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Autoradiography of ovaries after ($^3$H)uridine incubation has provided general information about RNA synthesis at this time. Chromosomal RNA synthesis increases dramatically from leptotene, through pachytene, to diplotene. It is accompanied by a similar increase in nucleolar DNA synthesis. Thus, throughout amplification, a site within the replicating nucleolar DNA remains active in RNA transcription. Towards the end of pachytene, more than one site of RNA synthesis in the amplified DNA is visible. The number of sites increases further during early diplotene.

Biochemical investigations have shown that meiotic amplification is intimately dependent upon concurrent protein synthesis. Partial inhibition of ovarian protein synthesis leads to an equivalent inhibition of nucleolar DNA synthesis. This implies that the rate of amplification is normally limited by the rate of protein synthesis in the oocyte.

Similar studies using an inhibitor of RNA synthesis produced less clear cut results, though it is likely that RNA synthesis is less intimately linked with the amplification process.

The possibility that amplification occurs by an RNA-dependent mechanism of DNA synthesis has been investigated. Whilst not conclusively discounting such a mechanism, the results do not confirm any of the predictions of this hypothesis.

The exact molecular mechanism for amplification remains unknown. Various proposals are discussed, and an extrachromosomal mechanism is found to be most likely.
THE CYTOLOGY AND BIOCHEMISTRY OF DNA AMPLIFICATION
IN THE OVARY OF XENOPUS LAEVIS

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SUMMARY

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CHAPTER I

A review of the literature on gene amplification

(1) Introduction

The selective amplification of nucleolar DNA during oogenesis is now one of several well documented exceptions to the principle of genome constancy. For some time it was held that all differentiation in multicellular organisms occurs against the background of an unvarying genome. However, the discovery that polyploidy, chromosome diminution or elimination, and polyteny can all be valid differentiation mechanisms demonstrated that the organism is relatively free to carry out gross alterations in the somatic genome. Amplification is the most recently accepted of such genome modifications and is unusual in that it occurs in the germ line. The chromosomes destined for the zygote are preserved in an unmodified state by accumulating the excess nucleolar DNA extrachromosomally. The free multiple nucleoli containing amplified DNA are jettisoned by the nucleus at maturation.

(2) Amplification during oogenesis in Amphibia.

First reports of a build up of extrachromosomal DNA in amphibian oocytes were made as a result of cytological staining of "chromatin" and DNA in young ovaries of anurans (King, 1908; Painter and Taylor, 1943; Brachet, 1940). It was noted that Feulgen positive granules accumulate at the "bouquet" stage of meiosis
(pachytene), and are apparently present in free nucleoli in the lambrush stage. In older oocytes the nucleus enlarges to form a "germinal vesicle" which may be as much as 1.5mm in diameter. The multiple nucleoli in these oocytes can be released after manual isolation of the germinal vesicle. Using such preparations from urodèles, Miller (1964, 1966) and Kezar (1965) demonstrated that each nucleolus contains a circle of DNase sensitive material, and Ebstein (1967) showed that (\(^3\)H)actinomycin D, which is known to attach specifically to double helical DNA, will bind to a site in each nucleolar core. There was thus a correlation between the presence of extra-chromosomal DNA in pachytene oocytes and the presence of DNA in multiple extra-chromosomal nucleoli late in oocyte development.

The correlation was strengthened by a more detailed cytochemical analysis of early meiosis in *Xenopus* (Perkowska et al., 1966; Macgregor, 1968). It was established autoradiographically that pachytene nuclei indeed engage in extrachromosomal DNA synthesis in the absence of chromosomal DNA synthesis, and that each nucleus accumulates 30μg of DNA compared with a 4c chromosomal value of 12μg. The DNA is stable and is passed on to multiple nucleoli in early diplotene. In the adult *Xenopus* ovary there are about 1,500 nucleoli per germinal vesicle many of which contain several "cores", (Perkowska et al., 1968).

More direct proof of the nature of the extra DNA was possible by taking advantage of the distinctive properties exhibited by ribosomal DNA (rDNA), namely its abnormally high guanine + cytosine (G+C) content, and its ready hybridisation to rRNA once denaturated (Wallace and Birnstiel, 1966; Birnstiel 1967). By virtue of the high G+C
content it was possible to separate rDNA from the remainder by ultracentrifugation in CsCl solution. These properties were exploited to demonstrate that rDNA incorporates \( ^{3}H \) thymidine in Xenopus ovaries which contain many pachytene nuclei, and that this amplified rDNA persists in the adult ovary (Gall, 1968). Using DNA prepared from isolated germinal vesicles of three species of amphibia (both anuran and urodele), Brown and Dawid (1968) demonstrated that all possess a conspicuous satellite which hybridises with rDNA. From the levels of hybridisation under conditions of saturation, it was concluded that in Xenopus most, if not all, the 3Oµg of amplified DNA was rDNA. In addition it was found that maturation of the oocyte prior to ovulation is accompanied by the disappearance of all the multiple nucleoli and only the somatic level of rDNA is present in subsequently formed gastrulae. Evans and Birnstiel (1968) finally linked rDNA with multiple nucleoli in the adult ovary by isolation of nucleoli in bulk and characterisation of the DNA which they contained.

An unequivocal corroboration of these experiments was achieved through the development of a technique for hybridising RNA to DNA denatured in situ, i.e. in cytological preparations (Gall, 1969; Gall and Pardue 1969; John et al, 1969). When \( ^{3}H \) rDNA was used, autoradiography showed specific hybridisation to the "cap" of extra DNA in pachytene nuclei and to the forming nucleoli in diplotene oocytes. Alternatively, when \( ^{3}H \) RNA, synthesised in vitro upon the Xenopus mainband DNA using a bacterial DNA-dependent RNA polymerase, was hybridised, it was found that hybrid formation was now restricted to the chromosomes. Since only reiterated or amplified
DNA is capable of hybridising significantly under these conditions, it is possible to state that no significant amount of non-ribosomal DNA is present in the pachytene cap.

Apart from the extensive hybridisation to pachytene cells, \(^{3}\text{H}\)rRNA was also bound to the nucleoli of oogonia and prepachytene oocytes (Gall, 1969). Since no detectable hybridisation was found over somatic (follicle, blood, and connective) cell nuclei in these preparations, it was concluded that some amplification of nucleolar DNA occurs in oogonia. Similar levels of hybridisation have been found in spermatogonia, and in both cases the excessive rDNA is often associated with up to 12 multiple nucleoli (Pardue and Gall, 1971). Whether the production of these gonial multiple nucleoli is a single early event and is followed by replication and segregation of the extra DNA at each subsequent oogonial mitosis, or whether amplification of the nucleolus organiser region occurs throughout oogonial division is not, as yet, known.

The need for nucleolar DNA amplification in amphibian oocytes is readily justified. As pointed out by Gall (1968), ribosomes must be supplied to a mass of cytoplasm which in other tissues would contain several thousand nuclei, without interference in the balance of the maternal meiotic chromosome set. Biochemical and cytological evidence supports the view that multiple nucleoli are needed in the production of massive quantities of 18S and 28S rRNA. It is known that after vitellogenesis 98% of RNA synthesised and stored in the oocyte nucleus is rRNA (Davidson and Mirsky, 1965). At this time the nucleoli are arranged adjacent to the nuclear membrane and can be seen to incorporate \(^{3}\text{H}\)uridine very rapidly (Macgregor 1967).
Isolated nucleoli contain an RNA component which is similar in base composition to rRNA (Edstrom and Gall, 1963). Miller and Beatty (1969) obtained pictures of amplified DNA in the act of transcription through osmotic disaggregation of the nucleolar cores. Naked DNA segments, corresponding to "spacer" DNA (see Birnstiel et al, 1968), alternate with "matrix units" displaying many growing chains of presumptive 40S precursor. These pictures imply that the nucleolar rDNA is saturated with RNA polymerase molecules.

In addition to ribosomes required by the highly active, growing cytoplasm, it is likely that amplified DNA in the nucleoli also furnishes a storage pool of ribosomes to be used in the early stages of embryogenesis.

Amplified rDNA of Xenopus laevis has been rigorously compared with the somatic variety (Dawid et al, 1970). The need for this comparison was occasioned by the unusual bouyant density of amplified rDNA compared with somatic rDNA. Nucleotide analysis, coupled with hybridisation and thermal denaturation studies showed that the sole distinction between the two DNAs is the presence of a methyl group on 13% of the cytidylic acid residues of somatic rDNA.

