNAs (M, D, Y, G, L_{UUR}, I)	Jacobs et al, 1988
pRB3	pUC19	EcoRI-BglII	3.3	Genomic subclone	ND5, ND6, Cytb	Jacobs et al, 1988
pH1.3	pUC19	HindIII	1.3	Genomic subclone	16S rRNA, ND2	Jacobs et al, 1988
pZH2.0	pUC19	SstI-HindIII	2.0	Genomic subclone	16S rRNA, COI, ND4L, COII, tRNA (R)	Jacobs et al, 1988
pPZ0.6	pUC19	PstI-SstI	0.6	Genomic subclone	12S rRNA	Jacobs et al, 1988
λmt1	XCharon4A	EcoRI	15.6	Genomic clone	The complete S. purpuratus mitochondrial genome	Jacobs and Grimes, 1986

Table 2.2 The recombinant plasmids and bacteriophages used in this study. The orientation of ssDNA clones are given tRNAs are denoted by the standard 1 letter amino acid code, also showing the codon recognition groups of leucyl and seryl tRNAs. Other abbreviations are as given at the start of this thesis.

Recombinant clones

The recombinant plasmids and bacteriophages used, including those constructed in this study, are described in Table 2.2.

2.3 Oligonucleotides

The oligonucleotides used in this study are described in Table 2.3. These were synthesised as described in Jacobs *et al* (1988), by V.B. Math of the Biochemistry Department, University of Glasgow.

2.4 Sea urchins

Adult *Strongylocentrotus purpuratus* were obtained from Pacific Biomarine, Venice, California.

2.5 Chemicals

Chemicals	Source
General chemicals and solvents (analytical grade unless stated)	BDH, Hopkins and Williams, Kochlight Laboratories, May and Baker, Fluka.
Microbiological media	Difco, Oxford.
Biochemicals	Sigma, Pharmacia, BRL.
Agaroses	BRL.
Radiochemicals	NEN (Dupont).
Antibiotics	Sigma.
Restriction enzymes	BRL, Boerhinger Mannheim.
DNA modifying enzymes	BRL, Boerhinger Mannheim, NEN (Dupont), Pharmacia, NBL.

2.6 Microbiological culture media

2.6.1 Media

L-broth: 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose, and 20 mg thymine were made up to 1 litre in distilled water and adjusted to pH7.0 with sodium hydroxide.

L-agar: As L-broth without glucose plus 15 $g.l^{-1}$ agar.

Identity number	Sequence	Length	Gene to which oligonucleotide is complementary	Map position	Strand
106	ATATTTAGCACTGCAG	16	12S	269	Non-coding
108	AAAGGCCCTACTACA	15	ND1	2301	Non-coding
111	TCCATTATACACTGG	15	16S	4456	Non-coding
118	AGGGGGCTGGGGAGG	15	ND2	3692	Coding
128	TTATATATAATTACA	15	ND2	4243	Noncoding
159	TCATAGCTGTGCCTA	15	COI	5884	Non-coding
195	GGTTCCTACAGTTAC	15	None	1205	Major coding strand, but in intergenic region
214	AGTGAATTCGAGCTC-GAGCATTTGAGGCAGAGT	30	M13 lacZ and $tRNA^{phe}$	..1065	Coding
281	AAGCTCTAAGCCACC	15	ND2	3305	Non-coding
282	CACCTTTCGCTCTAC	15	ND1	2254	Non-coding
290	AACTTTTCTATGTAA	15	16S/ND2	4266	Non-coding
301	GAGTCATAACTTTCC	15	16S/ND2	4273	Non-coding
372	GAATTCGGAAAATTG	15	Cytb	14551	Non-coding
374	GCGAGAGACTATAAA	15	ND6	14418	Non-coding
390	AGGCAGGGCATGAGC	15	ND3	10264	Non-coding
393	GAAACCTCTATCAAA	15	$tRNA^{ser}$	10130	Non-coding

Table 2.3: Oligonucleotides used during this study. The map position given represents the most 5' nucleotide of the oligonucleotide on the sequence of the S. purpuratus mitochondrial genome (Jacobs et al, 1988), except in the case of oligonucleotide 214, in which it represents the most 5' sequence complementary to sea urchin mitochondrial DNA.

Water agar: 2% agar in distilled water.

Top agar: 2 parts of L-broth and 1 part of L-agar, mixed under sterile conditions before use.

4X Davis-Mingioli (D&M) salts: 28 g K_2HPO_4, 8 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 1 g sodium citrate, 0.4 g $MgSO_4.7H_2O$ made up to 1 litre with distilled water.

2x TY Broth: 10 g bacto-tryptone, 10 g yeast extract, 5 g NaCl, made up to 1 litre with distilled water.

2.6.2 Supplements

When required, supplements were added to minimal media, by dilution of sterile stock solutions, at the following concentrations:

Glucose	2 $mg.ml^{-1}$
Amino acids	40 $\mu g.ml^{-1}g$
Thymine	50 $\mu g.ml^{-1}g$
Thiamine (vitamin B1)	20 $\mu g.ml^{-1}g$
Casamino acids	10 $\mu g.ml^{-1}g$

2.6.3 Indicators

X-gal and IPTG were added to molten agar (42°C) in order to detect recombinant clones. The M13 and pUC vectors encode the α-fragment of the *lacZ* gene, which is able to complement the lacZ, M15 deletion in appropriate *E. coli* host strains. The resulting β-galactosidase activity will act on the chromogenic substrate X-gal, when induced by IPTG, to give a blue colour. Insertion of DNA into the polylinkers of these vectors leads to disruption of the LacZ gene, and therefore a loss of β-galactosidase activity, and a white colour on X-gal/IPTG.

X-gal was dissolved in dimethylformamide, and IPTG in distilled water. Both were stored at -20°C as 20 $mg.ml^{-1}$ solutions, and used at a final concentration of 20 $\mu g.ml^{-1}g$.

Antibiotics: Ampicillin was the only antibiotic used for

bacterial work in this study. It was stored at -20°C as a 25 $mg.ml^{-1}$ dilution in distilled water. The working concentration was 50 $\mu g.ml^{-1}g$.

2.6.4 Sterilisation

All growth media and glassware were sterilised by autoclaving at 120°C for 15 minutes, supplements and buffer solutions at 108°C for 10 minutes, and 100 mM $CaCl_2$ at 114°C for 10 minutes. Heat sensitive reagents were sterilised by filtration using disposable 0.45 µm membrane filters. Plasticware was sterilised by autoclaving at 120°C for 90 minutes.

2.7 Buffer Solutions

2.7.1 Electrophoresis buffers

50X A-buffer: 242 g Tris-base, 57.1 g glacial acetic acid, 100 ml 0.5 M EDTA pH8.0, made up to 1 litre in sterile distilled water.

10X TBE Buffer: 109 g Tris-base, 55 g boric acid, 40 ml 0.5 M EDTA pH 8.0, made up to 1 litre in sterile distilled water.
10X MOPS Buffer: 21 g NaMOPS, 5.44 g sodium acetate trihydrate and 1.85 g EDTA were made up to 1 litre with distilled water and adjusted to pH7.0 with sodium hydroxide. The solution was stored at 4°C in the dark.

Sequencing gel tracking dye (10X): 90% de-ionised formamide, 10 mM EDTA, 10 mM NaOH, 0.1-0.3% each of xylene cyanol and bromophenol blue.

Agarose gel tracking dye (10X): 0.25% bromophenol blue, 0.25% xylene cyanol, 0.25% orange G, 25% ficoll 400.

Formaldehyde gel tracking dye (10X): 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol.

2.7.2 Enzyme buffers

BRL REact restriction buffers were generally used for

restriction digests, as follows.

10X REact 1: 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$.

10X REact 2: 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$, 500 mM NaCl.

10X REact 3: 500 mM Tris/HCl pH8.0, 100 mM $MgCl_2$, 1M NaCl.

10X XhoII Digestion buffer: 600 mM Tris/HCl pH7.5, 600 mM $MgCl_2$, 700 mM β-mercaptoethanol, 1% (v/v) Triton X-100. Stored at -20°C.

10X Ligation buffer: 660 mM Tris/HCl pH7.6, 60 mM $MgCl_2$, 100 mM DTT. Stored at -20°C.

5X S1 nuclease digestion buffer: 0.25 M NaCl, 0.03M sodium acetate pH 4.5, 1 mM $ZnSO_4$, 5% (v/v) glycerol. Stored at -20°C.

10X Nick-translation buffer: 500 mM Tris/HCl pH7.2, 100 mM $MgSO_4$, 1 mM DTT, 500 $ug.ml^{-1}$g BSA (enzyme grade). Stored at -20°C.

10X HIN buffer (or 10X core buffer): 500 mM NaCl, 70 mM Tris/HCl pH7.5, 100 mM $MgCl_2$, 30 mM DTT. Stored at -20°C.

5X Reverse transcriptase buffer (BRL): 250 mM Tris/HCl pH8.3, 375 mM KCl, 50 mM DTT, 15 mM $MgCl_2$. Stored at -20°C.

2.7.3 Plasmid preparation buffers

Birnboim-Doly I: 50 mM glucose, 25 mM Tris/HCl pH8.0, 10 mM EDTA.

Birnboim-Doly II: 0.2 M NaOH, 1% SDS. This solution was made freshly each time.

Birnboim-Doly III: Made by mixing 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml distilled water. The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate.

Cleared lysate method lytic mix: 2% Triton X-100, 50 mM Tris/HCl pH8.0, 60 mM EDTA.

2.7.4 General buffers

20X SSC: 3 M NaCl, 300 mM sodium citrate. The pH was adjusted to 7.0. with HCl.

20X SET: 400 mM Tris/HCl pH 7.8, 3 M NaCl, 20 mM EDTA.

1X TE: 10 mM Tris/HCl pH 8.0, 1mM EDTA.

1X TES: 20 mM NaCl, 10 mM Tris/HCl pH8.0, 0.1 mM EDTA.

Sodium Phosphate Buffer: Made by mixing 1 M Na_2HPO_4 with 1M NaH_2PO_4 until the pH was 6.8.

EDTA: Made by dissolving solid $Na_2EDTA.2H_20$ in dH_2O, and adjusting the pH to 8.0 with sodium hydroxide.

2.7.5 Filter washing buffers

5X W: 0.01% SDS, 0.1% sodium pyrophosphate, 0.025 M sodium phosphate buffer pH6.8, 5X SET.

1X W: As for 5X W but with 1X SET.

0.1X W: As for 5X W but with 0.1X SET.

2.8 Nucleotide stocks

4 mM ATP: 60 mg ATP (disodium salt) was dissolved in 0.8 ml distilled water and adjusted to pH7.5 with 0.1 M NaOH. 4 mM ATP was stored at -20°C in aliquots and thawed once only.

dNTPs: Made up as concentrated stocks at 20 mM in distilled water and stored at -20°C.

ddNTPs: Made up as concentrated stocks at 20 mM in distilled water and stored at -20°C.

2.9 Organic solvents

Phenol: Phenol was equilibrated several times with 1 M Tris/HCl pH8.0, and then with 0.1 M Tris/HCl pH 8.0. In some cases water saturated phenol was used. 8-hydroxyquinoline was added to 0.1% (w/v). Aliquots were stored in the dark under 0.1 M Tris/HCl pH 8.0 at -20°C.

Sevag: Chloroform was mixed with isoamyl alcohol (24:1, v/v). Sevag was stored at room temperature.

Formamide; Analytical grade formamide was stored at 4°C.

Other solvents: Ethanol, butanol, dimethyl formamide and isopropanol were stored at room temperature.

2.10 Microbiological growth conditions

Liquid cultures for DNA transformation and DNA preparations were grown at 37°C with vigorous shaking. JM101 and JM109 were grown in 2X TY and were used to propagate M13 recombinants; DS 941 and JM 83 were grown in L-broth and were used to propagate plasmids derived from pBR322; NM261 was grown in L-broth containing 0.2% maltose and 10 mM $MgCl_2$, and was used to propagate lambda DNA.

Plate cultures were grown at 37°C overnight. DS 941, NM621 and JM83 were grown on L-agar, the other strains were grown on water agar plus D&M salts, thiamine (vitamin B1), glucose and tryptophan. Plates contained 20 ml of agar solution to which antibiotics and indicators were added as required.

Bacterial strains were stored long term in 85% L-broth, 15% glycerol (v/v) at -20°C. In each case, bacteria were grown up from single colonies.

2.11 Transformation of competent E. coli.

2.11.1 Preparation of competent cells

20 ml of L-broth was inoculated with 400 ul of an overnight culture of *E. coli*, and grown with vigorous shaking at 37°C for 90 minutes, by which time the cells had entered the

logarithmic growth phase. The cells were then pelleted at 5000 rpm (JA 20 rotor) for 5 minutes at 4°C in a 40 ml Oakridge tube, and resuspended in 10 ml pre-chilled 50 mM Calcium chloride solution. After a 20 minute incubation on ice the cells were respun as above, and then resuspended in 2 ml 50 mM Calcium chloride. Competent cells were either used fresh, or after 24 hours at 4°C.

2.11.2 Transformation of E. coli

50-200 ng of DNA in a volume of 10 ul TE was mixed with 300 ul of competent E. coli in a 5 ml polypropylene tube, and placed on ice for 40 minutes. The mixture was then heat-shocked at 42°C for 2 minutes and returned to ice. For transformations of M13 recombinants, 200 µl of E. coli cells were added, and the cells were plated out mixing with top agar at 42°C and pouring onto a prewarmed agar plate; or by spreading on an agar plate in the case of transformation of pBR322 based plasmids.

2.12 Large scale preparation of plasmid DNA

Two methods were used for the initial preparation of plasmid DNA. In either case covalently closed circular DNA was subsequently purified by centrifugation through a caesium chloride gradient.

2.12.1 Alkaline lysis method

The alkaline lysis method (Birnboim and Doly, 1979) was used as described by Maniatis et al, (1982). 200 ml of an overnight culture of plasmid-containing E. coli were transferred to a 250 ml centrifuge bottle and pelleted by centrifugation (JA-14 rotor, 5 krpm, 4°C, 10 minutes). The pellet was resuspended in 8 ml of Birnboim and Doly solution I in a 40 ml oakridge tube, and kept at room temperature for 5 minutes. 16 ml of freshly made Birnboim and Doly solution II was then added, mixed gently, and the mixture was kept on ice for 10 minutes. 12 ml of ice cold Birnboim and Doly solution III was added, and mixed by inversion. Chromosomal DNA and cellular debris was removed by centrifugation (JA-20 rotor, 18 krpm, 4°C, 20 minutes, no brake), and the

supernatant was transferred to a fresh tube by filtration through siliconised glass wool. Plasmid DNA was precipitated at room temperature for 15 minutes by the addition of an equal volume of isopropanol, and pelleted by centrifugation (JA-20 rotor, 18 krpm, 20°C, 20 minutes). The pellet was then washed in 70% ethanol, vacuum dried and resuspended in TE.

2.12.2 Cleared lysate method

100 ml of a plasmid-containing E. coli was harvested from a culture by centrifugation (10 krpm, 4°C, 5 minutes), resuspended in 1.65 ml 25% sucrose/50 mM Tris/HCl pH8, and kept on ice. 350 ul of ice cold lysozyme solution (20 $mg.ml^{-1}$ in 50 mM Tris/HCl pH8.0) was added, followed by a 15 minute incubation on ice. 650 ul EDTA was added, followed by a 15 minute incubation on ice. 2.5 ml of the cleared lysate lytic mix was then added, followed by a 15 minute incubation on ice and centrifugation (JA-20 rotor, 20 krpm, 4°C, 25 minutes). The supernatant was then treated with 50 µl 5 $mg.ml^{-1}$ RNase A (which had been preboiled) for 25 minutes at room temperature.

2.12.3 Caesium chloride gradient centrifugation

7.5 g solid caesium chloride and 500 ul of 10 $mg.ml^{-1}$ ethidium bromide were added to 7.5 ml of a final solution from either 2.12.1 or 2.12.3. The mixture was then clarified by centrifugation (JA-20 rotor, 5 krpm, 10 minutes), and the supernatant was transferred to a 13.5 ml ultracentrifuge tube. Density gradients were formed by ultracentrifugation in a Beckman Ti-70 fixed angle rotor (49 krpm, 16 hours, 20°C), and viewed under a long wave (366 nm) u.v. source. The lower band, containing covalently closed circular DNA, was recovered and the ethidium bromide removed by successive extractions with isopropanol. Finally the aqueous fraction was dialysed against TE overnight with 3 changes of buffer. The DNA was then concentrated by ethanol precipitation, washed in 70% ethanol, and redissolved in TE.

2.13 Preparation of single-stranded M13 DNA

2.13.1 'Minipreparations'

A single M13 plaque was used to infect 1.5 ml of 2X TY broth containing 15 µl of an overnight culture of an appropriate strain of *E. coli*. This was grown at 37°C for 6 hours with vigorous shaking, and then transferred to a microfuge tube and centrifuged at room temperature for 5 minutes. The supernatant, containing the phage particles, was recovered and respun. 1 ml of the remaining supernatant was then mixed with 200 µl of a solution of 20% PEG, 2.5 M NaCl, and left at room temperature for 15 minutes to precipitate the phage particles. These were recovered by centrifugation at room temperature in a microfuge for 10 minutes. The supernatant was discarded, the pellet respun and all traces of supernatant removed. The pellet was then resuspended in 100 ul of TES, extracted twice with phenol-sevag, and twice with sevag. The DNA was then ethanol precipitated from the aqueous phase, and the DNA recovered by centrifugation in a microfuge. The DNA was then washed with 70% ethanol, dried *in vacuo* and resuspended in 20 µl TES.

2.13.2 'Maxipreparations'

The same basic procedure was followed as for 'minipreparations', but all the volumes were scaled up 20-fold. The cells from a 30 ml culture were spun out (JA-20 rotor, 11.5 krpm, 2 minutes). The supernatant were recovered and respun as before. 20 ml of the remaining supernatant were then PEG precipitated with 5 ml of 20% PEG,2.5 M NaCl for 10 minutes at room temperature, and the phage spun out (11.2 krpm, JA-20 rotor, 15 minutes). The phage pellet was resuspended in 1 ml of TES, and then reprecipitated and processed as for a miniprep, with all the volumes scaled up 30-fold.

2.14 Lambda DNA preparations

Bacteriophage plaques were placed into 1 ml of SM (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris/HCl pH7.5, 0.01% gelatin) and left at 4°C for 1 hour. 5 µl of this phage suspension was

incubated for 20 minutes at 37°C with 100µl of E. coli NM261, stored in 10 mM $MgSO_4$ to allow the phage to adsorb, and then added to 5 ml of supplemented L-broth and grown overnight at 37°C with vigorous shaking. Cellular debris was then removed by centrifugation (JA 20 rotor, 4°C, 10 minutes, 10 krpm).

For large scale phage DNA preparations, 5 ml of an overnight NM261 culture were innoculated into 250 ml L-broth supplemented with Magnesium and maltose, and were grown with shaking for 3 hours. The bacteria were then collected by centrifugation (JA-14 rotor, 5 krpm, 4°C, 10 minutes), and resuspended in 250 ml L-broth supplemented with Magnesium but not maltose. The 5 ml of phage supernatant was added, and the culture was grown until full lysis had occured (>8 hours). After this time, 1 ml of chloroform was added to the culture, which was then shaken for a further 15 minutes. Unlysed cells and cellular debris were removed by centrifugation (JA 20 rotor, 5 krpm, 10 minutes), after which DNase and RNase were added to the supernatant at a final concentration of 1 $\mu g.ml^{-1}$, and incubated at 37°C for 1 hour. Solid Sodium chloride was then added to the supernatant to a concentration of 1 M, dissolved by swirling, and the supernatant was left on ice for 1 hour, after which it was centrifuged (JA14 rotor, 6 krpm, 30 minutes, 4°C). After centrifugation, PEG 8000 was added to the supernatant to a concentration of 10% w/v, and the phage were precipitated at 4°C for 1 hour. The phage were then pelleted by centrifugation (JA14 rotor, 6 krpm, 30 minutes, 4°C), and the supernatant fully drained off and discarded. The dried pellet was then resuspended in 6 ml of TM (50 mM Tris-HCl pH8.0, 10 mM MgSO), transferrred to a 40 ml polypropylene tube and extracted with an equal volume of chloroform. Following centrifugation (JA20 rotor, 10 krpm, 15 minutes, 4°C), the aqueous phase was recovered and EDTA (to a final concentration of 20 mM), proteinase K (to a final concentration of 50 $\mu g.ml^{-1}$) and SDS (to 0.5%) were added. Protein digestion was carried out for 1 hour at 50°C. The solution was then extracted with an equal volume of phenol, phenol/Sevag, and Sevag, the aqueous phase being separated at each stage by centrifugation (JA 20 rotor, 10 krpm, 10 minutes, room temperature). Finally, 1/10 volume of 3M sodium acetate was added to the aqueous phase, and the phage DNA was precipitated with 2 volumes of ethanol for 30

minutes on ice, pelleted by centrifugation (JA 20 rotor, 10 krpm, 10 minutes, 4°C), washed with 70% ethanol, dried *in vacuo*, and then resuspended in 500 ul of TE. Recovery was quantitated by electrophoresis.

2.15 General nucleic acid extraction procedures

2.15.1 Removal of protein from nucleic acids with organic solvents

This was done using Tris-saturated phenol-sevag and sevag. In both cases extractions were carried out by adding an equal volume of the solvent, mixing by inversion, and centrifuging in a microfuge for 3 minutes. Proteins were retained in the organic phase, and residual solvent in the aqueous phase was removed by isopropanol or ethanol precipitation.

2.15.2 Concentration of DNA by precipitation with ethanol and isopropanol

Precipitation with isopropanol was achieved by adding an equal volume of isopropanol. Precipitation with ethanol was achieved by adding 0.1 volumes of 3 M sodium acetate pH6.0 and 2.5 volumes of ethanol. Ethanol precipitation mixtures were left for at least 1 hour at -20°C, and isopropanol precipitation mixtures for the same amount of time at room temperature. After precipitation, the nucleic acids were then recovered by centrifugation in a microfuge for 15 minutes, washed with 70% ethanol, dried *in vacuo* and then resuspended.

2.16 Restriction digests of DNA

Restriction digests were usually carried out in a final volume of 20 µl, containing between 0.25 and 1 µg of DNA, 2 ul of the appropriate restriction digest buffer (10X concentrate), 1-10 units of restriction enzyme, and distilled water. Reactions were allowed to proceed for 1-2 hours at 37°C, and then stopped either by the addition of agarose gel tracking dye, or by phenol-Sevag extraction and precipitation.

2.17 Ligation of DNA fragments

The concentration of the restriction fragments to be ligated was such that the insert was in 3-fold excess over the vector (only sticky-end ligations were performed in this study). 4 µl of 5X ligation buffer and 2 µl of 4 mM ATP were added to the DNA (concentrations given in figure legends), and the volume made up to 20 µl with distilled water. T_4 DNA ligase was then added (0.01 units per ug of DNA), and the solution incubated at room temperature for 1 hour, or overnight at 14°C.

2.18 DNA sequencing

Both single-stranded M13 DNA and double-stranded plasmid DNA were used for sequencing by the method of Sanger et al (1977). Single-stranded DNA was used for sequencing directly, whereas double-stranded DNA had to be denatured before the sequencing reactions.

2.18.1 Denaturation and annealing of double-stranded DNA

Plasmid DNA was routinely purified through a biogel A-50 column, and then ethanol precipitated prior to sequencing. 2 ug of plasmid DNA and 25 ng of oligonucleotide were then added to 40 µl of a denaturing solution (0.2 M NaOH, 0.2 mM EDTA pH 8.0), and left at room temperature for 5 minutes. This mixture was then neutralised with 4 µl 2 M sodium acetate pH4.5, ethanol precipitated at -70°C for 5 minutes, centrifuged and the pellet washed with 70% ethanol. 25 ng of oligonucleotide and 1 ul 10X HIN were added to the DNA, to give an annealing mix which was incubated at 50°C for 5 minutes.

2.18.2 Annealing of single-stranded DNA

0.5-1 ug of single-stranded DNA in in 4 ul of TES was mixed with 5 ng of a priming oligonucleotide, 1 ul of 10X HIN and 4 µl of distilled water. Annealing was carried out at 50°C for 5 minutes.

2.18.3 Sequencing reactions

Immediately prior to the reactions, 1 ul 100 mM DTT, 1 ul α-^{32}P dATP and 1 µl of large subunit DNA polymersae (Klenow polymerase) were added to the annealed DNA solutions. 3 µl of these template-primer mixes were then dispensed into each of 4 tubes containing 1 ul of the diluted ddNTPs (dilutions determined empirically, but generally 0.1 mM ddATP, 0.3 mM ddCTP, 0.5 mM ddGTP and 1 mM ddTTP), and 1 µl of one of the 4 dNTP mixes which lack dATP and contain a 1:20 dilution of the approriate dNTP (N^o reaction cocktails). After mixing, the tubes were incubated at 50°C for one minute, then 1 µl of 0.5 mM dATP was added and the tubes incubated for a further minute. The reactions were stopped by the addition of 10 µl of sequencing gel tracking dye, and heated at 90°C for one minute prior to loading on a sequencing gel.

N^o reaction cocktail mixes

	A^o	C^o	G^o	T^o
0.5 mM dCTP	20 µl	1 µl	20 µl	20 µl
0.5 mM dGTP	20 µl	20 µl	1 µl	20 µl
0.5 mM dTTP	20 µl	20 µl	20 µl	1 µl
10X HIN	20 µl	20 µl	20 µl	20 µl

2.19 Gel electrophoresis

Gel electrophoresis was used for the separation of nucleic acids. Electrophoretic mobility is inversely proportional to the $\log_{10}$ of the molecular weight: the molecular weights of fragments were calculated on the basis of this, with regard to the mobility of standards of known molecular mass.

2.19.1 Agarose gel electrophoresis

DNA was electrophoresed on agarose gels. Generally 20 cm X 16 cm gels containing 300 ml of agarose were run, using agarose concentrations of 0.5-2.0% (w/v) depending on the sizes of fragments to be resolved (Maniatis et al, 1982). Applied voltages varied between 2 and 10 $V.cm^{-1}$, depending on the time of running. Agarose was dissolved by boiling in 1X A

buffer, and the gels were run in 1X A buffer. Before loading, 0.1 volumes of agarose gel tracking dye solution was added to the DNA samples. Gels were stained in 0.5 $\mu g.ml^{-1}$ ethidium bromide, followed by destaining overnight in dH_2O.

2.19.2 Formaldehyde/agarose gel electrophoresis

This technique was used for the separation of RNA. Agarose was dissolved in 200 ml of distilled water, followed by the addition of 50 ml pre-warmed formaldehyde, 30 ml of pre-warmed (40°C) MOPS and 20 ml of pre-warmed distilled water. Before loading, RNA (in 4.5 ul DEPC-treated water- see below for details of DEPC-treatment) was denatured by the addition of 10 µl formamide, 2 µl 5X MOPS, 3.5 µl formaldehyde, and heating at 55°C for 15 minutes. Gels were run in 1X MOPS. RNA was visualised by soaking the gel in 0.5 $\mu g.ml^{-1}g$ ethidium bromide, followed by destaining overnight in distilled water.

2.19.3 Sequencing gels

Generally 60 cm gels were run, containing 5% polyacrylamide, 50% urea (w/v), and 1X TBE. Gels were poured between plates separated by Whatman 3MM paper strips, and polymerised by the addition of 35 ul TEMED and 1 ml ammonium persulphate. After polymerisation, gels were pre-run for at least 1 hour in 1X TBE. Gels were run for various lengths of time depending on the sizes of DNA to be resolved, but at least until the bromophenol blue had reached the bottom of the plates. The sequencing ladders were then visualised by autoradiography.

2.19.4 Visualisation and photography of gels

DNA and RNA were visualised by u.v. induced fluorescence on either a short wave (254 nm) or long wave (302 nm) transilluminator after staining agarose gels with ethidium bromide . Gels were photographed using either a polaroid camera loaded with 545- or 667- land film and fitted with a Kodak Wratten filter No. 9, or with a Pentax camera.

2.20 Preparation of RNA

Two techniques were used to isolate sea urchin RNA. All solutions (except those which contained amine groups), and glassware, were treated with 0.01% DEPC for at least 1 hour followed by autoclaving, prior to use.

2.20.1 By centrifugation through a caesium chloride cushion

This technique was based on that of Birnie and Graham (1986). Sea urchin eggs or embryos were resuspended in a greater than 10 volumes of guanidium isothiocynanate (GuTc) solution (4.5 mM guanidium isothiocyanate, 50 mM EDTA, 50 mM HEPES pH7.0 and 5% v/v b-mercaptoethanol). Solid N-lauroyl sarcosine was added to a final concentration of 2%, and the mixture was layered over 1 ml of a caesium chloride pad solution (5.7 M CsCl, 50 mM EDTA, 50 mM HEPES pH7.0) in an SW50 centrifuge tube. Samples were spun at 35 krpm for 16 hours at 20^{o}C in an SW50 rotor. The GuTc solution was then removed by aspiration, and the tubes were inverted and cut around the bottom to prevent RNase containing solution from running onto the pellets. The pellets were then resuspended in DEPC-treated water, phenol-Sevag extracted, ethanol precipitated, washed in 70% ethanol, dried *in vacuo* and stored in DEPC-treated water at -20^{o}C.

2.20.2 By acid phenol extraction

This technique was based on that of Chomcynski and Sacchi (1987), scaled down so that it could be performed in microfuge tubes. Sea urchin eggs or embryos were resuspended in 0.5 ml solution D (4 M guanidium isothiocyanate, 25 mM sodium citrate pH7.0, 0.5% sarcosyl, 0.1 M b-mercaptoethanol). Then 50 µl sodium acetate pH4.0, 0.5 ml water-saturated phenol and 0.1 ml sevag were added, with thorough mixing after the addition of each reagent. The final mixture was incubated on ice for 15 minutes, and then spun in a microfufuge for 15 minutes at 4^{o}C. RNA was precipitated from the aqueous phase by adding an equal volume of isopropanol, incubation at -20^{o}C for 1 hour, and then

centrifugation in a microfuge. RNA was then washed in 70% ethanol, dried *in vacuo*, and then resuspended in either formaldehyde gel tracking dye or DEPC-treated water. The RNA was stored at -20°C.

2.21 S1 nuclease mapping

S1 nuclease mapping was carried out using single-stranded M13- derived probes (Lee *et al*, 1986; Calzone *et al*, 1987).

2.21.1 Probe synthesis

1-2 ug of template ssDNA was annealed with an oligonucleotide primer in a volume of 28 µl (containing 4 µl 10X HIN buffer), by heating at 50°C for 5 minutes, and then 37°C for 10 minutes. The extension reaction was then carried out at 37°C for 30 minutes, by adding 4 ul each of 0.5 mM dCTP, dGTP, and dTTP; 50 µCi dATP; and one unit of Klenow. The reaction was then 'chased' for 30 minutes by adding 4 ul 0.5 mM dATP. After synthesis, probes were cut at a site within or beyond the insert by the addition of 20 ul restriction digest solution containing the appropriate enzyme and buffer. To purify the single-stranded probe, the restriction digest solution was denatured by the addition of 4 ul 3:1 5 M NaOH, 0.5 M EDTA and 20 µl agarose gel tracking dye, and electrophoresed on a 1.5% agarose gel. The position of the probe was determined by placing a piece of Kodak X-Omat film over the gel for 10 minutes. The probe was eluted from the gel by electrophoresing it onto Whatman DE81 paper, and mashing the paper in 1.5 M NaCl, 0.2 M NaOH, 5 $\mu g.ml^{-1}$g salmon sperm DNA.

2.21.2 Hybridisation

The probe and RNA were precipitated together, washed in 70% ethanol, dried *in vacuo*, and resuspended in 20 ul of formamide and 5 ul of 2 M NaCl,5 mM EDTA,0.2 M PIPES pH6.4. This was then denatured at 80°C for 5 minutes, and hybridised overnight at 37°C.

2.21.3 S1 nuclease digestion

This was carried out at 14°C for 30 minutes in 300 ul of S1 digestion buffer, containing 5 µg of denatured salmon sperm DNA, 5 ug of native salmon sperm DNA, and generally 250 units of S1 nuclease (Sigma). Reactions were stopped by the addition of 40 µl 4M sodium acetate, 50 mM EDTA, and 4 µl of 20% SDS, 10 $\mu g.ml^{-1}g$ tRNA. The reaction mixes were phenol-sevag extracted, ethanol precipitated, washed in 70% ethanol and resuspended in sequencing gel loading buffer. Products were analysed on 5% sequencing gels.

2.22 Primer extension mapping of transcript 5' ends

10 ng of oligonucleotide primer and 2-20 ug of sea urchin RNA were denatured together by heating at 90°C for 1 minute, and then annealed by adding 4 ul reverse transcriptase buffer and incubating at 50°C for 5 minutes. Following this incubation, 10 uCi ^{32}P dCTP (3000 Ci/ mmol); 5 ul of a mix of 5 mM dATP, dGTP, dTTP; and 200 units of Mu-MLV reverse transcriptase (BRL) were added. Primer extension was carried out for 5 minutes at 37°C, and 'chased' for 5 minutes with the addition of 2 ul 0.5 mM dCTP. The reaction mixes were extracted with phenol-Sevag, ethanol precipitated, washed in 70% ethanol, dried *in vacuo* and resuspended in 5 µl sequencing gel tracking dye. The products were analysed on sequencing gels.

2.23 Hybridisational analysis of nucleic acids immobilised on nylon filters

2.23.1 Southern Blotting

Southern blotting was carried out by a method adapted from Southern (1975), and described in Maniatis *et al* (1982). After electrophoresis, staining and photography, gels were soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl), for 30 minutes then in neutralising solution (0.5 M Tris/HCl pH7.4, 1.5 M NaCl) for 30 minutes, and then in 20X SSC for 30 minutes. DNA was transferred to Pall Biodyne nylon membrane filters, by capillary action of 20X SSC from a reservoir through the gel and filter membrane and into subsequent

layers of tissue paper. Transfer was carried out for 16 hours, after which the filter was air dried and baked for 2 hours at 80°C.

2.23.2 Northern Blotting

After electrophoresis, formaldehyde/agarose gels were immediately set up for capillary blotting as for Southern blots, without prior treatment with 0.5 M D, 1XN or 20X SSC.

2.23.3 Dot blotting

RNA or DNA was denatured in 100 ul DEPC-treated distilled water, 60 ul DEPC-treated 20X SSC and 40 µl 37% (w/v) formaldehyde, at 60°C for 15 minutes. After quenching on ice, 20 µl of the denatured sample was added to 80 µl of DEPC-treated 15X SSC, and blotted onto a Pall Biodyne membrane, using a hybridot apparatus (BRL) under moderate vacuum. Samples were washed through with 15X SSC and then baked at 80°C for 2 hours. For accurate calibration, DNA standards were dotted in parallel on the same membrane as the RNA samples.

2.23.4 Plaque lifts

Dry, circular nylon filters were placed for 30 seconds on dry, chilled plates containing phage plaques. The orientation of the filter was marked with a needle dipped in Indian ink. Filters were then soaked in 0.1 M D (0.1 M NaOH, 1.5 M NaCl) for 30 seconds, in 1XN (0.5 M Tris/HCl pH7.4, 1.5M NaCl) for 1 minute, and in 4X SSC for two 5 minute periods. The filters were then air dried, baked for two hours at 80°C, pre-hybridised in 5X SET, 5X Denhardts for one hour, and then hybridised in aqueous hybridisation buffer.

2.23.5 Aqueous hybridisations

DNA to DNA hybridisations were carried out at 68°C overnight in aqueous hybridisation buffer (5X Denhardts solution, 0.5 $mg.ml^{-1}$ salmon sperm DNA, 2.5% dextran sulphate, 4X SET, 0.2 M phosphate buffer pH6.8., 0.1% SDS, 20 ug.ml poly(A) and poly(C)). 50X Denhardts is made by

dissolving 5g of each of Ficoll, polyvinylpyrrolidone, and BSA (Pentax Fraction V) in 500 ml of dH_2O. Filters were pre-hybridised for 1 hour in the same buffer as was used for the hybridisation. Hybridisations were carried out in a shaking water bath.

2.23.6 50% formamide hybridisations

DNA to RNA hybridisations were carried out at 43°C overnight, in 50% formamide hybridisation buffer (5X SET, 50% formamide, 1X Denhardts solution, 5% dextran sulphate, 50 $\mu g.ml^{-1}g$ poly(A) and poly(C), 0.02 M phosphate buffer pH6.8, 0.01% SDS). Before addition of the probe the filters were wet in in 4X SET, 0.1% SDS for 20 minutes, and then pre-hybridised for 1 hour in the same buffer as was used for the hybridisation. Hybridisations were carried out in a shaking water bath.

2.23.7 Washing of filters

Filters from both types of hybridisation were washed for 20 minutes in 5X W at 65°C, twice for 20 minutes in 1X W at 65°C, and then twice for 20 minutes in 0.1X W at 65°C. washing was carried out in a shaking water bath.

2.24 Preparation of ^{32}P-labelled probes

2.24.1 Nick-translation

This was carried out by the procedure of Rigby et al (1977). The nick-translation mix was set up as follows:

5 ul 10X nick-translation buffer
24 ul distilled water
2 ul 500 $\mu g.ml^{-1}g$ DNA
1 ul 20 mM b-mercaptoethanol
10 ul 5XNT-N (500µM each of the 3 dNTPs without the labelled nucleotide)
50 uCi α-^{32}P dATP (800 Ci/mmol)
3 ul 100 $ng.ml^{-1}$ DNase I
5 units DNA polymerase I

This reaction mix was incubated at 14°C for 2 hours. As a general rule probes were used without removing the unincorporated nucleotides, an equal volume of 50% formamide was added, and the probes were denatured at 80°C for 10 minutes prior to use.

2.24.2 Hu/Messing probes

Effectively single-stranded probes were synthesised by the method of Hu and Messing (1982). 50 ng M13 17bp probe primer were annealed to 2 µg of M13 recombinant ssDNA, in a final volume of 11 ul 1X HIN buffer, by heating for 5 minutes at 90°C and then slowly cooling to room temperature. The primer was then extended for 1 hour at room temperature, by adding 1 ul of 0.1 M DTT; 1 ul of a mix of 0.5 mM each of dCTP, dGTP, dTTP; 1 µl ^{32}P dATP (800 Ci/ mmol); and one unit of Klenow polymerase. Reactions were stopped by the addition of 1 µl 0.5 M EDTA, and probes were used without denaturation.

2.24.3 In vitro capping of sea urchin RNA

Two techniques were used for *in vitro* capping of sea urchin RNA.

Using the method of Gherke (1986), around 40 ug of sea urchin RNA was labelled in a volume of 20 µl containing 2 µl 10X capping buffer I (500 mM Tris/HCl pH7.9, 12.5 mM $MgCl_2$, 60 mM KCl, 25 mM DTT), 1 µl 1 mM S-adenosyl methionine, 10 µCi α-^{32}P GTP (3000 Ci/mmol) and 5 units of guanylytransferase (BRL). Labelling was carried out for 30 minutes at 37°C.

Using the method of Barat-geuride *et al* (Barat-geuride *et al*, 1987), RNA was denatured prior to *in vitro* capping with 5mM methyl mercuric hydroxide, for 10 minutes at room temperature. Labelling was then carried out by adding 2 µl 10X capping buffer II (500 mM Tris/HCl pH7.9, 1.35 mM $MgCl_2$, 6mM KCl, 2.5 mM DTT), 2.5 units of GTP transferase, 80 µCi α-^{32}P GTP, and 5ul of dH_2O. Labelling was carried out at 37°C for 30 minutes, after which GTP was added to 0.5 mM, and a further incubation for 30 minutes at 37°C was carried out.

RNA *in vitro* capped using either protocol was analysed either by gel elsctrophoresis, or used as a probe for filter

hybridisations.

2.25 Detection of labelled nucleic acids

2.25.1 Autoradiography

^{32}P-labelled nucleic acids were visualised by autoradiography at -70°C, with the use of two intensifying screens.

2.25.2 Fluorography

^{3}H-labelled nucleic acids were visualised by transfer to filter membranes, followed by fluorography at -70°C, after spraying with Enhance (NEN-Dupont). Filters were placed directly against Kodak X-Omat film. Kodak X-Omat film was developed using a Kodak X-Omat film processor.

2.26 Sea urchin in vivo techniques

2.26.1 Sea waters

Artificial sea water (ASW) was made by the method of Osonai (1984). 131.5 g NaCl, 3.5 g KCl, 59.5 g $MgSO_4.7H_2O$, 7.5 g $CaCl_2.2H_2O$, and 2.5 g $NaHCO_3$ were dissolved in 5 litres of distilled water.

Calcium Free Sea Water (CFSW) was made up by dissolving 26.9 g of NaCl, 0.7 g of KCl, 11.9 g of $MgSO_4.7H_2O$, and 0.5 g of $NaHCO_3$ in 1 litre of distilled water.

2.26.2 Spawning

Sea urchins were spawned by the injection of 0.5-1 ml 0.5 M KCl. Eggs were collected by inverting the sea urchins over a beaker of ASW (14 °C), and sperm were collected dry on a petri dish. Eggs were dejellied by passing through a 90 um nitrex filter and then settled under gravity at least twice in 1.5 litres ASW at 4°C. Before filtering, eggs were examined under the microscope for envelope activation, which is a sign of premature fertilisation or activation, and also fertilisability, by adding a drop of sperm diluted in ASW onto a sample of eggs on a slide and looking for envelope activation. Only batches of eggs showing greater than 95%

fertilisation were used in subsequent experiments.

2.26.3 Cultures

After washing, the eggs were resuspended in ASW to give a final density of 10^3-10^4/ml, in approximately 1.5 litres of sea water. Eggs were fertilised by the addition of 2.5 ml of fresh sperm (2 drops diluted in 10 ml ASW), and checked for fertilisation microscopically after 30 seconds. If less than 90% of eggs were fertilised they were given a second dose of sperm and rechecked. Once more than 95% had been fertilised, the excess sperm were removed by centrifugation (1 krpm, JA-14 rotor, 1 minute), and the eggs were gently resuspended in fresh ASW. Cultures were grown at 14°C in a 1.5 litre beaker, with constant stirring and aeration with a teflon paddle. After the first cleavage (90 minutes post-fertilisation) penicillin/streptomycin were added from a 1000-fold concentrate to a final concentration of 50 $ug.ml^{-1}$ of each. Embryos were harvested by centrifugation as above.

2.26.4 Preparation of enucleate fragments

1 ml aliquots of settled sea urchin eggs were loaded onto 0.5-1 M continuous sucrose gradients in SW28 tubes. The gradients were prepared from a gradient maker filled with 17.8 ml 0.5 M sucrose in ASW (1:1 ratio of ASW and 1 M sucrose in distilled water), and 17.5 ml 1 M sucrose in distilled water. These tubes were then centrifuged (SW28 rotor, 4°C) for 7 minutes at 7 krpm, and then for 10 minutes at 15 krpm. This procedure resulted in three bands being formed in the tubes, the lowest of which was the enucleate fragments. These were examined microscopically, and then collected. The enucleate fragments were then washed by adding an equal volume of ASW, centrifuging (1000g, 3 minutes, Jouan rotor), resuspending in the original volume of ASW and respinning as above. The pellet was kept on ice before use.

Parthenogenetic activation of enucleate fragments: 50 ul enucleate fragments were resuspended in 50 ml ASW pH9.0 (adjusted with 1 M Tris/HCl pH9.0) in a 50 ml Falcon tube. 1 M NH_4Cl pH9.0 was then added to a final concentration of 1 mM, and the culture was incubated on a rotary shaker at 16°C for 2-4 hours. The cultures were then spun down (JA-14 rotor,

2 krpm, 5 minutes), and used for in vivo labelling.

2.26.5 In vivo RNA labelling

0.4 ml eggs (or in some cases enucleate fragments) were treated with 10-25 mg pronase in a volume of 15 ml CFSW, for 15 minutes at 14°C prior to labelling. The eggs were then washed in 50 ml ASW, centrifuged (1000g, Jouan centrifuge), and resuspended in 3.6 ml ASW and 20 µl 50 $mg.ml^{-1}$ penicillin/streptomycin. 360 uCi ^{3}H-uridine (36.5 Ci/mmol) was then added, and the mixture incubated at 14°C on rotary shaker for 16 hours. The eggs were then aliquoted into microfuge tubes, and recovered by centrifugation. In vivo labelling of blastulae RNA was carried out for 4 hours, by adding 360 uCi ^{3}H-uridine (36.5 Ci/mmol) directly to 0.4 ml embryos in a volume of 3.6 ml ASW. RNA was then prepared as above.

2.26.6 Preparation of mitochondria from sea urchin eggs

Sea urchin eggs were resuspended in at least 10 volumes of ice-cold 1X DEV/0.25 M sucrose (1X DEV is 0.25M NaCl, 0.03M Tris/HCl pH7.8, 2mM EDTA). They were then homogenised with a dounce homogeniser on ice, rupture of eggs being checked microscopically. Nuclei and cortex fragments were spun out (2000g, 15 minutes, Jouan centrifuge, 4°C). The supernatant was decanted into Oakridge tubes, and centrifuged (JA-20 rotor, 10 krpm, 20 minutes, 4°C), The pellet was then resuspended in 1X DEV, 0.25 M sucrose, and layered over a discontinuous sucrose gradient in an SW28 tube (from bottom to top: 4 ml 2.0 M sucrose, 1X DEV; 4 ml 1.8 M sucrose, 1X DEV; 12 ml 1.2 M sucrose, 1X DEV; 10 ml 0.6 M sucrose, 1X DEV; 5 ml of the sample in 0.25 M sucrose/1X DEV). These tubes were then centrifuged (25 krpm, SW28 rotor, 4°C, 6 hours). The bands in the 1.2 M sucrose step were then harvested.

Before preparing RNA from these mitochondria, they were incubated for 1/2 hour with 200 units micrococcal nuclease in 10% glycerol/10 mM Tris HCl pH8.0/1 mM $CaCl_2$, at 14°C, to remove any contaminating cytoplasmic RNA.

2.26.7 Preparation of mitochondria from blastulae

RNA was prepared from sea urchin blastula according tp the method of Bogenhagen and Clayton (1974). All manipulations were carried out either at 4°C or on ice. 1.5l of hatching blastula were centrifuged at 1000 rpm for 1 minute in a JA 10 rotor. The embryos were then washed in 100 ml of TD buffer in two 50 ml falcon tubes (134 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 10 mM Tris/HCl pH7.4), and centrifuged at 2.5 krpm for 5 minutes in a JS 7.5 rotor. The embryos were then lysed by the addition of 100 ml MgRSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH7.5). After 10 minutes incubation 40 ml of a 2.5 fold concentrate of MSB (mannitol sucrose buffer) to give a final concentration of 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris/HCl pH7.5, 5mM EDTA. Nuclei were removed by two successive sedimentations at 2.5 krpm for 4 minutes in a JS 7.5 rotor. The supernatant was then centrifuged for 20 minutes at 15 krpm in a JA-20 rotor. The resulting pellet was resuspended in MSB, and layered over a sucrose 2-step gradient (1.0 M sucrose/ 1.5 M sucrose, 5 mM EDTA, 10 mm Tris/HCl pH7.5). The gradients were then centrifuged at 22 krpm for 30 minutes in an SW28 rotor. The mitochondria were collected at the interface between the sucrose steps, and resuspended in MSB.

No experiments were carried out to monitor nuclear contamination of these preparations, but nuclear contamination would not have influenced the results.

CHAPTER 3

TRANSCRIPTIONAL ORGANISATION AND DEVELOPMENTAL REPRESENTATION OF SEA URCHIN MITOCHONDRIAL TRANSCRIPTS

3.1 Introduction

Sea urchin mitochondria contain a higher steady-state level of rRNAs than mRNAs (Ruderman and Schmidt, 1981), like those of other characterised eukaryotes. Preliminary evidence has shown that the ratio of rRNA:mRNA is developmentally modulated, with the level of rRNA remaining approximately constant in early development, whilst the level of mRNA increases (Lasky *et al* 1980; Wells *et al*, 1982; Cabrera *et al*, 1983). In this chapter I describe experiments which measure, using RNA dot-blots, the change in the steady-state level of 16S rRNA relative to to COI mRNA between the egg and blastula stages of development. In order to get an insight into how this developmental difference in expression of the rRNAs and mRNAs is established, I have examined the structure and abundance both of mature transcripts, and of higher molecular weight species which are candidates for nascent sea urchin mitochondrial primary transcripts.

The location of the sea urchin mitochondrial promoter(s) is of central importance to the understanding of sea urchin mitochondrial gene expression. The transcriptional initiation sites for the major coding strand of vertebrate mitochondrial DNA seem to be located at the 5' end of the 12S rRNA gene (section 1.5 and 1.9). Although in *Xenopus* there are multiple core promoter motifs located around the mitochondrial genome, only the sites located in the single intergenic region seem to promote transcription detectably *in vivo* (as inferred from the presence of transcripts with 5' triphosphate groups; sections 1.4 and 1.5), or *in vitro* (section 1.9).

Because of the separation of the genes encoding the large and small subunit mitochondrial rRNAs in sea urchins, the sea urchin 16S rRNA gene might be expected to have its own local promoter. All other mitochondrial promoters characterised to date are in intergenic regions (section 1.9). In order to determine whether an intergenic region is present between the genes for 16S rRNA and ND2 mRNA I have subcloned and sequenced this region of DNA. This was part of a larger sequencing project which was going on in the lab, in which the whole genome was sequenced (Jacobs et al, 1988).

All mitochondrial genomes require a minimum of two promoter elements, i.e. one to transcribe each strand. The

degree of intraspecific sequence similarity between individual promoters of mitochondrial genomes is variable. For example, whereas the mouse LSP and HSP show no similarity, the human HSP and LSP show a low level of sequence similarity in core promoter sequence, and the Xenopus mitochondrial promoters show a high level of sequence similarity (Section 1.9). It was conceivable that there might be reiterated sequence motifs upstream of the 16S rRNA gene which are also present elsewhere in the genome, and which might be involved in regulating transcriptional initiation, or other aspects, of sea urchin mitochondrial gene expression.

