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Degree: **PH.D.**  
Year: **1987**

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MOLECULAR ANALYSIS OF XENOPUS

OOCYTE MATERNAL RNA

Darrell Sleep

Ph.D.
University of Edinburgh
1987
Living starts when you start doubting everything that came before you.

Socrates.
ACKNOWLEDGEMENTS

I would like to thank Dr. Peter Ford for his excellent supervision and support during the period in which I have worked in his laboratory, and Dr. Nora Hunter and David Tannahill for their support and encouragement. I would also like to thank Pamela Beattie for the production of the electron micrographs.

This research was funded by the Science and Engineering Research Council.
ABSTRACT

The early stages of embryonic development of the amphibian *Xenopus laevis* has been under close examination for many years. These studies indicate that embryonic growth post-fertilisation is entirely dependent upon reserves of macromolecules accumulated during oogenesis. Calculations indicate that only 4% of the large messenger RNA (poly(A) RNA) store is actively translated. The mechanism by which this translational control is achieved is unknown, however, three possibilities have received considerable scientific attention.

1. The rate of protein synthesis is apparently limited by the availability of components of the translational machinery.

2. The messenger RNA is associated with certain proteins in the form of ribonucleoprotein particles (mRNPs), and these can actively suppress translation.

3. As much as 70% of the total mass of poly(A) RNA apparently is associated with interspersed repetitive sequences and as such are untranslatable.

The experimental data described in this presentation suggests that interspersed repetitive sequences are in fact a relatively rare component of the maternal RNA pool. A plasmid cDNA library has been constructed from RNA participating in
intra- and intermolecular associations and therefore enriched for any interspersed repetitive sequences within the RNA.

Of the many sequences studied only one displayed properties characteristic of an interspersed repetitive sequence. The element apparently defines a series of related but not identical sequences, reiterated some 4000 times within the genome. The element is also apparently species specific. More importantly, mRNA transcripts with homology to the repetitive sequence are extremely rare within the ovarian poly(A) RNA populations. If the abundance characteristics of this sequence are typical then interspersed repetitive sequences are not abundant components of the mRNA store.
ABBREVIATIONS

Amp  Ampicillin
APS  ammonium persulphate
BClG  5-bromo-4-chloro-3-indoyl-beta-galactoside  
      (X-gal)
bp  base pair
BSA  bovine serum albumin
butyl-PBD  2-(4'-tert-butylphenyl)-5-(4''-biphenyl)1,3,4-oxadiazole
cDNA  complementary deoxyribonucleic acid
Ci  Curie
catab  cetyltrimethylammonium bromide
ddNTP  2',3' dideoxynucleoside triphosphate
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
dNTP  2' deoxyribonucleoside triphosphate
DTT  dithiothreitol
EDTA  diaminoethanetetraacetic acid
g  gram
G  gravity
GpU  guanylyl-3',5'-uridine
Hepes  N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
hr  hour
IPTG  isopropyl-beta-D-thio-galactopyranoside
M  molar
mA  milliamp
Ms  Molar Seconds
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<tr>
<td>Mes</td>
<td>2-[N-Morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MOPS</td>
<td>morpholinopropane sulphonic acid</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>pH</td>
<td>$-\log_{10}$ hydrogen ion concentration</td>
</tr>
<tr>
<td>Pipes</td>
<td>piperazine-N,N'-bis[2-ethane sulphonic acid]</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>r.p.m.</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Strep</td>
<td>streptomycin</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNN'N'-tetra-methyl-1,2-diamino-ethane</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxy methyl) aminomethane</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
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<td>w/v</td>
<td>weight per volume</td>
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CHAPTER ONE

INTRODUCTION
1.1 A general introduction to amphibian oogenesis, oocyte maturation and early embryonic development as exemplified by the genus Xenopus

1.1.1 Xenopus oogenesis

Xenopus germ cells derive exclusively from a small number of blastomeres which receive so called "germinal plasm" originating from near the vegetal pole of the unfertilised egg. The germinal plasm can be identified by light microscopy as yolk free areas within the egg cytoplasm, or as in electron microscopic studies as aggregates called germinal granules which are in turn associated with numerous mitochondria, ribosomes and glycogen granules. During the early embryonic cleavage stages there is a general reorganisation of the egg cytoplasm which results in the movement of the granules from their original location to a position near the base of the blastocoel. As a result the granules are restricted to 10-20 blastomeres of the 20,000 cells which constitute the Xenopus laevis late blastula (Whittington and Dixon, 1975). During subsequent gastrulation and neutralisation, these blastomeres remain in the posterior neural endoderm. Later, at stage 38 of embryonic development, the 20-30 primordial germ cells derived from these blastomeres, identified by their large size, the presence of germinal plasm and yolk platelets, migrate to an area destined to become the gonads of the adult animal, called the germinal ridges (Wylie and Roos, 1976). The mechanism
by which this migration is controlled is not well understood but nonetheless is completed be developmental stage 41.

Once they have arrived in the germinal ridges, the 20-30 primordial germ cells multiply rapidly, eventually reaching some 10-20,000 cells. The germ cells, or gonocytes, are sexually uncommitted and can enter either the processes of spermatogenesis or oogenesis regardless of their sex chromosome constitution, but rather dependent upon a number of extracellular cues received during development. Therefore "male" germ cell transplants placed into female recipient embryos develop into oocytes even though genetically these cells are male. Furthermore, Xenopus laevis tadpoles exposed to oestrogen (a female sex steroid) all develop in female frogs. Sexual differentiation of the gonocytes occurs concomitant with the appearance of the embryonic forelimbs. Not all germ cells undertake differentiation simultaneously. While a large reserve or stem cell population of primary oogonia is maintained by mitosis, some divide to produce secondary oogonia, the first cells irreversibly committed to the differentiative steps of oogenesis. Therefore the ovary simultaneously contains all stages of oogonial and oocyte development (Keen et al. 1979). The secondary oogonium undergo four divisions to produce 16 daughter cells. At the end of this period of mitotic division the oocyte proceeds through a final round of DNA synthesis reaching the 4C DNA complement. The oocyte (approximately 50 μm in diameter) then enters a number of specialised stages of
nuclear and chromosomal differentiation (Coggins, 1973) which extends over a period of several months. The replicated chromosomes become visibly condensed thickening and shortening, the chromatids partially separate being joined at several chiasmata and enter a form known as the lampbrush stage. The lampbrush chromosomes are a site of intensive RNA transcription and themselves undergo a number of visible changes, as will be discussed later (Section 1.2). Although oocyte development will continue through a number of different stages, further progression of the replicated chromosomes through the cell cycle is blocked. Meiotic arrest will only be lifted following progesterone stimulation signalling oocyte maturation (Section 1.1.2). A specific amplification of the 18S, 5.8S and 28S ribosomal RNA genes as extrachromosomal circles occurs early in oocyte development and is associated with a massive increase in the number of nucleoli per germinal vesicle and even primary oogonia contain elevated numbers of nucleoli, 4-16 as opposed to the normal 2. The *Xenopus laevis* genome contains approximately 500 copies of the 18S, 5.8S, 28S ribosomal RNA genomic repeat (Birnstiel et al. 1972), however, following gene amplification this has increased to approximately $2 \times 10^6$ copies, or 1000-1500 nucleoli per germinal vesicle. The process of ribosomal gene amplification enables the rapid synthesis of substantial quantities of ribosomal RNA (4000 ng per stage 6 oocyte) an essential component
of the massive \(10^{12}\) store of preformed ribosomes which accumulate during oogenesis and are essential for early embryonic development.

While the primary oogonium contains no identifiable yolk platelets, the fully developed stage 6 oocyte contains massive amounts, comprising at least two thirds of the total protein of the cell. The process of vitellogenesis or yolk deposition can be divided into two periods, namely the previtellogenic and vitellogenic periods. During the previtellogenic period the oocyte enlarges from 50 \(\mu\)m to approximately 300 \(\mu\)m in diameter with a proportional enlargement of the germinal vesicle and includes the stages of nuclear differentiation discussed above as well as the cytoplasmic changes discussed below. The previtellogenic oocyte is transparent and an opaque body adhering to one side of the germinal vesicle can clearly be observed by dark field illuminated light microscopy. This body, the mitochondrial cloud, consists of numerous closely packed mitochondria. There is a rapid increase in mitochondrial DNA during this period which plateaus when the oocyte is approximately 500 \(\mu\)m in diameter (Webb and Smith, 1977). Mitochondrial DNA synthesis is not re-established until stage 30 of embryogenesis. At the end of the previtellogenic period the mitochondria disperse to the oocyte periphery, to form a uniform layer beneath the plasma membrane.

During the previtellogenic period each oocyte becomes
surrounded by follicle cells. Shortly afterwards deposits produced from both the oocyte and the follicle cells accumulate between both cell types, in an area that will eventually designate the vitelline membrane. At the end of the previtellogenic period blood vessels invade the follicle and establish a rich vascular network.

During the vitellogenic period, oocytes (between 300 μm and 1200 μm in diameter) actively remove vitellogenin, a 200,000 dalton protein secreted by the liver, from the blood by pinocytosis. The protein is proteolytically cleaved to produce lipovitellin and phosvitin which then crystallise under the ionic conditions of the oocyte cytoplasm. Both proteins are heavily modified by the covalent attachment of phosphate and lipid moieties. The control of vitellogenic uptake starts by the stimulation of the hypothalamus by external environmental factors. By releasing certain factors the hypothalamus stimulates the pituitary gland to secrete follicle stimulating hormones (FSH) which in turn stimulate the follicle cells to secrete oestrogen, finally stimulating the liver to secrete vitellogenin.

The yolk platelets accumulate by the coalescence of a number of pinocytic vesicles containing vitellogenin as well as other proteins. During vitellogenesis the yolk platelets accumulate, eventually accounting for 60-70% of the cell protein, acting as the reservoir of essential amino acids, phosphate, calcium and lipid.

During early embryonic development the
majority of this yolk is distributed within the embryonic gut region where these reserves can be digested and distributed to the embryonic cells via the developing vascular system.

The oocyte not only accumulates molecular precursors (phosvitin, lipovitin, lipids and glycogen) for later use during embryogenesis, but also many preformed macromolecules including ribosomes, messenger RNA and many proteins (including histones, actins and tubulins). The accumulation of these essential components will be discussed in detail below, but nonetheless recognition of their existence and importance is necessary at this juncture. The oocyte accumulates a large store of preformed mRNA, the so-called maternal RNA (85 ng poly(A) RNA per oocyte). The majority of this pool is not translated during oogenesis, but rather is transferred to polysomes following fertilisation of the egg. Translation of these mRNAs during early embryogenesis requires the presence of a preformed pool of tRNAs and ribosomes since early embryonic development proceeds in the absence of RNA transcription (Newport and Kirschner, 1982). As mentioned previously genes encoding three of the ribosomal RNAs are amplified as extrachromosomal circles during oogenesis ensuring that a large pool of these particular RNAs accumulates during oogenesis. The 5S genes are also repeated many times within the genome, but their gene complement
does not change during development. The *Xenopus laevis* genome contains 20,000 oocyte specific 5S genes and 400 somatic type 5S genes. However, while both banks of 5S genes are active during oogenesis, post fertilisation only the latter set is actively transcribed. The many various protein and RNA components are assembled into ribosomes at the nucleoli which as previously mentioned are amplified within the germinal vesical. This arrangement ensures that a massive preformed store of some $10^{12}$ ribosomes accumulates during oogenesis.

Although the process of oogenesis is completed within a period of 3-4 months, the oocyte is not capable of fertilisation. The oocyte must undergo a number of complex nuclear and cytological changes before fertilisation can be achieved. This process, oocyte maturation, proceeds over a relatively short period, lasting some 6-8 hours, elicited in response to the steroid hormone progesterone released by the follicle cell.

1.1.2. *Xenopus* oocyte maturation

The cell cycle arrest of the fully developed oocyte is released upon exposure to progesterone leading to maturation of the oocyte. This begins with the migration of the germinal vesicle to the animal pole, where the
nuclear membrane disintegrates, releasing the contents of the germinal vesicle into the animal hemisphere cytoplasm. This region is identifiable by its low yolk content, high glycogen, ribosome and sulphydryl content. Externally this area can be identified as a light spot on a dark background at the animal pole caused by the local dispersal of pigment granules. Simultaneously the cell proceeds through the entire meiotic first cycle with the budding of the first polar body from the oocyte surface in the area of the light spot. The cell continues through meiotic division until arrested in metaphase of the second meiotic cycle. The meiotic cycle will subsequently be reinitiated by activation of the egg during sperm entry (fertilisation), as will be discussed in Section 1.1.3.

One of the earliest responses to progesterone is a change in plasma membrane ion permeability resulting in a change in intracellular \( \text{Ca}^{2+}, \text{K}^+ \) and \( \text{Na}^+ \) ion concentration, detectable within 3 minutes of steroid stimulation. Also within 30 minutes of exposures to progesterone a drop in the intracellular cAMP concentration is observed, which in turn has led to the suggestion (Mailer and Krebs, 1977) that the arrested state of the oocyte is maintained by a phosphoprotein whose phosphorylation state is directly or indirectly under the control of a cAMP dependent protein kinase, or by an increase in protein phosphatase activity. A substantial amount of work has
now been carried out on the mechanism responsible for the change in cAMP levels during maturation. Evidence very strongly suggests that progesterone inhibits the oocyte adenylate cyclase by decreasing the rate of activation of the stimulatory subunit ($N_s$) by GTP via a membrane bound steroid receptor (Sadler and Maller, 1981, 1983).

The early events that are part of the triggering mechanism for oocyte maturation lead to an increase in protein synthesis in later stages (Wasserman et al. 1982) although the exact mechanism by which this is achieved is not well understood. Elevated levels of protein synthesis are, however, associated with phosphorylation of a 40S ribosomal subunit protein, S6. Studies indicate that all the S6 protein in ribosomes become maximally phosphorylated during maturation, and that the increase is correlated with the increase in protein synthesis (Nelson, Thomas and Maller, 1982). The increase in S6 phosphorylation is also correlated with an increase in intracellular pH, by 0.3-0.4 pH units. However, even when such an increase is blocked, S6 phosphorylation and oocyte maturation occur normally (Stith and Maller, 1985) suggesting that the intracellular pH increase may be a consequence rather than a cause of some specific step involved in stimulating oocyte maturation.

The late events of oocyte maturation are primarily concerned with the activities of a protein, maturation.
promoting factor (MPF). MPF is a cytoplasmic factor that is capable of inducing nuclear envelope breakdown (GVBD) chromosome condensation and spindle formation (Wu and Gerhart, 1980). In vivo MPF activity is rapidly inactivated following fertilisation which is known to be associated with a large increase in intracellular calcium levels. Recent evidence suggests that the in vivo regulation of MPF by calcium may be indirect, related to the calcium sensitivity of another cytoplasmic factor found in unfertilised eggs called cytostatic factor (CSF). CSF is believed to maintain the metaphase arrest of the unfertilised egg. Following fertilisation the rise in calcium is believed to cause a calcium dependent inactivation of CSF associated with the re-initiation of the cell cycle. The re-establishment of cell cycle is believed to in turn result in the inactivation of MPF activity, following which the fertilised egg proceeds through nuclear envelope formation, chromosome decondensation and DNA synthesis. The mechanism by which MPF exerts its effects is not clear and awaits the purification of MPF to homogeneity.

1.1.3 Early embryonic development of the genus *Xenopus*

Although the experimental data examined later in this presentation does not directly address the involvement of maternally inherited cytoplasmic components in early embryonic development, an understanding of the morphological and biochemical changes which occur during the first 8 hours of embryonic development, up to and including the
midblastula transition (MBT), is required if the reader is to fully appreciate the vital role of the maternally inherited components accumulated during oogenesis.

Following oocyte maturation the eggs are released from the ovary into the body cavity where they are carried by cilia of the body wall to the oviduct. Here the egg surface is modified by the addition of layers of jelly and finally released from the body for external fertilisation.

Although the egg is radially symmetrical the position of sperm entry appear to define approximately the bilateral axis of the fully developed embryo in the plane constructed from the point of sperm entry and the animal and vegetal poles, with the anterior end of the embryo directed toward the side of sperm entry. Following sperm entry a number of processes prepared for during oogenesis and oocyte maturation are set into motion. These include a rapid block to polyspermal fertilisation, separation of the vitelline membrane from the egg plasma membrane, swelling of the previtelline space between these two membranes and the rotation of the egg to a position such that the animal pole is uppermost and the vegetal pole downwards, the reinitiation of meiosis with the explusion of the second polar body, the initiation of DNA synthesis, pronuclei contact, the formation of the grey crescent and the appearance of the first cleavage plane. The entire chain of events from fertilisation to the appearance of the first cleavage furrow is completed in 90 minutes. Indeed the second cycle of DNA synthesis begins
before the first cleavage furrow is visible and the second furrow begins before the first is completed.

Following fertilisation the zygote undergoes 12 synchronous mitotic divisions, which within a period of approximately 7 hours takes the single celled zygote to the 4000 cell midblastula stage. These synchronous divisions are known to persist in individual blastomeres even after disaggregation and separation, and hence each blastomere must carry within itself an accurate endogenous cell-cycle timer which is transmitted to daughter blastomeres. The 12 cleavages are unusual in that they occur in the absence of the G1 and G2 cell cycle phases, and hence except for the first cleavage cell division occurs on average every 35 minutes and are characterised by rapid DNA synthesis but negligible RNA synthesis. During this period the developing embryo is entirely dependent upon a variety of macromolecules accumulated during oogenesis. These include ribosomes (Woodland, 1974), the so-called maternal RNA (Golden et al, 1980) in the form of ribonucleoprotein particles (mRNP) (Darnbrough and Ford, 1981, Richter and Smith, 1983) and many diverse proteins including histones (Woodland, 1980), nucleoplasm (Laskey and Earnshaw, 1980), DNA polymerase (Zierler et al, 1985), RNA polymerase (Roeder, 1974), tubulin (Pestell, 1975), actin (Clark and Merriam, 1977). The stock piling of these components is of vital importance to the developing embryo, allowing a rapid increase in cell number over a very short period of time.
Following the 12th cleavage the developing embryo experiences a dramatic change in many of the features characteristic of early embryonic development. The timing of the midblastula transition (MBT) is of vital importance to embryonic development and has recently been reinvestigated (Newport and Kirschner, 1984). These experiments suggest that the new developmental program is initiated when each blastomere reaches a critical nucleus to cytoplasm volume ratio. The results presented by Newport and Kirschner (1984), have lead to a model whereby the titration of some cytoplasmic component by DNA reaches an end point, triggering a profound change in cell activity. Since blastomeres of the cleavage embryo differ as much as ten-fold in size, this limiting material must be distributed not at a constant concentration but in equivalent amounts per blastomere. The "exhaustion hypothesis" is supported cytologically by the reduction of nuclear volume during the midblastula stage. During the period leading up to MBT the total nuclear volume increases until the midblastula stage when it suddenly reaches a limit. Since the number of nuclei is still increasing the nuclear volume per nucleus decreases. The limit value for nuclear volume is close to but slightly less than that of the germinal vesicle. It is plausible that the contents of the germinal vesicle provides nuclear material for the nuclei of the developing embryo and that this becomes limiting at the midblastula stage (2000-4000 nuclei). Following MBT the cell cycle slows down with the
appearance of both G1 and G2 phases, reinitiation of RNA synthesis and asynchronous cell division (Newport and Kirschner, 1984). The reinitiation of RNA synthesis can easily be incorporated into the exhaustion hypothesis whereby the limiting availability of some essential component required for rapid DNA synthesis would enforce a requirement for both the G1 and G2 cell cycle phases. The incorporation of these periods would allow the initiation of RNA synthesis.

1.2 The accumulation of poly(A) RNA during oogenesis and its fate during oocyte maturation

During oogenesis the chromosomes of the Xenopus oocyte are replicated, having the 4C complement of DNA but are arrested in diplotene. A visual inspection of these chromosomes with the aid of a light microscope reveals the presence of many lateral loops a feature which has led to their designation as the lampbrush chromosomes. The morphology of these chromosomes changes during oogenesis. The lateral loops lengthen during the early stages, maximal extension being observed during stage 3 but gradually retract during the latter stages of oogenesis. Experimental evidence suggests that these structures are the sites of intense RNA synthesis (MacGregor, 1980) and that the RNA produced contains both repetitive and non-repetitive transcripts (Sommerville and Malcolm, 1976, Varley et al. 1980, Jamrich et al. 1983). While the majority of non-ribosomal RNA synthesised is
rapidly turned over within the nucleus, approximately 15% is exported to the oocyte cytoplasm as relatively stable poly(A) RNA (Dolecki and Smith, 1979). There the poly(A) RNA accumulates, reaching a plateau equivalent to approximately 85 ng per oocyte prior to the onset of vitellogenesis, stage 2 according to Dumont (1982). Indeed, evidence presented by Golden et al. (1980) indicates that the accumulation of transcripts homologous to 16 cDNA sequences derived from oocyte poly(A) RNA exactly parallels that of total poly(A) RNA. No sequences were detected which appeared during some specific stage(s) of oogenesis and were then eliminated or significantly reduced in concentration.

As yet the correlation between the extent of lateral loop extension and the rate of RNA synthesis is unclear, since a large fraction of the poly(A) RNA is synthesised long before the loops are maximally extended (stage 3) by which time the size of the maternal RNA pool has stabilised (Rosbash and Ford, 1974, Golden et al. 1980). Any subsequent synthesis of poly(A) RNA is therefore presumed to be balanced by RNA turnover, although whether this involves the entire poly(A) RNA pool, or just the newly synthesised RNA is unknown. Nonetheless, calculations described by Anderson et al. (1982) suggest that the unique transcriptional activity of the lampbrush chromosomes is essential to sustain the massive pool of maternal RNA during the vitellogenic stages of oogenesis.

The existence of a maternal messenger RNA pool within
the oocytes of many organisms has been demonstrated unequivocally in different ways by a number of authors, reviewed by Davidson (1976). Thus enucleated eggs or egg fragments have been shown to synthesise proteins at rates identical to those of control embryos, as have actinomycin and α-amanitin treated eggs. Rosbash and Ford (1974) estimated the contents of poly(A) RNA in *Xenopus* oocytes to be 40 ng, based on the amount of $[^3H]$ poly(U) hybridising with oocyte poly(A) sequences, while Dolecki and Smith (1979) estimated the quantity of RNA able to bind to oligo-dT cellulose as approximately 85 ng per oocyte. Rosbash and Ford (1974) also demonstrated that the poly(A) RNA is also of typical messenger RNA size, 2.1 kb. Approximately 20,000 different sequences are to be found in immature (stage 1-2) and mature (stage 6) oocytes, and these sequences are the same in both types of cell, Perlman and Rosbash (1978). Approximately 1100 sequences, (5%) define the moderately abundant class and are represented at some $21 \times 10^6$ copies per cell accounting for approximately 50% of the total mass of poly(A) RNA, while the remaining 19,000 sequences (95%) defining the rare class, are presented at some $1.3 \times 10^6$ copies per cell. The oocyte contains no highly abundant mRNA polyA$^+$ sequences, Perlman and Rosbash (1978). The segregations of the maternal poly(A) RNA into two classes of moderate and low abundance is supported by the hybridisation studies of Golden et al. 1980, Dworkin.
and Dawid 1980, Krieg and Melton (1985). The length of poly(A) sequences within the poly(A) RNA of the oocyte is not however homogeneous. Cabala et al. (1977) and Sagata et al. (1980) describe experiments demonstrating that the RNA of stage 6 oocytes contains two distinct size classes of poly(A) designated poly(A)_S containing 15-30 A residues and poly(A)_L containing 40-80 A residues. Both classes are however active as templates for protein synthesis and yield identical patterns of proteins in vitro. It has also been demonstrated that there is a dramatic change in the amount and length of poly(A) tails during oocyte maturation (Darnbrough and Ford, 1976, Sagata et al. 1980). These studies indicate that the absolute amount of poly(A) and poly(A) RNA per oocyte decreases approximately 2 fold. However, data presented by Colot and Rosbash (1982), Dworkin and Dworkin-Rastl (1985) suggest that the polyadenylation status of different poly(A) RNAs are not co-ordinately regulated during oocyte maturation. While some poly(A) RNA transcripts become more polyadenylated, others become depolyadenylated. Both capping and polyadenylation of transcripts are believed to be important factors in the control of stability and translation efficiency of synthetic and naturally occurring messenger RNAs (Nudel et al. 1976, Marbaix et al. 1975, Drummond et al. 1985).

Interestingly, evidence presented by Anderson et al. (1982) suggests that the majority of the maternal RNAs possess both repetitive and single copy sequences. By
analogy to evidence presented by Costantini et al. (1980) relating to the abundance of interspersed repetitive sequences within the poly(A) of the sea urchin egg, Anderson et al. (1982) demonstrate that up to 70% of the mass of Xenopus laevis oocyte cytoplasmic poly(A) RNA can be visualised participating in multimolecular complexes and that while 80% of the mass consists of single copy sequences, 20% represents covalently linked repetitive sequences. More importantly only 15% of tadpole (stage 41) cytoplasmic poly(A) RNAs harbour repetitive sequences. These findings are in conflict with the observations made earlier by Perlman and Rosbash (1978), which suggest that less than 3% of the mass of Xenopus laevis oocyte poly(A) RNA is complementary to genomic repetitive sequences. Furthermore of the 16 individual cDNA sequences described by Golden et al. (1980) all hybridise to the same size transcript in oocyte and somatic poly(A) RNA, indicating that these RNA species are almost certainly stored as fully processed mature poly(A) RNAs. Kinetic reassociation experiments between cDNA and RNA from oocyte and stage 41 tadpoles, described by Perlman and Rosbash (1978), suggest that the majority of RNA sequences are shared between oocyte and tadpole. Approximately 80% of the tadpole cDNA hybridises with ovary RNA and almost 100% of the ovary cDNA hybridises with tadpole RNA, suggesting that only a small fraction of tadpole RNAs are newly expressed or increase from very low levels to substantially higher levels during development. These conclusions are supported by

The messenger RNA of eukaryotes is associated in vivo with specific proteins giving rise to ribonucleoprotein particles (mRNP) of 40-200S. The maternal poly(A) RNA of the Xenopus oocyte is no exception and a number of very basic proteins have been identified, Darnbrough and Ford (1981), Dearsley et al. (1985), which bestow the mRNA with certain RNase resistant properties. Four of these proteins appear to be oocyte specific, being synthesised well before the onset of vitellogenesis (Dixon and Ford, 1982) concomitant with the accumulation before vitellogenesis, of the majority of maternal RNA. As yet, however, the role of these proteins and of the mRNP particles themselves in the various developmental control processes is uncertain.

Although the majority of poly(A) RNA transcripts are evenly distributed throughout the egg cytoplasm (Carpenter and Klein, 1982, Rebagliati et al. 1985) a small number (3-5%) of transcripts have been identified which are enriched within certain portions of the egg and are subsequently inherited by particular blastomeres (Carpenter and Klein, 1982, Rebagliati et al. 1985). The partitioning of certain cytoplasmic determinants has previously been expounded as a mechanism by which certain domains of the developing embryo become determined. The partitioning of localised maternal RNA to a defined subset of blastomeres would run with this preconception.
Although oocyte poly(A) RNA is deficient in abundant poly(A) RNA species by comparison to tadpole RNA (Perlman and Rosbash, 1978, Rosbash, 1981) the oocyte does contain a high proportion of transcripts of mitochondrial origin, accounting for 15% of the total oocyte poly(A) RNA (Anderson et al. 1982). The *Xenopus laevis* mitochondrial DNA is known to encode two rRNAs and ten major poly(A) RNAs (Rastl and Dawid, 1979). Unlike the total poly(A) RNA pool, mitochondrial transcripts continue to accumulate throughout oogenesis, Golden et al. (1980).

The importance of maternally inherited cellular components in the programming and execution of early embryonic development cannot be overstressed. This is exemplified by the changes which occur in the accumulation of histones during oogenesis and early embryonic development (reviewed by Woodland, 1980). Following fertilisation the rates of histone synthesis vastly exceeds that of DNA synthesis. However, such synthesis proceeds in the absence of RNA transcription, probably due to the high rate of DNA synthesis. The embryo therefore requires a pool of preformed histone mRNA, approximately 200 pg, as demonstrated by Ruderman and Pardue (1977). Although the rate of histone synthesis will increase 2-3 fold during the period leading up to the mid- blastula transition, the rate of DNA synthesis will increase some 10,000 fold during the same period. Measurements indicate that the pool of preformed histone
mRNA is not entirely sufficient and can only supply enough histone proteins to accompany the ever increasing levels of DNA up until stage 7 of embryonic development (Adamson and Woodland, 1974). Since RNA synthesis is only resumed following the midblastula transition (MBT) (Newport and Kirschner, 1982) there is a deficit. However, measurements reveal a pool of approximately 135 ng (Adamson and Woodland, 1977) of preformed histone protein, accumulated during oogenesis. This pool is sufficient to assemble over 20,000 nuclei, a figure equivalent to that of the early gastrula, by which time histone accumulation is again supported by de novo synthesis from newly transcribed histone mRNA. Since many weeks are required to accumulate the histone mRNA pool within the oocyte, these RNAs must be stable within the oocyte. Oocyte histone mRNAs are atypical in that 50-75% are polyadenylated. However, following oocyte maturation, greater than 90% of the egg histone mRNA is non polyadenylated (Ruderman et al. 1979) as a direct result of depolyadenylation of the pre-existing histone mRNA pool (Ballantine and Woodland, 1985). The pool is then believed to decay at a rate characteristic of non-polyadenylated RNA, t\textsubscript{1/2} approximately 3 hours (Woodland and Wilt, 1980).

The Xenopus oocyte is an extremely specialised cell, which during its development accumulates a large reserve of numerous cellular components, of which the maternal RNA pool is just one. These components facilitate an extraordinarily rapid increase in cell number within a
few hours, ultimately enabling the swimming tadpole to emerge after approximately 2½ days.

1.3 Regulation of protein synthesis during oogenesis

As has previously been discussed the Xenopus oocyte accumulates a large preformed store of messenger RNA, the so-called maternal RNA. The oocyte also accumulates a large store of preformed ribosomes of which only 1.5% are actively engaged in protein synthesis (Woodland, 1974). Using this figure Richter et al. (1984) calculated that 3 ng, or 4% of the maternal poly(A) RNA is actively engaged in translation. Analysis of the in vitro translation products of Xenopus laevis previtellogenic and mature oogenic poly(A) RNA indicates that no difference exists between the coding capacities of these two developmental stages (Darnbrough and Ford, 1976, Ruderman and Pardue, 1977). However, the spectrum of in vivo translation products of the endogenous messenger RNA does change substantially during oogenesis (Darnbrough and Ford, 1976). This would suggest that some form of mRNA selectivity or translational control exists since not all mRNAs are translated with equal efficiency. This is in part supported by evidence relating to the efficiency of translation of exogenous poly(A) RNA injected into oocytes (Gurdon et al. 1973), since α-haemoglobin mRNA is translated only 20% as efficiently as β haemoglobin mRNA.

The rate of protein synthesis is low in stage 6
oocytes 23 ng/hr. (Wasserman et al. 1982, Taylor and Smith 1985), however during oocyte maturation there is a two fold increase in the polysome content (Woodland, 1974) and only a small further increase following fertilisation. During the subsequent period of development from fertilised egg to the late blastula, a period of some 9 hours, the polysome content gradually increases from 3% to 15%. Over the next 25 hours, during which the embryo reaches the neurula stage, the polysome content remains essentially constant, approximately 15% (Woodland, 1974), following which it gradually increases again until the embryo has reached the swimming tadpole stage, whereby the polysome content is approximately 75%, a value typical of somatic cells (Woodland, 1974). Since the protein synthetic rate increases so dramatically during the period from oocyte maturation to gastrulation (Wasserman et al. 1982, Woodland, 1974), the oocyte maternal RNA must be under some form of translational control. Although the mechanism(s) by which this is achieved is unknown, three possibilities have received considerable attention.

1. The rate of protein synthesis in oocytes is limited by the availability of components of the translational apparatus other than mRNA, activation of maternal mRNA translation coincides with the increased availability of the limiting components.

2. The structural organisation of maternal mRNA prevents translation, activation requires
structural modifications.

3. Proteins associated with maternal mRNA (mRNP) prevent translation, activation involves removal or degradation of these proteins.

These three possibilities are not mutually exclusive, translational control may involve a complex interplay between all three mechanisms.

As has been mentioned previously, injection of exogenous poly(A) RNA results in the translation of this mRNA, however as discovered by Laskey et al. (1977), Richter and Smith (1981), this occurs at the expense of endogenous mRNA, demonstrating that a component(s) of the translational machinery is limiting in the oocyte. However, since the protein synthetic rate increases during maturation and early embryonic development (Woodland, 1974) whatever the rate-limiting component may be, it must be provided in increased amounts during development as more mRNA is released into the translatable pool, and must always keep slightly ahead of the mRNA supply.

As much as 70% of the mass of oocyte poly(A) RNA can be visualised with the electron microscope participating in multimolecular complexes. According to Anderson et al. (1982) this is a direct result of intermolecular hybridisation between interspersed repetitive sequences, although this conclusion is in direct conflict with data presented by Perlman and Rosbash (1978), Golden et al. (1980). Nonetheless that portion of the maternal poly(A) RNA which participates in those complexes is untranslatable
both in vitro and in vivo. This would of course explain the observed failure of the bulk of the maternal poly(A) RNA to be translated. The occurrence of interspersed repetitive sequences within the maternal poly(A) RNA population will be discussed later, Section 1.5.