Before leaving the subject of amplification in amphibia it is necessary to describe the unusual variation on this process which is found in the "primitive" anuran Ascaphus (Macgregor and Kezer, 1970). During the last 3 oogonial mitoses all nuclei remain within the same cell and give rise to an 8 nucleate oocyte. Each nucleus amplifies less than 5μg of DNA in contrast to the values per nucleus for Xenopus, Bufo, and Necturus of 27-30μg. However, the occurrence
of amplification in all 8 nuclei of an Ascaphus oocyte removes this discrepancy.

(3) Amplification in Insects

Apart from the amphibia, and Xenopus laevis in particular, amplification is well documented in some species of insect. Most completely studied in this respect are oocytes of the cricket, Acheta (Gryllus) domesticus which is a member of the order Orthoptera. The cytology of the process has been reported by a number of investigators (lima-de-Faria et al, 1968; Kunz, 1968; Cave and Allen, 1969) and it will be described here to illustrate the quite striking similarity to amphibian amplification.

Extra DNA is first detectable as several small Feulgen-positive bodies in oogonia. These bodies replicate and pass through oogonial mitoses, appearing ultimately as a single body at the leptotene stage of meiosis. During leptotene the peripheral DNA body increases in size. (3H)thymidine autoradiography shows that DNA synthesis is occurring within the body and is not synchronous with premeiotic S phase. Synthesis is complete at pachytene and the body assumes a less tightly packed, "puff-like" appearance. At this time an RNA containing "shell" is formed around the body and this is active in RNA synthesis. Synthesis continues into an arrested diplotene and the DNA body eventually disperses. It is possible that considerable RNA synthesis occurs after dispersal (Kato, 1968).

The presence of "nucleoli" inside the body suggested that amplification of rDNA might be occurring, and this has been confirmed
biochemically by Lima-de-Faria et al., (1969). DNA isolated from ovaries in which the DNA body is at its largest, is enriched for a high density satellite compared with testis DNA. Hybridisation with Acheta \(^{32}\text{P}\)RNA showed that this satellite contains the ribosomal RNA genes.

At this level there are few dissimilarities between amplification in Acheta and in Xenopus, the 2 organisms most completely studied in this respect.

The Acheta ovary is referred to as "panoistic" because the oocyte nucleus furnishes all the materials involved in differentiation of the egg. In this type of oogenesis lampbrush chromosomes and amplified nucleoli are always present in the oocyte (Bier et al., 1967). An evolutionarily later, and more sophisticated process is exhibited by "meroistic" ovaries. Here a series of oogonial divisions gives rise to abortive "nurse cells". These are closely connected with each growing oocyte and engage in heavy RNA synthesis on its behalf. The advantages which this mechanism bestows on the organism can be appreciated by the difference in time required to complete oogenesis in the panoistic ovary of Acheta, 100 days, and in the meroistic ovary of Calliphora, 6 days (Ribbert and Bier, 1969).

Several variations on the meroistic process have been described. Young oocytes in Dystiscid water beetles (Bauer, 1933; Bier et al., 1967) and in Tipulid flies (Lima-de-Faria and Moses, 1966; Bayreuther, 1956), whilst receiving RNA from nurse cells, also possess a conspicuous DNA body which behaves in many ways like that in Acheta. The body gives rise to numerous nucleoli at diplotene and has been shown,
in Dytiscids, to correspond to rDNA by CsCl ultracentrifugation and hybridisation with rRNA (Gall et al., 1969).

The blowfly Calliphora shows a more extreme form of meroistic oogenesis. The oocyte nucleus remains relatively inactive in RNA synthesis, the chromosomes being condensed in a "karyosphere". rRNA synthesis is entrusted to amplified nucleoli in the already polytene nurse cells.

Other species of insect for which there is some evidence of amplification are listed by Gall (1969).

(4) Other examples of amplification in oocytes

Extra copies of nucleolar DNA have been found in a fish, Roccus saxatilis (Vincent et al., 1968), and in two mussels, Spisula solidissima (Brown and Dawid, 1968) and Sepia officinalis (Ribbert and Kuns, 1969). The most "primitive" organism to show signs of amplification is the echinoid worm Urechis caupo (Brown and Dawid, 1968; Dawid and Brown, 1970). The level of amplification was found to be 6 times the level of rDNA in the sperm which is relatively low compared with other cases which have been observed. Accordingly, only a single nucleolus is visible in these oocytes. The starfish (an echinoderm) also possesses a single nucleolus in the oocyte, but does not show detectable amplification as judged by hybridisation (Vincent et al., 1968). To my knowledge this is the only report of amplification in oocytes being looked for, and not found.

To summarise, it is clear that amplification of nucleolar DNA in animal eggs is a widespread phenomenon. It argues for an unusually heavy requirement for the 28S and 18S rRNA components of
9. ribosomes during the differentiation of these cells. Presumably the complicated amplification procedure is advantageous because it brings oogenesis down in time to a level commensurate with that required to accumulate the other constituents of the mature egg. For example, in Xenopus nucleoli are seen to be transcribing at, or near, capacity (Miller, 1969) for much of oogenesis. Since multiple nucleoli contain rDNA equivalent to 3000-4000 chromosomal nucleolus organisers, and the meiotic chromosomes contain only 4 nucleolus organisers, we can conclude that, in the absence of amplification the mature oocyte would take many years, as opposed to several months, to differentiate.

The need for meiotic chromosomes to remain inviolate has meant that similar short cuts in the lambrush stage are not feasible. In this respect the development of the meroistic ovary of insects, in which the transcriptional capacity of nurse cells is harnessed to the cause of egg development, represents a considerable advance. The chromosomes in these abortive cells can be freely modified and there are several instances of both polyplaid (Basile, 1969), and polytene (Riebert and Bier, 1969) nurse cells. In Dytiscus and Tipula amplification of nucleolar DNA continues to be a feature of the oocyte rather than the nurse cell nucleus. This is not the case in Calliphora where, in addition to polytene chromosomes, there are free nucleoli in nurse cell nuclei which may be a product of amplification over and above that achieved by polytenisation. (The occurrence of nutrient cells for the growing oocyte is apparently universal though the extent to which they contribute to the storage pools in the egg cytoplasm varies (Raven, 1961). Thus even the
panoistic oocyte is surrounded by a layer of follicle cells and receives materials from them during vitellogenesis (Rees and van Weel, 1934). A similar situation occurs in all vertebrate ovaries.)

(5) Somatic amplification

There are some parallels between amplification of nucleolar DNA during oogenesis and the occurrence of repeated chromosomal DNA replication as exhibited by polytene nuclei in various somatic tissues of insects. Both occur in the absence of cell division and presumably serve to increase template availability for the transcription of RNA. However, "endoreduplication" involves virtually the whole genome (though centromeric heterochromatin does not take part (Gall et al, 1971)) and will not be considered here.

More akin to specific gene amplification is the "DNA-puffing" which has been observed in the polytene chromosomes of Rynchosciara and Sciara (Breuer and Pavan, 1955; Rudkin and Corlette, 1957; Swift, 1962; Crouse and Keyl, 1968). After polytenisation of the chromosome set in the salivary glands of these organisms, autoradiography reveals disproportionate synthesis of DNA at several specific bands. Feulgen microspectrophotometry has demonstrated that this synthesis results in quantities of DNA at these sites in excess of the polytene amount. Unlike germ line amplification the extra DNA remains at the site of synthesis and does not hybridise with rRNA (Gerbi, 1971). cRNA synthesised in vitro upon Satellites of reiterated DNA from Rynchosciara does
not hybridise either (Eckhardt et al, 1971). There is some biochemical evidence that amplification of transcriptionally active DNA has occurred in these puffs (Meneghini et al, 1971).

Predominantly nuclear RNA from post-puff salivary glands hybridises to a greater degree with post-puff DNA than pre-puff and ovarian DNA. Saturation hybridisation with rRNA showed that nucleolar DNA is not present in disproportionate amounts (Gerbi, 1971).

Brown and Dawid (1968) have remarked that specific gene amplification might occur in the differentiation of cells destined to produce large amounts of a single gene product. The differentiating oocyte is a case in point as the great majority of ribosomes synthesised by multiple nucleoli are stored in the egg cytoplasm. So far, however, amplification has not been detected in other examples of this kind of situation. In one case, the silk fibrin producing gland of a larval insect (Brown et al., unpublished), amplification has been shown to be absent. Preliminary results also indicate that in the nucleated erythrocyte of birds only between 1 and 9 copies of the globin gene are present (Bishop, unpublished). Histone messenger RNA isolated from sea urchin anneals equally well with the DNA from sperm and pluteus despite their different requirements for histones (Kedes and Birnstial, 1971).