3.2 Results

3.2.1 Developmental changes in the representation of COI mRNA and 16S rRNA between the egg and blastula stages of development

The steady-state levels of COI mRNA and 16S rRNA were semi-quantitatively measured using filter hybridisations (Anderson and Young, 1985). Samples from a dilution series of egg and blastula total cellular RNA were dotted onto a Pall Biodyne filter, alongside a dilution series of the plasmid DNA used to generate the probe for the hybridisations. Filters were then probed with either nick-translated SpP144 (which is complementary to 16S rRNA), or nick-translated SpG30 (which is complementary to COI mRNA), both of which are cDNA clones (Table 2.2).

After autoradiography, the dots on the autoradiograph were scanned densitometrically. From the DNA dots, the autoradiographic signal corresponding to the known concentration of filter-bound insert DNA was calculated (not shown). This value was then used to calculate the mass of reactive filter-bound RNA for each of the dots of the RNA dilution series. The mass of filter-bound reactive RNA was then plotted against the total input RNA for each of the probes, at both developmental stages (Fig. 3.1). The best-fit line (fitted by eye) was a straight line in each case. This is important, since it shows that the probe was in excess of the filter-bound RNA during hybridisation. If the probe had not been in excess, a plateau value would have been reached

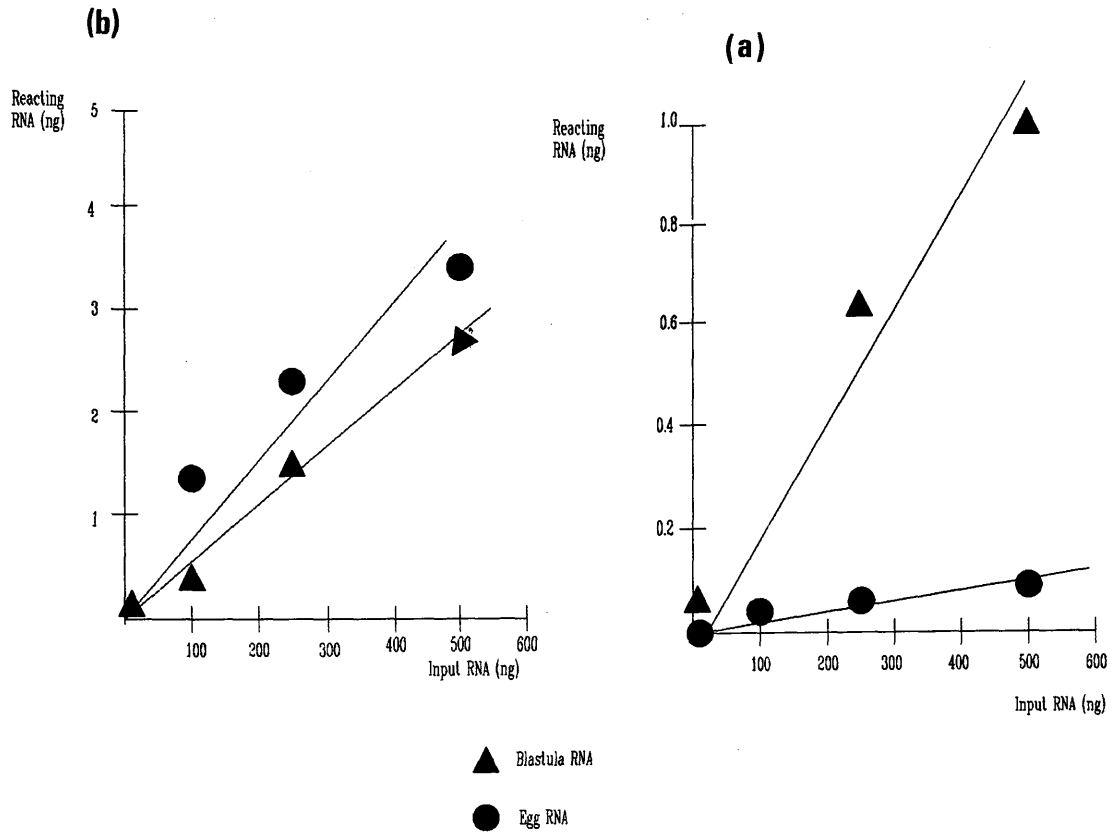


Figure 3.1: Semi-quantitative measurements of the prevalence of 16S rRNA and COI mRNA from sea urchin egg and embryo RNA, measured by RNA dotblots. (a) Graphic representation of the level of RNA reacting with SpG30 plotted against the total level of input RNA in the dots. The level of reacting RNA was calculated by comparison with the autoradiographic signal obtained with known amounts of SpG30 dotted on the same filter. (b) Graphic representation of the level of RNA reacting with SpP144, plotted against the total level of input RNA in the dots. The level of RNA reacting with SpP144 was calculated as for SpG30. Autoradiographic signals were measured densitometrically, using a Hoefer Scientific Instruments GS300 transmittance/refectance scanning densitometer. The units of area measured for the DNA standards were plotted against the known DNA concentrations. This calibration curve was then used to calculate the values shown in the figure.

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above which an increase in the amount of filter-bound RNA would not have led to an increase in autoradiographic signal (or apparent level of reactive RNA).

This value represented an underestimate of the true level of the transcripts in the dots, since SpG30 and SpP144 contain DNA corresponding to only portions of the COI and 16S rRNA transcripts repectively. After correction for this factor (see legend for table 1.1), the slopes of these graphs represent the fraction of the total mass of RNA that these transcripts contribute at the two developmental stages (Table 3.1). The ratio between these slopes is a measure of the relative abundances of these transcripts at the egg and blastula stages of development (Table 3.1). This shows that while the level of COI mRNA increases approximately 10-fold between the egg and blastula stages of development, the steady-state level of 16S rRNA decreases to around 0.7 of its original level during this period.

3.2.2 Transcript mapping by northern hybridisation of RNA from different developmental stages

The sizes of some mitochondrial transcripts were measured at the egg and blastula stages of development, by gel electrophoresis on formaldehyde/agarose gels, followed by transfer to nylon filters by northern blotting and hybridisation with various ^{32}P-labelled molecular probes.

cyt *b* mRNA was detected using a nick-tranlated probe synthesised from the clone K34 (Fig. 3.2a). The K34 probe detected a prominent transcript of 1.35 kb in both egg and blastula RNA, which is presumably the mature cyt *b* transcript. Since the size of the cyt *b* gene is 1157 nucleotides, a transcript size of 1.35 kb is consistent with a poly(A) tail in the cyt *b* transcript of around 200 nucleotides. An increase in the representation of cyt *b* mRNA between the egg and blastula stages of development is apparent. However, part of this increase is due to the fact that a blastula RNA preparation which had been enriched for mitochondrial RNA was used in this experiment. Purification of blastula RNA was achieved by preparing the RNA from mitochondria which had been purified by subcellular fractionation (see Materials and Methods). Other blots which have used total cellular RNA from both egg and blastula

Transcript	Mass fraction in egg	Mass fraction blastula	Accumulation factor between egg and blastula
16S rRNA	1.4×10^{-2}	1×10^{-2}	0.7
COI mRNA	5×10^{-4}	0.55×10^{-2}	11

Table 3.1 Summary of fluctuations in the prevalence of 16S rRNA and COI mRNA determined from the graphs shown in Fig. 3.1. Mass fractions were calculated as the slopes of the graphs, corrected to account for the fact that the probes used were only complementary to a fraction of the transcripts. The correction factor for SpG30 was 2.5, and for SpP144 was 1.75. The ratio between the mass fractions of each of the transcripts at the egg and blastula stages of development is a measure of the accumulation of the transcripts between the egg and blastula stages.

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stages have shown that there is an increase in the representation of cyt b mRNA between the egg and blastula stages (data not shown, and Elliott and Jacobs, 1989). The fact that purification of mitochondrial RNA enhances the autoradiographic signal obtained for these higher molecular weight transcripts shows that they are bona fide mitochondrially-located transcripts, and not cytosolic transcripts. The nuclear genome of Strongylocentrotus purpuratus is known to contain sequences homologous with mitochondrial genes (Jacobs and Grimes, 1986), some of which might be transcribed into RNA.

In addition to the mature cyt b transcript, a number of higher molecular weight transcripts were detected. A prominent transcript of 2.3 kb was detected in blastula RNA. This may correspond to the 2.3 kb transcript detected with an SpG410 probe (which is specific for 12S rRNA), which showed a decrease in representation between the egg and blastula stages (Elliott and Jacobs, 1988). In addition, transcripts of around 3.5 kb-3.8 kb (which comigrated on this gel) and 6.0 kb were visible in both blastula RNA, and much more faintly in egg RNA. Although there is a slight size difference between the transcripts from egg and blastula RNA in the 3.5-6.0 kb size range, this is most probably a gel artefact. Athough the 3.5 to 6.0 kb transcripts appear to increase in representation between the egg and blastula stages of development in the experiment shown in Figure 3.2a, this is most probably since the blastula RNA preparation was enriched for mitochondrial RNA. Hence, they may correspond to transcripts in the 3.5 to 6.0 kb size range detected with an SpG410 probe, which decrease between the egg and blastula stages (Elliott and Jacobs, 1989).

ND1 mRNA was detected using a Hu/Messing probe generated from clone T1. Three predominant size ranges of transcript were detected (Fig. 3.2b). The most abundant of these was a transcript of 1.25 kb, which is consistent with the size of the ND1 gene (970kb), with a poly(A) tail of 200-250 kb. A 2.4 kb transcript was also detected, which may represent a precursor molecule of the mature ND1 mRNA. There was also a hybridisation signal in the 0.1 kb size range of the filter, which most probably represents detection of 1 or more of the 4 tRNA species which are encoded by the region of the genome contained within T1. The egg tracks are underloaded in this

Figure 3.2: Analysis of mitochondrial transcripts by northern blotting (a) Analysis of cyt b mRNA. Autoradiograph of northern blot of total cellular egg RNA (E), and RNA prepared from purified blastula (B), and probed with nick-translated K34. Around 10 ug of egg and blastula RNA was electrophoresed on a 1% formaldehyde/agarose gel, and blotted onto a Pall Biodyne membrane. (b) Analysis of ND1 and ND2 mRNA by northern blotting. An autoradiograph of a northern blot of total cellular egg (E) and total cellular blastula (B) RNA probed with Hu/Messing probes made from clones T1 and R8. Around 10 ug of egg and blastula RNA was electrophoresed on a 2% formaldehyde/agarose gel, and blotted onto a Pall Biodyne membrane. Hybridisations were carried out in 50% formamide hybridisation buffer, and washed as described in Chapter 2. Transcripts were sized by comparison with the BRL size ladder. The egg track in (b) is underloaded. Hybridisations were carried out in 50% formamide hybridisation buffer, and washed as described in Chapter 2. Transcripts were sized by comparison with a BRL RNA size ladder.

Sizes are given in Kb.

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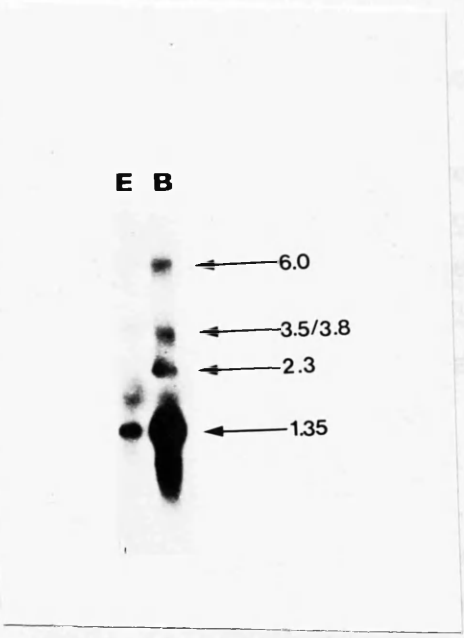
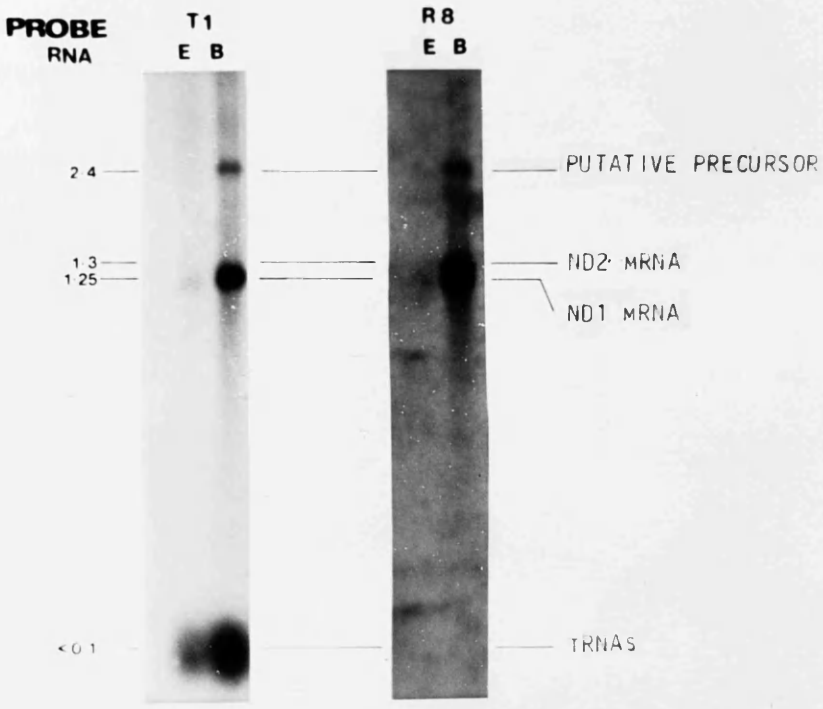
(a)


E B
6.0
3.5/3.8
2.3
1.35

(b)


PROBE
RNA
T1
E B
R8
E B
2.4
1.3
1.25
<0.1
PUTATIVE PRECURSOR
ND2 mRNA
ND1 mRNA
TRNAS

experiment, so it is difficult to make absolute statements about relative transcript prevalences at the egg and blastula stages. The level of the tRNAs showed an apparent increase between the egg and blastula stages. The level of the 1.25 kb transcript and the 2.4 kb trancript showed a greater level of increase than the tRNA(s) between the egg and blastula stages of development, which suggests that these transcripts show a real increase between the egg and blastula stages, whilst ths apparent increase in tRNA level might be indicative of the underloading in the lane loaded with egg RNA.

ND1 and ND2 mRNA were both detected using a Hu/Messing probe synthesised from clone R8 (Fig. 3.3b). In addition to the 1.25 kb transcript, an equally abundant transcript of about 1.3 kb was detected. This is presumably the mature message of ND2, since the gene for ND2 is 90 nucleotides longer than the gene for ND1. In addition, R8 hybridised with a transcript which comigrated with the 2.4 kb transcript detected with T1, and showed a similar apparent pattern of developmental increase in abundance between the egg and blastula stages of development. Since the R8 probe contains sequences which are complementary to both ND1 and ND2, the 2.4 kb transcript might have been detected only as a result of its complementarity to the ND1 containing region of the probe, and so might not contain ND2 reactive sequences. This possibility could be tested by using an ND2-specific single-stranded probe, for example, generated from primer extension from oligonucleotide 128 on clone H3, followed by restriction digestion at the end of the insert and gel purification (in a similar way to how single-stranded probes are made for S1 nuclease experiments- see Section 2.21.1). A faint hybridisation signal in the tRNA region of the filter was also detected. The higher tRNA signal detected by T1, as compared with that detected by R8 (Fig. 3.2b), might be due to the fact that 4 tRNAs rather than one were being detected by the T1 probe, or that the tRNAs detected by T1 are more abundant. This might be especially true of $tRNA^{met}$, which is the translational initiator tRNA, sequences complementary to which are contained within T1.

On longer autoradiographic exposure of these northern blots, additional higher molecular weight transcripts of around 3.5 kb, 5.0 kb and 5.4 kb were also visible detected by T1 and R8 probes (not shown). Higher molecular weight

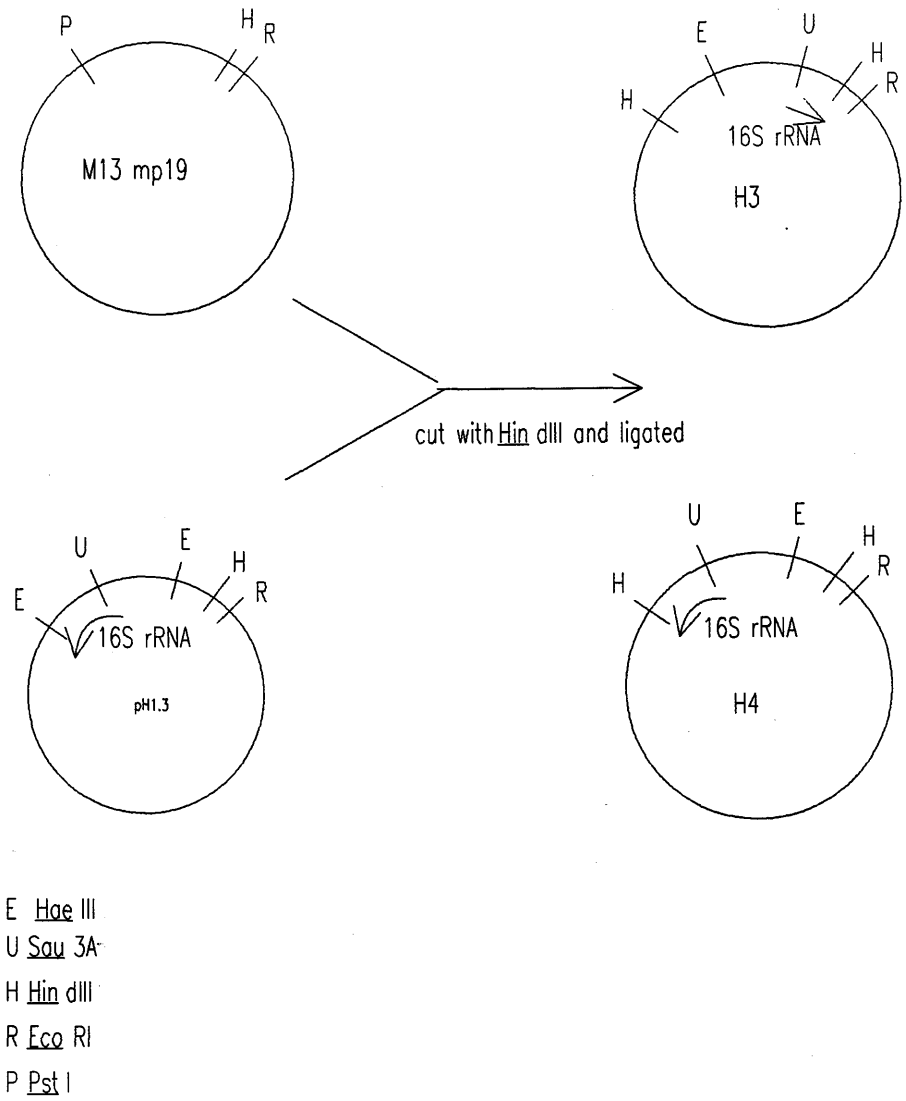


Figure 3.3: Construction of subclones H3 and H4. Subcloning strategy. 1 ug of HindIII-cut M13 mp19 was ligated with 1 ug of HindIII-cut pH1.3.

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transcripts which contain 16S rRNA sequences have been detected in Strongylocentrotus purpuratus RNA (Elliott and Jacobs, 1989). These higher molecular weight transcripts detected by 16S rRNA-specific probes are similar in size to the 3.5-5.4 kb transcripts detected with T1 and R8 probes, and so might contain both 16S rRNA and COI sequences. Higher molecular weight transcripts containing both 16S rRNA and ND2 reactive sequences have been reported by Cantatore et al (1990) for the sea urchin Paracentrotus lividus.

3.2.3 Subcloning the 16S rRNA gene/ ND2 gene junction into M13

In order to determine whether the 16S rRNA and ND2 genes are directly adjacent, or separated by an intergenic region, I sub-cloned and sequenced the junction between these two genes. This gene junction is within the clone pH1.3. Initially the clone pH1.3 was restriction mapped, and orientated with respect to 16S rRNA by hybridisation of a Southern blot of restriction-digested pH1.3, to a probe generated by nick-translation of SpP144 (not shown). The reason for this was to find restriction sites which would be suitable for subcloning the ND2/16S rRNA junction in a minimum size for sequence analysis. However, I was unable to obtain subclones using restriction sites within the pH1.3 insert. For this reason, I subcloned the entire 1.3 kb HindIII insert of pH1.3 into M13mp19, in both orientations, to generate the subclones H3 and H4 (Fig. 3.3). In order to avoid picking spurious white plaques, which had resulted from deletion of vector sequences rather than insertions of pH1.3 sequences, recombinant clones were detected by a plaque filter hybridisation, using a gel-purified, nick-translated pH1.3 insert as a probe (not shown). Single-stranded DNA was purified from 12 plaques and characterised by (A) sequencing ladders (not shown). From a sequence comparison with known S. purpuratus 16S rRNA sequence (H.T. Jacobs, unpublished information), and the upstream part of ND2 in the clone H4 with human ND2 sequence (Anderson et al, 1981), 2 clones were identified with the insert DNA in opposite orientations. The single-stranded DNA of subclone H3 contains the sense-strand of the 16S and ND2 genes, whereas the single-stranded DNA of H4 contains the antisense strand.

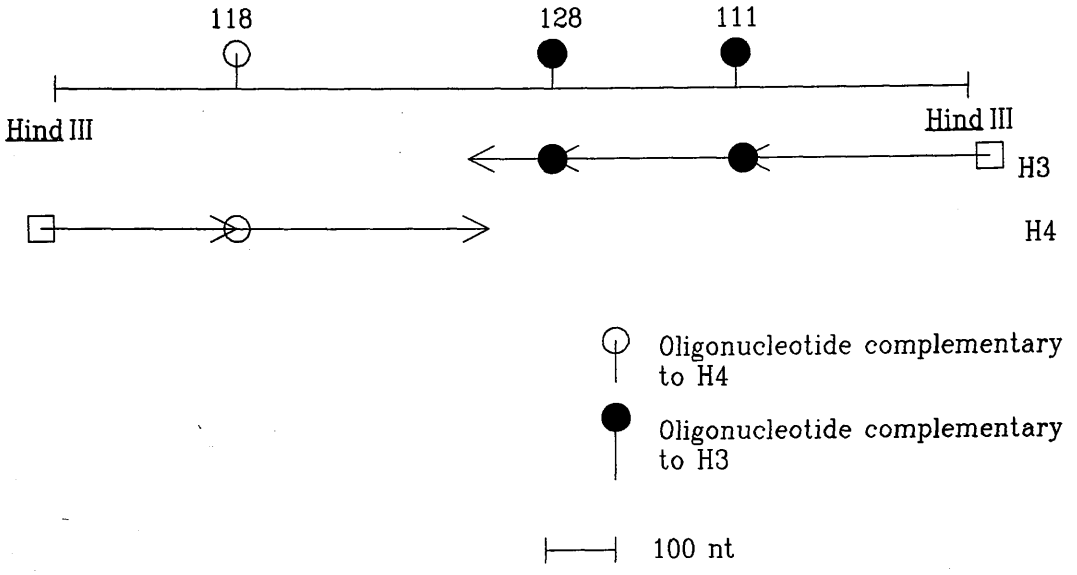

Figure 3.4 Sequencing strategy for the pH1.3 insert, showing the position of the oligonucleotides used to prime sequencing relative to the insert DNA. Filled circles, oligonucleotides complementary to H3; open circles, oligonucleotides complementary to H4; box, universal sequencing oligonucleotide. The extent of sequence obtained from a given oligonucleotide primer is shown as an arrow.

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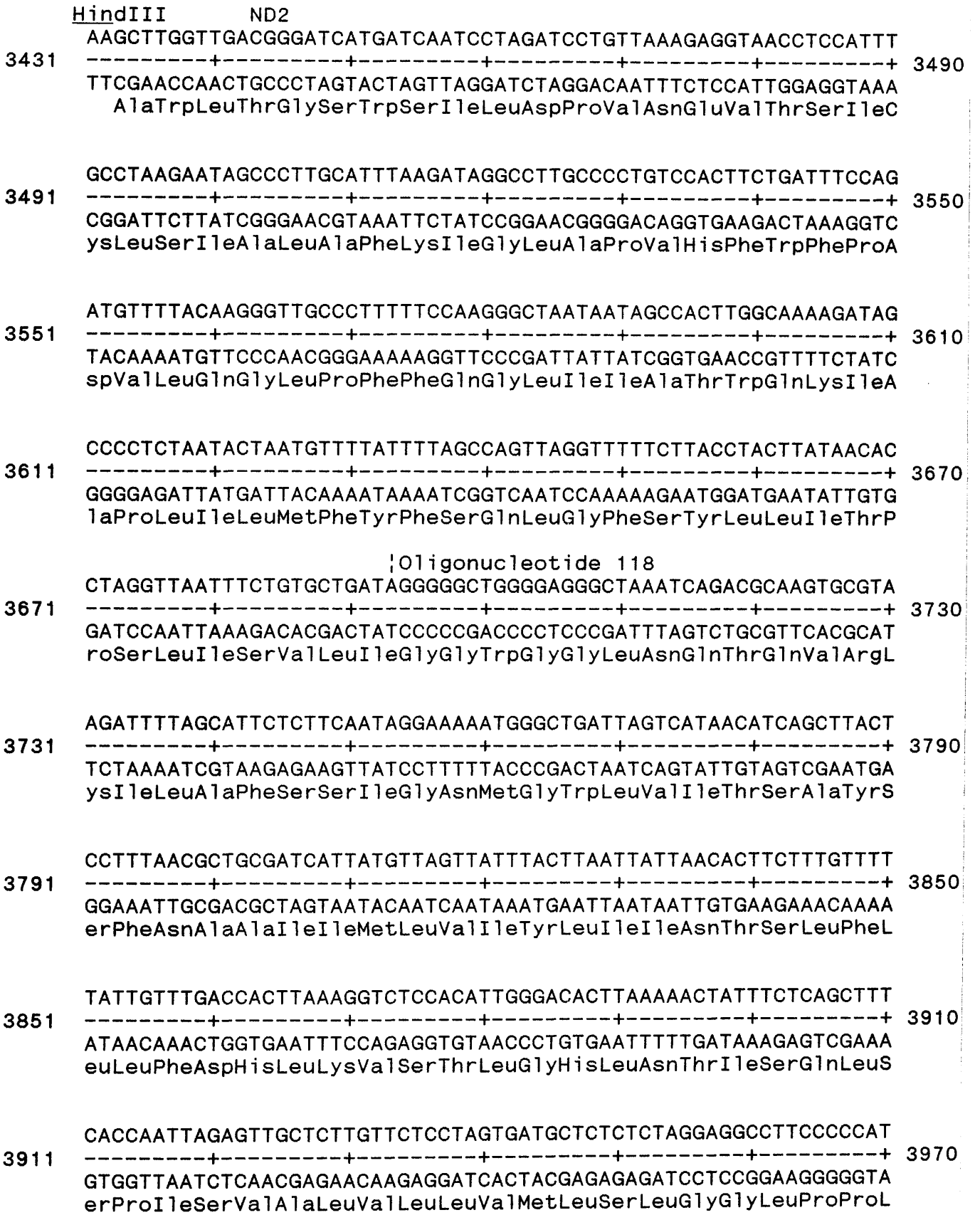
3.2.4 Sequence characterisation of clones H3 and H4.

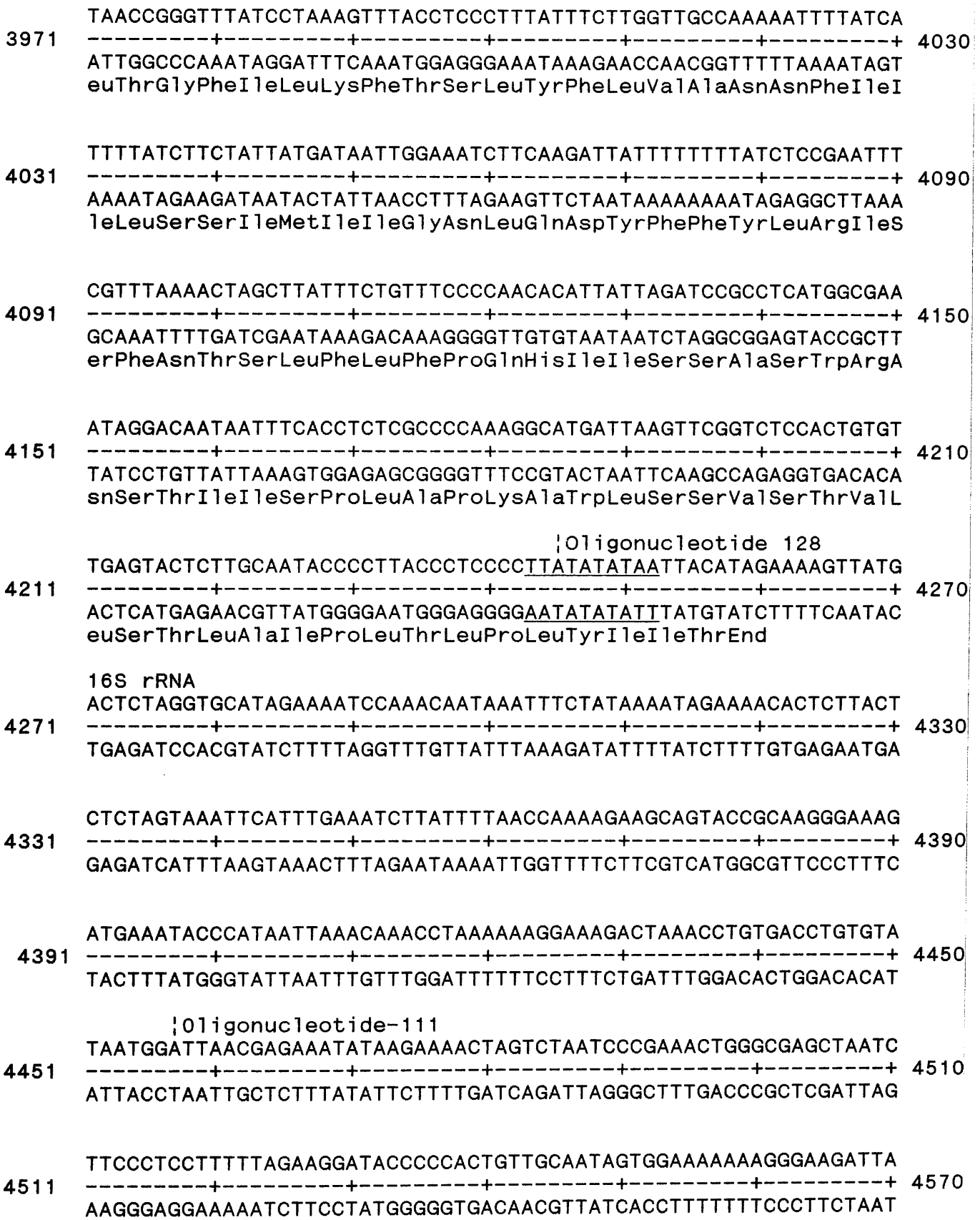
Both H3 and H4 were sequenced using dideoxy sequencing reactions (Sanger et al, 1977) primed from the universal M13 sequencing primer and other synthetic oligonucleotides which were complementary to sequences within the insert (Fig. 3.4). Only a finite amount of sequence data can be obtained from extension of a given oligonucleotide, since during sequencing components of the reaction mix may be used up and so limit extension at a distance from the priming oligonucleotide, and also higher molecular weight bands tend to be poorly resolved on sequencing gels (although resolution can be improved to a certain extent by using buffer gradient or wedge gels). There are two ways to extend sequencing data beyond the limits imposed by these constraints. One of these is to create deletions using exonuclease III or mung bean nuclease, which will shorten the distance between the priming oligonucleotide and the distant portion of the DNA to be sequenced. The other possibility, which I used, is to extend the sequence by priming from an oligonucleotide which is designed to be complementary to the most downstream part of DNA which can be read clearly from the reactions primed from the previous oligonucleotide.

3.2.5 Analysis of the sequence of the pH1.3 insert

The complete nucleotide sequence of the pH1.3 insert is given in Fig. 3.5. The reading frame for the ND2 portion of the pH1.3 insert was deduced from a comparison of the S. purpuratus sequence with known ND2 sequences from other animals (citations given below), and by extending the reading frame from the adjacent, upstream fragment (Jacobs et al, 1988). This reading frame was translated using the S. purpuratus mitochondrial genetic code (Jacobs et al, 1988). The upstream Hind III site (Fig. 3.4) used to generate pH1.3 was internal to this reading frame. The predicted reading frame of ND2 ended at a TAG stop codon at nucleotide 4258 (Fig. 3.5). The sequences flanking this TAG codon presumably represent the region of the junction between the 16S rRNA and ND2 genes.

The GAP programme is a University of Wisconsin Genetics Computer Group (UWGCG) programme which makes an optimal


HindIII
ND2
AAGCTTGGTTGACGGGATCATGATCAATCCTAGATCCTGTTAAAGAGGTAACCTCCATTT
3431
TTCGAACCAACTGCCCTAGTACTAGTTAGGATCTAGGACAATTTCTCCATTGGAGGTAAA
AlaTrpLeuThrGlySerTrpSerIleLeuAspProValAsnGluValThrSerIleC
3490
GCCTAAGAATAGCCCTTGCATTTAAGATAGGCCTTGCCCCTGTCCACTTCTGATTTCCAG
3491
CGGATTCTTATCGGGAACGTAAATTCTATCCGGAACGGGGACAGGTGAAGACTAAAGGTC
ysLeuSerIleAlaLeuAlaPheLysIleGlyLeuAlaProValHisPheTrpPheProA
3550
ATGTTTTACAAGGGTTGCCCTTTTTCCAAGGGCTAATAATAGCCACTTGGCAAAAGATAG
3551
TACAAAATGTTCCCAACGGGAAAAAGGTTCCCGATTATTATCGGTGAACCGTTTTCTATC
spValLeuGlnGlyLeuProPhePheGlnGlyLeuIleIleAlaThrTrpGlnLysIleA
3610
CCCCTCTAATACTAATGTTTTATTTTAGCCAGTTAGGTTTTTCTTACCTACTTATAACAC
3611
GGGGAGATTATGATTACAAAATAAAATCGGTCAATCCAAAAAGAATGGATGAATATTGTG
laProLeuIleLeuMetPheTyrPheSerGlnLeuGlyPheSerTyrLeuLeuIleThrP
3670
Oligonucleotide 118
CTAGGTTAATTTCTGTGCTGATAGGGGGCTGGGGAGGGCTAAATCAGACGCAAGTGCGTA
3671
GATCCAATTAAAGACACGACTATCCCCCGACCCCTCCCGATTTAGTCTGCGTTCACGCAT
roSerLeuIleSerValLeuIleGlyGlyTrpGlyGlyLeuAsnGlnThrGlnValArgL
3730
AGATTTTAGCATTCTCTTCAATAGGAAAAATGGGCTGATTAGTCATAACATCAGCTTACT
3731
TCTAAAATCGTAAGAGAAGTTATCCTTTTTTACCCGACTAATCAGTATTGTAGTCGAATGA
ysIleLeuAlaPheSerSerIleGlyAsnMetGlyTrpLeuValIleThrSerAlaTyrS
3790
CCTTTAACGCTGCGATCATTATGTTAGTTATTTACTTAATTATTAACACTTCTTTGTTTT
3791
GGAAATTGCGACGCTAGTAATACAATCAATAAATGAATTAATAATTGTGAAGAAACAAAA
erPheAsnAlaAlaIleIleMetLeuValIleTyrLeuIleIleAsnThrSerLeuPheL
3850
TATTGTTTGACCACTTAAAGGTCTCCACATTGGGACACTTAAAAACTATTTCTCAGCTTT
3851
ATAACAAACTGGTGAATTTCCAGAGGTGTAACCCTGTGAATTTTTGATAAAGAGTCGAAA
euLeuPheAspHisLeuLysValSerThrLeuGlyHisLeuAsnThrIleSerGlnLeuS
3910
CACCAATTAGAGTTGCTCTTGTTCTCCTAGTGATGCTCTCTCTAGGAGGCCTTCCCCCAT
3911
GTGGTTAATCTCAACGAGAACAAGAGGATCACTACGAGAGAGATCCTCCGGAAGGGGGTA
erProIleSerValAlaLeuValLeuLeuValMetLeuSerLeuGlyGlyLeuProProL
3970


TAACCGGGTTTATCCTAAAGTTTACCTCCCTTTATTTCTTGGTTGCCAAAAATTTTATCA
3971
4030
ATTGGCCCAAATAGGATTTCAAATGGAGGGAAATAAAGAACCAACGGTTTTTAAAATAGT
euThrGlyPheIleLeuLysPheThrSerLeuTyrPheLeuValAlaAsnAsnPheIleI
TTTTATCTTCTATTATGATAATTGGAAATCTTCAAGATTATTTTTTTTATCTCCGAATTT
4031
4090
AAAATAGAAGATAATACTATTAACCTTTAGAAGTTCTAATAAAAAAAATAGAGGCTTAAA
leLeuSerSerIleMetIleIleGlyAsnLeuGlnAspTyrPhePheTyrLeuArgIleS
CGTTTAAAACTAGCTTATTTCTGTTTCCCCAACACATTATTAGATCCGCCTCATGGCGAA
4091
4150
GCAAATTTTGATCGAATAAAGACAAAGGGGTTGTGTAATAATCTAGGCGGAGTACCGCTT
erPheAsnThrSerLeuPheLeuPheProGlnHisIleIleSerSerAlaSerTrpArgA
ATAGGACAATAATTTCACCTCTCGCCCCAAAGGCATGATTAAGTTCGGTCTCCACTGTGT
4151
4210
TATCCTGTTATTAAAGTGGAGAGCGGGGTTTCCGTACTAATTCAAGCCAGAGGTGACACA
snSerThrIleIleSerProLeuAlaProLysAlaTrpLeuSerSerValSerThrValL
¦Oligonucleotide 128
TGAGTACTCTTGCAATACCCCTTACCCTCCCCTTATATATAATTACATAGAAAAGTTATG
4211
4270
ACTCATGAGAACGTTATGGGGAATGGGAGGGGAATATATATTTATGTATCTTTTCAATAC
euSerThrLeuAlaIleProLeuThrLeuProLeuTyrIleIleThrEnd
16S rRNA
ACTCTAGGTGCATAGAAAATCCAAACAATAAATTTCTATAAAATAGAAAACACTCTTACT
4271
4330
TGAGATCCACGTATCTTTTAGGTTTGTTATTTAAAGATATTTTATCTTTTGTGAGAATGA
CTCTAGTAAATTCATTTGAAATCTTATTTTAACCAAAAGAAGCAGTACCGCAAGGGAAAG
4331
4390
GAGATCATTTAAGTAAACTTTAGAATAAAATTGGTTTTCTTCGTCATGGCGTTCCCTTTC
ATGAAATACCCATAATTAAACAAACCTAAAAAAGGAAAGACTAAACCTGTGACCTGTGTA
4391
4450
TACTTTATGGGTATTAATTTGTTTGGATTTTTTCCTTTCTGATTTGGACACTGGACACAT
¦Oligonucleotide-111
TAATGGATTAACGAGAAATATAAGAAAACTAGTCTAATCCCGAAACTGGGCGAGCTAATC
4451
4510
ATTACCTAATTGCTCTTTATATTCTTTTGATCAGATTAGGGCTTTGACCCGCTCGATTAG
TTCCCTCCTTTTTTAGAAGGATACCCCCACTGTTGCAATAGTGGAAAAAAAGGGAAGATTA
4511
4570
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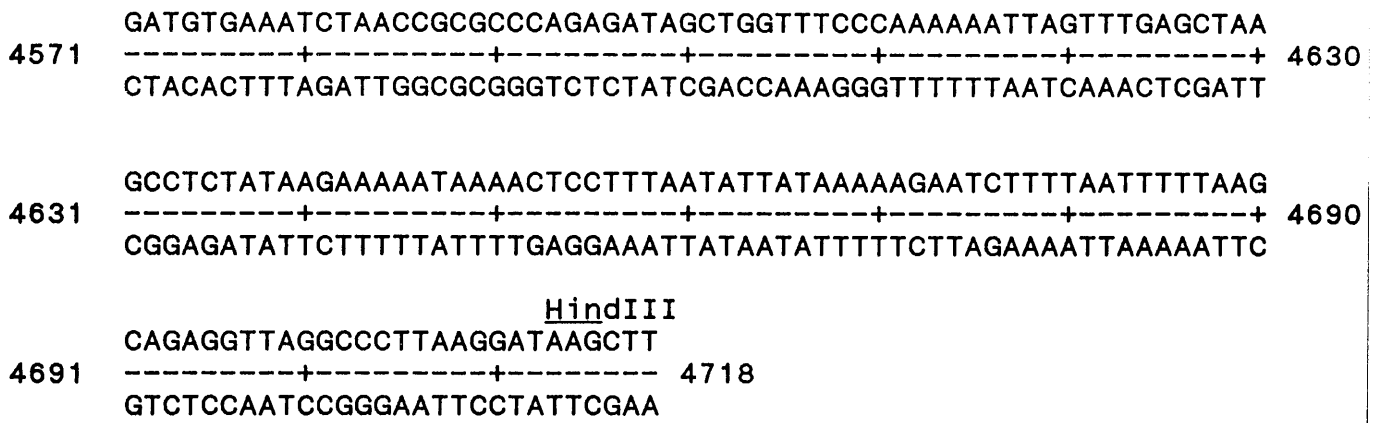

Figure 3.5: Sequence analysis of subclones H3 and H4. Complete sequence of the 1.3 kb HindIII fragment containing the ND2/16S rRNA junction (as deduced from sequence similarities and the reading frame of ND2). The sequences of both of the strands are shown. The upper strand is the coding strand, the lower strand is the non-coding strand. The corresponding amino acid sequence of ND2 is shown according to the standard 3 letter abbreviations for amino acids. The most 5' nucleotide of the synthetic oligonucleotides used to prime sequencing reactions are shown by a vertical line. The TTATATATAA motif upstream of the gene for 16S rRNA is underlined. The nucleotides are numbered as in the complete nucleotide sequence of the sea urchin mitochondrial genome (Jacobs et al, 1988).

(a)

```
                    4262                                                4304
                     |                                                   |
Spurp       ------AAA GTTATGACTC TAGGTGCATA GAAAATCCAA ACAATAAATT
  Hum       --------- --GC.A.AC. ...CCC..A. CCC.C....C CTT.CT.CCA
  Xel       --------- -.CCAA.AA. CTA.CATTCC A.TT..AAT. .......CC.
  Dry       TTATTAT.T A...AT.A.A .TCT.AT... A...TATA.T TATT.T...A

            4305
             |
Spurp       TCTATAA--- --
  Hum       GAC.ACCTTA GC
  Xel       CA...TCTCA T-
  Dry       .--------- --
```

(b)

```
              4211                                                  4258
               |                                                     |
Spurp       --TGAGTACT CTTGCAATAC CCCTTACCCT CCCCTTATAT ATAATTACAT
  Hum       CC.T.CC..G ..ACTCC... .TA.CT...C TTTTA..CTA .....CTT..
  Xel       -------.T. AA.C.T..C. T.A..TATTA TT..AAT.TC .CC....AC.
  Dry       -T.TTTC.A. T..CGG..TA TTT...ATT. .TTTA.T.T. T.TTA...T.

            4259
             |
Spurp       AG-----
  Hum       -------
  Xel       TTAACAT
  Dry       T------
```

Figure 3.6 (a) Alignment of the 5' ends of the predicted S. purpuratus (Spurp) 16S rRNA gene with the corresponding regions of the human (Hum), Xenopus laevis (Xel), and Drosophila yakuba (Dry) mitochondrial 16S rRNA genes. (b) A similar alignment of the predicted 3' end of the sea urchin ND2 gene with the same range of other animal ND2 genes. Nucleotide coordinates of the sea urchin mitochondrial sequence are given as in the complete nucleotide sequence (Jacobs et al, 1988). Nucleotide similarities between the S. purpuratus mitochondrial sequence and each of the other animal mitochondrial sequences are shown as dots, and in the case of differences, by the nucleotide found in the corresponding positions of the Xenopus, human, and Drosophila genomes. Gaps, which have been inserted to give the maximum degree of sequence similarity, are shown as dashes. The sequences are shown in 10 nucleotide blocks, with a space between each block.

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alignment between two complete sequences to maximise the number of matched bases, and minimise the number of gaps (Devereux et al, 1984). Using this programme, I aligned 50 nucleotides at the presumptive 3' end of the presumptive ND2 gene with the sequence of the known 3' ends of the ND2 genes of of the human (Anderson et al, 1981), Xenopus laevis (Roe et al, 1985) and Drosophila yakuba (Clary and Wolstenholme, 1985) mitochondrial genomes, obtained from the EMBL database. The presumptive terminal 50 nucleotides of the S. purpuratus ND2 gene showed a low level of nucleotide similarity with the 3' ends of the human (47.9%), Xenopus (42.2%) and Drosophila yakuba (40.1%) ND2 genes. In particular, there were a number of gaps in the alignment at the termini of the 50 nucleotide segements compared. The predicted C-terminal portion of the ND2 protein, which this 50 nucleotides of the S. purpuratus mitochondrial genome would be predicted to encode, is not highly conserved when compared with the corresponding regions of either the Xenopus, Drosophila or bovine proteins, although the length of the C-terminal portions are fairly similar (Jacobs et al, 1988).

I carried out a similar GAP programme analysis on the 50 nucleotides downstream of the S. purpuratus ND2 TAG putative stop codon. This region of the S. purpuratus mitochondrial genome showed a low level of nucleotide sequence similarity with the 5' terminal 50 nucleotides of the human (37.8%), Xenopus laevis (41.3%) and Drosophila yakuba (45.5%) 16S rRNA genes.

These alignments are illustrated in figure 3.6, using the UWGCG programme LINEUP. Since the predicted 3' end of the S. purpuratus ND2 gene, and the predicted 5' end of the S. purpuratus 16S rRNA gene, show such a low level of nucleotide sequence similarity with the known 16S rRNA and ND2 gene termini from other animal mitochondrial genomes, it is not possible from nucleotide sequence alone to determine whether the sea urchin 16S rRNA and ND2 genes are directly adjacent. For this reason I carried out fine transcript mapping at the 16S rRNA/ND2 gene junction. The results of these experiments are described in the next chapter.

Another reason for the sequence analysis of the pH1.3 insert was to look for sequence motifs which are also found elsewhere in the genome, and which may be involved in the control of mitochondrial gene expression. The sequence

Consensus	TTATATATAA
Putative O_R	TaATATATAA | 1186
tRNA(val)/tRNA(met)	TTATATATAA | 1785
ND2/16S rRNA	TTATATATAA | 4241
tRNA(ser-UCN)/ND3	TcATATATAA | 10187
ND6/cyt b	TTATATATAA | 14487

Figure 3.7 Genomic locations of a reiterated sequence motif found at several locations around the sea urchin mitochondrial genome, including the ND2/16S rRNA gene junction. The motifs are indicated by the genes which flank them, or in the case of the motif found within the single extended intergenic region, as O_R, since this motif may prime replication (Jacobs et al, 1989).Nucleotide coordinates are given as for the complete nucleotide sequence (Jacobs et al, 1988). In each case the nucleotide sequence of the major coding strand is given. The genomic positions of these motifs can be seen on Figure 1.5. The proposed consensus for this sequence is given, and for those motifs in the genome which differ from this consensus, the nucleotides which differ are indicated by lower case letters.

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TTATATATAA, is found within the presumptive coding sequence of ND2, and also between the divergently transcribed genes encoding cyt b and ND6; and between the divergently transcribed genes encoding $tRNA^{met}$ and $tRNA^{val}$ (Fig. 3.8). Sequences with 9/10 matches to TTATATATAA are also found at two locations in the genome: within the single large intergenic region, and between the divergently transcribed genes encoding ND3 and $tRNA^{ser}$(UCN) (Fig. 3.7). Jacobs et al (1989) have calculated that a sequence with at least 9/10 matches to TTATATATAA would be expected to occur at random once every 7000 nucleotides, and from Poisson probability the chance of it occurring 5 or more times would be less than 0.07 in a genome of 15650 bp which was 60% (A+T) rich (like the S. purpuratus mitochondrial genome). Both TTATATATAA motifs, and sequences with 9/10 matches to it, are referred to in this thesis as TTATATATAA-like motifs.

TTATATATAA-like motifs are also found in the mitochondrial genome of the starfish Asterias forbesii in 7 locations, including those mentioned above for Strongylocentrotus purpuratus (Jacobs et al, 1989). The fact that this sequence is also found in Asterias, which is an echinoderm diverged from S. purpuratus by 500-520 milion years (Smith, 1989), suggests that the TTATATATAA-like motifs might have an important functional role, particularly since it is predominantly found in intergenic sequences (at least in S. purpuratus). TTATATATAA and TTATATATAA-like motifs are also found in the mitochondrial genome of the sea urchin Paracentrotus lividus at identical positions to those in S. purpuratus (Cantatore et al, 1989). The distribution of TTATATATAA-like motifs appears to be non-random in that they are found, except in one case (the motif described above, which is apparently within the coding sequence of ND2), in non-protein coding sequence.

In contrast, there are no obvious nucleotide sequence similarities upstream of 16S rRNA and 12S rRNA, and the upstream border of the 12S rRNA gene is not closely adjacent to a TTATATATAA-like motif (Figure 1.5).

3.3 Discussion

3.3.1 Developmental alterations in transcript levels

The experiments described in this chapter have shown that the steady-state level of COI mRNA increases approximately 10- fold between the egg and blastula stages of development, whereas during the same period in development the steady-state level of 16S rRNA decreases around to around 0.7 of its original level. The steady-state level of 16S rRNA exceeds that of COI mRNA by around 30-fold in the unfertilised egg, but this decreases to only around 2-fold at the hatching blastula stage. Although the levels of only 16S rRNA and COI mRNA have been quantitated, increases in transcript abundance between the egg and blastula stages of development in the steady-state levels of ND1 and ND2 mRNAs were suggested from northern blots, although to a certain extent this can be accounted for by an underloading of the egg tracks. From the northern blot data presented in this chapter it is difficult to draw definite conclusions about cyt b mRNA, since in the northern blot shown in Fig. 3.2a total cellular egg RNA was electrophoresed in parallel with mitochondrially enriched blastula RNA. However, a developmental increase in the level of cyt b mRNA has been observed in experiments performed by H.T. Jacobs which have compared the levels of total cellular egg and blastula RNA on northern blots (Elliott and Jacobs, 1989). The time between the egg and blastula represents the first 18 hours of embryonic development in S. purpuratus. The differences in RNA steady-state levels may occur as a result of differences in RNA stability or synthesis between the egg and embryonic stages of development, or a combination of these levels. A northern blot prepared from RNA made from several time-points after fertilisation, by H.T. Jacobs, and then probed for COI mRNA showed that the developmental increase in the steady-state level of COI mRNA may begin shortly after fertilisation (Elliott and Jacobs, 1989).