Darnbrough and Ford (1981) have reported the existence of a series of oocyte specific proteins which can associate with poly(A) RNA in vivo, in the form of mRNPs. Richter and Smith, (1983, 1984) have similarly identified a series of proteins which bind radiolabelled globin mRNA. These proteins can be subdivided into two groups (Richter and Smith, 1983). The abundance per oocyte of the first group increases five fold as the oocyte progresses through oogenesis and these proteins also cosediment with monosomes and polysomes. The abundance of the second group, however, decreases by approximately 75% during the same period. These developmentally regulated RNA binding proteins are also believed to be associated with non-translating poly(A) RNAs in vivo, as mRNPs. Moreover, mRNP reconstitution experiments with globin mRNA indicate that these oocyte specific proteins have the ability to efficiently suppress translation of globin mRNA in vivo (Richter and Smith, 1984). These developmentally regulated proteins therefore have the properties expected of proteins which can mask oocyte mRNA. Since the increase in protein synthesis during oogenesis (0.18 ng/hr - 22.8 ng/hr) is a gradual process and not a single dramatic event
which occurs in response to a single stimulus, a gradual reduction in the abundance of these proteins during oogenesis as identified by Richter and Smith (1983) is consistent with a hypothesis involving the unmasking of mRNA from the untranslatable mRNP store allowing an increase in the observed level of protein synthesis during oogenesis (Taylor and Smith, 1985).

The observations described above can be summarised in the form of a hypothesis (Woodland, 1982) whereby the control of translation is exerted almost wholly by the release of mRNA from the inactive mRNP pool. The supply of active mRNA is the major factor limiting the rate of protein synthesis. There is however, a vast store of unused translational capacity kept in check by a limiting supply of some factor(s). As development progresses the translatable pool of mRNA expands but without a significant decrease in the efficiency of protein synthesis. The supply of any rate limiting factors must therefore be kept slightly ahead of this expansion.

1.4 Gene expression from heterologous sequences micro-injected into the Xenopus eggs and oocytes

The Xenopus oocyte is an extremely specialised cell. As discussed previously, Sections 1.1-1.3, the oocyte accumulates massive stores of numerous cellular components in vast excess of its immediate needs. The macromolecules are essential to support the period of rapid cellular
division characteristic of early embryonic development. This has enabled researchers to exploit Xenopus eggs and oocytes as "cellular test tubes" for the expression of various heterologous genes from a wide variety of sources. With this in mind, it is extremely important to remember that biochemically the Xenopus oocytes and eggs are very different cells. Oocytes actively transcribe RNA, possess a nucleus (the germinal vesicle) and can, even when microinjected with various cellular components, be maintained in culture for several weeks. Furthermore, oocytes will actively accumulate radiolabelled amino acids and nucleosides from the culture medium. By contrast, radiolabelled precursors can only be introduced into the Xenopus egg by microinjection which will in the process activate the egg. The egg will then degenerate within a few hours unless it has previously been fertilised. Therefore oocytes represent the best recipient for microinjected nuclei, DNA and mRNA.

The transfer of genetic information into Xenopus oocytes and eggs by microinjection has been reviewed by Gurdon and Melton (1981) and Gurdon and Wickens (1983). DNA microinjected into the Xenopus oocyte cytoplasm is ultimately unstable and is completely degraded within a few days. However, DNA microinjected into the nucleus is stable for several weeks. Although the DNA is not integrated into or associated with the oocyte chromosomes it is rapidly assembled with nucleosomes into a chromatin like structure.
As far as can be determined the transcription of microinjected DNA within the oocyte nucleus utilises the same type of RNA polymerase as that used to transcribe the gene at its normal site of synthesis. However, results from various researchers (summarised by Gurdon and Melton, 1981) indicate that initiation and termination at the correct sites is not achieved. The effects on transcription fidelity from heterologous genes by various vector/plasmid sequences are not fully understood. Furthermore, while *Xenopus laevis* α1 and β1 globin genes are actively transcribed but incorrectly initiated when injected into oocytes, these same genes are correctly initiated when microinjected into eggs although the rate of transcription is much reduced (Bendig and Williams, 1984). However, the transcription of microinjected DNA is not entirely non-specific since the tRNA genes of *Escherichia coli* and human mitochondria are transcribed at least 1000 times less efficiently than genomic tRNA genes.

The *Xenopus* oocyte has also been used quite successfully as a research tool aimed at dissecting the various post-transcriptional intermediates associated with RNA processing. These include the removal of 5' leader sequences, 3' trailer sequences, splicing of intervening sequences and the ribonucleotide modifications associated with processing of tRNA precursors and the molecular analysis of RNA processing associated with RNA polymerase II derived transcripts (Green et al. 1984). These studies
result from the microinjection of cloned genes as well as mRNA precursors synthesised in vitro.

Xenopus oocytes have also been adapted by researchers whose interests concern the later events of gene expression, including translation, protein modification and compartmentation. Interest in this field was first excited by the demonstration that Xenopus oocytes could translate mRNA with greater efficiency than cell-free translation systems. Subsequently many proteins have been successfully synthesised from microinjected mRNA. More importantly however, the oocyte offers an excellent opportunity to examine in detail the processes of protein post-translational modification. These include N-acetylation, glycosylation, phosphorylation, peptide cleavage (including the removal of secretory leader sequences), protein assembly (including disulphide bridge formation and non-covalent associations) and protein compartmentation reviewed by Colman et al. (1984).

Although Xenopus oocytes can actively translate and secrete many proteins with the removal of the secretory leader sequence the oocyte appears to be deficient in some endopeptidases responsible for the removal of certain "pro" sequences from protein precursors although this does not impair the progress of most protein through the secretory pathway. The oocyte can also secrete oligomeric proteins whose biological activity requires correct disulphide bridge formation, these include IgG
tetramers (Colman et al. 1982) and rat prostatic binding protein (Mous et al. 1982). However the oocyte can also fail to or incorrectly assemble other oligomeric proteins (reviewed by Colman et al. 1984).

Finally, many secreted proteins are glycosylated and glycosylation of secreted proteins by Xenopus oocytes derived from microinjected mRNA has been demonstrated (Mous et al. 1982; Colman et al. 1984). However in many cases although the correct sites have been selected for core glycosylation the final modification stages involving the removal of specific mannose residues and the addition of sialic acid and fucose sugar residues is either incomplete or inefficient.

The results demonstrate that although the oocyte is a specialised cell, primed and programmed for rapid cellular division, it is in fact by virtue of its very nature extremely versatile.
1.5 The interspersed repetitive sequence organisation of sea urchin egg poly(A) RNA and Xenopus oocyte poly(A) RNA

As previously mentioned in sections 1.2 and 1.3, up to 70% of the mass of Xenopus oocyte poly(A) RNA is believed to be associated with interspersed repetitive sequences (Anderson et al. 1982). Since much of the experimental data relating to this observation is made with reference to data pertaining to the abundance of interspersed repetitive sequences within sea urchin egg poly(A) RNA, a résumé of the experimental data relating to the sequence organisation of sea urchin egg poly(A) RNA is required.

A number of sea urchin genomic interspersed repetitive sequences have previously been isolated from a portion of the sea urchin genome which is S1 nuclease resistant after renaturation to a C\_o\_t equivalent to 40 Ms (Klein et al. 1978). Sequence analysis indicates that 6 of 8 clones studied (Posakony et al. 1983) cannot be translated since translation stop codons are detected in all six possible reading frames. This is interesting in the light of previous work (Costantini et al. 1978) demonstrating that transcripts complementary to 10-20% of these repetitive sequences, or 500-1000 different sequence families, are present in the sea urchin egg RNA. These repetitive sequences have since been shown to be restricted to the sea urchin maternal RNA and can
therefore be enriched by selecting for poly(A) RNA. The repetitive sequences are less than 200 bp in length (Costantini et al. 1980), or 159 bp in one example where the boundaries of an element have been defined (Posakony et al. 1983). Since each repetitive sequence family is reiterated many times throughout the genome (1000-10,000 copies per haploid genome, Posakony et al. 1983) many different single copy genes are related by their association with a genomic repetitive sequence. Furthermore, since repetitive sequences do not appear to be asymmetrically orientated in relation to coding sequences, hybridisation of individual cloned repetitive sequences to sea urchin egg poly(A) RNA reveals a unique pattern of transcripts which as expected is also strand specific (Posakony et al. 1983). When sea urchin egg poly(A) is renatured and examined with the electron microscopy, numerous multimolecular complexes are observed (Costantini et al. 1980). It is the contention of the authors that these complexes are stabilised by intermolecular hybridisation between homologous interspersed oppositely oriented repetitive sequences. The multimolecular complexes are unfortunately extremely entangled and it is impossible to interpret the structures unambiguously, unlike similar structures described for HeLa hnRNA (Federoff et al. 1977) and Xenopus laevis oocyte hnRNA (Sommerville and Scheer, 1982). Moreover, since 65% of the poly(A) RNA is associated with these complexes the repetitive sequences are believed to be
very prevalent within the egg poly(A) RNA population. Indeed, since many of the complexes contain more than two RNA molecules, many transcripts must contain more than one repetitive sequence. Unfortunately this estimate (65%) derived, as it is, by a visual inspection of the RNA complexes is far higher than that portion of the egg poly(A) RNA which hybridises to sea urchin genomic repetitive DNA, 11-16% (Costantini et al. 1980), although the authors suggest that this latter figure seriously underestimates the true fraction of poly(A) RNA molecules which harbour repetitive sequences.

A more detailed molecular analysis of individual sea urchin repeat containing maternal RNA is described by Posakony et al. (1983). Although each repetitive sequence hybridises to many hundreds of transcripts within the sea urchin egg poly(A) RNA, only 10-20 prominent transcripts can be detected with each repetitive sequence element. Therefore only a small percentage of those mRNA containing repetitive sequences are transcribed into prevalent poly(A) RNAs. Furthermore, those transcripts which are detected are extremely large, 3-15 kb. By virtue of their large size these transcripts more closely resemble hnRNA than typical cytoplasmic or polysomal poly(A) RNAs which by comparison have an average length closer to 2 kb (Wilt, 1977). Moreover, experimental evidence suggests that the majority of those large, repetitive sequence containing transcripts are stably maintained during the early stages of embryogenesis, at least up until the
gastrula stage (Posakony et al. 1983) and therefore are unlikely to be rapidly converted by RNA processing to transcripts typical of polysomal mRNAs, as suggested by Thomas et al. (1982).

By comparison a molecular analysis of Xenopus oocyte interspersed repetitive sequences is far less advanced. Since the interspersed sequence organisation of the Xenopus genome is similar to that of the sea urchin (Davidson, 1976) attempts were made to demonstrate the existence of interspersed repetitive elements with the maternal poly(A) RNA of the Xenopus oocyte. When renatured to $C_t 600$ Ms approximately 70% of the mass of Xenopus laevis oocyte cytoplasmic poly(A) RNA can be observed in large multimolecular complexes (Anderson et al. 1982) of which 20% is S1 nuclease resistant. As mentioned previously, Section 1.2, these findings are in direct conflict with the observations of Perlman and Rosbash (1978), which suggest that less than 3% of Xenopus laevis oocyte poly(A) RNA is complementary to Xenopus laevis repetitive genomic sequences. Since the majority of RNA molecules are associated with complexes with greater than 4 ends, a large proportion of the transcripts also harbour more than one repetitive sequence element. Moreover, since when renatured only 15% of the mass of Xenopus laevis stage 41 tadpole cytoplasmic poly(A) RNA can be observed participating in intermolecular associations, embryonic development must be associated with a reduction in the abundance of repetitive sequences within
the cytoplasmic compartment (Anderson et al. 1982). As yet, however, the mechanism by which these interspersed repetitive sequence transcripts accumulate is unknown, nor is there sufficient data available to conclusively support a hypothesis as to their function, if any, in early embryonic development. Evidence does however, suggest that these transcripts are not translatable in vivo or in vitro (Richter et al. 1984). Since transcripts of this type apparently account for the majority (70%) of the oocyte poly(A) RNA pool, but are nonetheless untranslatable, it seems unlikely that without subsequent sequence modification, these poly(A) RNAs can function as bona fide maternal messenger RNA, i.e. messenger RNA destined to be translated during the early stages of embryonic development.

It is therefore the aim of this thesis to independently verify the existence of interspersed repetitive sequences within the maternal RNA pool of the related species Xenopus borealis, and to characterise the repetitive elements as far as possible in relation to their genomic organisation, sequence characteristics and prevalence within the maternal RNA pool.
CHAPTER TWO

MATERIALS AND METHODS
2.1 Standard Solutions

**NETS** 1 x is 10 mM Tris-HCl pH 7.4
- 100 mM NaCl
- 10 mM EDTA
- 0.2% SDS

**NTE** 1 x is made as NETS but without SDS.

**TE** 1 x is 10 mM Tris-HCl pH 7.4
- 1 mM EDTA
  
  Made as a 10 x stock solution.

**SSPE** 20 x 3.6 M NaCl
- 0.2 M NaH₂PO₄ pH 7.4
- 0.02M EDTA

**Denhardt's**
- 50 x is 1% (w/v) Ficoll
- 1% (w/v) Polyvinyl pyrrolidone
- 1% (w/v) B.S.A.

**Barth** (Barth and Barth, 1959)

**Solution A:** 51.50 g NaCl
- 0.75 g KCl
- 2.04 g MgSO₄·7H₂O
- 0.62 g Ca(NO₃)₂·H₂O
- 0.60 g CaCl₂·2H₂O

Make up to 1000 ml - store in 100 ml aliquots.

**Solution B:** 2 g NaHCO₃

Make up to 500 ml - store in 50 ml aliquots.
Solution C: 24.22 g Tris-HCl pH 7.6

20 mg phenol red

Make up to 500 mls - store in 50 ml aliquots.

Mix one aliquot of each solution, dilute to 1000 mls to give 1 x Barth X solution.

**T4 DNA ligase buffer**

10 x is 0.66M Tris-HCl pH 7.6

66 mM MgCl$_2$

0.1 M DTT

10 mM ATP

**Universal Restriction Buffer**

10 x is 0.33 M Tris-acetate pH 7.9

0.66 M KAcetate

0.1 M MgAcetate

5 mM DTT

1 mg/ml B.S.A.

10 x TBE 0.9 M Tris-borate pH 8.3

25 mM EDTA

### 2.2 Microbiological Strains and Media

#### 2.2.1. Microbiological Strains

All the bacterial strains and bacteriophage vectors described in this study are listed in Table 2.1. Stocks of each bacterial strain were maintained as liquid cultures stored frozen at -70°C, in a modified L-broth nutrient media (Gergen et al., 1979) described in Section 2.2.2.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM 514</td>
<td>$\text{lyc}^7 \ hsdR^{-M+S^+} \ hf1$</td>
<td>Murray, N. (1983)</td>
</tr>
<tr>
<td>NM259</td>
<td>$\text{supE supF hsd(R^-M+S^+) met trpR}$</td>
<td>Murray et al. (1977)</td>
</tr>
<tr>
<td>NM522</td>
<td>$\text{hsd} \Delta (M^-S^-R^-) \Delta \text{lac} \Delta \text{pro} \text{ supE}$</td>
<td>Gough and Murray (1983)</td>
</tr>
<tr>
<td></td>
<td>$\text{thi F'}\text{proA^+B^+ lacI}^q \ lacZ \Delta \text{M15 traD36}$</td>
<td></td>
</tr>
<tr>
<td>RR1 M15</td>
<td>$\text{leu pro thi strA hsd(R^- M^-)}$</td>
<td>Ruther (1982)</td>
</tr>
<tr>
<td></td>
<td>$\text{lacZ} \Delta \text{M15 F'} \text{ lacI}^q, \text{ lacZ} \Delta \text{M15 pro^+}$</td>
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<table>
<thead>
<tr>
<th>Vector</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>NM1149</td>
<td>$\text{imm}^{434} \ b(538)$</td>
<td>Murray, N. (1983)</td>
</tr>
</tbody>
</table>
Bacteriophage lambda vectors were stored at 4°C as phage particles in the caesium chloride ultracentrifugation buffer, section 2.13.2.

2.2.2. Microbiological Media

**L-broth** - 10 g Difco Bacto Tryptone
5 g Difco yeast extract
10 g NaCl
distilled water to 1000 ml pH 7.2

**L-broth agar** - Made as for L-broth, plus
15 g Difco agar per 1000 mls.

**BBL agar** - 10 g Baltimore Biological Laboratories trypticase
5 g NaCl
10 g Difco agar
distilled water to 1000 ml.

**BBL top agar** - Made as for BBL agar, but only 6.5 g
Difco agar per 1000 ml.

**Spitzen minimal salts** - 100 g \((\text{NH}_4)_2\text{SO}_4\)
200 g \(\text{K}_2\text{HPO}_4\)
300 g \(\text{KH}_2\text{PO}_4\)
50 g tri sodium citrate
10 g \(\text{MgSO}_4\)
distilled water to 10 litres.
**Minimal agar** - 6 g Difco Bacto agar
100 ml Spitzen salts
4 ml 20% (w/v) glucose
0.2 ml 1 mg/ml vitamin B1
distilled water to 400 ml.

**Phage buffer** - 3 g KH$_2$PO$_4$
7 g Na$_2$HPO$_4$
5 g NaCl
10 ml 0.1 M MgSO$_4$
10 ml 0.1 M CaCl$_2$
1 ml 1% (w/v) gelatin
distilled water to 1000 ml.

**M13 Phage buffer** - 20 mM Tris-HCl pH 7.5
20 mM NaCl
1 mM EDTA

**Antibiotics** - Amp/Strep plates contain L-broth agar supplemented with 50 µg/ml ampicillin and 200 µg/ml streptomycin.

200 mg/ml stock of streptomycin in distilled water stored at 4°C.

5 mg/ml stock of ampicillin in distilled water stored at 4°C.

**L broth freezing medium** (Gergen et al. 1979)

6.3 g K$_2$HPO$_4$
1.8 g KH$_2$PO$_4$
0.45 g Na citrate
0.09 g MgSO$_4$·7H$_2$O
0.9 (NH$_4$)$_2$SO$_4$
36 ml glycerol
1000 ml L-broth pH 7.2
2.3 General Laboratory Procedures

2.3.1. Phenol/Chloroform extraction

Commercially available phenol was first redistilled under nitrogen, adjusted to 0.1% (w/v) 8-hydroxyquinoline and saturated with TE. The purified phenol was then stored in 100 ml aliquots at -20°C until required.

The aqueous solution to be extracted was adjusted to 20 mM EDTA and 0.4 M NaCl. An equal volume of phenol, preheated to 37°C was added, vortexed and incubated at 37°C for 5 minutes with occasional vortexing to maintain the emulsion. After cooling on ice, one volume of chloroform was added, vortexed again, and kept on ice for a further 5 minutes. The aqueous phase was removed and stored, while the organic phase was back extracted with one volume of TE. The two combined aqueous phases were re-extracted with 2 volumes of chloroform on ice for 5 minutes. After a further centrifugation for 5 minutes to separate the two phases the aqueous phase was removed and the nucleic acid precipitated with ethanol or isopropanol.

2.3.2. Precipitation of nucleic acids

DNA or RNA was precipitated from aqueous solutions by adjusting the solution to 0.3M sodium chloride, or 0.3M sodium acetate, followed by the addition of 2 volumes of ethanol and incubating at -20°C. For nucleic acid concentrations greater than 5 µg/ml, the precipitated nucleic acid can be recovered after 2 hrs, while for maximal recovery of small quantities of nucleic acid, a
longer incubation at -20°C is desirable. The precipitate was recovered by centrifugation at greater than 10,000 X G in a Sorvall centrifuge or in a bench top microfuge if smaller volumes were employed.

DNA can also be recovered from aqueous solutions by adjusting the solution to 2M ammonium acetate, followed by the addition of an equal volume of propan-2-ol. After incubating at -20°C the nucleic acid precipitate was collected as described above.

2.3.3. Autoradiography

Autoradiography was preformed using Cronex 4 x-ray film and cassettes with, or without lightening plus intensifying screens. Cronex 4 x-ray films were usually preflashed, Laskey and Mills (1977), and stored at -70°C Koren et al. (1970), for the appropriate time.

2.3.4. Deionising formamide

Formamide was deionised with BioRad analytical grade mixed bed resin AG 501-X8(D), 20-50 mesh. 10 g of resin per 100 mls of formamide were stirred at room temperature for 30 minutes. The deionised formamide was filtered through Whatman No. 1 filter paper and stored frozen at -20°C until required.

2.3.5. Digestion of DNA with restriction endonucleases

DNA was routinely digested to an end point of 2-4 fold over-digestion according to the optimal enzymatic conditions recommended by the commercial suppliers (Boehringer Mannheim GmbH, Amersham, BRL, Pharmacia).
Where possible multiple enzyme digests were preformed simultaneously in 1 x Universal buffer. Where optimal enzymatic conditions were incompatible DNAs were digested sequentially, being phenol/chloroform extracted and precipitated between each digest.

2.3.6. **Agarose gel electrophoresis**

(a) **DNA gels**

Digested DNA was fractionated by electrophoresis through 0.7-2.5% (w/v) agarose gels as follows. Horizontal slab gels of various sizes were used, submerged in electrophoretic tanks containing gel electrophoresis buffer (40 mM Tris-acetate pH 8.2, 20 mM glacial acetic acid, 1 mM EDTA, 0.5 µg/ml ethidium bromide). Agarose (Miles) was heated under reflux in this buffer at the desired agarose concentration, allowed to cool to approximately 50-60°C and poured into perspex gel formers.

DNA samples were prepared for electrophoresis by the addition of 1/5th volume of sample buffer (50% (v/v) glycerol, 1% (w/v) ficoll, 0.1% SDS (w/v), 25 mM EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol). DNA samples were electrophoresed at various voltages (5-100 volts cm⁻¹) and for different lengths of time (30 mins to 18 hr) depending on the separation required.

Various restriction digestion of bacteriophage lambda cI857 were routinely used as molecular weight markers.
(b) RNA gels (Derman et al. 1981)

RNAs were fractionated by electrophoresis through 1% (w/v) agarose gels as follows. Horizontal slab gels (16 x 9 x 0.7 cm) were routinely used. 1 g agarose (Pharmacia, N.A.) was melted in 72 mls of distilled water under reflux and cooled to 60°C. 10 mls of 10 x gel buffer (200 mM MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA) and 18 mls 12.3 M formaldehyde (37% (v/v) stock solution) was added, mixed, and poured into perspex gel formers.

Up to 20 μg of RNA was precipitated and dried down under vacuum in a rotary dessicator. The RNA was resuspended in 30 μg sample buffer (50% deionised formamide, 2.2 M formaldehyde, 1 x gel buffer) and heat denatured at 55°C for 15 min. After cooling on ice, 3 μg of 10 x dyes (0.2%(w/v) bromophenol blue, 20% (w/v) ficoll) were added and the RNAs were electrophoresised at 5 V cm\(^{-1}\) for 18 hrs.

Gels were stained with 5 μg/ml ethidium bromide in 1 x gel buffer for 30 min at room temperature, and destained for 3 hrs in 1 x gel buffer.

2.3.7. Gel photography

Gels were photographed under ultraviolet light using Ilford HP5 professional 5" x 4" sheet film, and a red filter. Exposures were from 15 secs. to 2 min, followed by 5 min in Ilford Microphen developer, stopped in 3% (v/v) acetic acid and fixed for 5 min in Ilford Hypam fixer.
The relative mobilities of RNA and DNA were measured directly from the negative.

2.4 Maintenance of Xenopus stocks

All Xenopus borealis, Xenopus tropicalis and Xenopus laevis stocks used in this study have been raised from our own matings. The animals were kept in large glass sided tanks, at a density of 20-30 adults per tank. The tanks were emptied, cleaned and refilled with fresh top water (preheated to 23°C) 3 times a week. Animals were fed on food pellets supplied by Xenopus Ltd.

2.5 Isolation of nucleic acids from Xenopus

2.5.1. Preparation of Xenopus erythrocyte DNA

DNA was prepared from Xenopus erythrocyte nuclei essentially as described by Blinn and Stafford (1976). Cells were pelleted and resuspended in 1 volume 0.1 M NaCl repelleted and resuspended in 10 volumes of lysis buffer (0.5 M EDTA) prior to the addition of sarkosyl (0.5% w/v final) and proteinase K (100 μg/ml final) and incubated at 50°C for 60 min. The DNA was extracted three times with one volume of phenol and then extracted with two volumes chloroform on ice. DNA was dialysed extensively against TE and incubated with RNAase A at 10 μg/ml for 3 hr at 37°C, following which the DNA was treated with proteinase F (20 μg/ml) for 60 min at 37°C. The DNA was then phenol/chloroform extracted three times and dialysed extensively against TE.
2.5.2. Preparation of ovarian RNA

*Xenopus borealis* RNA was extracted, essentially as described by Darnbrough and Ford (1976), from pre-intellogenic ovaries of immature animals containing mainly stage 1 and stage 2 oocytes (Dumont, 1972), and mature animals containing mainly stage 5 and stage 6 oocytes.

**Modified Kirby Salt RNA extraction buffer:** (Kirby, 1965)

- 0.1 M Tris-HCl pH 9.0
- 10 mM EDTA
- 0.5% (w/v) SDS
- 1.0% (w/v) Tri-iso-propynaphthalene sulphonate
- 6.0% (w/v) 4-aminosalicylic acid
- 1.0% (w/v) NaCl
- 6.0% (v/v) phenol

The following procedure is suitable for the extraction of RNA from both immature and mature animals.

Female animals were anaesthetised in MS222 (ethyl-M-aminobenzoate). The ovarian tissue was dissected, washed in Barth X solution (Barth and Barth, 1959), weighed and homogenised in Kirby salts (10 ml/g tissue) until large tissue fragments could no longer be observed. Following the addition of an equal volume of phenol, the RNA was incubated at 37°C for 15 min. The emulsion was resolved by centrifugation at 10,000 rpm in a Sorvall HB4 swing out rotor at 18°C for 15 min. The upper aqueous phase
containing the RNA was removed, 1/10 volume of 3M NaCl was added as was an equal volume of phenol, shaken and incubated at 37°C for a further 15 min. The emulsion was separated by centrifugation as before, the aqueous phase was removed and extracted twice with 2 volumes of chloroform on ice. Following centrifugation RNA was precipitated from the aqueous phase by addition of a volumes of ethanol (-20°C). Finally, RNA was recovered by centrifugation, resuspended in NETS and re-precipitated with ethanol.

An alternative procedure for the preparation of RNA from previtellogenic oocytes, previously described by De Robertis et al. (1982), is described below. It is however unsuitable for preparation of RNA from mature oocytes, since the extraordinarily high levels of yolk protein, contained within these oocytes, interferes with the extraction procedure.

0.5 g of tissue was homogenised in 1.5 ml of proteinase K-SDS solution (1.7 % (w/v) SDS, 300 mM NaCl, 2 mg/ml proteinase K, 40 mM Tris-HCl pH 7.5), phenol/chloroform extracted, backextracted with 1 ml TE, and the combined aqueous phases extracted with 2 volumes of chloroform. Nucleic acids were recovered by ethanol precipitated.

2.5.3. Preparation of poly(A) RNA

Selection of poly(A) RNA was achieved by affinity chromatography on oligo(dT)-cellulose columns equilibrated in binding buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA,
0.1% SDS, 0.5 M NaCl). Bound RNA was washed extensively with wash buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS, 0.1 M NaCl), and finally eluted in elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS). Poly(A)RNA was recovered by ethanol precipitation and subjected to a second cycle of oligo(dT)-cellulose affinity chromatography. Poly(A)RNA was stored at -20°C as an ethanol precipitate. RNA stored in this way remained stable for at least 12 months.

2.6 Sucrose density centrifugation

100 µg of total ovarian RNA in 250 µl of TE was heat denatured at 80°C for 60 sec, cooled rapidly on ice and adjusted to NETS by the addition of an appropriate amount of SDS and NaCl. An additional 100 µg of total ovarian RNA was similarly treated but without denaturation. Each RNA sample was loaded on to a 12 ml 30% (w/v)-7% (w/v) sucrose in NETS gradient. Centrifugation was in a 6 x 14 Ti rotor at 40,000 rpm, 18°C for 6 hrs.

The gradients were fractionated and the RNA distribution measured at 254 nm by an ISCO continuous UV analyser. The poly(A) content of each fraction was determined by [3H] poly U hybridisation, (Bishop et al. 1974), see Section 2.8.

2.7 Synthesis of [3H] poly U

Mix A is 0.5 M Tris-HCl pH 8.5 at 37°C

0.1 M KCl
5 mM MgCl₂
1 mCi of [5-\textsuperscript{3}H]UDP (13.6 Ci/mmol) was dried down under vacuum and resuspended in 2460 \( \mu l \) of distilled water. 90 \( \mu l \) of 1\% (w/v) UDP was added and the mixture incubated briefly at 37°C. Then 300 \( \mu l \) Mix A, 6 \( \mu l \) primer (GpU (Miles Laboratories), 500 \( \mu g \)/100 \( \mu l \) in distilled water), 60 \( \mu l \) polynucleotide phosphorylase (Boehringer Mannheim 5 mg/ml in 50\% (v/v) glycerol and 10\% (v/v) mix A) and 90 \( \mu l \) distilled water were added. The enzymic polymerisation of [\textsuperscript{3}H]UDP was monitored by ctab precipitation (see Section 2.10.5) every 10 mins. The incorporation of radiolabelled UDP into [\textsuperscript{3}H]poly U ceased after 60 min with 50\% of the [\textsuperscript{3}H]UDP being incorporated into ctab precipitable material. 300 \( \mu l \) of 2M sodium acetate (pH 5.0) and 150 \( \mu l \) 10\% (w/v) SDS were added, and the reaction phenol/chloroform extracted. The aqueous phase was adjusted to 0.3 M NaCl, 10 mM sodium acetate (pH 5.0) and fractionated on a Sephadex G-50 column equilibrated in 0.3 M NaCl, 10 mM sodium acetate (pH 5.0). The fractions containing [\textsuperscript{3}H]poly U were pooled and stored frozen in aliquots at -20°C.

The specific activity of the [\textsuperscript{3}H]poly U was determined in a standard [\textsuperscript{3}H]poly U hybridisation procedure (Section 2.8) with known amounts of poly A. In this way the specific activity of the [\textsuperscript{3}H]poly U was calculated to be 2 \( \times \) 10\textsuperscript{6} cpm/\( \mu g \) poly U.
2.8 Standard $[^3]$Hpoly U hybridisation procedure

Annealing buffer:  
- 50 mM Tris-HCl pH 7.0
- 1 mM EDTA
- 300 mM NaCl

The RNA sample was diluted to 100 μl with distilled water. 400 μl of annealing buffer and excess $[^3]$Hpoly U (1-10 μl of $[^3]$Hpoly U, specific activity 2 x $10^6$ cpm/μg) were added and annealed at 45°C for 30 min. The annealed RNA was then cooled on ice for 5 min, and then incubated with 10 μg of ribonuclease A on ice for 20 min. Ribonuclease resistant material was ctab precipitated (Section 2.10.5).

The amount of $[^3]$Hpoly U used in each experiment was dependent upon the quantity of poly(A) present in the sample, but as a general rule 5 x $10^4$ cpm of $[^3]$Hpoly U (specific activity 2 x $10^6$ cpm/μg) was used with 10 μg poly(A) RNA.

2.9 Analysis of Renatured poly(A) RNA

2.9.1. Renaturation of poly(A) RNA

Poly(A) RNA was resuspended in 68% (v/v) deionised formamide, 0.058 M Pipes (pH 6.8), 0.116 mM EDTA, at an RNA concentration of 580 μg/ml, denatured by heating to 90°C for 2 min and cooled rapidly on ice. Denatured RNA was adjusted to 0.75 M NaCl by addition of 5 M NaCl and incubated at 30°C, in sealed glass capillary tubes, to the required equivalent RNA C$_0$ t value (Britten et al. 1974), see Appendix 1.
2.9.2. **Cellulose/Ethanol chromatography of RNA**

Renatured poly(A) RNA was fractionated on cellulose/ethanol columns as described by Federoff et al. (1977). 2 ml cellulose (Sigmacell Type 50) columns, with a binding capacity of 100 μg poly(A) RNA (Anderson et al. 1982), were equilibrated in equilibration buffer (0.1 M NaCl, 0.1 M Tris-HCl pH 6.85, 2 mM EDTA, 35% (v/v) ethanol). Renatured poly(A) RNA was diluted with column equilibration buffer to 10 μg/ml and applied to the column. Unbound RNA was eluted in equilibration buffer, while bound RNA was sequentially eluted with equilibration buffer containing 15% (v/v) ethanol and then 0% (v/v) ethanol. The proportion of poly(A) RNA eluted at each stage of fractionation was monitored at 260 nm. Approximately 20% of the RNA input mass was never recovered from the column, as previously described by Anderson et al. (1982).

2.9.3. **Electron microscopic analysis of renatured RNA**

Renatured RNA was prepared for analysis essentially as described by Davis et al. (1971). 0.5 μg RNA (in 1 μl annealing buffer was diluted into 200 μl hyperphase solution (50% (v/v) formamide, 100 mM Tris-HCl pH 8.6, 10 mM EDTA, 0-250 mM NaCl, 100 μg/ml cytochrome C, 10 μg pAT153 DNA, 10 ng M13 mp 8 DNA) and spread on to hypophase solution (10% formamide, 10 mM Tris-HCl pH 8.6, 1 mM EDTA). RNA was transferred to copper grids, rotary shadowed and observed under a Siemen electron microscope.
2.10. **Radioactive labelling of nucleic acids**

2.10.1. **Random primer labelling of DNA restriction fragments**

Solution O: 0.125 M MgCl$_2$·6H$_2$O
1.25 M Tris HCl pH 8.0

Solution A: 470 μl solution O
9 μl 2-mercaptoethanol
12.5 μl 20 mM dATP
12.5 μl 20 mM dTTP
12.5 μl 20 mM dGTP

Solution B: 2 M Hepes pH 6.6

Solution C: Hexadeoxyribonucleotides (Pharmacia) evenly suspended in TE, 90 00 units/ml.