Oocytes and spermatozoa represent unique examples of differentiation in multicellular organisms. The functional unit in these cases is a single cell, the egg or the sperm. Other functional units in multicellular organisms are the result of cooperation between millions of cellular units. It may be that in the face of a
selective pressure for larger quantities, or faster synthesis of a particular gene product, it is simpler to raise the number of stem cells destined for that function, than to incorporate a gene amplification mechanism in each component cell of the tissue responsible.

(6) The origin of amplified DNA in Xenopus

Two origins are possible for amplified rDNA in each generation

(1) The chromosomal nucleolus organizer region (Miller, 1966)

(2) A nucleolar DNA episome carried in the germ line (Wallace et al., 1971).

The second hypothesis was proposed to explain certain aspects of nucleolar behaviour during oogenesis which have been discovered in Xenopus; notably the occurrence of extra nucleoli in both oogonia and spermatogonia without obvious justification, and the very great variation in size of the DNA circles from multiple nucleoli (Miller and Beatty, 1969). Wallace et al suggested that both these phenomena would be expected if this rDNA was always episomal and separate from the chromosomal rDNA. They put forward the following scheme: At maturation the DNA from multiple nucleoli is released into the egg cytoplasm. There it is sequestered in the region in which the germ cells will arise and may act as a germ cell determinant. As germ cells appear the episomal DNA is distributed amongst them, and upon entering the nucleus is capable of replication and organisation of functional nucleoli. At pachytene, amplification occurs using this DNA as a template.

Brown and Blackler (in the press) have tested these two
alternatives by the use of interspecies hybrids between *Xenopus laevis* and *Xenopus mulleri*. Ribosomal DNAs from these species can be distinguished biochemically, and it was found that in all progeny of hybrid crosses, only *laevis* rDNA was amplified. This remained so whichever species was maternal. The episomal theory requires that amplified DNA should be maternally determined since the sperm carries negligible cytoplasm and no detectable rDNA in excess of the haploid amount. Since this requirement is not fulfilled, it is difficult to countenance an episomal theory of this kind, and it may be concluded that amplification in each egg originates at the chromosomal nucleolus organiser.

### 17) The mechanism of amplification

The molecular mechanism which is responsible for amplification is discussed later in this thesis.

The various proposed mechanisms are outlined in the discussion following Chapter II. The mechanism proposed by Tocchini Valentini and Crippa (1971) is the subject of experimental work in Chapter VII and is there described in detail.

In the general discussion at the end of this thesis the mechanism is considered in the light of results reported here. Some anomalies which must still be explained are presented, and tentative explanations are suggested.
CHAPTER XI

The cytology of amplification: an autoradiographic study of the morphological sequence and timing of events during early meiotic prophase.

Introduction

Amplification of nucleolar DNA in amphibia has been the subject of several cytological studies. Macgregor (1968) has extended early studies by Painter & Taylor (1942) and Brachet (1940) by a combination of Feulgen microspectrophotometry and \(^3\)H-thymidine autoradiography of squash preparations from young ovaries. He found that about 30 \(\mu g\) of DNA are synthesised in the pachytene oocyte. Conclusive evidence that this DNA is nucleolar was supplied by the technique of cytological hybridisation. When squash preparations containing a high proportion of pachytene oocytes were exposed to \(^3\)H-RNA, after denaturation of their DNA, under conditions favourable to RNA/DNA hybrid formation, hybridisation was specific to the pachytene cap (John, Birnsteil & Jones, 1969; Pardue and Gall, 1969; Gall 1969).

Cytological hybridisation has also demonstrated the equivalent of 20-40 nucleolus organisers in oogonia, and pre-pachytene oocytes. This suggests that amplification is not unique to pachytene cells. There are also reports of occasional \(^3\)H-thymidine incorporation into non-S phase oogonial and leptotene nucleoli (Gall, 1969). In order to put these findings in some perspective, it was decided to study in some detail the timing and sequence of events during early meiotic prophase in the oocyte.

The timing of early meiosis may also yield an exact duration for the major amplification phase which occurs during pachytene.
Any investigation of the mechanism of amplification must necessarily take into account the length of time required for the accumulation of 30 μg of rDNA per nucleus. It is possible that this knowledge might allow discrimination between the various mechanisms which have been suggested to account for the amplification process.

**Materials and Methods**

*Xenopus* tadpoles were grown at 19-21°C. The water was supplemented with estradiol at a concentration of 0.05 mg/litre to raise the proportion of females above 90% (Gallien, 1953). For the timing study, injections were made into the abdominal cavity of 30 animals nearing the end of metamorphosis, i.e. those in which the tail had been almost completely resorbed. Each tadpole received 5 μCi of (³H)thymidine (20 Ci/mM) in 5μl of aqueous solution.

At various times following the injections, animals were killed and the ovaries fixed in 3:1 ethanol-acetic acid for 15 minutes, transferred to a drop of 45% acetic acid, teased apart with needles to disperse the cells and squashed between slide and coverslip. The coverslip was removed with dry-ice and the preparation hydrated before immersing in 5% trichloroacetic acid for 10 minutes at 4°C. Slides were coated with Kodak AR-10 stripping film and exposed for 1-3 weeks. Before staining, autoradiographs which had been developed, washed and dried were saturated with phosphate buffer pH 5.75 as described by Adamik (1960), by passing through the following solutions for 3 minutes each: (1) ethanol-phosphate buffer pH 5.75, (2) 95% aqueous ethanol, (3) phosphate buffer pH 5.75. The cycle was repeated and after a final buffer rinse slides were stained in a 5% phosphate buffered solution of Giemsa.
In some later experiments, ovaries were dissected from freshly killed tadpoles and cultured in vitro at 25°C in the presence of (\(^3\)H)thymidine (26 Ci/mM). The medium used consisted of 75% Eagles "minimal essential medium", 10% calf serum, 15% water, and included 10 units/ml penicillin and 130 units/ml streptomycin. It is referred to as "Xenopus" medium. Autoradiographs were prepared and stained exactly as above.

In experiments to determine the length of the (\(^3\)H)thymidine pulse, DNA was prepared from a whole tadpole by homogenising in 3 ml of 0.4 M sucrose, 0.6 mM CaCl\(_2\), 0.08M KCl, 0.02 M NaCl, in a Sorvall Omnimixer. The homogenate was incubated at 60°C with 10 ml of 0.4 M NaCl, 0.1 M EDTA, pH 8 at 9.5% sodium dodecyl sulphate for 20 minutes and shaken twice with chloroform-isoamyl alcohol (24:1) to deproteinise nucleic acids. The aqueous layer was then precipitated with ethanol and washed with 70% and absolute ethanol. The pellet was resuspended in 0.1 x SSC (0.015 M NaCl, 0.0015 M tri-sodium citrate, pH 7) and RNased (100 µg/ml RNase A for 1 hour at 37°C) and pronased (100 µg/ml for 1 hour at 37°C). After a further chloroform-isoamyl alcohol shake, the DNA was precipitated with ethanol and dissolved in 0.1 x SSC. The specific activity was determined from optical density estimates of the DNA concentration in solution, and counts of trichloroacetic acid precipitated material upon Oxoid filters in a Packard Tri-Carb scintillation counter.

Results

1. The timing of amplification and early meiotic prophase

The method used to determine the length of the meiotic ampli-
Amplification period can be summarised in the following way. Amplifying tadpoles were pulsed with 5 μCi of $^{3}$H-thymidine by injection and allowed to chase naturally. Autoradiographs of ovarian squashes were prepared at various times afterwards. Nuclear size and morphology change dramatically as the oocyte passes from premeiotic S-phase, through leptotene, zygotene and pachytene to diplotene. In autoradiographs prepared 1 day after the injection it is possible to correlate this change with nucleolar and cap labelling and thereby establish the range of stages which are amplifying nucleolar DNA (see Macgregor, 1968). In particular a morphological stage may be identified at which amplification ceases. Once the labelled pulse has ended, new oocytes continue to enter meiosis and accumulate unlabelled nucleolar chromatin. Similarly, late pachytene cap DNA disperses and gives rise to labelled diplotene nuclei. By scoring the proportion of labelled "cap nuclei" which have not yet reached the end-point of amplification at various times following the injection, the decline of labelled cells in the amplifying cell population can be monitored. The time at which this proportion reaches zero is the time required for those nuclei which were at the earliest detectable stage of amplification during the pulse, to pass through the complete amplifying phase. In other words it is the time taken for 1 oocyte to amplify its nucleolar DNA.