Developmental differences in the steady-state levels of mitochondrial transcripts have also been described in Xenopus and mice, although these organisms show several differences from the situation in sea urchins. By an analysis of the steady-state Xenopus mitochondrial RNA levels by filter-

hybridisations, El Meziane et al (1989) showed that after fertilisation the level of mitochondrial rRNAs remained constant, whereas the level of mitochondrial mRNAs decreased 5-10 fold after fertilisation and then remained at a low level until organogenesis. In early Xenopus development the differences in steady-state levels of mitochondrial RNA probably occur as a result of the differences in half-life between the two classes of RNA (50 hours and 3 hours for the rRNAs and the mRNAs respectively), rather than as a result of differential transcription, since in vivo-labelling experiments failed to detect any mitochondrial transcription during the early stages of Xenopus embryonic development (Chase et al, 1972; El Meziane et al, 1989). It is, of course, conceivable that Xenopus mitochondria might be impermeable to label at the egg stage of development. After organogenesis, an increase in the level of certain mitochondrial mRNAs was observed, whereas the level of the rRNAs remained constant. In order to establish this increase in the mRNA steady-state level after organogenesis, new RNA synthesis would be required.

A rather different situation is seen in the case of early mouse development (Piko and Taylor, 1987). Between the oocyte and the 2-cell stage the level of both rRNA and at least some mRNAs (COI mRNA and COII mRNA) decreased, but the level of all these transcripts then increased between egg and blastula (the rRNAs by 25% and the mRNAs by 50%). Similarly to the case in sea urchins, the steady-state levels of rRNA and mRNA change differently. Between the egg and blastula stages of development in mice, the ratio of rRNA:mRNA decreased from 32:1 to 10:1, although the ratio increased again to 20:1 in late blastula. These differences in steady-state transcript levels seem to be at least partly established at the level of transcription, since mitochondrial RNA synthesis can only be detected in stages of development after the 2-cell stage, although again it is possible that mitochondria are impermeable to label at and before the 2-cell stage (which would prevent the detection of transcription).

In summary, mitochondrial RNA synthesis in early sea urchin development shows differences from that in both mice and Xenopus. Unlike in mice, mitochondrial RNA is synthesised in the egg (Chapter 5), although the apparent lack of synthesis in mouse eggs might result from an impermeability

to label. Sea urchin eggs are normally impermeable to label, and require protease treatment to allow incorporation of radioactive RNA precursors into mitochondrial RNA (see Chapter 5). Similarly to mice, but totally unlike Xenopus, the ratio of sea urchin mitochondrial rRNA:mRNA decreases between the egg and blastula stages. In mice, however, the steady-state level of rRNA increases between egg and blastula, whereas in sea urchins the steady-state level of the rRNAs decrease slightly.

Despite these apparent differences, there may be underlying similarities between the expression of mitochondrial transcripts in Xenopus, sea urchins, and mice. In all three species, nuclear transcription is minimal in the egg, but increases shortly after fertilisation. Mitochondrial mRNA accumulation seems to begin at an equivalent point in the embryonic development of each species, shortly after the increase in nuclear transcription (El Meziane, 1990). Thus, mitochondrial mRNA accumulation in sea urchins starts soon after fertilisation, in Xenopus between stages 10 and 19 of embryonic development, and in mice at the 4-cell stage. This suggests that de novo nuclear transcription is required for mitochondrial mRNA accumulation to occur. This de novo nuclear transcription might be required for the synthesis of a protein specifically stimulating mitochondrial mRNA (as opposed to rRNA) synthesis. This protein might act at the transcriptional or the post-transcriptional level.

Although the examples discussed above are from early development, differential control of mitochondrial rRNA and mRNA might be a general phenomenon. In rats after birth the levels of rRNA and mRNA fluctuated differently, and were differentially affected by thyroid hormone levels (Cantatore et al, 1985, 1986).

3.3.2 Examination of higher molecular weight transcripts

The higher molecular weight species detected on northern blots may be precursors to the mature RNA species. Higher molecular weight transcripts of a similar size were detected by probes specific for 12S rRNA and cyt b mRNA. This may also be the case for the transcripts detected by probes specific for ND1 and ND2 mRNA. It is important to remember that only the most abundant higher molecular weight transcripts would

be detected on northern blots. In addition to the rate of synthesis, the abundance of higher molecular weight transcripts would also be determined by the rate of transcript turnover, with only stable, slowly processed transcripts being detected. It is therefore conceivable that only a fraction of the existing higher molecular weight transcripts are being detected. This point is discussed further in chapter 5.

The observed developmental increase in some higher molecular weight transcripts is consistent with their synthesis between the egg and blastula stages, and, by inference, the use of the synthetic pathways in which they are putative intermediates, to produce mature mitochondrial transcripts. The abundance of the 2.3 kb transcript detected with SpG410 and K34 probes increases between the egg and blastula stages of development, during which time the steady-state level of cyt b mRNA also increases (Elliott and Jacobs, 1989). The steady-state level of 12S rRNA decreases between the egg and blastula stages, as does the level of the 3.5 kb, 3.8 kb and 6.0 kb transcripts (Elliott and jacobs, 1989). The 2.3 kb transcript might therefore represent a precursor to cyt b mRNA, rather than a precursor to 12S rRNA. The 3.5 kb, 3.8 kb and 6.0 kb may represent 12S rRNA precursors rather than cyt b mRNA precursors.

The 2.4 kb transcript detected with T1 and R8 probes shows a developmental increase in abundance between the egg and blastula stages of development, as do ND1 and ND2 mRNAs, and at least some of the tRNAs encoded by this region of the genome. Thus, the 2.4 kb transcript may represent a precursor for ND1 and ND2 mRNA.

The higher molecular weight transcripts detected contain RNA corresponding to several genes, and in some cases are larger than the distance between adjacent TTATATATAA-like motifs. These motifs therefore cannot simply demarcate transcription units by acting as sites for transcriptional initiation/termination, or RNA processing sites. In contrast, some of the higher molecular weight transcripts are of the correct size range to have 5' ends within TTATATATAA-like motifs, if their 3' ends correspond to the 3' ends of genes. It is possible that the 2.4 kb transcript, detected with T1 and R8 probes, might initiate at the TTATATATAA motif within the tRNA cluster between $tRNA^{met}$ and $tRNA^{val}$. A transcript

which initiated at this TTATATATAA motif, and contained the coding information for ND1 and ND2, would be predicted to be approximately 2.4kb in size, if it was not polyadenylated. The polyadenylation status of this 2.4 kb transcript is clearly open to experimental investigation by oligo(dT)-cellulose chromatography. Whether the 2.4 kb transcript contains information for ND2 as well as ND1 could be determined by the approach described above (making a single-stranded probe specific for ND2 mRNA). The 2.3 kb transcript detected with SpG410 and K34 probes, which contains 12S rRNA and cyt b sequences, could similarly have initiated at the TTATATATAA-like motif upstream of the cyt b gene, and terminate at the 3' end of the 12S rRNA gene.

The fact that higher molecular weight transcripts are detected at all suggests that, at least to a certain extent, endonucleolytic cleavage of the primary transcripts does not occur concomitantly with transcription, and endonucleolytic cleavage of some sites must occur some time after they have been transcribed. This is interesting, since the mitochondrial matrix is not compartmentalised into membrane-bounded spaces, and so the RNA processing machinery is not spatially separated from the nascent transcripts. Although the existence of these molecular weight transcripts might reflect an inherently slow rate of transcript processing, their existence might alternatively suggest that large regions of flanking RNA sequences are required for the endonucleolytic processing machinery to select, and cleave, its target sites.

This idea is particularly intriguing when one considers the physical organisation of the sea urchin mitochondrial genome (Fig. 1.5). Unlike most other animal mitochondrial genomes characterised (Section 1.2), the tRNA genes are predominantly clustered in one region of the genome, and not dispersed around the genome between other (i.e. mRNA and rRNA) genes. It has been suggested that for those genomes with a dispersed arrangement of tRNA genes, such as the vertebrate mitochondrial genome, RNA processing might depend on the local secondary structure induced by the tRNA portion of the primary transcripts (section 1.11.1). The paucity of higher molecular weight transcripts detected in mammalian mitochondria might be consistent with endonucleolytic cleavage occurring concomitantly with transcription, although

not necessarily being the first event in RNA processing (Clayton, 1984; section 1.10.1; Figure 1.2). In contrast, a number of higher molecular weight transcripts are detected in the case of S. cerevisiae mitochondria, the genome of which, like that of S. purpuratus, is also notable for its clustering of tRNA genes (Fig 1.2). The endonucleolytic cleavage machinery of mitochondria such as those of S. cerevisiae and S. purpuratus, might depend on the secondary structure of large nascent transcripts for target site selection, rather than short regions of secondary structure. It is also notable in this context that in mouse mitochondria, endonucleolytic processing at junctions within primary transcripts which did not contain tRNAs was less rapid than those which did, again suggesting that the primary transcript as a whole might be the precursor for endonucleolytic cleavage reactions, rather than just local secondary structure conformations around the gene junction (Section 1.11.1). The functional implications of this point are discussed in chapter 6.
It is important to note, however, that a product/precursor relationship has not been established between these higher molecular weight transcripts and the mature mitochondrial transcripts.

3.3.3 Sequence of the ND2/ 16S rRNA gene junction

This has been discussed in some detail above, and for this reason I will not comment further upon it at this point.

CHAPTER 4

FINE MAPPING OF THE PRIMARY STRUCTURE OF SEA URCHIN MITOCHONDRIAL TRANSCRIPTS

4.1 Introduction

Although mitochondrial genes can be identified on the basis of sequence homology, fine transcript mapping is required to locate transcript termini unambiguosly. For example, the sequence analysis of the 16S rRNA/ND2 junction described in the previous chapter suggested that these two genes are directly adjacent, both on the basis of sequence similarity between these genes and their homologues in other organisms, and the extension of the ND2 reading frame up to the predicted 5' end of the 16S rRNA gene. However, this is not conclusive evidence, since the termini of these genes are not highly conserved.

The exact location of sea urchin mitochondrial transcript termini is of interest for two reasons. Firstly, non-coding intergenic sequences have been shown to contain elements involved in mitochondrial gene expression in other organisms. The sequence elements involved in the promotion of vertebrate mitochondrial transcriptional initiation are located in the major intergenic region of the genome (section 1.5.1 and 1.9.1), and the shorter promoter elements of yeast mitochondria are located in intergenic sequences (section 1.6.1 and 1.9.2). Intergenic sequences are thus candidates for containing such elements in sea urchin mitochondrial DNA.

Secondly, it was of interest to determine whether the developmental changes in transcript abundance observed in the previous chapter could be correlated with changes in transcript primary structure. It was conceivable that the termini of mature transcipts would differ at different developmental stages so as to contain flanking sequences which might influence RNA stability, either to stabilise a transcript from RNases (for example a hairpin structure, which might act to block exonuclease action), or to destabilise them (for example by including a binding site for an RNA endonuclease). This is an important point, since the steady-state level of a transcript is determined both by its rate of synthesis and its rate of decay. No gross differences in transcript size were apparent from the northern blots described in the previous chapter.

I have concentrated fine transcript mapping on the transcripts encoded by regions of the genome investigated by northern hybridisation in the previous chapter, i.e. the

region between cyt b and COI (Fig.1.5). I have also mapped transcripts adjacent to the TTATATATAA-like motifs described in the previous chapter.

4.2 Results

4.2.1 Mapping of the 5' ends of the 16S and 12S rRNA transcripts

Synthetic oligonucleotide primers 111 and 106 were annealed to total sea urchin RNA and extended using the enzyme Mu-MLV reverse transcriptase to map the 5' ends of the 16S and 12S rRNA transcripts respectively (Fig. 4.1). Primer extension reaction products were electrophoresed on 5% polyacrylamide sequencing gels in parallel with sequencing reactions primed from the same oligonucleotide on template clones H3 and pP12, which contain genomic sequence for the 5' junctions of 16S and 12S rRNA respectively. This means that, in the absence of RNA editing or splicing, primer extension products should run exactly alongside the nucleotide corresponding to the 5' end of the gene.

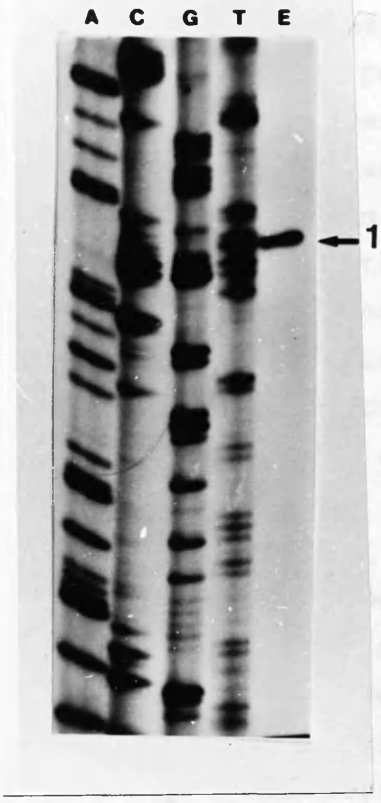
In the case of 12S rRNA a single primer extension product was obtained with sea urchin egg RNA, which defined a 5' end which mapped to a position in the genomic sequence to the nucleotide downstream of the 3' boundary of the $tRNA^{phe}$ gene (Fig. 4.1a and b), as predicted from the secondary structure of $tRNA^{phe}$ (Jacobs et al, 1988). This implies that there is no intergenic sequence between $tRNA^{phe}$ and 12S rRNA. As numbered in the genomic sequence (Jacobs et al, 1988), the 5' end of the 12S rRNA gene was mapped by this experiment to nucleotide G-64. Transcript termini are given as map coordinates in this chapter according to the nucleotide numbering of Jacobs et al, (1988). The complete nucleotide sequence of the S. purpuratus mitochondrial genome is reproduced in the appendix at the end of this thesis.

Two major primer extension products were seen in the case of 16S rRNA: a predominant product mapped 2 nucleotides downstream of the presumptive ND2 stop codon (nucleotide A-4261), and a second minor 5' end mapped to the adjacent 5' nucleotide (Fig. 4.1 c and d). The mechanism resulting in the formation of two 5' ends for 16S rRNA is not clear. A possibility is that they may arise due to an imprecision in

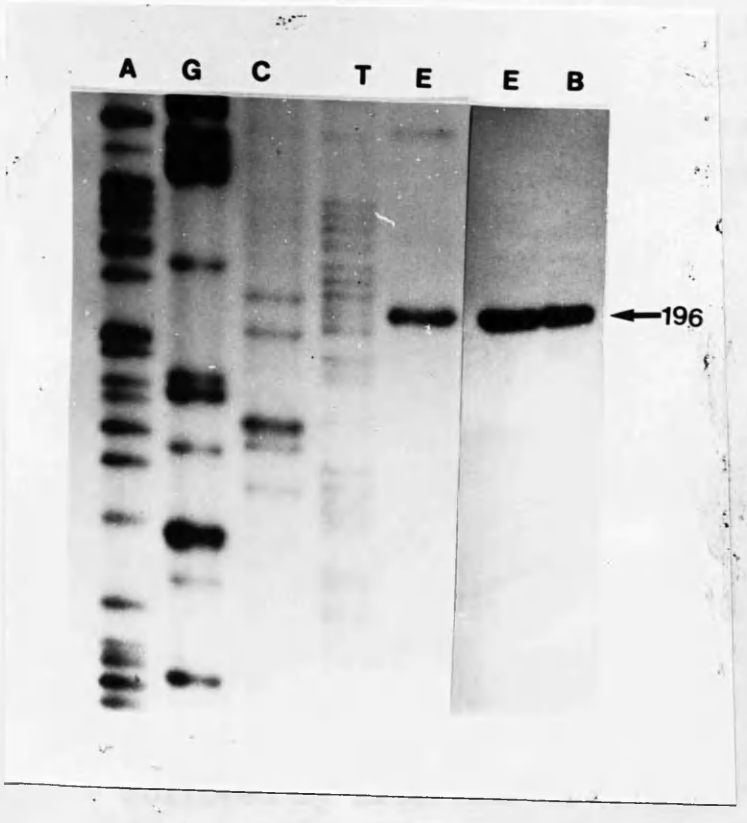
Figure 4.1: Primer extension mapping of the 5' ends of 16S rRNA and 12S rRNA. (a) Extension product of egg RNA primed with oligonucleotide 106, and electrophoresed in parallel with sequencing reactions (lanes ACGT) primed from oligonucleotide 106 on template pP12. (b) Summary diagram (not to scale) to show the mapped 5' end of 12S rRNA with respect to the SalI-SacI insert of pP12. (c) Extension products of blastula (B) and egg (E) RNA primed with oligonucleotide 111, and electrophoresed in parallel with sequencing reactions (lanes ACGT) primed from oligonucleotide 111 on clone H3. Two autoradiographs are spliced together in this figure, by aligning the primer extension products, and the sequencing ladders (not shown), to show that the primer extension products obtained from egg and blastula RNA were identical, and that the higher molecular weight primer extension products obtained by extending oligonucleotide 111 on egg RNA were not reproducible. (d) Summary diagram (not to scale), showing the mapped 5' end of 16S rRNA with respect to clone H3. The summary diagrams are annotated as described in the figure.

(Facing page 94)

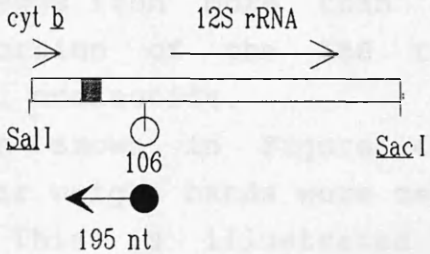
(a)


A C G T E
195

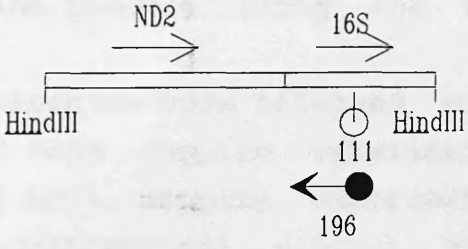
(b)

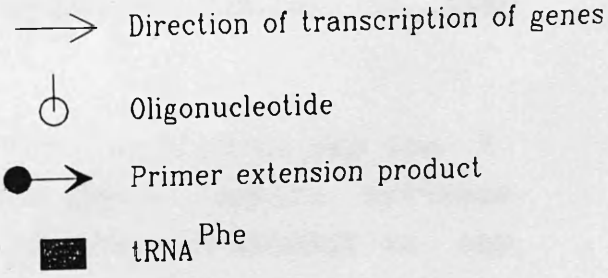

A G C T E E B
196

(b)


cyt b
12S rRNA
SalI
Sac I
106
195 nt

(d)


ND2
16S
HindIII
HindIII
111
196


Direction of transcription of genes
Oligonucleotide
Primer extension product
tRNA Phe

the processing machinery, which would have to distinguish between 4 adjacent (A) residues to select the most ND2 proximal nucleotide (if this is indeed a site of RNA processing). Another possibility is that there might be some genomic sequence polymorphism for the 16S rRNA gene, which, since the RNA was prepared from eggs from more than one female, might result in a proportion of the 16S rRNA transcripts containing an additional nucleotide.

On one of the autoradiograph shown in Figure 4.1a additional higher and lower molecular weight bands were seen, but these were not reproducible. This is illustrated in Figure 4.1a, where the reaction products from a different primer extension experiment are also shown. Hence the additional higher molecular weight transcripts might have arisen as a result of spurious hybridisation of the priming oligonucleotide, perhaps as a result of a slight difference in hybridisation conditions used in this experiment. The lower molecular weight bands can be explained as being premature termination products arising as a result of RNA secondary structure, or RNA degradation, stopping the extension of the primer extension product along the RNA template.

Identical primer extension products were obtained with egg and blastula RNA for the 16S rRNA mapping experiments (Fig. 4.1c), and also for the 12S rRNA mapping experiments (not shown). Control reactions carried out without RNA, without primer and without either primer or RNA gave no signal (not shown).

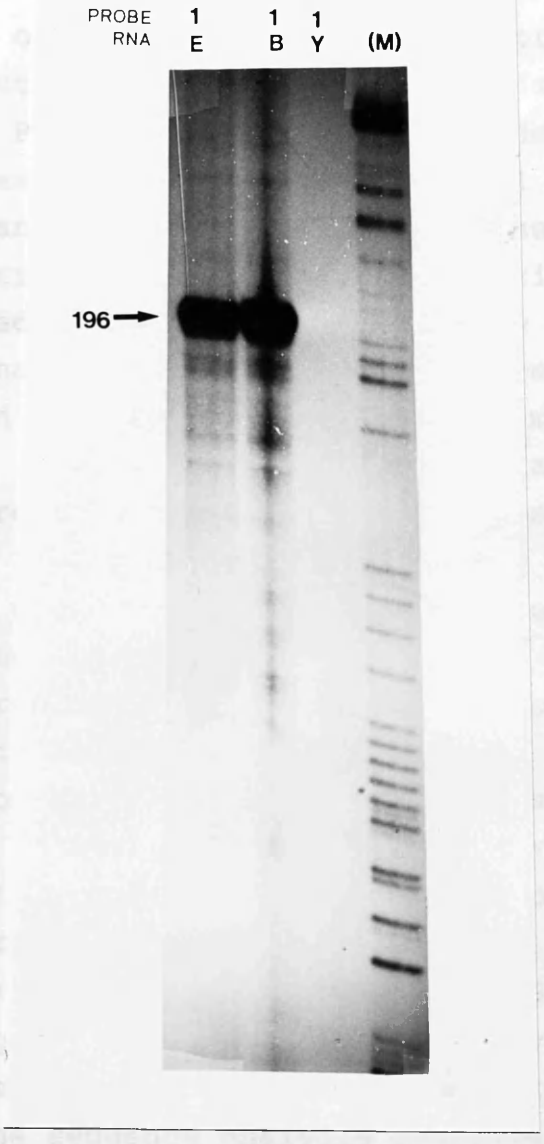
4.2.2 S1 nuclease mapping of transcripts at the ND2/16S rRNA junction

Primer extension mapping can only be used to map the 5' termini of transcripts. In order to map 3' termini nuclease protection assays are necessary. In order to attempt to map the 3' end of the ND2 transcript, I used an S1 nuclease experiment, using a single-stranded radio-labelled probe (probe 1). This was generated from the extension of oligonucleotide 111, using the template ssDNA of clone H3, followed by digestion with XhoII (Fig. 4.2a). This probe was internally labelled and so should have been able to detect both transcripts across the junction. A 196 nucleotide

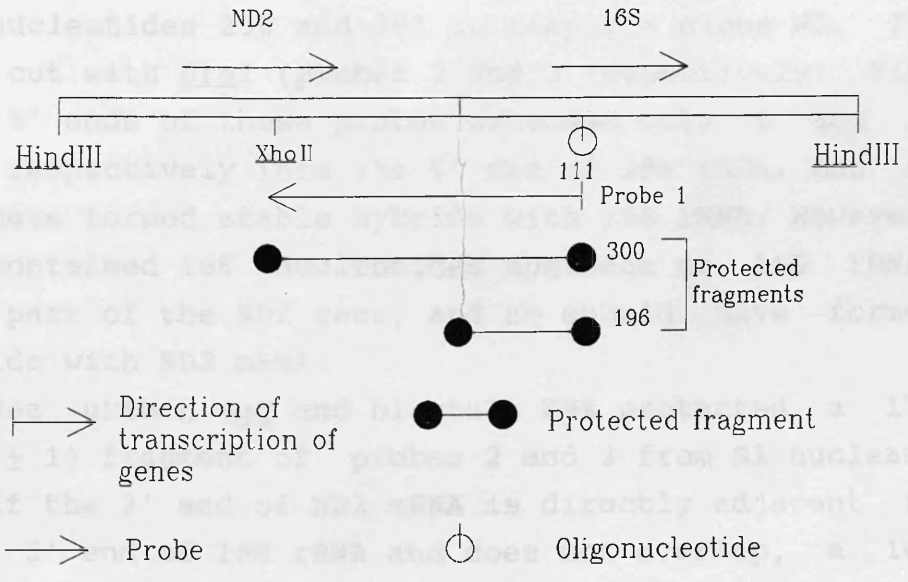
Figure 4.2: S1 mapping of the ND2/16S rRNA junction. (a) Summary diagram (not to scale) to describe how probe 1 was synthesised, showing the position of oligonucleotide 111, which was used to prime probe synthesis, and the XhoII restriction site which was used to generate the probe 3' end, on the template clone H3. The fragments of the probe protected by sea urchin RNA from digestion from S1 nuclease are shown, with their sizes in nucleotides indicated. Reaction products were analysed on a 5% polyacrylamide/urea (sequencing) gel. (b) An autoradiograph of the 5% polyacrylamide/urea gel. The 196 nt (±1) reaction product was sized by reference to a sequencing ladder. Of this ladder, the (G) track obtained by extension of the universal M13 oligonucleotide (17-mer) on clone HP1, is shown in the figure (M). Other lanes are indicated as to the reacting RNA: Y (yeast tRNA negative control), B (sea urchin blastula RNA) and E (sea urchin egg RNA).Symbols are as indicated on the figure.

(Facing page 96)

(a)


PROBE
RNA
1
E
1
B
1
Y
(M)
196

(b)


ND2
16S
HindIII
XhoII
111
HindIII
Probe 1
300
196
Protected fragments
Direction of transcription of genes
Protected fragment
Probe
Oligonucleotide

fragment of probe 1 was protected from S1 nuclease digestion by both egg and blastula stage sea urchin RNA (Fig. 4.2b). This is the size of fragment expected from protection by 16S rRNA, as predicted by the primer extension experiment described above. Probe protection was dependent on sea urchin RNA since it was not observed when probe 1 was hybridised with yeast tRNA and digested with S1 nuclease.

No band corresponding to probe protection by ND2 mRNA alone was observed. There are three possible explanations for this. One is that a weaker signal would be expected from probe protection by ND2 mRNA, and this might have been swamped out by lower molecular weight labelled material resulting from probe degradation. Another possibility is that ND2 mRNA and 16S rRNA are butt-joined together, or overlapping, as would be suggested by the sequence analysis described in the previous chapter. This would lead to complete probe protection, without an ND2-protected band, if probe were not in excess. Higher molecular weight material was protected from S1 nuclease digestion by sea urchin RNA in this experiment (not shown). The reasons why complete probe protection might be expected are discussed below in section 4.2.4. The third possibility is that the probe fragments resulting from protection by ND2 mRNA and 16S rRNA comigrated on sequencing gels. This last possibility is the least likely, from the sizes of protected fragment which would be predicted from the sequence analysis described in Chapter 3.

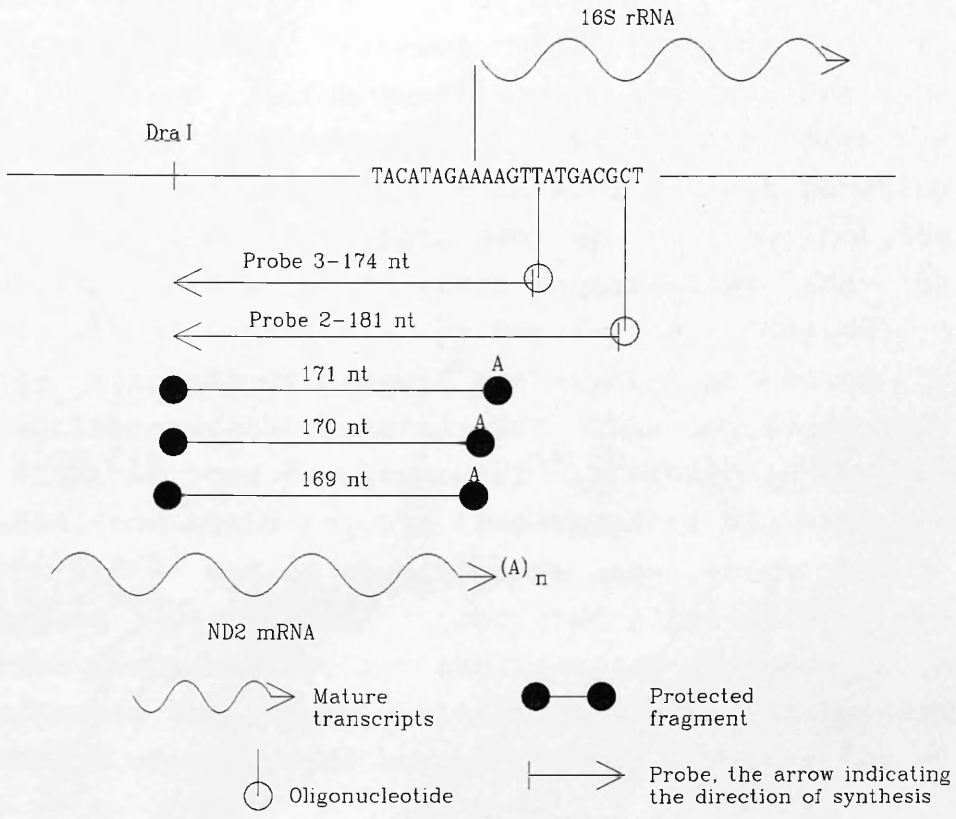
In order to map the 3' end of ND2 mRNA directly, I carried out an S1 nuclease experiment using probes primed from oligonucleotides 290 and 301 on template clone H3. The probes were cut with DraI (probes 2 and 3 respectively: Fig. 4.3). The 5' ends of these probes extended only 6 and 13 nucleotides respectively into the 5' end of 16S rRNA, and so should not have formed stable hybrids with 16S rRNA. However, they also contained 168 nucleotides upstream of 16S rRNA, containing part of the ND2 gene, and so should have formed stable hybrids with ND2 mRNA.

Both sea urchin egg and blastula RNA protected a 170 nucleotide ($\pm$ 1) fragment of probes 2 and 3 from S1 nuclease digestion. If the 3' end of ND2 mRNA is directly adjacent to the mapped 5' end of 16S rRNA and does not overlap, a 168 ($\pm$1) nucleotide protected fragment would have been predicted. However, the putative stop codon of ND2 is followed by 4 (A)

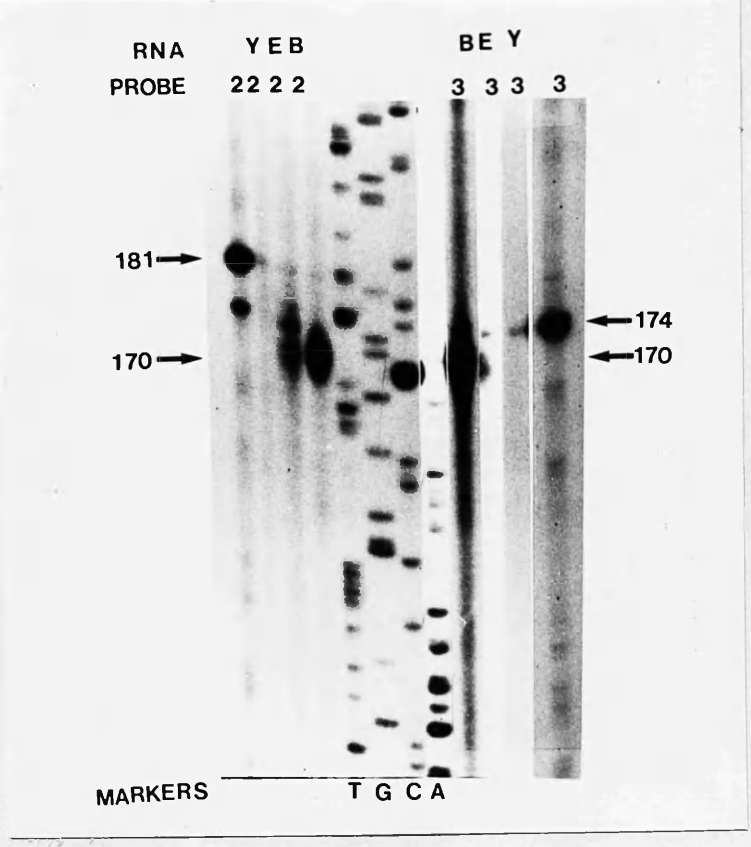
Figure 4.3: S1 mapping of the 3' end of ND2 mRNA. (a) Summary diagram (not to scale) showing how probes 2 and 3 were synthesised by extension from two oligonucleotides 290 and 301, which are complementary to sequences just within the 5' region of the 16S rRNA gene, on the template clone H3. The 3' ends of the probes were created by cutting, after extension, at the DraI site. The fragments of the probes protected after S1 nuclease digestion are shown. The (A) residues at the 5' end of both the probes, which were the most 5' residues in the probes which were protected, are indicated. The symbols used are indicated on the figure. (b) The reaction products were analysed on a 5% polyacrylamide/urea (sequencing) gel. Lanes are indicated as: ACGT (the (A), (C), (G) and (T) reactions of a T3 sequencing ladder primed with the universal M13 17-mer primer); Y, E, B (probe hybridised with yeast tRNA, sea urchin egg RNA and blastula RNA respectively, and digested with S1 nuclease). The probe (2 or 3) used in each lane is indicated, as is the size in nucleotides of the protected fragments and the unreacted probes. A number of photographs of different autoradiographic exposure times of the same sequencing gel are spliced together in the figure, to give a more comparable exposure time for each lane.

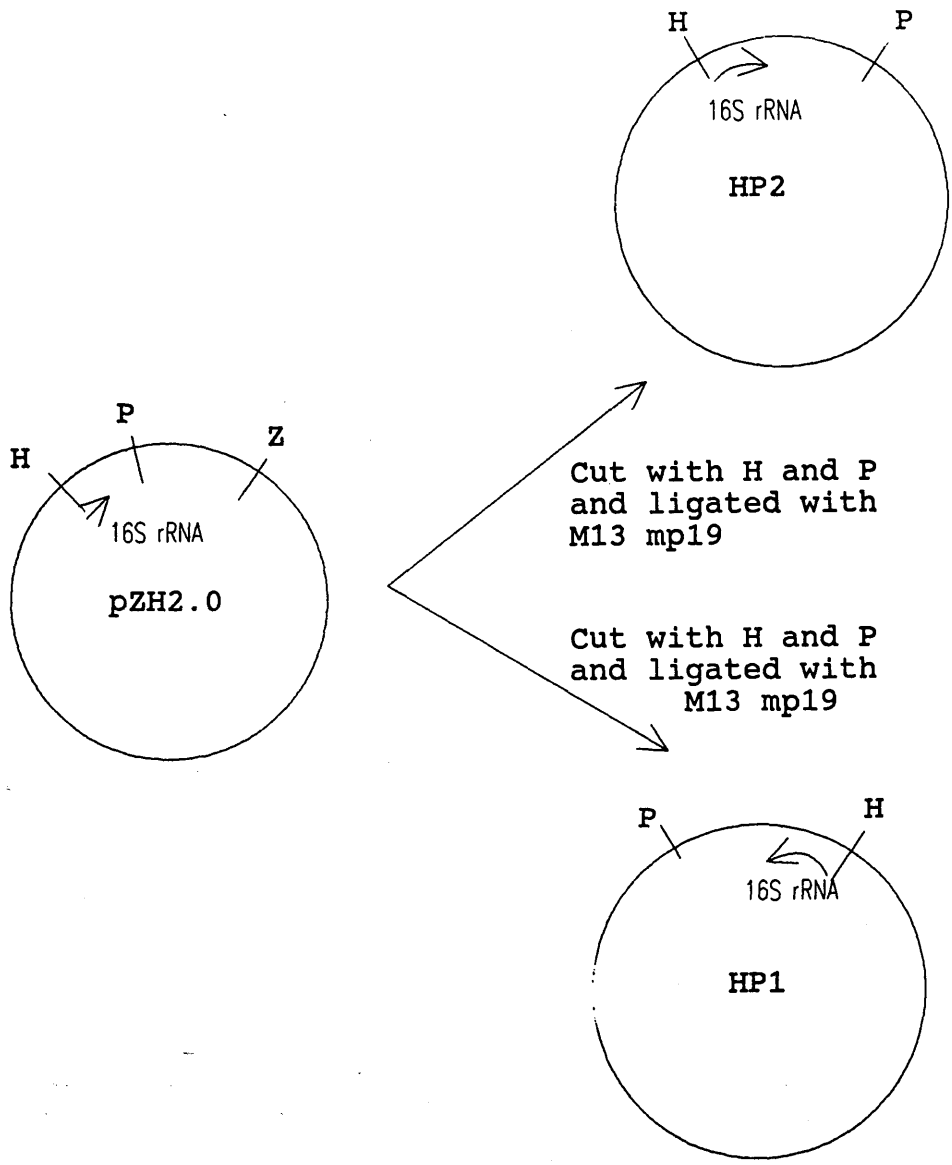
(Facing page 98)

(a)


16S rRNA
Dra I
TACATAGAAAAGTTATGACGCT
Probe 3-174 nt
Probe 2-181 nt
171 nt
170 nt
169 nt
A
(A) n
ND2 mRNA
Mature transcripts
Protected fragment
Oligonucleotide
Probe, the arrow indicating the direction of synthesis

(b)


RNA
PROBE
YEB
22 2 2
BE Y
3 3 3 3
181
170
174
170
MARKERS
T G C A



H - HindIII

P - Pst I

Z - Sac I

Figure 4.4: Subcloning of the 16S rRNA/COI junction. pZH2.0 was cut with HindIII and PstI, and ligated into HindIII/PstI-cut M13mp18 and mp19 R.F., to create the subclones HP1 and HP2 respectively.

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residues in the genomic sequence. These would have corresponded to 4 (T) residues in the probe which would have been protected by the poly(A) tail of ND2 mRNA. This would result in the observed fragment size of 170 (± 1) nucleotides. If this is the case, the 3' end of the ND2 transcript prior to polyadenylation might map directly adjacent to the 5' nucleotide of 16S rRNA. It cannot be ruled out, however, that the transcripts overlap 1-3 nucleotides prior to polyadenylation (which would require that they be synthesised by mutually exclusive pathways: see dicussion). A 174 nucleotide fragment of probe 2 was visibly protected by sea urchin egg RNA, but not blastula RNA. This is suggestive that there might be some developmental difference in the 3' end of ND2 mRNA, and would require the 3' end of ND2 mRNA to overlap with the 5' end of the 16S rRNA gene (Fig. 4.3a). Both probes 2 and 3 were of their predicted sizes of 181 and 174 nucleotides respectively, and the reaction products were shorter, confirming that there was no stable hybrid formation with 16S rRNA, which would have led to complete probe protection.

4.2.3 Subcloning the 16S rRNA/COI gene junction into M13

Plasmid pZH2.0 was restriction-mapped (not shown) in order to locate sites which could be used to subclone the 3' end of 16S rRNA into M13, in a size suitable for making probes for S1 nuclease experiments (i.e. which were of a size which could be analysed conveniently on a sequencing gel, the insert of pZH2.0 being too large to fulfil this criterion). The 800bp HindIII-PstI fragment of pZH2.0 was subcloned into M13mp18 and mp19 to create the subclones HP1 and HP2 (Fig. 4.4). These subclones were initially characterised by running (A) sequencing ladders, then sequencing and comparison of this data with known sea urchin mitochondrial DNA sequences (Jacobs and Grimes, 1986). Although they both contain the 3' end of 16S rRNA, they differ in orientation as a result of the asymmetry of the polylinkers of M13mp18 and mp19.

4.2.4 Mapping of transcript termini at the 16S rRNA/ COI mRNA junction

Transcript termini at the 16S rRNA/COI junction were mapped using an S1 nuclease protection assay. Single stranded-probes were synthesised from clones HP1 and HP2, primed from the M13 universal (17 mer) sequencing primer (Fig. 4.5a). Probe 5, synthesised from HP1, was of the same sense as both 16S rRNA and COI mRNA. This probe was not protected from S1 nuclease digestion by sea urchin RNA (Fig. 4.5b and c), so there is no significant amount of antisense transcripts with discrete termini encoded by this region of the genome. In contrast, probe 4, synthesised from HP2, was protected to give 2 fragments: one of 162 ±1 nucleotides, and another fragment of approximately 828 (± 1) nucleotides (Fig. 4.5b and c). The 828 ±1 nucleotide fragment corresponds to the size of the complete probe (867 nt), minus the polylinker sequence (39 nt) which it contains. Neither probe 4 or 5 was protected by yeast tRNA from digestion by S1 nuclease, showing that the protected bands seen for probe 4 were a result of protection by sea urchin RNA.

In order to determine the map positions of the reaction products, the transcript termini at the junction were S1 mapped using probe 6 (Fig. 4.6). This was primed from oligonucleotide 159, which is complementary to a region within the insert of HP2. Probe 6 was cut at the same HindIII site as probe 4. This means that probes 4 and 6 were 3' coterminal. Two fragments of probe 6 were protected from S1 nuclease digestion by sea urchin RNA (Fig. 4.6b); one of 162 (± 1) nucleotidess, and another of 258 (± 1) nucleotides which corresponds in size to complete protection of the probe. A faint band of 258 nucleotides (± 1) was also seen in the yeast tRNA negative control lane, but at a much lower intensity in comparison to the 258 nucleotide band for the reactions containing sea urchin RNA. The latter therefore probably results from specific protection by sea urchin RNA.

As well as complete protection of the sea urchin mitochondrial DNA sequences in the probes (which is explained below), 162 nucleotide (± 1) fragments of probes 4 and 6 were protected by egg and blastula RNA. Since probes 4 and 6 were 3' coterminal, these common 162 nucleotide fragments must represent probe protection by 16S rRNA. This size

Figure 4.5: S1 mapping of the 16S rRNA/CO1 junction.(a) Summary diagram showing probe synthesis (not to scale). Probes 4 and 5 were synthesised by extension of the universal M13 sequencing 17-mer oligonucleotide (square boxes) on templates HP2 and HP1 respectively. Since they were primed from the universal oligonucleotide, both these probes contained M13 polylinker sequences. The 3' ends of the probes were generated by restriction with HindIII and PstI, for probes 4 and 5 respectively. The fragments of the probes protected from S1 nuclease digestion are shown with their sizes indicated in nucleotides, and their predicted position with regard to the genomic organisation the template clone indicated (see text for explanation). The symbols used are explained on the figure. The reaction products were analysed on a 5% polyacrylamide/urea (sequencing) gel. (b) A photograph of a portion of an autoradiograph, in an experiment where RNA was analysed with probes 4 and 5, to show the 162 nucleotide protected fragment of probe 4. Two photographs of different autoradiographic exposure times of the same gel are spliced together, so as to give the ideal exposure of both the sequencing ladder, and the S1 nuclease reaction products. (c) The complete gel from (b), showing the additional 828 nucleotide protected fragment of probe 4. Two photographs of the autoradiograph are spliced together, to remove some of the lanes in the centre of the gel which were not loaded. Lanes are indicated to show the probe used (4 or 5), the reacting RNA (B, blastula; E, egg; Y, yeast tRNA), the digestion time in hours for (b), and the presence (+) or absence (-) of S1 nuclease in the digestion. Probe fragments were sized by reference to a sequencing ladder generated by extending oligonucleotide 159 on HP2 (indicated as ACGT).

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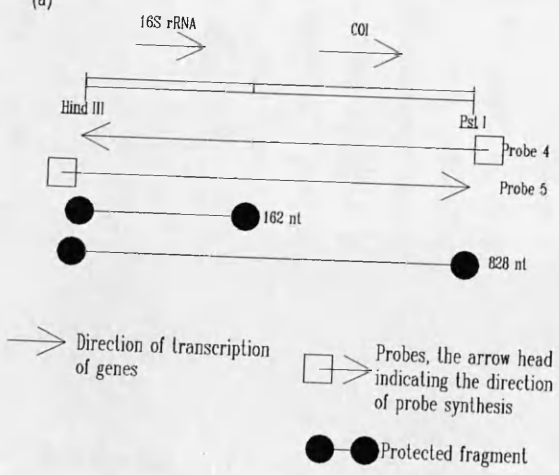
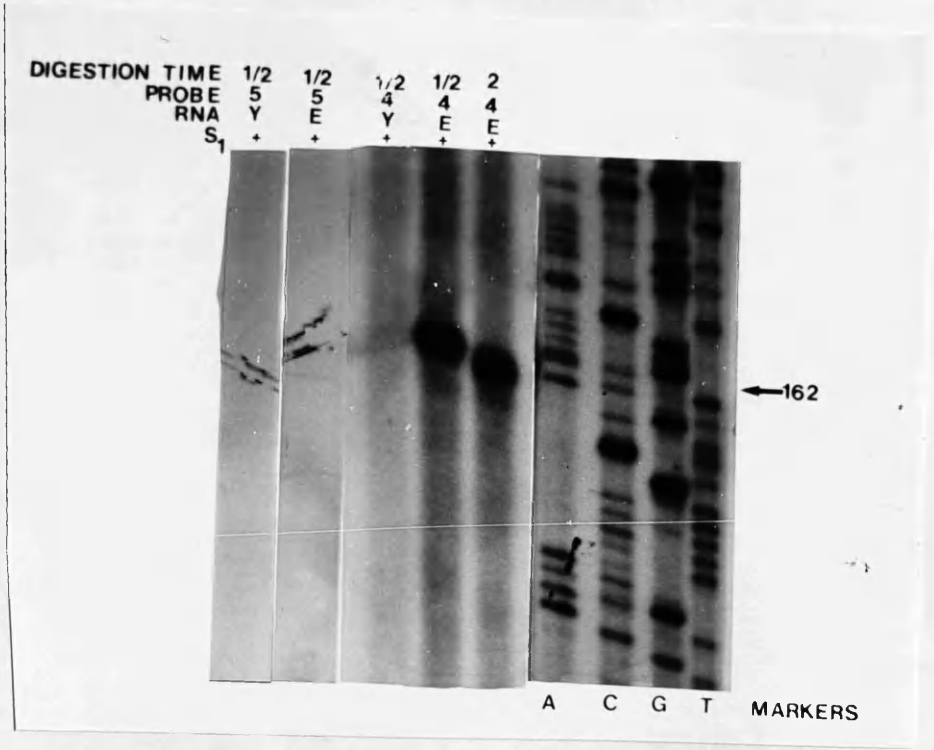
(a)


16S rRNA
COI
Hind III
Pst I
Probe 4
Probe 5
162 nt
828 nt
Direction of transcription of genes
Probes, the arrow head indicating the direction of probe synthesis
Protected fragment

(b)


DIGESTION TIME 1/2 1/2 1/2 1/2 2
PROBE 5 5 4 4 4
RNA Y E Y E E
S1 + + + + +
162
A C G T MARKERS

(c)

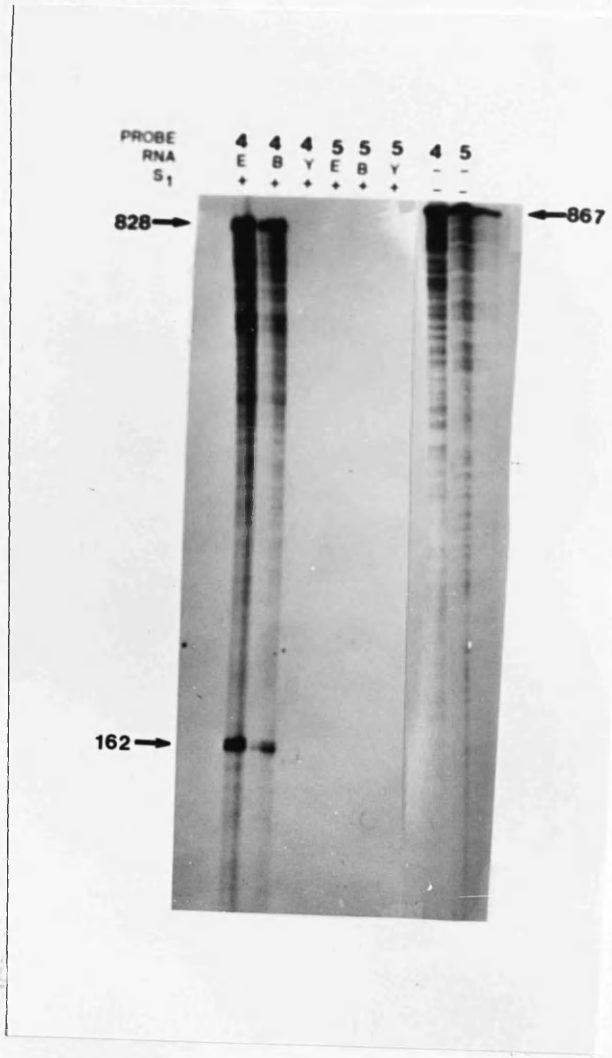

PROBE 4 4 4 5 5 5 4 5
RNA E B Y E B Y
S1 + + + + + + - -
828
867
162

Figure 4.6: S1 nuclease mapping of the 16S rRNA/COI gene junction. (a) Summary diagram (not to scale) showing how probe 6 was synthesised, by extension of oligonucleotide 159 on clone HP2. The 3' end of probe 6 was generated by cutting at the HindIII site. The reaction products are shown, with their sizes indicated in nucleotides. The predicted positions of the S1 nuclease protected fragments, with regard to the genomic organisation of the template clone used for probe synthesis, are indicated (see text for explanation). The symbols used are explained in the figure.Reaction products were analysed by electrophoresis on a 5% polyacrylamide/urea sequencing gel, followed by autoradiography. (b) S1 nuclease digestion products obtained after hybridisation RNA with probe 6. (c) Reaction products obtained by probing RNA with a 10-fold higher level of probe 6, to show the additional 106 (±1) nt reaction product. A 10-fold higher level of probe 6 was generated by using 10 times as much template HP2 in the probe synthesis reactions, as in that used for the experiment shown in (b) [see Materials and Methods for details of probe synthesis for experiment (b) and other experiments in this chapter]. Lanes are indicated to show the reacting RNA (E, egg; B, blastula; b, smaller loading of B; O, RNase-treated blastula RNA; Y, yeast tRNA; - , no reacting RNA). The markers used to size the probe fragments were for (b) sequencing ladders obtained from oligonucleotide 159 extended on clone HP2 (C track shown, indicated as M), and for (c) from the universal M13 17-mer extended on clone H3 (A, T and G tracks shown).