Solution OLB: 50 μl solution A
125 μl solution B
75 μl solution C

All radiolabelled DNA restriction fragments were prepared by a modification to the technique described by Feinburg and Vogelstein (1984). DNA restriction fragments were separated by electrophoresis through 0.7%-2% low melting point agarose (BRL) gels as previously described in Section 2.3.6. Under UV light the desired restriction fragment was identified and excised from the gel with a scalpel blade. The gel slice was weighed and diluted with water (3 ml/g gel). The agarose fragment was boiled for 7 min to dissolve the gel and denature the DNA. Subsequently if the DNA was
required for another labelling reaction, the agarose slurry, containing the DNA, was reboiled for only 3 min. The labelling reaction was carried out at 22°C as follows.

10 µl OLB solution
1 µl 20 mg/ml BSA
35 µl agarose/DNA slurry
5-50 µCi \([\alpha-^32P]dCTP\) (3000 Ci/mmol)
2 U "Klenow" DNA Polymerase Fragment

If 10-20 ng of DNA were being labelled, as was usually the case, reactions were left for 18 hr. Following which they were phenol/chloroform extracted and isopropanol precipitated with the addition of 25 µg E.coli tRNA carrier. Radiolabelled DNA prepared in this way routinely yielded DNA at greater than 1 x 10^9 cpm/µg input DNA.

2.10.2. Strand specific probes

Strand specific probes were prepared from M13 sequencing templates (preparation of which is described in Section 2.20.4). M13 17-mer sequencing primer (5 ng) was heated to 100°C for 2 min, cooled on ice, mixed with 10 µl of 10 mM Tris-HCl (pH 7.9), 60 mM NaCl, 6.6 mM MgCl_2, 8 mM DTT, 0.5 µg M13 template DNA, and incubated at 65°C for 15 min. After being allowed to cool to room temperature, 10-40 µCi \([\alpha-^32P]dCTP\) (3000 Ci/mmol) 1 µl of 2 mM dGTP, 1 µl 2 mM dATP, 1 µl of 2 mM dTTP and 0.5U "Klenow" were added and incubated at 15°C for 90 min. Following which the radiolabelled
DNA was phenol/chloroform extracted and ethanol precipitated.

2.10.3. Radio-labelling of mung bean digested RNA

Mung bean digested poly(A) RNA, Section 2.14, was labelled with cytidine 3',5'-[5'-32P]bisphosphate, as described by England et al. (1980). 10 μl reactions containing 20-50 μCi cytidine 3',5'[5'-32P]bisphosphate (3000 Ci/mmol), 0.5 μg RNA, 6 μM ATP, 50 mM Hepes (pH 7.5), 20 mM MgCl₂, 3.3 mM DTT 10 μg/ml BSA, 10% (v/v) DMSO and 1U T4 RNA ligase (New England Biolabs) were incubated at 15°C. The incorporation of radiolabel was monitored by ctab precipitation. When the incorporation of radiolabel had been completed, the reaction was phenol/chloroform extracted and ethanol precipitated with 5 μg of E.coli tRNA carrier.

2.10.4. Phosphorylation of DNA linkers

10 x Kinase buffer: 0.6 M Tris-HCl pH 8.0
0.1 M MgCl₂
0.05M DTT

10 x T4 DNA ligase buffer: 0.66 M Tris-HCl pH 7.6
66 mM MgCl₂
0.1 M DTT
10 mM ATP

10 x TBE: 0.9 M Tris/borate pH 8.3
25 mM EDTA

10% acrylamide gel mix: 50 mls 10 x TBE
97 g acrylamide
3 g bis-acrylamide
distilled water up to 1000 ml.
Commercially available linkers were resuspended at 0.5 μg/μl in TE and stored at -20°C. 4 μl of linkers (2 μg) was diluted with 1.5 μl 10 x Kinase buffer, 50 μCi [\(^{32}\)P]ATP, 10 μl distilled water, 10 units polynucleotide kinase (NBL Enzymes Ltd.) and incubated at 37°C for 60 min. The phosphorylation of the DNA was monitored by ctab precipitation. The incorporation of radiolabel was usually equivalent to 1-2 x 10^6 cpm/μg DNA. Following the radiolabelled phosphorylation the remaining sites were phosphorylated by the addition of 7.5 μl distilled water, 0.5 μl 20 mM ATP, 1 μl 10 x Kinase buffer, 10 U polynucleotide kinase and incubated at 37°C for a further 60 min. The phosphorylated DNA linkers were stored at -20°C until required.

It proved necessary to check each batch of linkers in trial ligation and restriction reactions following phosphorylation, since one batch of DNA linkers failed to self blunt-end ligate. This was done as follows, a trial 10 μl ligation reaction was set up with 2.5 μl phosphorylated linkers, 1 μl 10 x T4 DNA ligase buffer, 0.5 μl 20 mM ATP, 6 μl distilled water, 1 unit T4 DNA ligase (Pharmacia) and incubated at 15°C for 2 hr. Following which the ligation reaction was terminated by heating to 65°C for 15 min. Following ligation a sample of the ligated linkers was digested as follows. 3 μl ligated linkers was diluted with 10 μl distilled water, 2.5 μl 10 x restriction buffer of the required restriction
endonuclease, 10 units of restriction enzyme and incubated for a further 60 min at 37°C. Finally, 0.5 μl of the phosphorylated linkers, 3 μl of the ligated linkers and 3 μl of the restricted ligated linkers were electrophoresed through a 10% polyacrylamide gel prepared as follows. 50 mls of 10% acrylamide gel mix was polymerised by the addition of 100 μl 25% (w/v) APS and 100 μl TEMED, and allowed to set for 2-3 hrs. The radiolabelled linkers were electrophoresed through the polyacrylamide gel in 0.5 x TBE. The gel was dried down and autoradiographed.

2.10.5. Ctab precipitation

The incorporation of radiolabel into nucleic acids was monitored by ctab precipitation. A sample is distilled into 0.5 ml distilled water, then 0.5 ml yeast tRNA carrier (2 mg/ml in 1M sodium acetate pH 4.6) and 1 ml of 4% (w/v) ctab are added and mixed. The precipitate was collected by vacuum filtration on Whatman GF/C glass fibre filters. The filter was first washed with 1 ml 5% (w/v) sodium pyrophosphate, (Na₄P₂O₇) then the precipitate was passed through the filter, washed with 2 mls of distilled water, and finally washed with 1 ml 5% (w/v) sodium pyrophosphate. The filters were dried at 80°C, put into scintillation vials, covered in scintillation fluid (8 g/litre butyl-PBD in toluene) and counted.
2.11. Preparation of an ovarian poly(A) RNA cDNA library

2.11.1. **cDNA synthesis**

2 µg of poly(A) RNA, prepared from ovaries containing mainly stage 6 oocytes (Dumont, 1972), was resuspended in 20 µl distilled water, denatured for 30 sec at 80°C, cooled rapidly and diluted to 120 µl with 50 mM Tris-HCl (pH 8.3 at 43°C), 20 mM DTT, 6 mM MgCl₂, 60 mM NaCl, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 0.33 mM dCTP, 60 µCi [5'-'H]dCTP (26.5 Ci/mmol), 5 µg/ml oligo(dT12-18), 25U placental ribonuclease inhibitor (Amersham). 20 Units super RT (Anglian Biotechnology), and incubated at 43°C for 4 hrs. The incorporation of radiolabel was monitored by ctab precipitation. The reaction was terminated by the addition of EDTA to 20 mM. Following phenol/chloroform extraction the cDNA hybrid was isopropanol precipitated with 2M NH₄Ac. Calculations described in Appendix II, indicate that cDNA equal to 50% of the RNA input mass, or 1 µg of cDNA was synthesised. Indeed this experiment was repeated on a number of occasions with the same efficiency.

Second strand cDNA synthesis was achieved when up to 700 ng cDNA/RNA hybrid was processed in 100 µl reactions, essentially as described by Gubler and Hoffman (1983), containing 20 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM NAD, 20 µCi [3H]dCTP (26.5 Ci/mmol), 50 µg/ml BSA, 80 µM dNTPs, 8.5 units/ml E.coli RNase H (Pharmacia), 10 units/ml E.coli DNA ligase
(Pharmacia), 800 Units/ml DNA polymerase. Highest efficiencies of conversion into double-stranded cDNA were achieved when the reactions were incubated for 15 min on ice prior to addition of DNA polymerase. Following the addition of DNA polymerase reactions were incubated sequentially at 12°C for 60 min, then 23°C for 2-3 hrs. The efficiency of conversion to double stranded cDNA was monitored by ct&ab precipitation. Calculations described in Appendix II indicate that the incorporation of radiolabel into the second cDNA strand was equal to 90-95% of the theoretical maximum, calculated from the cDNA/RNA hybrid input mass. Synthesis was terminated by the addition of 20 mM EDTA, phenol/chloroform extraction and isopropanol precipitation from 2 M NH₄Ac.

Double stranded cDNA was size fractionated on a 7 ml Sepharose CL-4B column, equilibrated in column buffer (10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA). 0.5 ml fractions were collected and the cDNA ethanol precipitated.

2.11.2. cDNA EcoRI methylation and end repair

The cDNA was methylated with EcoRI methylase as follows. 1-2 μg cDNA was resuspended in 20 μl containing 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 80 μM S-adenosyl-methionine, 0.5 mg/ml BSA and 2U EcoRI methylase (New England Biolabs), incubated for 2 hrs at 37°C, phenol/chloroform extracted and ethanol precipitated.

Methylated cDNA was end repaired as follows. 1-2 μg of methylated cDNA was resuspended in 20 μl containing
33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 μg/ml BSA and 5 units "Klenow" fragment DNA polymerase 1 (Boehringer Mannheim GmbH), and incubated for 18 hrs at 15°C. Following phenol/chloroform extraction the cDNA was ethanol precipitated.

2.11.3. Ligation of EcoRl linkers to methylated cDNA

765 ng methylated, and repaired cDNA was resuspended in 20 μl distilled water and diluted to 35 μl by the addition of 3.5 μl 10 x T4 DNA ligase buffer, 1.5 μl 20 mM ATP and 8 μl phosphorylated EcoRl linkers (prepared as in Section 2.10.4) equivalent to 625 ng linkers. The reaction was incubated overnight at 15°C in the presence of 1U T4 DNA Ligase (Pharmacia). Linker polymerisation could be monitored by ctab precipitation, since the efficiency of linker precipitation increases as the chain length increases. Linker ligation was terminated by incubating the reaction at 65°C for 15 min. The reaction conditions were then modified as to be suitable for the restriction enzyme EcoRl. The 35 μl linker ligation buffer was diluted with 60 μl distilled water, 11 μl 10 x EcoRl restriction buffer (1M Tris-HCl pH 7.5, 70 mM MgCl₂, 500 mM NaCl), 30 units of EcoRl and incubated at 37°C for a further 6 hrs. The restriction of the polymerised linkers could again be monitored by ctab precipitation, since the efficiency of precipitation gradually decreased as the reaction proceeded. The reaction was phenol/chloroform
extracted and isopropanol precipitated from 2 M NH₄Ac.

Unligated EcoRl linkers and unincorporated \( [\alpha^{32}\text{P}]\text{ATP} \) was removed from the cDNA by size fractionation on a 7 ml Sepharose CL-4B column equilibrated in column buffer (10 mM Tris-\(\text{HCl}\) (pH 7.4), 0.1 M NaCl, 1 mM EDTA), as described previously in section 2.11.1. The fractions containing the cDNA were ethanol precipitated in the presence of 5 \( \mu \text{g} \) of EcoRl restricted NM1149 vector DNA.

2.11.4. cDNA cloning into bacteriophage lambda strain NM1149

50 ng of cDNA were ligated with 7.5 \( \mu \text{g} \) of EcoRl restricted NM1149 vector DNA as follows. 50 ng of cDNA in 10 \( \mu \text{l} \) was diluted with 30 \( \mu \text{l} \) EcoRl restricted NM1149 DNA (7.5 \( \mu \text{g} \)), 5 \( \mu \text{l} \) 10 \( \times \) T4 DNA ligase buffer, 3 \( \mu \text{l} \) distilled water, 2 \( \mu \text{l} \) 20 mM ATP and 1 unit T4 DNA ligase (Pharmacia) and incubated for 18 hr. at 15°C.

Ligated lambda DNA was packaged in vitro using a procedure described by Hohn and Murray (1977). Both the sonicated extract and freeze thaw lysate were gifts from Dr. Nora Hunter, Department of Molecular Biology, Edinburgh.

Buffer A 20 mM Tris-\(\text{HCl}\) pH 8.0

3 mM MgCl₂

0.05% (v/v) 2-mercaptoethanol

1 mM EDTA

Buffer M1 110 \( \mu \text{l} \) distilled water

6 \( \mu \text{l} \) 0.5 M Tris-\(\text{HCl}\) pH 7.5

300 \( \mu \text{l} \) 0.05 M spermidine, 0.1 M putrescine

9 \( \mu \text{l} \) 1M MgCl₂
75 μl 0.1 M ATP
1 μl 2-mercaptoethanol.

DNA was packaged as follows, 7 μl buffer A was mixed with the ligated lambda DNA (up to 0.5 μg DNA maximum), 1 μl buffer M1, 6 μl sonicated extract and 10 μl freeze thaw lysate. The packaging mixture was incubated at 20°C for 60 min. Maximum packaging efficiency was obtained when extra freeze thaw lysate (10 μl) and buffer M1 (1 μl) were added after 60 min and incubated for a further 2 hr. The packaging was terminated by the addition of 500 μl phage buffer and 10 μl chloroform. Packaged DNA was stored at 4°C.

The percentage of phage particles which contained recombinant DNA was determined as follows. An overnight culture of the selective host NM514 and the non-selective host NM259 were prepared in 5 ml of L-broth supplemented with 0.2% (w/v) maltose. Cells from this culture were harvested by centrifugation and resuspended in 1.25 ml phage buffer. Sequential dilutions of packaged DNA was incubated for 20 min on ice with 50 μl resuspended cells and then at 37°C for 20 min. The infected cells were mixed with 2 ml of liquid BBL top agar supplemented with 10 mM MgCl₂ and poured into 9 cm diameter BBL agar plates. Plates were inverted and incubated at 37°C for 12 hr.
2.12. **Screening of a bacteriophage cDNA library**

Preparation of a cDNA library has been described previously in Section 2.11. An overnight culture of *E.coli* strain NM514 was prepared in 5 ml L-broth supplemented with 0.2% (w/v) maltose. Cells (5 ml) were harvested by centrifugation and resuspended in 1.25 ml phage buffer. 200 μl of *in vitro* packaged phage, equivalent to 20,000 recombinant pfu, were incubated on ice with 200 μl resuspended cells for 20 min and then at 37°C for a further 20 min. The infected cells were then mixed with 8 ml of 0.7% (w/v) agarose (Miles) supplemented with 10 mM MgCl₂ and poured into a 12 cm x 12 cm BBL agar petri dish. After being allowed to cool, the plate was incubated at 37°C for 12 hrs.

Phage plaque lifts were carried out as follows. A single sheet of nitrocellulose (Schleicher and Schuell) cut to the dimensions of the petri dish was laid on to the agarose surface and incubated at 4°C for 5 min. The nitrocellulose was removed and laid, plaque side up, on blotting paper soaked in 0.5 M NaOH, 1.5 M NaCl for 5 min, then 0.5 M Tris-Cl (pH 7.0), 1.5 M NaCl for 5 min, 2 x SSPE for 5 min and finally air dried and baked at 80°C for 90 min. Duplicate filters were prepared from each petri dish.

Nitrocellulose filters were prehybridised for 6 hrs at 65°C in 6 x SSPE, 5 x Denhardtts, 0.1% SDS (w/v) and 100 μg/ml denatured herring sperm DNA. Conditions of hybridisation were exactly as for prehybridisation but
with the addition of denatured radiolabelled DNA, see Section 2.10.1. Filters were usually hybridised for 18 hrs at 65°C.

Filters were washed at 2 x SSPE, 37°C for 2 x 20 min, then at 2 x SSPE, 65°C for 2 x 20 min. Hybridisation of the radiolabelled DNA to recombinant plaques were detected by autoradiography, see Section 2.3.3.

2.13. Preparation of bacteriophage lambda DNA

2.13.1. Plate lysates

An individual and well isolated phage plaque was picked from a fresh plate and diluted into 1 ml phage buffer. Enough phage particles were taken from this isolate to give 5 x 10^5 pfu per plate when incubated with 50 μl of NM259 (when working with non-recombinant phage) or NM514 (when working with recombinant phage) plating cells. The phage particles were recovered by covering the agar with 4 mls L-broth supplemented with 10 mM MgCl_2, and incubating overnight at 4°C. The L-broth was removed and the phage concentration titrated.

2.13.2. Liquid lysates

A 200 ml culture of NM259 or NM514 cells was grown to an absorbance of 0.45-0.50 (OD_{650}) in L-broth supplemented with 10 mM MgCl_2. Infect cultures with 2 x 10^7 pfu of phage particles and follow absorbance at 650 nm. Absorbance increased up to OD_{650} 1.7-2.0, but dropped to approximately OD_{650} 0.3 after 5 hrs, 500 μl of chloroform was added and the lysate shaken for a further
10 mins. Then 8 g NaCl was added as was RNase A and DNase to 1 µg/ml, and incubated at 23°C for a further 60 min. The lysate was clarified by centrifugation at 3000 rpm in a HS4 Sorvall rotor for 4 min. The supernatant was recovered, 50 mls of 50% (w/v) polyethylene-glycol 6000 was added, and the mixture incubated at 4°C for 18 hrs.

2.13.3. Purification of phage particles

The precipitated phage particles were recovered by centrifugation at 8000 rpm in a Sorvall HS4 rotor. The particles were resuspended in 10 mls of phage buffer, 7.3 g CsCl was added and the solution placed in a 12 ml heat seal centrifuge tube. The phage particles were centrifuged at 38,000 rpm in a Ti50 rotor at 4°C for 16 hrs. After centrifugation an opaque band, containing the phage particles, could be observed. The band was removed through the side of the tube with a syringe needle.

2.13.4. Extraction of phage DNA

The caesium chloride phage suspension was dialysed extensively against 10 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂. Following dialysis the suspension was diluted to 2 ml with TE, then 100 µl of 0.4 M EDTA, 10 µl 20 mg/ml proteinase K and 100 µl 10% (w/v) SDS were added and suspension incubated at 65°C for 60 min with occasional shaking. Following which the phage DNA was phenol/chloroform extracted and precipitated with isopropanol from 2M NH₄Ac.
2.14. Mung bean nuclease digestion of poly(A) RNA

2.14.1. Small scale nuclease titration

Small scale mung bean nuclease titration experiments were carried out as follows. 15 μg of poly(A) RNA was renatured to an equivalent RNA C₀₇t of 600 Ms, as described in Section 2.9.1, diluted with 0.3 M NaCl to a final formamide concentration of 10% (v/v) and ethanol precipitated. After resuspension at 500 μg/ml in 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM Zinc acetate, 0.001% (v/v) Triton X-100, the RNA was incubated in a siliconised glass test tube for 100 min at 24°C with 0.25-10 units mung bean nuclease (Pharmacia). At these low enzyme concentrations Triton X-100 is essential for enzyme stability (Kowalski et al. 1976). Digestion was terminated by the addition of SDS to 0.01% (w/v) and EDTA to 20 mM, the RNA was phenol/chloroform extracted and ethanol precipitated. The resuspended RNA was then fractionated on a cellulose/ethanol column, as described in Section 2.9.2, and the percentage of RNA eluted at each fractionation stage was monitored at 260 nm.

2.14.2. Large scale nuclease digestion

160 μg poly(A) RNA prepared from ovaries containing mainly stage 5 and stage 6 oocytes was renatured to an equivalent RNA C₀₇t of 600 Ms diluted and precipitated as described above. After being resuspended at 500 μg/ml as described above the RNA was incubated for 100 min at 24°C in the presence of 16μ
mung bean nuclease. The digestion products were phenol/chloroform extracted, precipitated and fractionated on a cellulose/ethanol column.

Double stranded RNA was fractionated on a Sephacryl S-300 column (28 cm x 0.5 cm x 0.5 cm) equilibrated in 10 mM NH₄Ac (pH 7.4), 0.5 ml fractions were collected and lyophilised. Alternatively the column can be equilibrated in NTE, and DNA recovered by ethanol precipitation.

2.15. Preparation of a cDNA library from the double stranded mung bean nuclease resistant poly(A) RNA

2.15.1. Tailing double stranded RNA

The double stranded RNA was converted to a suitable substrate for conventional cDNA cloning procedures by the addition of a 60-80 nucleotide polyA tract to the 3' hydroxyl end of the RNA.

1.8 μg of double stranded RNA was resuspended in 50 μl of 50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 2.5 mM MnCl₂, 0.25 M NaCl, 0.216 mM ATP, 5 μCi [2,5',8³-H]ATP (55 Ci/mmol), 500 μg/ml BSA and incubated at 37°C with 2 units E.coli polyA polymerase (BRL) until the correct poly(A) tail length had been achieved. This was monitored by the incorporation of radiolabel, as detected by ctab precipitation, Appendix III. Following polyA tailing the RNA was phenol/chloroform extracted and ethanol precipitated in the presence of 5 μg of E.coli tRNA carrier.
2.15.2. **cDNA synthesis**

Double stranded cDNA was synthesised from the double stranded polyA tailed RNA in essentially the same way as previously described in Section 2.11.1, except that the double stranded cDNA was size fractionated on a Sephacryl S-300 column equilibrated in NTE as described in Section 2.14.2, replacing the Sepharose CL-4B fractionation described in Section 2.11.1.

The fractionated cDNA was end repaired as described previously in Section 2.11.2, phenol/chloroform extracted and ethanol precipitated.

2.15.3. **Cloning in pUC19**

20 μg of pUC19 (Yanisch-Perran, 1985) was digested to completion with SmaI, phenol/chloroform extracted and ethanol precipitated.

The end repaired cDNA was blunt end ligated into the Sma I restricted pUC19 vector. In an attempt to reduce the proportion of clones containing two cDNA inserts a series of small scale test ligations were performed covering a range of insert to vector DNA ratios. In this way a mass ratio was established in which no more than 10% of the religated vector molecules contained a cDNA insert.

20 μl ligation reactions containing 20 ng Smal restricted pUC19, 1 x T4 DNA ligase buffer, 2 mM ATP and 12 μg cDNA was incubated for 18 hr at 15°C with 1 unit T4 DNA ligase (Pharmacia).
2.15.4. **Bacterial Transformation**

Bacterial transformation was performed as described by Hanahan, D. (1983).

TFB: 10 mM MES-KOH pH 6.2
- 100 mM RbCl
- 45 mM MnCl$_2$.4H$_2$O
- 10 mM CaCl$_2$.2H$_2$O
- 2 mM Hexamine Cobalt Chloride

DTT: 2.25 M in 40 mM potassium acetate pH 6.0

DMSO: 500 µl aliquots stored under nitrogen, -20°C

SOB: 100 mls L-broth
- 0.25 mls 1M KCl
- 2 mls 1M MgCl$_2$

SOC: SOB plus 1 ml 20% (w/v) glucose

An overnight culture of RR1 M15 was prepared in 5 mls of SOB. 1 ml of this culture was diluted in 100 mls SOB and incubated at 37°C until an OD$_{556}$ - 0.5 to 0.5 was achieved. The cells were rapidly cooled on ice for 15 min. Cells were harvested by centrifugation at 5000 rpm in a HS4 rotor for 4 min at 4°C. The supernatant was removed and cells gently resuspended in 32 mls TFB at 0°C, and incubated on ice for a further 10 min. The cells were again harvested, as before, and resuspended in 8 mls TFB. Cells were incubated on ice for 5 min with the addition of 280 µl DMSO, then for a further 10 min with the addition of 280 µl 2.25 M DTT, then finally for a further 5 min with the addition of 280 µl DMSO to produce competent...
cells. 60 µl of competent cells were incubated for 30 mins on ice with the transforming DNA. Cells were then heat shocked at 42°C for 90 sec, diluted with 1 ml SOC (preheated to 37°C) and incubated at 37°C for 1 hr.

Transformed cells were diluted with 2 ml of liquid BBL-top agar containing 50 µl X-gal, 30 µl IPTG and 30 µl 5 mg/ml ampicillin, and poured on to ampicillin containing L-broth agar plates. Recombinant white colonies were stored at -70°C as individual frozen cultures in 96 well microlitre plates as described by Gergen (1979).

2.16. Preparation of plasmid DNA
2.16.1. "Mini" plasmid preparation technique

5 ml L-broth medium was inoculated with a single bacterial colony and grown overnight at 37°C. 1.5 ml of overnight culture was placed into a microfuge tube, centrifuged for 30 sec and the supernatant discarded. The cell pellet was resuspended in 350 µl lysis buffer (8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, 10 mM Tris-HCl pH 8.0), 25 µl of lysozyme (10 mg/ml in 10 mM Tris-HCl pH 8.0) was added and the suspension vortexed briefly. Lysis was achieved at 100°C for 40 sec and the lysate cleared in a microfuge for 10 min to remove chromosomal DNA and cellular debris. The supernatant was recovered, mixed with 40 µl 2.5 M sodium acetate, 420 µl isopropanol and incubated at -70°C for 60 min. Nucleic acids were recovered by centrifugation
in a microfuge and the pellet incubated in 50 μl TE containing 50 μg/ml RNase A at 37°C for 15 min. Following phenol/chloroform extraction the DNA was precipitated at -70°C for 60 min by the addition of 5 μl 2.5 M sodium acetate, 50 μl 4M ammonium acetate and 100 μl isopropanol.

2.16.2. "Maxi" plasmid preparation technique

5 ml L-broth medium was inoculated with a single bacterial colony and grown overnight at 37°C. 1 ml of this culture was used to inoculate 25 ml L-broth medium and incubated at 37°C for a further 3 hr. Finally this culture was transferred to 500 ml L-broth medium, incubated for 2 hr at 37°C, 75 mg of chloramphenicol was added and the culture incubated at 37°C for a further 16 hrs. Cells were harvested by centrifugation at 4000 rpm for 5 min and resuspended in 5 ml sucrose buffer (25% (w/v) sucrose, 50 mM Tris-HCl pH 8.1, 40 mM EDTA). Incubation continued on ice for 15 min following addition of 1 ml 10 mg/ml lysozyme (in sucrose buffer), and 1 ml 0.5 EDTA. Lysis was achieved by incubation on ice for 10 min after addition of 13 ml Triton buffer (0.1% (w/v) Triton X-100, 60 mM EDTA, 50 mM Tris-HCl pH 8). Chromosomal DNA and cellular debris was removed by centrifugation at 13,000 rpm for 30 min. The supernatant was recovered and phenol/chloroform extracted. Nucleic acids were recovered by addition 1/10th volume of precipitation salts (2.5 M sodium acetate pH 5.0, 0.1 M MgCl₂) and 2 volumes of ethanol.
Nucleic acids were recovered by centrifugation, resuspended in 3 ml TE containing 1 mg/ml RNase A and incubated at 37°C for 30 min followed by phenol/chloroform extraction and recovery by ethanol precipitation. Plasmid DNA was purified by caesium chloride density centrifugation to remove chromosomal DNA fragments. 3 g of caesium chloride and 150 μl 5 mg/ml ethidium bromide in TE, were added to the RNase treated DNA previously resuspended in 3 ml TE. 6.15 g were removed to a vertical rotor centrifuge tube, overlaid with paraffin and centrifuged in a Sorvall vertical rotor at 45,000 rpm, 18°C for 18 hr. The lower plasmid DNA band was recovered from the caesium chloride gradient via a syringe needle pushed through the centrifuge tube wall. Ethidium bromide was removed from plasmid DNA by extraction four times with an equal volume of butanol (saturated in 4 M NaCl TE). The DNA was then diluted to 2 ml with TE, precipitated with 2 ml of isopropanol, recovered and precipitated with isopropanol twice more from 2M ammonium acetate.

2.17. **Colony hybridisation**

Colony hybridisation was performed as described by Gergen *et al.* (1979). As stated previously, Section 2.15.4, individual recombinants were stored separately in freezing medium in individual wells of a 96 well microlitre plate. Replicas of each plate were made as follows. 12 cm x 12 cm L-broth agar-ampicillin plates were prepared and on single sheet of Whatman 540 chromatography paper, shaped to fit the dimensions of the plate,
was placed on to the agar. A 96 multi-pronged device, designed to fit to 8 x 12 well array, was used to transfer culture samples of each well on to the 540 paper. The plates were incubated at 37°C for 18 hrs. The bacterial colonies were lysed by immersing the 540 filters in the following solutions, 0.5 M NaOH, 0.5 M Tris-Cl pH 7.4, 2 x SSPE and finally 95% (v/v) ethanol. The filters were air dried.

Filters were prehybridised in 6 x SSPE, 5 x Denhardt, 0.1% SDS, 5 µg/ml poly U, 100 µg/ml denatured herring sperm DNA, at 65°C for 4 hrs. Filters were hybridised in the same buffer containing a denatured radiolabelled DNA for 18 hrs at 65°C. The filters were washed in 2 x SSPE, 0.1% SDS at 65°C, 2 washes for 20 min each.

2.18. Southern hybridisation

15 µg of each genomic DNA sample (derived from Xenopus borealis, Xenopus tropicalis and Xenopus laevis) was digested to completion with the desired restriction enzyme, followed by phenol/chloroform extraction and ethanol precipitation. Digested DNA was resuspended in 20 µl TE, 5 µl sample buffer (Section 2.3.6) and electrophoresed through a 1% (w/v) agarose (NA agarose, Pharmacia) Tris-acetate gel, Section 2.3.6. The gel was photographed to record the migration of the molecular weight markers. DNA was depurinated, denatured, and transferred on to nitrocellulose filters (Schleicher and Schull), as previously described by
Southern (1975) and Wahl et al. (1979). The gel was first soaked in 0.25 M HCl for 15 min (depurination); then 1.5 M NaCl, 0.5 M NaOH for at least 60 min (denaturation) and finally 1 M ammonium acetate, 0.02 M NaOH for at least 2 hr. DNA was transferred to nitrocellulose by blotting with 1 M ammonium acetate, 0.02 M NaOH, for at least 18 hr to allow efficient transfer to the high molecular weight DNA. Blotting paper was removed and the filter at first air dried and then baked at 80°C for 90 min in a vacuum oven.

Filters were prehybridised, hybridised with radiolabelled DNA and washed as described in Section 2.17.

2.19. **Northern hybridisation**

2.19.1. **Agarose gels**

RNAs were fractionated as previously described in Section 2.3.6a. The migration of RNA markers, run in a parallel track, was recorded by staining the gel slice containing the marker RNAs, with ethidium bromide, destaining and photographing the markers under UV irradiation.

RNA was transferred to nylon membranes (Bio-dyne) by blotting from 20 x SSPE, as previously described by Thomas (1980). The filters were air dried and then baked at 80°C for 60 min.

2.19.2. **Polyacrylamide gels**

Low molecular weight RNAs, prepared from previtellogenic ovaries, as described in Section 2.5.2, were analysed on polyacrylamide gels as described by
De Robertis et al. (1982)

10 x TBE is 0.9 M Tris/borate pH 8.3

25 mM EDTA

0.5 x TBE Gel Mix 50 ml 10 x TBE

76 g acrylamide

4 g bis-acrylamide

420 g urea

distilled water up to 1000 ml

20 μg of RNA was resuspended in 10 μl loading buffer (95% (v/v) deionised formamide, 0.01% (w/v) SDS, 0.025% bromophenol blue, 0.025% xylene cyanol), heat denatured for 10 min at 55°C, cooled and electrophoresed through an 8%/7 M urea gel in 0.5 x TBE as follows. 70 mls of 0.5 x TBE gel mix was polymerised by the addition of 140 μl 25% (w/v) APS and 140 μl TEMED for at least 2.3 hrs.

To ensure efficient transfer of the RNA from the polyacrylamide gel, the RNA was electroblotted onto nylon membranes (Biodyne) in a Bio-Rad Transblot apparatus. A sheet of membrane was cut to the dimensions of the gel and laid on to the gel. The gel and membrane were sandwiched between six sheets of Whatman 3MM paper and inserted into the apparatus, containing 25 mM Sodium phosphate pH 5.5, so that the membrane was on the anode or positive side. DNA was electroblotted for 4 hrs at 16 volts (2 volts/cm), then at 27 volts for 1 hr. The membrane was removed, air dried and baked at 80°C for 60 min.
Filters prepared by both procedures were prehybridised in 0-50% formamide, 6 x SSPE, 0.1% SDS, 100 μg/ml denatured herring sperm DNA, 5 μg/ml poly U for 4 hrs at 37-65°C depending on the stringency required. Filters were hybridised for 18 hrs using the same conditions.

2.20. Dideoxynucleotide DNA sequencing

DNA sequencing was performed by the dideoxynucleotide chain termination method described by Sanger et al. (1977), Sanger and Coulson (1978) and subsequently modified by Biggin et al. (1983).