Initially it was necessary to determine the length of time for which injected $^{3}$H-thymidine was available to the cells in these tadpoles. Whole tadpole DNA was isolated at various times following a 5 μCi injection. Figure II.1. shows that its specific activity reaches a plateau within 1 day. We have considered this to be the
FIGURE II.1. Incorporation of $^3$H-thymidine to whole tadpole DNA at various times after a 5μCi injection. The curve represents the average of two experiments.
maximum duration of the pulse.

Autoradiographs prepared 1 day after injections served to define the range of cell types which are amplifying. Figures II 2-6 show oocyte nuclei in the various stages. During leptotene the chromosomes condense into a mass of slender strands and the nucleolus (or occasionally 2 or 3 different sized nucleoli) appears at the periphery of the nucleus (Figure II.2). Nucleolar label was rarely found at this time. In zygotene cells (Figure II.3) the chromosomes have begun to synapse. Silver grains are associated with the nucleolus in these nuclei and this suggests that amplification begins in early zygotene. By pachytene (Figure II.4) the chromosomes are more distinct due to complete pairings of homologues, and the nucleolus has given rise to a cap. The latter is more heavily labelled than the zygotene nucleolus in agreement with Macgregor (1968). By late pachytene, cap labelling has declined (Figure II.5) and has ceased altogether in diplotene (Figure II.6). The end point of amplification lies between these two stages. The gradual transition from pachytene to diplotene as well as squashing and staining differences between preparations tended to make strict definition of this stage difficult, but it was found that the large size compared to other cap cells, the broken appearance of the cap, and the elongated and whiskery state of the chromosomes, together allowed recognition of the end-point with reasonable certainty.

Further autoradiographs were prepared at 3, 7, 10, 14, 21 and 28 days. Table II.1 (A) shows how label was chased from amplifying cells over this period. For convenience of presentation
**TABLE II.1.**

The fate of \(^{3}H\)thymidine pulse labelled chromosomal and nucleolar DNA. Percentages represent an average value after scoring at least 2 preparations. Strict comparison between values is not possible as the distribution of amplifying stages in different ovaries may vary.

<table>
<thead>
<tr>
<th>Time (days) since (^{3}H)-thymidine injection</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) % nucleolar or cap labelled amplifying nuclei</td>
<td>100</td>
<td>98</td>
<td>90</td>
<td>72</td>
<td>45</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>(B) % chromosome labelled zygotene nuclei</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>94</td>
<td>93</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>(C) % chromosome labelled pachytene nuclei</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>27</td>
<td>50</td>
</tr>
</tbody>
</table>
FIGURES II.2 - II.6 present autoradiographs of the various stages of amplification in Xenopus ovaries squashed 1 day after injection with 5 μCi (³H)thymidine.

FIGURE II.2. Leptotene nuclei showing unlabelled peripheral nucleoli (arrows) and a mass of slender chromosomes.

FIGURE II.3. A zygotene nucleus. The arrows indicate chromosomes apparently in the process of synapsis. Nucleolar grains suggest that amplification has just begun.

FIGURE II.4. A pachytene nucleus. Considerable growth has occurred since zygotene and the nucleolus has given rise to a cap.

FIGURE II.5. Late pachytene nucleus after 3-3½ weeks of amplification. The level of (³H)thymidine incorporation has decreased and the cap shows signs of disintegration.

FIGURE II.6. A diplotene nucleus. Nucleolar DNA shows no labelling and is no longer localised in a cap. The chromosomes have become indistinct.
the proportion of labelled nuclei is expressed as a percentage of all the nuclei found at that stage, though preparations are not strictly comparable between different preparations with possibly different distributions of the various amplifying stages. In 21 day preparations late pachytene cells showed light cap labelling probably incorporated during zygotene or early pachytene. By 28 days no nuclei with intact labelled caps were found. All labelled nucleolar DNA was clearly present in nuclei in the process of cap dispersal. It is concluded that amplification lasts for 3-4 weeks in each oocyte.

All zygotene nucleoli became unlabelled between 3 and 7 days after the injection. Less than 7 days of amplification are therefore spent in zygotene, the majority occurring at pachytene. Making the assumption that pachytene ends at roughly the same time as amplification, the length of pachytene can be estimated at about 20 days.

Apart from amplifying nuclei, the ($^3$H)thymidine injection also labels S-phase (the DNA replication phase of the cell cycle) nuclei. Amongst these are S-phase oogonia (Figure II.7), and oocytes in the premeiotic S-phase. Chromosomal label acquired by these last cells during the pulse is present in leptotene cells in 3 day autoradiographs (Figures II.8 and II.9) implying that premeiotic G2 (second growth phase) is less than 3 days. Callan (1968) found that premeiotic S-phase in the large Triturus vulgaris spermatocytes did not end until after the beginning of leptotene. This may well be the case in Xenopus oocytes, but the smaller size of these nuclei made it difficult to distinguish the early stages
FIGURES II.7 - II.10 show the fate of label acquired by S-phase cells during the (3H)thymidine pulse.

FIGURE II.7. Oogonial nucleus. The centrally placed nucleolus already possesses multiple copies of the nucleolar DNA. Light labelling suggests that these oogonia were just starting or finishing an S-phase during the pulse.

FIGURE II.8. Prophase nucleus 1 week after injection. This may be either a leptotene nucleus overlayed by premeiotic S-phase labelling, or an oogonial prophase.

FIGURE II.9. Leptotene or early zygotene nuclei (compare figures II.2 and II.3). Chromosomal label acquired during premeiotic S has arrived at this stage 7 days after injection.

FIGURE II.10. Chromosome labelled pachytene nuclei. Premeiotic S-phase label has reached these cells in 3 weeks, after passing through leptotene, zygotene and about half of pachytene.
FIGURE II.11. Diagramatic representation of the timing of early meiotic prophase in *Xenopus laevis*. The length of each horizontal line represents the duration of the stage concerned. Broken lines show the difference between maximum and minimum estimates. Data are largely derived from Table II.1.
of leptotene in my preparations

By 7 days labelled chromosomes have appeared in sygotene cells (Table II.1. (B) and Figure II.9). This points to a $5 \pm 2$ day duration for (G2) leptotene. Similarly, the time taken for chromosomal label to arrive in pachytene cells (Table II.1 (C) and Figure II.10) gives a (G2) leptotene plus sygotene estimate of $12 \pm 2$ days. By subtraction sygotene becomes approximately 7 days in length.

The length of premeiotic S-phase can be determined by the difference in the time taken for chromosomal label to appear in sygotene nuclei, and the time when the proportion of such labelled cells begins to decline. The first represents arrival at sygotene of those cells at the end of premeiotic S-phase during the pulse, and the second represents arrival of oogonia which started premeiotic S-phase just after the pulse. From Table II.1 (B) it is apparent that this difference is 1-2 weeks. The data do not permit a more accurate estimate than this.

Our findings are summarised in Figure II.11.

(2) Additional studies on meiotic amplification by autoradiography of in vitro pulse-labelled ovaries.

The results of the timing study have shown quite clearly the sequence of events during early meiotic prophase. Of particular interest is the delay of several days between the end of premeiotic S-phase and the start of amplification. It might have been expected that nucleolar DNA synthesis would occur late in premeiotic S-phase
and gradually develop into the large scale process of amplification. However, the finding that there is a gap of about 5 days before nuclear incorporation is seen suggests that premeiotic S-phase and amplification are distinct events. It is not known whether nucleolar DNA is replicated during premeiotic S.

The level of labelling in sygotene nucleoli after injection with (3H)thymidine was relatively low, and it was therefore possible that some undetected nucleolar DNA replication preceded the sygotene amplification. To determine just how sharp is the cut off between the period of nucleolar labelling and the preceding stages of meiosis, (3H)thymidine labelling of amplifying ovaries was carried out in vitro. The uptake of label is far greater in culture than in vivo.

The conditions of the experiment were designed to investigate simultaneously another aspect of the amplification process. It is possible that nucleolar DNA synthesis is not occurring throughout the cap but is localised within it. Long labelling incubations such as those which have been performed previously might cause incorporation all over the cap if rDNA accumulates at a place different from the site of its synthesis. Short pulse might detect the presence of localised synthesis within the cap if this is occurring.