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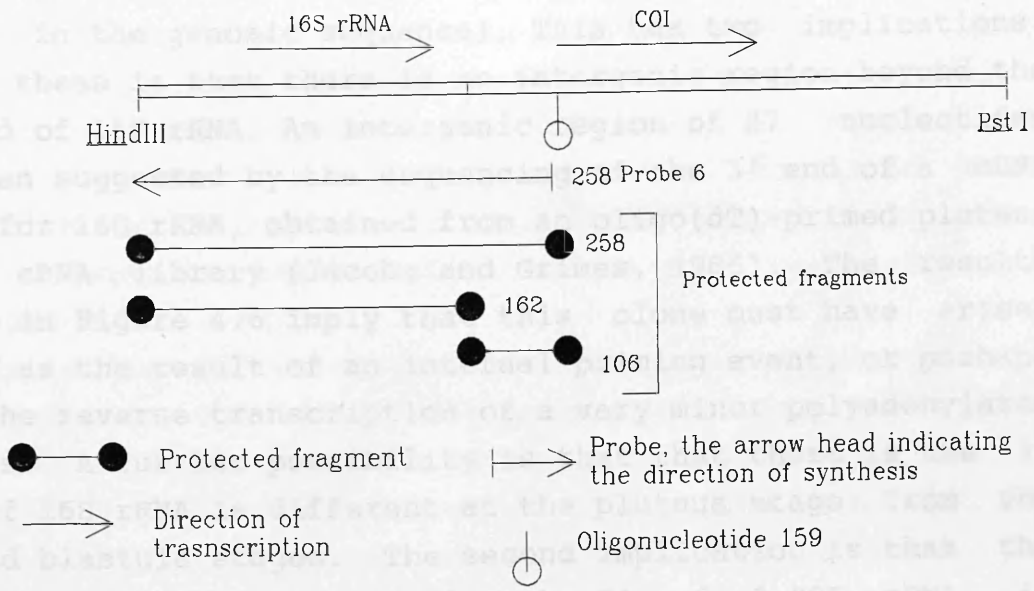
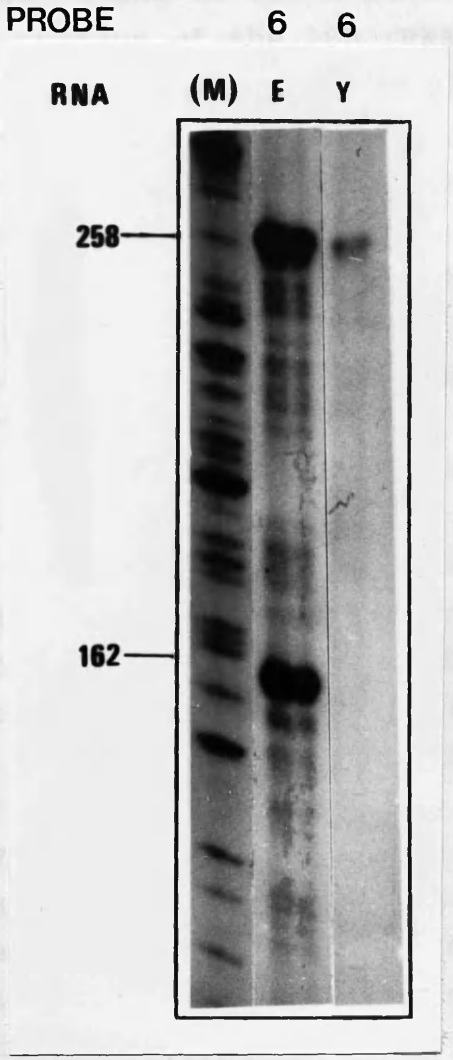
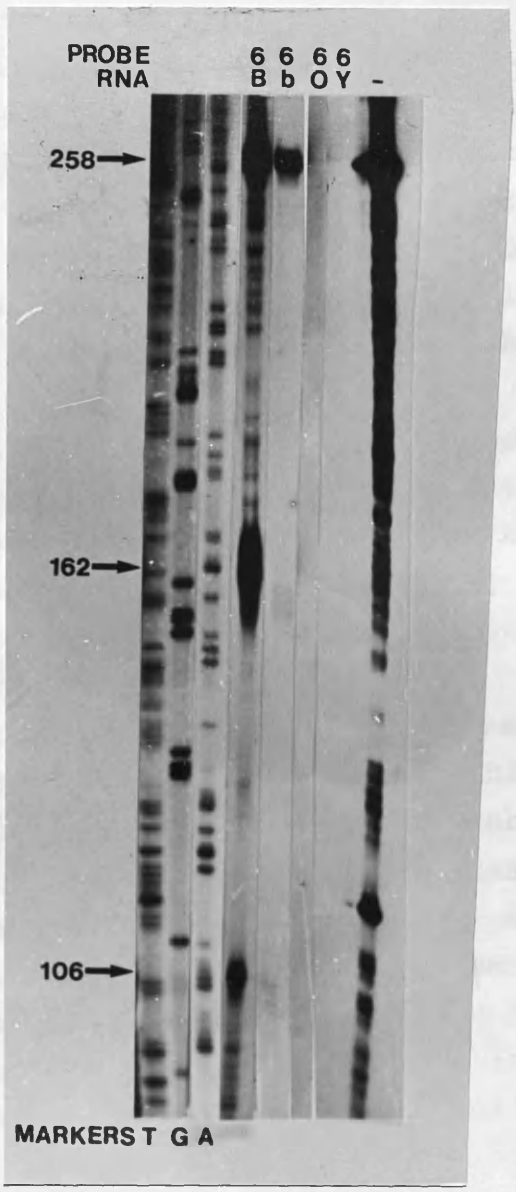
(a)


16S rRNA
COI
HindIII
Pst I
258 Probe 6
258
162
106
Protected fragments
Protected fragment
Probe, the arrow head indicating the direction of synthesis
Direction of trasnscription
Oligonucleotide 159

(b)


PROBE
6
6
RNA
(M)
E
Y
258
162

(c)


PROBE
RNA
6 6 6 6
B b O Y -
258
162
106
MARKERS T G A

corresponds with a 3' end of 16S rRNA mapping to the nucleotide downstream of the initiation codon of COI (to nt C-5788 in the genomic sequence). This has two implications. One of these is that there is no intergenic region beyond the 3' end of 16S rRNA. An intergenic region of 27 nucleotides had been suggested by the sequencing of the 3' end of a cDNA clone for 16S rRNA, obtained from an oligo(dT)-primed pluteus stage cDNA library (Jacobs and Grimes, 1986). The results shown in Figure 4.6 imply that this clone must have arisen either as the result of an internal priming event, or perhaps from the reverse transcription of a very minor polyadenylated species. A further possibility is that that there is the 3' end of 16S rRNA is different at the pluteus stage from the egg and blastula stages. The second implication is that the 3' end of 16S rRNA must overlap the 5' end of COI mRNA, if this ATG represents the initiation codon, which is suggested by sequence data (Jacobs et al, 1988).

There are several possible explanations for complete protection of the sea urchin mitochondrial DNA sequences of probes 4 and 6. (1) A precursor RNA could exist which spans the junction of the 16S rRNA and COI genes. However, northern blotting experiments (Elliott and Jacobs, 1989) did not detect any such transcripts which which might be sufficiently abundant to account for the level of complete probe protection observed.

(2) The activity of the S1 nuclease might have been inhibited, preventing (in the case of probe 4) complete digestion of probe not duplexed with RNA. However, the polylinker region of probe 4, which was not complementary to sea urchin RNA, was digested, suggesting that protection of the probe was sea urchin RNA-dependent.

(3) Contaminating DNA complementary to the probe may have protected it from S1 nuclease. There were two possible sources of contaminating DNA. One of these was the probe synthesis reaction, but this is unlikely since in this case the contaminating DNA would have also led to protection of the polylinker of probe 4. In the case of the experiment shown in Fig. 4.6b, there might have been trace levels of such contamination, since a fully protected band of low intensity was seen in the yeast tRNA lane (this fully protected band in the yeast negative control was not reproducible, as can be seen from Fig. 4.6c, which is

described below). The band representing complete probe protection in the yeast tRNA lane was of a much lower intensity than the band resulting from fully protected probe after hybridisation with sea urchin RNA. The other possible source of contaminating DNA is the sea urchin RNA preparation, which conceivably might have contained trace quantities of mitochondrial DNA. This second explanation would account for the digestion of the polylinker sequences in probe 4.

In order to establish whether trace quantities of mitochondrial DNA were responsible for complete probe protection, I carried out an S1 nuclease experiment in which the sea urchin RNA was pre-treated with boiled RNase A in BRL REact 2 buffer, and then recovered by phenol-sevag extraction and ethanol precipitation, before hybridisation with probe 6 and S1 nuclease digestion. None of the probe was protected from S1 nuclease digestion by this RNAse treated RNA (Fig. 4.6c). Since the RNA sample used for this experiment was an aliquot from that used in the above experiments, contaminating mitochondrial DNA cannot have been the cause of the probe protection observed.

(4) Complete protection of the sea urchin mitochondrial DNA-complementary region of the probe might have resulted from hybrid formation by both 16S rRNA and COI mRNA with single probe molecules, if these transcripts overlap or directly abut. The additional shorter 16S rRNA-protected fragment would be expected, since 16S rRNA is the more abundant transcript (Chapter 3), and so would form hybrids with probe molecules in amounts over and above those hybridised by COI mRNA. One prediction of the interpretation that complete probe protection was due to hybrid formation with both 16S rRNA and COI mRNA, would be that a band representing probe protection by COI mRNA alone should be visible if the probe were present in excess of RNA. If probe were not in excess, any probe hybridised to COI mRNA would also be hybridised to 16S rRNA, as a result of the lower abundance of COI mRNA. To test this possibility, I carried out a further experiment using a 10-fold higher level of probe. An additional band of 106 ($\pm$1) nucleotides was observed, which corresponds to the expected size of probe protection by the 5' end of COI mRNA (Fig. 4.6c; see also below). Hence the most probable explanation for complete

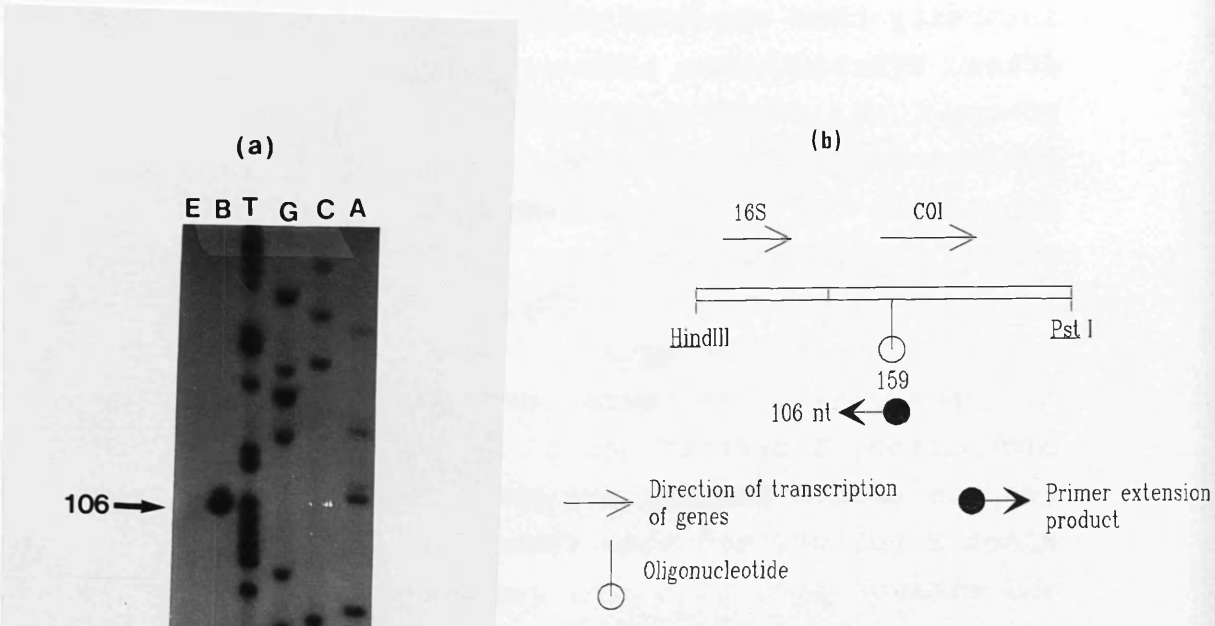


Figure 4.7: Primer extension mapping of the 5' end of COI mRNA. (a) 5% polyacrylamide/urea (sequencing) gel electrophoresis of extension products of egg (E) and blastula (B) RNA, primed with oligonucleotide 159, run alongside sequencing ladders (ACGT) primed from oligonucleotide 159 on clone HP2. (b) Summary diagram (not to scale) showing the primer extension product with respect to the clone HP2.

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probe protection in the previous experiments (Figure 4.5b, Figures 4.6 b and c) is (4).

4.2.5 Mapping of the 5' end of COI mRNA

The 5' end of COI mRNA was directly mapped using primer extension from oligonucleotide 159. The primer extension reaction products were electrophoresed in parallel with sequencing reactions, primed from oligonucleotide 159 on clone HP2 (Fig. 4.7). A clear, major reaction product of 106 nucleotides was obtained, which defined a 5' end which mapped 5 nucleotides upstream of the A of the ATG start codon of COI. This position corresponds exactly with the size of the S1-protected fragment in Fig. 4.6c, and so confirmed the 5' end of COI mRNA as being 5 nucleotides upstream of its ATG start codon (at nt A-5779 in the genomic sequence). The reaction products of egg and blastula RNA were identical in size, but the autoradiographic signal from the egg reaction product was weaker, since COI mRNA is less prevalent at the egg than the blastula stage (Chapter 3). An additional, minor primer extension product, one nucleotide longer (which defined a 5' end mapping to nt T-5780), was visible for blastula RNA.

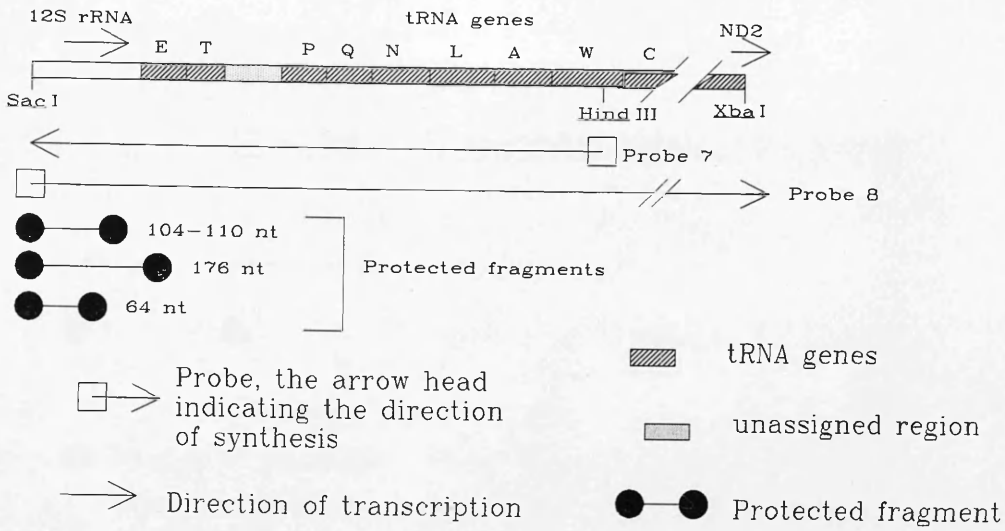
4.2.6 Mapping of transcript termini at the 3' end of 12S rRNA

Transcript termini at the 12S rRNA/tRNA^{glu} gene junction were mapped by an S1 nuclease experiment, using single-stranded probes primed from the M13 universal oligonucleotide (17-mer) on templates Q4 (probe 8) and CD5 (probe 7) (Fig. 4.8a), and a 3' coterminal probe made from extension of oligonucleotide 195 on template CD5 (probe 9) (Fig. 4.9a). Probe 8 was of the same strandedness as 12S rRNA, and was not protected by sea urchin RNA. Hence there are no detectable, discrete, antisense transcripts encoded by this region of the genome. Probe 7 was protected by sea urchin RNA to give fragment of 3 sizes (Fig. 4.8b and c): a group of fragments of 103-111 ($\pm$1) nucleotides; a larger fragment of 176 ($\pm$1) nucleotides; and a smaller fragment of 64 nucleotides. Probe 9 gave a group of fragments, of 110-113 ($\pm$1) nucleotides (Fig. 4.9b). None of the probes were protected from S1 nuclease by yeast tRNA.

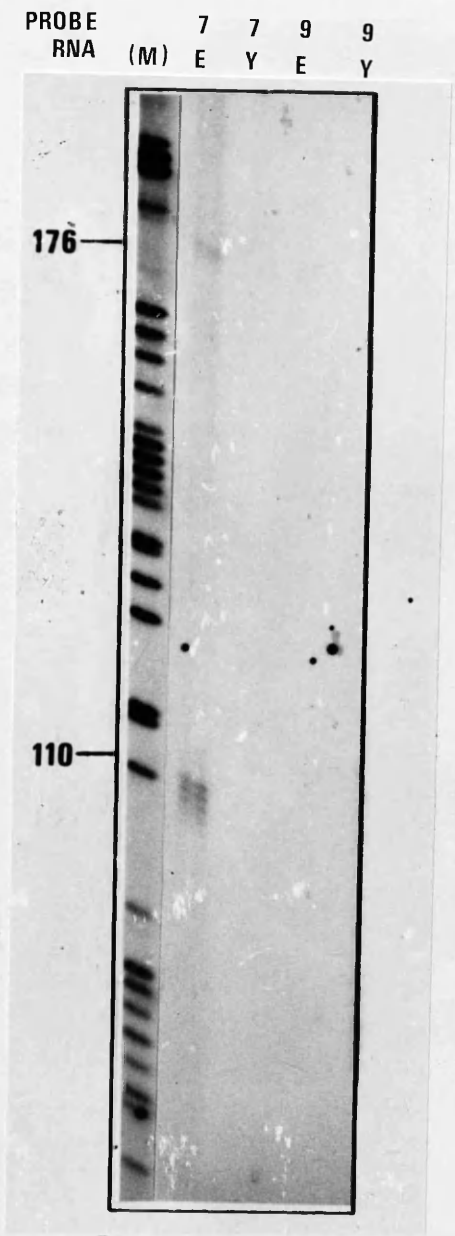
Figure 4.8: S1 mapping of transcript termini at the 12S rRNA/tRNA ^{glu} boundary. (a) Summary diagram (not to scale) to show how the probes were made. Probes 7 and 8 were primed from the universal M13 17-mer sequencing primer on clones CD5 and Q4 respectively. The 3' ends of probes 7 and 8 were formed by cutting at the SacI site and the HindIII site respectively. The fragments of probe 7 protected by sea urchin RNA from S1 nuclease are shown, with their sizes indicated in nucleotides. The predicted positions of these protected fragments are not indicated on the figure, but are discussed in the text. The symbols used are as indicated in the figure. The reaction products after S1 nuclease digestion were analysed by electrophoresis on a 5% polyacrylamide/urea (sequencing) gel, and sized by comparison with a sequencing ladder obtained by extending the universal M13 sequencing primer (17-mer) on clone HP2. (b) Autoradiograph of sequencing gel of reaction products after analysing RNA with probes 7 and 8. (c) A larger portion, and longer exposure, of an autoradiograph of a different gel which was prepared as in (b), to show the 64 nucleotide protected fragment of probe 7. Lanes are indicated as M (sequencing markers, (G) track shown); (E), probe reacted with sea urchin egg RNA, (b) blastula RNA, and (Y) yeast tRNA; and as to which probe was used (7 or 8).

(Facing page 106)

(a)


12S rRNA
tRNA genes
E T P Q N L A W C
ND2
Sac I
Hind III
Xba I
Probe 7
Probe 8
104–110 nt
176 nt
64 nt
Protected fragments
Probe, the arrow head indicating the direction of synthesis
Direction of transcription
tRNA genes
unassigned region
Protected fragment

(b)


PROBE
RNA
(M)
7 E
7 Y
9 E
9 Y
176
110

(c)

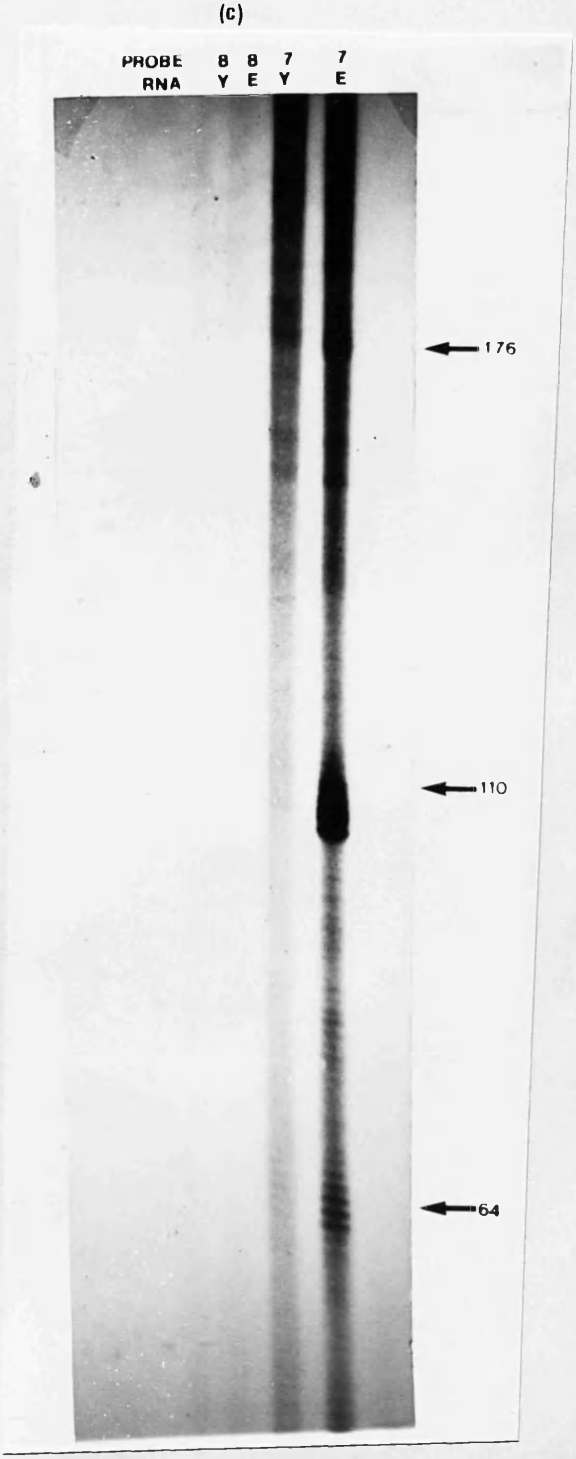
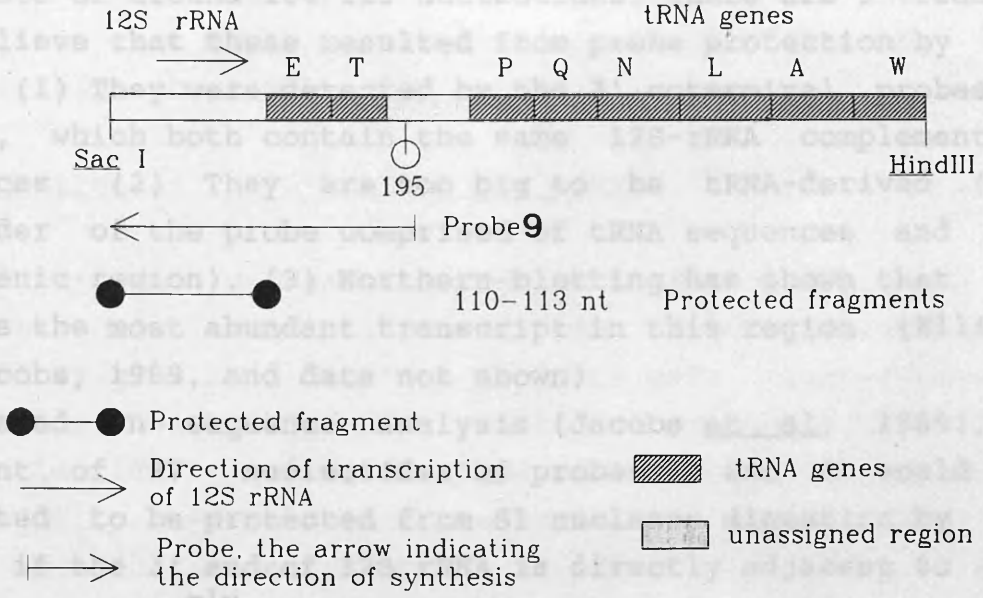

PROBE
RNA
8 Y
8 E
7 Y
7 E
176
110
64

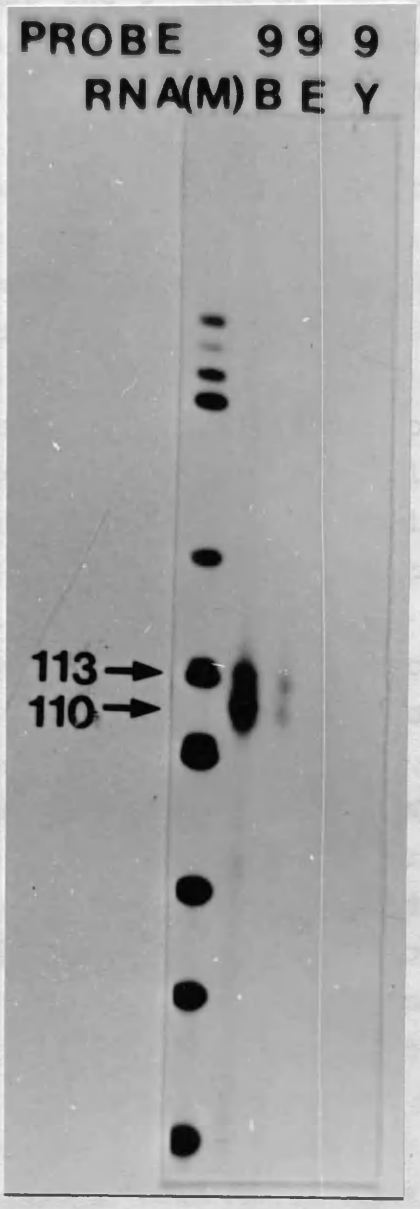
Figure 4.9 S1 nuclease mapping of the 3' end of 12S rRNA. (a) Summary diagram (not to scale) to show how probe 9 was made, by extension of oligonucleotide 195 on clone CD5. The 3' end of probe 7 was generated by cutting at the SacI site. The protected fragment of probe 7 after digestion with S1 nuclease is shown with its size in nucleotides indicated. The symbols used are indicated on the figure. The reaction products were analysed by electrophoresis on a 5% polyacrylamide/urea gel, and sized by comparison with a sequencing ladder obtained by extending clone HP2 with the M13 universal primer (17-mer) The G track is shown (labelled M). (b) Reaction products were analysed by gel electrophoresis on a 5% sequencing gel. Lanes are indicated as to the probe (9 in each case); and as to the reacting RNA (B, blastula; E, egg; and Y, yeast).

(Facing page 107)

(a)


12S rRNA
tRNA genes
E T P Q N L A W
Sac I
195
HindIII
Probe 9
110–113 nt
Protected fragments
Protected fragment
Direction of transcription of 12S rRNA
Probe, the arrow indicating the direction of synthesis
tRNA genes
unassigned region

(b)


PROBE 9 9 9
RNA(M) B E Y
113
110

The predominant fragments of probes 7 and 9 protected by sea urchin RNA were therefore a heterogeneous group of fragments of around 104-113 nucleotides. There are 3 reasons to believe that these resulted from probe protection by 12S rRNA. (1) They were detected by the 3' coterminal probes 7 and 9, which both contain the same 12S-rRNA complementary sequences. (2) They are too big to be tRNA-derived (the remainder of the probe comprised of tRNA sequences and the intergenic region). (3) Northern blotting has shown that 12S rRNA is the most abundant transcript in this region (Elliott and Jacobs, 1989, and data not shown).

Based on sequence analysis (Jacobs et al, 1988), a fragment of 97 nucleotides of probes 7 and 9 would be predicted to be protected from S1 nuclease digestion by 12S rRNA, if the 3' end of 12S rRNA is directly adjacent to the 5' end of $tRNA^{glu}$. Protection of 104-113 nucleotides of probe means that the 3' end of 12S rRNA must map at least 7 (to nt A-945 in the genomic sequence), and perhaps as many as 13 nucleotides into the gene encoding $tRNA^{glu}$, based on the boundaries of this gene as have been deduced from the predicted secondary structure of $tRNA^{glu}$ (Jacobs et al, 1988).

Probe 7 contained sequence not present in probe 9 (the part of the tRNA cluster on the far side of the intergenic region from the 12S rRNA) (Fig. 4.8a and 4.9a). A comparison of the fragments of probe 7 and probe 9 protected by sea urchin RNA suggests that the tRNA cluster at the other side of the intergenic sequence from the 12S rRNA gene, encodes at least the major portion of two detectable transcripts. Since sea urchin RNA protected 64 and 176 nucleotide fragments of probe 7, but not probe 9, this protection must have resulted from hybridisation with the region of probe 7 not found in probe 9. The 64 nucleotide fragment detected by probe 7 may result from probe protection by a tRNA molecule, although it is slightly too short to represent probe protection by a full length tRNA molecule, the tRNAs encoded in this region being predicted to be about 70 nucleotides in length from secondary structure predictions (Jacobs et al, 1988).

The fact that no antisense transcripts are detected in the intergenic region is somewhat surprising, since such transcripts have been reported in the sea urchin Paracentrotus lividus (Cantatore et al, 1990): this apparent

difference might be as a result of a lower sensitivity of my experiments, or an interspecific difference. The primer for replication might be expected to be encoded by the intergenic region, but this might be annealed to mitochondrial DNA, and copurify with it (Jacobs et al, 1989).

4.2.7 Mapping of the 5' end of ND1 mRNA

An attempt was made to map the 5' end of ND1 mRNA by primer extension reactions primed from oligonucleotides 167 and 282 (Fig. 4.10). Reaction products were electrophoresed in parallel with sequencing reactions primed from either oligonucleotide on clone R6. These oligonucleotides gave a number of primer extension products (Fig. 4.10a). Some of these were in the region of the 5' end of ND1 as predicted from sequence data (Jacobs et al, 1988). Two primer extension products of oligonucleotide 282 defined 5' ends which mapped 1 and 2 nucleotides upstream of the predicted ATG translational start codon of ND1 mRNA (nts C-2160 and T-2161 in the genomic sequence, Fig. 4.10b). The major 5' end mapped to the upstream nucleotide. The primer extension product of oligonucleotide 167 closest the predicted 5' end of ND1 mapped to the (G) residue of the predicted ND1 ATG codon (nt G-2164), and/or to the nucleotide downstream of this (these two nucleotides were compressed on the sequencing gel shown in Fig. 4.10c).

However, these were not the major reaction products using either oligonucleotide 167 or 282. The major reaction products defined 5' ends which mapped downstream of the predicted 5' end of the ND1 gene, and their position was dependent on the oligonucleotide used in the primer extension reaction (Fig. 4.10a). The major reaction products of RNA primed with oligonucleotide 282 defined 5' ends which mapped to nucleotides T-2205 and C-2206 in the genomic sequence. The major reaction products of RNA primed with oligonucleotide 167 defined 5' ends which mapped to nucleotides T-2214 and T-2227 in the genomic sequence. These major reaction products resulted from extension of developmentally regulated transcripts, since the primer extension products of egg RNA were stronger than those obtained from blastula RNA. This is the reverse of the changes in RNA representation of mature ND1 mRNA (chapter 3).

Figure 4.10: Primer extension mapping of the 5' end of ND1 mRNA. (a) Extension of oligonucleotide 167 and 282 on egg (E) or blastula (B) RNA, electrophoresed in parallel with sequencing ladders. The entire gel is shown to show all the reaction products. The reaction products shown in (b) and (c) are indicated by their sizes. (b) Enlarged view of part of the gel shown in (a), to show the reaction product closest to the size predicted for a 5' end mapping to the putative start of the ND1 transcript (see text) obtained from extension from oligonucleotide 282. (c) Enlarged view of part of the gel shown in (a) to show the reaction product closest to the predicted size for a 5' end mapping to the predicted start of the ND1 transcript (see text), obtained by extension from oligonucleotide 167. Lanes are indicated as ACGT (marker sequencing reactions, primed on clone R6 with the same oligonucleotide as was used in the primer extension reactions); or to indicate the RNA used in the primer extension reactions (B, blastula RNA; E, egg RNA). (d) Summary diagram (not to scale) showing the predicted 5' end of ND1 mRNA in relation to the priming oligonucleotides 167 and 282, and the insert of the clone R6 which was used for the sequencing ladders. The symbols used are as indicated in the legend.

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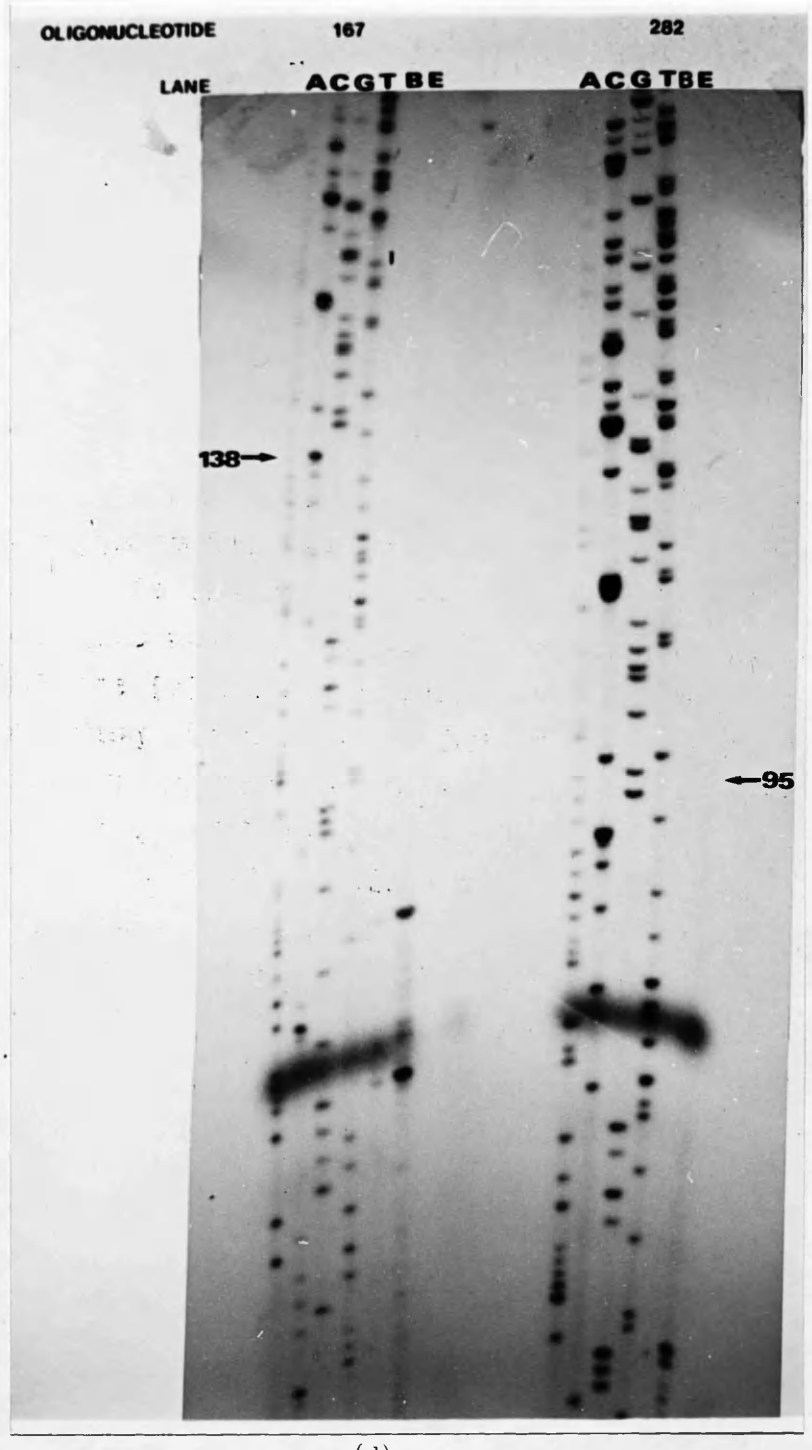
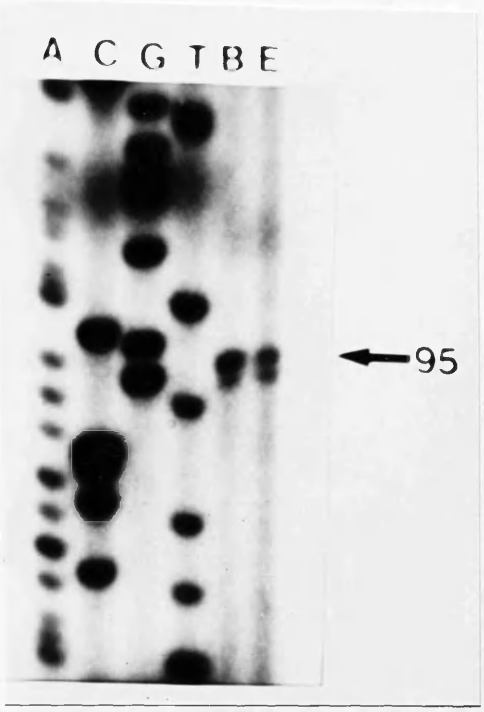
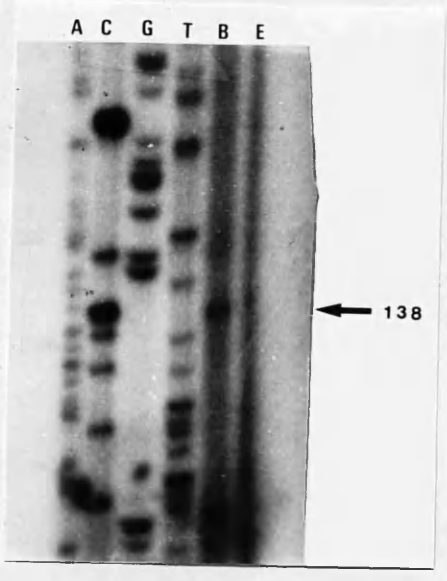
(a)


OLIGONUCLEOTIDE
167
282
LANE
ACGTBE
ACGTBE
138
95

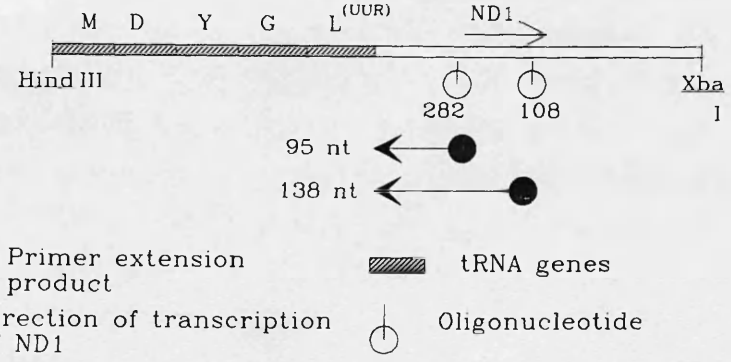
(b)


A C G T B E
95

(c)


A C G T B E
138

(d)


M D Y G L(UUR)
ND1
Hind III
282
108
Xba I
95 nt
138 nt
Primer extension product
tRNA genes
Direction of transcription of ND1
Oligonucleotide

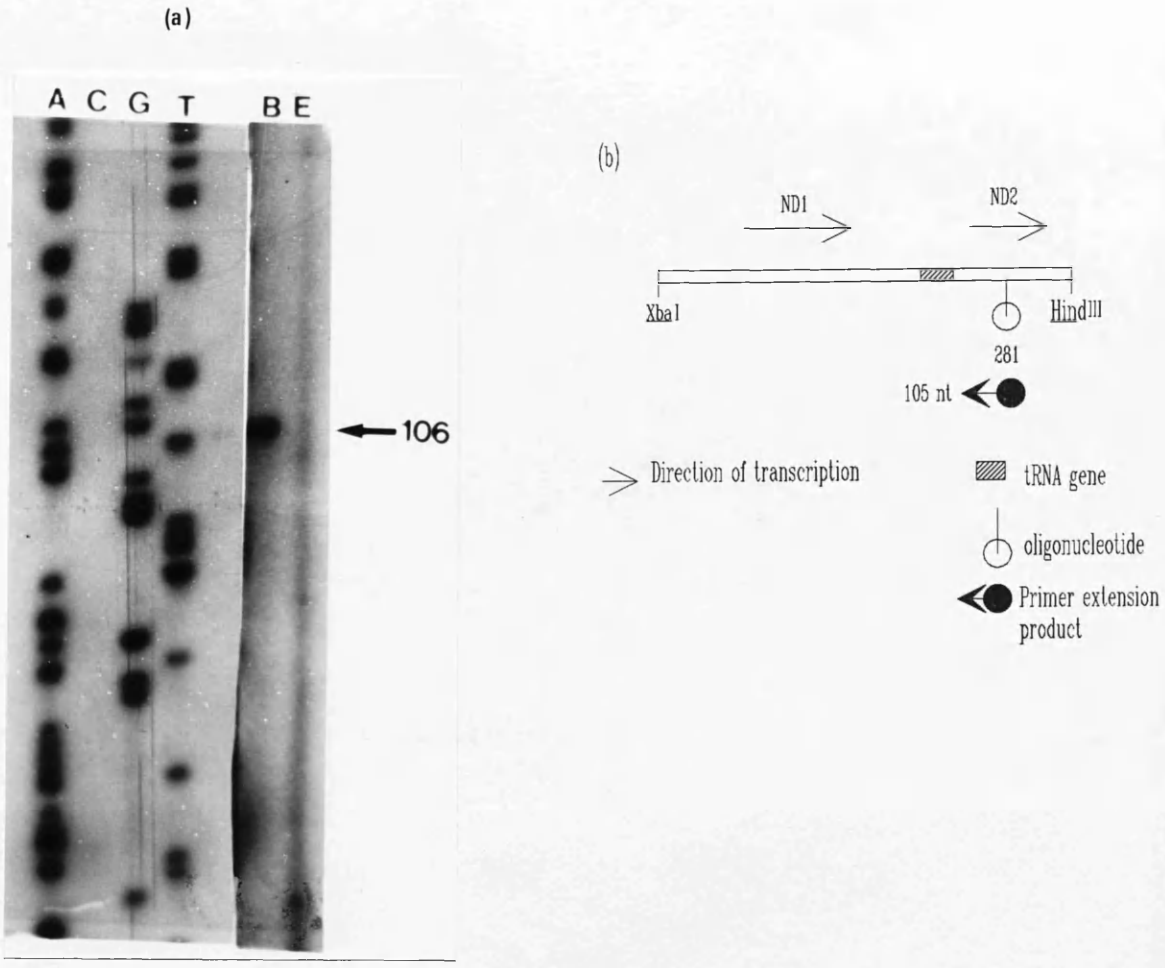


Figure 4.11: Primer extension mapping of the 5' end of ND2 mRNA. (a) Primer extension product obtained by priming egg and blastula RNA with oligonucleotide 281, run alongside sequencing ladders primed from the same oligonucleotide on clone T3. Lanes are indicated ACGT (sequencing ladders); and to indicate the RNA used in the primer extension reactions (B, blastula RNA; E, egg RNA). (b) Summary diagram (not to scale) showing the primer extension product with regard to the insert of the clone T3 and the oligonucleotide 281, which were used for the sequencing reactions. A full explanation of the symbols used is given on the figure. Two autoradiographs are spliced together in this figure to give comparable exposures of each.

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The reason for the ambiguities in the 5' mapping of ND1 mRNA, or why this has only occurred for ND1 mRNA is not clear. Which of the primer extension products (if any) represents the true 5' end of ND1 is not known.

4.2.8 Mapping of the 5' end of ND2 mRNA

The 5' end of ND2 mRNA was mapped using primer extension from oligonucleotide 281, and the reaction products were sized by electrophoresis in parallel with a sequencing ladder primed with oligonucleotide 281 on clone T3 (Fig. 4.11). In blastula RNA, a clear single reaction product of 106 nucleotides was obtained which mapped to the A of the ATG start codon of ND2 (nt A-3201 in the genomic sequence). An equivalent primer extension product was not seen in the case of egg RNA, although this could be since ND2 mRNA is less abundant at the egg stage, so a weaker primer extension product would be predicted. A number of weak bands are apparent in the egg track shown in Fig. 4.11, however. These might mean that the 5' end of ND2 mRNA is different in the egg than in tha blastula stage.

4.2.9 Mapping of 5' ends adjacent to a TTATATATAA-like motif

In Chapter 3 I described a TTATATATAA-like sequence motif which is found at several positions in the sea urchin mitochondrial genome, which are suggestive of a functional role in mitochondrial gene expression. In order to see if transcript ends were directly associated with it, I carried out a series of primer extension experiments.

4.2.9.1 Mapping of the 5' end of cytb mRNA

The 5' end of cyt b mRNA was mapped using primer extension of oligonucleotide 372, electrophoresed alongside a sequencing ladder of the genomic clone N18 primed with the same oligonucleotide. Two reaction products were seen (Fig. 4.12). The predominant reaction product was 43 nucleotides in length, and defined a 5' end which mapped to the (A) of what is presumably the ATG translational initiation codon of cyt b. This is downsteam of the 5' end which had been predicted on the basis of the open reading frame, but fits

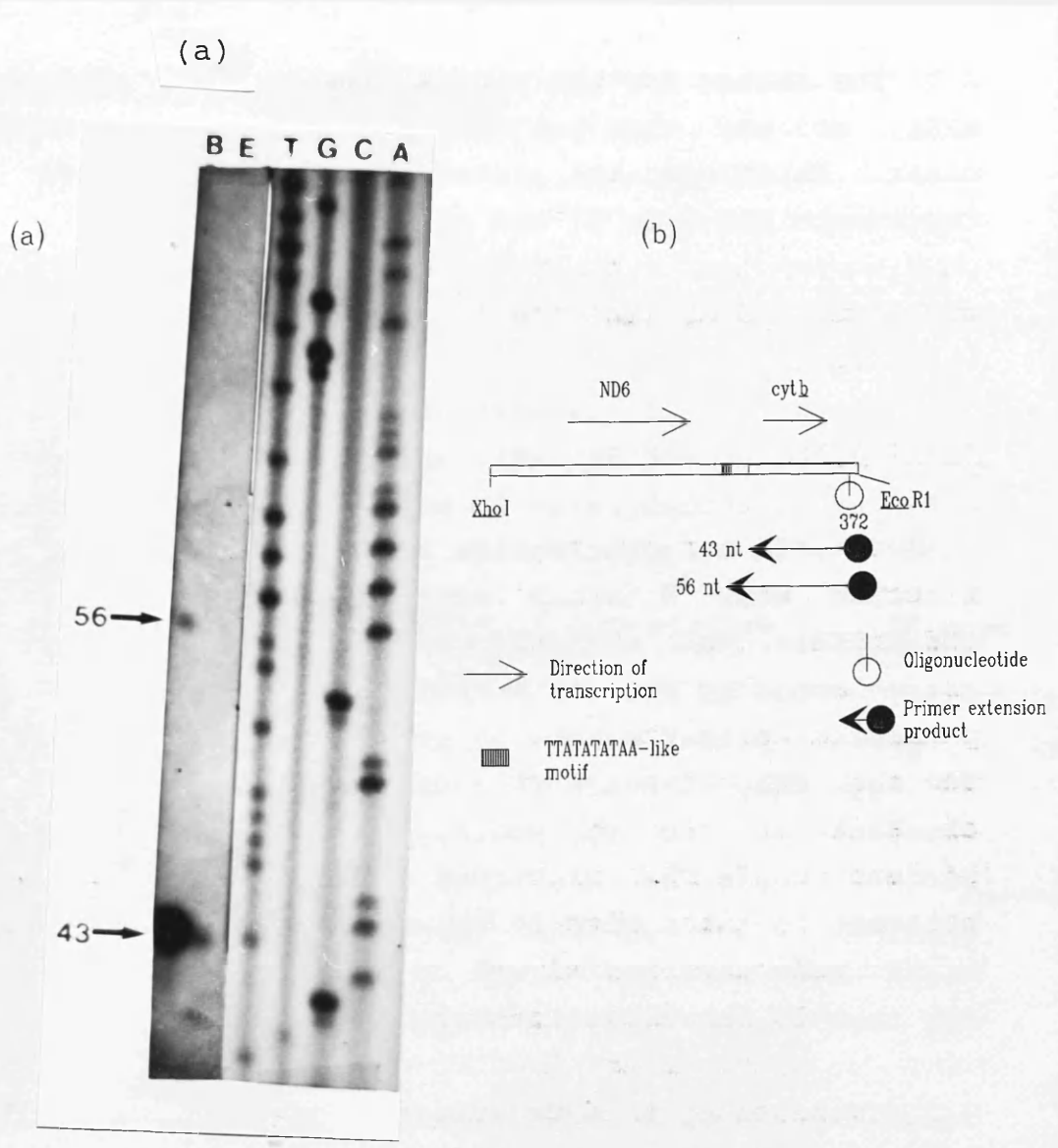

Figure 4.12 Primer extension mapping of the 5' end of cyt b mRNA. (a) Primer extension products obtained by extending oligonucleotide 372 on sea urchin RNA, run alongside sequencing reactions primed from oligonucleotide 372 on clone N18. Lanes are indicated as ACGT (sequencing ladder, from reactions primed with the same oligonucleotide as that used for the primer extension reactions); and as to the RNA used in the primer extension reactions (B, blastula RNA; E, egg RNA). Photographs of two autoradiographic exposures of the same gel are spliced together to give the ideal autoradiographic exposure for the primer extension products and the sequencing ladder. (b) Summary diagram (not to scale) to show the mapped 5' ends in regard to the template clone used for the sequencing reactions. The symbols used are indicated on the figure.

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better with arguments based on sequence similarity (Jacobs et al, 1988). On longer exposure, an additional product of 56 nucleotides was visible, which defines a 5' end which maps to the final (A) of a TTATATATAA-like motif. This longer primer extension product possibly represents a precursor transcript of cyt b mRNA, with the mature 5' end being generated by RNA processing.