2.20.1. Stock solutions

<table>
<thead>
<tr>
<th>BCIG (x-gal)</th>
<th>5-bromo-4-chloro-3-indoyl-beta-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg/ml in dimethylformamide</td>
<td></td>
</tr>
</tbody>
</table>

**IPTG**

| Isopropyl-beta-D-thio-galactopyranoside |
| 20 mg/ml in distilled water |

**M13 phage buffer**

<table>
<thead>
<tr>
<th>20 mM Tris-HCl pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM NaCl</td>
</tr>
<tr>
<td>1 mM EDTA</td>
</tr>
</tbody>
</table>

**TM buffer**

<table>
<thead>
<tr>
<th>100 mM Tris-HCl pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM MgCl</td>
</tr>
</tbody>
</table>

**Sequencing TE**

<table>
<thead>
<tr>
<th>10 mM Tris-HCl pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM EDTA</td>
</tr>
</tbody>
</table>

**M13 sequencing primer**

17-mer universal sequencing primer

5'-GTAAAACGACGGCCAGT-3'

2.5 μg lyophilite resuspended in 1000 μl sequencing TE
^35S-ATP  deoxyadenosine 5'-(α-[^35S]thio) triphosphate
8.1 mCi/ml
410 Ci/mmol (Amersham International)

dNTP stock solutions  10 mM of each dNTP in TE pH 8.5.
ddNTP stock solutions  (ddNTPs supplied by Boehringer
Mannheim GmbH)
10 mM of each ddNTP in TE pH 8.5

**ddNTP termination buffer composition**

The dNTP stock solutions were diluted down to
0.5 mM with TE pH 8.5.

The composition of each ddNTP termination buffer is
tabulated below, all values given are in microlitres (µl)

<table>
<thead>
<tr>
<th>Termination buffer</th>
<th>ddTTP</th>
<th>ddCTP</th>
<th>ddGTP</th>
<th>ddATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM ddTTP</td>
<td>25</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0.5 mM ddCTP</td>
<td>500</td>
<td>25</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0.5 mM ddGTP</td>
<td>500</td>
<td>500</td>
<td>25</td>
<td>500</td>
</tr>
<tr>
<td>10 mM ddTTP</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM ddCTP</td>
<td></td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM ddGTP</td>
<td></td>
<td></td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>10 mM ddATP</td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>TE pH 8.5</td>
<td>935</td>
<td>969</td>
<td>959</td>
<td>500</td>
</tr>
</tbody>
</table>
Chase Mix

0.25 mM dGTP
0.25 mM dCTP
0.25 mM dATP
0.25 mM dTTP
in distilled water

Formamide dyes

10 mls deionised formamide
0.01 g xylene cyanol
0.01 g bromophenol blue
0.25 ml 0.4M EDTA

10 x TBE

0.9 M Tris-borate pH 8.3
25 mM EDTA

0.5 x TBE Gel Mix

50 ml 10 x TBE
460 g urea
57 g acrylamide
3 g bis-acrylamide
make up to 1000 ml with distilled water

2.5 x TBE gel Mix

125 ml 10 x TBE
230 g urea
28.5 g acrylamide
1.5 g bis-acrylamide
25 g sucrose
25 mg bromophenol blue
make up to 500 ml with distilled water.

Gel Fix

250 ml glacial acetic acid
250 ml methanol
make up to 2.5 litres with distilled water.
2.20.2 **Preparation of insert DNA**

DNA was digested with the appropriate enzyme(s) to generate the restriction fragment of interest. The fragment was purified by electrophoresis through a 1% agarose gel, as described in Section 2.3.6, identified under UV irradiation and excised from the gel. The gel slice was placed into a dialysis bag containing 2 ml 0.5x TBE immersed in an electrophoresis tank containing 0.5x TBE, and electrophoresed for 2 hr at 100 mA. After 2 hrs the polarity was reversed for 60 sec and the bag removed from the tank. The solution within the dialysis bag was removed and passed down a DE52 ion exchange column pre-equilibrated as follows. 2 ml of DE52 slurry was packed into a column and washed sequentially with 0.6 M NaCl TE, then TE, then 0.1 M NaCl TE. 1 ml of packed DE52 has a binding capacity of 20 µg DNA. The band DNA was washed with 3 ml 0.3 M NaCl TE, finally eluted with three 0.5 ml washes with 0.6 M NaCl TE and recovered by ethanol precipitation.

2.20.3 **Subcloning into M13 mpl8 and mpl9**

Those DNA fragments which were no greater than 200 base pairs in length could be ligated directly into M13 mpl8 or M13 mpl9 vector DNA (Yanish-Perron et al. 1985) which had been digested with the appropriate restriction enzyme. However, to enable complete sequencing of DNA fragments greater than 200 base pairs, these had first to be digested with a series of 4 base cutter
restriction enzymes, to generate a series of overlapping fragments. Favourable enzymes were Taq 1, (compatible with the AccI site in mp 18/19), MboI (compatible with BamHI) and AluI (compatible with Smal), which proved effective in generating a series of overlapping restriction fragments covering all of the larger clones.

Typically 5 µg of vector DNA was digested to completion with the required enzymes in the presence of 1 unit of calf intestinal phosphatase, phenol/chloroform extracted and precipitated. DNA fragments were ligated into the linearised vector DNA, at a ratio of fragment DNA to vector DNA which varied between individual subcloning reactions since the efficiency of ligation was dependent upon the nature of the restriction sites being ligated. Optimum ligation conditions were defined empirically for each fragment. However, typically 500 ng of vector DNA and 100 ng of insert DNA were ligated in 20 µl reactions containing 1 x T4 DNA ligase buffer, 1 mM ATP, 1 unit T4 DNA ligase and incubated at 15°C for 18 hr. 5 µl of the ligation reaction were used to transform competent NM522. On subsequent transformations the amount of DNA was adjusted so that there were 50-100 plaques per plate. Competent NM522 cells were prepared as follows. 5 ml of L-broth was inoculated with a single NM522 colony picked from a minimal medium agarose plate, and grown up overnight at 37°C. 1 ml of this culture was used to inoculate 100 ml L-broth which was again grown up at 37°C until an absorbance of 0.2 (OD$_{650} = 0.2$) was reached.
The cells were cooled for 10 min on ice and then harvested by centrifugation. The cells were resuspended in 50 ml of cold 50 mM CaCl$_2$ and incubated on ice for 30 min. The cells were again harvested by centrifugation and resuspended in 10 mls of cold 50 mM CaCl$_2$ and incubated on ice for 60 min. 200 μl of competent cells were incubated with the transforming DNA for 40 min on ice, heat shocked at 42°C for 2 min and then returned to 0°C. The transformed cells were mixed with 3 ml of liquid BBL-top agar containing 30 μl x-gal, 30 μl IPTG and 200 μl competent cells, poured on to a BBL agar plate, allowed to set and then incubated, inverted, for 18 hrs at 37°C.

Individual plaques were either used directly to produce template DNA or were picked and diluted into 500 μl M13 phage buffer.

2.20.4. Preparation of templates from M13 recombinants

100 mls of L-broth medium was inoculated with 1 ml of a NM522 fresh overnight culture. The culture was incubated at 37°C until absorbance (OD$_{650}$) of 0.2 was reached. The cells were then placed on ice for 2 min, and 2 mls dispensed into small culture bottles. A M13 plaque was picked from a fresh overnight plate, mixed with an aliquot of the NM522 culture and incubated at 37°C for 6 hrs. 1.5 ml of this culture was transferred to a microfuge tube and centrifuged for 5 min to remove bacterial cells. 1.2 ml of the supernatant was transferred
to another microfuge tube, 200 µl 2.5 M NaCl, 20% (w/v) polyethylene glycol 6000 was added, vortexed and incubated on ice for 30 min to precipitate the phages. The precipitation mix was centrifuged for 5 min and the supernatant removed. The tube was recentrifuged and the last remnants of the supernatant removed. The phage pellet was resuspended in 100 µl NTE, phenol/chloroform extracted and precipitated by the addition of 10 µl 3M sodium acetate pH 5.5, 200 µl ethanol. A DNA pellet was recovered by centrifugation, washed in 95% ethanol and resuspended in 30 µl sequencing TE.

2.20.5. Chain termination dideoxynucleotide DNA sequencing

Primer Mix - per template
1 µl 17-mer M13 sequencing primer (2.5 ng/µl)
1 µl TM buffer
3 µl distilled water

Annealing mixture - per template
5 µl template DNA (in sequencing TE)
5 µl primer mix.

The annealing reactions were incubated at 65°C for 60 min in capped tubes, following which they were allowed to cool slowly to room temperature.

Radiolabel mixture
0.62 µl [³⁵S-thio]dATP (8 µCi/µl)
1 µl 80 mM Tris-HCl pH 8.5
1 µl 80 mM DTT
5.1 µl distilled water
0.31 µl Klenow fragment (5 units/µl, Boehringer Mannheim)
All components of the radiolabel mix, except the enzyme, were dispensed and held on ice prior to requirement. The Klenow fragment was added just before the mixture was dispensed.

**Primed DNA synthesis**

2 μl of annealed template was dispensed into four capless 1.5 ml eppendorf tubes. 2 μl of ddTTP termination buffer was added to one tube, similarly 2 μl of the remaining ddNTP termination buffers was added to each of the other tubes. Finally 2 μl of the radiolabel mixture was added to each tube. The tubes were centrifuged to mix the components and incubated at 22°C for 25 min. At the end of this period 2 μl chase mix was added to each tube, centrifuged and incubated for a further 20 min at the same temperature. The reactions could be stored overnight at -20°C or alternatively the sequencing products were analysed immediately on a 8% acrylamide/7M urea polyacrylamide gel as follows. 2 μl of formamide dyes were added to each tube, centrifuged, boiled for 3.5 min, and cooled on ice for 10 min. Half of each reaction was then electrophoresed through one of the two alternative gel systems.

**Polyacrylamide gel electrophoresis**

The sequencing products were analysed on two different polyacrylamide gel systems. The gradient buffer gel, Biggin et al. (1983), is designed to optimise the separation of sequence information close to the sequence primer,
while the straight buffer gel is designed to optimise the separation of sequence information 200-400 nucleotides from the primer.

The gradient gel employs a buffer gradient at the base of the gel, prepared by mixing 4 mls 0.5 x TBE gel mix with 6 ml 2.5 x TBE gel mix, the rest of the gel contains 0.5 x TBE gel mix. The straight gel employs no buffer gradient and only contains 0.5 x TBE gel mix. Straight gels were prepared with 35 mls 0.5 TBE Gel mix, 70 µl TEMED and 70 µl 25% (w/v) APS, poured into 20 cm x 40 cm x 0.35 mm glass gel formers, and left to polymerise for at least 2 hrs. The non-gradient, upper section, of the buffer gradient gels was prepared in the same way. This was overlaid onto a 10 ml buffer gradient prepared as follows. 4 mls of polymerising 0.5 x TBE gel mix was taken up into a 10 ml glass pipette, 6 mls of polymerising 2.5 x TBE gel mix (by the addition of 12 µl TEMED, 12 µl 25% (w/v) APS) was then taken up into the same pipette. The lower 2.5 x TBE gel mix contains 5% sucrose and is therefore denser. A buffer gradient is produced by introducing 4-5 bubbles into the base of the pipette, and poured quickly into the base of glass gel former. 1.0 x TBE gel buffer is used in both the upper and lower gel tanks. Samples were electrophoresed at 25 watts constant power. Samples loaded on to gradient gels were electrophoresed until the bromophenol blue dye from the samples had reached the base of the gel. Samples loaded on to straight
buffer gels were initially electrophoresed in the same way, however, when the bromophenol blue had migrated to the base of the gel, extra loading dyes were added to two wells and electrophoresis continued until the xylene cyanol from this addition of dyes had migrated to the base of the gel.

The gels were dismantled and soaked in gel fix for 15 min, transferred to blotting paper, dried under vacuum and autoradiographed with non-preflash cronex 4 film at room temperature.

2.20.6. Single dideoxynucleotide sequence screening

Single dideoxynucleotide sequence screening was used as a rapid screening procedure to identify a number of M13 sequence templates.

3 μl of template DNA and 3 μl of primer mix were incubated at 80°C for 5 min and then allowed to cool to room temperature. 2 μl of a single dideoxynucleotide termination buffer (in this case ddCTP) and 2 μl of the radiolabel mixture were added, centrifuged and incubated at 22°C for 25 min. 2 μl of chase mix was added, centrifuged and incubated at 22°C for a further 10 min. 2 μl of formamide dyes were added and electrophoresed on a gradient buffer gel.
CHAPTER THREE

MOLECULAR ANALYSIS OF RENATURED

XENOPUS BOREALIS OVARIAN POLY(A) RNA
3.1 Introduction

The maternal RNA populations of sea urchin eggs and Xenopus laevis oocytes include poly(A)RNA transcripts which contain short interspersed repetitive sequences (Cotton et al. 1980, Anderson et al. 1982). A number of sea urchin interspersed repetitive sequences have been defined (Posakony et al. 1981) and their expression at different developmental stages studied (Posakony et al. 1983). As yet however, similar detailed investigations relating to the interspersed repetitive sequences of the Xenopus laevis oocyte maternal RNA have not been reported.

Before attempting to analyse the repetitive sequences of the maternal RNA from the related species Xenopus borealis it was necessary to repeat and extend the investigations described by Anderson et al. (1982) to show similar or identical structures for this species also. In this chapter I describe experiments which show that Xenopus borealis oocyte poly(A)RNA contains sequences which form duplexes during RNACoT hybridisation.

3.2 Sedimentational analysis of ovarian RNA

Total ovarian RNA prepared from adult animals, containing mainly stage 5 and stage 6 oocytes, was analysed on two 30% (w/v) -7% (w/v) sucrose density gradients as described in Materials and Methods. The RNA on one gradient had been heat denatured prior to centrifugation, while in a control experiment run in parallel, RNA was analysed without prior denaturation.
The uv absorbance profiles of the two gradients after centrifugation are shown in Figure 3.1A. Since ribosomal RNAs account for the vast majority (greater than 98%) of the ovarian RNA, the absorbance traces reflect the sedimentation profile of ribosomal RNAs. Although the profiles differ quantitatively, the migration of intact 28S, 18S and 5S ribosomal RNA appears to be unaffected by heat denaturation. This is in contrast to the distribution of poly(A)RNA, as detected by the formation of nuclease resistant polyA:[$^{3}$H]polyU hybrids, Figure 3.1B. Before heat denaturation the majority (64%) of poly(A)RNA migrates with an apparent sedimentation coefficient greater than 28S, compared to only 14.5% when the RNA is denatured prior to centrifugation.

The change in poly(A)RNA sedimentation profiles observed in Figure 3.1B can be reversed. Purified poly(A)RNA (5 µg) was denatured, allowed to anneal under controlled conditions to various equivalent RNA C_{o}t values (Appendix I) and the products analysed on 30% (w/v) – 7% (w/v) sucrose density gradients as before. However, since the renaturation buffer contained 50% formamide, the Tris buffer of the sucrose gradients was replaced by MOPS. Otherwise the composition of the gradients was identical to those employed previously, Figure 3.1. The poly(A)RNA distribution, as detected by [$^{3}$H]polyU hybridisation is shown in Figure 3.2. The migration of 28S and 18S ribosomal RNA markers, measured on a separate gradient, is indicated. The proportion of poly(A)RNA sedimenting with
Figure 3.1. **Sedimentational analysis of ovarian RNA**

100 µg of *Xenopus borealis* ovarian RNA was analysed on two 30% (w/v) - 7% (w/v) sucrose gradients as described in Materials and Methods. RNA samples were either heat denatured prior to centrifugation (--- --- ---) or centrifuged without denaturation (-----).

A. Following centrifugation the gradients were fractionated on an ISCO continuous UV analyser. The sedimentation profiles of the ribosomal RNAs are shown.

B. Fractionated gradients were analysed for poly(A) contented by $[^3H]$polyU hybridisation. Hybridisation, RNase A digestion and recovery of the nuclease resistant material was carried out as described in Materials and Methods. The amount of protected $[^3H]$polyU in each fraction is plotted against its sedimentational position. Sedimentation is from right to left.
an apparent sedimentation coefficient greater than 28S increases in a RNA C_{o}t dependent manner. 56% of the poly(A)RNA annealed to a C_{o}t of 600Ms migrates with an apparent sedimentation coefficient greater than 28S, compared with only 6.5% when the poly(A)RNA is denatured prior to centrifugation. These volumes are slightly lower than the percentages calculated from Figure 3.1B, but confirm the observation that structures with a high sedimentation coefficient, and therefore a high molecular weight, are present in both the ovarian RNA preparation and renatured poly(A) RNA, but these structures, however stabilised, are sensitive to heat denaturation.

As can also be seen in Fig. 3.2 a peak of [^3H]polyU hybridisation can be detected in poly(A)RNA annealled to 60Ms and 600Ms which sediments more slowly than 18S ribosomal RNA and does not appear to undergo any alteration in sedimentation during annealing.

3.3 Cellulose/Ethanol column fractionation of annealed poly(A)RNA

Ovarian poly(A)RNA prepared from adult animals containing mainly stage 5 and stage 6 oocytes was heat denatured and annealed to various RNA C_{o}t values (Appendix I). The annealed RNA was diluted and fractionated on cellulose/ethanol columns as previously described by Fedoroff et al. (1977) and Anderson et al. (1982). This form of adsorption chromatography allows double stranded nucleic acids to be separated from single
Figure 3.2 Sedimentational analysis of renatured poly(A)RNA

*Xenopus borealis* ovarian poly(A)RNA was renatured to the required RNA C<sub>o</sub>t value and analysed on 30% (w/v) - 7% (w/v) sucrose gradients buffered in METS (10 mM MOPS pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% SDS), as described in Materials and Methods. Following centrifugation the gradients were fractionated and the poly(A) content of each fraction determined by [³H]polyU hybridisation, as described in Materials and Methods. Sedimentation was from right to left.
Table 3.1  Chromatographic separation of renatured poly(A)RNA on cellulose/ethanol columns

Xenopus borealis ovarian poly(A) RNA was renatured to the required RNA $C_{ot}$ value and fractionated on cellulose/ethanol columns, as described in Materials and Methods.

The mass of RNA in each fraction, calculated from its absorbance at 260 nm, is given (1).

The mass of RNA eluted from the column (2) is given by the sum of the masses eluted in each fraction (1).

The mass of RNA bound to the column (3) is given by the sum of the double stranded and single stranded RNA.

The percentage of RNA eluted in each fraction (4), is given by the mass eluted in each fraction (1) divided by the total eluted from the column (2).

The percentage of bound RNA eluted from each fraction (5), is given by the mass of the double stranded or single stranded RNA (1), divided by the total mass of RNA bound to the column (3).
<table>
<thead>
<tr>
<th>Chromatographic Fractionation</th>
<th>Unbound RNA (μg)</th>
<th>Single Stranded RNA (μg)</th>
<th>Double Stranded RNA (μg)</th>
<th>Total eluted from the column (μg) (2)</th>
<th>Total bound to the column (μg) (3)</th>
<th>Percentage of eluted RNA in each fraction</th>
<th>Percentage of bound RNA in each fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01</td>
<td>0.768</td>
<td>3.934</td>
<td>0.112</td>
<td>4.814</td>
<td>4.046</td>
<td>16%</td>
<td>97%</td>
</tr>
<tr>
<td>60</td>
<td>0.472</td>
<td>2.514</td>
<td>0.492</td>
<td>3.478</td>
<td>3.006</td>
<td>13.5%</td>
<td>84%</td>
</tr>
<tr>
<td>535</td>
<td>0.645</td>
<td>1.954</td>
<td>1.348</td>
<td>3.947</td>
<td>3.302</td>
<td>16%</td>
<td>59%</td>
</tr>
<tr>
<td>600</td>
<td>5.128</td>
<td>16.166</td>
<td>17.931</td>
<td>39.225</td>
<td>34.097</td>
<td>13%</td>
<td>48%</td>
</tr>
<tr>
<td>3000</td>
<td>0.477</td>
<td>1.500</td>
<td>1.407</td>
<td>3.384</td>
<td>2.907</td>
<td>14%</td>
<td>51%</td>
</tr>
</tbody>
</table>

TABLE 3.1
stranded nucleic acids. Table 3.1 describes the results of a series of such chromatographic experiments performed as described in Materials and Methods.

A variable percentage, 8-16% of the RNA input remained unbound to the column, while approximately 20% of the RNA input was never recovered from the column. These figures are in line with the operational characteristics for cellulose/ethanol columns outlined by Anderson et al. (1982).

The percentage of RNA eluted in the double stranded fraction increases in a RNA Cₜ dependent manner, up until 600 Ms, whereupon essentially all the sequences involved in complex formation have renatured. The values presented in Table 3.1 agree well with the values described for *Xenopus laevis* (Anderson et al. 1982) oocyte poly(A)RNA and suggest that there are no gross differences between the poly(A) RNA populations from oocytes of these two species as far as fractionation of renatured poly(A)RNA is concerned.

3.4 **Electron microscopic analysis of renatured poly(A)RNA**

Interspersed repetitive sequences have been visualised directly by electron microscopic analysis of renatured sea urchin egg poly(A)RNA (Costantini et al. 1980). Since sequences homologous to both strands of sea urchin genomic repetitive elements are represented in the egg poly(A)RNA (Posakony et al. 1983), large, highly complex, multi-molecular structures can be observed in renatured egg
Figure 3.3  Electron microscopic analysis of renatured poly(A) RNA

*Xenopus borealis* ovarian poly(A) RNA was renatured to a RNA C_0t of 600Ms and spread for analysis under the electron microscope as described in Materials and Methods.

A) poly(A) RNA, spread from hyperphase containing 0.25M NaCl

B) poly(A) RNA, spread from hyperphase containing 0.1M NaCl

C) poly(A) RNA, spread from hyperphase containing 0.05M NaCl

D) poly(A) RNA, spread from hyperphase containing 0.05M NaCl
poly(A)RNA under the electron microscope (Costantini et al. 1980). Attempts to understand the physical basis by which these structures are stabilised is hampered by the high degree of intermolecular and intramolecular secondary structure observed within individual molecular networks. However, it has been suggested (Costantini et al. 1980) that the networks are stabilised by the formation of RNA-RNA duplexes between the complementary repetitive sequences known to be present within the egg poly(A)RNA. Similar structures have also been observed in renatured Xenopus laevis oocyte poly(A)RNA (Anderson et al. 1982) and the existence of interspersed repetitive sequences within the oocyte poly(A)RNA has been inferred by analogy. Attempts have therefore been made to observe such structures within renatured Xenopus borealis poly(A)RNA.

Poly(A)RNA was annealed and prepared for examination under the electron microscope as described in Materials and Methods. Initial attempts to observe multimolecular poly(A)RNA networks met with complete failure. No difference could be observed between denatured poly(A)DNA and renatured poly(A)RNA when RNA samples were prepared for electron microscopy as described by Costantini et al. (1980). Multimolecular networks could, however, be observed in abundance when 0.25 M NaCl was incorporated into the formamide hyperphase, Figure 3.3A. The presence of single stranded DNA size markers (M13 mp8 7229 b) and double stranded DNA size markers (pAT153 3658 bp) can also be observed, Figure 3.3. The
structures are indistinguishable from those observed by Anderson et al. (1982).

By gradually reducing the hyperphase sodium chloride concentration it is possible to relax the large multimolecular complexes observed at higher salt concentrations, Figure 3.3 B-D. At lower sodium chloride concentrations the networks appear to dissociate, until at a hyperphase concentration of 50 mM sodium chloride, no large multimolecular complexes are observed, only isolated bimolecular structures can be seen, Figure 3.3.D. If it is assumed that the map length of double stranded RNA is the same as double stranded DNA under the spreading conditions employed, then in bimolecular structures where the RNA-RNA duplex is clearly visible, the length of the duplexed region is approximately 250-300 bp. This figure is in agreement with measurements performed on complexed Xenopus laevis hn RNA previously reported by Sommerville and Scheer (1982).

3.5 Nuclease digestion of renatured poly(A) RNA

In an attempt to probe further the physical basis by which the large multimolecular networks are stabilised complexes were exposed to mild digestion with a single strand specific nuclease. Mung bean nuclease was chosen
Table 3.2  Chromatographic separation of nuclease digested poly(A) RNA

15 μg Xenopus borealis ovarian poly(A) RNA was renatured to 600 M$s$, digested with increasing amounts of mung bean nuclease (0.25-10 units) for 100 min at 24°C, and the products fractionated on cellulose/ethanol columns as described in Materials and Methods.

The percentage of RNA which remained as double stranded RNA (1) after digestion with x units of nuclease, is the mass of RNA remaining double stranded (after digestion with x units of nuclease) divided by the total mass of RNA which remained bound to the column (after digestion with x units of nuclease).

The percentage of double stranded RNA remaining nuclease resistant (2), is the mass of RNA remaining nuclease resistant after digestion with x units of nuclease, divided by the mass of double stranded RNA after no nuclease treatment (48.5%).
TABLE 3.2

<table>
<thead>
<tr>
<th>Mung Bean Nuclease (Units)</th>
<th>10</th>
<th>2.25</th>
<th>1.33</th>
<th>1.0</th>
<th>0.89</th>
<th>0.66</th>
<th>0.4</th>
<th>0.26</th>
<th>0.25</th>
<th>0*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatographic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unbound RNA (µg)</td>
<td>1.32</td>
<td>0.92</td>
<td>0.80</td>
<td>0.64</td>
<td>0.56</td>
<td>0.56</td>
<td>0.72</td>
<td>0.47</td>
<td>1.01</td>
<td>0.52</td>
</tr>
<tr>
<td>Double Stranded RNA (µg)</td>
<td>0.83</td>
<td>0.99</td>
<td>1.05</td>
<td>1.09</td>
<td>1.44</td>
<td>2.11</td>
<td>2.05</td>
<td>2.44</td>
<td>2.61</td>
<td>4.29</td>
</tr>
<tr>
<td>RNA eluted from column (µg)</td>
<td>11.30</td>
<td>12.65</td>
<td>13.41</td>
<td>11.86</td>
<td>12.12</td>
<td>12.84</td>
<td>11.84</td>
<td>11.93</td>
<td>12.84</td>
<td>8.86</td>
</tr>
<tr>
<td>RNA bound to column (µg)</td>
<td>9.97</td>
<td>11.73</td>
<td>12.61</td>
<td>11.22</td>
<td>11.56</td>
<td>12.28</td>
<td>11.12</td>
<td>11.46</td>
<td>11.83</td>
<td>8.34</td>
</tr>
<tr>
<td>Percentage of eluted RNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unbound RNA</td>
<td>11.7%</td>
<td>7.3%</td>
<td>6.0%</td>
<td>5.4%</td>
<td>4.6%</td>
<td>4.4%</td>
<td>6.1%</td>
<td>3.9%</td>
<td>7.8%</td>
<td>5.8%</td>
</tr>
<tr>
<td>Single stranded RNA</td>
<td>80.9%</td>
<td>84.8%</td>
<td>86.2%</td>
<td>85.5%</td>
<td>83.5%</td>
<td>79.2%</td>
<td>76.6%</td>
<td>75.6%</td>
<td>71.8%</td>
<td>45.7%</td>
</tr>
<tr>
<td>Double stranded RNA</td>
<td>7.4%</td>
<td>7.9%</td>
<td>7.8%</td>
<td>9.1%</td>
<td>11.8%</td>
<td>16.4%</td>
<td>17.3%</td>
<td>20.5%</td>
<td>20.4%</td>
<td>48.5%</td>
</tr>
<tr>
<td>Double stranded RNA (with x units of nuclease)</td>
<td>8.4%</td>
<td>8.5%</td>
<td>8.3%</td>
<td>9.6%</td>
<td>12.4%</td>
<td>17.2%</td>
<td>18.4%</td>
<td>21.3%</td>
<td>22.1%</td>
<td>51.4%</td>
</tr>
<tr>
<td>Total bound to column (with x units of nuclease)</td>
<td>9.6%</td>
<td>12.4%</td>
<td>17.2%</td>
<td>18.4%</td>
<td>21.3%</td>
<td>22.1%</td>
<td>51.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double stranded RNA (at x units of nuclease)</td>
<td>15.3%</td>
<td>16.3%</td>
<td>16.1%</td>
<td>18.7%</td>
<td>24.3%</td>
<td>33.8%</td>
<td>35.7%</td>
<td>42.3%</td>
<td>42.0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Only 10 µg of poly(A)RNA was renatured in this reaction.
Figure 3.4  **Chromatographic separation of nuclease digestion poly(A)RNA**

The percentage of double stranded RNA remaining nuclease resistant (see (2), Table 3.2) is plotted against the number of units of mung bean nuclease used in each digestion (A). Alternatively the percentage of double stranded RNA remaining nuclease resistant is plotted against $\log_{10}$ the number of units of mung bean nuclease used in each digestion (B).
Log_{10}(percentage(%) double stranded RNA remaining nuclease resistant)

Percentage(%) double stranded RNA remaining nuclease resistant

Units of nuclease
in preference to other single strand nucleases because the enzymic activity is only stabilised in the presence of 0.1 mM $2n^{2+}$ and 0.001% (w/v) Triton X-100 at pH 5.0 and can be completely inactivated by the addition of 0.1% SDS (w/v) and 20 mM EDTA (Kowalski et al. 1976).

In a series of trial digestions 15 μg aliquots of ovarian poly(A)RNA were renatured to 600 Ms, ethanol precipitated, digested with increasing concentrations of mung bean nuclease and the resulting products fractionated on cellulose/ethanol columns as described in Materials and Methods. Table 3.2 described the results of a series of such experiments, including the percentage of RNA remaining resistant to nuclease digestion. The results have also been displayed graphically in Figure 3.4.

As the extent of nuclease digestion increases the proportion of double stranded RNA decreases exponentially. This is matched by an increase in the proportion of single stranded RNA, while the overall level of unbound RNA remains constant. This trend continues, until a ratio approximately 1 unit of mung bean nuclease to 15 μg of complexed poly(A)RNA is reached. Thereafter, no further decrease in the proportion of double stranded RNA is observed, while the proportion of single stranded RNA begins to decrease and the proportion of unbound RNA increases. The results presented in Table 3.2 and Figure 3.4 indicate that a nuclease resistant, double stranded RNA component, equivalent to approximately 8%
of the recoverable RNA mass, exists within the complexed poly(A)RNA, and that the nuclease sensitive, single stranded RNA, which was initially associated with the resistant fraction, can be displaced into the single stranded RNA fraction. This fraction is, as expected, sensitive to further digestion and can be displaced into the unbound fraction, which probably contains short oligonucleotides, since it has previously been demonstrated that RNase A treated RNA remains unbound to the column (Anderson et al. 1982).

The molecular analysis of double stranded, nuclease resistant products was extended by radiolabelling the RNA and examining the products on non-denaturing, Tris-acetate agarose gels. In similarity to other single strand specific nucleases from Aspergillus oryzae (S1) and Penicillium citrinum (P1), mung bean endonuclease generates 5'-phosphorylated polynucleotides (Kowalski et al. 1976). Therefore the 3'-hydroxyl terminus can be radiolabelled with cytidine-3'-5'-[5'-32P]bisphosphate, as described in Materials and Methods.

Double stranded, nuclease resistant RNA, prepared under digestion conditions just sufficient to generate the nuclease resistant component (1.0 unit/15 μg RNA, under standard assay conditions) contains a heterogeneous population of molecules in the order of 80-200 bp, Figure 3.5A. However, RNA digested less extensively (0.26 units/15 μg RNA, under standard assay conditions)
Figure 3.5 Analysis of radiolabelled double stranded RNA

Double stranded, nuclease resistant RNA was radiolabelled with cytidine-3'-5'-[5'-32P]bisphosphate as described in Materials and Methods. The radiolabelled DNA markers are derived from a HindIII/BamH1 digested of lambda DNA (20,000 cpm).

A) Track 1. 15 µg renatured poly(A)RNA digested with 1.0 unit of nuclease. Double stranded RNA prepared on a cellulose/ethanol column, radiolabelled and 29000 cpm analysed on agarose gel.

Track 2. 15 µg renatured poly(A)RNA digested with 0.26 units of nuclease. Double stranded RNA prepared on a cellulose/ethanol column, radiolabelled and 23000 cpm analysed on agarose gel.

B) 15 µg renatured poly(A) RNA digested with 0.26 units of nuclease. Double stranded RNA prepared on a cellulose/ethanol column and then fractionated on a sephacryl S-300 column. The RNA from each fraction was then radiolabelled with cytidine-3'5'-[5',32P],bisphosphate and analysed on a 2.5% agarose gel.
contains two heterogeneous populations of double stranded, nuclease resistant molecules. The first has a similar size distribution to that just described, while the molecules of the second population are approximately 300-450 bp in length, Figure 3.5A. Moreover, following size fractionation on a Sephacryl S-300 column, a band migrating with an apparent size of 310 bp, is clearly visible within the second slower migrating population, Figure 3.5B. Furthermore, this band is similar in length to the RNA-RNA duplexes reported previously by Sommerville and Scheer (1982) and to the duplexes measured in this study, although it is not known if the observed band is derived from the RNA-RNA duplexes observed by electron microscopy. The slower migrating nuclease resistant population is clearly not itself completely resistant to nuclease digestion, suggesting a degree of base mismatch within the double stranded duplexes, and consistent with the observation that RNA duplexes are rarely seen by electron microscopy when low sodium chloride concentrations are employed during spreading in 50% (v/v) formamide.