In order to investigate these two points, whole ovaries were cultured in 0.5 ml of Xenopus medium containing 50 μCi/ml of (3H)thymidine for 15 minutes.

Figures II.12 - II.16 show sygotene and pachytene nuclei from 15 minute labelled preparations. All sygotene nuclei with
completely peripheral nucleoli are visibly incorporating $^{3}\text{H}$-thymidine. This agrees with the results obtained by injecting label. Moreover, it is usual to find incorporation at more than one peripheral site (Figure II.12) and often, though to a lesser extent, over the chromosomal material (e.g. Figure II.13). There are several possible explanations for this finding. One is that some chromosomal DNA synthesis accompanies synapsis (Roth and Ito, 1967; Stern and Hotta, 1969). This has been suggested by Watson Coggins and Gall (in the press). Another possibility is that this extra incorporation is present in other, smaller nucleoli; multiple nucleolar DNA copies exist already at this stage (Gall, 1969). An argument that at least some of the additional incorporation is taking place in small nucleoli is provided by nuclei of slightly later stages in which nucleoli have developed into small caps. Thus the appearance of several labelled sites on a single nucleus in Figures II.14 - II.16 correlates with the presence of light staining, cap-like material at the same sites. Pachytene nuclei all possess a single cap suggesting that the amplifying nucleoli coalesce during zygotene (Figures II.17 - II.19).

One significant objection to this interpretation is that the squashing process either exaggerates or causes the dispersal of these early amplification sites. This objection cannot be entirely ruled out until similar experiments have been performed on sectioned material.

In these preparations incorporation into zygotene nucleoli is well above background, and it is apparent that only peripheral nucleoli show labelling. No label is visible in any nucleoli which
FIGURES 11.12 to 11.19 show autoradiographs of amplifying nuclei after incubation for 15 minutes with 50 μCi/ml (3H)thymidine.

FIGURE 11.12 Zygote nuclei all showing more than one site of (3H)thymidine incorporation. All sites are peripheral. Synthesis is often associated with a forming "cap" structure, though in some cases no such structure is visible.

FIGURE 11.13. Two zygote nuclei in which a major site of thymidine incorporation is the nucleolus and smaller sites are seen near the nuclear periphery and also over the chromosome mass.

FIGURE 11.14 Zygote nucleus in which DNA synthesis appears confined to nucleolar material at two peripheral regions.

FIGURE 11.15. Early pachytene nucleus displaying an amplifying cap and a small additional site of nucleolar DNA synthesis.

FIGURE 11.16. Early pachytene nucleus with two small caps active in DNA synthesis.
FIGURE II.17  Mid-pachytene nuclei, each with a large "cap". DNA synthesis is apparently occurring throughout the cap. The bottom left hand nucleus shows what may be a smaller site of amplification coalescing with the main cap.

FIGURE II.18  Late pachytene nucleus. Once again label covers the entire cap.

FIGURE II.19  Late pachytene nucleus in which a pale staining region is visible. This region is not covered by silver grains.
which was not at the edge of the meiotic chromosome mass. It is likely that towards the end of leptotene the nucleolus appears near the periphery of the nucleus and by zygotene it has emerged from the chromosome mass. It is at this last stage that amplification is first detectable. The coincidence of nucleolar emergence and the start of amplification may be fortuitous, or it may be that some association between nucleoli and the nuclear membrane is required for amplification to begin.

Figures II.17 - II.19 show pachytene nuclei. Even after 15 minutes exposure to \(^3\)H\)thymidine no localisation of label is apparent within the cap. It seems that DNA synthesis is not confined to specific regions but occurs throughout the cap. However, the pachytene nucleus shown in Figure II.19 displays a prominent pale staining region in the centre of the amplified material which shows a distinct absence of silver grains. It is possible that this region is the distorted nucleolus observed in the E.M. by Watson Coggins (1971). She has observed that after an injected \(^3\)H\)thymidine pulse, grains are not found by E.M. autoradiography over this nucleolar area. The fact that this also appears true after much shorter labelling periods suggests that amplification might not occur at this site. (Results reported in Chapter IX suggest that the cap "nucleolus" is active in RNA synthesis.)

Most pachytene nuclei do not exhibit the pale staining region with its absence of grains. This is probably because, in squash preparations, the "nucleolar" region is usually sandwiched between replicating cap material which would obscure the inactivity of this area. The particular nucleolus shown in Figure II.19 has
presumably squashed with the nucleolus close to the cap surface and hence, to the emulsion.

A further notable feature of these preparations is the distribution of labelling amongst the various amplifying stages. In these short pulses zygotene nucleoli incorporate more label than pachytene caps. Longer \textit{in vivo} and \textit{in vitro} labelling shows the reverse. Equilibrium between endogenous and exogenous thymidine pools has clearly not yet been reached, and the more rapid labelling of early cells may be a result of higher initial permeability or smaller endogenous thymidine pools in these cells.

\textbf{Discussion}

There is some evidence that amplification of nucleolar DNA may have occurred before the onset of meiosis. The technique of cytological hybridization (Gall \& Pardue, 1969) has shown that extra copies of the ribosomal genes are already present in oogonia. Gall (1969) has estimated 20-40 nucleolus organizer equivalents of ribosomal DNA in these cells, or less than 0.5 \(\mu\)g compared with the 30 \(\mu\)g synthesised during zygotene and pachytene. Wallace, Morray \& Langridge (1971) have suggested that these early copies originate from non-chromosomal nucleolar DNA carried in the germ cell cytoplasm, though work done by Brown and Blackler (in the press) using \textit{Xenopus} interspecies hybrids implies that the bulk of the amplified DNA, and perhaps - though not necessarily - these early copies, arises by a chromosome copying amplification process. Leptotene nucleoli show a similar enrichment for ribosomal DNA (Gall \& Pardue 1969). This DNA may either be derived from oogonial copies by S-phase duplication and segregation at each oogonial mitosis, or there
may be some amplification during oogonial proliferation. Certainly it is not as yet possible to distinguish any cytological stage at which premeiotic amplification may be occurring, and this study has therefore only been concerned with the major amplification phase which takes place during meiotic prophase.

This period begins less than a week before the oocyte enters pachytene, as nucleolar label is completely chased from syngamy nuclei between 3 and 7 days after injection. Since syngamy is estimated to be approximately 7 days in length, this implies that the process starts during this stage. It then proceeds continuously throughout the 21-3 weeks of pachytene and eventually ceases as diplotene begins.

In vitro labelling studies suggest that nucleolar DNA synthesis starts as the nucleolus emerges from the chromosomes, since no non-peripheral nucleolar thymidine incorporation is apparent in meiotic cells. This would imply that the start of amplification is coincident with the beginning of syngamy.

If the frequent presence of several sites of early nucleolar DNA synthesis in syngamy nuclei is not an artefact caused by the squashing procedure, then it appears that in the course of leptotene, nucleoli are pushed out from amongst the chromosomes to the nucleolar membrane, and as they emerge at various points around the periphery they begin to amplify. It is possible that the mechanical forces set up in the nucleus by chromosome condensation and pairing are sufficient to force the nucleolus to the edge.

As syngamy progresses to pachytene "multiple" amplification sites must coalesce, for by early pachytene only a single, small cap is present.
The approximate timings of premeiotic S, (G2) leptotene, and sygotene give durations similar to those observed by Callan (1968) in his precise studies on spermatocyte meiosis in *Triturus vulgaris* at 16°C. He estimates a 9-10 day premeiotic S-phase followed by 4-5 days of leptotene and 7-8 days of sygotene. This compares with my estimates, on *Xenopus* grown at 19-20°C, of 1-2 weeks, 5 ± 2 days, and 7 ± 2 days respectively. Beyond synapsis amphibian oocyte and spermatocyte meioses differ considerably. Pachytene and diplotene in oocytes see considerable growth and accumulation of materials, whilst spermatocytes pass through these stages in under a week and go on to complete both meiotic divisions. In *Xenopus* oocytes pachytene lasts for 2½-3 weeks before a gradual transition to diplotene and the beginning of a diffuse lampbrush stage lasting several months.