4.2.9.2 Attempt to map the 5' end of ND6 mRNA

The ND6 and cyt b genes are divergently transcribed from a common site (Fig. 1.5). I was unable to map the 5' end of ND6 mRNA either by primer extension mapping, or by S1 nuclease mapping (not shown). These experiments were performed using the same procedures as those described above. The failure of these experiments might have been as a result of a low abundance of the ND6 transcript. Alternatively the 5' end of ND6 mRNA might not be discrete, in which case any primer extension signal would be distributed among a number of positions when analysed by electrophoresis, each one of which would be weaker than the signal which would be obtained from a single band. Another explanation might be that the oligonucleotide used to prime extension might not anneal to ND6 mRNA efficiently, either as a result of secondary structure or sequence (the oligonucleotide used for the primer extension reactions was designed from genomic sequence information, which as a result of RNA editing or base modification might differ from the RNA sequence). It might also be the case that the 5' end of ND6 maps within the coding sequence of cyt b, in which case the cyt b mRNA 5' sequences might compete with either the priming oligonucleotide in a primer extension experiment, or a single-stranded probe in an S1 nuclease mapping experiment.

4.2.9.3 Mapping of the 5' end of ND3 mRNA

The 5' end of ND3 mRNA was mapped using primer extension on sea urchin RNA from oligonucleotide 390, run alongside sequencing reactions primed from oligonucleotide 390 on template clone N15. A single reaction product was obtained in egg and blastula-stage RNA, which defined a 5' end which mapped to the T_8 of a TTATATATAA-like motif (nt T-10195 in

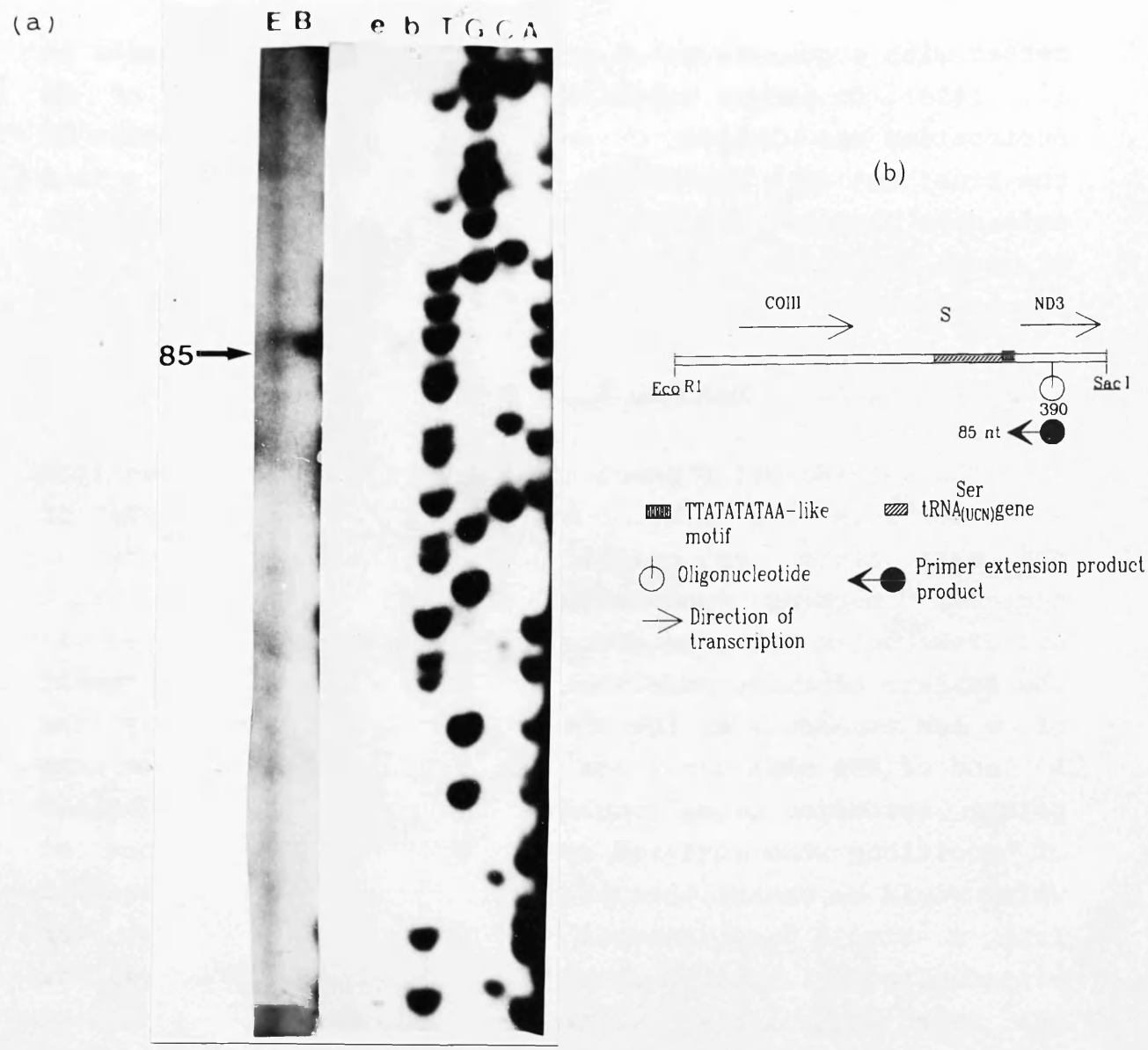

Figure 4.13 Primer extension mapping of the 5' end of ND3 mRNA. Primer extension products obtained by extending oligonucleotide 390 on egg and blastula RNA, electrophoresed in parallel with sequencing reactions primed from the same oligonucleotide on clone N15. Lanes are labelled as ACGT (sequencing reaction); and to indicate the RNA used in the primer extension reactions (B, blastula RNA; E, egg RNA; b, short exposure of the blastula reaction products; e, short exposure of the egg reaction products). (b) Summary diagram (not to scale) to show the mapped 5' end of ND3 mRNA, with regard to the template clone N15 used for the sequencing reactions and oligonucleotide 390. The symbols used are as indicated on the figure.

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the genomic sequence, Fig. 4.13a and b). This is 6 nucleotides upstream of the predicted start codon of ND3 (Jacobs et al, 1988). The implications of this observation are discussed below. A weaker reaction product was obtained from egg-stage RNA, presumably because ND3 mRNA is less prevalent at the egg stage of development.

4.2.9.4 Mapping of the 5' end of $tRNA^{ser}(UCN)$

In order to map the 5' end of $tRNA^{ser}(UCN)$ I used primer extension on sea urchin RNA from oligonucleotide 393, electrophoresed alongside sequencing reactions primed from the same oligonucleotide on the genomic template clone ZH3A. A very weak signal was seen which defined a 5' end which mapped to the predicted (from the sequence data of Jacobs et al, 1988) 5' end of the tRNA (not shown). It is possible that the tRNA secondary structure, or base modification, prevented efficient annealing of the priming oligonucleotide, which might explain this low primer extension signal, or $tRNA^{ser}(UCN)$ might be a rare transcript. This latter explanation is unlikely given the frequency of the UCN codon in the genome. No band which would correspond to a precursor with a 5' end mapping within the TTATATATAA-like motif was detected. The signal resulting from the mature end of the gene was so weak, that the detection of a precursor molecule, if it was a rarer species, would be impossible.

4.3 Discussion

4.3.1 Validity of experimental results

The validity of the results described in this chapter are dependent on the reliability of the mapping techniques used. S1 nuclease tends to give heterogeneous reaction products as a result of the transient thermal denaturation of probe/RNA hybrids during the digestion, which makes the termini susceptible to 'nibbling' by the enzyme (Williams and Mason, 1985). The extent of these effects depends on the reaction conditions (particularly the temperature of digestion and enzyme concentration), base composition [(A+T)-rich regions are more susceptible to melting], and the

secondary structure of the terminal regions of the transcript. The heterogeneity observed when mapping the 3' end of 16S rRNA was within the range expected from S1 nuclease reactions (2-3 nt), and increasing the amount of enzyme or altering the reaction conditions did not affect the result (see Fig. 4.5b, and other data not shown).

In contrast, mapping of the 3' end of 12S rRNA gave a level of heterogeneity outside that expected from S1 nuclease nibbling (8-10 nucleotides). The 176 nt fragment of probe 7 was not heterogeneous, which provides an internal control to this experiment (as far as the temperature of digestion, or other experimental conditions, affecting heterogeneity). The 3' end of the 12S rRNA gene is not particularly (A+T) rich, so the 3' end of 12S rRNA may be heterogeneous <u>in vivo</u>, rather than the heterogeneity representing a mapping artefact. This property of heterogeneity is unique among those sea urchin mitochondrial transcripts mapped to date. There are two ways that this heterogeneity might have arisen. (1) It might arise as a result of heterogeneous processing. In animal mitochondria transcripts bounded by tRNAs are thought to be excised by an exact processing mechanism based on recognition of their secondary, and/or tertiary structure (section 1.11.1). This type of processing presumably cannot be operating at the 12S rRNA/$tRNA^{glu}$ junction, since the mapped 3' end of 12S rRNA lies within the $tRNA^{glu}$ gene sequence, unless $tRNA^{glu}$ sequences direct processing in a different manner from other tRNAs. However, it is possible that the original endonucleolytic event is at the 3' end of $tRNA^{glu}$, with the mature 3' end of 12S rRNA being formed by 3' clipping by ribonucleases. (2) The 3' end of 12S rRNA may result from a transcriptional attenuation event. It has been proposed that the 3' heterogeneity which is present in mammalian 16S rRNA arises as a result of transcriptional attenuation, with this being a less precise mechanism as compared to endonucleolytic cleavage (discussed in section 1.5.1).

A problem of 3' end analyses (which must be performed using nuclease protection experiments, as opposed to 5' ends which can be mapped using either nuclease protection or primer extension mapping) is that it is not possible to generate a sequencing ladder with products having an identical structure to the protected fragment(s). Since the

electrophoretic migration of fragments is not only related to their size but also to their nucleotide composition and other features (for example some sequences can cause compression on gels), a given fragment may not necessarily comigrate with a band in a sequencing ladder of identical molecular weight. However, experiments to map the 3' ends of 16S and 12S rRNA used two unrelated sequencing ladders to size the protected fragments, which gave identical results in each case (not shown). When I carried out transcript mapping by both S1 nuclease and primer extension the results I got were identical (Figures 4.6 and 4.7 for COI mRNA, and Figures 4.1 and 4.2 for 16S rRNA).

Primer extension mapping gives different artefacts from S1 nuclease mapping. Predominant among these are that the primer used in the extension reactions need only be homologous with a short region of the transcript. Since it is known that the nucleotide composition of mitochondrial transcripts of some organisms can be altered post-transcriptionally by RNA editing (section 1.11.4) and splicing (section 1.11.5), the 5' end of a transcript as mapped by primer extension may represent only the distance from the primer to the 5' end of the transcript, rather than its position in the genomic sequence. Additionally it has been shown in Vaccinia virus that residues can be added post-transcriptionally to the 5' end of a transcript by stuttering of the RNA polymerase on the most 5' nucleotide (Schwer and Stunnenberg, 1988). However there is no evidence that any of these processes occur in animal mitochondria, or that the structure of animal mitochondrial genes imposes any such requirement. In the case of primer extension mapping of COI mRNA, a longer primer extension product was obtained than was expected from alignment of the predicted amino acid sequence of the gene, since the 5' ends of most animal mitochondrial mRNAs map to the first nucleotide of their translational initiation codon (Sections 1.2 and 1.11.1). The fact that the mapping data from primer extension agreed exactly with that derived from S1 nuclease mapping precludes RNA editing, or a short RNA sequence being spliced onto the 5' end of the mitochondrial message. The only way that this could occur would be if the residues added to the 5' end of the transcript were identical to those which are genomically encoded. In the case of the COI transcript this would be a

run of 5 (A) residues, and in the case of the minor 5' end an additional (T) residue. 5' polymerase stuttering would lead to a run of residues identical to those genomically encoded in the case of the major COI mRNA 5' end, but not the minor 5' end mapping just upstream.

A disadvantage of the short primer lengths used in these experiments is that they might bear enough sequence homology with other transcripts to anneal to them, and be extended by reverse transcriptase. Additional minor products were seen on long exposure in the case of primer extension mapping of 16S rRNA. However, the reaction products in the case of primer extension mapping of ND1 mRNA seemed to be oligonucleotide dependent, and mainly defined 5' ends which did not not map to the expected 5' terminus of the gene, and so almost certainly represent spurious priming events. It is not clear why these spurious reaction products were only seen for ND1 mRNA, which is a question which deserves further analysis. It might be the case that there is a population of nuclear-encoded transcripts which are homologous enough with ND1 to cross-hybridise with the priming oligonucleotide. Another possibility might be that the 5' end of ND1 mRNA is extensively edited, or spliced, and the two priming oligonucleotides were hybridising to alternative forms of the mRNA, or its precursor.

A possible approach which could be used to further map the 5' end of ND1 mRNA, would be to use oligonucleotides 108 and 282 to generate single-stranded probes from clone R6, and to use these to S1 nuclease map this junction. These probes would be longer, and so should have an increased specificity. Another possibility would be to carry out the primer extension reactions using purified mitochondrial RNA, which would contain a lower complexity of sequences to compete for the oligonucleotide.

4.3.2 Fine structure of mature sea urchin mitochondrial transcripts

In general, no developmental alterations in primary transcript structure were detected by the experiments described in this chapter. This means that the developmental alterations in the relative abundance of different functional classes of transcript are unlikely to have

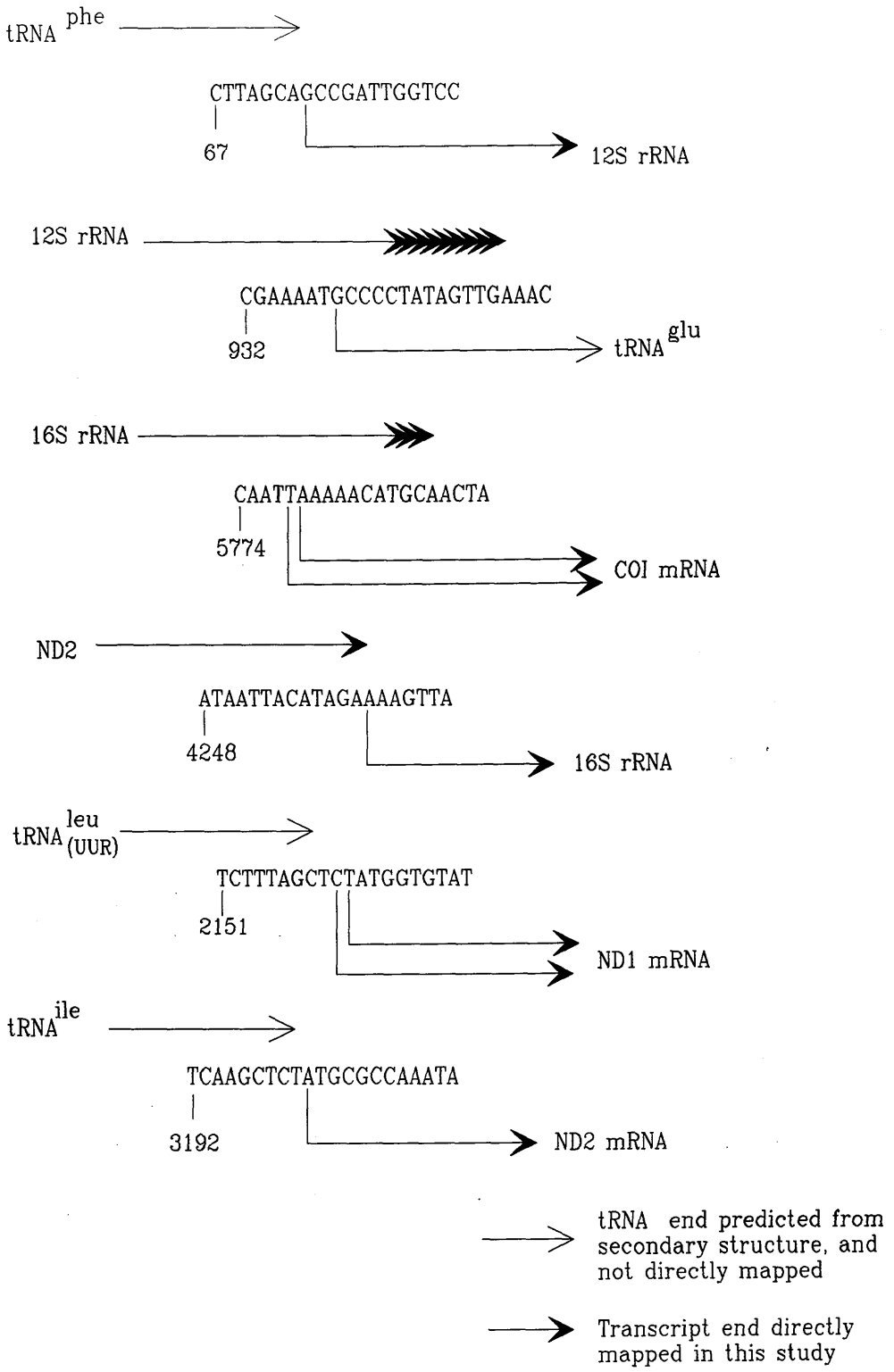

Figure 4.14 Summary diagram showing the termini of transcripts mapped in this chapter. Solid circles denote 5' ends, and arrowheads denote 3' ends and transcriptional direction. The map coordinates are as in Jacobs et al (1988).

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occurred as a result of either the inclusion, or exclusion, of sequence elements in mature sea urchin mitochondrial transcripts at different developmental stages, affecting RNA stability. There is a possibility that there might be developmental differences in the termini of ND2 mRNA.

The transcript termini mapped in this study are summarised in figure 4.14. The termini of several genes have been shown to be butt-joined to their adjacent transcripts with either no intervening nucleotides (in the case of the $tRNA^{ile}$/ ND2, $tRNA^{phe}$/ 12S rRNA, and possibly ND2/16S rRNA gene junctions), or with very few intergenic nucleotides (probably 1 or 2 in the case of the $tRNA^{leu}$/ ND1 gene junction). The absence of intergenic nucleotides of any significant length suggests that these termini are generated by RNA processing, unless there are cryptic intragenic promoter sequences or transcriptional termination sites.

In contrast the transcripts for several genes have been shown to overlap with their neighbours. The 3' end of 16S rRNA has been shown to map 3-5 nucleotides into the 5' coding region of COI, and 8-10 nucleotides from the mapped 5' end of COI mRNA. The 3' end of 12S rRNA maps 7-13 nucleotides into the predicted sequence of tRNA ^{glu}, a region which contains the sequences necessary for base-pairing in the dihydroxyuridine and aminoacyl stems of the tRNA. Selection of the 3' ends of either of the mature rRNAs must preclude synthesis of a functional transcript of the downstream gene. Similarly, selection of the COI mRNA 5' end removes sequences which are part of the mature 16S rRNA. Hence functional transcripts of the rRNAs and their downstream genes must be synthesised by distinct, mutually exclusive pathways.

These pathways may operate at the transcriptional level, with transcription terminating optionally at the 3' ends of the rRNA genes. In this case transcripts for the downstream genes could be generated either by a transcriptional attenuation/readthrough mechanism similar to that found downstream of the vertebrate 16S rRNA gene (in which case an RNA processing event would additionally be required to generate the mature 5' ends of COI mRNA and $tRNA^{glu}$, for the precursor transcripts which readthrough the attenuator); or by initiation of transcription at the 5' end of the downstream genes (which would imply that the sequences

responsible for promoting transcription would be intragenic).

Alternatively, the pathways may operate at the post-transcriptional level, with functional transcripts of the rRNA or the downstream genes being produced by either alternative processing of the same precursor transcript, or processing of different precursors. This latter case would require alternative transcriptional and post-transcriptional pathways. Selection at the post-transcriptional level would result in a population of truncated, nonfunctional transcripts. Although such transcripts were not detected by the experiments described in this chapter, it is conceivable that they would be rapidly turned over in vivo, which would make their detection difficult. The 64 nucleotide band which was detected by probe 7 (section 4.6) is of the correct size range to represent protection by a portion of a tRNA molecule such as $tRNA^{glu}$. However, a 64 nucleotide protected band was not observed with probe 9, which suggests that the RNA species giving rise to this protected band is transcribed from the other side of the unassigned region from 12S rRNA, which means that it could not be $tRNA^{glu}$-derived.

In addition to the overlaps at the RNA level which have been mapped in the above experiments, a number of overlaps between sea urchin mitochondrial genes on the same strand have been predicted on the basis of sequence homologies (Jacobs et al, 1988). These have yet to be tested at the RNA level, which is particularly important since the sequences from which the location of these termini were predicted are not very well conserved. The predicted overlaps are a 1 nucleotide overlap between $tRNA^{leu}$(CUN), and $tRNA^{ala}$, an 11 nucleotide overlap between ND4 and $tRNA^{his}$, 1 nucleotide between ND4L and COII (although this could be avoided if the termination codon of ND4 is created by polyadenylation), and 4 nucleotides between A6 and A8. This last case is unlikely to represent a mutually exclusive synthesis of transcripts, if, as in mammals, these two genes are transcribed as a bicistronic message containing two overlapping reading frames (Fearnly and Walker, 1986).

There is only one other reported example of overlapping mitochondrial transcripts similar to that of the sea urchin rRNAs and their downstream genes. This is from murine mitochondria, where a proportion (30%) of the 3' ends of 16S rRNA molecules map 1-7 nucleotides within the sequence of

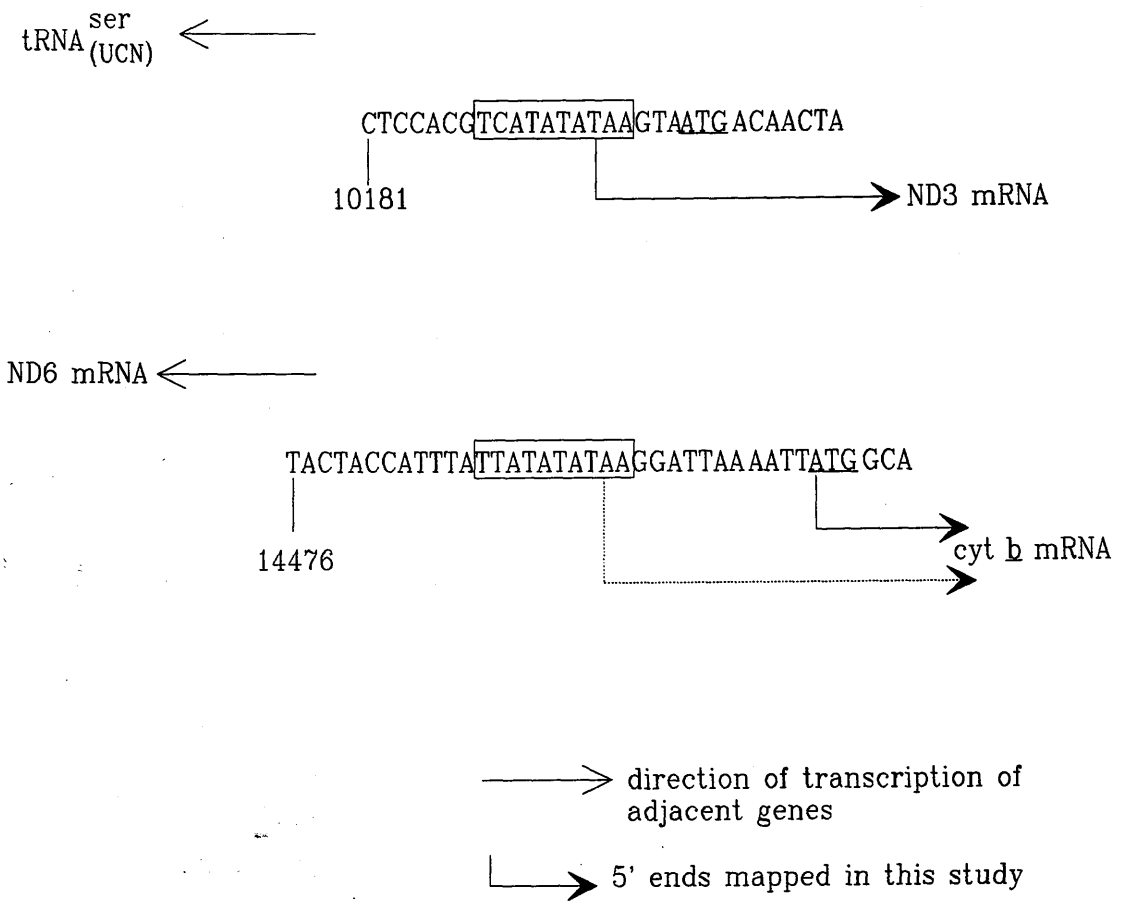

Figure 4.15 Summary diagram showing the fine mapping of transcripts adjacent to a TTATATATAA-like motif, other than the mapping of the 16S rRNA/ND2 junction summarised in Fig. 4.14. The broken line indicates the putative primary leader of cytb mRNA, and the solid lines indicate mature transcripts. The map coordinates are as in Jacobs et al, 1988. The TTATATATAA-like motif is boxed. The proposed translational initiation codons are underlined.

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$tRNA^{leu}$, the remainder mapping 1-2 nucleotides upstream of the tRNA (Van Etten et al, 1983). This gene junction is known to be a site of transcriptional attenuation (Christianson and Clayton, 1986, 1988). A more extreme example of overlapping transcripts is thought to occur in of HeLa cell mitochondria, where rRNAs and transcripts linking the mRNA genes with their promoter grossly overlap over most of their length (Section 1.5.1).

4.3.3 Mapping of transcript termini adjacent to TTATATATAA-like motifs

The 5' ends of at least two transcripts have been shown to map within copies of an TTATATATAA-like motif (Fig. 4.15), and the 5' end of $tRNA^{met}$ is predicted to map to a copy of this motif on the basis of predictions of its secondary structure (Jacobs et al, 1988). From these experiments it is not possible to conclude whether these transcript 5' ends result from RNA processing, or transcriptional initiation. No transcript end mapping to the TTATATATAA motif upstream of 16S rRNA was detectable, either by primer extension or S1 nuclease mapping.

Experiments to map the 5' ends of ND6 mRNA and $tRNA^{ser}$(UCN) were less successful, possibly because these transcripts are less abundant in sea urchin RNA. This might result from shorter half lives than ND3 and cyt b mRNAs. ND6 mRNA and $tRNA^{ser}$(UCN) are encoded by the minor coding strand of sea urchin mitochondrial DNA. Most of the RNA encoded by the minor coding strand in mammalian mt is rapidly turned over (section 1.5.2). Alternatively, it is conceivable that ND6 and $tRNA^{ser}$(UCN) are transcribed at a lower level than either ND3 or cyt b. If the TTATATATAA-like motifs are core promoters for sea urchin mitochondrial DNA, then they may promote transcription predominantly in one direction. The human mitochondrial promoters have been shown to promote transcription asymmetrically (section 1.9). The TTATATATAA-like motif is essentially symmetrical, and may be the basic sequence requirement to promote transcription. The asymmetry of the transcripts encoded adjacent to the TTATATATAA-like motifs, might be determined by the sequences flanking the motifs.

4.3.4 Implications for RNA stability

In the absence of a transformation system for sea urchin mitochondria, it is not possible to insert and remove RNA sequences from mitochondrial transcripts, and determine whether these have a functional role in *in vivo* in controlling steady-state RNA levels. However, from the data described above, it is possible to speculate about sequence elements which may be involved in RNA metabolism. Truncated transcripts of 16S rRNA, 12S rRNA and COI mRNA were not detected, although their existence would be predicted if the mature transcripts are synthesised by mutually exclusive processing pathways. If this is the case, therefore, the elements missing from the truncated transcripts are presumably of importance for RNA stability. In the case of COI mRNA this includes the translational start codon- if this, perhaps along with the 5 (A) residues encoded upstream, was missing from the transcript it would prevent ribosome loading and so leave the transcript open to attack by ribonucleases. The requirement for the mature 3' ends of the rRNAs is less explicable, although these sequences may be again involved in RNA protection from ribonucleases, or interactions with ribosomal proteins necessary for ribosome assembly or stability.

In the case of ND3 mRNA the mature end of the transcript mapped to the TTATATATAA-like motif, whereas in the case of cyt *b* only the putative primary 5' end mapped to the adjacent TTATATATAA-like motif (a precursor/product relationship has not been demonstrated between these two transcripts). In neither case was a potential translational initiation codon present in these leader sequences. The 5' end of ND3 mRNA must contain a leader sequence which does not contain protein coding information, and so might have a role in stability of the transcript, or in ribosome binding. However it could be the case that the retention of the 5' leader of ND3 mRNA is tolerated in the mature message since it is neutral in its effect. In either case, presumably there must be some reason why the putative leader sequence is removed from cyt *b* mRNA.

The overlapping regions of COI mRNA and 16S rRNA are identical in both *S. purpuratus* (Jacobs *et al*, 1988), *P. lividus* (Cantatore *et al*, 1990), and in the starfish *P. ochraceous* and *A. amurensis* (Jacobs *et al*, 1989b). This

conservation might indicate that this sequence has an important functional role. In contrast, the non-protein coding regions of the 5' termini of ND3 and cyt b mRNAs, from S. purpuratus and P. lividus, are not identical, although they are similar.

4.3.5 Summary of salient conclusions from Chapter 4, and their implications for future work

No differences have been detected in the primary structure, at different developmental stages, of most of the transcripts examined in this chapter. This eliminates the hypothesis that such differences might contribute to the developmental fluctuations in the transcript levels of these transcripts. It is possible that there are developmental differences in some transcript termini, for example those of ND2 mRNA. Although differences in polyadenylation level would not have been detected by these experiments, no gross differences were apparent from the experiments described in Chapter 3, or reported by Elliott and Jacobs (1989).

No intergenic sequences flank either of the rRNA genes, so if either of these have local promoters these would have to be intragenic.

The 3' ends of both of the rRNA genes overlap with their downstream transcripts. This means that functional transcripts of the rRNAs, or the transcripts encoded by genes lying downstream of them, would have to be synthesised by mutually exclusive pathways.

A number of transcript 5' ends have been shown to map to a reiterated TTATATATAA-like motif, which might have a functional role in RNA processing or transcriptional initiation.

These conclusions raise a number of questions. The most important of these are (1) whether the mutually exclusive synthetic pathways generating the rRNAs and their downstream transcripts operate at the transcriptional level, or at the post-transcriptional level; (2) how these pathways are modulated to give rise to the observed fluctuations in the levels of different functional classes of transcript; and (3) whether the TTATATATAA-like motifs are involved in transcriptional initiation, RNA processing, or both. Once these questions are answered, at least to a certain level, it

will be possible to design *in vitro* assays to reproduce these processes, and determine their biochemical basis.

The mapping of the site(s) of transcriptional initiation, and the definition of the associated transcription units on the sea urchin mitochondrial genome is central to answering these questions. This could be achieved in several ways. In the past, mitochondrial transcriptional initiation sites have been mapped by *in vitro* capping of RNA with guanyl transferase (Section 1.4), and by run-off experiments with homologous RNA polymerases on cloned mitochondrial templates (Section 1.9). Another possible approach would be to analyse the transcription products of sea urchin mitochondria either *in vivo* or *in organello*, before and after treatment to cause premature termination of transcription. Experiments which address these questions are descibed in the next chapter.

CHAPTER 5

MITOCHONDRIAL RNA SYNTHESIS IN SEA URCHIN EGGS AND EMBRYOS

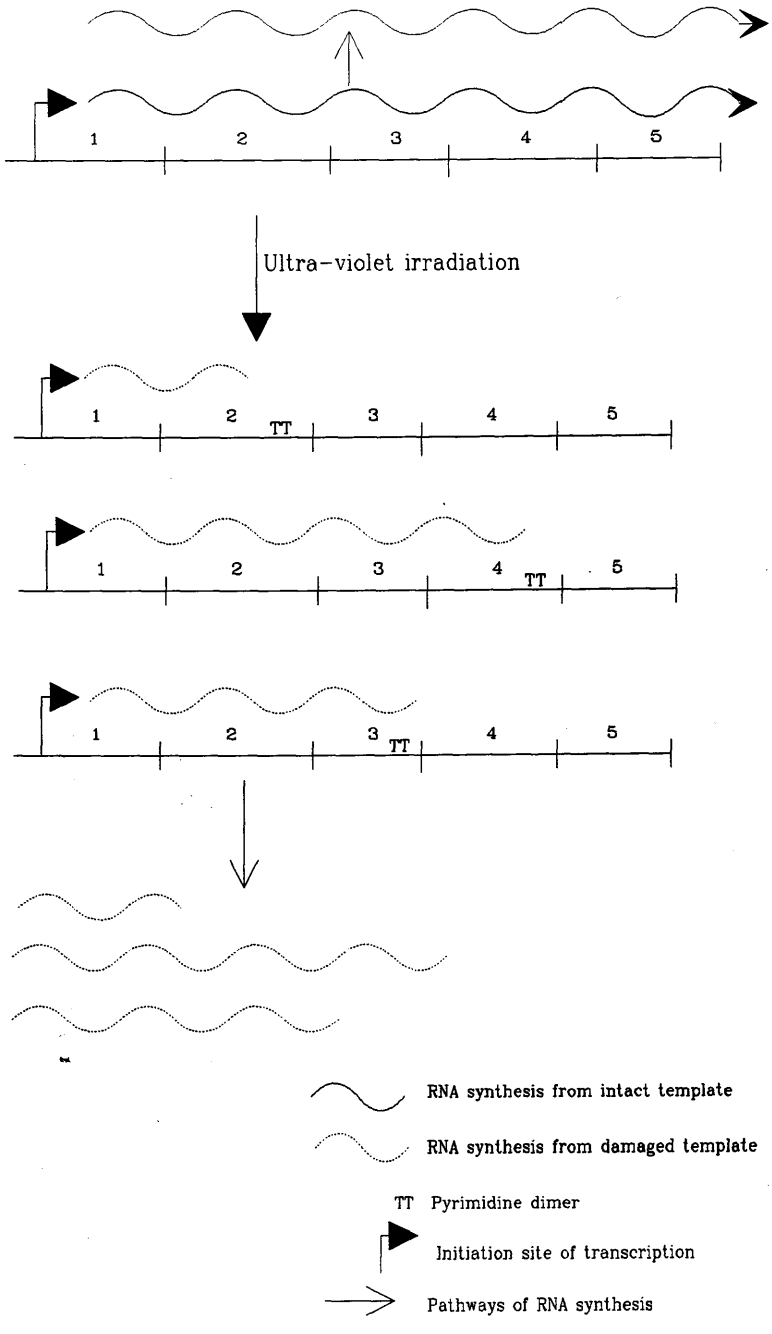

Figure 5.1: Diagrammatic representation of u.v. mapping, for 5 genes (1-5) in a hypothetical polycistronic transcription unit. Ultra-violet irradiation causes the formation of pyrimidine dimers (TT), which act to block the passage of RNA polymerase. With increasing distance from the transcriptional initiation site the chance of an RNA polymerase molecule encountering a pyrimidine dimer increase exponentially, leading to a relative decrease in the transcription of genes encoded further from the promoter.

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5.1 Introduction

One of the major aims of this project was to map the site(s) of transcriptional initiation in the sea urchin mitochondrial genome. The results described in the previous chapter did not directly address this issue, since the mapping techniques used did not differentiate between 5' ends resulting from transcriptional initiation and those created by RNA processing. The mapping of transcriptional initiation sites would enable some of the questions raised in the previous chapter to be answered: whether the TTATATATAA-like motifs are involved in transcriptional initiation or RNA processing, and whether the mutually exclusive pathways responsible for the synthesis of the rRNAs and the transcripts encoded downstream of them are achieved at the level of transcriptional initiation or alternative RNA processing. The mapping of transcriptional initiation sites would also enable the mitochondrial promoter elements to be localised. In other organisms, mitochondrial promoters are associated with transcriptional initiation sites (section 1.8). In an attempt to map transcriptional initiation sites on the sea urchin mitochondrial genome, I have carried out two complementary lines of investigation: ultra-violet (u.v.) mapping, and *in vitro* capping of sea urchin RNA.

Radio-labelled precusors can be incorporated *in vivo* into sea urchin egg and embryo RNA. Most of the labelling is of mitochondrial-encoded transcripts (Ruderman and Schmidt, 1981; Hartman *et al*, 1971; Dworkin and Infante, 1978; Devlin, 1976). In this chapter I describe experiments in which I have carried out *in vivo*-labelling in order to analyse mitochondrial RNA synthesis.

Ultra-violet irradiation causes the formation of pyrimidine dimers in DNA, which act to block the passage of RNA polymerase, thereby causing premature chain termination. After irradiation, RNA species which are encoded close to a promoter are more likely to be transcribed before the polymerase encounters a pyrimidine dimer, than RNA species which are located distant to the promoter (Fig. 5.1). After chain termination, RNA polymerase is able to re-initiate at the start of the transcription unit, and resume elongation until it encounters another pyrimidine dimer. Hence u.v. light causes a polar effect on RNA synthesis. After

irradiation, sequences located close to transcriptional initiation sites are transcribed at a higher level than those which are distant. The frequency of pyrimidine dimer formation, and therefore the effect of u.v. light on transcription, is dose-dependent and can be used to order genes within transcription units (Sauerbier and Hercules, 1978). I have used u.v. mapping to attempt to order sea urchin mitochondrial genes within transcription units, by analysing the products of mitochondrial transcription *in vivo*, before and after various doses of u.v. irradiation.

In vitro capping of RNA with guanylyl transferase and GTP (section 1.4) has been widely used to identify mitochondrial transcriptional initiation sites, since it labels the 5' ends resulting from transcriptional initiation, but not the monophosphate groups resulting from RNA processing. In this chapter I describe experiments in which I have applied this technique to sea urchin RNA.

5.2 Results

5.2.1 In vivo synthesis of sea urchin egg RNA

RNA was labelled *in vivo* with ^{3}H-uridine. Labelling conditions were based on those described by Ruderman and Schmidt (1981) for the sea urchin *Lytechinus pictus*, in which the level of incorporation was increased by pronase treatment prior to incubation with the label. In order to establish the best conditions for *in vivo*-labelling of *Strongylocentrotus purpuratus* RNA in eggs, I carried out labelling for 2 different times (3 and 16 hours), with or without prior pronase treatment. After labelling, RNA was isolated by acid phenol extraction, electrophoresed on a formaldehyde agarose gel, and visualised by transfer to a nylon filter membrane, followed by fluorography (Fig. 5.2A). RNA was only labelled to a high enough specific activity to allow visualisation by fluorography in the case of pronase-treated eggs incubated for 16 hours. For this reason, subsequent reactions were carried out for 16 hours after pronase treatment. This incubation time differs from that reported for *Lytechinus pictus*, in which labelling was carried out for 2-4 hours (Ruderman and Schmidt, 1981).

Figure 5.2 In vivo RNA synthesis in sea urchin eggs and enucleate egg fragments. Fluorograph of 1% agarose/formaldehyde gel electrophoresis of in vivo transcription reaction products, after transfer to a nylon filter membrane. (A) Optimisation of conditions for ^{3}H labelling in vivo in whole eggs. Lanes are indicated as 1 (3 hours labelling without pronase pre-treatment), 2 (3 hours labelling after pronase pre-treatment), 3 (16 hours labelling without pronase pre-treatment), 4 (16 hours labelling after pronase pre-treatment). 0.4 ml of packed eggs were labelled as described in Chapter 2. (B) RNA synthesised by enucleate egg fragments. Lanes are indicated as to their treatment prior to labelling (P: pronased; A: parthenogenetically activated; U: untreated). The activated fraction was activated in 50 ml of ASW (pH9.0) for 1/2 hour (see Chapter 2). Pronaseing was carried out in 10 ml CFSW with 10 mg pronase, for 1/2 hour (see Chapter 2). In each case, after 30 minutes preincubation under the same conditions as the labelling was carried out in, 45 ul of ^{3}H uridine was added. 50 ul aliquots of packed enucleate egg fragments (prepared as described in Chapter 2) were labelled for 16 hours in 0.5 ml ASW containing 5 ul penicillin/streptomycin (50 ug/ml). Transcripts were sized in (A) and (B) by comparison with RNA size markers (BRL).

Sizes are given in kb.

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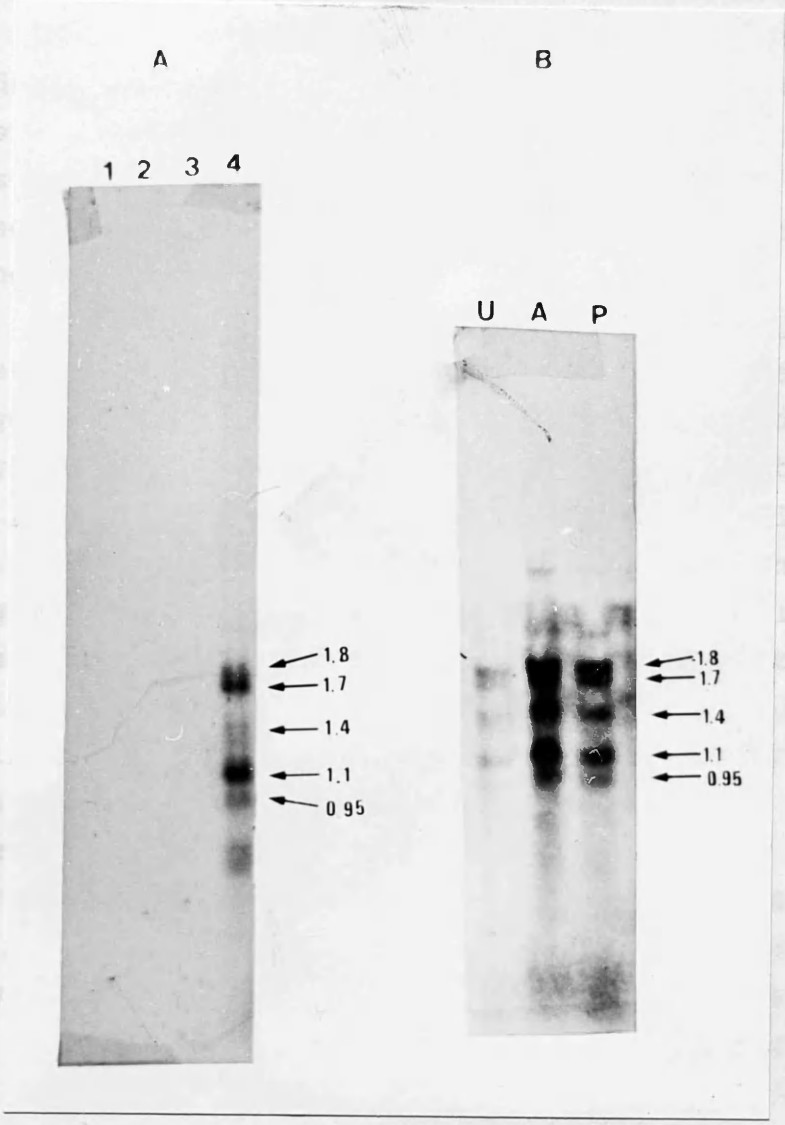
A
1 2 3 4
1.8
1.7
1.4
1.1
0.95
B
U A P
1.8
1.7
1.4
1.1
0.95

5.2.2 Analysis of in vivo-labelled RNA

I carried out 3 different types of experiment in order to determine which of the *in vivo*-labelled transcripts were mitochondrial in origin, and to identify these transcripts.

5.2.2.1 In vivo RNA labelling in enucleate egg fragments

Enucleate egg fragments are eggs from which the nuclei have been removed by fractionation on a continuous sucrose gradient (Wilt, 1973; Rinaldi *et al*, 1977), leaving the mitochondria as the only known remaining source of RNA synthesis. Hence all RNA labelled *in vivo* in enucleate egg fragments must be mitochondrial in origin.

Transcription in enucleate egg fragments can be activated by parthenogenetic activation (Rinaldi *et al*, 1977). To optimise conditions for *in vivo*-labelling in *S. purpuratus*, enucleate egg fragments were either pronase-treated, parthenogenetically activated, or left untreated prior to labelling. In each case the pattern of transcripts, and the relative intensity of bands, was similar (Fig. 5.2B). However, the overall level of incorporation differed according to the pre-treatment. Parthenogenetically activated fragments showed the highest level of incorporation, while pronase-treated enucleate egg fragments showed a slightly lower level of incorporation. Untreated enucleate egg fragments incorporated much less ^{3}H-uridine.

A comparison of the pattern of labelled transcripts obtained in enucleate egg fragments and whole eggs, shows that the majority of highly labelled discrete products in the latter were also synthesised in enucleate fragments, which suggests that they are mitochondrial in origin (Fig. 5.2). A possible criticism of this conclusion is whether the egg fragments were in fact enucleate. They appeared to be enucleate on microscopic examination, and they responded to parthogenetic activation in a similar fashion (i.e. mitochondrial transcription was stimulated) as has been reported for enucleate egg fragments (Rinaldi *et al*, 1977). Since mitochondrial transcription cannot be activated in whole eggs (Rinaldi *et al*, 1977), this suggests that the enucleate fragment preparations were at least significantly enriched for fragments over intact eggs. This enrichment

would mean that any nuclear transcription products which were being synthesised in the enucleate egg fragment preparations, would be ^{3}H-labelled at a comparitively lower level than in an equal volume of whole eggs. The fact that none of the major ^{3}H-labelled products from the enucleate egg fragment preparations was labelled at a lower level than that obtained in whole eggs, is consistent with their site of synthesis being the mitochondria. A similar pattern of labelling was reproduced several times from independent preparations of enucleate egg fragments. Conclusive proof of the absence of nuclei in these enucleate egg fragment preparations could be obtained by probing a Southern blot of DNA with a probe for a nuclear DNA repetitive element, using a Southern blot of DNA prepared from an aliquot of enucleate egg fragments, another aliquot of which had been used in an *in vivo* RNA synthesis reaction.

When analysed by gel electrophoresis, the only labelled products in whole eggs which did not comigrate with labelled products from enucleate egg fragments are two lower (about 0.85 kb) molecular weight species (Fig. 5.2). Two low molecular weight RNA species were also ^{3}H-labelled in *L. pictus* eggs, where they were shown to comigrate with histone transcripts, detected with ^{32}P-labelled molecular probes (Ruderman and Schmidt, 1981). However, the experiments described below (see Fig. 5.3b) show that a breakdown product of 16S rRNA also migrates in this region of the gel (i.e. with a length of about 0.85 kb). The fact that the 0.85 kb species were not detected on the fluorograph shown in Figure 5.2A might be related to its exposure time, which was considerably shorter than that for the autoradiographs shown in Fig. 5.3B.

5.2.2.2 Hybridisation with ^{32}P-labelled probes

In vivo-labelled RNA from whole eggs was electrophoresed on a 1% formaldehyde/agarose gel, and then transferred to a nylon filter membrane. Parallel lanes were fluorographed, and then hybridised with ^{32}P-labelled probes for specific mitochondrial transcripts. The location of the ^{32}P-labelled bands after probing was determined by autoradiography. Contribution of the ^{3}H-label to the autoradiographic signal was prevented by sealing the filters in bags prior to

exposure, and by the use of intensifying screens (Fig. 5.3). A limitation to this approach is that transcripts detected by ^{3}H-labelling are not necessarily the same transcripts as detected by ^{32}P-labelling. In other words, comigration is not definitive proof of identity.

To detect 16S rRNA I used a nick-translated SpP144 probe. Several transcripts were detected by autoradiography, the predominant one of which comigrated with the 1.7 kb transcript detected by fluorography, which presumably represents the mature transcript of 16S rRNA (Fig. 5.3A, lane 3). A set of shorter transcripts were also detected by autoradiography which comigrated with ^{3}H-labelled bands, and probably represent breakdown products of 16S rRNA. These lower molecular weight transcripts were 1.4 kb, 0.95 kb, 0.85 kb in size, and there was a doublet of around 1.1 kb. Although these transcripts have not been examined in detail, similar lower molecular weight transcripts have been detected in rat mitochondria where they have been shown to be non-random poly(A)$^{-}$ fragments of 16S rRNA (Mazo et al, 1984), and in Xenopus mitochondria (El Meziane et al, 1989).

COI mRNA was detected by hybridisation with nick-translated SpG30. This probe hybridised to a band comigrating with the 1.8 kb ^{3}H-labelled transcript, which presumably represents the mature form of COI mRNA (Fig.3A, lane 4).