3.6 Discussion

The observations reported confirm and extend previous studies (Anderson et al. 1982) of maternal poly(A)RNA in Xenopus. Large multimolecular networks can be observed by electron microscopy and on sucrose gradients in renatured poly(A)RNA of Xenopus borealis.
ovaries. These structures form in a RNA $C_{ot}$ dependent manner up until 600 Ms whereupon essentially all the sequences involved in complex formation have renatured. However, these large multimolecular structures are probably relatively unstable by a number of criteria. First, large complexes are only observed by electron microscopy when 0.25M sodium chloride is present in the spreading hyperphase. Complexes show a high degree of secondary structure, foldback structures are clearly discernible, but obvious duplexes between interspersed repetitive sequences are not easily observed. At sodium chloride concentrations lower than 0.25M, the large complexes relax, but relatively few repetitive duplexes are visible. Where they are clearly discernible, length measurements made relative to both double and single strand DNA standards indicate a RNA-RNA duplexed region of approximately 300 bp, which is similar to the values reported for RNA-RNA duplexes in renatured *Xenopus laevis* oocyte hnRNA (Sommerville and Scheer, 1982). High molecular weight poly(A) RNAs are also observed in ovarian RNA preparations analysed on sucrose gradients. These structures are heat labile and following denaturation the size distribution of poly(A) RNA (as detected by
[\textsuperscript{3}H]polyU hybridisation) is similar to that previously described for \textit{Xenopus laevis} ovarian poly(A)RNA (Rosbash and Ford, 1974). The sedimentation characteristics of the ribosomal RNA component is, however, essentially unchanged by heat denaturation. It is not known if such multimolecular structures occur \textit{in vivo}, since the observation (Kohne \textit{et al.} 1977) that aqueous-phenol emulsions can accelerate the rate of nucleic acid hybridisation by concentrating the nucleic acids of the emulsion interface, would allow interspersed repetitive sequences within the poly(A)RNA component of ovarian RNA to anneal \textit{in vitro} during RNA extraction. Similar changes in sedimentation profiles have been documented in association with the Alu short interspersed repeat of HeLa hnRNA (Federoff \textit{et al.} 1977). Secondly, a double stranded RNA component of renatured poly(A)RNA, equivalent to 8% of the RNA mass, is resistant to extensive digestion by mung bean nuclease. This RNA is heterogeneous in size but somewhat smaller than RNA-RNA duplexes observed by electron microscopy. However, renatured poly(A)RNA digested less extensively does contain a band of double stranded RNA migrating with an apparent size (310 bp) similar to the duplexes observed in the electron microscope. This suggests that a degree of sequence heterogeneity exists between different members of any given repetitive sequence family. Similar observations (Federoff \textit{et al.} 1977) have been described for renatured HeLa hnRNA. While renatured hnRNA is observed to contain
300 bp RNA-RNA duplexed, the nuclease resistant, double stranded RNA component is much smaller, being 50-150 bp in length. Moreover, it has been shown that individual members of the human Alu family do not contain exactly the same sequence. Instead they are divergent from a consensus sequence by about 13% (Deininger et al. 1981) which makes them a family of similar, but not identical sequences. The existence of nuclease sensitive base mismatches within double stranded duplexes is consistent with the observation that RNA duplexes are rarely seen by electron microscopy when renatured *Xenopus borealis* ovarian poly(A)RNA is spread from a hyperphase employing 50 mM sodium chloride.

It has been observed (Figure 3.2) that a peak of \[^{3}H\]polyU hybridisation does not appear to undergo any alteration in sedimentation during annealing. It is possible that this fraction is identical to that fraction of renatured poly(A)RNA which can be separated as single stranded RNA on cellulose/ethanol columns.

The basis for the discrepancy between the apparent stability of renatured *Xenopus laevis* poly(A)RNA networks (Anderson et al. 1982) and the renatured *Xenopus borealis* poly(A)RNA networks, described earlier, is unknown. However, since renatured *Xenopus laevis* RNA was diluted directly from the annealing buffer (Anderson et al. 1982) some sodium chloride must have been present in the hyperphase. Unfortunately, no details are available as
to the dilution factor involved and so the final hyperphase sodium chloride concentration is unknown. But it is conceivable that sufficient sodium chloride was present to stabilise the networks. Alternatively the interspersed repetitive sequences of the *Xenopus borealis* ovarian poly(A)RNA could be significantly more diverged and thus less stable than their *Xenopus laevis* counterparts.
CHAPTER FOUR

ANALYSIS OF A CDNA LIBRARY PREPARED FROM

A MUNG BEAN NUCLEASE RESISTANT COMPONENT

OF RENATURED POLY(A) RNA
4.1 Introduction

As previously discussed in Chapter 3, large multi-molecular networks can be observed in renatured *Xenopus borealis* ovarian poly(A) RNA. These networks appear extremely complex and apparently extensive secondary structure can be observed. Studies (section 3.5) indicate that a double stranded, single strand nuclease resistant, component, accounting for approximately 8% of the RNA mass, can be purified from renatured ovarian poly(A) RNA. However, a second transiently nuclease resistant component is observed when annealled poly(A) RNA is digested less extensively with mung bean nuclease. Studies (section 3.5) also indicate that this component is enriched for a 310 bp double stranded RNA species, which is similar in size to the RNA-RNA duplexes observed in renatured ovarian poly(A) RNA and oocyte hnRNA (Sommerville and Scheer 1982).

In an attempt to analyse the sequences responsible for the formation and stabilisation of the multimolecular networks, a cDNA library has been prepared from the nuclease resistant, double stranded, RNA component enriched for the 310 bp RNA band.

4.2 Construction of a cDNA library from a double stranded, nuclease resistant, component of renatured poly(A) RNA

Figure 4.1 outlines the strategy employed to construct a cDNA library from double stranded nuclease resistant RNA. In the experiments described in Section 3.5, sufficient nuclease resistant material was produced to quantitate
Figure 4.1  A cDNA cloning strategy

This outline summarises the cDNA cloning rationale employed to select for regions of poly(A) RNA participating in intramolecular and intermolecular hybridisation, *in vitro*. Details of this procedure are described in Section 2.15.
Xenopus Ovarian Poly(A) RNA

Renaturation To Equivalent RNA (C_0 > 600 Ms)

i. Digestion With Mung Bean Nuclease
ii. Fractionation On Cellulose/Ethanol Columns

Purified Double Stranded RNA

i. S300 Fractionation
ii. Homopolymer Tailing With *E. coli* Poly A Polymerase

A Tailed Double Stranded RNA

i. Oligo-dT Primed First Strand cDNA Synthesis
ii. Second Strand cDNA Synthesis (RNase H, DNA Polymerase, *E. coli* Ligase)

Double Stranded cDNA

i. Treatment With T4 DNA Polymerase
ii. Blunt-end Ligation Into SmaI Cut pUC19
iii. Transform Into RRΔM15

Selection Of Recombinants
the nuclease titration, but this was insufficient RNA from which to construct the cDNA library. In the titration experiments, described in Table 3.2, 15 μg annealled RNA was digested, under standard assay conditions, with increasing amounts of mung bean nuclease. However, when larger quantities of annealled RNA was digested under identical conditions, relatively more units of nuclease were required to produce similar cellulose/ethanol column fractionation characteristics. Hence the conditions described in Materials and Methods (section 2.14.2) in which 160 μg annealled RNA, resuspended in 320 μl, was digested with 16 units mung bean nuclease (1.5 units/15 μg RNA) was sufficient to allow 21% of the RNA which bound to the cellulose/ethanol column to elute as double stranded RNA. This is comparable to the values produced when 15 μg renatured RNA was digested with 0.25-0.26 units of mung bean nuclease (Table 3.2) when 21.3%-22.1% of the RNA which bound to the column was eluted as double stranded RNA, in which a band migrating with an apparent size of 310 bp could later be detected in Sephacryl S-300 column fractionated material.

Double stranded RNA prepared from 160 μg of renatured, nuclease digested, RNA was size fractionated and the enriched 310 bp RNA band tailed with ATP as described in Materials and Methods. Calculations described in Appendix III indicate that on average 86 A residues were added to each 3' hydroxyl terminus. This figure is only an estimate and
assumes that the average length of the RNA molecules was 300 nucleotides; that only one poly(A) tail is added per molecule and that each 3' hydroxyl terminus is equally efficient as a primer for \textit{E.coli} polyA polymerase.

Double stranded cDNA was synthesised, end repaired, size fractionated and ligated into vector pUC19 (digested with SmaI) as described in Materials and Methods. Competent \textit{E.coli} cells (strain K12 RR\textsubscript{1}ΔM15, Rüther, 1982) were then transformed with ligated cDNA:pUC19 DNA. \textit{E.coli} strain K12 RR\textsubscript{1}ΔM15 is derived from \textit{E.coli} strain RR\textsubscript{1} and is deleted for its endogenous β-galactosidase gene, it does however harbour an episomal copy of a defective β-galactosidase gene on a F plasmid. Functional β-galactosidase enzymic activity can therefore be generated by intercistronic complementation between the defective episomally encoded β-galactosidase gene and the N-terminal portion of the β-galactosidase gene encoded by pUC19. Recombinant and non-recombinant colonies can therefore be distinguished when cells are grown on nutrient agar containing the lactose analogue BCIG in the presence of the inducer IPTG. In this way a library of 10\textsuperscript{12} recombinant clones was generated from 16 ng of cDNA ligated with 27 ng SmaI digested pUC19. The transformation efficiency of competent \textit{E.coli} K12 RR\textsubscript{1}ΔM15 cells ranged from 1-4 x 10\textsuperscript{7} transformants/μg supercoiled pUC19 DNA. The recombinants were stored as individual cultures in freezing medium in the wells of eleven microtitre plates as described in Materials and Methods.
Each plate contains 96 wells arranged as 8 rows (labelled A-G) of 12 wells (labelled 1-12). Individual clones, referred to in this text, were identified by their position on a particular plate (1-11). For example clone pA9-6, is therefore to be found on row A, column 9, plate 6 (also see the legend to appendix IV). Four control cultures were included on each plate; positions A1 and A4 were inoculated with cells harbouring the plasmid pXlr101 (Dawid and Wellauer, 1976) which contains a complete HindIII ribosomal genomic repeat from *Xenopus laevis*; position A2 was inoculated with cells harbouring the plasmid pUC19 and position A3 was inoculated with cells harbouring the plasmid pAT153 (see appendix IV).

4.3 **The ribosomal cDNA clones**

The library was screened by the colony hybridisation procedure described by Gergen (1979), which immobilises DNA on to Whatman 540 filter paper. The filters proved very durable, being successfully screened and washed in excess of 10 times without significant loss of signal strength. Initially the library was screened with the insert from the plasmid pxlr101 (Dawid and Wellauer, 1976). The presence of internal ribosomal positive controls, enabled a comparison of hybridisation signal strength to be made over all eleven plates. Approximately 60% of the cDNA clones showed a positive signal with the radio-labelled ribosomal DNA, although the signal strength did vary between individual clones. An assessment of signal
strength was made by eye and scored relative to the hybridisation strength observed with the control plasmids. Details of these results are given in Appendix IV.

Secondly, the library was screened with inserts prepared from members of the library which had previously been shown a positive signal with radiolabelled *Xenopus laevis* ribosomal DNA. Ligation of random DNA sequences into the Smal site of pUC19 does not usually reconstruct the Smal site. Inserts were therefore prepared by digesting the plasmid DNA with the restriction endonucleases EcoRl and PstI, they therefore also contain 37 bp of DNA derived from the pUC19 polylinker. Those members of the library which displayed a positive signal with a particular radiolabelled plasmid insert were grouped together and referred to collectively by the name of the original plasmid from which the cDNA insert was derived. The results of a series of such screens are described in Appendix IV and summarised in Table 4.1.

Of the 10 ribosomal clones described in Table 4.1, six are representative of groups which show no overlap between their respective populations. Two of the remaining populations, represented by pD4-11 and pG8-1, show an extensive overlap, 54 clones are common to both groups. This suggests that the 11 pD4-11 and 6 pG8-1 specific recombinants, which do not cross-hybridise, are derived from different regions of the RNA molecule, separated by the region which is common to both groups. For the two remaining populations, pG6-10 and pA10-8, 39 clones
Table 4.1. Summary of the experimental data relating to the cDNA clones derived from ribosomal RNA

The 10 cDNA clones described in Table 4.1 have been placed into groups according to the identity of those clones within the cDNA library to which they show cross-hybridisation. Therefore, the cDNA clone pH3-9 hybridises to 32 clones within the library, none of which can be detected by any other of the sequences examined, while the cDNA clone pAl0-8 defines a sequence which hybridises to 41 clones within the library. However, 39 of these clones are also identified by hybridisation with another clone, pG6-10. Together, therefore, 60 clones hybridise either to pA10-8, pG6-10, or both. Where available other data pertaining to these clones is also given. This includes the cDNA insert length as measured by gel electrophoresis or DNA sequencing, the identity of the ribosomal RNA type from which the cDNA was derived and the strength of cross-hybridisation with radiolabelled plasmid pxlr101 insert DNA. This latter figure was estimated by eye relative to the intensity of autoradiographic signal from the positive plasmid control (pxlr101) and the negative plasmid control (pUC19).

See also Appendix IV.
### TABLE 4.1

<table>
<thead>
<tr>
<th>CLONE</th>
<th>Signal Strength with Radiolabelled pXL101 insert DNA</th>
<th>Number of clones within cDNA library displaying cross-hybridisation to cloned insert</th>
<th>Insert size, EcoRI-Pst Digest (bp)</th>
<th>Northern Analysis - primary signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pA10-8</td>
<td>1</td>
<td>41</td>
<td>260</td>
<td>not determined</td>
</tr>
<tr>
<td>pG6-10</td>
<td>1</td>
<td>60</td>
<td>340</td>
<td>18 S rRNA</td>
</tr>
<tr>
<td>pD4-11</td>
<td>2</td>
<td>65</td>
<td>240</td>
<td>28 S rRNA</td>
</tr>
<tr>
<td>pG8-1</td>
<td>1</td>
<td>71</td>
<td>180</td>
<td>not determined</td>
</tr>
<tr>
<td>pH3-9</td>
<td>1</td>
<td>32</td>
<td>210</td>
<td>28 S rRNA</td>
</tr>
<tr>
<td>pE5-7</td>
<td>2</td>
<td>25</td>
<td>280</td>
<td>not determined</td>
</tr>
<tr>
<td>pA9-71</td>
<td>2</td>
<td>18</td>
<td>280</td>
<td>28 S rRNA</td>
</tr>
<tr>
<td>pA9-6</td>
<td>2</td>
<td>17</td>
<td>280</td>
<td>28 S rRNA</td>
</tr>
<tr>
<td>pG3-11</td>
<td>1</td>
<td>12</td>
<td>210 (119)*</td>
<td>28 S rRNA</td>
</tr>
<tr>
<td>pB7-7</td>
<td>1</td>
<td>6</td>
<td>240</td>
<td>28 S rRNA</td>
</tr>
</tbody>
</table>

* Brackets indicate that the nucleotide sequence has been determined, and that the cDNA insert size is accurately known.
Figure 4.2. Northern blot analysis of the ribosomal cDNA clones

5 μg of *Xenopus borealis* previtellogenic (A) and mature (B) ovarian poly(A)RNA was electrophoresed and blotted on to nitrocellulose filters as described in Section 2.19. The filters were then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardt's, 100 μg/ml Herring sperm DNA 5 μg/ml poly U, 37°C) with radiolabelled insert DNA, washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed, Section 2.3.3 for 16 hours.

The specific activity of the radiolabelled insert fragments is given below.

<table>
<thead>
<tr>
<th>Insert</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pG6-10</td>
<td>2.0 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>pH3-9</td>
<td>1.3 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>pA9-6</td>
<td>1.6 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>pB7-7</td>
<td>1.1 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>pD4-11</td>
<td>1.4 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>PE10-4</td>
<td>1.2 x 10^9 cpm/μg</td>
</tr>
<tr>
<td>pG3-11</td>
<td>0.7 x 10^9 cpm/μg</td>
</tr>
</tbody>
</table>
are common to both groups, while there are 19 pG6-10 specific clones but only 2 pAl0-8 specific clones. These observations also confirm that the nuclease digestion pattern of ribosomal RNA is not random, since apparently a discrete series of non-overlapping fragments have been recovered.

Seven of the ribosomal clones have been further analysed by Northern blot hybridisation to poly(A) RNA prepared from mature and previtellogenic ovaries (Table 4.1 and Figure 4.2). All but one show an intense hybridisation to a band that co-migrates with 28 S rRNA, but which appears to be more intense within poly(A) RNA derived from mature ovaries reflecting a greater contamination by 28S rRNA, probably due to the higher levels of 28S rRNA found in fully developed oocytes. The seventh clone, pG6-10, shows an intense hybridisation to a band which co-migrates with 18S rRNA suggesting that clone pAl0-8, which shows cross hybridisation to pG6-10, is derived from 18S rRNA. Unlike the 28S rRNA derived clones, the intensity of hybridisation appears to be the same in both poly(A) RNA preparations. All six clones also display a less intense hybridisation to a series of other bands, whose signal intensity and size distribution varies between individual clones. These minor signals reflect the existence of nicked rRNA molecules within the poly(A) RNA preparations as previously suggested by the sedimentation analysis at native and denatured ovarian RNA, section 3.2 (Figure 3.1). However, in an attempt to confirm this conclusion and to dispel any possibility that the minor
Figure 4.3 Southern blot analysis of the ribosomal cDNA clone, pG3-11

15 μg of *Xenopus tropicalis*, *Xenopus borealis* and *Xenopus laevis* genomic DNA was digested with EcoR I or Pst I, gel electrophoresed and southern blotted as described in Section 2.18. The filter was then hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardt's, 100 μg/ml Herring Sperm DNA 5 μg/ml poly U, 65°C) with radiolabelled pG3-11 insert DNA (specific activity 1.2 x 10^9 cpm/μg) washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed, Section 2.3.3, for 16 hours.

The migration of bacteriophage lambda HindIII digested DNA acting as DNA size markers is also indicated.
X. borealis  X. tropicalis  X. laevis  X. borealis  X. tropicalis  X. laevis

kbp

23.15
9.42
6.56
4.38
2.32
2.02
0.56

Eco R1  Pst 1
bands represent poly(A) RNAs containing interspersed repetitive elements which share sequence homology to ribosomal RNAs, radiolabelled pG3-11 insert DNA was hybridised to a Southern blot of EcoR1 and Pst1 digested Xenopus genomic DNA (Figure 4.3). In each case only a single band of hybridisation was detected, confirming that the sequence defined by the pG3-11 insert is only located on one EcoR1 or Pst1 endonuclease digested DNA fragment.

The nucleotide sequence of the ribosomal clone, pG3-11 and four members of the library which cross hybridise to pG3-11 (Table 4.1, Appendix IV) have been determined. The sequences have been arranged in such a way as to maximise sequence homologies between each clone and to a region of the Xenopus laevis 28S rRNA sequence, to which they show extensive homology, Figure 4.4. The DNA sequence homologous to the RNA from which the cDNA clone was originally derived is easily established, since the nuclease resistant RNA was modified by the addition of a poly(A) tail to the 3' end of the RNA. The length of the poly(A) tail displayed in Figure 4.4 is only an estimate since the long stretch of A residues causes imprecise termination during the sequencing reaction. However, it is believed that the figures given in Figure 4.4 are accurate to plus or minus 10 A residues. The length of the poly(A) tails are similar to that calculated from the poly(A) addition reaction as discussed previously (Section 4.2 and Appendix II).
Figure 4.4 DNA sequence alignment of five members of the pG3-ll family

The DNA sequences have been arranged to maximise sequence homology between each clone and to a region of the *Xenopus laevis* 28S rDNA sequence to which they show extensive homology. Each of the five cDNA sequences terminates in a poly dA region. This is derived from the polyadenylation of the RNA during the cloning procedure, as described in Section 4.2, and therefore defines the orientation of the cDNA coding sequence.
The size of the insert as determined by dideoxy sequencing (119 ± 10 bp) is somewhat smaller than the insert size determined by agarose gel electrophoresis (210 ± 30 bp), even after allowing for the extra 37 bp derived from the parental plasmid (pUC19). This discrepancy is probably due to the inaccuracies associated with determining insert length by the relative migration of the insert DNA to the migration of known DNA size markers by agarose gel electrophoresis.

It may be that the clones pD5-5 and pH8-4 are siblings, since they share exactly the same 3' and 5' termini and, as far as can be determined, an identical poly(A) tail length. However, the insert boundaries and poly(A) tail length of the other 3 clones are completely different and are therefore derived from independent cloning events. Except for the 5' terminus of clone pC9-6 and the 3' terminus of clone pD5-5 (and its sibling, pH8-4) the termini of all the clones are very close to each other, in fact plus or minus four nucleotides. This would suggest that in four independent cloning events a similar region has been selected as a nuclease resistant fragment.

The sequences, as displayed in Figure 4.4 are identical to a region of the *Xenopus laevis* 28S rDNA except at 3 positions where a single base and at 1 position where a two base deletion or insertion has to be made to allow maximal sequence homology.
4.4 The non-ribosomal clones

A number of cDNA clones have been identified as being non-ribosomal in origin by virtue of their non-cross hybridisation to radiolabelled pXlr1ol insert DNA. Four of these clones have been further analysed by screening the cDNA library with inserts prepared by digesting plasmid DNAs with the endonucleases PstI and EcoRl, as previously described in Section 4.3. The results of these screens are described in Appendix IV and summarised in Table 4.2. The four clones are represented between two and five times in the library, but as yet it is not known if any of the other positives are siblings of the original plasmid or are derived from independent cloning events. None of the populations identified overlap in any way.

"Mini" plasmid preparations were prepared from 83 non-ribosomal clones, and the DNA digested with the endonucleases EcoRl and PstI. Analysis of the digested DNA by agarose gel electrophoresis indicates that the average length of the liberated fragments was 240 bp (5.0 ± 60).

Of the 400 non-ribosomal clones, 19 have been analysed by Northern blot hybridisation to poly(A) RNA prepared from both mature and previtellogenic ovaries, Figure 4.5. The results of this study have been summarised in Table 4.3. Surprisingly a signal can not be detected with 10 of the radiolabelled cDNA inserts, even after a 5 day autoradiographic exposure. However, when Northern blots, hybridised with radiolabelled cDNA
Table 4.2. Summary of the experimental data relating to four cDNA clones derived from non-ribosomal RNA

Table 4.2 summarises some of the experimental data relating to the four non-ribosomal cDNA clones for which most experimental data has been collected.

All four display a level of cross-hybridisation to radiolabelled pxlr101 insert DNA similar to that observed with the negative plasmid control (pUC19). They also display cross-hybridisation to a small but unique family of other cDNA clones. All four cDNA clones have also been sequenced and these are described: pD9-1 Section 6.3, pC6-3 Section 4.4, pF8-6 Section 5.2 and pB5-6 Section 4.4.

See also Appendix IV.
<table>
<thead>
<tr>
<th>CLONE</th>
<th>Signal Strength with radiolabelled pxirlul insert DNA</th>
<th>Number of clones within cDNA library displaying cross-hybridisation to cloned insert</th>
<th>Insert size (bp)</th>
<th>Northern analysis size of transcript(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD9-1</td>
<td>0</td>
<td>5</td>
<td>450 (575*)</td>
<td>Abundant 300 base transcript</td>
</tr>
<tr>
<td>pC6-3</td>
<td>0</td>
<td>4</td>
<td>330 (292)</td>
<td>Abundant 410 base transcript</td>
</tr>
<tr>
<td>pF8-6</td>
<td>0</td>
<td>3</td>
<td>240 (149)</td>
<td>Two transcripts, 1230 of 860 bases</td>
</tr>
<tr>
<td>pB5-6</td>
<td>0</td>
<td>2</td>
<td>280 (316)</td>
<td>Single transcript 2600 base</td>
</tr>
</tbody>
</table>

* Figures in brackets indicate size of cloned cDNA insert as determined by DNA sequencing
Figure 4.5. Northern blot analysis of the non-ribosomal cDNA clones

5 μg of *Xenopus borealis* previtellogenic (A) and mature (B) ovarian poly(A)RNA was electrophoresed and blotted on to nitrocellulose filters as described in Section 2.19. The filters were then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardt's, 100 μg/ml Herring sperm DNA 5 μg/ml polyU, 37°C) with radiolabelled insert DNA, washed (0.2 x SSPE, 0.1% SDS, 65°C) and autoradiographed, Section 2.3.3.

The specific activity of the radiolabelled insert fragments and the length of autoradiographic exposures is given in Table 4.3.

The migration of *Xenopus borealis* ovarian ribosomal RNA markers is indicated.
Table 4.3. Summary of the data relating to the detection of transcripts homologous to the non-ribosomal cDNA clones

Radiolabelled DNA was prepared from a number of non-ribosomal cDNA clones and hybridised to poly(A) RNA prepared from previtellogenic and mature *Xenopus borealis* ovaries. Hybridisation was conducted under the conditions described in Figure 4.5. Table 4.3 summarises the data relating to the specific activity of the radiolabelled DNA (cpm/μg), the length of autoradiographic exposure required to detect a transcript and the estimated molecular size of that transcript relative to the migration of 28S and 18S ribosomal RNA.
### TABLE 4.3A

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specific Activity of Probe (cpm/μg)</th>
<th>Transcript Size (kb)</th>
<th>Autoradiographic Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD12-1</td>
<td>0.23 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pH10-1</td>
<td>0.23 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pB7-2</td>
<td>1.00 x 10^9</td>
<td>1.30</td>
<td>5 days</td>
</tr>
<tr>
<td>pC3-3</td>
<td>0.36 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pD12-3</td>
<td>0.50 x 10^9</td>
<td>0.99</td>
<td>5 days</td>
</tr>
<tr>
<td>pD5-4</td>
<td>0.29 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pH1-5</td>
<td>0.60 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pH8-5</td>
<td>0.87 x 10^9</td>
<td>2.75</td>
<td>5 days</td>
</tr>
<tr>
<td>pD10-6</td>
<td>1.10 x 10^9</td>
<td>1.61</td>
<td>5 days</td>
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<td>1.50 x 10^9</td>
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<td>5 days</td>
</tr>
<tr>
<td>pE2-8</td>
<td>1.06 x 10^9</td>
<td>N.D.</td>
<td>5 days</td>
</tr>
<tr>
<td>pG12-9</td>
<td>1.10 x 10^9</td>
<td>1.23</td>
<td>5 days</td>
</tr>
</tbody>
</table>

### TABLE 4.3B

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity of Probe (cpm/μg)</th>
<th>Transcript Size (kb)</th>
<th>Autoradiographic Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB5-6</td>
<td>0.70 x 10^9</td>
<td>2.60</td>
<td>4 weeks</td>
</tr>
<tr>
<td>pE10-7</td>
<td>0.45 x 10^9</td>
<td>1.75</td>
<td>4 weeks</td>
</tr>
<tr>
<td>pD9-1</td>
<td>2.5 x 10^9</td>
<td>0.30</td>
<td>90 minutes</td>
</tr>
<tr>
<td>pC6-3</td>
<td>1.86 x 10^9</td>
<td>0.41</td>
<td>90 minutes</td>
</tr>
<tr>
<td>pF8-6</td>
<td>1.30 x 10^9</td>
<td>1.28 and 0.86</td>
<td>18 hours</td>
</tr>
</tbody>
</table>
inserts prepared from either clone pB5-6 or pE10-7, were autoradiographed for 4 weeks, a single transcript could just be detected above the background radiation, Figure 4.5. The background signal from the remaining 5 Northern blots, from which a signal could not be initially detected, was significantly higher and they were therefore not autoradiographed for a longer period. Since the 19 non-ribosomal clones used in this study were chosen at random from the library, it is reasonable to assume that transcripts homologous to approximately \( \frac{9}{19} \times 400 = 47\% \) clones in all are represented in the poly(A) RNA preparations at levels which are below the detection threshold of the hybridisation procedure. Moreover, when signals could be detected, usually only a single transcript was observed, Figure 4.5, and the signal intensity appeared to be higher in poly(A) RNA prepared from previtellogenic ovaries. It is possible that this is due to different levels of ribosomal contamination, since it has previously been observed, Figure 4.2, that poly(A) RNA prepared from mature ovaries is more contaminated with 28S rRNA than poly(A) RNA prepared from previtellogenic ovaries.

The nucleotide sequence of the cDNA insert from clone pB5-6 has now been determined, Figure 4.6. The insert (316 bp) is slightly longer than that estimated from agarose gel electrophoresis (240 bp) and possesses a 36 bp poly(A:T) homopolymer at one end, thus establishing which strand is identical to the pregenitor RNA sequence. No homology was detected between the nucleotide sequence
The nucleotide sequence of the cDNA clone pB5-6 has been determined as described in Materials and Methods, Section 2.20. The sequence terminates in a poly(dA:dT) region. Since the RNA was polyadenylated enzymatically at the 3' end during the cloning procedure, as described in Section 4.2, the sequence displayed in Figure 4.6 is homologous to the RNA molecule present within the mung bean nuclease resistant RNA fraction described in Section 4.2.
AGAACTGACA GAATGGGCAG ACCTTCAGCA TACTGGATGA GCAACGGTTT
TCGAAAGAGA TCCTTCACAG ATACTTCAAG CACAGCAATT TGTCCAGCCT
TCAGCTTAAT AGTATGGCTT CCGTAAGGTG TGTGGTAGT TTGGAGAATG
GTCTTTGTAA AATCTGAGAG TGCGGCCCCG ACGTTTGAGA GCATAGACCA
CATCCATAGC GGTCAGGTCT TCCTCTTGGC GTGCTCGGTG TAGGXGACGG
CGTCCGGTAA CACATTCTCC AGGAAACTTC(A)36
of clone pB5-6 and the EMBL (Release 8) or Genbank (Release 40) nucleotide sequence databanks.

A single, low molecular weight transcript can be detected in ovarian poly(A) RNA with radiolabelled DNA prepared from the non-ribosomal cDNA clone pC6-3, Figure 4.7. However, unlike the non-ribosomal clones so far discussed, the transcript homologous to clone pC6-3 is very abundant in poly(A) RNA prepared from previtellogenic ovaries while barely detectable in poly(A) RNA prepared from mature ovaries, Figure 4.7. In longer autoradiographic exposures a band of the same molecular weight can be detected in the poly(A) RNA prepared from mature ovaries. Estimates, derived from densitometer scans of a series of autoradiographic exposures of increasing duration, suggest that the transcript is 20 times more prevalent in the previtellogenic ovarian poly(A) RNA preparation. The length of the low molecular weight transcript was more accurately determined by hybridising radiolabelled pC6-3 insert DNA to a Northern blot prepared by electrophoresis of total previtellogenic ovarian RNA through an 8%/7M urea polyacrylamide gel, Figure 4.7. The suRNAs, tRNAs and ribosomal RNAs present within the RNA preparation served as molecular weight standards. A single transcript was detected in ovarian RNA prepared from both *Xenopus tropicalis* and *Xenopus laevis*. Unfortunately the band of hybridisation is significantly larger than the RNA molecular weight markers, and therefore any estimate of transcript size will necessarily be inaccurate.
Figure 4.7. Northern blot analysis of the cDNA clone pC6-3.

Northern 1: 5 µg of *Xenopus borealis* previtellogenic (A) and mature (B) ovarian poly(A) RNA was electrophoresed and blotted on to a nitrocellulose filter as described in Section 2.19. The filters were then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 37°C) with radiolabelled pC6-3 insert DNA, washed 2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 60 minutes.

The specific activity of the radiolabelled pC6-3 insert DNA was $1.86 \times 10^9$ cpm/µg.

Northern 2: 20 µg of *Xenopus tropicalis* and *Xenopus borealis* total previtellogenic ovarian RNA was electrophoresed through a polyacrylamide gel and electroblotted on to a nitrocellulose filter as described in Section 2.19.2. The filter was hybridised and washed as described above and autoradiographed for 2 hours.

The specific activity of the radiolabelled pC6-3 insert DNA was $2.5 \times 10^9$ cpm/µg.
Figure 4.8. Southern blot analysis of the cDNA clone pC6-3.

15 μg of *Xenopus tropicalis*, *Xenopus borealis* and *Xenopus laevis* genomic DNA was digested with EcoRI, gel electrophoresed and southern blotted as described in Section 2.18. I would like to thank David Tannahill for the production of this southern blot filter. Subsequently I hybridised the filter (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 65°C) with radiolabelled pC6-3 insert DNA (specific activity, 1.5 x 10⁹ cpm/μg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 16 hours.

The migration of bacteriophage lambda HindIII digested DNA acting as DNA size markers is also indicated.
<table>
<thead>
<tr>
<th>km</th>
<th>X. borealis</th>
<th>X. laevis</th>
<th>X. tropicalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.15</td>
<td></td>
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</tr>
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<td>9.42</td>
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<tr>
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</tr>
</tbody>
</table>
Figure 4.9. DNA sequence of the cDNA clone, pC6-3

The nucleotide sequence of the cDNA clone, pC6-3 has been determined as described in Materials and Methods, Section 2.20. The sequence terminates in a poly(dA:dT) region. Since the RNA was polyadenylated enzymatically at the 3' end during the cloning procedure, as described in Section 4.2, the sequence displayed in Figure 4.6 is homologous to the RNA molecule present within the mung bean nuclease resistant RNA fraction, described in Section 4.2.

The DNA sequence is identical to a region of the *Xenopus borealis* histone H4 cDNA, pXbH4W1, previously described by Turner and Woodland (1982). The region of homology is also shown.
CCGCGTCTG GCCGCAGAG GTGGAGTTAA GCGCATCTCT GGCCTCATCT

ACGAGAAAC TCGCGGGGTG CTGAAAGTTT TCCTGGAGAA TGTTATCCGG

GACGCCGTCN CCTACACCGA GCACGCCAAG AGGAAGACCG TGACCGCTAT

GGATGTGGTC TATGCTCTCA AACGTCAGGG CCGCACTCTC TACGGTTTCG

GAGGTTAACG CATCGCTCCT CT(A)70
However, measurements indicate that the transcript is approximately 410 ± 50 nucleotides in length.

Radiolabelled pC6-3 insert DNA was also hybridised to a genomic Southern blot of Xenopus DNA digested with endonuclease EcoRl, Figure 4.8. The hybridisation pattern observed is unique to each species. A single major band (10.0 kbp) is detected in the genome of Xenopus borealis while two major bands are detected in the genome of Xenopus laevis (9.3 kbp and 5.9 kbp) in addition to a number of other less intense bands. By contrast, the hybridisation pattern observed with Xenopus tropicalis DNA is very complex, consisting of numerous bands, spread over a wide size range (13.5 kbp - 4.0 kbp).