There are currently a number of proposals for the mechanism of nucleolar DNA amplification. Miller (1966) originally suggested 2 possible extremes: (1) that the tetraploid nucleolus organisers replicate repeatedly and the released DNA copies accumulate passively in the cap region; (2) that each detached product copy itself serves as a template for further nucleolar DNA syntheses. The discovery that extra copies of the nucleolar DNA are already present at leptotene (Gall, 1969) has meant that either of the above possible mechanisms, or a combination, may apply to meiotic nucleolar DNA replication at these multiple sites. Circumstantial evidence offers some support for mechanism (2). Macgregor (1968) noted autoradiographically that incorporation of (3H)thymidine during a 6 hour incubation increased as pachytene proceeded. This would be expected if
the "cascade" mechanism progressively made more templates available for replication, though an increase in rate may also result from changes in the permeability or metabolic state of the growing oocyte. In addition to these possibilities, Tocchini-Valentini and Crippa (1971) have recently proposed an RNA-dependent DNA polymerase driven mechanism. They suggest that DNA is synthesised on an RNA copy of the ribosomal genes via an RNA-DNA hybrid. Evidence for such a process is as yet equivocal.

Of these possibilities Miller's mechanism (1) is potentially the slowest, and it is of interest to determine whether or not 3-3½ weeks is sufficient to permit amplification by this means. Data on the rate of S-phase DNA synthesis in Xenopus has been obtained by Callan (unpublished) through autoradiography of DNA fibres. He finds a replication rate in cultured Xenopus cells at 25°C of 9 μ/hour in two directions from many points along the DNA. It is possible to use this data in estimating the feasibility of mechanism (1).

In Xenopus the nucleolus organising region constitutes 0.3% of the somatic genome (Grunstein, unpublished). Given a haploid somatic DNA value of 3.1 μg we can estimate a total of 9.3 x 10⁻³ μg of nucleolar DNA per haploid genome. Dividing this mass into the total of 30 μg of amplified nucleolar DNA gives the equivalent of 3400 nucleolus organisers in the post-amplification oocyte. With an amplification time of 24 days, it would be necessary for the 4 chromosomal organising regions to synthesise 35 copies per day, or 1 every 0.7 hours. Each organiser is about 2800 μ in length, so it is clearly not possible to synthesise 1 copy in less than 1 hour with a replication rate of 18 μ/hour (9 μ/hour in both directions) from a single
initiation point. The nucleolar DNA would have to be divided into many simultaneously replicating units with initiation sites less than $0.7 \times 18 = 12\mu$ apart if a complete copy is to be released every 0.7 hours. The shortest replicating units observed by Callan in his DNA fibre autoradiographs of cultured *Xenopus* cells were 20 $\mu$ in length. In *Triturus* he found considerable flexibility in average unit length between cell types, and it is likely that *Xenopus* cells are also capable of such flexibility. Our estimate then, is theoretically within the scope of a *Xenopus* cell, and it is not possible to rule out entirely a chromosome based mechanism on the grounds of insufficient available time.

Little is known about the RNA dependent mechanism which would allow an estimate of its potential rate, but both the "cascade" process (Miller's second mechanism) and serial duplications of the multiple leptotene nucleolar DNA copies must be capable of replicating DNA more rapidly than the chromosome based method examined above. The question of a molecular mechanism must therefore remain open until a more detailed analysis of the amplification process has been achieved.
CHAPTER III

An autoradiographic study of RNA synthesis in young oocytes

Introduction

RNA synthesis in post vitellogenic oocytes has been extensively studied using the available biochemical and cytological techniques. At this time meiotic chromosomes are in the lampbrush configuration (Callan and Lloyd, 1960) and are engaged in RNA synthesis at each lateral loop (Gall and Callan, 1962). The multiple nucleoli are situated adjacent to the nuclear membrane and incorporate \(^3\)H)uridine very rapidly (Macgregor, 1967). On the other hand little is known about RNA synthesis in previtellogenic oocytes. An autoradiographic study of \(^3\)H)uridine incorporation into oocyte nuclei in the early stages of meiotic prophase was performed to give some indication of the transcriptional activity of both chromosomal and newly amplified nucleolar DNA.

Materials and Methods

Whole ovaries were cultured in vitro in 1 ml of medium containing 100 \(\mu\)Ci/ml \(^3\)H)uridine (30 Ci/mM) at 25°C for either 30 or 60 minutes.

Autoradiographs of squash preparations were made as described previously (Chapter II, Materials and Methods).

Results

Initially, amplifying ovaries were placed in \(^3\)H)uridine containing medium for 1 hour. Figures III 1 to 8 show autoradiographs
of nuclei at various stages from oogonium to diplotene. All these pictures are taken from the same slide which was exposed for 3 weeks.

A typical oogonial nucleus is shown in Figure III.1. Both the nucleolus and the nucleoplasm appear active in RNA synthesis. In leptotene (Figure III.2) the level of chromosomal incorporation is similar and has increased slightly by zygotene (Figure III.3).

This contrasts with the situation in pachytene nuclei (Figure III.4 and III.5) where there is extensive incorporation into the chromosomes. Furthermore a conspicuous localisation of label is often present within the cap region. Cap localisation is not always evident in pachytene nuclei and this is probably because the position relative to the emulsion of the region responsible can vary with the squashing of each pachytene nucleus.

In many cases there is a comparatively low level of incorporation all over the cap in addition to localised label. Several explanations for this finding are possible:

1. Some RNA synthesis is occurring throughout this region;
2. Labelled RNA from either the chromosomes or the cap site mentioned above has moved into the cap either naturally, or as a result of the squashing procedure; 3. \(^{3}\text{H}\text{uridine is converted to a DNA precursor and incorporated into rDNA during amplification. Experiments described in Chapter VII show that this does occur to a considerable extent.}

Figure III.6 shows a late pachytene or early diplotene oocyte nucleus. In addition to the incorporation over the entire cap, there are several discrete foci of incorporation located in the
FIGURES III.1. to III.8 are taken from a single autoradiographed squash preparation of an ovary incubated with 100 μCi/ml (³H)-uridine for 1 hour.

FIGURE III.1. Oogonium in which both the nucleolus and the chromosomes are incorporating (³H)-uridine.

FIGURE III.2. Leptotene nucleus. (³H)Uridine incorporation is comparable with that in oogonial and follicle cell nuclei.

FIGURE III.3. Zygotene nucleus showing heavier labelling than at leptotene. Peripheral nucleolar material is also labelled. Amplification has just begun at this stage.

FIGURE III.4. Pachytene nucleus with heavily labelled chromosomes. In addition to light labelling over the entire cap, there is a marked localisation of label at a site within the cap.

FIGURE III.5. Pachytene nucleus showing similar localised cap label.

FIGURE III.6. Diplotene nucleus. Chromosome labelling has increased still further. Several sites of RNA synthesis are apparent within the cap.
FIGURE III.7. Later diplotene in which lambrush chromosomes are engaged in RNA synthesis. Amplified DNA is organised into many foci active in RNA synthesis.

FIGURE III.8. Large diplotene nucleus. Amplified DNA is still sequestered in a cap and sites of RNA synthesis have become more numerous in this region.
cap region. Each cap generally possesses more than one site of RNA synthesis at this stage; in Figure III.6, for example, there appear to be at least 3 sites.

Later diplotene nuclei have increased greatly in size (Figures III.7 and III.8) and exhibit very heavy chromosomal labelling. Considerable chromosomal RNA synthesis is to be expected as the nuclei are now in the lampbrush stage (Gall and Callan, 1962). Vestiges of the cap structure are still evident over one side of the nucleus. This shows that amplified DNA does not, in fact, disperse throughout the nucleus at the end of amplification, but merely fragments at the site of its synthesis. Many discrete sites of uridine incorporation have developed within the cap area.

Interpretation of autoradiographs during the amplification period is hampered by the finding that (\(^{3}\text{H}\)uridine can be converted to (\(^{3}\text{H}\)deoxyctydine - a DNA precursor. It is therefore possible that (\(^{3}\text{H}\)uridine label is being incorporated into both DNA and RNA in cap nuclei. To try and eliminate the utilization of labelled deoxycytidine during DNA synthesis, a large amount of cold deoxyctydine was included in the medium during a 30 minute incubation with 100 μCi/ml (\(^{3}\text{H}\)uridine.

Figures III.9 to III.13 are once more taken from a single slide, after 2 weeks exposure. Zygotene (Figure III.9) again displays normal RNA synthesis in both chromosomes and nucleolus.