Both the 1.7 and 1.8 kb transcripts were detected with a Hu/Messing probe synthesised from the clone HP1 (Fig. 3A, lane 5). The steady-state level of 16S rRNA is around 30-fold higher than COI mRNA (Chapter 3; Cabrera et al, 1986), but the probe synthesised from HP1 labelled both approximately equally, partly since it contains more COI-reactive sequence (655 nt) than 16S reactive sequence (155 nt). There must be some other contributory factor since this would be consistent with a steady-state level of 16S rRNA being 4-fold higher than COI mRNA, which is much lower than the value calculated in Chapter 3. A very probable contributory factor might be that the probe was not in excess of the filter-bound RNA, particularly of 16S rRNA. This would result in an under-estimation of the ratio of the level of 16S rRNA to the less abundant COI transcript. Another important point is that the breakdown products of 16S rRNA would have made a significant contribution to level of 16S rRNA calculated in Chapter 3 from dot blot data. These breakdown products would have been

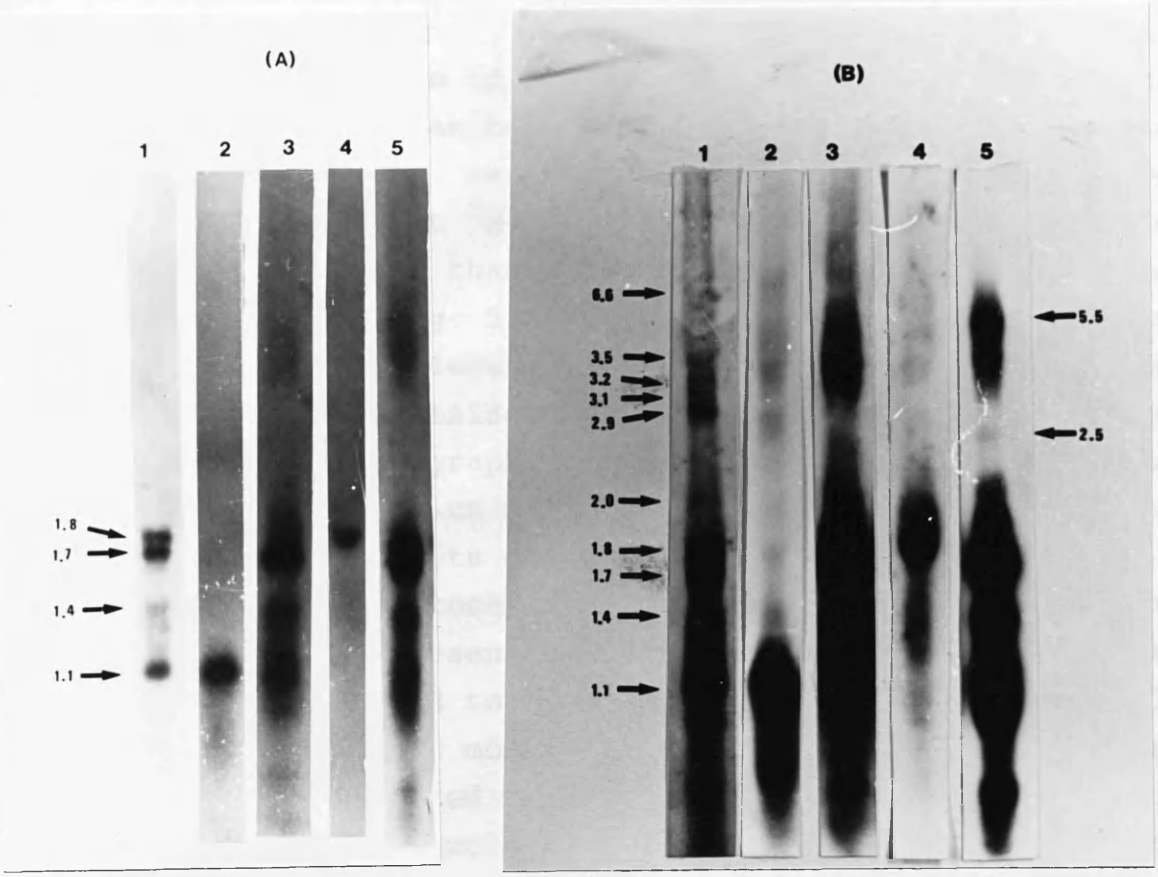

Figure 5.3 Comigration of in vivo-labelled RNA from pronase treated whole eggs, with mitochondrial transcripts detected using ^{32}P-labelled molecular probes. (A) Short exposures of (1) fluorograph of in vivo ^{3}H-labelled RNA, and autoradiographs of parallel lanes of the gel probed with ^{32}P-labelled (2) nick-translated pPZ0.6, (3) nick-translated SPp144, (4) nick-translated SpG30, and (5) a Hu/Messing probe synthesised from HP1. (B) Longer exposures of the fluorograph and autoradiographs shown in (A), labelled correspondingly. Hybridisations were carried out in 50% formamide hybridisation buffer. Other experimental details were as described in Chapter 2. Transcripts were sized by comparison with a BRL RNA size ladder.

Sizes are given in Kb.

detected as 16S rRNA reactive sequnces, as well as the mature 16S rRNA transcript, and so would have led to an overestimate of the level of the mature 16S rRNA transcript calculated.

12S rRNA was detected using nick-translated pPZ0.6. The band detected comigrated with the 1.1kb ^{3}H-labelled band (Fig. 3A, lane 2).

On longer exposure of the fluorograph, higher molecular weight transcripts can be visualised (Fig. 5.3B). In this experiment, labelling was carried out in whole eggs. More higher molecular weight ^{3}H-labelled transcripts are visible (Fig. 5.3B, lane 1), than in the RNA labelled in enucleate fragments, shown in Fig. 5.2B. Therefore, it is possible that some of the higher molecular weight transcripts visible in Fig. 5.3B were synthesised in the nucleus. However, the exposure in the fluorograph in Fig. 5.3B was much longer than that in Fig. 5.2B, which might be a contributory factor to the different transcripts detected.

The bona fide mitochondrial higher molecular weight transcripts might represent precursor transcripts, which are subsequently processed to generate the mature mitochondrial transcripts. The higher molecular weight transcripts detected by fluorography migrated in the same region of the gel as a proportion of the high molecular weight transcripts detected by long exposure of autoradiographs of the parallel filters probed with ^{32}P-labelled molecular probes specific for 16S rRNA, 12S rRNA and COI mRNA (Fig. 5.3B). It is reasonable to suppose that these higher molecular weight transcripts, if they do represent precursor mitochondrial transcripts, to be precursors of the most abundant mature mitochondrial transcripts, since these would be expected to be synthesised at a higher level than the less abundant mitochondrial transcripts. Given this assumption, the fact that only a proportion of the higher molecular weight transcripts detected by the probes specific for 16S rRNA, 12S rRNA and COI mRNA were detectably ^{3}H-labelled, might be indicative of distinct classes of sea urchin mitochondrial precursor transcripts which have different kinetic properties, determined at the level of RNA synthesis and turnover. The fact that they were detectably ^{3}H-labelled, would suggest that they were synthesised at a greater rate, but also turned over more rapidly, than the higher molecular weight transcripts not detectably ^{3}H-labelled, but detected by

hybridisation with a ^{32}P-labelled probe. This property is reminiscent of the HeLa cell mitochondrial rRNA precursors, as opposed to the mRNA precursors, both of which are transcribed from the same region of the HeLa cell mitochondrial genome (described in section 1.5.1). An alternative explanation, however, is that the higher molecular weight transcripts not detected by ^{3}H-labelling may represent long-lived mitochondrial precursor transcripts which have been synthesised in the oocyte.

In some experiments higher levels of the 2.9 kb transcript were detected by fluorography of RNA ^{3}H-labelled in vivo in both whole eggs, and enucleate egg fragments. The reason for this variability in signal is not clear: the relative intensities of the identified, mature ^{3}H-labelled transcripts were not affected. It is possible that the processing machinery responsible for metabolising this transcript contains a labile component, the activity of which has been compromised in some experiments. Other possibilities are that this 2.9 kb transcript is synthesised by bacterial contaminants in the sea water, the level of which might have differed between experiments (although the experiments were carried out in the presence of penicillin and streptomycin); an endoparasite infecting the sea urchin eggs; or only at certain stages of oogenesis. The egg preparations used most probably contained a certain level of contaminating oocytes, but the stage of development of these oocytes might have differed between experiments. A further possibility is that the level of this 2.9 kb transcript might have been affected by the environment the egg had been kept in, e.g. the length of time the animals had been kept in the aquarium. However, there did not seem to be any correlation between the length of time that animals had been kept in the aquaria, and the intensity of the 2.9 kb band.

5.2.2.3 Reverse northern hybridisation of in vivo-labelled RNA with sea urchin cloned mitochondrial DNA

In vivo-labelled RNA from whole eggs was hybridised to a Southern blot of restrction-digested cloned sea urchin mitochondrial DNA (Fig. 5.4). Subsequent fluorography of the blot showed that the labelled RNA specifically hybridised to insert (i.e. sea urchin mitochondrial) sequences. No cross-

Figure 5.4 Reverse northern analysis of in vivo-labelled transcription products synthesised in pronase-treated whole eggs, by hybridisation with filter-bound sea urchin mitochondrial DNA. (A) Photograph of an ethidium bromide-stained 1% agarose gel of a range of plasmid digests containing sea urchin sequences. Lanes are indicated as 1, pRB3 digested with TaqI; 2, pZH1.1 digested with TaqI and HindIII; 3, pZH1.4 digested with TaqI and HindIII; 4, pZ3 digested with TaqI and SacI; 5, pZ1A digested with HindIII, SacI and KpnI; 6, K34 (R.F.) digested with EcoRI and SalI; and 7, pP12 digested with HindIII and SacI, but with the 7.0 kb fragment (the highest molecular weight fragment) removed before blotting; (B) Fluorograph of filter derived by Southern blotting the gel shown in (A), after hybridisation with RNA ^{3}H-labelled in whole eggs. The lanes are indicated as in (A). (C) Restriction map of pZ1A showing the restriction fragments which gave the strongest fluorographic signal after hybridisation to ^{3}H-labelled RNA, and also showing the positions of the genes within the pZ1A insert. The positions of the major reactive fragments of pZ1A are indicated on parts (A) and (B) of the figure by their size in nucleotides. Experimental conditions are described in Chapter 2.

Sizes are given in kb.

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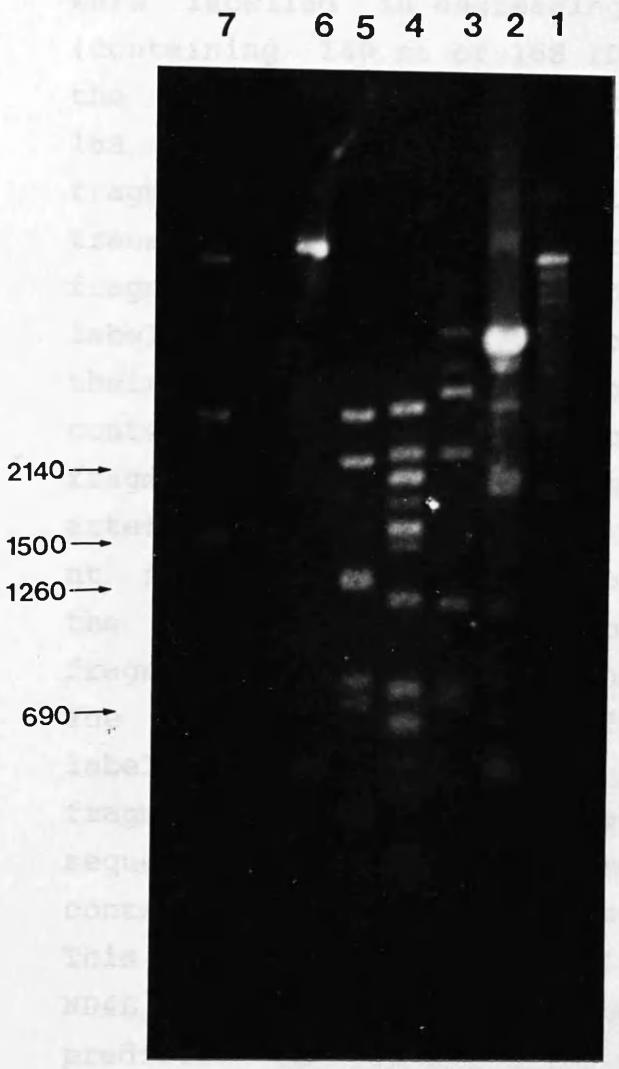
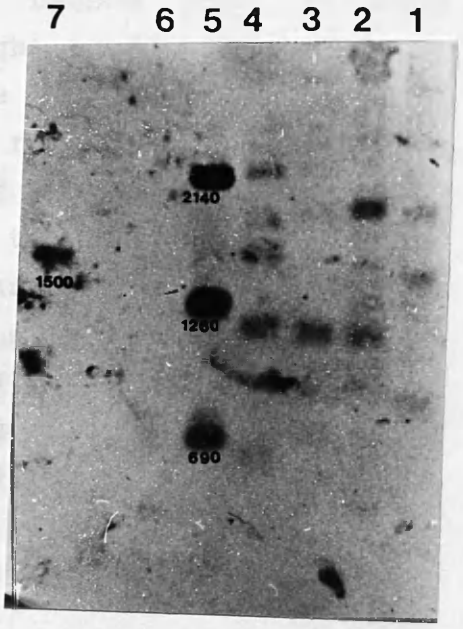
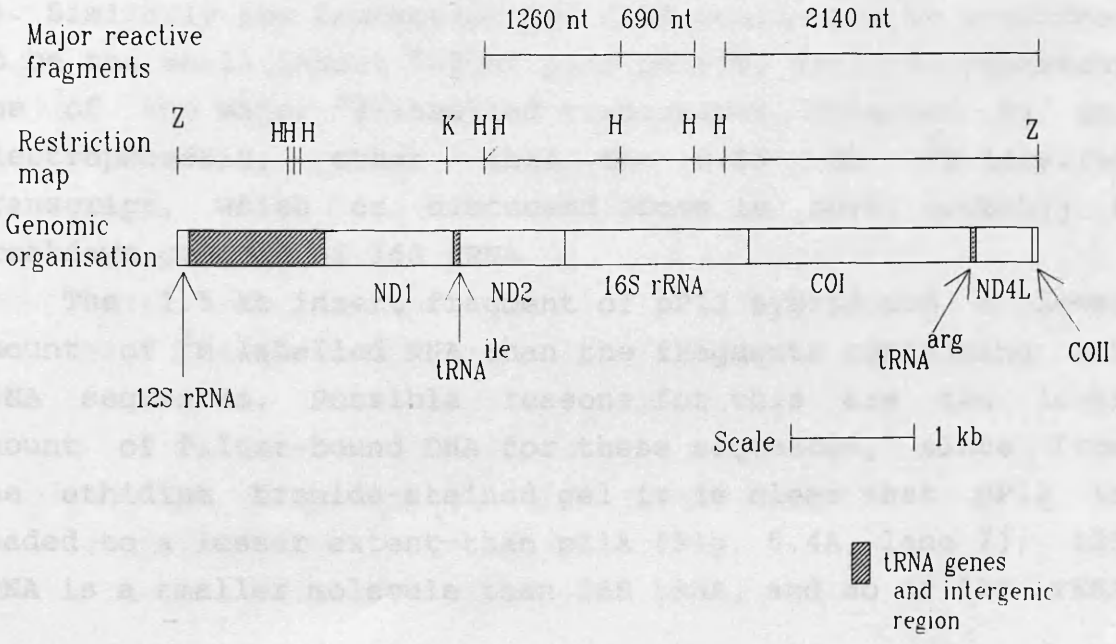
(A)


7 6 5 4 3 2 1
2140
1500
1260
690

(B)


7 6 5 4 3 2 1
2140
1500
1260
690

(C)


Major reactive fragments
1260 nt
690 nt
2140 nt
Restriction map
Z
HHH
K
HH
H
H
H
Z
Genomic organisation
ND1
ND2
16S rRNA
COI
ND4L
12S rRNA
tRNA ile
tRNA arg
COII
Scale
1 kb
tRNA genes and intergenic region

reaction was observed with either vector pUC or M13 (or lambda, not shown) DNA sequences.

The majority of the hybridisation signal was to three fragments of the pZ1A digest (summarised in Fig.5.4C). These were labelled in decreasing order of the 2140 nt fragment (containing 140 nt of 16S rDNA, all of the COI and part of the ND4L genes), the 1260 nt fragment (containing 300 nt of 16S rDNA and 960 nt of the ND2 gene), and the 690 nt fragment (containing only 16S rDNA). There is possibly a transfer artefact, in that the higher molecular weight fragments on the Southern blot have become more strongly labelled than the lower molecular weight bands, unrelated to their sequence content. For example the 690 nt fragment contains more of the sequence of 16S rRNA than the 1260 nt fragment, but is labelled to a lesser degree. This transfer artefact is unlikely to have affected the 2140 nt or the 1260 nt fragments, however, since there was little difference in the intensity of autoradiographic signal from the pZ3 fragments in the adjacent lane (lane 3), in this size range. The fact that the 2140 nt fragment has hybridised to more labelled RNA than either the 690 nt or the 1260 nt fragments, even though these contain more 16S rRNA-reactive sequences, suggests that some other labelled transcript is contributing to the hybridisation signal of the 2140 nt band. This is most likely to be COI mRNA, since the transcript for ND4L, which from sequence data (Jacobs et al, 1988) would be predicted to be 290 nt, plus poly(A) tail, is not of an appropriate size to be one of the predominantly labelled transcripts detected by gel electrophoresis (Fig. 5.2a and b). Similarly the transcript for COII would also be predicted to be too small (about 700 nt plus poly(A) tail) to represent one of the major ^{3}H-labelled transcripts detected by gel electrophoresis, other than the 0.95 kb ^{3}H-labelled transcript, which as discussed above is most probably a breakdown product of 16S rRNA.

The 1.5 kb insert fragment of pP12 hybridised a lower amount of ^{3}H-labelled RNA than the fragments containing 16S rDNA sequences. Possible reasons for this are the lower amount of filter-bound DNA for these sequences, since from the ethidium bromide-stained gel it is clear that pP12 is loaded to a lesser extent than pZ1A (Fig. 5.4A, lane 7); 12S rRNA is a smaller molecule than 16S rRNA, and so if 12S rRNA

and 16S rRNA are synthesised at an equimolar ratio there should be less labelled 12S rRNA available to contribute to the hybridisation signal. The remainder of the insert of pP12, which corresponds to the pZ1A insert, was removed prior to blotting, to prevent it titrating out labelled RNA.

The rest of the sea urchin mitochondrial genome was represented on the Southern blot as a series of plasmid digests. A low level of labelled RNA hybridised to the DNA in these digests, suggesting that the rest of the mitochondrial genome is expressed at a lower level than the regions discussed in the two preceding paragraphs. This corroborates the information obtained from formaldehyde/agarose gels, on which no transcripts corresponding to the genes other than 16S rRNA, COI mRNA and 12S rRNA were visualised.

An important factor to consider when trying to assess in vivo synthetic rates from reverse northern data, such as that described above, is whether during the hybridisation the filter-bound DNA, or the RNA, was in excess. A volume of 0.4 ml (which is the volume of packed sea urchin eggs used for the in vivo-labelling experiments) contains about $2X10^5$ eggs. If each of these eggs contained about 3.3 ng of total cellular RNA (Brandhorst, 1985), a volume of 0.4 ml would contain 0.6 mg of total cellular RNA. The results described in Chapter 3 showed that 1 ug of total egg RNA contains about 0.5 ng of a given mitochondrial mRNA, and around 15 ng of a mitochondrial rRNA. Hence 0.6 mg total cellular RNA would contain around 0.3 ug of a given mitochondrial mRNA, and around 9 ug of a given mitochondrial rRNA. This is probably an over-estimate, since not all the RNA used for the reverse northern was in solution (it is difficult to dissolve such high concentrations of RNA in 50% formamide hybridisation buffer). Around 0.5 ug of plasmid was used for the restriction digestions for the Southern blot, and only a proportion of this plasmid DNA (depending on the particular plasmid) was complementary to sea urchin RNA. This means that the filter-bound DNA is likely to be slightly in excess of the RNA in the case of the mRNA-reactive sequences, but possibly not the rRNA-reactive sequences. This would have the effect of leading to an under-estimate in the level of ^3H-labelled 16S rRNA relative to ^3H-labelled mRNA. This would thus compromise any calculation of in vivo synthetic rates on the basis of the data described in this section.

There are other factors which could affect any conclusion from these reverse northerns. Half-lives of transcripts might be important in affecting the proportion of a given transcript which was labelled (i.e. the fraction of the steady-state level of a transcript which was synthesised during the labelling period), particularly during the long labelling periods such as were used for in vivo-labelling sea urchin RNA in these experiments. If sea urchin mitochondrial mRNAs have shorter half-lives than rRNAs, which is the case in HeLa cells (Section 1.5.2: Gelfand and Attardi, 1981), then after a given duration of labelling time, a greater proportion of the short-lived transcripts (i.e. mRNAs) would be labelled than the long-lived transcripts (i.e. rRNAs). This would be the case since the mitochondrial mRNAs would be turned over and replaced more rapidly than the mitochondrial rRNAs, so the specific activity of the mitochondrial mRNAs would be higher. This would have the reverse affect on the conclusion than the caveat descibed in the paragraph above, but the extent of its effect would depend on the relative half-lives of the mRNA and rRNA. This argument concerning specific activity would also apply to the analysis of the ^{3}H-labelled RNA by gel electrophoresis, as described in the previous section.

In conclusion, it is not possible to draw quantitative conclusions about relative synthetic rates from this reverse northern experiment. However, it does confirm that mitochondrial RNA is being detected by in vivo-labelling, and the apparent level of labelling of 12S rRNA, 16S rRNA and probably COI mRNA, are higher than the other transcripts.

5.2.3 Effect of ethidium bromide on in vivo transcription in sea urchin eggs

The effect on transcription in vivo of a transcriptional inhibitor was tested. There were two reasons for doing this experiment. Firstly, since different classes of RNA polymerase differ in their sensitivity to transcriptional inhibitors (Penman et al, 1970), any differential affect on the individual in vivo transcription products would suggest that they are transcribed by different RNA polymerases. If this was the case, it would contradict the data obtained in the previous section, which suggested

that these transcription products were all mitochondrial in origin, and so most likely all synthesised by the mitochondrial RNA polymerase. The other reason for doing this experiment is that it would enable me to estimate what proportion of the total level of mitochondrial RNA was synthesised during the labelling period (and so ^{3}H-labelled). The idea behind this estimate is described below. The transcriptional inhibitor was added to cells 1 hour before the ^{3}H-label was added, to allow absorbtion of the drug, and any inhibition of RNA polymerase, to occur before the synthesis of labelled RNA began.

In vivo transcription was carried out in the presence of various concentrations of ethidium bromide, which intercalates into DNA. Incubations were carried out with the tubes wrapped in foil in order to prevent photocleavage of DNA. Concentrations of ethidium bromide above 0.2 ugml^{-1} totally abolished in vivo transcription, as assayed by formaldehyde gel electrophoresis, northern blotting and fluorography (Fig. 5.5a). A faint fluorographic signal corresponding to the 1.7 kb, the putative breakdown products of 16S rRNA (see above),and to a slightly lesser extent the 1.8 kb transcript was observed for RNA synthesised at 2ugml^{-1} ethidium bromide. This may represent some sort of artefact, since no fluorographic signal was obtained from RNA synthesised at 1ugml^{-1} ethidium bromide. A comparison of the signal of ^{3}H-labelled RNA detected by fluorography (which represents the RNA synthesised in vivo over the time-course of the labelling), with the autoradiographic signal detected with a ^{32}P-labelled probe (which represents the steady-state level of a given transcript), shows that the in vivo-labelled RNA only makes a very small contribution to the steady-state level, since at concentrations of ethidium bromide which lead to a complete suppression of in vivo-labelling, only a small decrease in the autoradiographic signal for 12S rRNA was observed (Fig. 5.5B).

Classes of RNA polymerase differ in their sensitivity to transcriptional inhibitors (Penman et al, 1970). In eggs of the sea urchin L. pictus, it has been reported that concentrations of ethidium bromide which led to a greater than 90% reduction in mitochondrial transcription (as assayed by comigration with mitochondrial transcripts) only led to a 30-60% reduction in nuclear RNA (histone mRNA) labelling

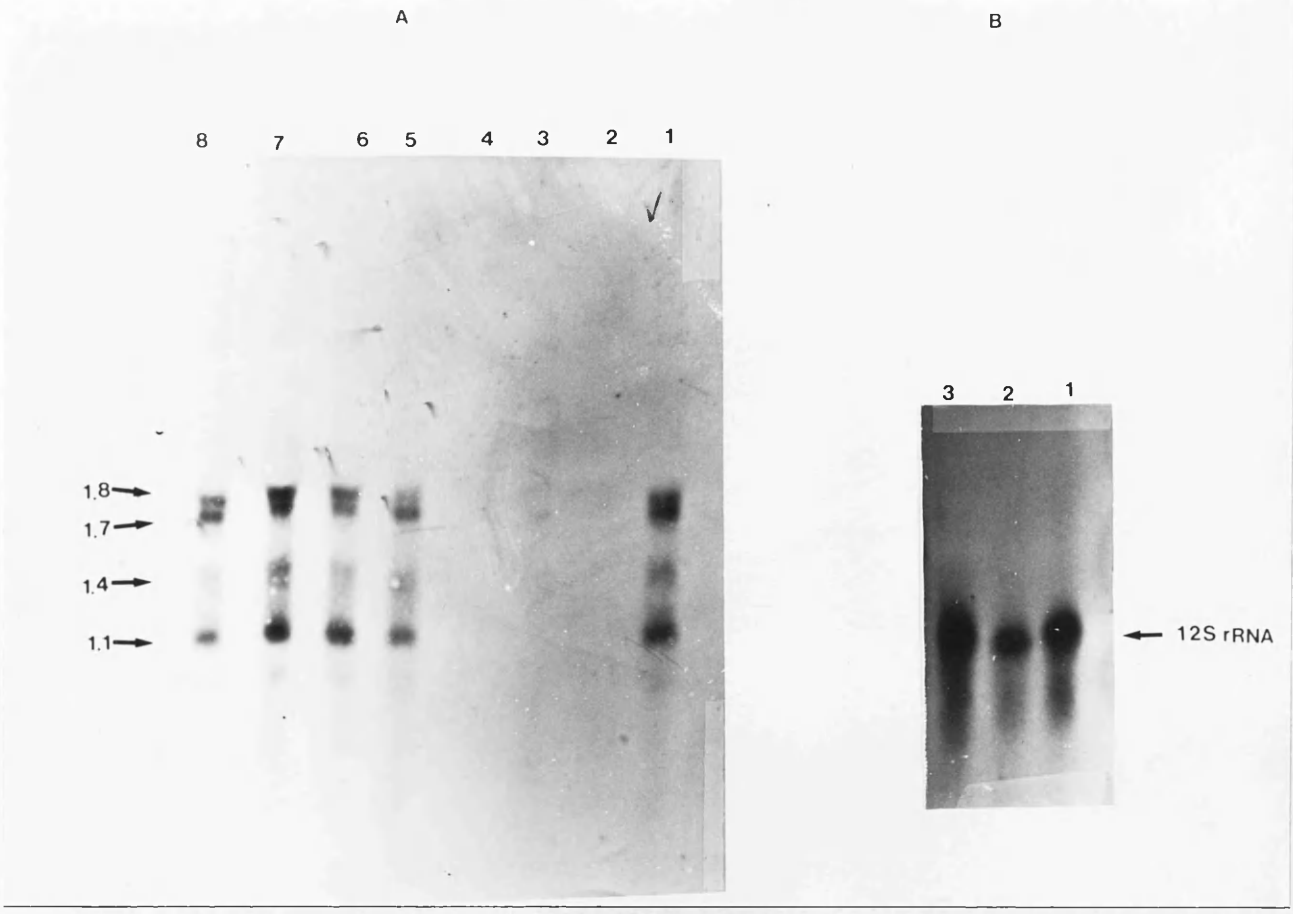


Figure 5.5 Effect of ethidium bromide on RNA synthesised in vivo in pronase-treated whole sea urchin eggs. (A) Fluorography of RNA synthesised in the presence of various concentrations of ethidium bromide, then electrophoresed on a 1% agarose/formaldehyde gel and transferred to a nylon filter membrane. Lanes are indicated as 1, no ethidium bromide; 2, RNA 20 ug/ml ethidium bromide; 3, ug/ml ethidium bromide; 4, 1ug/ml ethidium bromide; 5, 0.2 ug/ml ethidium bromide; 6, 0.1 ug/ml ethidium bromide; 7, 0.02 ug/ml ethidium bromide; 8, 0.01 ug/ml ethidium bromide. (B) Total level of 12S rRNA in 3 lanes from (A), detected by autoradiography after hybridisation of the filter in 50% formamide hybridisation buffer with a nick-translated pPZ0.6 probe. Lanes are labelled as in (A). Labelled transcripts were sized by comparison to an RNA size ladder (BRL). Experimental techniques were carried out as described in Chapter 2.

Sizes are given in Kb.

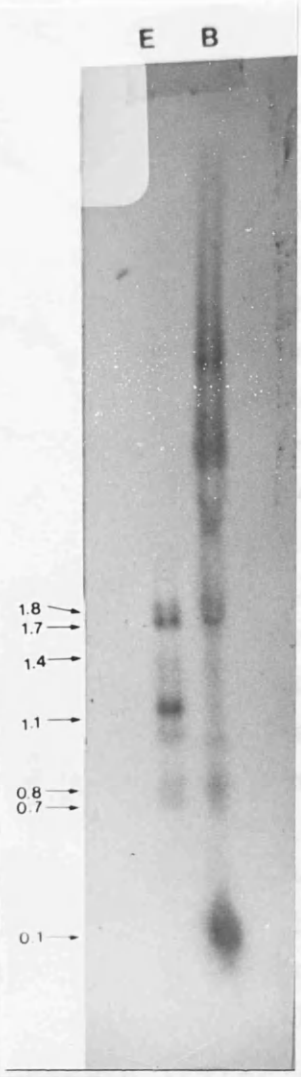


Figure 5.6 RNA synthesised in vivo in hatching blastula (B), and whole eggs (E) electrophoresed in parallel on a 1% agarose/formaldehyde gel, and transferred to a Pall Biodyne nylon filter prior to fluorography. Blastula RNA was labelled for 4 hours, and egg RNA was labelled for 16 hours. 0.4 ml of both packed eggs and blastulae were used for the labelling reactions. Egg RNA was labelled as described in the Materials and Methods section, and Blastula RNA was labelled by adding ^{3}H uridine to the blastulae without any prior pronase treatment. Transcripts were sized by comparison with an RNA size ladder (BRL).

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(Ruderman and Schmidt, 1981). The fact that the synthesis of all the major species of labelled transcript showed the same dose response to inhibition by ethidium bromide suggests that they are all transcribed from the same RNA polymerase. Since these labelled products are synthesised in enucleate eggs, this is almost certainly the mitochondrial RNA polymerase.

5.2.4 RNA synthesis in sea urchin embryos

In order to determine the extent of mitochondrial RNA synthesis which was going on at later stages of development from the egg, I carried out *in vivo* RNA labelling in hatching blastulae. *In vivo* transcription in hatching bastulae was monitored by adding ^{3}H uridine for 4 hours. A comparison of labelled RNA species with those synthesised in eggs electrophoresed alongside shows a large difference in the pattern of transcripts synthesised at the two developmental stages (Fig. 5.6). In ^{3}H-labelled blastula RNA, only a small amount of labelled material comigrated with that ^{3}H-labelled in the egg, and no *in vivo*-labelled mitchondrial RNA could be detected by hybridisation to cloned mitochondrial DNA, in a reverse northern experiment (not shown). Quantitatively, the bulk of RNA ^{3}H-labelled in hatching blastulae was of a higher molecular weight than that synthesised in the egg, and also RNA in the tRNA size range was labelled. A similar result has been obtained in *L. pictus* (Devlin, 1976), where the RNA labelled in blastulae was identified as being predominantly the result of nuclear transcription, on the basis of a decreased sensitivity to ethidium bromide, and since the level of this labelled RNA decreased when mitochondrial RNA which had been purified by sub-cellular fractionation was analysed.

These results suggest that mitochondrial RNA is not being actively synthesised during the blastula stage of development. Although the results described in Chapter 3 suggested that mitochondrial transcription occurred between the egg and blastula stages of development, this synthesis might have occurred at an earlier stage than that of the blastula itself. It should be noted, however, that the labelling period used to label hatching blastulae was shorter than that used to label eggs, necessarily so since this later developmental stage is shorter. Another important point is

that the mitochondrial precursor pool of uridine might not have been the same in the blastula as in the egg. The specific activity of the mitochondrial precursor pool at the blastula stage could have been reduced by the fact that a much greater level of transcription is obviously occuring in the nucleus than at the egg stage, which might reduce the amount of label transported to the mitochondria.

5.2.5 Ultra-violet mapping of sea urchin mitochondrial transcription units

The experiments described in Section 5.2 have shown that the bulk of RNA labelled *in vivo* in sea urchin eggs is mitochondrial in origin, and have allowed an identification of these transcripts (although this is not conclusive). I decided to use this *in vivo*-labelling system, along with u.v. mapping, to attempt to map sea urchin mitochondrial transcription units.

Sea urchin eggs were prepared for *in vitro* transcription by pronase treatment. Aliquots of 0.4 ml of packed sea urchin eggs were resuspended in 10 ml ASW, and placed on a petri dish on ice. The eggs were then exposed to short wave (254 nm) u.v. irradiation, from a calibrated Hannoveria mutagenic lamp, for between 30 seconds and 20 minutes with continual manual agitation. The Hannoveria mutagenic lamp was calibrated with a Macam u.v. monitor. Irradiation was performed from above, since short-wave u.v. is quenched by even short thicknesses of plastic. During and after irradiation eggs were kept in the dark to prevent photorepair of pyrimidine dimers, which is known to occur in sea urchin eggs (Rustad, 1975). After irradiation, the eggs were transferred to 15ml Falcon tubes, wrapped in foil, spun down (Jouan rotor, 1000g, 1 minute), and then resuspended in ASW containing penicillin and streptomycin as for a normal *in vivo* transcription incubation. The eggs were then incubated for 1 hour on a multimixer, in order to allow any polymerase which was engaged in transcription prior to irradiation to detach from the template DNA and re-initiate. Label was then added, and the eggs incubated as for a normal *in vivo*-labelling experiment.

^{3}H-labelled RNA was analysed by gel electrophoresis, transfer to a nylon filter membrane, and fluorography (Fig.

5.7). In 3 separate experiments, u.v. irradiation caused a differential decrease in certain RNA species, which have been assigned as being mitochondrial in origin and putatively identified on the basis of the experiments described in section 5.2.1 and 5.2.2. Synthesis of the putative mature 16S rRNA transcript, and also of the putative breakdown products of 16S rRNA, was resistant to even the largest doses of u.v. used, whereas synthesis of the putative COI mRNA transcript and 12S rRNA transcript were sensitive to low doses of u.v. used. Synthesis of the 2.9kb higher molecular weight transcript was resistant to u.v. irradiation, whereas synthesis of the 3.2kb transcript was u.v.-sensitive. Although the general trend of transcript sensitivities was the same in all experiments, in the experiment shown in Fig. 5.7c the synthesis of the putative COI transcript was less sensitive to u.v. irradiation than the putative 12S rRNA transcript, being still synthesised at 360 $\mu J.mm^{-2}$. However , in the experiment shown in Fig. 5.7b the synthesis of the putative COI transcript at this u.v. dose was drastically reduced. The implications of these results are discussed in detail below.

In order to examine the u.v.-sensitivity of synthesis of other mitochondrial transcripts which were too rare to be detected by fluorography of filter-bound RNA after formaldehyde/agarose gel electrophoresis, I carried out reverse northerns of u.v.-treated and untreated <u>in vivo</u>-labelled RNA with cloned mitochondrial DNA. To date these experiments have not been successful, as a result of a cross-reaction of labelled RNA in these experiments with vector sequences. The source of this cross-reaction is not clear. The pattern of transcripts obtained in the experiments described in Section 5.2.5 for the non-irradiated control was identical to that obtained previously (Section 5.2a and data not shown) when no cross-reaction was seen. It is possible, however, given the low hybridisation signal obtained in the reverse northern from non-irradiated DNA shown in Fig. 5.4), that reverse northerns might have not provided information on the u.v.-sensitivity of the transcripts other than the putative 12S rRNA, 16S rRNA and COI mRNA transcripts.

(a)

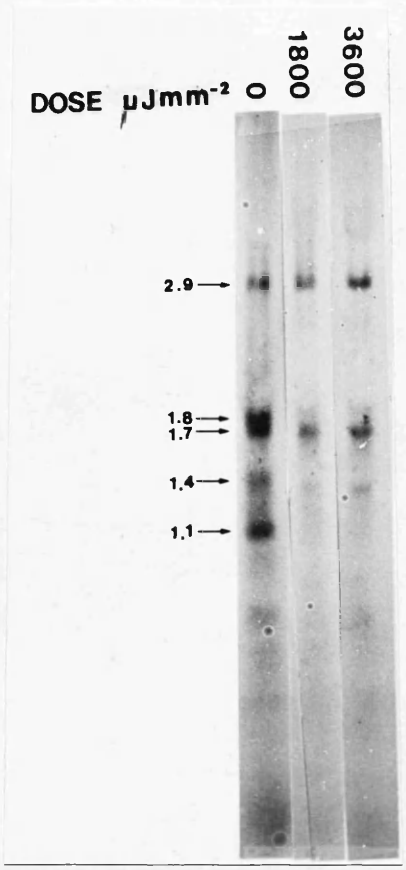

Figure 5.7 Ultra-violet mapping of sea urchin mitochondrial transcription units. The results from 3 separate experiments are shown in (a), (b) and (c). The doses of u.v. used are indicated above the lanes. RNA was electrophoresed on 1% formaldehyde agarose gels, transferred to a Pall Biodyne nylon filter membrane, and visualised by fluorography. Transcripts were sized by comparison with an RNA size ladder (BRL). Full details are given in the text and Chapter 2.

Sizes are given in Kb.

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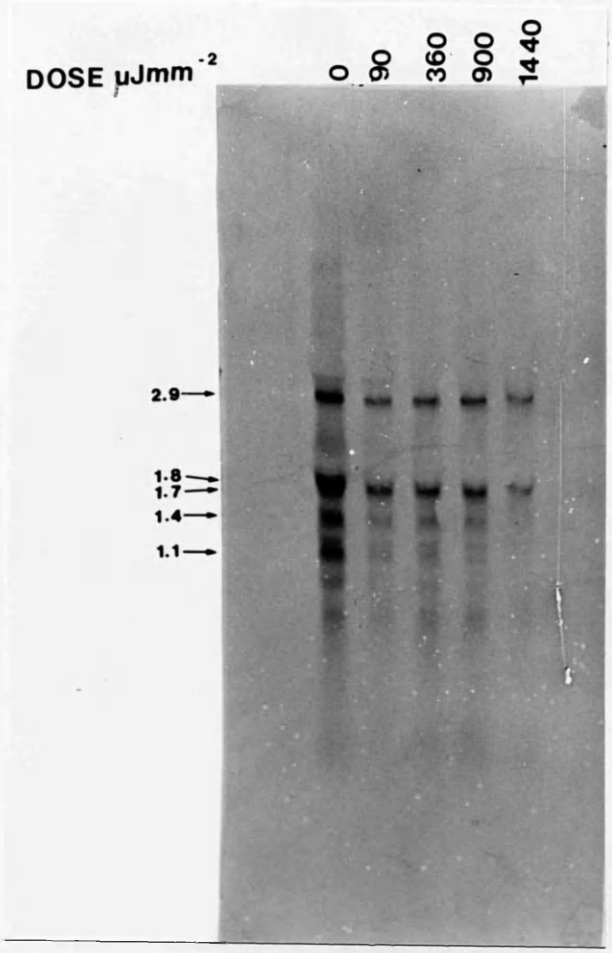
(b)


DOSE µJmm⁻²
0
90
360
900
1440
2.9
1.8
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1.4
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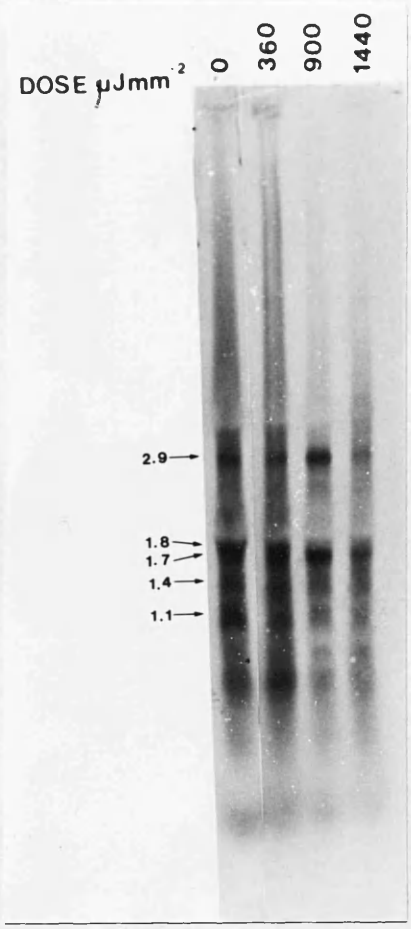
(C)


DOSE µJmm⁻²
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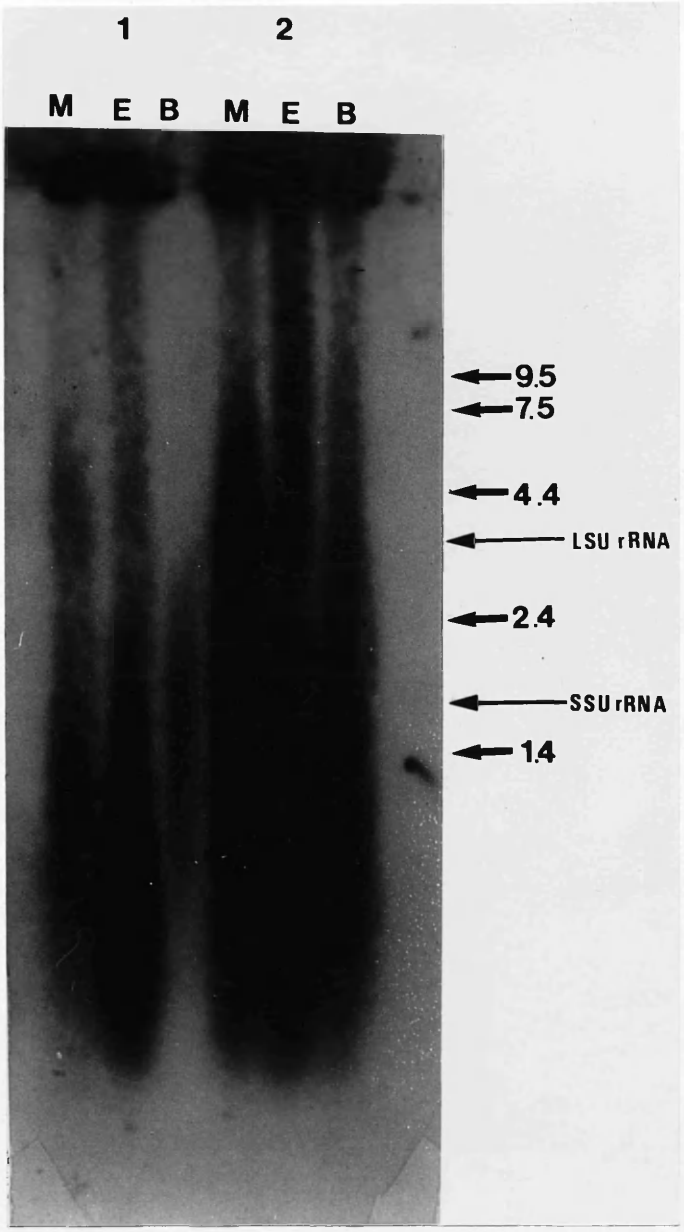

Figure 5.8: *In vitro* capping of sea urchin RNA followed by analysis of reaction products by formaldehyde/agarose gel electrophoresis. Lanes are indicated according to the RNA used in the reactions (B, blastula; E, egg; and M, mitochondrially enriched egg RNA), and as to the labelling protocol used (1: Barat-Geuride *et al*, 1987; 2: Gherke, 1986). After electrophoresis on a 1% agarose/formaldehyde gel, RNA was transferred to a Pall Biodyne filter, and autoradiographed. The positions of the BRL size ladder and the nuclear-encoded large and small subunit rRNAs are shown. Sizes are given in Kb.

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5.2.6 In vitro capping of sea urchin RNA

Published reaction conditions for in vitro capping experiments differ. In order to find the optimal conditions for labelling sea urchin mitochondrial RNA by in vitro capping, I carried out two different labelling protocols, one based on Gherke (1986) and the other based on Barat-Gueride et al (1987).

After labelling, total cellular RNA was analysed by electrophoresis on a agarose/formaldehyde gel, followed by blotting onto a nylon filter membrane (which eliminated unincorporated nucleotides), and autoradiography (Fig. 5.8). Labelling of sea urchin RNA by either technique resulted in a smear of reaction products which were predominantly of a size below 4.4 kb. Labelling by the technique of Gherke (1986) gave a higher level of ^{32}P-incorporation into RNA. In neither case were any distinct bands visible above the smear of reaction products.

When used to probe Southern blots of cloned mitochondrial DNA restriction fragments, reaction mixes from either protocol containing sea urchin egg or blastula RNA, or a control reaction carried out without added RNA, showed hybridisation of labelled RNA with vector pUC but not lambda DNA (Figures 5.9, 5.10, 5.11). Hence, the reaction mixes must have contained contaminating RNA which was homologous to pUC sequences. A possible source of this is the BRL guanylyl transferase enzyme, which is produced from a cloned gene in E. coli, and so might contain some pUC RNA which has a triphosphate group able to act as a substrate for the reaction. The hybridisation signal obtained from a reaction mixture without added sea urchin RNA is shown in Figure 5.9.

Sea urchin egg and blastula RNA in vitro capped by the Gherke method also showed a specific hybridisation signal to the insert of pH1.6, and blastula RNA also showed a hybridisation signal to the 1.7 kb EcoR1 fragment of the insert of lambda mt1 (Fig. 5.10). The insert of pH1.6 contains the genes coding for several tRNAs, ND1 and part of ND2. The 1.7 kb EcoR1 fragment of lambda mt1 contains the sequence of part of the gene for COII, all of the genes for A6 and A8, and part of COIII. The region of lambda mt1 corresponding to the pH1.6 insert (the 7.7kb EcoR1 fragment) did not hybridise to RNA at a detectable level. A possible

Figure 5.9 Hybridisational analysis of in vitro capping reaction carried out without added sea urchin RNA. (A) Photograph of an ethidium bromide-stained agarose gel. Lanes are indicated as: i, pPZ0.6 cut with PstI and SacI; ii, K34 cut with EcoRI and SalI; iii, pZH2.0 cut with HindIII and PstI; iv, pH1.3 cut with HindIII (partial digest); v, pH1.6 cut with HindIII; vi, SpG30, did not digest; vii, pZ3 cut with SacI; viii, pRB3 cut with EcoRI and BglII; ix, pPZ0.8 cut with SacI and HindIII; x, pR3 cut with EcoR1 (partial digest); xi, pZH1.4 cut with SacI and HindIII; xii, pZH1.1 cut with SacI and HindIII; xiii, mp18 cut with HinfI; xiv, pUC19 cut with HinfI; xv, lambda mt1 cut with EcoR1; xvi, lambda mt1 digested with PstI; xvii, lambda mt1 digested with HindIII. (B) Autoradiograph of a Southern blot prepared from the gel shown in (A), probed with an in vitro capping reaction (using method 1, see legend for Fig. 5.8), carried out without added RNA.

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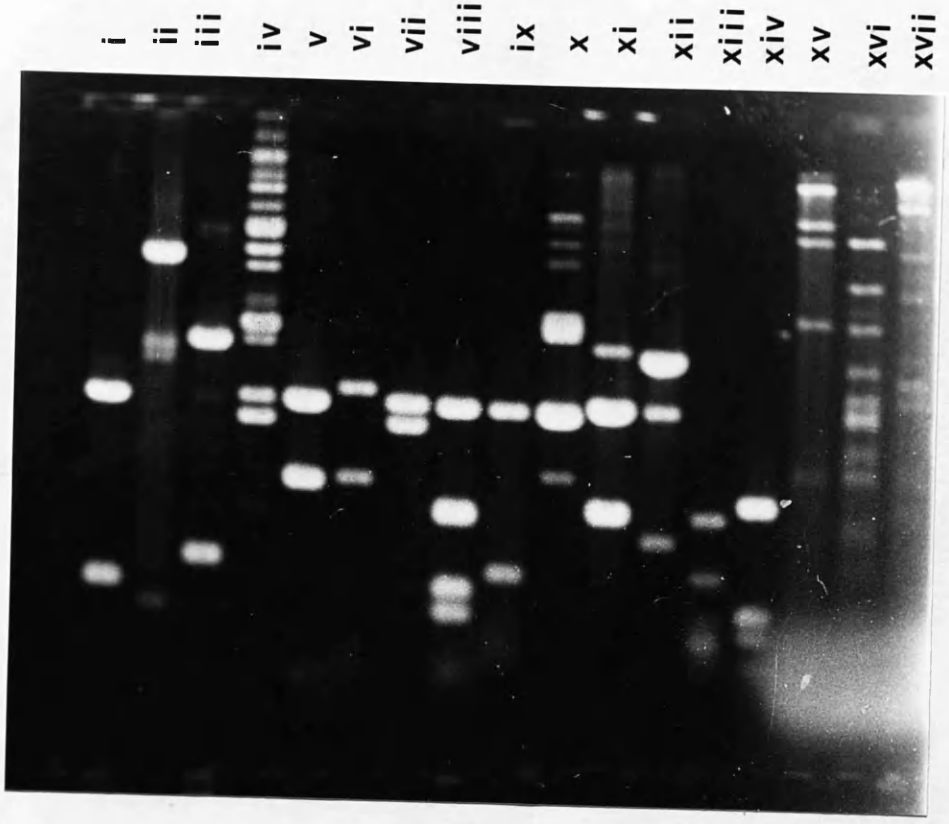
A


i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
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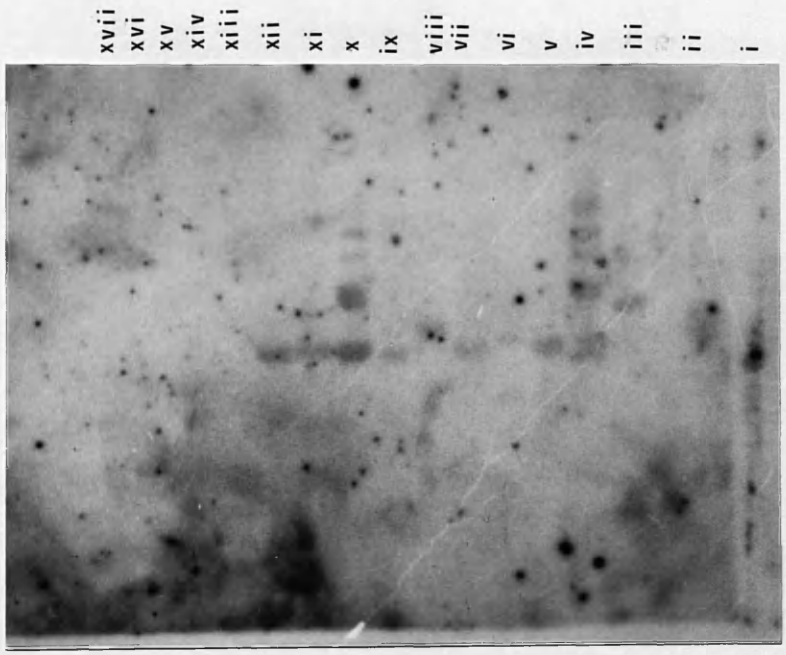
xv
xvi
xvii

B


xvii
xvi
xv
xiv
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A

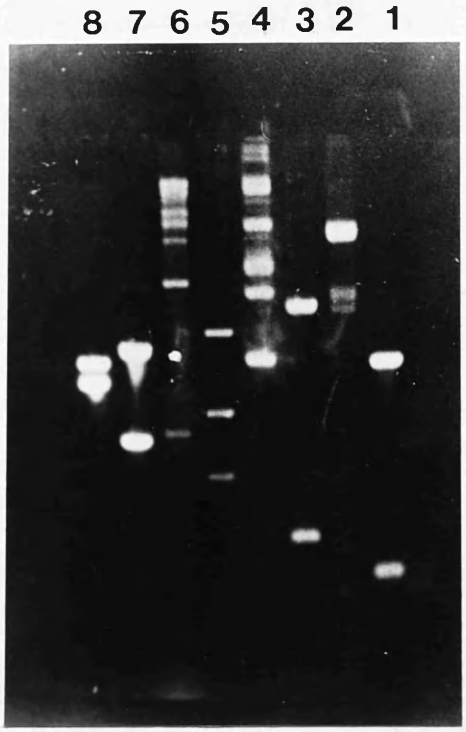

Figure 5.10 Hybridisation analysis of RNA labelled by *in vitro* capping using method 2 (see legend for Figure 5.8). (A) Ethidium bromide-stained fluorescent photograph of 1% agarose gel, used to make Southern blots of cloned sea urchin mitochondrial DNA. The lanes are indicated as: 1, pPZ0.6 digested with SacI and PstI; 2, K34 (R.F.) digested with EcoRI; 3, SpG30 digested with SalI and HindIII; 4, pZH2.0 digested with PstI and HindIII; 5, RB3 digested with EcoRI and BglII; 6, Lambda mt1 digested with EcoRI; 7, pH1.6 digested with HindIII; 8, pZ2 digested with SacI. (B) Southern blot prepared as in (A), probed with egg RNA capped *in vitro* using method 2. (C) Southern blot prepared as for (A) probed with blastula RNA labelled by method 2. (D) Longer autoradiographic exposure of Southern blot shown in (C).