The nucleotide sequence of clone pC6-3 has now been determined, Figure 4.9. The clone contains a 222 bp cDNA insert and a 70 bp poly(A:T) homopolymer at one end. A computer based sequence homology search indicates that clone pC6-3 is identical to a region of the Xenopus borealis histone H4 cDNA clone pc XbH4W1, previously described by Turner and Woodland (1982).

4.5 Discussion

In an attempt to analyse some of the properties of the sequences involved in the stabilisation of the multicomolecular networks formed by self annealing ovary A⁺ RNA a cDNA library was prepared from the nuclease resistant, double stranded RNA, after modification by addition of poly(A) homopolymer tails to their 3' ends. Calculations
described in Appendix III indicated that an average 86 adenosine residues were added per 3' hydroxyl end. The sequence of six independent cDNAs has been determined and in each case the length of the actual poly(A) sequence has been equal to, or less than, that calculated in Appendix III. The length of the poly(A) sequence of the cDNA clone will ultimately depend upon the position at which the oligodT primer bound. Since the length of the sequenced poly(A) tails are not greater than that calculated in Appendix III, the assumptions made during the calculations, outlined earlier in Section 4.2, are probably correct.

60% of recombinants in the library are derived from ribosomal RNA since they can be detected by hybridisation to radiolabelled pG3-101 DNA. They can also be subdivided into groups by virtue of their cross-hybridisation to radiolabelled DNA prepared from individual cDNA inserts sequences. Since a discrete series of non-overlapping fragments was recovered it seems probable that mung bean nuclease digests ribosomal RNA in a non-random manner. This has been confirmed in one case by comparing the nucleotide sequence of five clones which cross hybridise with ribosomal clone, pG3-11. Although two of these clones may be siblings, 4/5 have 5' boundaries within 3 nucleotides and 3/5 have 3' boundaries within 4 nucleotides. Thus the conditions used for mung bean nuclease digestion were capable of distinguishing regions of secondary structure in ribosomal RNA and
suggests therefore that the same is true for other RNA molecules including poly(A) RNA networks.

The cDNA library was screened with a number of inserts prepared from both ribosomal and non-ribosomal sequences, Table 4.1 and 4.2. In all 255 clones, 25% of the library, have been assigned to either of these two groups. Clearly any given ribosomal sequence is on average represented more frequently in the library than a non-ribosomal sequence. Therefore even though ribosomal sequences account for approximately 60% of the library, there are only a small number of different ribosomal sequence families. The 8 families described in Table 4.1 account for 241 out of the 600 ribosomal clones present, therefore 20 such families might be expected to account for all the ribosomal clones in the library. Consequently the non-ribosomal sequences although only accounting for 40% of the library, will represent many more families, perhaps as many as 100 (400/3.5), since any one sequence is represented far less frequently in the library than a ribosomal sequence.

As described previously (Section 3.2) ribosomal RNAs, in contrast to poly(A) RNAs, do not appear to undergo any significant intermolecular hybridisation. However, intramolecular forms of secondary structure have been observed within *Xenopus laevis* ribosomal RNA (Wellauer and Dawid, 1974). Indeed the position of these secondary structures are reproducible, enabling researchers to determine the processing pathway of the *Xenopus laevis*
40S ribosomal RNA precursor (Wellauer and Dawid, 1974). Furthermore, in the electron micrographs described by Wellauer and Dawid (1974), 28S rRNA possesses significantly more secondary structure than 18S rRNA, an observation supported by the results described in Figure 4.2 and Table 4.1, in which 6 of the 7 ribosomal families studied are derived from 28S rRNA.

A secondary structure model of *Xenopus laevis* 28S rRNA has been described by Clark et al. (1984). The *Xenopus laevis* 28S rRNA domain homologous to the region defined by the four members of the pG3-11 family is not predicted to be an area containing double stranded RNA. It is therefore unclear why this domain is represented in the library, unless the secondary structure model is incorrect.

A number of interspersed repetitive elements within the maternal RNA population of sea urchin egg poly(A) RNA have been described by Posakony et al. (1983). Characteristically, radiolabelled DNA prepared from these clones detect as many as 20 different transcripts when hybridised to a Northern blot of sea urchin egg poly(A) RNA. In an attempt to define *Xenopus borealis* interspersed repetitive elements by the same rationale, radiolabelled insert DNA was prepared from a number of non-ribosomal cDNA clones and hybridised to *Xenopus borealis* ovarian poly(A) RNA. Transcripts homologous to approximately half of the non-ribosomal clones were detected after an autoradiographic exposure of two to five days. However,
in all cases, so far discussed, only a single transcript was observed. None of the clones displayed a northern blot hybridisation pattern similar to these sea urchin interspersed retetitive sequences described by Posakony et al. (1983). It is therefore presumed that these sequences represent areas of intramolecular secondary structure within the poly(A) RNA transcripts. Transcripts homologous to the remaining clones could not be detected after a 5 day autoradiographic exposure. However, in two cases, clones pB5-6 and pE10-7, a single transcript could be detected after a 4 week exposure, suggesting that these sequences too are not representative of interspersed repetitive elements.

As has been noted previously (Dworkin and Dawid (1980), Golden et al. (1980), Krieg and Melton (1985)) a substantial fraction (perhaps 40%, Krieg and Melton (1985)) of the oocyte poly(A) RNA sequences are represented on low abundance transcripts. RNA-cDNA hybridisation kinetic studies, described by Perlman and Rosbash (1978), indicate that two different abundance classes of poly(A) RNA occur within the oocyte. Firstly, the moderately abundant class represent 1,100 different sequences (21 x 10^6 copies per oocyte) and secondly, the low abundance class represent 19,000 different sequences (1.3 x 10^6 copies per oocyte). These observations are in agreement with the data presented in Table 4.3, where transcripts homologous to 9 of the 19 (47%) non-ribosomal clones can only be detected after a 4 week
exposure, or cannot be detected at all.

One non-ribosomal clone, pC6-3 has been identified as a histone H4 cDNA. A low molecular weight transcript, 410 ± 50 nucleotides, can be readily detected in poly(A) RNA prepared from previtellogenic ovaries, however the amount of transcript is much reduced in poly(A) RNA prepared from mature ovaries. These observations agree with the published data available concerning histone H4 transcripts. Unlike somatic cell histone mRNAs, which belong exclusively to the poly(A)⁺ class, roughly half of the stored mRNA of amphibian oocytes is polyadenylated (Turner and Woodland (1982), Ballantine and Woodland (1985)). An almost full length *Xenopus borealis* histone H4 cDNA clone, pcXBH4W1, has been described by Turner and Woodland (1982), the cDNA is 384 bp long which agrees with the size of the transcript detected, as measured on a polyacrylamide gel. In a S1 nuclease protection experiment described by Ballantine and Woodland (1985), the level of histone H4 transcript also appears to be much reduced in stage 4-6 oocytes, when compared to the level of hybridisation detected with poly(A) RNA prepared from stage 1 and 2 previtellogenic oocytes. Documentation is also available concerning the genomic organisation of histone genes in *Xenopus borealis*, *Xenopus laevis* and *Xenopus tropicalis*. In *Xenopus borealis* at least 70% of the histone genes are present in a very homogeneous major cluster which contains all 5 histone genes and is at least 16.1 kbp in length (Turner and Woodland, 1983).
On a restriction endonuclease map presented by Turner and Woodland (1983) the histone H4 gene is situated on a single 9.9 Kbp EcoRl DNA fragment. This is in agreement with the observation that a single major 10.0 Kbp EcoRl histone H4 containing EcoRl genomic fragment was identified in Figure 4.8. It also appears that in *Xenopus laevis* there is an 8.9 Kbp major EcoRl fragment (Old et al. 1982, Van Dongen et al. 1981) containing all 5 histone genes and a 5.1 kbp EcoRl fragment which is not adjacent to the 8.9 kbp fragment, but is probably part of an unrelated cluster. This is in agreement with the pattern of hybridisation observed in Figure 4.8, where two major bands, 9.3 kbp and 5.9 kbp, were identified. The *Xenopus tropicalis* genomic histone organisation has also been described by Ruberti et al. (1982). Their observations, also in agreement with the results presented in Figure 4.8, indicate that although the 5 histone genes are clustered the EcoRl genomic fragments are highly heterogeneous in size. The similarities observed between the characteristics of clone pC6-3 and the *Xenopus borealis* histone H4 cDNA clone pcXbh4W1, positively identify pC6-3 as being a *Xenopus borealis* histone H4 cDNA.

According to the computer program developed by Zuker and Stiegler (1981) which constructs minimum free energy secondary structure maps the *Xenopus borealis* histone H4 mRNA can adopt a conformation with a high degree of secondary structure which would enable it to
remain nuclease resistant. In a similar way a domain of
the 28S rRNA, defined by the pG3-11 family, appears to be
nuclease resistant, since the same domain has been
recovered as cDNA clones on four independent occasions.
It therefore appears that the sequences represented in
the library are a specific subset of all sequences within
the ovarian poly(A) RNA population and probably represent
areas of secondary structure. Indeed for the cellulose/
ethanol column fractionation procedure to work correctly
these RNA's must be involved in either intramolecular
or intermolecular RNA-RNA interactions. While the cDNA
library contains sequences derived from moderately abundant
transcripts, e.g. Histone H4 (clone pC6-3) and 7S RNA
(clone pD9-1, discussed in Chapter 6) and others from
rare sequences, e.g. pC5-6 and pE10-7, none of the
19 clones studied (i.e. 19/110 families = 17%) shows
a pattern of hybridisation to RNA blots which is
indicative of a repetitive element. This does not
agree with previous observations (Anderson et al. 1982)
which suggest that 70% of poly(A) transcripts contain on
average 1.6 copies of repetitive sequence element with
an average size of approximately 300 bases (i.e. 480
bases of repetitive element per transcript). This is
equivalent to 15% of the total sequence present in the
poly(A) RNA \( \left( \frac{480}{2300} \times 0.7 = 15\% \right) \). Furthermore, data
described by Perlman and Rosbash (1978) suggests that a
maximum of 3% of cDNA derived from *Xenopus laevis* oocyte
poly(A) RNA is complementary to repeated DNA when \(^{3}\text{H}\) cDNA
was annealed to a vast excess of *Xenopus laevis* genomic DNA.
CHAPTER FIVE

MOLECULAR ANALYSIS OF THE CDNA

CLONE F8-6
5.1 Introduction

Analysis of a secondary structure cDNA library obtained from ovary polyA\(^+\) RNA indicates that transcripts homologous to approximately 40\% of the non-ribosomal clones cannot be detected easily by Northern blot hybridisation to total ovarian poly(A) RNA, while the remaining non-ribosomal clones detect only single transcripts. This latter class is therefore apparently derived from poly(A) RNA sequences represented only once in the RNA population and not from multiple sized transcript classes expected for repeated elements. It is therefore of interest to understand how such sequences have entered a cDNA library originally designed to select for intermolecular duplexes. Moreover, interspersed repetitive sequences are apparently absent from the set of non-ribosomal sequences which can be detected by Northern blot hybridisation. It is therefore of some concern that the cloning procedure outlined in Section 4.2 may have failed either for technical reasons during the handling of the DNA or RNA or because the assumptions on which the cloning strategy was developed were incorrect. The rational for the cloning strategy is the following:

(i) the large multimolecular complexes observed with the electron microscope are stabilised by intermolecular hybridisation between interspersed repetitive sequences, (ii) these sequences are mung bean nuclease resistant when double stranded while the majority of the multimolecular complexes are nuclease sensitive, (iii) these
Figure 5.1. Northern blot analysis of the cDNA clone pF8-6

**Northern 1:** 5 µg of *Xenopus borealis* previtellogenic (A) and mature (B) ovarian poly(A) RNA was electrophoresed and blotted on to a nitrocellulose filter as described in Section 2.19. The filters were then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 37°C) with radiolabelled insert DNA (specific activity 1.3 x 10⁹ cpm/µg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 16 hours.

**Northern 2:** as described above for northern 1, except that hybridisation was achieved in a solution containing 20% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 37°C). The specific activity of the radiolabelled insert DNA was 1.6 x 10⁹ cpm/µg, and the nitrocellulose filter was autoradiographed for 16 hours.
RNA duplexes remain double stranded and can be purified by cellulose/ethanol chromatography, (iv) these interspersed repetitive sequences, by analogy with those sea urchin interspersed repetitive sequences described by Costantini, et al. (1980) are an abundant component of the *Xenopus borealis* maternal RNA, enabling them to be detected by Northern blot hybridisation.

It was therefore decided to investigate one of the non-ribosomal sequences by obtaining and sequencing full length cDNA clones in order to understand the mechanism by which the original cloned sequence entered the secondary structure cDNA library.

5.2 **Cloning and sequencing of cDNA clones homologous to the transcripts detected by pF8-6**

Two transcripts homologous to pF8-6 can be detected in poly(A) RNA prepared from *Xenopus borealis* ovaries containing stage 1 and stage 2 oocytes, and ovaries containing mainly stage 5 and stage 6 oocytes, Figure 5.1. The approximate size of the transcripts was calculated relative to the migration of 28S and 18S rRNA run in a parallel track and indicates that the upper transcript is approximately 1230 bases in length while the lower transcript is approximately 860 bases in length. Both transcripts appear to be more abundant in the previtellogenic ovarian poly(A) RNA preparation, as has been previously observed for other non-ribosomal cDNA clones, Figure 4.5. By reducing the stringency of hybridisation and subsequent
washing conditions, a new low molecular weight transcript, approximately 300 bases in length, could be detected in the previtellogenic poly(A) RNA preparation, Figure 5.1. Nothing more is known about the low molecular weight transcript, but its appearance is reproducible, as is the preferential expression in previtellogenic oocytes.

A cDNA library in phage λ NM1149 equivalent to 6 x 10^6 phages was prepared from poly(A) RNA derived from Xenopus borealis ovaries containing mainly stage 5 and stage 6 oocytes, as described in Materials and Methods. A portion of this library, equivalent to 20,000 pfu, was screened with radiolabeled pF8-6 insert DNA. Three recombinant phages with homology to pF8-6 were detected. DNA sequences for these three lambda recombinants, λF8-6/2; λF8-6/3; λF8-6/4 and the original plasmid clone pF8-6, were determined, Figure 5.2. The cDNA inserts from λF8-6/4 and pF8-6 were small enough to allow the complete sequence of each to be determined by subcloning the entire insert into the M13 vectors mpl9 and mpl8. The cDNA insert from λF8-6/2 and λF8-6/3 were larger and the complete nucleotide sequences of both clones were determined by sequencing a series of overlapping endonuclease fragments subcloned into mp 19 and mp 18. The sequencing strategies employed for λF8-6/2 and λF8-6/3 are outlined in Figure 5.3. Analysis indicates that the nucleotide sequences of the original plasmid clone, pF8-6, and two lambda clones (λF8-6/2 and
Figure 5.2. DNA sequences of the plasmid cDNA clone, pF8-6 and the three lambda cDNA clones, λF8-6/2, λF8-6/3 and λF8-6/4

The nucleotide sequence of the cDNA clones was determined as described in Materials and Methods, Section 2.20.

The clone, pF8-6, results from the insertion of a cDNA molecule into the SmaI site of the plasmid pUC19, and only the DNA sequence of the portion is given.

The clones, λF8-6/2, λF8-6/3, λF8-6/4, result from the insertion of independent cDNA molecules into the unique EcoRl site of the lambda phage NM1149. The DNA sequences of the cloned inserts including the EcoRl cloning sites are given.
\textbf{pF8-6} \\
1 CTTTTGGAGG TTTGTGCAAT GCTAAGACTG GCTGGTTTTTC TTGAAGTCTG \\
51 AAATGGGAAGC TGAAGCAAAAA TCCTCATGCT GTGAAGCCTC AACGGGCAAA \\
101 CAACCTGTGC AACCAAAAGA ATCTGGATAA AAAAAAAAAA AAAAAAAAAA \\

\textbf{\lambda F8-6/2} \\
1 GAATTCATTG CTAAGACTGG CTGGTTTTTCT TGAAGTCTGA AATGGAAGCT \\
51 GAAGCAAAAAT CCTCATGCTG TGAAGCCTCA ACGGGCAAAC AACCTGTGCA \\
101 ACCAAAGAGA TCTGGATACC TGAATACTG CTGCTTTAAAA GTTACACAGA \\
151 TCTGGCCTGTT GAAATATGTG TTCAAGCCTTT TGAACACAGAT TCTGTCCTCA \\
201 GCCTGACTTT CCCCCAAAAAT CGAGACTGTA GGAACGTGGA GTCTAAAAAC \\
251 TCCCTATGGGC AGGCGTTTTTT TTTTGGAAAGAAAAAGCTTT GGCTGTCTTTG \\
301 CTCTGTCTCT ACTCGCGGTTT GCCCCTTAAA AACTACAGTG TGGCTGGGCC \\
351 TACACTCGGG CAGCAGATAT ACGGCAGTCT GCCAGGACGG ATGACATCAG \\
401 GAAGTCTCCT TCTGAAGCCAT GCGAAAAGG CAGCAAAGG AAGCAAGATG \\
451 ATCTTGAAATG AAGAGAGCAA CAAAGCTGGG TGGCTTTAAT GAATGAGGAT \\
501 CTCCAGATGG AGATGATGCA AGATGCTCCA ACTTCAAGAC ATGTAATAC \\
551 AGACACCGAT ACTGAAAGAC ACCATTCCAA AAATGACACT GAAAGTGATC \\
601 TTGAAGTTGG GGAATAAGAG GGGTTTGGGA TGCTGAAGGA AATCCCGACA \\
651 AAGAGATATG AAAACTGCT GTATGGAAGCC AATCGGAGTA CCACACAAAA \\
701 GTCAAGTCAT GAAAATCACA CCGATCAACA CCAAGACCAT AACTTTCTTG \\
751 CAGATAACTT CCTGGCATGC TTATCGCTTG GATGATGCTC CTTGAGATTTG \\
801 CAGATTTGGCA TGTGCTATTA ATGTAACGTA TGGACACTTC TCTTGCCAAC \\
851 TTGGTGTCTAA TGTATCATAA AATATTGCTC ACTGAAAAAA AAAAAAAAAA \\
901 AAAAAAAAAA AAGAATTC
\textbf{\( \lambda F8-6/3 \)}

\begin{verbatim}
1  GAATTCCCTT GAACACATAT TTAAGTGGTG CTTCAGTTC TTGAGGGTTT
51 GTGACTTACT TTGTCAATGC TAGTTTTTTT TTGTGGCCCTA AAATGGAATG
101 TGAAGCTAAA TCCCCACGCC GTGAGGCCTC AGAGAGCAAA CATCCTGTGC
151 AGACAAAAAGA ATCTGGGATAC CTAAAGTCAA TCTTCTTAAAG CGTTACTCAG
201 ATCTGGCCTC TAAATAATGT GTTCAAGATA TTGAAGAAGA TTCTGCTCCAT
251 AGCTGGACTTT TCTCCAAAAA TTGAGAAATG AGGAACTGCA GAGTCTAAAT
301 CTCCCTATGG CAGACGTTTT GTGATTGGAA GAAAAAGGCT TGGCCGCTTT
351 GCTCGTCTTC TGCTCTCCTT TGCCCCCTCA AGACTACAGT GTGCTCTGGG
401 CTACCCTCTCA GCAGAAAGTA TAGGGCAGTC TGCAGGGTCA GGAATTC
\end{verbatim}

\textbf{\( \lambda F8-6/4 \)}

\begin{verbatim}
1  GAATTCCTGA AATGGAAGCT GAAGCAAAAT CCTCATGCTG TGAAGCCTCA
51 ACGGGCAAAC AACCTGTGCA ACCAAAAGAA TCTGGATACC TGAAATCAGT
101 GGTCTTAAAA GTTACACAGA TCTGGCCTCT GAAATATGTG TTCAAGCTTT
151 TGAACAAGAT TCTGTCCCTA GCTGGACTTT CCCCAAAAAT CGAGACTGTA
201 GGAACCTGTTG AGTCTAAATG AATTC
\end{verbatim}
Figure 5.3. Strategies employed during the sequencing of the cDNA clones, λF8-6/3 and λF8-6/2

Elucidation of the complete DNA sequences of the cDNA clones, λF8-6/3 and λF8-6/2 was achieved by subcloning a series of overlapping DNA fragments into the M13 sequencing vectors mpl9 and mpl8, see Materials and Methods, Section 2.20.3. Such a strategy allows the sequence of relatively large fragments of DNA to be accurately determined in both orientations.

Figure 5.3 summarises the sequencing strategies employed for the two clones λF8-6/3 and λF8-6/2 and indicates the relative position of the various endonuclease recognition sites utilised. The arrows indicate regions over which reliable data was collected, from the subcloning site up to the position where the last unambiguous sequence data was recorded.
Figure 5.4. Nucleotide sequence comparison of the plasmid cDNA clone pF8-6 and the recombinant lambda cDNA clones, λF8-6/2 and λF8-6/4

Over certain nucleotide domains the sequences of the three cDNA clones, pF8-6, λF8-6/2 and λF8-6/4 are identical. These homologies are shown diagramatically in Figure 5.4. It is therefore believed that these three clones represent different domains of the same poly(A) RNA, the sequence of which is denoted as λF8-6/C.

For the purpose of this diagram, the EcoRI endonuclease cleavage recognition sequences, GAATTC, indicated in Figure 5.2, have been omitted since these sequences were not part of the original cDNA molecule synthesised from the poly(A) RNA. Likewise the 21 base poly(A) sequence present within the pF8-6 cDNA sequence has been omitted.
Figure 5.5. Nucleotide sequence comparison of
the cDNA clone λF8-6/3 and the
cconsensus sequence F8-6/C

Figure 5.5 diagramatically summarises the homology which exists between the nucleotide sequence of the lambda cDNA clone, λF8-6/3 and the consensus sequence, F8-6/C, derived from the homologies which exist between the plasmid clone pF8-6 and the lambda cDNA clone, λF8-6/2.
The longest open reading frame detected in the two sequences, extends from position 53 to position 767 in F8-6/C. A shorter open reading frame is also present in λF8-6/3, commencing at a position homologous to the open reading frame initiation site detected in F8-6/C. However, the λF8-6/3 clone sequence terminates before it reaches the translation stop codon detected in F8-6/C. 25 other open reading frames have also been
Figure 5.6  Summary of the DNA sequence comparisons aligned in Figures 5.4-5.5

This figure diagramatically summarises the sequence homologies between the four cDNAs, pF8-6, λF8-6/2, λF8-6/3 and λF8-6/14.

As has been shown in Figure 5.4, the sequences of the cDNAs, pF8-6, λF8-6/2 and λF8-6/4 probably represent different domains of the same nucleotide sequence, the position of these domains is indicated relative to each other. Similarly, the homology between λF8-6/3 and λF8-6/2 is also shown relative to the other two sequences.

As will be shown in Figure 5.7, the nucleotide sequence of the λF8-6/2 cDNA clone contains a long open reading frame. The relative position of the translation start and termination codons is indicated, as is the location of common ATG translation start codons in the other three sequences and their nucleotide positions.

As will also be shown later in Figure 5.8, an area of potentially important secondary structure has been identified within the nucleotide sequence of the sequence defined by λF8-6/3 and F8-6/C. The hairpin loop domain within λF8-6/3 is slightly larger than that observed within F8-6/C but they both occur within the same area as defined by the homology which exists between λF8-6/3 and F8-6/C (Figure 5.5). The extent of this domain is shown diagramatically as the foot of the figure.
Figure 5.7. Potential amino acid coding capacity of the cDNA clones λF8-6/3 and F8-6/C

The DNA sequence of F8-6/C contains a single large open reading frame. The entire potential amino acid coding capacity of the complete sequence within this reading frame shown opposite. The position of the potential translation start codon ATG is shown (*) as are the positions of any translation stop codons within the reading frame (given by small stars). Since the nucleotide sequence of F8-6/C also shows significant sequence homology to that of λF8-6/3, the potential amino acid coding capacity of this sequence within the same frame is compared with that of F8-6/C.
\( \text{XF8-6/3} \)

1. LNTYLSGASSFERFVTYFVIASFFLWPMDGEAKSPRASESKHPQVT

\( \text{F8-6/C} \)

1. FWRFVHC*DWLVFLKSEMAEAKSSCEASLASTGKPQPK

39

51. ESGYLKSIIFLSVQIWPVYFVKILKILKSIAGLSPIENVGTAESKSPY

100

40. ESGYLKSVVFLKVTQIWPVYFVKLKNKLSAGLSPIETVGTVESKSPY

89

101. GRRFVIGRRLGGRALLLSLAPQRLQCALGYSAGTSIGQASG

145

90. GRRFVIGRRLGCIFARLLLSLAPQLQCALGYSADSGQASGTDTE

139

140. PLKPCGKGSKRQDDLEVVEEOQSWVALMNEDELPPEDDEDDPTEPSNTDT

189

190. DSEEHSHKNDTESLIEEKEEVMLKESPAKKPEMVNGDPSNEEKS

239

240. TENTQDEHQHNSASH*LIACLSA*MVHLHVQICMCY*CNVWTLLPTFC

289

290. CINKYLSSLKSKKSKK

307
identified in F8-6/C, having a potential encoding capacity of between 4 and 54 amino acids. However, only two of all the potential initiating methionine codons are situated in a sequence environment similar to a consensus described by Kozak (1984, 1986), which has been shown to be the optimal sequence for initiation by eukaryotic ribosomes. The consensus sequence (PuXXATGG) is only observed at positions 53 and 677 of F8-6/C. It is not known if the open reading frames detected are used \textit{in vivo}. Nonetheless, the open reading frame initiating at position 53 has the capacity to encode a 238 amino acid protein of 26300 daltons molecular weight, while that initiating at position 677 has the capacity to encode a 30 amino acid polypeptide. In fact the shorter reading frame represents the carboxy terminal 30 amino acids of the larger 238 amino acid protein (Figure 5.6). Following the translation stop codon, TAA, common to both of these open reading frames, are 157 bp of untranslated 3' flanking sequence, which includes a polyadenylation consensus sequence AATAAA (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; Montell \textit{et al.} 1983), correctly positioned 12 bp upstream from the 28 bp poly(A) tail.

The amino acid sequences encoded by the open reading frames defined by the ATG methionine codon at position 53 in F8-6/C and at the homologous position in \(\lambda\)F8-6/3, nucleotide 87 are compared, Figure 5.7. The two potential amino acid sequences show 84% homology
from the methione until the end of \( \lambda F8-6/3 \), while 11 of the 19 amino acid substitutions are conservative with respect to amino acid chemistry. This region of amino acid coding sequence is very well conserved in contrast to potential amino acid sequences upstream from the methionine codon. Indeed, the \( \lambda F8-6/C \) amino acid sequence contains an in frame stop codon, making translation from contiguous upstream sequences impossible in this frame.

The nucleotide sequences of \( \lambda F8-6/C \) and \( \lambda F8-6/3 \) show no significant homology to documented sequences within either the Genbank (release 40) or EMBL (release 8) databanks. The amino acid sequences derived from the open reading frames, described above, also show no significant homologies to amino acid sequences contained within the NBRF (release 8) protein sequence databank.

Computer analysis employing a program developed by Zuker and Stiegler (1981) indicates that a portion of the nucleic acid sequences \( \lambda F8-6/3 \) and \( F8-6/C \) could adopt a conformation with a high degree of secondary structure. The predicted structures are shown diagramatically in Figure 5.8, and the AUG codon starting the open reading frames, described in Figure 5.6, are indicated. The "hairpin" structure observed in \( \lambda F8-6/3 \) sequence is bounded by an 18 bp inverted repeated, which itself possesses an imperfect diad symmetry, Figure 5.8. The free energy (\( \Delta G \)) which would be generated by the structures predicted for \( \lambda F8-6/3 \) and \( F8-6/C \) are -81.3 KJ and -45.7 KJ respectively. The domain of potential
A region within the sequences of F8-6/C and λF8-6/3 has been identified which has the potential to adopt a hairpin conformation. The domain within λF8-6/3 is much larger than that detected within F8-6/C and is bound by an 18 base pair inverted repeat which itself displays partial dyad symmetry (shown separately). Both sequences occur toward the 5' end of the cDNA sequences relative to the polyA tail detected within the λF8-6/2 sequence Figure 5.2 and 5.6.

The nucleotide positions from the 5' end of each sequence is indicated.
secondary structure detected in λF8-6/3 is larger than detected in F8-6/C and therefore more energetically stable. Unfortunately clones homologous to F8-6/C and λF8-6/3 which extend further upstream, are not yet available and it is therefore not known if the domains identified in Figure 5.8 represent the full extent of potential hairpin conformation within the RNA transcripts themselves. It is also not known if these "hairpin" structures occur in vivo, although it should be remembered that this is the same domain represented by the sequence of pF8-6, identified in the mung bean nuclease resistant cDNA library, Figure 5.6. Since the sequence of pF8-6 overlaps the nucleotide sequence of F8-6/C from position 1 to 128, pF8-6 encompasses the entire hairpin structure identified by the sequence folding program. If this structure occurs in vitro then the cloning procedure described in Section 4.2 is capable of cloning regions of double stranded RNA even though they may contain areas of sequence mismatch and thus is sufficient to have cloned double stranded repetitive elements had they been present.

5.3 Genomic sequences homologous with F8-6 and cross-hybridisation with Xenopus laevis and Xenopus tropicalis

Two fragments were detected by pF8-6 in Xenopus borealis genomic DNA digested with either PstI (11.6 kbp and 7.0 kbp) or EcoRI (5.9 kbp and 4.7 kbp), while
Figure 5.9. Southern blot analysis of the plasmid cDNA clone, pF8-6

15 µg of *Xenopus tropicalis*, *Xenopus borealis* and *Xenopus laevis* genomic DNA was digested with EcoRl or PstI, gel electrophoresed and Southern blotted as described in Materials and Methods, Section 2.18. The filter was then hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µl/ml Herring sperm DNA, 5 µg/ml poly U, 65°C) with radiolabelled pF8-6 insert DNA (specific activity 1.2 x 10^9 cpm/µg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 7 days.

The migration of bacteriophage lambda HindIII digested DNA acting as DNA size marker is also indicated.
Figure 5.10. Northern blot analysis of the cDNAs pF8-6 and λF8-6/2

Northern A: 20 μg of total (T), 20 μg of poly(A)$^-$ (1) and 2 μg of poly(A)$^+$ (2) RNA from various *Xenopus* ovarian sources was gel electrophoresed and northern blotted on to a nitrocellulose filter as described in Section 2.19. The RNA was prepared from either *Xenopus tropicalis* previtellogenic (A), *Xenopus borealis* previtellogenic (A) and mature (B) ovarian tissue. The filter was then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardt's, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 37°C) with radiolabelled λF8-6/2 insert DNA (specific activity 1.34 x 10$^9$ cpm/μg) and autoradiographed for 16 hours.

Northern B: As above except that the filter was hybridised with radiolabelled pF8-6 insert DNA (specific activity 0.9 x 10$^9$ cpm/μg) and autoradiographed for 16 hours.
a single fragment was detected in *Xenopus laevis* genomic DNA digested with PstI (2.2 kbp) or EcoRI (5.5 kbp). However, no homology to pF8-6 was detected with *Xenopus tropicalis* genomic DNA, Figure 5.9. A similar observation, reflecting the non-crosshybridisation of radiolabelled pF8-6 DNA to *Xenopus tropicalis* sequences has been obtained with *Xenopus tropicalis* ovarian RNA. Two transcripts homologous to both λF8-6/2 and pF8-6 were detected in total RNA, poly(A) RNA and poly(A)_- RNA prepared from either previtellogenic or mature *Xenopus borealis* ovaries, Figure 5.10A and Figure 5.10B. However, only a single transcript, comigrating with the 900 nucleotide *Xenopus borealis* transcript, could be detected in *Xenopus tropicalis* previtellogenic RNA hybridised with λF8-6/2 even after prolonged exposure, Figure 5.10A. Moreover, this single *Xenopus tropicalis* transcript was not detected when hybridised with radiolabelled pF8-6 DNA, even though both *Xenopus borealis* transcripts are clearly visible, Figure 5.10B. The sequence defined by pF8-6 is therefore absent, or diverged to the point of non-crosshybridisation, from both genomic and RNA sequences of *Xenopus tropicalis*, while the sequence defined by λF8-6/2, which includes the open reading frame identified in Figure 5.6 is clearly present in the *Xenopus tropicalis* ovarian RNA.

The amount of the two transcripts detected by pF8-6 appears to be constant between the two oocyte developmental stages so far analysed. However, a
Figure 5.11. Northern blot analysis of the cDNA clone pF8-6

5 μg of *Xenopus borealis* stage 1/2, stage 5/6 and *in vivo* matured oocyte poly(A) RNA was electrophoresed and blotted on to a nitrocellulose filter as described in Section 2.19. This filter was generously produced by David Tannahill. The filter was subsequently hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardt's, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 37°C) with radiolabelled pF8-6 insert DNA (specific activity 1.4 x 10⁹ cpm/μg), washed 2 x SSPE, 0.1% SDS 65°C) and autoradiographed for 16 hours.
significant change is observed in poly(A) RNA prepared from eggs following in vivo induction of ovulation by human chorionic gonadotrophin, Figure 5.11. The 1200 nucleotide transcript is no longer detected with radio-labelled pF8-6 DNA in the matured oocyte poly(A) RNA. The physical basis for this apparent differential transcript stability is not known.