A range of pachytene nuclei is shown in Figures III.10 to III.13. They have all incorporated considerable chromosomal label as before, and localised grains within the caps are once more evident. Silver grains were never distributed over the whole cap in this
FIGURES III.9 to III.13 are taken from a squash preparation of ovaries which had been incubated with 100 μCi/ml (³H)uridine for 30 minutes in the presence of a large quantity of deoxycytidine. Under these conditions incorporation of (³H)uridine counts into amplified rDNA is minimised and all grains reflect incorporation in RNA.

FIGURE III.9. Three late zygotene nuclei showing both chromosomal and nucleolar label.

FIGURES III.10 to III.13
Pachytene nuclei. Chromosomal label has increased compared to zygotene nuclei. Localised cap label is visible and, in Figures III.11 and III.13, is associated with pale staining regions of the cap.
preparation. This could be attributed to the effectiveness of deocycytidine treatment in preventing conversion of labelled uridine to the DNA precursor, though incorporation is generally lower than in the untreated preparation, and cap grains may therefore not have developed significantly above background. If cap grains were caused by diffusion of labelled RNA in the living cells, then one would also expect the spread of label to be less in 30 minutes than in the previous 60 minute incubation.

Whatever the explanation, it is clear in this preparation that cap labelling during pachytene is largely isolated in a single region. Often this label is correlated with the presence of a lightly staining region within the cap, (e.g. Figures III.11 and III.13).

Discussion

General conclusions about the progress of oocyte RNA synthesis during meiotic prophase can be drawn from these results. During leptotene and zygotene RNA synthesis is occurring at a low level comparable with interphase dividing cells (oogonia and follicle cells). As the cell enters pachytene, chromosomal RNA synthesis increases and is coupled with an increase in nucleolar RNA synthesis. During pachytene the chromosomes are heavily engaged in RNA synthesis. Cap label is confined to a single site within the amplifying rDNA. By late pachytene, when amplification has ceased or is declining, there are several sites of RNA synthesis in the cap. Diplotene nuclei now enter a period of rapid growth and show extensive chromosomal RNA synthesis. The integrity of the
cap region is not lost but it now contains many foci active in RNA synthesis.

There is good reason to believe that the RNA synthesis which occurs in the cap is taking place in a nucleolar structure. The evidence for this comes from an electron microscope (E.M.) study of amplifying oocytes carried out by Watson Coggins (1971). She has observed that the amplifying cap is mostly homogeneous, but for a rather large and irregularly shaped nucleolus embedded within it. This structure is derived from the orthodox nucleolus which can be identified in leptotene and zygotene cells. E.M. autoradiography implies that the irregular nucleolus does not participate in DNA synthesis and this is supported by an observation at the light microscope level (see Chapter II). Often the pachytene cap grains are localised over a pale staining site within the cap. Since the nucleolar structure is the only interruption in an otherwise homogeneous cap, then it is likely that RNA synthesis is occurring at this site.

Watson Coggins has also observed that at the end of amplification, in late pachytene or early diplotene, the nucleolar structure gives rise to multiple nucleoli. This again fits well with the autoradiographic data as multiple sites of (3H)uridine incorporation appear at this time.

The evidence discussed above suggests that cap RNA synthesis is nucleolar in origin and may well involve 40S rRNA precursor synthesis. In this case the correlation during meiotic prophase between chromosomal and nucleolar incorporation is striking. Starting from the leptotene and early zygotene nucleus in which
chromosomal and nucleolar incorporation is visible, chromosomal RNA synthesis increases through pachytene and culminates in RNA production on an unprecedented scale at diplotene. Nucleolar RNA synthesis shows a similar progression from the low level in early pachytene to the high level of multiple nucleolar activity in the lampbrush stage.

The accumulation of rRNA in large quantities does not begin in amphi-bia until the start of vitellogenesis (Davidson et al., 1964). At this time the nucleoli are arranged adjacent to the nuclear envelope (Macyregor, 1967) and presumably transfer product rRNA to the cytoplasm directly. The experiments reported here show that nucleoli are continuously active prior to this stage, presumably in the synthesis of 40S rRNA precursor.

It is of particular interest that rDNA amplification and rDNA transcription can occur simultaneously in the cap. \(^3\text{H}\)thymidine and \(^3\text{H}\)uridine autoradiography suggest that they may be taking place in different structures, however. It may be that only rDNA which is arranged in a nucleolar structure is capable of rRNA precursor synthesis, and is likewise incapable of amplification. As amplification is completed more rDNA is organised into nucleoli and hence becomes functional in RNA synthesis.

If indeed the cap nucleolus is derived directly from the leptotene nucleolus and does not take part in amplification, then it seems likely that amplification is not occurring through the release of DNA replicas from the nucleolus. This makes Miller's mechanism I (Miller, 1966) less likely and tends to favour a "cascade" process (mechanism II).
The occurrence of nucleolar RNA synthesis at the time of amplification would fit well with a "reverse transcription" mechanism which may require a continuous supply of RNA copies of the ribosomal genes as templates. It will be of interest to discover whether any of the RNA synthesised in the nucleus migrates into the outer cap region where DNA amplification is occurring.
APPENDIX TO CYTOLOGICAL STUDIES ON AMPLIFICATION

The autoradiographed squash preparations used for the cytological studies described in Chapters II and III can offer limited information about the behaviour of multiple nucleoli prior to meiosis. In addition to meiotic oocytes, oogonia were present in each squash, and a small fraction of these was in the prophase of mitosis.

It is known that 20-40 nucleolus organiser equivalents are present in oogonia (Gall, 1969) and that they can occur both as multiple nucleoli, and in a single nucleolus. Since, in ovaries which contain amplifying oocytes, all oogonia apparently possess multiple nucleoli, then the question arises as to whether the extra DNA is duplicated at S phase and segregated with the chromosomes at each oogonial mitosis, or whether amplification occurs de novo at each cell generation.

In Acheta domesticus the "DNA body" can be seen to pass through mitosis and associate with one anaphase chromosome group. Thus only one daughter cell retains the body. It is not known whether this also occurs in Xenopus.

DNA synthesis has been observed in multiple and single nucleoli both during S phase, and after its completion (Watson Coggins and Gall, 1971). Late nucleolar labelling of this kind is shown in Figures A.1. and A.2. Whether it constitutes rDNA duplication prior to segregation at meiosis, or further amplification of the nucleolar DNA is not known.
FIGURES A.1. and A.2. are (³H) thymidine labelled autoradiographs of interphase oogonial nuclei. Whole ovaries were labelled with 100 µCi/ml (³H) thymidine (20 Ci/mM) for 15 minutes at 25°C. Squash preparations were autoradiographed as described in Chapter II.

FIGURE A.1. Oogonial nucleus in which nucleolar DNA replication is occurring outside the S-phase of the cell cycles.

FIGURE A.2. This oogonial nucleus contains two nucleoli, each engaged in DNA synthesis.

FIGURES A.3. and A.4. are taken from an autoradiographed squash preparation of (³H) uridine treated ovaries. Both show nuclei in oogonial mitotic prophase. They are readily distinguishable from meiotic cells (compare Figures II.2, II.3 and II.9). Only one nucleolus is seen. It is attached to the chromosome mass at a single site.
Unfortunately it was not possible using these preparations to build a complete picture of the behaviour of multiple nucleoli at mitosis, as all mitotic nuclei which could be clearly identified as oogonial were at mitotic prophase. Presumably this stage is longer than metaphase and anaphase. Figures A.3 and A.4 present a selection of prophase nuclei. All possess a single nucleolus which is attached to the chromosome mass. These pictures are remarkably similar to those of oogonial prophase in Acheta obtained by Kunz (1969).

If the single nucleolus indeed contains amplified rDNA then the initial requirements for the transfer of the nucleolar body through mitosis (the aggregation of multiple nucleoli in a single body, and the attachment of that body to the chromosomes) has been fulfilled. How it behaves later in mitosis is not known. It is difficult to envisage an accurate division of the nucleolar body into 2 equal parts which separate at anaphase. A more thorough study, perhaps involving the use of mitotic inhibitors, will shed light on this problem.
CHAPTER IV

The amplifying ovary as material for biochemical studies;
Preparation and properties of an ovarian cell suspension.

Introduction

The *Xenopus laevis* ovary at the end of metamorphosis contains young oocytes and proliferating oogonia embedded in follicle and connective tissue. Many of the oocytes are in zygotene and pachytene, and consequently are synthesising extrachromosomal rDNA. However, even at this stage amplifying cells represent a minority of the total number of cells in the ovary, the majority being follicle cells and non-amplifying germ cells. Thus any biochemical approach to the study of amplification in oocytes must take into account the excess of contaminating cells which the ovary contains.