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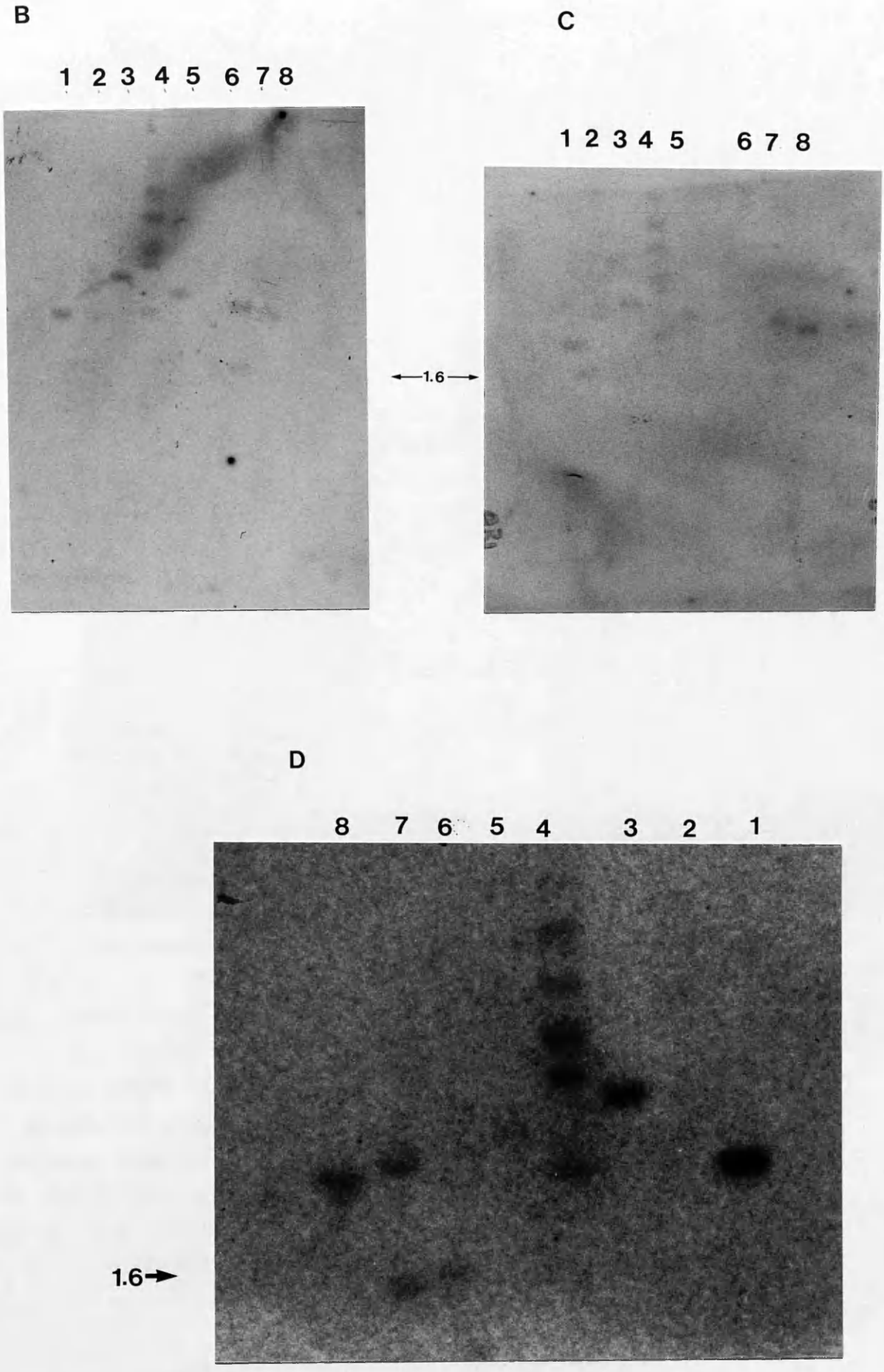
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8
1.6
D
8 7 6 5 4 3 2 1
1.6

Figure 5.11 Hybridisational analysis of RNA labelled by *in vitro* capping using method 1 (see legend for figure 5.8). (A) Photograph of an ethidium bromide-stained agarose gel. Lanes are indicated as: a, pUC19 digested with HinfI; b, lambda mt1 digested with EcoRI; c, RB3 digested with EcoRI and BglII (partial digest); d, A45 (R.F.) digested with SacI and HindIII; e, pZ3 digested with SacI and PstI; f pZ1B digested with PstI and SacI; g, pZ1B digested with SacI and XbaI; h, pZ1B digested with SacI; i, pP12 digested with SalI and SacI; j, K34 (R.F.) digested with EcoRI. (B) An autoradiograph of a Southern blot prepared from the gel shown in (A), and probed with *in vitro* capped blastula RNA labelled by method 1, with the lanes indicated as in (A).

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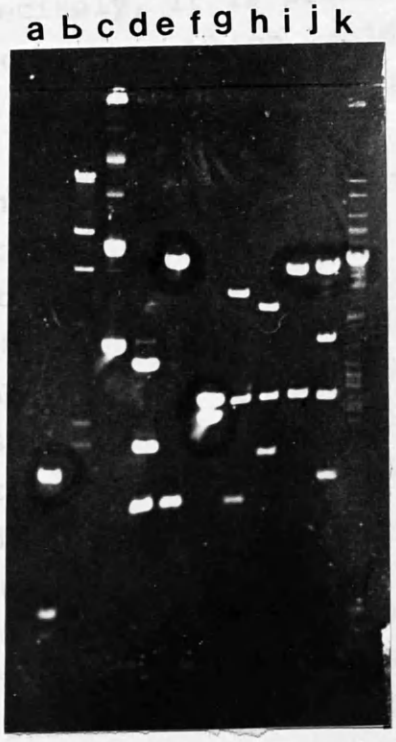
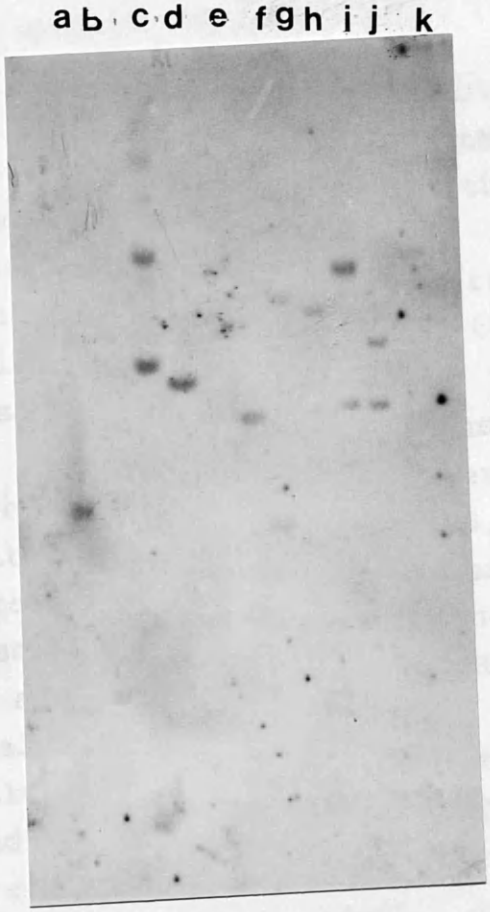
A



B



explanation for this discrepancy is that the filter-bound DNA concentration in the case of the lambda mt1 digest, but not the pH1.6 digest, was not in excess of the sea urchin RNA (which, in addition to ^{32}P-labelled RNA, would also contain a level of competing unlabelled RNA). Two factors could contribute towards this effect. Firstly, the lambda mt1 DNA preparation used for these Southern blots contained a proportion of deleted molecules (H.T. Jacobs, personal communication). Although this deletion has not been mapped precisely, it is probable that it maps to the pH1.6 region of the genome, which would lead to a reduction in the amount of filter-bound DNA of this region on the Southern blot.

Secondly, since lambda mt1 is a much larger molecule than pH1.6, for a given quantity of DNA on a filter, the molar amount of pH1.6 insert would be much higher than the corresponding region cloned in lambda mt1. lambda mt1 is 46.3 kb. Approximately 1/30 of this will correspond to the pH1.6 insert. Since 0.5 ug of lambda mt1 was used for the Southern blot, of which 16.7 ng should have corresponded to the pH1.6 insert. Around 40 ug of total cellular RNA was used in the capping reactions using the protocol based on Gherke (1986). On the basis of the results described in Chapter 3, 1 ug of total egg RNA should contain around 0.5 ng of each mitochondrial mRNA, and in 1 ug sea urchin blastula RNA there should have been around 5 ng of a given mitochondrial mRNA. Multiplying 5 ng of mitochondrial mRNA/ug total cellular RNA by the number of ug total cellular RNA used, shows that around 200 ng of each mitochondrial mRNA should have been available for hybridisation, as compared with around 16.7 ng of filter-bound DNA corresponding to the pH1.6 insert. Hence on the basis of this calculation, the RNA in solution should have been in excess over the filter-bound DNA. However, since it was filter-bound, some of this DNA may not have been available for hybridisation.

The 1.75 kb EcoR1 fragment of the insert of lambda mt1 was not represented in a plasmid digest on the Southern blot, so it is not possible to compare the autoradiographic signal resulting from hybridisation of *in vitro* capped RNA with the same sequences contained within a plasmid (which would contain a much higher proportion of DNA corresponding to the 1.75 kb insert).

A superficial conclusion for the fact that a

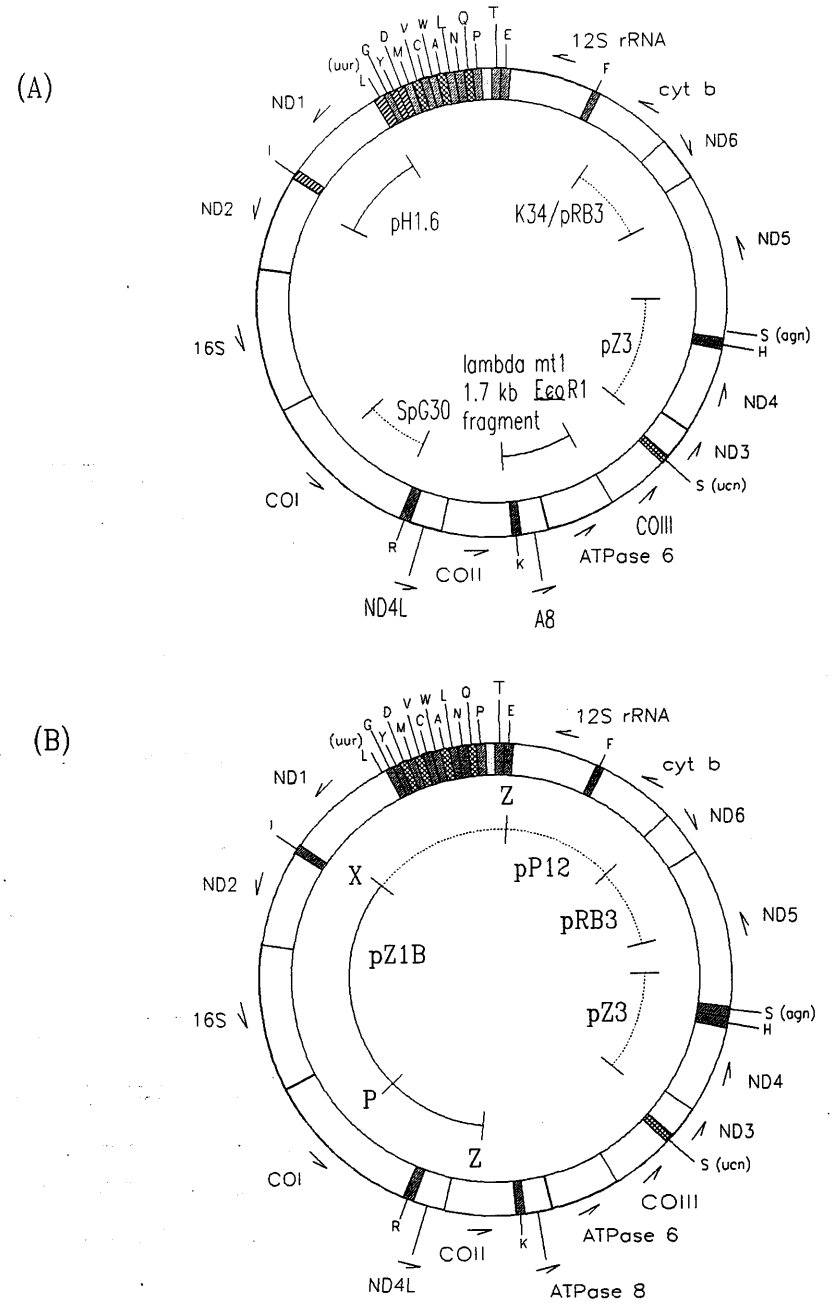


Figure 5.12 Summary of data obtained from the in vitro capping experiments shown in Figures 5.9-5.11. (A) Data from figure 5.10. (B) Data from figure 5.11. Restriction sites within pZ1B are shown as P (PstI), Z (SacI), and X (XbaI).

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hybridisation signal was observed to this lambda mt1 fragment, given the arguments in the peceding paragraph, would be that it was complementary to RNA with high levels of 5' triphosphate. However, there is an important caveat to this conclusion. The hybridisation signal observed to this restriction fragment, relative to the absence of hybridisation signal observed to other restriction fragments, would also be dependent on other factors, such as the relative levels of competing unlabelled RNA, and the ability of different 5' triphosphate termini to act as substrates for in vitro capping reactions.

In a separate experiment, where in vitro capped-blastula RNA (by the method of Gherke, 1986) was hybridised to a Southern blot, cross-reaction with pUC was again observed, particularly with the large fragment of the pUC/HinfI digest (Figure 5.11). In this experiment, the only region of the sea urchin mitochondrial genome to hybridise specifically with labelled RNA was the pZ1B insert. This insert contains the pH1.6 insert, but this region of the pZ1B insert (the 1.8kb SacI-XbaI fragment) did not hybridise labelled RNA on its own, whereas both the adjacent 3.5kb XbaI-PstI and 1.4kb PstI-SacI fragments did. Hence these experiments did not give wholly consistent results. The data which was obtained is summarised in Fig. 5.12.

A general problem encountered during these experiments was in obtaining, reproducibly, a high enough specific activity of labelled RNA in order to probe Southern blots, and to obtain an autoradiographic signal within a reasonable time period.

5.3 Discussion

5.3.1 In vivo transcription

The experiments described in this chapter, which compared mitochondrial RNA synthesis in whole and enucleate eggs, have shown that mitochondrial transcription products are the major RNA species labelled in vivo in pronase-treated eggs. There was no detectable labelling of the nuclear-encoded rRNAs. This result agrees with that obtained by Ruderman and Schmidt (1981), and Devlin (1976), who found that 80-90% of transcription occurring in the eggs of the sea

urchin Lytechinus pictus was mitochondrial in origin (as determined by comigration of the labelled transcripts with ethidium bromide-stained mitochondrial rRNA), with the remainder of the detectable transcription being of histone genes (as determined by comigration of the labelled transcripts with those detected with a molecular probe for histone mRNA). However, this result differs from that of Dworkin and Infante (1978), who found that 65-80% of RNA labelled in both L. pictus and S. purpuratus was restricted to the nucleus upon cell fractionation. The reason for this discrepancy is not clear. It cannot be accounted for by the technique used to purify sea urchin nuclei by Dworkin and Infante (differential centrifugation), since their labelled RNA species are too big to represent mature mitochondrial transcripts.

A similar pattern of ^{3}H-labelled transcripts were detected here, as were reported by Ruderman and Schmidt (1981) for L. pictus, although the duration of labelling used for labelling S. purpuratus RNA was significantly longer. I have made a preliminary identification of these major labelled RNA species on the basis of their comigration with transcripts detected with gene specific ^{32}P-labelled probes, and using the ^{3}H-labelled RNA species as a probe for a reverse northern against cloned mitochondrial DNA sequences. Comigration is not definitive proof of identity, since the ^{3}H-labelled transcripts detected by fluorography of northern blots might merely have comigrated with, but not been identical to, the transcripts detected by northern hybridisation. Given this possibility, the major labelled species have been identified as 16S rRNA, 12S rRNA and COI mRNA. 16S rRNA and 12S rRNA are the most abundantly synthesised mitochondrial transcripts in HeLa cells (Gelfand and Attardi, 1982). Another reason for believing that the proposed identification of 16S rRNA is correct, is that an the breakdown products of 16S rRNA detected with ^{32}P-labelled probes comigrate exactly with ^{3}H-labelled transcripts. The chances of such a number of transcript comigrating must be slim.

The high level of labelling of the putative COI mRNA is somewhat surprising, since the results described in Chapter 3 showed that the steady-state level of COI mRNA in eggs is 30-fold lower than 16S rRNA. Moreover, COI mRNA is expressed at

a 100-fold lower level in HeLa cells than 12S rRNA (Gelfand and Attardi, 1982). Probing northern blots with ^{32}P-labelled probes measures the level of COI mRNA stably accumulated over the egg and oocyte stages of development. The experiment shown in Fig. 5.5B shows that the 12S rRNA synthesised over 16 hours represents only a very small fraction of the total steady-state level of 12S rRNA. Hence the difference in the relative steady-state levels and the in vivo-synthesised levels of COI mRNA and 16S rRNA might be explained by the longer term stability (i.e. greater than 16 hours) of 16S rRNA rather than COI mRNA. It is interesting to note that the levels of labelled COI mRNA and 16S rRNA, labelled in vivo in HeLa cells with ^{32}P-orthophosphate over a 4 hour period , were similar (Attardi et al, 1985). This might suggest that differential stability contributes to the differential levels of COI mRNA and 16S rRNA in HeLa cells as well. As discussed above, the specific activity of in vivo-labelled transcripts may be affected by transcript half-lives as well as synthetic rates. Another possible reason for the observed difference in the in vivo-labelled and steady-state levels of 16S rRNA and COI mRNA, could be that RNA synthesis in pronase-treated eggs might not be entirely physiological.

In addition to the mature mitochondrial transcripts detected by ^{3}H-labelling, a number of higher molecular weight transcripts were labelled. The origin of these has been discussed above. In future experiments it might be possible to enrich for these transcripts using the methylation inhibitor cycloleucine, which has been shown to cause an accumulation of mitochondrial rRNA precursors in chinese hamster cells in culture (Dubin et al, 1985; Prince et al, 1986 ; Section 1.11)

A conclusive identification of these RNA species will rely on the use of a form of hybrid-selection mapping, for example by hybrid-selecting ^{3}H-labelled RNA with filter-bound DNA, then eluting this filter-bound RNA and analysing it by gel electrophoresis. A more efficient alternative might be to hybridise in vivo-labelled RNA either to a transcript-specific oligonucleotide, or to specific cloned single-stranded DNA in solution, then to cleave with RNase H and look for loss of the appropriate transcript by gel electrophoresis.

A general problem of using in vivo-labelling in order to

monitor transcription is that only transcripts showing a high level of both transcription and stability over the labelling period can be detected, and, during hybridisation experiments, labelled RNA has to compete for hybridisation with high levels of unlabelled RNA. A comparison of the hybridisation signals obtained by probing northern blots of RNA from eggs in which in vivo transcription had been blocked using ethidium bromide, and untreated controls, revealed only slight differences in the level of 12S rRNA. This suggests only a small fraction of the steady-state mass of 12S rRNA was synthesised during the labelling period used.

5.3.2 Ultra-violet mapping

The experiments described in this chapter have shown that the synthesis of different transcripts encoded by the sea urchin mitochondrial genome show a different sensitivity to u.v. irradiation. Synthesis of the putative transcript for 16S rRNA has been shown to be insensitive to the highest doses of u.v. irradiation used in these experiments, whereas the synthesis of the putative COI mRNA and 12S rRNA transcripts were sensitive to the lowest doses used. Irradiation with u.v. inhibits transcription in a dose-dependent manner depending on distance from the site of transcriptional initiation. These results therefore suggest that 16S rRNA is synthesised by transcription initiating closely upstream of the 16S rRNA gene, whereas COI mRNA and 12S rRNA are synthesised using transcriptional initiation sites which initiate far upstream of their respective genes. Since the genes encoding 16S rRNA and COI mRNA are adjacent (Fig. 1.5), and ultra-violet inactivation of transcription increases exponentially with distance from the transcriptional initiation site, the difference in the effect of u.v. on the synthesis of 16S rRNA and COI mRNA suggests that they must be contained within different transcription units, the initiation site for COI being located far upstream of the 16S rRNA initiation site. A 40-fold higher dose of u.v. irradiation than that which suppressed the synthesis of the putative COI mRNA, had no effect on the synthesis of the putative 16S rRNA transcript. Additionally, synthesis of the 2.9 kb putative precursor transcript was u.v. resistant. This means that transcription 2.9 kb downstream of at least one

site of transcriptional initiation is largely unaffected by the u.v. doses used in these experiments, which is approximately the same distance as the 5' end of 16S rDNA to the 3' end of the COI gene.

Based on the data presented in this chapter, 12S rRNA and 16S rRNA may either be in the same or in different transcription units, a point which is discussed further in chapter 6. In any case the transcriptional order of the 12S rRNA and 16S rRNA genes must differ from all other animal mitochondrial transcription systems characterised, with the gene for 16S rRNA being located nearer a transcriptional initiation site than the gene for 12S rRNA.

The reliability of these conclusions is dependent on several factors. One of these is the correct identification of the mitochondrial transcripts detected after in vivo RNA synthesis. The evidence supporting their identification is discussed above.

Ultra-violet mapping is also dependent on a random distribution of pyrimidine dimers being induced by u.v. irradiation. In practice, however, non-random formation of pyrimidine dimers is likely to only be a problem in molecules much shorter than the sea urchin mitochondrial genome, or where there is a very non-random distribution of sequences of different base composition, which is not the case for sea urchin mitochondrial DNA (Sauerbier and Hercules, 1978).

Another important factor is the reliability of u.v. mapping per se as a technique for mapping transcription units. In the past, results obtained using u.v. mapping to map transcription units have been corroborated by using other techniques (Sauerbier and Hercules, 1978; Johnston et al, 1987). However, recent data has shown that, in addition to its effect on transcription, u.v. irradiation also inhibits nuclear RNA processing in Trypanosomes, as judged by an increase in the stability of normally short-lived processing intermediates after irradiation (Coquelet et al, 1989). A number of mitochondrial RNA processing enzymes have been shown to contain RNA components (section 1.11), the activity of which could be affected by cross-linking by u.v. irradiation. In the experiments described in this chapter no build-up of higher molecular weight transcripts was detected, suggesting u.v. irradiation was not exerting a global effect on RNA processing.

In order for u.v. mapping to work, it is necessary both for pyrimidine dimers to be formed in the DNA, to remain unrepaired during the time course of the experiment, and for the appropriate RNA polymerase enzyme to be unable to traverse them during the course of transcription. The target of u.v. irradiation in these experiments is most probably cytoplasmic rather than nuclear, since the nucleus of a sea urchin egg is shielded by 30-50 um of cytoplasm (Rustad, 1977). It is possible to detect pyrimidine dimers in DNA using a class of enzymes called u.v. endonucleases. These enzymes are produced in response to u.v. irradiation by a number of organisms such as phage T4 (Fried and Clayton, 1972) and Micrococcus luteus (Resnick et al, 1987). Ultra-violet endonucleases make single-stranded endonucleolytic cuts at the 5' side of pyrimidine dimers, which lead to a reduction in the intensity of restriction fragments of u.v.-irradiated as opposed to untreated DNA, when electrophoresed on an alkaline agarose gel (Berk and Sharp, 1977a; Resnick et al, 1987). It would be possible to detect the presence of pyrimidine dimers, and their level for the u.v. doses used, by purifying DNA from sea urchin eggs, cutting with a restriction enzyme and a u.v. endonuclease, electrophoresing the DNA on an alkaline agarose gel, and then monitoring digestion by Southern blotting the gel and probing for mitochondrial DNA. I have not done this experiment since u.v. endonucleases are not commercially available, and so have to be purified prior to use (Resnick et al, 1987).

Different classes of RNA polymerase differ in their sensitivity to transcriptional termination by pyrimidine dimers. The HeLa cell mitochondrial polymerase has been reported as being 5 times less sensitive to pyrimidine dimers than nuclear RNA polymerases (Vesco and Penman, 1969). These early experiments showed that of the two major labelled species in HeLa cell mitochondria, synthesis of the larger RNA species (21S) was more inhibited after u.v. irradiation (75% of control, non-irradiated cells) than synthesis of the smaller RNA species (12S, 93% of control, non-irradiated cells). On the basis of the reported data it is not possible to conclude whether these predominantly labelled species are the mitochondrial rRNAs, although these would be expected to be the major in vivo-labelled RNA species as described in section 1.5. The discrepancy in size between the larger

species with the large rRNA subunit (21S as opposed to the true size of 16S rRNA), may be due to a sizing artefact.

If these two labelled RNA species do represent the RNA components of the large and small ribosome, then the differential sensitivity of their synthesis to u.v. irradiation would correspond to their known order of transcription (12S rRNA being located upstream of 16S rRNA in the same transcription unit: Section 1.5 and 1.9). The results of Vesco and Penman (1969) thus would seem to validate u.v. mapping as a technique for the mapping of mitochondrial transcription units.

5.3.3 In vitro capping

In the experiments described in this chapter, 5' triphosphate termini associated with transcripts of two regions of the sea urchin mitochondrial genome have been detected by hybridisation of *in vitro*-capped sea urchin egg RNA and blastula RNA with Southern blots of cloned mitochondrial DNA (Fig 5.10). These represent the region of the genome containing, on one hand, the genes for ND1, ND2 and several tRNAs (detected in both egg and blastula RNA); and, on the other, the region of the genome containing part of the genes for COII and COIII, and the entire genes for A6 and A8 (detected only in blastula RNA). This result suggests that these regions of the genome are complementary to an RNA species with a 5' triphosphate group. A problem in interpreting these data is that the capped transcripts detected might have been long molecules, which overlapped with several restriction fragments. In the case of the *in vitro*-capped RNA which hybridised to the 1.7 kb *Eco*R1 fragment of lambda mt1, this is unlikely to have been the case, since the rest of the lambda mt1 digest was available for hybridisation. This was not the case for the of the pH1.6 insert, and any overlapping capped RNA species could have been complementary to DNA adjacent, and on either side in the sea urchin mitochondrial genome.

A general problem experienced in these experiments was getting a high enough level of incorporation into RNA in order to be able to probe a Southern blot, and get a hybridisation signal within a reasonable length of time. Other workers have also reported problems with these

experiments. Capping reactions are inefficient. RNA species containing 5' triphosphates could not be detected in mouse mitochondria, although putative primary 5' ends could be detected by primer extension of RNA and by *in vitro* initiation with purified mitochondrial RNA polymerase (no RNA processing activities were detected in these extracts by assaying processing of an SP6-generated transcript) (Chang and Clayton, 1986a, 1986b, 1986c; section 1.5 and 1.9.1). In *Neurospora* consistent results were not obtained using *in vitro* capping (Kennel and Lambowitz, 1989).

5.3.4 Comparability of the techniques used to analyse transcription

For a number of reasons, the results obtained by *in vitro* capping and u.v. mapping are not directly comparable. *In vitro* capping does not reflect physiological use of a transcriptional initiation site, since triphosphate containing 5' ends might be rapidly processed, and the triphosphate itself might be unstable. In contrast, the u.v.-sensitivity of synthesis of a given transcript should not be dependent on the rate of processing of RNA sequences upstream of it in the same transcription unit, unless these influence the rate of elongation. The u.v. mapping experiments suggested a transcriptional initiation site upstream of the 16S rRNA gene, but this might not be detected by *in vitro* capping if this 5' end were rapidly processed. Also, the u.v. mapping experiments were only able to determine the approximate location of the most highly abundant transcripts with regard to their transcriptional initiation sites, whereas *in vitro* capping detects the 5' end resulting from transcriptional initiation. This means that an initiation site for a relatively rare transcript, which would not be detectably labelled *in vivo*, might have a 5' triphosphate group which was an abundant substrate for *in vitro* capping reactions. Hence, whereas the synthesis of this transcript would not be detected as being insensitive to u.v. irradiation, it might be detected by *in vitro* capping as corresponding to a transcriptional initiation site.

Ultra-violet mapping cannot be used to map transcription units in the stages of development after fertilisation, since it causes a dose-dependent inhibitory effect on development

(Rustad, 1975 and not shown). A possible reason for this inhibitory effect might be cross-linking of RNA inhibiting translation. This would prevent the translation of molecules such as cyclin, which are required for cell division, but are short-lived proteins and are newly synthesised in each cell cycle.

CHAPTER 6

CONCLUDING REMARKS

The aim of this project was to gain an understanding of how mitochondrial RNAs are differentially expressed in early sea urchin development. In order to achieve this aim I have carried out exeriments to investigate the organisation of the sea urchin mitochondrial genome, to map sea urchin mitochondrial transcripts, and to examine mitochondrial RNA synthesis in vivo in sea urchin eggs and embryos.

6.1 The rRNA:mRNA ratio is modulated in early sea urchin development

RNA filter hybridisations have shown that the levels of at least some mitochondrial RNAs alter differentially early in development. The levels of those mitochondrial mRNAs examined were found to increase around 10-fold between the egg and blastula stages of development, whereas the level of 16S rRNA was found to fall slightly over this period. Mitochondrial translation increases after fertilisation in sea urchins (Innes and Craig, 1978). The increase in mitochondrial mRNA and tRNA synthesis can be thus rationalised as being necessary for this increase in translation. The slight decrease in rRNA synthesis might suggest that the steady-state levels of mitochondrial rRNAs in sea urchin eggs are in excess of requirements for translation after fertilisation. Hence mitochondrial translation in sea urchin eggs might be regulated at least partly at the level of mRNA/tRNA availability.

The higher molecular weight transcripts detected with gene-specific probes on northern blots most probably represent precursor mitochondrial transcripts. Different higher molecular weight transcripts were detected at different developmental stages, which suggests that alternate synthetic pathways are used at various stages of development.

6.2 There are no intergenic sequences upstream of the rRNA genes

Fine transcript mapping has shown that the 5' end of 16S rRNA maps two nucleotides downstream of the coding sequence of ND2 (its reading frame deduced from sequence analysis). S1 nuclease mapping of the 3' end of ND2 mRNA showed that ND2 is

either butt-joined to 16S rRNA, or overlaps a short distance (as a result of the sequence of the 5' end of the 16S rRNA gene, these possibilities cannot be distinguished). Primer extension mapping has shown that the 5' end of 12S rRNA is directly adjacent to the downstream boundary of $tRNA^{phe}$, as predicted from the $tRNA^{phe}$ secondary structure (Jacobs et al, 1988).

These results imply that, if either of the rRNA genes are directly adjacent to a promoter element, then this element would have to be intragenic. The evidence for an element of this kind upstream of the 16S rRNA gene is discussed below.

6.3 Multiple, overlapping transcription units in sea urchin mitochondrial DNA

6.3.1 In vivo mapping of sea urchin mitochondrial transcription units

By monitoring in vivo RNA synthesis with ^{3}H uridine in pronase-treated sea urchin eggs, and enucleate egg fragments, I have shown that the major ^{3}H-labelled transcripts in Strongylocentrotus purpuratus eggs are mitochondrial in origin. I have identified these ^{3}H-labelled transcripts both on the basis of their comigration with mitochondrial transcripts detected with ^{32}P-labelled probes, and by the fluorographic signal detected after hybrididisation of the in vivo-labelled RNA with a Southern blot of cloned sea urchin mitochondrial DNA. On the basis of this identification, the major in vivo-labelled transcripts are 16S rRNA, 5 non-random fragments of 16S rRNA, 12S rRNA, and COI mRNA. Conclusive identification will require hybrid-selection of these transcripts with specific mitochondrial probes. In addition, a number of higher molecular weight transcripts, which remain to be identified, were detectably ^{3}H-labelled.

I have used this in vivo RNA-labelling system along with u.v. mapping in order to map sea urchin mitochondrial transcription units. Ultra-violet irradiation exerts a polar effect on transcription, with the synthesis of RNA species encoded close to their site of transcriptional initiation being affected less than the synthesis of transcripts encoded further downstream of their transcriptional initiation site.

Ultra-violet irradiation caused a differential effect on the synthesis of sea urchin mitochondrial transcripts. The synthesis of 16S rRNA, along with the non-random fragments of 16S rRNA, was resistant to even the highest doses of u.v. irradiation used in these experiments, whereas the synthesis of 12S rRNA and COI mRNA were inhibited at low doses of u.v. irradiation.

The u.v. mapping data are consistent with transcription of the 16S rRNA gene being initiated just upstream of its gene, whilst transcription of 12S rRNA and COI mRNA are initiated at sites located far upstream of their genes. The genes encoding 16S rRNA and COI are adjacent. This means that, at least in some cases, adjacent genes are not necessarily co-transcribed from the same promoter. This point is discussed below in more detail.

Transcript mapping has shown that there is no intergenic sequence upstream of 16S rRNA. This means that the core promoter (defined in Section 1.9) directing transcriptional initiation around the 5' end of 16S rRNA must be either intragenic (within the gene for ND2), or promote transcriptional initiation at a distance. Of these possibilities, the former seems more likely. Mitochondrial transcriptional initiation sites tend to be internal to the core promoter elements directing them (Section 1.9, Figure 1.3). An exception to this is the murine HSP, which appears to select sites of transcriptional initiation stereospecifically, 1 and 2 turns of the DNA helix downstream (Section 1.9, Figure 1.4).

The intragenic location of the proposed sea urchin mitochondrial promoter element, within the ND2 gene, would be a novel feature. All other mitochondrial promoter elements characterised to date are in at least partially intergenic sequences. In contrast, a class of nuclear promoters, from which RNA polymerase III initiates transcription, are known to be intragenic (Bogenhagen et al, 1980; Sakonju et al, 1980).

6.3.2 Evidence for multiple promoters in the sea urchin mitochondrial genome

A conserved TTATATATAA-like sequence motif is found upstream of the sea urchin mitochondrial 16S rRNA gene,

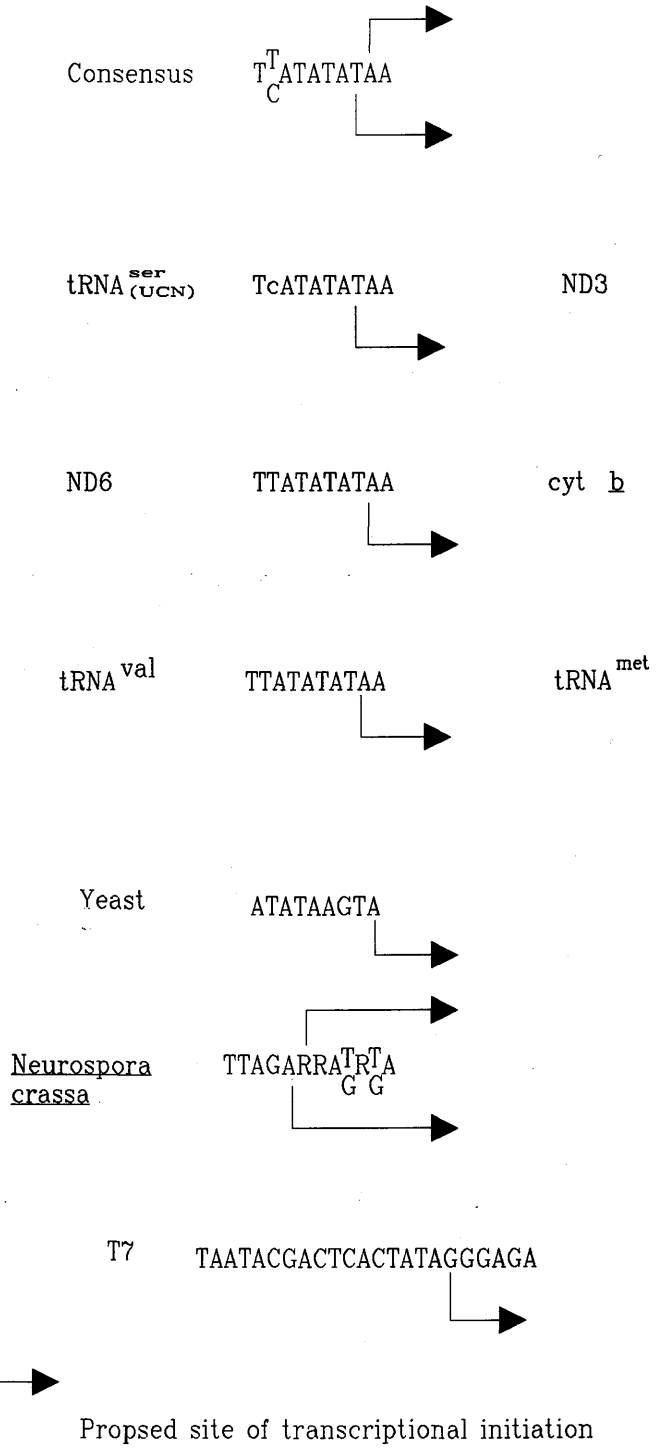

Figure 6.1: Organisation of the prosed sea urchin mitochondrial promoter sequence, along with the known core promoter sequences of yeast, phage T7 and Neurospora.

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within the coding sequence of the ND2 gene, which might be the core promoter element directing 16S rRNA transcription (Fig. 1.5). There are a number of reasons for believing that this sequence motif is the core sea urchin mitochondrial promoter. TTATATATAA-like motifs are also found at a number of other strategic locations in the genome, which are strongly suggestive of it being a promoter element. A TTATATATAA-like motif is found within the single extended intergenic region of the genome, just upstream of the leading strand of DNA replication (A.G. Mayhook, personal communication; Jacobs et al, 1989a), which it might prime. TTATATATAA-like motifs are also found between several pairs of divergently transcribed genes: $tRNA^{val}$ and $tRNA^{met}$; $tRNA^{ser}$ and ND3; and between ND6 and cyt b. Further support for a functionally important role for these sequences are that they are found at similar positions within the mitochondrial genomes of other echinoderms, including the sea urchin Paracentrotus lividus (Cantatore et al, 1990), and the starfish Asterias forbesii (Smith et al, 1990). Definitive proof that the TTATATATAA-like motifs are the sea urchin mitochondrial core promoter sequences, along with the characterisation of adjacent sequences which might be important in modulating transcription, will require the development of a homologous in vitro transcription system.

Using primer extension mapping I have shown that the 5' end of ND3 mRNA, and the 5' end of a possible precursor transcript to mature cyt b mRNA, map within an TTATATATAA-like motif (Fig. 6.1). In addition, the 5' end of $tRNA^{met}$, as predicted from the $tRNA^{met}$ secondary structure (Jacobs et al, 1988), also maps to a TTATATATAA-like motif. A 5' end mapping to the TTATATATAA-like motif found upstream of the 16S rRNA gene could not be detected, although this does not exclude the possibility that a molecule with such a 5' end exists, if only transiently. If RNA processing at the 5' end of 16S rRNA is rapid, then detection of such a precursor transcript 5' end would be extremely difficult.

The 5' end which was mapped just upstream of the cyt b gene, and the predicted 5' end of $tRNA^{met}$, map to the penultimate 3' nucleotide of the associated TTATATATAA-like motifs, whereas the most 5' nucleotide of ND3 mRNA maps to the nucleotide upstream of this in the TTATATATAA-like motif adjacent to the ND3 gene (Fig. 6.1). This difference in 5'

ends might be indicative of a physiological difference between motifs, despite their apparent sequence similarity. This physiological difference might be either mediated by their local sequence environment, or by slight differences between the TTATATATAA-like motifs. With regard to this latter possibility, whereas the core TTATATATAA motifs upstream of cyt b and $tRNA^{met}$ are identical, they both differ by one nucleotide from the core motif upstream of ND3 (Figure 6.1). A similar flexibility in the nucleotide selected as the transcriptional start site has been observed in Neurospora crassa mitochondria, where promoters utilise either one or both of two adjacent sites of transcriptional initiation (Kennel and Lambowitz, 1989; Section 1.9; Figure 1.4). It has been suggested that in this case the choice of which site(s) are used for transcriptional initiation is determined by subtle differences in promoter sequence (Kennel and Lambowitz, 1989).

6.3.3 Possible functional asymmetry of the TTATATATAA-like sequences

The fact that several of the TTATATATAA-like motifs in the sea urchin mitochondrial genome are located between divergently transcribed genes, strongly suggests that the motif functions bidirectionally as a promoter element. A number of animal mitochondrial promoter elements have been shown to be bidirectional (Section 1.9). The human mitochondrial promoters are both weakly bidirectional, in that they promote transcriptional initiation predominantly in one direction (Chang et al, 1986), whereas 2 out of the 3 Xenopus mitochondrial promoters have been shown to be fully bidirectional (Bogenhagen and Yoza, 1986).

Although the putative core promoter motifs for sea urchin mitochondrial DNA are symmetrical (or almost symmetrical in the case of some of the the TTATATATAA-like motifs, as shown in Fig. 6.1), there is an asymmetry in the steady-state levels of transcripts encoded on either side of them. The most extreme case of this is for the TTATATATAA-like motif situated upstream of the 16S rRNA gene, from which transcription in only one direction would be expected to result in a functional transcript (i.e. 16S rRNA), whereas transcription in the other direction would give rise to a

transcript antisense to the ND2 mRNA. Mature 16S rRNA was easily detected by S1 nuclease mapping, but no transcript antisense to ND2 mRNA was detected (although this does not rule out the fact that such a transcript might exist, but be turned over rapidly). Similarly, I was able to detect by primer extension the 5' ends of both cyt b mRNA, and ND3 mRNA, but not ND6 mRNA, and that of $tRNA^{ser}$(UCN) only very weakly. Tranfer RNA^{met} might be expected to be required at a higher concentration than $tRNA^{val}$, since the former is probably the tRNA responsible for translational initiation. In all these cases, the transcripts encoded by the major coding-strand are synthesised at a higher level than those transcribed from the minor coding-strand. It is important to note that on the basis of the current data, these differences in transcript level might be determined at either the transcriptional or the post-transcriptional level. However, this difference in steady-state levels might be indicative of a functional asymmetry in the sea urchin mitochondrial promoter, with transcription being initiated more frequently in one direction (the clockwise direction in Fig. 1.5).

If the TTATATATAA-like motifs do represent core promoter elements, which is consistent with current evidence, then this would imply that there are multiple transcription units on the sea urchin mitochondrial genome, with widely separated initiation sites. The sea urchin mitochondrial genome might resemble yeast mitochondrial genomes (Section 1.6.1) in this respect, rather than those of vertebrates (Section 1.5.1). This similarity might be particularly close with yeasts such as Torulopsis glabrata, which have mitochondrial genomes in a similar size range to the sea urchin mitochondrial genome, considerably smaller than that of S. cerevisiae (Section 1.2; Table 1.1). A further similarity with yeast is that the proposed transcriptional units on sea urchin mitochondrial DNA would contain genes encoding a number of different functional classes of RNA, and possibly the two rRNA genes would be in different transcription units (Section 1.6.1; Figure 1.2). There is a sequence similarity between the yeast (Section 1.9.2) and putative sea urchin mitochondrial promoters (Figure 6.1), but the implications of this observation are not clear. Having said this, the mitochondrial promoter of the sea urchin may be bidirectional, although to date 5' ends mapping to only one

end of the motif have been detected in this study. The mitochondrial promoters of S. cerevisiae (and most probably T. glabrata, where the core promoter motif seems to be the same as in S. cerevisiae) act unidirectionally.

6.4 The 3' ends of both rRNAs overlap with the adjacent downstream transcripts

Using both primer extension and S1 nuclease mapping, I have shown that both 16S rRNA and 12S rRNA overlap with the transcripts encoded immediately downstream of them. The 3' end of 16S rRNA overlaps 3-5 nucleotides into the COI coding sequence, extending 8-10 nucleotides from the mapped 5' end of COI mRNA. The 3' end of 12S rRNA extends 7-13 nucleotides into the gene encoding $tRNA^{glu}$. This means that neither of the sea urchin rRNAs can be synthesised from the same primary transcript which gives rise to functional transcripts from the immediately downstream genes.

The u.v. mapping has suggested that 16S rRNA is synthesised from an initiation site just upstream of the gene encoding it, whereas COI mRNA is synthesised from an initiation site far upstream of its gene (Fig. 6.2). This rules out a model in which COI mRNA is synthesised from a transcript initiating within the 16S rRNA gene. It does not rule out the possibility that some form of transcriptional attenuation limits RNA polymerase readthrough past the 3' end of 16S rRNA, but the absence of 3' heterogeneity in the 16S rRNA transcript suggests that this is not occurring.

Hence the evidence supports the idea that 16S rRNA and COI mRNA are synthesised from alternative processing at the 16S rRNA/COI gene junction, within precursor mitochondrial transcripts containing the sequence information for either mature transcript. However, the evidence is consistent with the idea that 16S rRNA and COI mRNA may have distinct precursors, and that the sites of transcriptional initiation might influence which mature transcript is selected by the mutually exclusive pathways. The u.v. sensitivity of the synthesis of COI mRNA suggests that it is transcribed from an initiation site far upstream of the transcriptional initiation site which results in the synthesis of 16S rRNA. These 16S rRNA and COI mRNA precursors would have distinct properties, which could be recognised by the mitochondrial

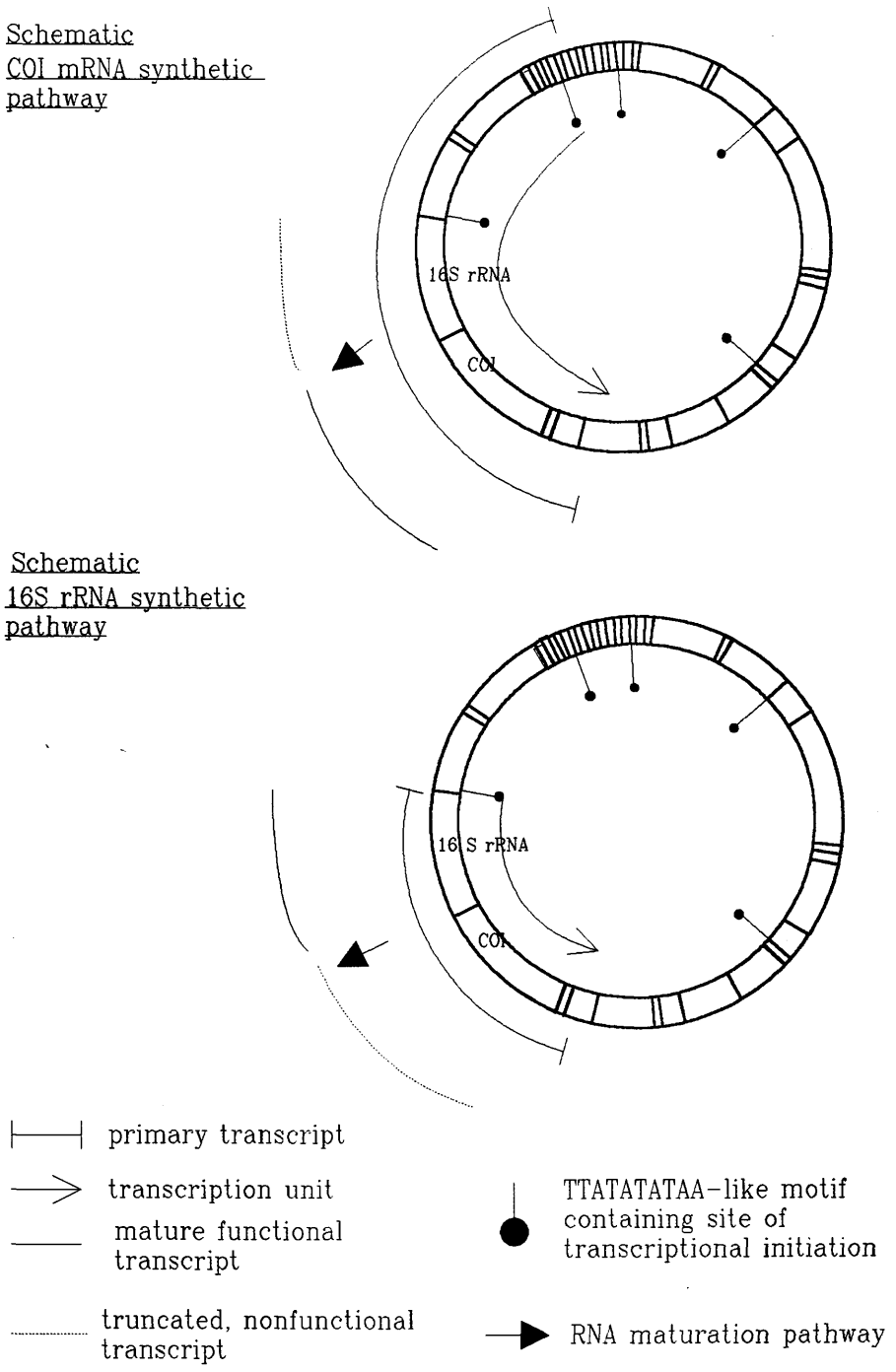

Figure 6.2: Schematic model to explain the mutually exclusive synthesis of 16S rRNA and COI mRNA, based on the use of alternative transcription units. The initiation site for COI mRNA synthesis is implied on the figure to map to the TTATATATAA-like motif upstream of ND1 for the sake of argument, but could map to any of the TTATATATAA-like motifs upstream of the one used for 16S rRNA synthesis. The implied transcription units are also hypothetical, and probably generate additional transcripts as well as 16S rRNA and COI mRNA.