5.4 Discussion

Three clones which cross to the cDNA clone, pF8-6 have been isolated from a phage lambda cDNA library prepared from intact *Xenopus borealis* ovarian poly(A) RNA. On the basis of sequence data these four clones can be organised into two sets, which it is believed are derived from two transcripts detected in both previtellogenic and mature *Xenopus borealis* ovarian poly(A) RNA. Although the nucleotide sequence of each set is unique, extensive sequence homology does exist between them over a region which also contains an open reading frame, encoding a 238 amino acid protein of 26300 dalton molecular weight. Upstream from the open reading frame the nucleotide sequences are more divergent and maximum sequence homology is obtained by the inclusion of three gaps into the primary sequence of one set. It seems unlikely that the region upstream of the potential initiating codon defining the reading frame identified in F8-6/C is translated since a stop codon is present 30 nucleotides upstream from this AUG. No significant homology was detected between either the nucleotide or the
hairpin sequence which may have properties which allow it to remain mung bean nuclease. Also \( \lambda F8-6/3 \) contains a similar but not identical 118 bp domain which could form a hairpin structure, bounded by a perfect 18 bp inverted repeat, which is itself an imperfect inverted repeat. The presumptive AUG start codon is positioned close to the apex of this hairpin. It is apparent that the nuclease digestion procedure outlined in Figure 4.1 may allow a certain degree of sequence mismatch to be tolerated, since the predicted secondary structure of F8-6/C (Figure 5.8) covers a region of 119 bases, of which 41 bases (34%) do not participate in Watson and Crick base pairing. Likewise, the secondary structure domain identified in \( \lambda F8-6/3 \) covers a region of 221 bases, of which 69 bases (31%) do not participate in base pairing. The region of potential secondary structure observed in F8-6/C is not as extensive as that detected in \( \lambda F8-6/3 \), but this may be a reflection of the fact that \( \lambda F8-6/3 \) extends further upstream than F8-6/C. It is not known if the hairpin conformations identified within F8-6/C and \( \lambda F8-6/3 \) exist in vivo or in vitro. However, the observations that the 5' terminal sequences of all three lambda clones terminate in close proximity to each other, and within the domain defined by the hairpin structure of \( \lambda F8-6/3 \), lends support to the existence of these structures in vitro, if such a secondary structure causes reverse transcriptase to terminate prematurely. Such structures would also account for appearance of
these sequences within the mung bean nuclease resistant cDNA library, and together with other forms of intramolecular secondary structure within poly(A) RNA transcripts may account for the majority of clones within this cDNA library, for which only one, or a limited number of transcripts can be detected.

pF8-6 sequences in both *Xenopus borealis* and *Xenopus laevis* do not appear to have undergone significant divergence, since they are detectable in the genomic DNA of both species, whereas a sequence homologous to pF8-6 is completely absent from the genome of *Xenopus tropicalis*. Since the pF8-6 sequence is also absent from the RNA transcript homologous to λF8-6/2 in *Xenopus tropicalis* ovarian poly(A) RNA this may indicate that the sequence has diverged significantly from the sequences detected in the other two species. Alternatively, the sequence domain represented by pF8-6 could have been completely lost from the *Xenopus tropicalis* genome following the genome duplication which occurred some 30 million years ago (Bisbee *et al.* 1977).

It is interesting to compare the absence of a pF8-6 like sequence from the 900 nucleotide *Xenopus tropicalis* transcript, but which can nevertheless be detected by λF8-6/2, with the observation concerning the divergence of the two sequence classes described for the pF8-6 containing *Xenopus borealis* cDNAs. The two *Xenopus borealis* sequences are most diverged towards their 5' end, whereas the sequences become progressively more
conserved toward their 3' end. This may reflect a greater selective pressure to maintain the amino acid sequences, while the 5' sequences are under less stringent pressure, to the extent that they can be completely absent or non-crosshybridising as in the case of the 900 nucleotide *Xenopus tropicalis* transcript.

It has also been observed that the 900 nucleotide and 1200 nucleotide transcripts detected in *Xenopus borealis* ovarian RNA and the 900 nucleotide transcript detected in *Xenopus tropicalis* ovarian RNA are present in both the poly(A) RNA and oligo(dT) cellulose flow through fraction. It has been previously demonstrated, Cabada et al. (1977), that two size populations of poly(A) exist in the poly(A) RNA population of *Xenopus laevis* ovarian RNA, one with a short poly(A) sequence (mean = 20A residues) and another with a long poly(A) sequence (mean=61A residues). Only poly(A)$_L$ RNA binds to oligo(dT) columns, whereas up to 50% of the poly(A)$_S$ RNA can be isolated from the flow through fraction by binding to poly(U) Sepharose. *In vitro* translation of both classes yielded identical patterns of *in vitro* protein products, suggesting that the two classes of poly(A) RNA are similar. These results are supported by the observations indicated above, since both *Xenopus borealis* transcripts even though slightly diverged are represented in both poly(A) RNA classes. Results presented also suggest that a segregation of the poly(A) RNA population into two subclasses may be a general feature of *Xenopus*
ovarian RNA, since the 900 nucleotide *Xenopus tropicalis* transcript is also detected within the poly(A) RNA and oligo(dT) cellulose flow through RNA populations.

Although the total pool size of both *Xenopus borealis* F8-6 transcripts stays constant during the later stages of oogenesis, a significant departure is observed following chorionic gonadotrophin induced maturation of oocytes. The upper 1200 nucleotide transcript is apparently lost from the population while the 900 nucleotide transcript remains at the same level. The molecular basis of this observation is unknown, although two possible explanations exist. Firstly, the upper transcript could be processed to a size equivalent to the smaller 900 nucleotide transcript. A sequence TTTCTTGAAGTC, similar to the consensus 3' intron accepter site $\text{PyPyPyPyPyPyPyPy}_{\text{C}}^{T} \text{AGG}_{\text{T}}^{G}$ (Breathnach and Chambon, 1981) is detected 6 bp upstream from the presumptive ATG translation start codon identified earlier in F8-6/C (Figure 5.6). Moreover, a further 21 bp upstream from the potential 3' accepter site is the sequence TGCTAAG, which is similar to the Lariat branch consensus sequence $\text{PyPyTPuAPy}$, (Ruskin et al. 1984). If processing were to occur at this site by the introduction of as yet unidentified 5' sequences, intron removal would result in the loss of all sequences 6 bp 5' to the ATG initiating codon. There are however a number of other potential 3' intron accepter sites, some of which have sequences upstream of them which resemble lariat consensus sequences, but all are downstream of the presumptive ATG
initiating codon and processing involving these sites would result in removal of potential amino acid coding sequences. Alternatively the RNA transcript could be lost from the poly(A) population by deadenylation and/or degradation of the transcript. It has been previously observed, Darnbrough and Ford (1979), that the amount of poly(A) 10 hours post germinal vesicle breakdown is approximately 35-50% of the amount prior to maturation. This is in line with the observed loss of one of the two transcripts from the poly(A) RNA preparation.
CHAPTER SIX

MOLECULAR ANALYSIS OF THE
cDNA CLONE pD9-1
6.1 Introduction

Molecular analysis of the cDNA clones described so far supports the belief that the nuclease digestion procedure outlined in Figure 4.1 has correctly selected for sequences which could adopt conformations rendering them resistant to nuclease digestion. It is also apparent that the procedure may also allow a certain degree of sequence mismatch within duplexed regions to be tolerated. Therefore if 70% of the poly(A) RNA transcripts contain interspersed repetitive sequences as described by Anderson et al. (1982) such sequences should be detectable within a cDNA library derived from the nuclease digested poly(A) RNA as described in Chapter 4, even if intermolecular duplexes contain regions of sequence mismatch. As discussed in Section 4.4, analysis of 19 non-ribosomal clones indicates that none of these clones have properties indicative of an inter­spersed repetitive element within the oocyte poly(A) RNA.

In this chapter I discuss a cDNA clone, pD9-1, a portion of which does display properties characteristic of a genomic repetitive sequence but which is only very rarely detected in the poly(A) RNA transcripts of the Xenopus borealis oocyte.

6.2 Detection of a transcript homologous to pD9-1

A single low molecular weight transcript can be detected within Xenopus borealis ovarian poly(A) RNA by radiolabelled pD9-1 insert DNA, Figure 6.1. Like the transcript detected by clone pC6-3 (Section 4.4),
Northern blot analysis of the cDNA clone pD9-1

Northern 1: 5 μg of Xenopus borealis previtellogenic (A) and mature (B) ovarian poly(A) RNA was electrophoresed and blotted on to a nitrocellulose filter as described in Section 2.19. The filters were then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 37°C) with radiolabelled pD9-1 insert DNA (specific activity $2.5 \times 10^9$ cpm/μg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 60 minutes.

Northern 2: 20 μg of Xenopus tropicalis and Xenopus borealis total previtellogenic ovarian RNA was electrophoresed through a polyacrylamide gel and electroblotted on to a nitrocellulose filter as described in Section 2.19.2. The filter was hybridised with radiolabelled pD9-1 insert DNA (specific activity $1.0 \times 10^9$ cpm/μg) washed as described above and autoradiographed for 2 hours.
The transcript detected by pD9-l is approximately 20 times more prevalent within poly(A) RNA prepared from ovaries containing only stage 1 and stage 2 oocytes, than within poly(A) RNA prepared from ovaries containing mainly stage 5 and stage 6 oocytes. This estimate was made by comparing the length of autoradiographic exposures required to obtain approximately equal signal intensities from each RNA sample.

In an attempt to determine more accurately the size of the transcript detected by pD9-l, RNA prepared from Xenopus borealis and Xenopus tropicalis previtellogenic ovaries was electrophoresised through an 8%/7M urea polyacrylamide gel, electroblotted on to a nylon transfer membrane and hybridised with radiolabelled pD9-l insert DNA as described in Materials and Methods. The presence of SnRNAs, tRNA and ribosomal RNAs within the ovarian RNA preparations served as internal low molecular weight size standards. A single 300 ± 30 base transcript was detected in both RNA preparations, Figure 6.1.

Since the different levels of pD9-l transcript detected within the two poly(A) RNA preparations may simply reflect differences in the extent of polyadenylation of these RNAs, radiolabelled pD9-l insert DNA was also hybridised to a northern blot containing poly(A) RNA, oligo-dT cellulose flow through RNA and total ovarian RNA prepared from Xenopus borealis and Xenopus tropicalis previtellogenic ovaries, and Xenopus borealis mature ovaries, Figure 6.2. Clearly, the majority of transcripts appear to be non-polyadenylated
Figure 6.2 Northern blot analysis of the cDNA clone pD9-1 and the cDNA subfragment D9-la

Northern A: 20 μg of total (T), 20 μg of poly(A)$^-$(1) and 2 μg of poly(A)$^+$ (2) RNA from various Xenopus ovarian sources was gel electrophoresed and northern blotted on to a nitrocellulose filter as described in Section 2.19. The RNA was prepared from either Xenopus tropicalis previtellogenic (A) Xenopus borealis previtellogenic (A) and mature (B) ovarian tissue. The filter was then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 5 x Denhardtts, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 37°C) with radiolabelled pD9-1 insert DNA (specific activity 0.9 x 10$^9$ cpm/μg) and autoradiographed for 5 hours.

Northern B: 20 μg of Xenopus tropicalis previtellogenic (A), Xenopus borealis previtellogenic (A) and mature (B) poly(A) RNA was gel electrophoresed and blotted on to nitrocellulose. The filter was hybridised with radiolabelled D9-la subfragment DNA (see Figure 6.3, specific activity 8 x 10$^9$ cpm/μg), washed as described above and autoradiographed for 2 days.
since the signal intensity observed in the flow through fraction was similar to that observed with total ovarian RNA but much higher than that observed with poly(A) RNA prepared from either *Xenopus borealis* or *Xenopus tropicalis* previtellogenic ovaries. Furthermore, a very much reduced signal was detected with *Xenopus borealis* mature ovarian total and oligo-dT cellulose flow through RNA, while no signal could be detected with the poly(A) RNA prepared from the same source. It therefore appears that although the amount of transcript homologous to pD9-1 detected with the *Xenopus borealis* mature ovarian poly(A) RNA was much reduced when compared to the *Xenopus borealis* previtellogenic ovarian poly(A) RNA, Figure 6.1 and 6.2, this is accompanied by a reduction in the overall level of D9-1 transcripts whether they be polyadenylated or not.

6.3 Sequence analysis of pD9-1

The nucleotide sequence of pD9-1 has now been determined, Figure 6.3. The 575 bp sequence is substantially larger than the 300 base transcript detected by northern blot hybridisation, Figure 6.1, and since the cDNA insert also contains a centrally located 85 bp polyA tract, it seems likely that the cDNA clone, pD9-1, has arisen from a double insertion of two independent cDNA molecules. Accordingly these subfragments have been renamed D9-1a for the larger 308 bp sequence, and D9-1b for the smaller 182 bp sequence.
The sequence of the cDNA pD9-1 has been determined and consists of a 575 bp insert with an internal poly(dA:dT) region.

For various reasons explained in detail within the text, the sequence has been divided into two regions D9-la and D9-lb. Practically these two regions can be separated by digesting the isolated D9-1 insert fragment with the endonuclease Hhal for which a unique cleavage signal exists 6 bp from the polyA sequence at the 5' end of D9-1b. A partial separation can also be achieved by digesting the isolated D9-1 insert fragment with Hinfl for which a unique cleavage signal exists 58 bp from the polyA sequence at the 5' end of D9-1b. This, however, results in a slightly larger DNA subfragment containing all of the D9-la subfragment sequence but also a portion of the D9-lb subfragment sequence.
A computer search failed to detect any significant homology between D9-la and the nucleotide sequences contained within either the Genbank (release 40) or EMBL (release 8) databases. However a closer examination of the D9-la sequence reveals that all six reading frames contain multiple stop codons, 5 of the possible 6 frames contain 3 translation stop codons while the sixth has 4 translation stop codons.

A computer search of the Genbank and EMBL databanks revealed that D9-lb shows significant homology to the 295 base Xenopus laevis 7SL cDNA described by Ullu and Tschudi (1984). D9-lb is not a complete 7SL cDNA and may have undergone a rearrangement relative to the Xenopus laevis 7SL sequence. The D9-lb nucleotide sequence extending from position 394-487 (Figure 6.3) is 78% homologous to the Xenopus laevis 7SL (Ullu and Tschudi, 1984) between positions 10-102, while between position 489-577 D9-lb is 88% homologous to the complementary sequence of the 7SL cDNA from position 184-96. After allowing for the possible molecular rearrangement, D9-lb is 83% homologous to the published Xenopus laevis 7SL cDNA sequence (Ullu and Tschudi, 1984).

The two subfragment sequences were separated by digestion with the endonuclease HhaI since a unique recognition site for this enzyme is situated 6 bp from the polyA sequence of the 5' end of D9-lb. In an attempt to quantitate the level of crosscontamination between the two subfragment preparations, gel purified D9-la and D9-lb DNA
Figure 6.4  Separation and purification of D9-la and D9-lb subfragments

As indicated in Figure 6.3 the pD9-1 insert can be subdivided into two fragments, D9-la and D9-lb by digestion with the endonuclease Hhal.

0.1 µg pD9-1 insert DNA (Lane 1), 0.1 µg pD9-1 insert DNA digested with Hhal (Lane 2) or 0.1 µg pD9-1 insert DNA digested with Hinfl (Lane 3) was gel electrophoresed and southern blotted on to a nitrocellulose filter. The filter was hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 65°C) with either radiolabelled D9-la subfragment DNA (Filter A, specific activity 0.8 x 10⁹ cpm/µg), or radiolabelled D9-lb subfragment DNA (Filter B, specific activity 0.6 x 10⁹ cpm/µg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 20 minutes.
1: D9-1 insert
2: " "
3: " "
digested with Hha1
digested with Hinf1

B
A
was prepared, radiolabelled and hybridised to pD9-1 insert DNA immobilised on to nitrocellulose filters, Figure 6.4. By comparing the intensities of hybridisation it was estimated that the D9-1a subfragment preparation contains approximately 2%-4% (w/w) D9-1b sequences. However, as far as can be determined, the D9-1b preparation is not contaminated by D9-1a sequences.

Northern blots of *Xenopus borealis* previtellogenic and mature ovarian poly(A)RNA were hybridised independently with radiolabelled D9-1a and D9-1b subfragment DNA, Figure 6.5. Clearly only the D9-1b subfragment preparation hybridises to the low molecular weight transcript detected by the full length pD9-1 insert DNA, confirming that the homology to the low molecular weight RNA is indeed limited to the D9-1b subfragment as suggested by the computer homology search. However, no transcript(s) can be detected when radiolabelled D9-1a subfragment DNA was hybridised under identical conditions.

Northern blots of *Xenopus borealis* previtellogenic and mature ovarian poly(A) RNA were also hybridised with radiolabelled strand specific DNA prepared from M13 dideoxy sequencing templates (Figure 6.5) as described in Materials and Methods. These two templates, D9-112 and D9-101, were prepared by digesting the pD9-1 DNA with EcoR1 and Pst1, purifying the insert DNA and recloning the pD9-1 insert sequence into the M13 sequencing vectors mpl8 and mpl9 which had been similarly digested with EcoR1 and Pst1. Therefore, although strand specific
Northern blot analysis of the cDNA clone pD9-1

**Northern 1:** 5 μg of *Xenopus borealis* previtellogenic (A) or mature (B) ovarian poly(A) RNA was gel electrophoresed and northern blotted as described in Section 2.19. The filter was then hybridised (50% formamide, 6 x SSPE, 0.1% SDS, 100 μg/ml Herring sperm DNA, 5 μg/ml poly U, 37°C) with either radiolabelled D9-1b subfragment DNA (specific activity 0.5 x 10⁹ cpm/μg) or radiolabelled D9-1a subfragment DNA (specific activity 0.6 x 10⁹ cpm/μg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 6 hours.

**Northern 2:** As above, but the filter was hybridised with strand specific probes, prepared as described in Section 2.10.2, from the M13 sequencing templates D9-112 or D9-101. Both sequencing templates contain the entire pD9-1 cDNA insert, but orientated in opposite orientations relative to the universal M13 dideoxy sequencing primer hybridisation site. The nitrocellulose filter was autoradiographed for 16 hours.
radiolabelled DNA prepared from these two templates contain complementary sequences, they both detect the same low molecular weight transcript previously detected by radiolabelled pD9-1 insert DNA and D9-1b subfragment DNA.

6.4 Genomic organisation of sequences homologous to the pD9-1 subfragments D9-la and D9-lb

Radiolabelled pD9-1 insert DNA was hybridised to a genomic southern blot of Xenopus DNA digested with either EcoRl or PstI. The detected hybridisation pattern is very complex, Figure 6.6. A series of bands of various sizes can be detected in both the Xenopus laevis and Xenopus tropicalis DNAs, while an intense smear of hybridisation can be detected in the Xenopus borealis DNA. Since it has been shown, Figure 6.3 and Figure 6.5, that only pD9-1 subfragment D9-lb shows sequence homology to 7SL RNA, suggesting that the two pD9-1 subfragments may have arisen from independent RNAs (discussed later in Section 6.6), identical genomic southern blots were independently hybridised with radiolabelled D9-la and D9-lb subfragment DNA, Figure 6.7. Clearly the hybridisation pattern observed in Figure 6.6 is a composite of the genomic organisation of the two subfragment sequences, D9-la and D9-lb.

The hybridisation pattern observed with radiolabelled D9-lb DNA is unique to each species and to the endonuclease with which the DNA was digested. As many as 10 bands of varying size (29.2 kbp to 1.9 kbp) are detected with
Figure 6.6. Southern blot analysis of the cDNA clone pD9-1

15 µg of *Xenopus tropicalis, Xenopus borealis* and *Xenopus laevis* genomic DNA was digested with EcoRl or Pstl, gel electrophoresed and Southern blotted as described in Section 2.18. The filter was hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 65°C) with radiolabelled pD9-l insert DNA (specific activity 1.2 x 10^9 cpm/µg) washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 16 hours.

The migration of bacteriophage lambda HindIII digested DNA acting as DNA size markers is also indicated.
Figure 6.7  Southern blot analysis with the cDNA subfragment D9-la and D9-lb

15 µg of *Xenopus tropicalis*, *Xenopus borealis* and *Xenopus laevis* genomic DNA was digested with EcoRI or PstI, gel electrophoresed and southern blotted as described in Section 2.18. The filter was hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 65°C) with radiolabelled D9-la subfragment DNA (Filter A, specific activity $0.44 \times 10^9$ cpm/µg) or radiolabelled D9-lb subfragment DNA (Filter B, specific activity $0.66 \times 10^9$ cpm/µg), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for 16 hours.

The migration of bacteriophage lambda HindIII digested DNA acting as DNA size markers is also indicated.
Xenopus borealis and Xenopus laevis DNA digested with either EcoRl or Pstl. However, the Xenopus tropicalis genomic organisation of sequences homologous to D9-1b differs in a number of ways. Firstly, fewer bands are detected in each digest, 5 bands in the EcoRl digested DNA and only 4 bands in the Pstl digested DNA. Secondly, unlike the pattern observed with either Xenopus borealis or Xenopus laevis, there does appear to be a major 17.2 kbp and a slightly less intense 20.1 kbp Pstl genomic fragment with homology to D9-1b, while no major EcoRl genomic fragment can be detected, 4 moderately intense bands (22.2 kbp, 18.2 kbp, 11.0 kbp and 6.9 kbp) are observed. Overlaying the various arrangement of bands detected in the EcoRl or Pstl digested Xenopus borealis or Xenopus laevis DNAs, there also appears to be a faint smear of hybridisation (290 kbp to 1.9 kbp), which is not detectable in the Xenopus tropicalis digested DNA.

Figure 6.7 clearly demonstrates that sequences homologous to D9-1a are reiterated and dispersed within the Xenopus borealis genome. An intense smear of hybridisation is detected over a wide range of DNA fragment sizes (20.1 kbp to 0.9 kbp). Furthermore, a far weaker smear of hybridisation is also detected within the EcoRl and Pstl digested Xenopus laevis genomic DNA. However, as far as can be determined, no homology exists between D9-1a and sequences within the Xenopus tropicalis genome.
Table 6.1 Estimate of the reiteration frequency of the repetitive element D9-la within the Xenopus borealis genome

Table A: Increasing amounts of Xenopus borealis genomic DNA were immobilised on to two strips of nitrocellulose. The filters were then hybridised (6 x SSPE, 0.1% SDS, 5 x Denhardts, 100 µg/ml Herring sperm DNA, 5 µg/ml poly U, 65°C) with either radiolabelled D9-la subfragment DNA or pG3-11 insert DNA (specific activities as indicated), washed (2 x SSPE, 0.1% SDS, 65°C) and autoradiographed for the periods indicated. An estimate of the reiteration frequency was obtained by comparing the intensity of autoradiographic signal recorded on the X-ray film for any given mass of genomic DNA. This was achieved by scanning the autoradiographic film and recording the output on an automatic chart recorder. The area under individual peaks is therefore proportional to the signal intensity. The peak area was measured by cutting out the area under peak and weighing the paper. The figures recorded in Table 6.1A are given in milligrams (mg) for the weight of paper. Since it requires approximately a seven fold longer exposure
time to achieve comparable signal intensities
the element defined by D9-la is represented
approximately 3500 (7 x 500) times within the
haploid genome.

Theoretically, providing the labelled
DNA is in excess over the number of target
genes on the filter and that the filter
can covalently bind all the DNA, the amount
of hybridisation is proportional to the
amount of DNA on the filter and a straight
line would be expected. Therefore if the data
presented in Table 6.1A is subjected to
linear regression analysis the slope of the
line is dependent on the number of target
genes within the DNA.

For pG3-ll  \( y = 4.809 + (2.430 \times x) \)
For D9-la  \( y = 1.374 + (2.728 \times x) \)
Where \( y \) = mass of paper (mg)
and \( x \) = amount of DNA (g)
The reiteration of the D9-la element at this
stringency is therefore equivalent to

\[
\begin{align*}
7 \times 500 & \quad 2.728 = 3929 \text{ copies per haploid} \\
2.430 & \quad \text{genome}
\end{align*}
\]
The filters described above were then rewashed (0.2 x SSPE, 0.1% SDS, 65°C). The filters were then cut up as to separate each area of immobilised DNA and the amount of radiolabelled DNA recorded by placing each piece of filter into a liquid scintillation counter. The figures given in Table 6.1B are therefore in counts per minute (cpm). The amount of hybridised radiolabelled D9-la DNA is approximately twice that recorded for radiolabelled pG3-ll insert DNA. Therefore at this stringency, sequences homologous to D9-la are represented approximately some 1000 times per haploid genome.

Similarly these figures can be subjected to linear repression analysis and the slope of the line is proportional to the number of genes within the DNA.

For pG3-ll \( y = 298 + (25.68 \times x) \)

For D9-la \( y = 468 + (67.47 \times x) \)

Where \( y = \text{cpm in hybrid (cpm)} \) and \( x = \text{amount of DNA in µg.} \)

The reiteration frequency of the D9-la element at this stringency is therefore equivalent to

\[
500 \times \frac{67.47}{25.68} = 1319 \text{ copies per haploid genome.}
\]
### TABLE 6.1A

Stringency = 2 x SSPE, 0.1% SDS, 65°C

<table>
<thead>
<tr>
<th>Radiolabelled DNA</th>
<th>Specific Activity (cpm/μg)</th>
<th>Exposure Time (hr.)</th>
<th>Mass of Immobilised <em>Xenopus borealis</em> Genomic DNA (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9-1a</td>
<td>1.4 x 10⁹</td>
<td>1</td>
<td>5.25 10.75 15 29.5 55.5</td>
</tr>
<tr>
<td>G3-11</td>
<td>1.38 x 10⁹</td>
<td>7</td>
<td>5 14.5 19.25 30 52.5</td>
</tr>
</tbody>
</table>

### TABLE 6.1B

Stringency = 0.2 x SSPE, 0.1% SDS, 65°C

<table>
<thead>
<tr>
<th>Radiolabelled DNA</th>
<th>Specific Activity (cpm/μg)</th>
<th>Mass of Immobilised <em>Xenopus borealis</em> Genomic DNA (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9-1a</td>
<td>1.4 x 10⁹</td>
<td>461 cpm 655 cpm 752 cpm 1269 cpm 1766 cpm</td>
</tr>
<tr>
<td>G3-11</td>
<td>1.38 x 10⁹</td>
<td>245 cpm 338 cpm 463 cpm 669 cpm 750 cpm</td>
</tr>
</tbody>
</table>
An estimate of the reiteration frequency of the repetitive element defined by D9-la within the *Xenopus borealis* genome was achieved by independently hybridising radiolabelled D9-la and pG3-11 insert DNA with serial dilutions of *Xenopus borealis* DNA immobilised on to nitrocellulose filters. Since the pG3-11 cDNA defines a region of the *Xenopus borealis* 28S rRNA (Section 4.3), which is itself repeated some 500 times within the genome (Birnstiel et al. 1972), a direct comparison of the length of autoradiographic exposure required to achieve identical signal intensities on an autoradiograph was used to estimate the reiteration frequency of D9-la relative to 28S rDNA. When such filters were washed at 2 x SSPE, 0.1% (w/v) SDS, 65°C and autoradiographed, it was recorded that filters hybridised with radiolabelled pG3-11 inserted DNA (specific activity, $1.38 \times 10^9$ cpm/µg) required a seven fold greater exposure time to produce a similar autoradiographic signal intensity to identical filters hybridised with radiolabelled D9-la subfragment DNA (specific activity, $1.40 \times 10^9$ cpm/µg) Table 6.1A. Therefore at this stringency, (2 x SSPE, 0.1% SDS, 65°C), sequences homologous to D9-la occur some 3500 times within the *Xenopus borealis* genome. The same nitrocellulose filters were then rewashed at a higher stringency (0.2 x SSPE, 0.1% SDS (w/v), 65°C), dried and counted in a scintillation counter, Table 6.1B. The amount of radioactivity detected with radiolabelled D9-la DNA with any given mass of immobilised *Xenopus*
borealis DNA was approximately twice that detected with the radiolabelled pG3-11 insert DNA. Therefore it appears that at a stringency of 0.2 x SSPE, 0.1% SDS, 65°C, approximately 1000 genomic sequences homologous to D9-la can be detected within the Xenopus borealis genome.

6.5 Detection of poly(A) RNAs homologous to the D9-la subfragment DNA within an ovarian poly(A) RNA preparation

The cDNA subfragment D9-la defines a sequence which is moderately reiterated and dispersed within the Xenopus borealis genome, Figure 6.7. However, under conditions sufficient to detect RNA homologous to D9-lb no transcript(s) homologous to D9-la could be detected within the Xenopus borealis previtellogenic or mature ovarian poly(A) RNA preparations, Figure 6.5, even though transcripts homologous to subfragment D9-la must be represented within ovarian poly(A) RNA since the cDNA pD9-1 was constructed from Xenopus borealis ovarian poly(A) RNA. Attempts were therefore made to detect D9-la related transcripts by increasing the amount of poly(A) RNA immobilised on the transfer membrane, preparing very highly radiolabelled D9-la DNA and increasing the length of autoradiographic exposure. In achieving just such conditions it was possible to just detect hybridisation to transcripts homologous to D9-la, Figure 6.2. A smear of hybridisation was detected within the Xenopus borealis mature ovarian poly(A) RNA preparation, 1.45 kb - 8.7 kb, relative to the migration of 28S and 18S rRNA. No transcripts homologous
to D9-la could be detected within Xenopus borealis or Xenopus tropicalis previtellogenic ovarian poly(A) RNA preparations. An extremely intense hybridisation to a low molecular weight RNA was also detected within the Xenopus tropicalis and Xenopus borealis previtellogenic ovarian poly(A) RNA, but not within the Xenopus borealis mature ovarian poly(A) RNA.

6.6 Discussion

Experimental evidence presented in this chapter suggests that the cDNA, pD9-1, has arisen from two independent sequences which have fortuitously coupled during cDNA cloning. Firstly, the genomic organisation of sequences D9-la and D9-lb is very different and therefore as far as can be determined sequences homologous to each subfragment are not generally represented on identical PstI or EcoRI endonuclease genomic fragments, although this is not excluded for a subset of these sequences. Secondly, transcripts homologous to D9-la and D9-lb show very different characteristics as far as abundance and transcript size are concerned. Finally, sequence analysis reveals the presence of an internally located 85 base poly(A) sequence. The length and position of this sequence is consistent with the hypothesis that it was synthesised on to the 3' end of the D9-la RNA prior to cDNA synthesis, following mung bean nuclease digestion, and subsequently blunt-end ligated with the D9-lb sequence to produce the cDNA sequence,
pD9-1. No poly(A) sequence associated with D9-1b was detected and presumably this sequence was lost during the synthesis of double stranded cDNA. Therefore for the purpose of this discussion the properties of these two sequences, D9-1a and D9-1b, are described independently.

The sequence defined by subfragment D9-1a represents a *Xenopus borealis* genomic repetitive element. The element is apparently represented on many differently sized genomic PstI or EcoRI endonuclease fragments and therefore suggests that sequences homologous to D9-1a are not generally clustered in tandem arrays like the OAX sequences (Ackerman, 1983 and Lam and Carroll, 1983a) or the 388 bp repeats (Lam and Carroll, 1983b; Garrett and Carroll, 1986) but dispersed throughout the genome in a similar fashion to the Alu repetitive sequences in primates and the "Alu equivalent" sequences or B1 sequences of rodents (reviewed by Rogers, 1986). Measurements indicate that at a stringency of 2 x SSPE, 0.1% SDS, 65°C approximately 3500 copies of the element can be detected in the *Xenopus borealis* genome. However, after more stringent washing (0.2 x SSPE, 0.1% SDS, 65°C) fewer sequences, approximately 1000 can be detected. This suggests that sequences within the *Xenopus borealis* genome homologous to D9-1a will display a certain degree of divergence from each other and therefore the subfragment D9-1a defines a series of related but not identical elements. Interestingly no homology is detected between
D9-la and *Xenopus tropicalis* genomic sequences, while only a very weak homology is detected between D9-la and *Xenopus laevis* genomic sequences. If this low level cross hybridisation between *Xenopus borealis* and *Xenopus laevis* genomic sequences simply reflected a lower copy number of D9-la sequences within the *Xenopus laevis*, one might expect resolution of individual EcoRl or Pstl endonuclease fragments to be visualised. However, since a weak smear of hybridisation is detected rather than individual bands, Figure 6.7, the observation suggests that interspersed repetitive sequences homologous to D9-la are present within the *Xenopus laevis* genome, but that they have undergone significant divergence, such that only a small percentage are now detectable by hybridisation to radiolabelled D9-la subfragment DNA. Interestingly no homology can be detected between D9-la and sequences within the *Xenopus tropicalis* genome. At this time it is impossible to determine the exact cause of this observation. It may reflect a difference in reiteration frequency within the *Xenopus tropicalis* genome; a divergence from the *Xenopus borealis* sequence, or a combination of both effects. Ryffel *et al.* (1981) have previously described a similar observation with a series of intron specific subclones derived from the *Xenopus laevis* vitellogenin A1 gene. Two subclones are highly reiterated within the *Xenopus laevis* genome and produce a smear with EcoRl digested genomic DNA. However, these same sequences, like D9-la, are completely absent from
the *Xenopus tropicalis* genome.