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RNA processing machinery. For example, the processing machinery might distinguish between these primary transcripts on the basis of alternative secondary structures at the 16S rRNA/COI junction, which might be influenced by other sequences within the primary transcript, so as to produce either COI mRNA or 16S rRNA. This hypothesis is further supported by the observation that higher molecular weight transcripts containing the coding information for more than one gene exist in sea urchin mitochondria. This is consistent with the idea that these higher molecular weight transcripts are substrates for the RNA processing machinery responsible for endonucleolytically cleaving primary transcripts, rather than these endonucleolytic cleavage events occuring concomitantly with transcription, as has been suggested to occur in vertebrates (section 1.11.1).

The u.v. mapping data is consistent with the hypothesis that 12S rRNA, like COI mRNA, is synthesised from an initiation site far upstream of the gene encoding it (Fig. 6.2). From the current data it is not possible to conclude whether 12S rRNA is synthesised from the same initiation site as 16S rRNA, although the absence of detectable higher molecular weight transcripts containing both 16S rRNA and 12S rRNA sequences is suggestive that they are not. Since the synthesis of 12S rRNA is sensitive to the lowest doses of u.v. irradiation which I have tested, it is likely that 12S rRNA is synthesised from a transcriptional initiation site much further upstream than the closest TTATATATAA-like motif which is located just upstream of the cyt b gene (Fig. 1.5).

In addition to an alternative processing event giving rise to a functional $tRNA^{glu}$ transcript, there is tentative evidence that some form of transcriptional attenuation might occur at the 3' end of the 12S rRNA gene. This evidence is from the fact that the 3' end of the 12S rRNA gene is heterogeneous, unlike the other transcript ends mapped in this study. It has been suggested that 3' heterogeneity might be a characteristic feature of transcript ends generated from transcriptional attenuation (Section 1.5.1).

6.5 Large precursor transcripts may be substrates for the RNA processing machinery in sea urchin mitochondria

The sea urchin mitochondrial genome contains an extended

tRNA gene cluster (Fig. 1.5). This means that tRNA genes are not present at the junctions between many other (i.e. rRNA and mRNA) genes. It has been proposed that the endonucleolytic processing machinery responsible for the processing of primary transcripts in the mitochondria of other organisms recognises tRNA sequences in the primary transcript, most probably on the basis of their secondary structure (Section 1.11.1). The organisation of the sea urchin mitochondrial genome precludes this mechanism for the generation of most of the mature transcript termini. The existence of high molecular weight transcripts suggests that sites of RNA cleavage may be selected in sea urchin mitochondrial primary transcripts on the basis of the secondary structure of these large transcripts. As discussed in Chapter 3, this mode of RNA processing may be similar to that operating in S. cerevisiae.

6.5 The frequency of transcriptional initiation may be important in controlling sea urchin mitochondrial transcript expression during early development

The evidence described above suggests that the developmental control of sea urchin mitochondrial gene expression might be achieved by the differential selection of transcriptional initiation sites. The sites of initiation selected would affect the subsequent physiological properties of the precursor transcripts, and determine which mature transcripts were synthesised from them.

In addition to the mature transcripts, distinct higher molecular weight transcripts containing the sequences for more than one gene were detected by probing northern blots with gene- specific probes. The levels of these higher molecular weight transcripts showed developmental changes, which corresponded with changes in the levels of the corresponding mature transcripts. The detection of these higher molecular weight transcripts might be indicative of the use of different synthetic pathways of which they are the intermediates. For example, between the egg and blastula stages of development, the level of cyt b mRNA increases, while the level of 12S rRNA declines. During this stage in development, the steady-state level of a 2.3 kb transcript detected with probes specific for 12S rRNA (Elliott and

Jacobs, 1989) and cyt b increases, and the steady-state level of the 5' end which maps to the TTATATATAA-like motif upstream of cyt b also increases. A precursor-product relationship has not been demonstrated between this 2.3 kb transcript and the mature cyt b mRNA, and the 5' end mapping within the TTATATATAA-like motif upstream of cyt b has not been shown to be associated with the 2.3 kb transcript. None the less, these results suggest developmental accumulation of cyt b mRNA may be the result of increased transcriptional initiation at the TTATATATAA-like motif immediately upstream of the cyt b gene, which gives rise to the 2.3 kb precursor. In contrast, the steady-state of the higher molecular weight transcripts of between 4 and 7 kb which were detected on northern blots with probes for 12S rRNA (Elliott and Jacobs, 1989), and cyt b decline between the egg and blastula stages. This is also consistent with the u.v.-sensitivity of 12S rRNA synthesis, in suggesting that the precursor transcripts which give rise to 12S rRNA initiate far upstream of the site of initiation which is responsible for cyt b mRNA synthesis. This model would predict a reduced rate of transcription from the upstream promoters during embryogenesis (and therefore a decline in the 5' ends associated with their TTATATATAA-like motifs), resulting in the observed net decline in the steady-state levels of the associated 12S rRNA precursor transcripts. This possibility could be experimentally tested by mapping 5' ends adjacent to the remaining TTATATATAA-like motifs, which were not mapped in this study, in egg and blastula RNA.

ND1 and ND2 mRNA increase in representation between the egg and blastula stages of development, as does the 2.4 kb higher molecular weight transcript detected with probes for these genes. This is consistent with the 2.4 kb transcript being a precursor for ND1 and ND2 mRNA synthesis. The 2.4 kb transcript is of an appropriate size to have initiated at the TTATATATAA-like motif within the tRNA cluster, just upstream of ND1, and terminating at the 3' end of ND2. Moreover, a triphosphate end mapping to this region of the genome was detected by capping reactions in sea urchin RNA (although these experiments did not give wholly consistent results).

If transcriptional initiation frequencies of TTATATATAA-like motifs vary, and are developmentally modulated, then the sea urchin promoters cannot be equivalent

despite the apparent similarity of the proposed core motif. Although it has not been documented in this thesis, the frequncy of transcriptional initiation at the TTATATATAA-like motif in the major intergenic region might be important in determining the frequency of transcriptional initiation at the origin of replication, to which it is thought to be adjacent (Jacobs et al, 1989a).

The frequency of transcriptional initiation is also thought to make an important contribution to the control of mitochondrial transcript expression in S. cerevisiae, where the frequency of transcriptional initiation at different promoter elements can vary as much as 20-fold (Mueller and Getz, 1986; Section 1.6.1).

6.7 Sea urchin mitochondrial gene expression in perspective

The sea urchin mitochondrial genome is organised in a very economical fashion, with most of the genes butt-joined together, and there is only one major intergenic sequence, which is relatively short when compared with other characterised animal mitochondrial genomes (Section 1.2; Table 1.1). However, the work described in this thesis would tend to suggest that the RNA synthetic systems are more elaborate. This presents an anomaly: why have a small, compactly organised genome, but use mechanisms to express it which are apparently wasteful in that they involve grossly overlapping transcription units, and then discard large regions of the primary transcripts? A possible explanation for this might be that the RNA sequences in the primary transcripts which are not used subsequently for mature transcripts, might be involved in some other aspect of RNA metabolism. For example, these sequences could contribute to alternative secondary structures in the sea urchin mitochondrial primary transcripts, and so influence the processing pathways to which they are directed. These sequences might thus contribute to the economical organisation of the genome. Since sequences involved in RNA metabolism are themselves potentially utilised within mature transcripts, this means that specific regions of the genome do not need to be set aside for such a role.

Whereas the mechanisms which seem to control mitochondrial gene expression in sea urchin eggs appear to be

highly complex, and superficially bear little resemblance to those which have been shown to operate in other animal mitochondria, there is the possibility that there might also be fundamental similarities between the control of sea urchin mitochondrial gene expression and that in other organisms. For HeLa cell mitochondria it has been suggested that the rRNAs and mRNAs are synthesised from partially overlapping transcription units with closely spaced transcriptional start sites (Montoya et al, 1983; Section 1.5.1). In this case the basis of the different physiological properties of these primary transcripts, and how they lead to one or other of two mutually exclusive fates i.e. the synthesis of mRNA or rRNA, is not known. It is possible that they may be determined by the short sequence present in the rRNA-destined precursors, but which is absent in the mRNA-destined precursors. The transcripts which are part of the mRNA transcription unit, but which are synthesised from the rRNA region of the genome, seem to be discarded in HeLa cell mitochondria (Section 1.5).

There are also examples of overlapping transcription units in fungi. In Neurospora mitochondria it has been shown that there might be two routes of synthesis of the Neurospora mitochondrial small subunit rRNA, one involving transcriptional initiation directly upstream, and another involving transcriptional initiation far upstream (Kennel and Lambowitz, 1989).

Although a system of RNA synthesis which discards much of the RNA it synthesises might seem wasteful, examples of such systems are not confined to mitochondria. Such systems are also found in the nucleus, where they seem to be the rule rather than the exception. A great deal of the nuclear non-ribosomal RNA which is synthesised (heterogeneous nuclear RNA), is apparently discarded, and does not leave the nuclear compartment for the cytoplasm (reviewed by Darnell, 1982). The choice between the discard of a heterogenous nuclear transcript, or its processing into a mature mRNA and export to the cytoplasm, may be affected by its nuclear polyadenylation status (Salditt-Georgieff and Darnell, 1981). Other slightly different examples of the discard of nuclear RNA sequences (where parts of transcripts, as opposed to entire transcripts, are discarded) occur as a result of RNA splicing (the removal of introns from a primary transcript), alternative splicing (the removal from a primary transcript

of alternative introns and/or exons) and differential polyadenylation (the use of alternative 3' cleavage/polyadenylation sites in a primary transcript) (reviewed by Breitbart et al, 1987).

6.8 Future work

There are a number of future lines of investigation which would give useful information on the control of RNA metabolism in sea urchin mitochondria. Following directly on from the work described in this thesis, it will be necessary to confirm the identification of the major ^{3}H-labelled transcripts by hybrid selection mapping. Another kind of experiment would be to examine the effect of cycloleucine on the pattern of sea urchin transcripts which are synthesised in vivo, since cycloleucine has been shown to inhibit the endonucleolytic processing of rRNA precursors in Chinese hamster mitochondria, and result in an increase in their steady-state level (Section 1.11.1).

The development of an in vitro transcription system from soluble extracts of sea urchin mitochondria would allow confirmation of the identity of the sea urchin mitochondrial promoter(s), and also allow identification of other sequences important for transcription by deletion analyses. Purification of mitochondria from different developmental stages would enable the factors governing the different rates of transcriptional initiation to be determined, both by examining the role of flanking sequences at different developmental stages, and by examining the components of the polymerase itself. It is conceivable that different transcription factors might be associated with the polymerase at different developmental stages. It has been shown that there are probably multiple transcription factors in yeast mitochondria (Section 1.8). Additionally, as described above, it would be useful to map transcript ends associated with the TTATATATAA-like motifs not examined in this study.

DNA-binding proteins might well play a role in determining the frequency of transcriptional initiation at mitochondrial promoters. As described above, as well as affecting the steady-state levels of transcripts, these might also influence the rate of replication of the genome. The presence of such proteins could be detected by incubating

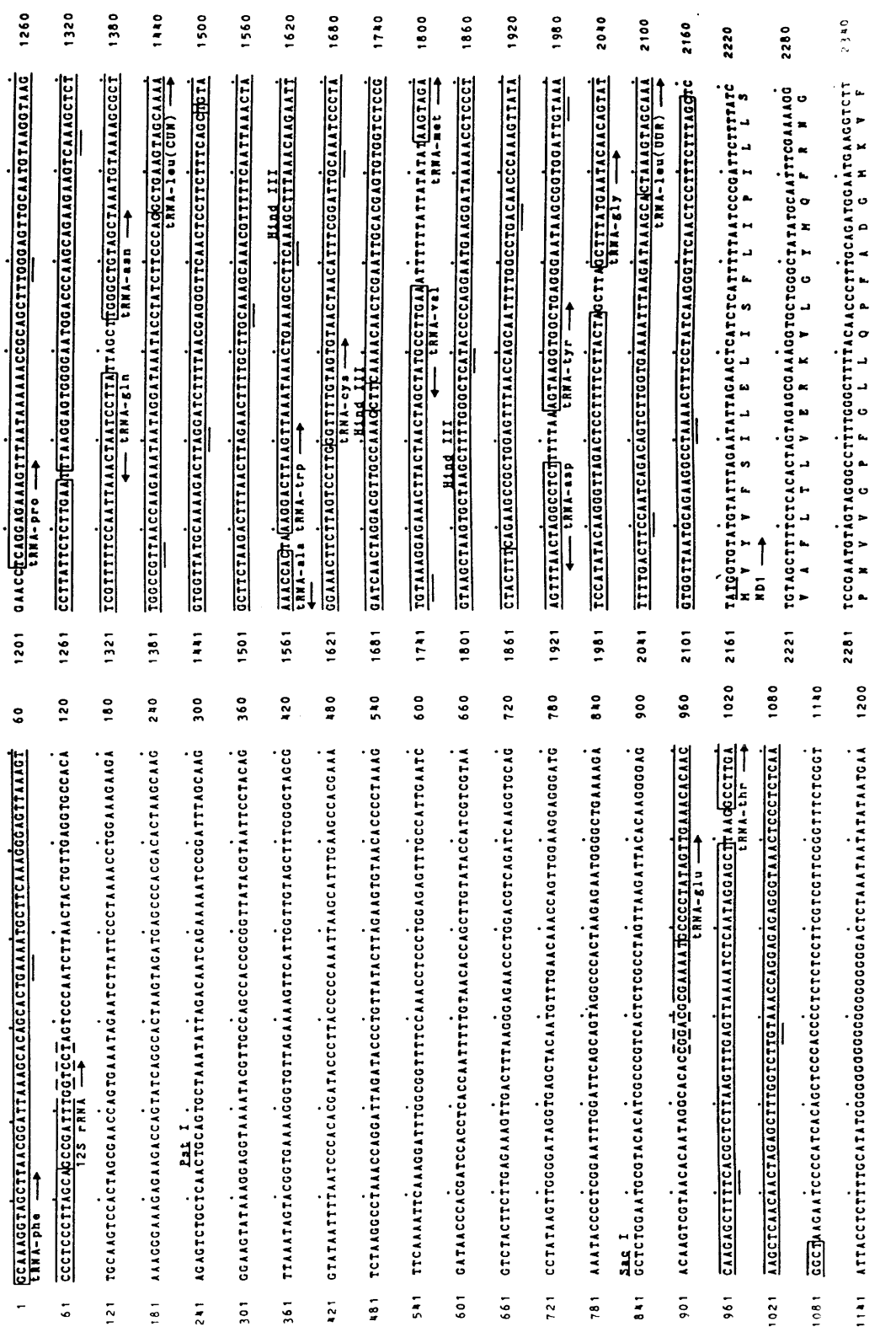
radio-labelled probes containing specific regions of the genome, with soluble extracts of sea urchin mitochondria, and then cross-linking these with u.v. irradiation (Eekelen et al, 1982). The proteins labelled in this fashion could then be analysed by gel electrophoresis. The genes encoding these proteins could be screened for in a lambda gt11 library, using an appropriate sea urchin mitochondrial DNA probe (Singh et al, 1988; Vinson et al, 1988).

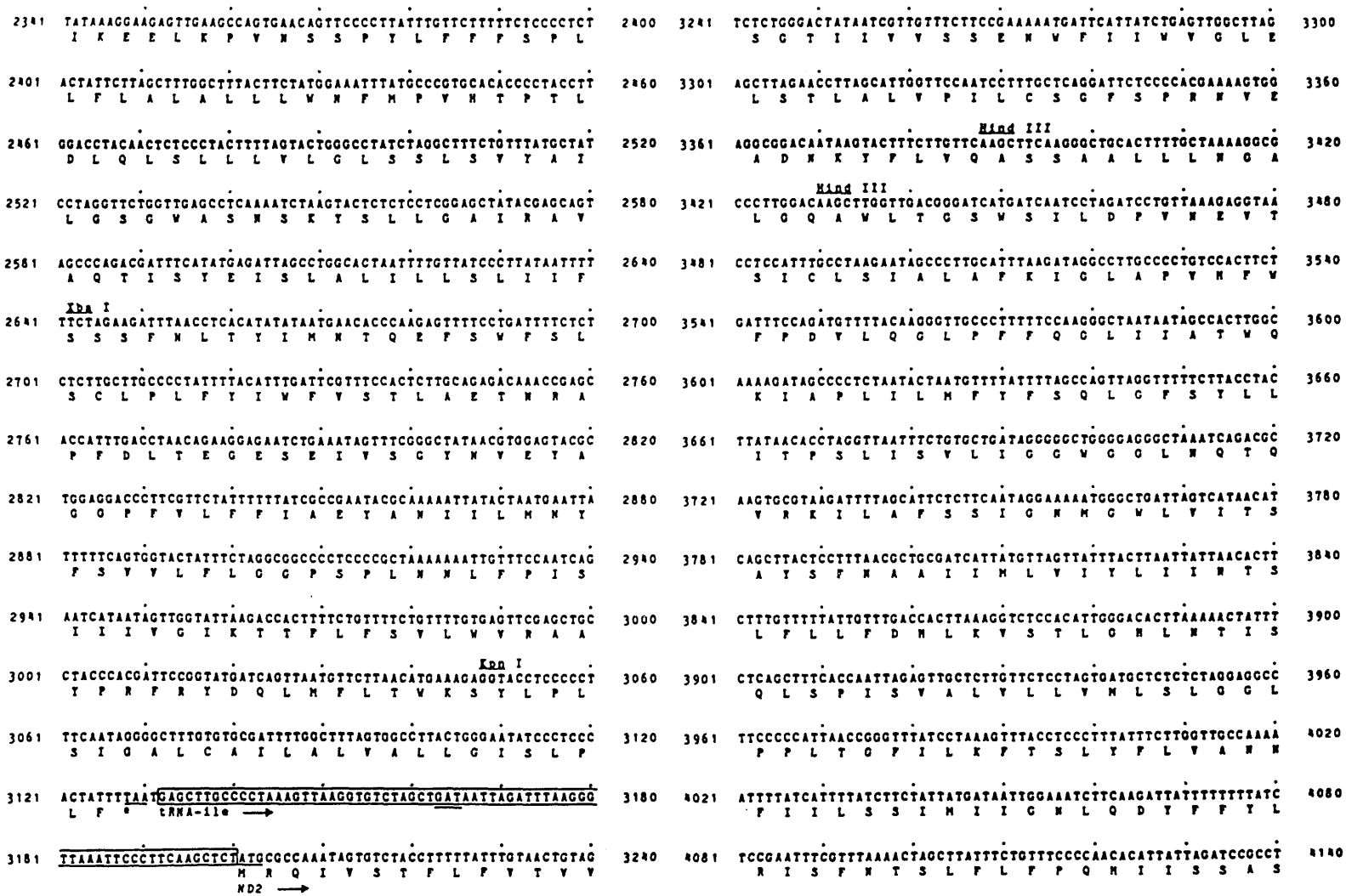
Another possible line of future experiments would be to examine RNA processing in vitro using fractions from solubilised mitochondria. However, the fact that large precursor transcripts might be the substrates for such reactions may complicate their analysis in vitro. For example, the transcriptional initiation site leading to COI mRNA synthesis has not been localised. Its position might have important implications for examination of the 16S rRNA/COI mRNA processing event in vitro, if the primary mitochondrial transcript is the substrate for this processing reaction. Alternatively, the processing step generating the mature COI mRNA 5' end might be one of a number of consecutive processing reactions.

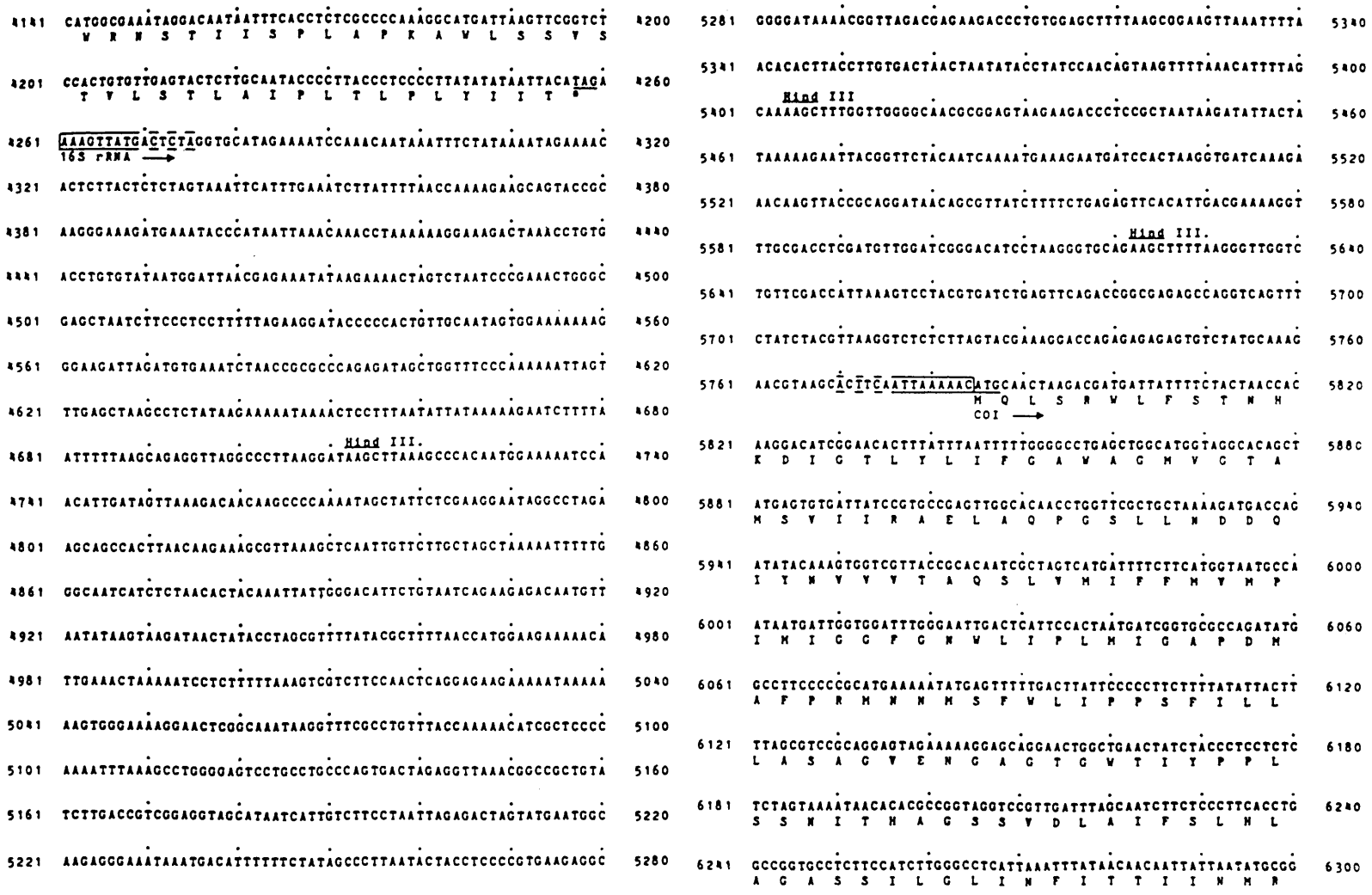
In conclusion, many questions concerning sea urchin mitochondrial RNA synthesis and its developmental regulation remain to be answered. In addition to answering these, future research will no doubt raise yet more questions which will have to be answered before this complex biological system can be understood in detail.

APPENDIX

The complete sequence of the sea urchin mitochondrial genome is given below, taken from Jacobs et al (1988). Nucleotides within this sequence are referred to in the text of this thesis.




Xba I
Kpn I
Hind III
Hind III
tRNA-Ile
ND2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W R W S T I I S P L A P K A W L S S V S
4201 CCACTGTGTTGAGTACTCTTGCAATACCCCTTACCCTCCCCTTATATATAATTACATAGA 4260
T V L S T L A I P L T L P L Y I I T *
4261 AAAGTTATGACTCTAGGTGCATAGAAAATCCAAACAATAAATTTCTATAAAATAGAAAAC 4320
16S rRNA
4321 ACTCTTACTCTCTAGTAAATTCATTTGAAATCTTATTTTAACCAAAAGAAGCAGTACCGC 4380
4381 AAGGGAAAGATGAAATACCCATAATTAAACAAACCTAAAAAAGGAAAGACTAAACCTGTG 4440
4441 ACCTGTGTATAATGGATTAACGAGAAATATAAGAAAACTAGTCTAATCCCGAAACTGGGC 4500
4501 GAGCTAATCTTCCCTCCTTTTTAGAAGGATACCCCCACTGTTGCAATAGTGGAAAAAAAG 4560
4561 GGAAGATTAGATGTGAAATCTAACCGCGCCCAGAGATAGCTGGTTTCCCAAAAAATTAGT 4620
4621 TTGAGCTAAGCCTCTATAAGAAAAATAAAACTCCTTTAATATTATAAAAAGAATCTTTTA 4680
HindIII.
4681 ATTTTTAAGCAGAGGTTAGGCCCTTAAGGATAAGCTTAAAGCCCACAATGGAAAAATCCA 4740
4741 ACATTGATAGTTAAAGACAACAAGCCCCAAAATAGCTATTCTCGAAGGAATAGGCCTAGA 4800
4801 AGCAGCCACTTAACAAGAAAGCGTTAAAGCTCAATTGTTCTTGCTAGCTAAAAATTTTTG 4860
4861 GGCAATCATCTCTAACACTACAAATTATTGGGACATTCTGTAATCAGAAGAGACAATGTT 4920
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4981 TTGAAACTAAAAATCCTCTTTTTAAAGTCGTCTTCCAACTCAGGAGAAGAAAAATAAAAA 5040
5041 AAGTGGGAAAAGGAACTCGGCAAATAAGGTTTCGCCTGTTTACCAAAAACATCGCTCCCC 5100
5101 AAAATTTAAAGCCTGGGGAGTCCTGCCTGCCCAGTGACTAGAGGTTAAACGGCCGCTGTA 5160
5161 TCTTGACCGTCGGAGGTAGCATAATCATTGTCTTCCTAATTAGAGACTAGTATGAATGGC 5220
5221 AAGAGGGAAATAAATGACATTTTTTCTATAGCCCTTAATACTACCTCCCCGTGAAGAGGC 5280
5281 GGGGATAAAACGGTTAGACGAGAAGACCCTGTGGAGCTTTTAAGCGGAAGTTAAATTTTA 5340
5341 ACACACTTACCTTGTGACTAACTAATATACCTATCCAACAGTAAGTTTTAAACATTTTAG 5400
HindIII
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5461 TAAAAAGAATTACGGTTCTACAATCAAAATGAAAGAATGATCCACTAAGGTGATCAAAGA 5520
5521 AACAAGTTACCGCAGGATAACAGCGTTATCTTTTCTGAGAGTTCACATTGACGAAAAGGT 5580
HindIII.
5581 TTGCGACCTCGATGTTGGATCGGGACATCCTAAGGGTGCAGAAGCTTTTAAGGGTTGGTC 5640
5641 TGTTCGACCATTAAAGTCCTACGTGATCTGAGTTCAGACCGGCGAGAGCCAGGTCAGTTT 5700
5701 CTATCTACGTTAAGGTCTCTCTTAGTACGAAAGGACCAGAGAGAGAGTGTCTATGCAAAG 5760
5761 AACGTAAGCACTTCAATTAAAAACATGCAACTAAGACGATGATTATTTTCTACTAACCAC 5820
M Q L S R W L F S T N H
COI
5821 AAGGACATCGGAACACTTTATTTAATTTTTGGGGCCTGAGCTGGCATGGTAGGCACAGCT 5880
K D I G T L Y L I F G A W A G M V G T A
5881 ATGAGTGTGATTATCCGTGCCGAGTTGGCACAACCTGGTTCGCTGCTAAAAGATGACCAG 5940
M S V I I R A E L A Q P G S L L N D D Q
5941 ATATACAAAGTGGTCGTTACCGCACAATCGCTAGTCATGATTTTCTTCATGGTAATGCCA 6000
I Y N V V V T A Q S L V M I F F M V M P
6001 ATAATGATTGGTGGATTTGGGAATTGACTCATTCCACTAATGATCGGTGCGCCAGATATG 6060
I M I G G F G N W L I P L M I G A P D M
6061 GCCTTCCCCCGCATGAAAAATATGAGTTTTTGACTTATTCCCCCTTCTTTTATATTACTT 6120
A F P R M N N M S F W L I P P S F I L L
6121 TTAGCGTCCGCAGGAGTAGAAAAAGGAGCAGGAACTGGCTGAACTATCTACCCTCCTCTC 6180
L A S A G V E N G A G T G W T I Y P P L
6181 TCTAGTAAAATAACACACGCCGGTAGGTCCGTTGATTTAGCAATCTTCTCCCTTCACCTG 6240
S S N I T H A G S S V D L A I F S L H L
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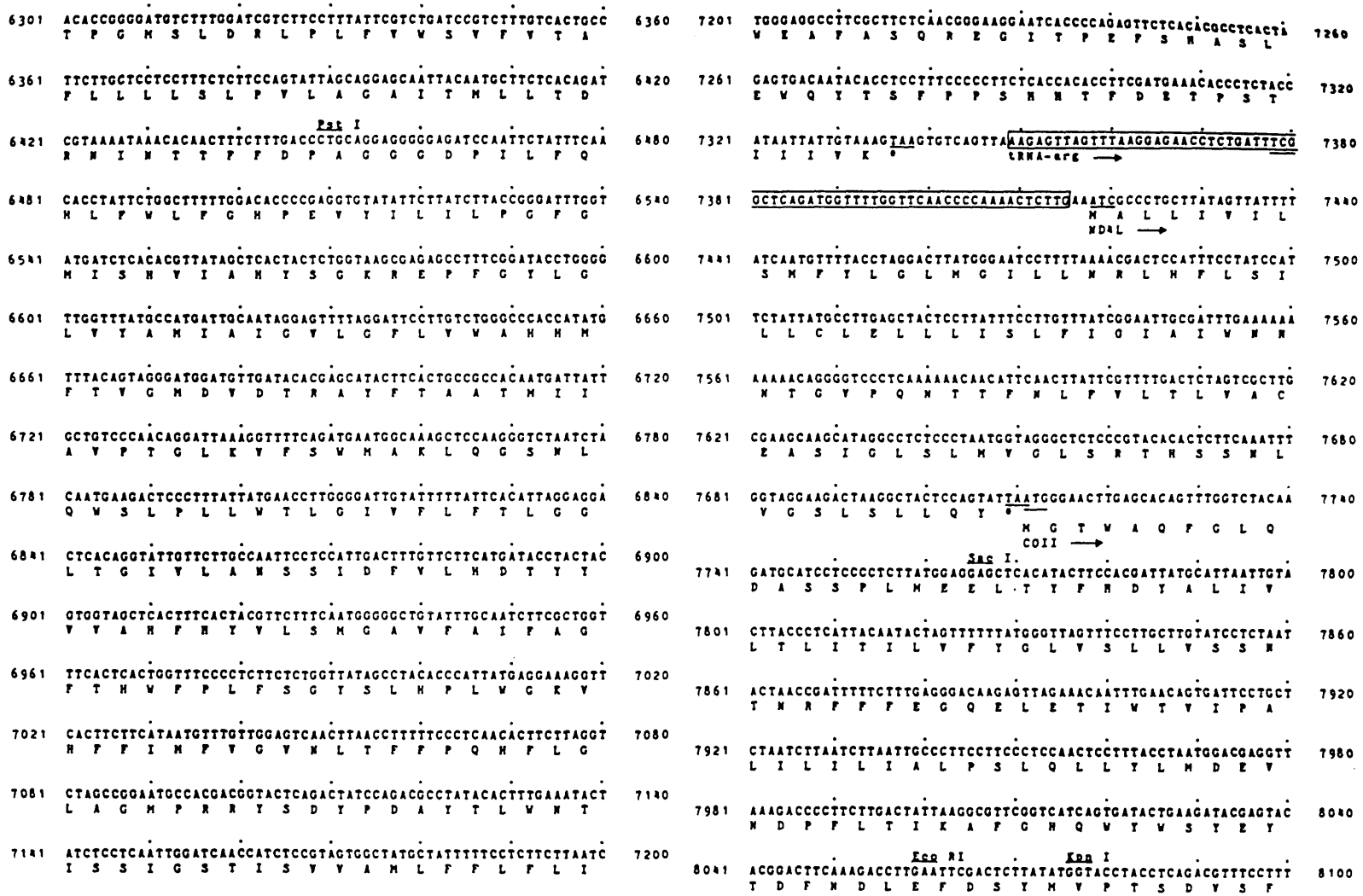
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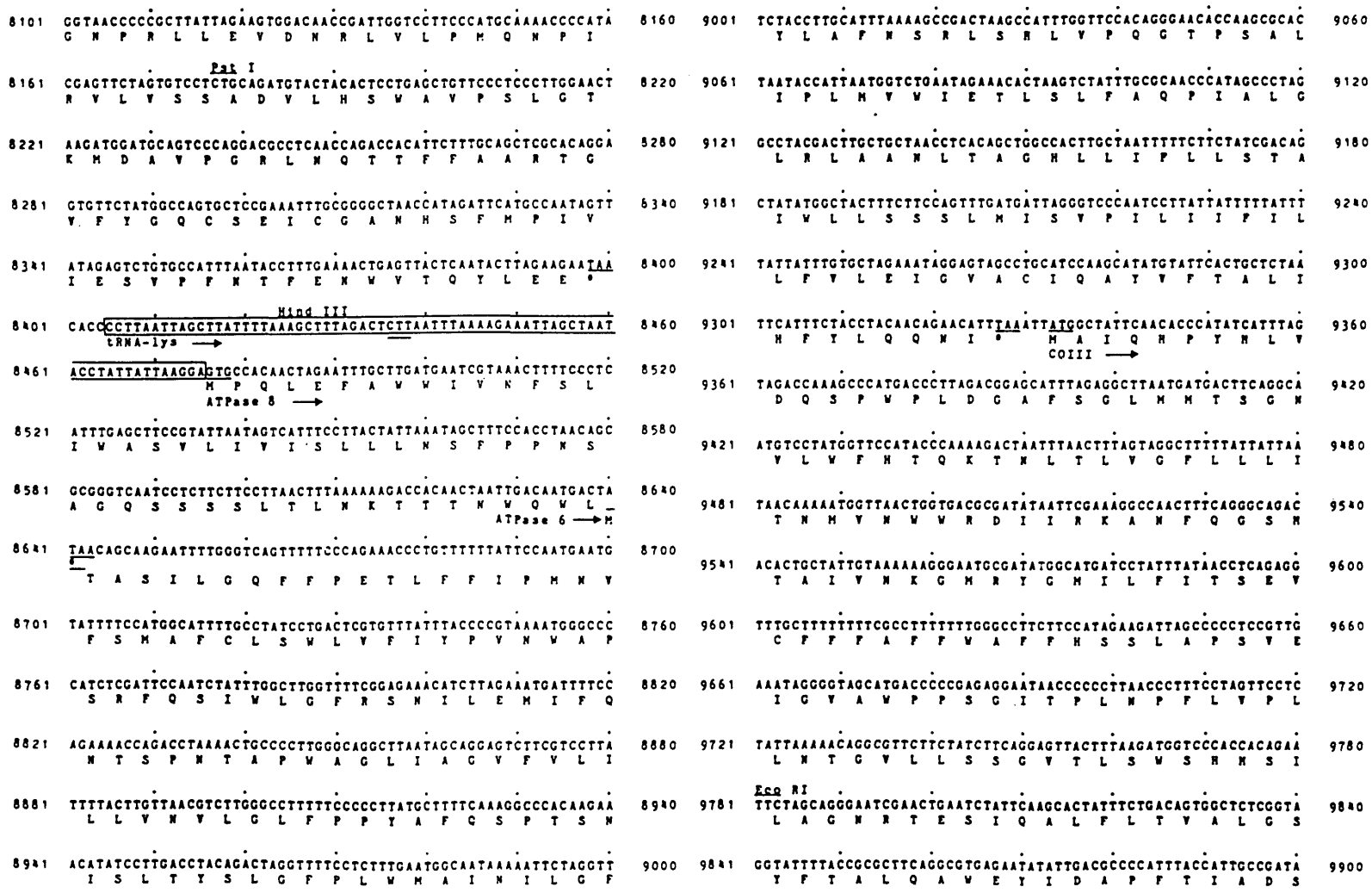
NDaL
COII
Sac I.
Eco RI
Kpn I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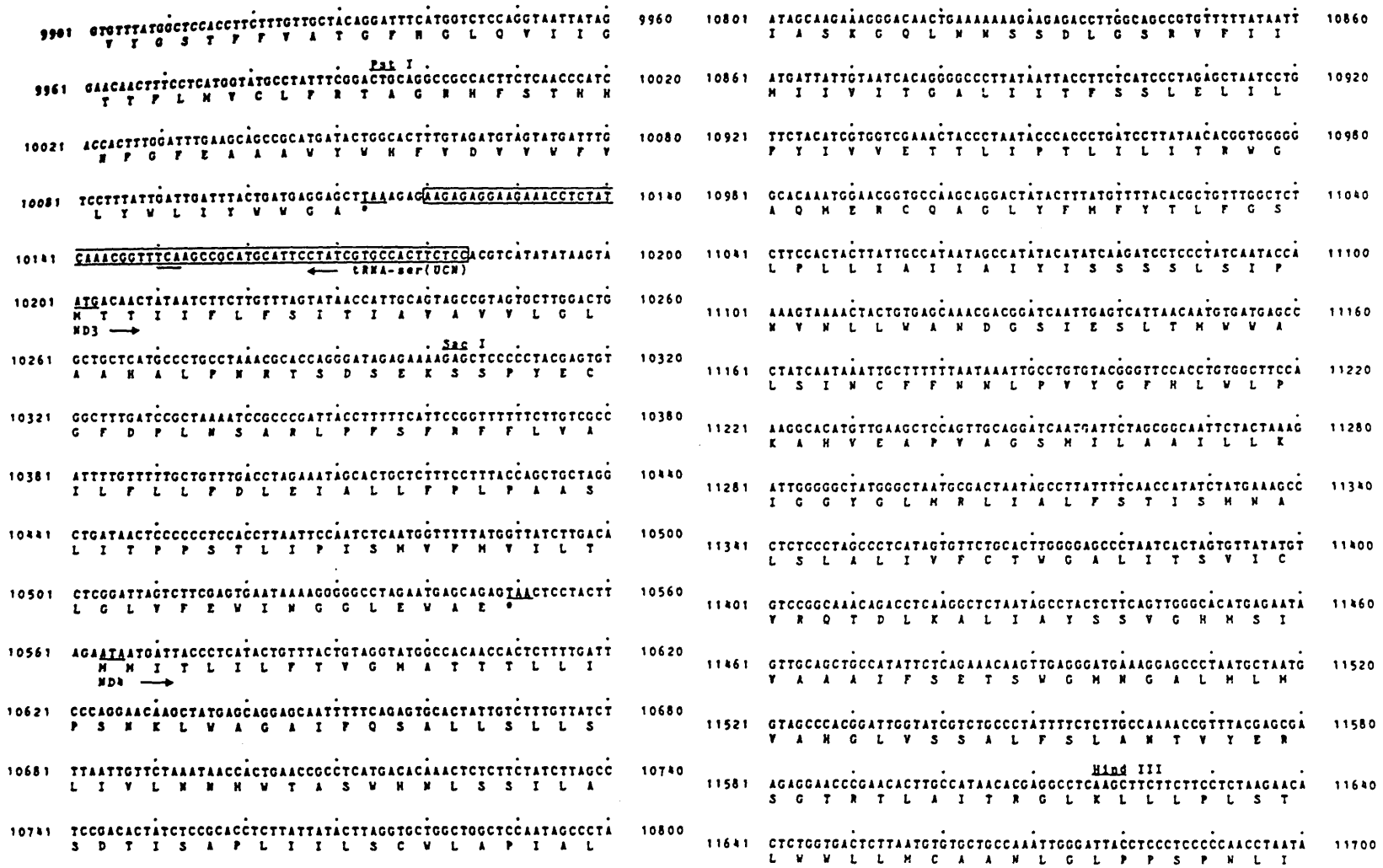
ATPase 8
ATPase 6
COIII
Eco RI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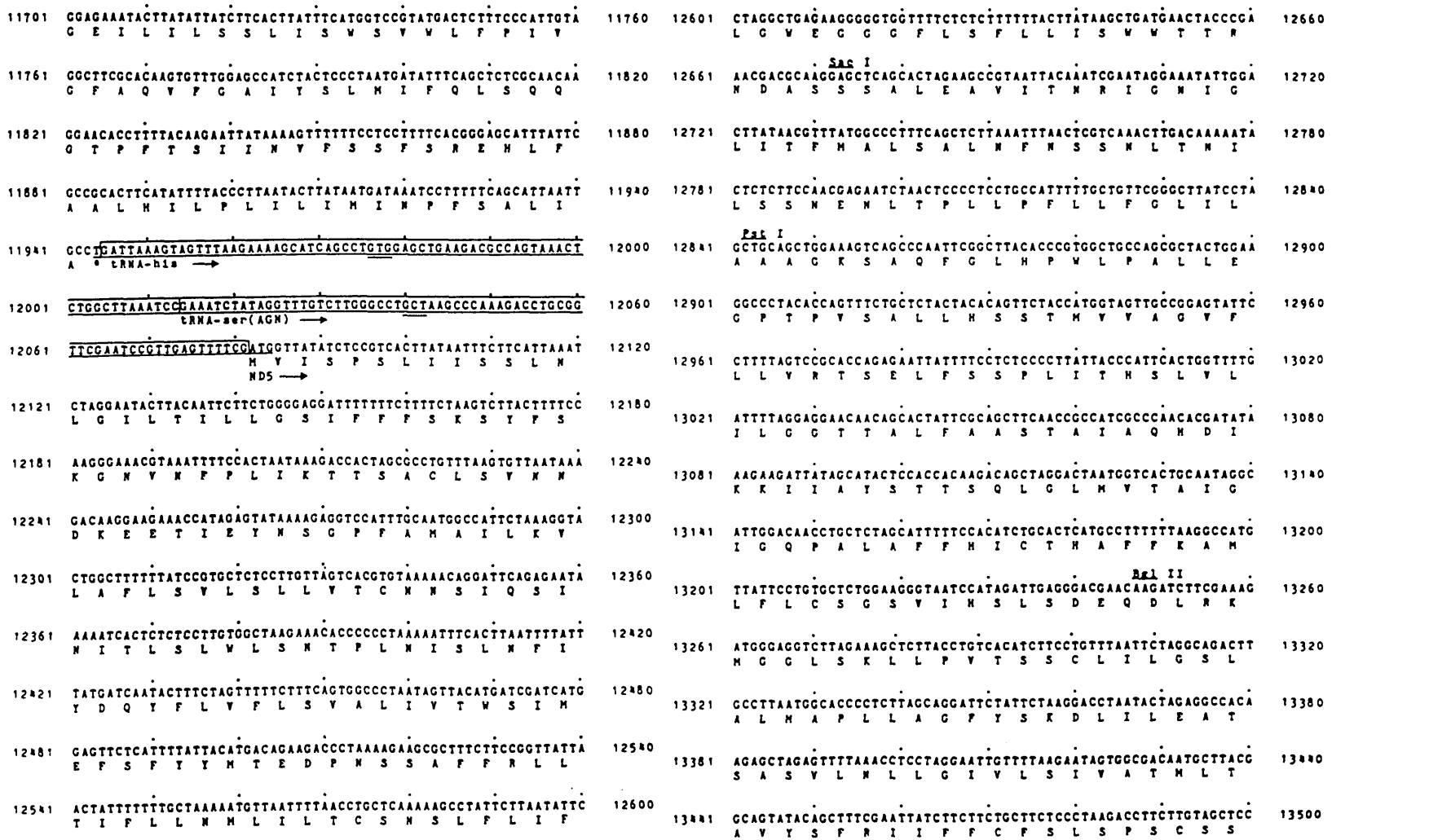
ND3
ND5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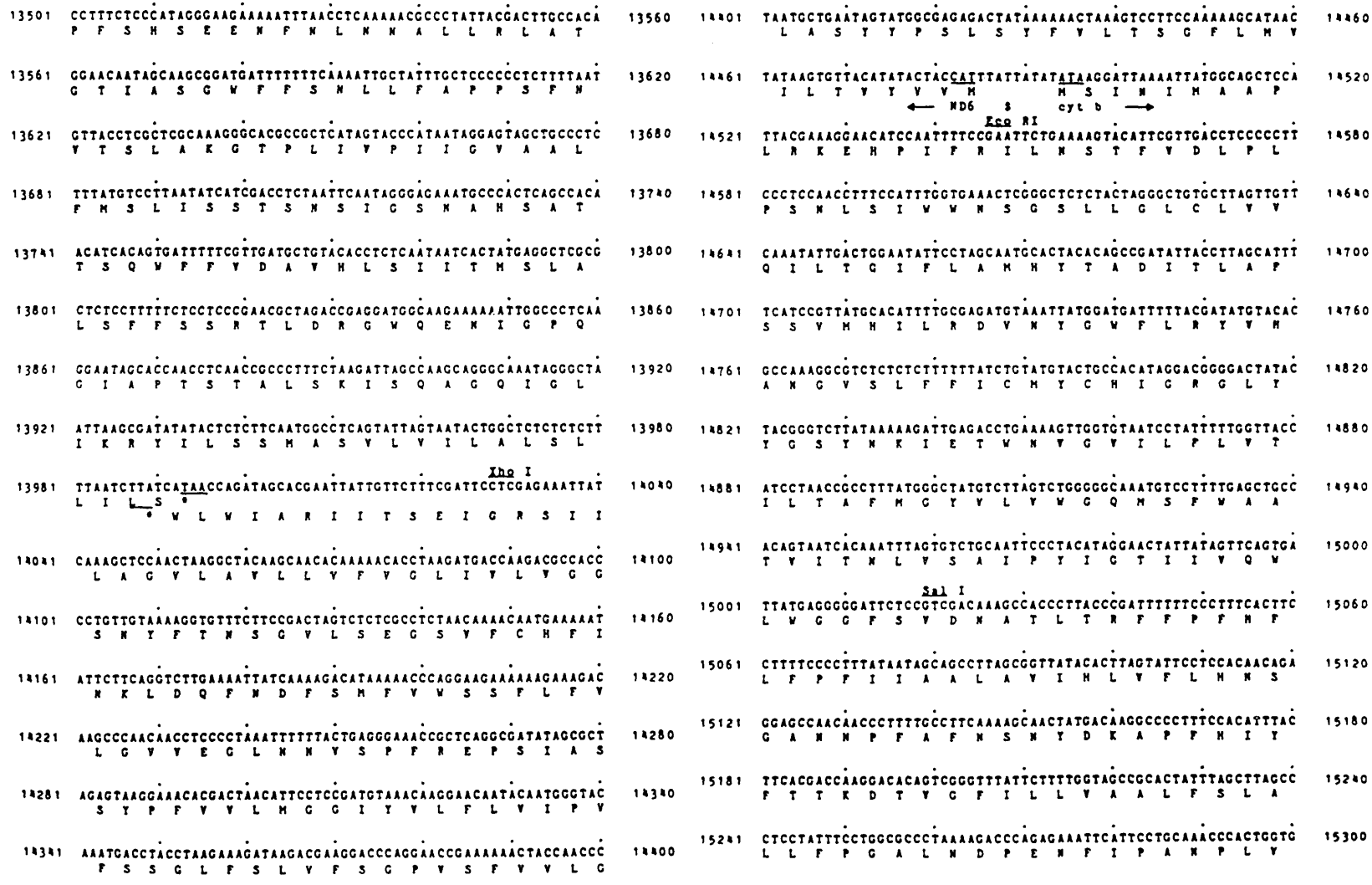
Pst I
Bgl II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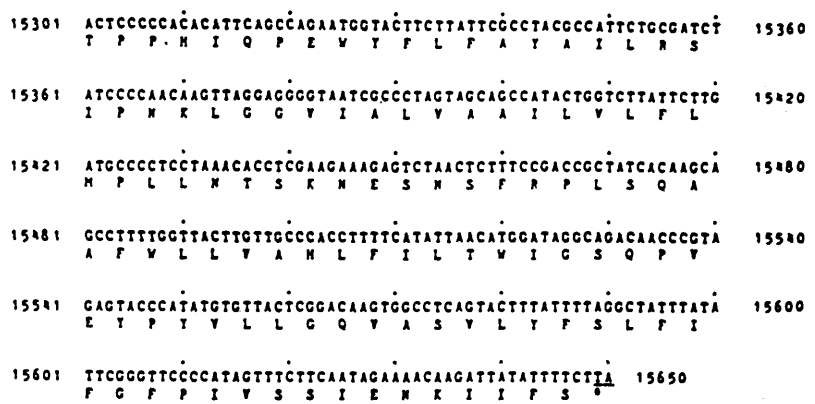
ND6
cyt b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15421 ATGCCCCTCCTAAACACCTCGAAGAAAGAGTCTAACTCTTTCCGACCGCTATCACAAGCA 15480
M P L L N T S K N E S N S F R P L S Q A
15481 GCCTTTTGGTTACTTGTTGCCCACCTTTTCATATTAACATGGATAGGCAGACAACCCGTA 15540
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15601 TTCGGGTTCCCCATAGTTTCTTCAATAGAAAACAAGATTATATTTTCTTA 15650
F G F P I V S S I E N K I I F S *

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