Although subfragment D9-la defines a *Xenopus borealis* interspersed repetitive element, transcripts homologous to D9-la are only very rarely detected within *Xenopus borealis* ovarian poly(A) RNA, Figure 6.2. Evidence suggests that the intense hybridisation to a low molecular weight transcript detected in Figure 6.2 is entirely due to the sequence homology between 7S DNA and the D9-lb subfragment, while the smear of hybridisation is detected in Figure 6.2 is due to hybridisation between radiolabelled D9-la and sequences homologous to D9-la within the poly(A) RNA population. Firstly, sequences homologous to D9-1b are present at only a limited number of genomic locations, while sequences homologous to D9-la are present many thousand times within the genome. It is therefore very unlikely that D9-1b sequences could be present within so many different poly(A) RNA transcripts. Secondly, no smear was detected when a northern containing *Xenopus borealis* ovarian poly(A) RNA was hybridised with D9-1b subfragment DNA, Figure 6.5. Finally, the low molecular weight transcript observed in Figure 6.2 is most prevalent within the previtellogenic ovarian poly(A) RNAs, reflecting the differential expression observed with radiolabelled D9-1b DNA, Figure 6.5. Since the D9-la subfragment preparation is known to be contaminated at a low level with D9-1b sequences (Figure 6.4) in preparing highly radiolabelled D9-la subfragment DNA significant levels of radiolabelled D9-1b DNA would also be synthesised
and therefore hybridisation to the low molecular weight transcript would most certainly be expected.

Transcripts with homology to D9-la are detected over a size range of 1.4 kb-8.7 kb, Figure 6.2 and are therefore skewed toward the larger transcripts found within the oocyte poly(A) RNA population which have an average size of 2.1 kb (Rosbash and Ford, 1974). A similar phenomenon has previously been documented by Posakony et al. (1983) in relation to the size distribution of sea urchin egg poly(A) RNA which possess homology to genomic interspersed repetitive elements. These transcripts occur over a range of 3-15 kb while the average size of sea urchin egg poly(A) RNA is 2-3 kb (Wilt, 1977). However, two major differences between the hybridisation pattern observed by Posakony et al. (1983) and that shown in Figure 6.2 are evident. Firstly, 10-20 prominent bands with homology to each element are detectable within the sea urchin egg poly(A) RNA, while no such pattern is observed within the *Xenopus borealis* ovarian poly(A) RNA. Secondly the sea urchin transcripts with homology to the genomic repetitive sequences are more prevalent within the sea urchin egg poly(A) RNA than the *Xenopus borealis* transcripts which show homology to the D9-la subfragment, which appear to belong to the rare class of poly(A) RNA transcripts described by Perlman and Rosbash (1978).

It is not known if sequences homologous to both strands of the repetitive element D9-la occurs within
poly(A) RNAs which then accumulate within the oocyte. Although considering the number of repeats detected within the genome and poly(A) RNA preparations it seems likely that they will. Nonetheless, although the D9-la nucleotide sequence from which the cDNA clone was originally derived is defined by the sequence given in Figure 6.3, the transcripts displaying homology to D9-la or its complementary sequence could not be translated through the region defined by D9-la as multiple translation stop codons are present in all six possible reading frames. A similar observation has previously been documented (Posakony et al. 1981 and 1983) in association with sea urchin genomic repetitive elements.

Although the D9-la subfragment defines a *Xenopus borealis* interspersed repetitive element the exact boundaries of this element are unknown. Such information requires the isolation and sequence comparison of a number of such sequences from a *Xenopus borealis* genomic library where these elements are to be found in reasonable abundance. This would also enable an estimate of the sequence divergence of the D9-la repetitive family to be determined. Indeed sequence information from the right hand and left hand terminal domains of the element may give some indications as to the mode of amplification by which this element has become dispersed throughout the genome.

Finally, a major difference is detected in the prevalence of transcripts with homology to the D9-la
subfragment DNA detected within poly(A) RNA prepared from ovaries containing only stage 1 and stage 2 oocytes and poly(A) RNA prepared from ovaries containing mainly stage 5 and stage 6 oocytes, Figure 6.2. The basis for this observation is unknown and must await experiments designed to determine the cytological location of these high molecular weight repeat containing transcripts.

The D9-lb subfragment has been tentatively identified as a *Xenopus borealis* 7SL cDNA. The sequence shows a very strong sequence homology to a portion of the *Xenopus laevis* 7SL cDNA described by Ullu and Tschudi (1984). However, the homology detected by computer search of the Genbank (release 40.0) and the EMBL databank (release 8.0) is split into two portions. The first (positions 394-487 of the pD9-l sequence, Figure 6.3) shows extensive homology to the 5' end of the *Xenopus laevis* 7SL cDNA between nucleotides 10-102 (Ullu and Tschudi, 1984). However, the second portion of the D9-lb sequence (positions 489-577) shows extensive homology to the complementary sequence of the *Xenopus laevis* 7SL cDNA between positions 184-96. There appears therefore to have been a rearrangement of the 7SL RNA sequence involving an inversion and a duplication. A small region of the proposed *Xenopus borealis* 7SL sequence, between nucleotides 96-102, and its complement appear in each portion of the *Xenopus borealis* cDNA subfragment D9-lb. It is not known if the proposed molecular rearrangement occurred prior to cDNA synthesis or during the cDNA
cloning procedure. Ullu and Weiner (1984), have described a number of the 500 7SL pseudogenes which occur in the human genome, two of these pseudogenes are polyadenylated at their 3' ends. It is therefore conceivable that polyadenylated transcripts homologous to 7SL pseudogenes, which may or may not have undergone some form of molecular rearrangement, could arise within the oocyte, promoted from the 7SL internal RNA Polymerase III promoter.

The purified D9-lb subfragment DNA shows homology to a 300 ± 30 nucleotide RNA, in good agreement with the documented molecular length (296 nucleotides) of *Xenopus laevis* 7SL RNA described by Ullu and Tschudi (1984), present within the poly(A)$^-$, as well as the poly(A)$^+$ previtellogenic ovarian RNA preparations. It is not known whether this is due to contamination of the poly(A) RNA preparation by non-polyadenylated 7SL RNA as has been previously described in relation to 28S and 18S rRNA, section 4.3, or by the presence of a small population of polyadenylated 7SL RNA within the ovarian poly(A) RNA. The retention of a significant fraction of 7SL has also recently been reported by Weiner et al. (1986) and the existence of 7SL pseudogenes with poly(A) tails within the human genome strongly suggest that a small fraction of 7SL RNA may well be polyadenylated in primates as well as in *Xenopus borealis*.

A reduction in the prevalence of 7SL RNA is detected within the total ovarian RNA and poly(A) RNA following the appearance of mature stage 6 oocytes within the ovary.
This may however simply reflect a dilution of 7SL RNA synthesised during oogenesis by the massive accumulation of 18S and 28S rRNA during the latter stages of oogenesis rather than a real reduction in the overall amount of 7SL RNA per oocyte.

The genomic organisation of sequences with homology to the subfragment D9-lb is species specific. The *Xenopus tropicalis* genome has by far the simplest organisation of the three species studied. There appears to be a single 17.2 kbp *PstI* repeating unit along with 3 other less major fragments. It is not known if this major repeat unit is scattered throughout the genome or arrayed tandemly. The genomic organisation of sequences homologous to D9-lb in both *Xenopus borealis* and *Xenopus laevis* appears to be slightly more complex consisting of numerous bands overlaid with a background smear. Ullu *et al.* (1982) describe the detection of Alu like elements within the *Xenopus laevis* genome. The primate Alu interspersed repetitive sequence monomer is believed to have arisen from 7SL RNA by the removal of a centrally located 155 bp domain. This region is therefore specific to the 7SL RNA. Hybridisation with this 7SL specific sequence from a human 7SL cDNA to EcoR1 digested *Xenopus laevis* DNA (Ullu *et al.* 1982) reveals a pattern of hybridisation identical to the pattern of bands detected in Figure 6.7. Moreover, hybridisation of EcoR1 digested *Xenopus laevis* DNA with that portion of the human 7SL cDNA which is also found as part of the primate Alu repeat reveals a smear
of hybridisation over a range of EcoRI fragments, Ullu et al. (1982).

The observation made in Section 6.4 (Figure 6.7) that hybridisation of radiolabelled D9-1b subfragment DNA to a genomic southern blot of *Xenopus laevis* DNA reveals a series of bands overlaid with a faint smear of hybridisation is in agreement with the observations of Ullu et al. (1982) since the D9-1b subfragment contains sequences homologous to both the Alu repetitive element and the 7SL specific regions of human 7SL RNA. The results presented in Figure 6.7 therefore confirm and extend the observations of Ullu et al. (1982) since Alu-like sequences are apparently dispersed within the *Xenopus borealis* as well as the *Xenopus laevis*, genomic sequences. Moreover, since a similar smear of hybridisation is apparently not detected with EcoRI digested *Xenopus tropicalis*, genomic DNA sequences homologous to those Alu-like sequences observed within the *Xenopus borealis* and *Xenopus laevis* genome (Ullu et al. (1982) and the observations presented in Figure 6.7), if present at all, can not be dispersed through the *Xenopus tropicalis* genome in the same way as in the *Xenopus laevis* and *Xenopus borealis* genomes.
CHAPTER SEVEN

DISCUSSION
7.1 Introduction

The *Xenopus* oocyte accumulates a large pool of poly(A) RNA during oogenesis (Golden et al. 1980). Since early embryonic development proceeds in the total absence of any RNA transcription until the mid-blastula transition, MBT, (Newport and Kirschner, 1982), the so-called maternal RNA pool is of vital importance in maintaining and supplementing the pool of proteins accumulated during oogenesis. For example, calculations indicate (Adamson, E.D. and Woodland, H.R., 1974) that the maternal pool of *Xenopus laevis* histone RNA is sufficient to equip the developing embryo until the mid-blastula transition (or approximately 10,000 nuclei). Following MBT the rapid synchronous cell division characteristic of early embryonic development is replaced by a slower asynchronous cell division and by the initiation of RNA synthesis (Newport and Kirschner, 1982). Histone requirement post MBT is therefore met by histone RNAs synthesised de novo. Measurements also suggest that only 4% of the maternal RNA pool is active in translation (Richter et al. 1984), while only 2% of the 10^{12} ribosomes present within the stage 6 oocyte are present within polysomes (Woodland, 1974). The mechanism of this translational block is unknown. It was therefore of great interest when results presented by Anderson et al. (1982) suggested that up to 70% of the maternal poly(A) RNA contained interspersed repetitive sequences and that these RNAs were apparently untranslatable (Richter et al. 1984).
1984) while the remaining 30% which apparently did not contain interspersed repetitive sequence elements could be translated in vitro.

The results presented in Chapters 3, 4, 5 and 6 describe the molecular analysis of a cDNA library prepared from a subfragment population of total ovarian poly(A) RNA designed to be enriched for such interspersed repetitive sequences. The significance of these results in relation to the abundance of interspersed repetitive sequences within the poly(A) RNA population and to the mechanism of the translational block are now discussed.

7.2 Discussion

The construction of a plasmid cDNA library designed to enrich for poly(A) RNA sequences participating in intramolecular or intermolecular associations has been described in section 4.2. Confidence in the cloning procedures ability to select for such sequences can only be sustained by a detailed molecular analysis of individual clones. Firstly, studies indicate that a group of clones, identified by cross hybridisation to the cDNA clone pG3-11, defines a specific domain of the *Xenopus borealis* 28S rRNA the boundaries of which are shared by the majority of pG3-11 group clones sequenced, Figure 4.4. Since the domain defined by the pG3-11 cDNA does not cross-hybridise with any of the other seven 28S rRNA groups defined in Section 4.3 it would, therefore, appear that the sequences represented within the library are a specific subset of all
sequences within the ovarian poly(A) RNA population. Secondly, a sequence defined by the cDNA pF8-6 exactly coincides with a region of an ovarian poly(A) RNA which by computer modelling (Zuker and Stiegler, 1981) has the potential to adopt a hairpin conformation. Finally, a sequence which defines an interspersed repetitive element, D9-la, has been cloned. Unfortunately since no other examples of this element have yet been sequenced and until such times as this has been achieved the 3' and 5' boundaries of this repetitive element can not be defined. Moreover, the effectiveness of the procedure to accurately select for those sequences participating in intramolecular and intermolecular associations will be clearly demonstrated by the comparison of the D9-la sequence with a complete genomic repeat for which the boundaries have been precisely defined. Since, if the procedure has been 100% efficient the 3' and 5' ends of the D9-la sequence would exactly correspond with the boundaries of the genomic repeat. Nonetheless, it is felt that because of the examples cited above the cloning procedure developed in Section 4.2 has correctly selected for sequences which do participate both in intramolecular and intermolecular associations.

While the majority of sequences recovered from the library are derived from 28S rRNA, approximately 400 non-ribosomal sequences have been identified. The vast majority of the non-ribosomal sequences have not been characterised to any degree. However, of those clones which have, two show significant homology to previously
characterised sequences. The cDNA, pC6-3 has been positively identified as a histone H4 transcript, Section 4.4, while the subfragment D9-lb is believed to be derived from a *Xenopus borealis* 7SL RNA, Sections 6.3 and 6.4. Molecular analysis of these two clones confirms and extends the previously documented data pertaining to these transcripts and their genomic organisation.

In all, 19 non-ribosomal cDNAs have been characterised by northern blot hybridisation. Transcripts homologous to 9 (47%) clones could only be detected after a 4 week exposure or not at all. Therefore these sequences are probably representatives of the low abundance class of oocyte transcripts identified by Perlman and Rosbash (1978), which represent 51% of the oocyte poly(A) RNA. More importantly, those remaining clones (10/19) derived from the moderately abundant class of oocyte transcripts, Perlman and Rosbash (1978), show homology to a single transcript within the ovarian poly(A) RNA. Therefore it is believed that these sequences are not members of dispersed repetitive sequence families, like subfragment D9-la, but come from intramolecular secondary structure conformations like the hairpin conformation detected within the cDNAs F8-6/C and F8-6/3. This is not to say that these transcripts could not themselves contain repetitive sequences, but that the region defined by any one of these clones is itself not a repetitive sequence within the ovarian poly(A) RNA. It is not known if such structures have any importance in relation to cellular control.
mechanisms (e.g. rates of translation, or initiation of particular transcripts) or even if such structures occur in vivo.

The results presented in Chapter 3 confirm that *Xenopus borealis* ovarian poly(A) RNA like *Xenopus laevis* oocyte poly(A) RNA (Anderson et al. 1982) and sea urchin egg poly(A) RNA (Constantini et al. 1980) will aggregate into large molecular complexes in a RNA C_0t dependant manner. Sedimentation analysis, Section 3.2, indicates that while ribosomal RNA and a certain portion of the poly(A) RNA do not apparently undergo any reassociation the greater proportion of ovarian poly(A) RNA does undergo a change in apparent molecular weight depending on the extent of renaturation. In association with this change in molecular weight, large multimolecular structures can be observed in the electron microscope, Section 3.4.

Like these complexes observed with renatured *Xenopus laevis* oocyte poly(A) RNA (Anderson et al. 1982) and sea urchin egg poly(A) RNA (Constantini et al. 1980) it is impossible to interpret most of the RNA complexes unambiguously. However, by reducing the sodium chloride concentration in the spreading hyperphase it is possible to relax the complexes into more easily interpreted structures. Under these conditions the complexes which remain are much reduced in size (Figure 3.3) and contain mainly intra-molecular foldback regions, only very rarely are inter-molecular duplexes observed. These observations are therefore
in disagreement with earlier reports (Anderson et al. 1982, Costantini et al. 1980) where up to 70% of Xenopus laevis oocyte poly(A) RNA and 65% of the sea urchin egg poly(A) RNA can be observed participating in multimolecular complexes even in the absence of sodium chloride. It seems unlikely that the apparent difference in complex stability observed in the presence or absence of sodium chloride reflects a genuine departure in the case of Xenopus borealis ovarian poly(A) RNA but rather an underestimation of the sodium chloride content of the spreading hyperphase by Anderson et al. (1982) and Costantini et al. (1980). Therefore a revision of the data supporting the existence of interspersed repetitive sequences as a major component of both sea urchin egg poly(A) RNA and Xenopus laevis oocyte poly(A) RNA is urgently required.

The existence of interspersed repetitive sequences within Xenopus laevis oocyte poly(A) by Anderson et al. (1982) was inferred by analogy with data (Costantini et al. 1980, Posakony et al. 1983) which demonstrates the existence of interspersed repetitive elements within the sea urchin egg poly(A) RNA and suggests that the multimolecular complexes observed within renatured poly(A) RNA are stabilised by intermolecular hybridisation between the interspersed repetitive elements. The only direct evidence presented by Anderson et al. (1982) for the existence of interspersed repetitive elements within the Xenopus laevis oocyte poly(A) RNA is the observation that approximately 20% of the renatured poly(A) RNA is nuclease
resistant and can be purified by cellulose/ethanol chromatography. While this figure is approximately twice that calculated in Table 3.2 (8.4%) for the nuclease resistant component of *Xenopus borealis* poly(A) RNA and that reported by Constantini et al. (1980) for the nuclease resistant component of renatured sea urchin egg poly(A) RNA (10%), the premise that this component is solely due to repetitive sequences is not supported by the molecular analysis of the cDNA library described in Chapter 4. If it is assumed that the abundance of any given sequence within the mung bean nuclease resistant fraction of renatured *Xenopus borealis* ovarian RNA is similar to its reiteration frequency within the cDNA library, then 60% of the nuclease resistant mass is derived from ribosomal RNA. Furthermore, since 12 of the 19 (63%) non-ribosomal cDNA clones studied by northern blot hybridisation detects only a single transcript, these sequences therefore do not define repetitive elements within the ovarian poly(A) RNA. Thus of the 8.4% mung bean nuclease resistant renatured *Xenopus borealis* ovarian poly(A) RNA (Section 3.5, Table 3.2) only 1.4% remains unaccounted for, this fraction representing these 7 non-ribosomal clones for which no transcript could be detected. Indeed, the only example of an interspersed repetitive element so far identified within the cDNA library, D9-la described in Chapter 6, is only very rarely represented within the ovarian poly(A) RNA, Figure 6.2. If this sequence is representative of
the major interspersed repetitive elements occurring within *Xenopus borealis* ovarian poly(A) RNA, interspersed repetitive sequences cannot be a major component of the ovarian poly(A) RNA. This conclusion is in agreement with cDNA:DNA excess hybridisation kinetic studies reported by Perlman and Rosbash (1978) which indicate that a maximum 3% of cDNA prepared from *Xenopus laevis* ovarian poly(A) RNA is complementary to *Xenopus laevis* genomic repetitive sequences, an observation which suggests that there are no major differences in the abundance of interspersed repetitive sequences within *Xenopus laevis* and *Xenopus borealis* ovarian poly(A) RNA. If other interspersed repetitive sequences are represented within the *Xenopus borealis* genome with a reiteration frequency similar to that described for D9-la, then it should be possible to detect cDNA clones homologous to such sequences by hybridising sheared (to approximately 300 bp) radiolabelled *Xenopus borealis* genomic DNA to the cDNA library in a way previously described in Section 4.3 and 4.4.

Whilst several authors, Constantini et al. (1980), Thomas et al. (1982) and Posakony et al. (1983), have clearly demonstrated that interspersed repetitive sequences do exist within the sea urchin egg poly(A) RNA, a significant difference is observed in relation to the abundance of interspersed repetitive elements as estimated by visual inspection of renatured poly(A) RNA and those estimates derived from other molecular techniques. While 65% of renatured sea urchin egg poly(A) RNA appears to
participate in multimolecular complexes (Constantini et al. 1980) only 11-16% of sea urchin egg poly(A) RNA hybridises to repetitive sea urchin genomic DNA (Constantini et al. 1980). The authors suggest that due to a number of technical difficulties this latter estimate (11-16%) is a serious underestimate of the proportion of transcripts which possess repetitive sequences.

Posakony et al. (1981) have previously characterised a number of sea urchin genomic repetitive sequences. These sequences detect 10-20 prominent maternal poly(A) RNAs of moderate to low abundance (Posakony et al. 1983). These transcripts are unusual in that they are very large, 3-15 kb and by virtue of their size more closely resemble hnRNA than polysomal poly(A) RNA. By way of contrast the average size of sea urchin egg poly(A) RNA is 2-3 kb (Wilt, 1977). Similarly Scouzo et al. (1974) have previously described sea urchin egg cytoplasmic poly(A)RNAs over 9 kb in length. Northern blot hybridisation has identified a number of transcripts (1.4-8.9 kb) within Xenopus borealis ovarian poly(A) RNA and with homology to the Xenopus borealis repetitive element, D9-1a (Chapter 6). Like the sea urchin repetitive sequences described by Posakony et al. (1981, 1983) the majority of these transcripts are somewhat larger than the average Xenopus laevis oocyte poly(A) RNA, 2.1 kb (Rosbash and Ford, 1974). If both the Xenopus borealis and Xenopus laevis interspersed repetitive elements are usually confirmed to ovarian poly(A) RNAs that in size resemble hnRNA, then experimental
data pertaining to the abundance of large poly(A) RNA molecules within the *Xenopus* oocyte maternal RNA pool might give an indication as to the proportion of transcripts harbouring repetitive sequences. Anderson and Smith (1977) demonstrate that the majority of newly synthesised oocyte hnRNA is rapidly turned over within the nucleus ($t_{1/2} = 30$ minutes), however a small proportion approximately 5% (equivalent to 0.1 ng/hr) enters the cytoplasm as large weight transcripts to supplement the maternal RNA pool. These transcripts are stable and turn over relatively slowly ($t_{1/2} > 90$ hours). If these transcripts equilibrate efficiently with the total pool of poly(A) RNA, since the total pool of poly(A) RNA remains constant (85 ng/oocyte, Golden *et al.* 1980), the flow of poly(A) RNA into the cytoplasm is met by an equivalent loss of RNA from the cytoplasm by degradation. If so, the total pool of cytoplasmic poly(A) RNA which resembles hnRNA by virtue of its size represents approximately 15% of the total poly(A) RNA. Interestingly, this figure is identical to the estimate of sea urchin egg poly(A) RNAs which harbour repetitive sequences (11-16%, Constantini *et al.* 1980) or determined by hybridisation to sea urchin genome repetitive sequences, but significantly less than the proportion of renatured sea urchin egg transcripts (65%) or *Xenopus laevis* oocyte transcripts (70%) which can be observed within multimolecular complexes in the electron microscope.
If, as is suggested by molecular analysis of the repetitive clone D9-la, interspersed repetitive sequences are relatively rare within the *Xenopus borealis* oocyte poly(A) RNA population, it is very unlikely that the multimolecular complexes observed within renatured ovarian poly(A) RNA are stabilised solely by inter-molecular hybridisations between interspersed repetitive sequences as suggested by Costantini *et al.* (1980) and Anderson *et al.* (1982). It is therefore unclear how such multimolecular complexes came to be stabilised.

It is, however, conceivable that intermolecular duplexes between interspersed repetitive sequences, perhaps as exemplified in Figure 3.3.D, act as nucleation sites for molecular associations but that the majority of the complexes observed in the presence of 0.25M sodium chloride (Figure 3.3.A) result from some other form of molecular association. If so, this secondary stabilisation can apparently be destabilised by reducing the sodium chloride concentration (Figure 3.3 B-D).

The observations discussed in Chapters 3 and 6 do not directly address the basis for the apparent untranslatability of the majority of oocyte poly(A) RNA (Richter and Smith, 1981, Richter *et al.* 1984) in relation to the observation that a portion of renatured *Xenopus laevis* oocyte poly(A) RNA which can be purified as complexed RNA
by cellulose/ethanol chromatography is apparently untranslated, while that portion which remains uncomplexed and can be purified as single stranded RNA is apparently translatable (Richter et al. 1984). If the abundance of the interspersed repetitive element defined by D9-la is typical of the majority of interspersed repetitive sequences within the ovarian poly(A) RNA population it is unlikely, as previously suggested by Richter et al. (1984), that the majority of untranslated maternal RNA represents poly(A) RNA with interspersed repetitive sequences. However, since the nucleotide sequence of D9-la does reveal the presence of multiple translation stop codons in all six reading frames, a characteristic of sea urchin interspersed repetitive sequences described by Posakony et al. (1981), those domains of the ovarian poly(A) RNAs displaying homology to D9-la (Figure 6.2) cannot be translated.

An understanding of the role, if any, of interspersed repetitive sequences within *Xenopus* oocyte maternal RNA pool in various aspects of embryonic developmental control is only at an early stage. The cloning procedure described in Section 4.2 was based on the premise that interspersed repetitive sequences are an abundant component of the *Xenopus* oocyte maternal RNA pool as suggested by Anderson et al. (1982) and that a cDNA library prepared from size fractionated mung bean nuclease resistant double stranded RNA would be highly enriched for such sequences. As has been demonstrated in Chapter 4, 5 and 6 this is clearly not the case and if the abundance of element D9-la is
typical of interspersed repetitive sequences within the oocyte poly(A) RNA population then such sequences are not an abundant component of the maternal RNA pool, perhaps accounting for 1/19 of the non-ribosomal cDNA clones, or 2% of the clones within the library.
APPENDIX I

The reassociation of nucleic acid sequences has for many years facilitated the molecular analysis of both sequences within the eukaryotic genome and within specific RNA populations. The environmental factors which influence the rate of reassociations have been described (Britten et al. 1974). The extent of RNA reassociation is a product of the initial RNA concentration, $C_0$ (Molarity) and the time elapsed (seconds). Since the rate of reassociation is also influenced by the sodium ion concentration the rate observed under the experimental conditions described in section 2.9.1 (0.75M Na$^+$) is, according to Britten et al. (1974), accelerated by a factor of 5.8157 relative to the standard sodium ion concentration (0.18 M Na$^+$).

The extent of RNA reassociation (RNA $C_0 t$) is therefore given by the equation

$$RNA\ C_0 t = M \times s \times 5.8157$$

where $M$ = nucleotide concentration (Molar)
$s$ = time (seconds)

The nucleotide concentration in all renaturation experiments was 500 $\mu$g/ml. If the average molecular weight of a RNA nucleotide is 302, then the initial RNA concentration was $1.6556 \times 10^{-3}$ M.

Following substitution and rearrangement the time in seconds required to achieve the desired RNA $C_0 t$ is described by the equation
= \frac{RNA \, C_{t}}{1.6556 \times 10^{-3} \times 5.8157}

Using this equation the incubation period, under the conditions described in Section 2.9.1, required to achieve any RNA $C_{t}$ can be calculated.

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<thead>
<tr>
<th>RNA $C_{t}$ (Ms)</th>
<th>Time period</th>
</tr>
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<tbody>
<tr>
<td>60</td>
<td>6231 (1 hr. 44 min)</td>
</tr>
<tr>
<td>600</td>
<td>62314 (17 hr. 18 min)</td>
</tr>
<tr>
<td>3000</td>
<td>311570 (3 days, 14 hr. 33 min)</td>
</tr>
</tbody>
</table>
APPENDIX II

The synthesis of cDNA as described in Section 2.11.1 was monitored by the incorporation of [\textsuperscript{3}H] dCTP into both the first and second strands.

First strand synthesis:

The total dCTP pool equivalent to $4.186 \times 10^{-8}$ moles is derived from both the radiolabelled dCTP (equivalent to $2.26 \times 10^{-9}$ moles) and the non-radiolabelled dCTP (equivalent to $3.96 \times 10^{-8}$ moles).

If the molecular weight of dCTP is 340 and if the C content of the Xenopus genome is 22%, then mass of cDNA synthesised in ng is given by:

$$x = \frac{t_1 - t_0}{t_t} \times 4.186 \times 10^{-8} \times \frac{340}{0.22} \times 10^9$$

where $t_0 =$ ctab precipitable [\textsuperscript{3}H] dCTP in 1 µl of reaction buffer at time zero (in cpm).

$t_1 =$ ctab precipitable [\textsuperscript{3}H] dCTP in 1 µl of reaction buffer at time 1 (in cpm)

$t_t =$ total [\textsuperscript{3}H] dCTP in 1 µl of reaction buffer (in cpm).

In one reaction the values for $t_0$, $t_1$ and $t_t$ were as follows: $t_1 = 5774$

$t_0 = 119$

$t_t = 344958$
The mass of cDNA (first strand) synthesised is thus given by

\[
\frac{5774-119}{344958} \times 4.186 \times 10^{-8} \times \frac{340}{0.22} \times 10^9 = 1061 \text{ ng}
\]

Described as a percentage of poly(A) RNA input this is equivalent to \(\frac{1060}{2000} \times 100 = 53\%\)

Second strand synthesis:

The synthesis of second strand cDNA was again monitored by the incorporation of radiolabelled dCTP C\(^{3}\text{H}\)dCTP into a ctab precipitable form.

In this reaction 645 ng of cDNA/RNA hybrid (equivalent to 322 ng of cDNA) was processed in a 100 \(\mu\)l reaction as described in Section 2.11.1. In this instance the total dCTP pool was equivalent to \(1.77 \times 10^{-8}\) moles. The mass of second strand cDNA synthesised (in ng) is given by:

\[
\frac{t_1 - t_0}{t_t} \times 1.77 \times 10^{-8} \times \frac{340}{0.22} \times 10^9
\]

The values of \(t_1\), \(t_0\) and \(t_t\) were as follows

\[
t_1 = 2433 \\
t_0 = 619 \\
t_t = 162835
\]

Therefore the mass of cDNA (second strand) synthesised was equivalent to
\[
\frac{2433-619}{162835} \times 1.77 \times 10^{-8} \frac{340}{0.22} \times 10^9 = 304 \text{ ng}
\]

Input mass of cDNA (first strand) = 322 ng.

Second strand synthesis was therefore equal to 94% \[
\frac{340}{322} \times 100 \text{ of the theoretical maximum.}
\]
APPENDIX III

The addition of polyA tails was monitored by the incorporation of radiolabelled $[^3H]$ATP into a ctab precipitable form.

The RNA on to which the polyA tails were to be polymerised was double stranded and was derived from a sephacryl S-300 column fraction with an average molecular size of approximately 300 base pairs.

An estimate of the polyA tail length can be calculated if the number of RNA molecules is known. If the average molecular size of the 1.8 µg of RNA is 300 bp, and it is assumed that the average molecular weight of a RNA base pair is 604, then the number of molecules is given by

$$\frac{1.8 \times 10^{-6}}{300 \times 604} = 9.934 \times 10^{-12} \text{ moles}$$

The number of ends available for polymerisation is therefore twice this figure, or $1.986 \times 10^{-11}$ moles.

The average length of the polyA tail is therefore defined as

$$\text{number of moles of ATP incorporated into ctab precipitable material}$$
$$\text{number of 3' hydroxyl ends (1.986 \times 10^{-11} Moles)}$$

The number of moles of ATP incorporated can be calculated as follows:

$$\frac{t_1 - t_0}{t_t} \times \text{total ATP pool}$$

where $t_0 = \text{ctab precipitable } [^3H] \text{ATP in 1 µl of reaction buffer at time zero (cpm)}$
t₁ = ctab precipitable $[^3H]ATP$ in 1 µl of reaction buffer at time 1 (cpm)

tₜ = total $[^3H]ATP$ in 1 µl of reaction buffer.

The total ATP pool, equivalent to $1.089 \times 10^{-8}$ moles, is derived from both the radiolabelled ATP (equivalent to $9 \times 10^{-11}$ moles) and the non-radiolabelled ATP (equivalent to $1.08 \times 10^{-8}$ moles).

The average poly(A) tail length is therefore given by

$$\frac{11898 - 315}{73419} \times 1.089 \times 10^{-8} \quad \frac{1.986 \times 10^{-11}}{1.089 \times 10^{-8}} = 86 \text{ A per end}$$
APPENDIX IV

Summarised in Appendix IV are the cumulative results of three experimental protocols described and referred to in Chapter 4.

First, the library was screened with radiolabelled DNA prepared from the plasmid pXlr101 (Dawid and Wellauer, 1976). The strength of hybridisation was recorded for each clone and given a score of 0, equivalent to the signal achieved with the negative control plasmids pAT153 and pUC19, to 5, equivalent to the signal achieved with the positive control plasmid pXlr101.

Secondly, an estimate of cDNA insert size was achieved by digesting "mini" plasmid preparations of selected clones with the endonuclease EcoR1 and Pst1. This was successful in liberating the cloned insert plus 37 bp of flanking polylinker DNA. The figures presented for insert length include the 37 bp of flanking, pUC19 derived, DNA.

Thirdly, radiolabelled DNA prepared from isolated DNA from individual cDNAs, was hybridised to the library. For the purpose of this appendix, each clone used in this final study has been represented by a simple number code (1-14) described below. Where a positive signal was detected the number code of the radiolabelled cloned insert is indicated.
<table>
<thead>
<tr>
<th>Code No.</th>
<th>Clone No.</th>
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<tr>
<td>2</td>
<td>p G6-10</td>
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<tr>
<td>3</td>
<td>p D4-11</td>
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<td>14</td>
<td>p B5-6</td>
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The diagrams have been arranged as to reflect the layout of the microtitre plates described in Sections 2.15.4 and 4.2. Information pertaining to any given clone is recorded at its position within the library. The information derived from the three experiments just outlined is arranged within each box as follows:

- **0 - 5**: Hybridisation signal strength with radiolabelled pXlr101 insert DNA
- **INSERT SIZE**: Insert size in bp (if known)
- **1 - 14**: Clones showing hybridisation to this cDNA
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**PLATE 1**
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- **Column B (pUC19)**: 6, 1, 6, 1
- **Column C (pAT153)**: 1, 4, 1, 4
- **Column D (pXl101)**: 1, 1, 210, 1
- **Column E (pAT153)**: 2, 2, 240, 2
- **Column F (pXl101)**: 1, 1, 210, 1
- **Column G (pAT153)**: 1, 1, 2, 180
- **Column H**: 8, 12, 1, 8
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**Notes:**
- **A:** pXlr101
- **B:** pUC19
- **C:** pAT153
- **D:** pXlr101

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**PLATE 5**

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- **PUC19**
- **pAT153**
- **pXlr101**

*Table columns labeled A to H, rows labeled 1 to 12.*

*Table entries include numbers, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, and letters, such as B, C, D, E, F, G, and H.*
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REFERENCES