Fortunately amplifying cells are different from the surrounding cells in 2 main ways:

1. In squash preparations they are seen to be morphologically distinct from other cells. In zygotene the chromosomes are condensed and the nucleolus or nucleoli are peripheral, whilst pachytene cells show the bouquet arrangement of chromosomes and a large chromatin cap over one side of the nucleus. Furthermore, both the pachytene cap and the zygotene nucleoli incorporate (³H) thymidine which can be detected autoradiographically.

2. The amplified DNA is rDNA and can therefore be studied in isolation by virtue of its high bouyant density in CsCl (Wallace
and Birnstiel, 1966). When intact ovaries are incubated with \(^{3}H\)thymidine, all replicating DNA becomes labelled. If the isolated DNA is now centrifuged to equilibrium in CsCl, rDNA is separable from the main band of DNA (Evans & Birnstiel, 1968; Gall, 1968; Brown & Dawid 1968) and the amount of incorporation into this peak during the incubation period can be measured. In addition, rDNA can be specifically detected amongst other DNA species by hybridisation to radioactive rRNA.

Advantage has been taken of both these features of the amplifying oocyte in the previous work on this process. (Gall, 1968; Evans and Birnstiel, 1968; Brown and Dawid, 1968).

It was my intention to analyse biochemically the relationship between amplification and protein and RNA synthesis using specific inhibitors. This approach necessarily involves comparison of inhibitor treated and untreated samples and raises another difficulty if whole ovary is to be used. Ovaries from different animals - even from a single mating - may vary considerably both in size and in the stage of development reached. Furthermore, the 2 halves of an ovary are not comparable as they rarely give the appearance of mirror-images. It was therefore necessary to devise some means of comparing amplifying cell preparations in order to simplify quantitative biochemical investigations.

Several solutions were possible:

1. By using large numbers of ovaries in each experiment the variation between individual gonads could be statistically reduced.

2. Ovaries could be "standardised" by estimation of the initial differences between them for the particular parameter to
be measured. This could be achieved by prelabelling all the material for a period before the experiment proper with an isotope different from the one to be used later. Thus RNA synthesis might be standardised in 2 ovaries which are to be treated differently by preincubating for, say, 2 hours in \(^{32}\text{P}\) phosphate medium. The ovaries would then be thoroughly washed and transferred to \(^{3}\text{H}\) uridine medium for the experiment. The levels of uridine incorporation into RNA preparations from these ovaries could then be compared by dividing \(^{3}\text{H}\) counts per minute by \(^{32}\text{P}\) counts per minute.

(3) A suspension of ovarian cells could be prepared so that equal volumes of the suspension contain equal numbers and proportions of the various cell types. The preparation could be obtained either by mechanical disruption of the ovaries or enzymic digestion of the intercellular links.

I have used all of these methods at some time during my studies on amplification. Method (1) suffers from the disadvantage that stocks of amplifying toads are limited, and therefore consistent use of large numbers of ovaries would seriously deplete the supply. Method (2) has 2 main disadvantages. Firstly it relies upon most prelabel being washed out before the second labelling, if significant prelabel is incorporated during the main part of the experiment then the results will be distorted. Secondly, \(^{32}\text{P}\) phosphate is not incorporated efficiently into ovarian DNA, and it also reduces the viability of the tissue in culture. \(^{14}\text{C}\) labelled precursors are an expensive alternative and are obligatory if protein synthesis is to be standardised in this way.
The most satisfactory solution to the problem of comparison of ovaries has been the ovarian cell suspension. Such a preparation also has advantages in other directions which make it more convenient as a material for studying some biochemical aspects of amplification. I shall therefore describe in detail its preparation and properties.

Materials and Methods

(a) Animals

Animals were kept in the same way as described in Chapter II.

(b) DNA Extraction

2 basic methods of DNA extraction have been used during the course of this work. Extraction method (1) was used in early experiments, but was superseded by extraction method (2).

**DNA extraction method (1):** Whole ovaries were washed in ice-cold SSC (0.15M NaCl, 0.015M sodium citrate, pH 7) and homogenised in 1 ml of 0.4M sucrose, 0.4M NaCl, 0.1M EDTA. The homogenate was made 0.5% SDS (sodium dodecyl sulphate) and 100 µg/ml predigested pronase and incubated for at least 3 hours at 37°C. After addition of \( \frac{1}{10} \) volume of saturated Tris (pH 8.5), the solution was shaken for 20 minutes with an equal volume of chloroform–isoamyl alcohol (24:1). The layers were separated at 10,000 revs/min for 10 minutes and the top layer was precipitated overnight with 2 volumes of ethanol at -20°C. The invisible precipitate was pelleted at 10,000 revs/min for 10 minutes and dissolved in 0.1xSSC.

**DNA extraction method (2):** This method was used both for cells in suspension and whole ovaries. It is based on the technique
of Gall, Macgregor and Kidston (1969). The material was suspended in 1 ml of 0.4M NaCl, 0.1 M EDTA (pH). SDS was added to a final concentration of 0.5% and pronase to a final concentration of 100μg/ml. The suspension was then incubated at 37°C for a minimum of 2 hours. In this time whole ovaries disintegrate completely and the solution becomes viscous. 25-50μg of either Xenopus erythrocyte DNA or Micrococcus lysodeicticus DNA were then added as carrier, and the solution shaken for at least 30' with an equal volume of water saturated phenol. After spinning for 10 minutes at 10,000 revs/min, the aqueous layer was removed and precipitated with 2 volumes of ethanol. It was not necessary to allow precipitation in the deep freeze as the carrier DNA brought newly extracted DNA out of solution immediately as part of a visible precipitate. Impure DNA was collected by centrifugation and dissolved in 1 ml of 0.1 x SSC. The solution was made up to SSC and incubated with 100μg/ml RNase A and 330 units/ml RNase T1 for 1 hour. DNA was finally precipitated with ethanol and pelleted by centrifugation. (c) Density gradient centrifugation

DNA was analysed by equilibrium centrifugation in a concentrated CsCl solution using a fixed angle rotor to improve resolution (Flamm, Birnstiel and Walker, 1969). 4 ml's of DNA dissolved in 0.1 x SSC were mixed with 5.20 g of solid CsCl. The density of the solution was checked by measurement of its refractive index (the desired value being 1.05101). Centrifugation was carried out in an M.S.E. 50 ultracentrifuge at 42,000 revs/min, 25°C, for at least 30 hours. 10 drop fractions were collected by puncturing the bottom of the tube. Often the optical density of each fraction at 260μm
was determined in a Beckman spectrophotometer to determine the position of marker DNAs. The radioactivity in each fraction was measured by precipitating the DNA in the presence of 100 μg of yeast RNA with cold trichloroacetic acid (TCA) at a final concentration of 5%. Precipitate was collected by suction through Oxoid filters. Filters were dried with washes of ethanol and finally ether and counted in 5 mls of scintillation fluid in either a "Nuclear Chicago" or a "Packard Tricarb" scintillation counter.

(d) Preparation of the cell suspension

Metamorphosing tadpoles were killed and their ovaries dissected into *Xenopus* medium at 25°C. After washing they were transferred to 1-3 mls of fresh medium in a small sterile plastic petri dish. The ovaries were then gently teased apart with a pair of fine tungsten needles under a low power dissection microscope. When all the ovaries had been dissaggregated in this way, there remained visible pieces of fibrous tissue. All such visible material was scrupulously removed from the medium. The teasing operation takes about 5 minutes per ovary.

Results and Discussion

Microscopical examination of the cell suspension shows many oogonia and oocytes released from the ovaries both singly and in small groups. There are usually a few larger cell aggregates which appear to be undisaggregated pieces of ovary surrounded by an envelope of follicle cells.

In an attempt to estimate what proportion of amplifying cells are released by this operation, 3 whole ovaries were dissected into...
FIGURE IV.1. Ovaries which had been labelled with \(^{3}H\) thymidine were teased apart as described in the text. DNA was isolated separately from the teased out cells and the fibrous remains, and analysed by CsCl centrifugation.
2 ml of "Xenopus medium" containing 50 μCi/ml (3H) thymidine and incubated for 23 hours at 25°C. When the incubation was completed they were washed free of labelled medium and disaggregated. All the fibrous remains were transferred to a separate dish of medium. The suspended cells and the fibrous remains were collected by centrifugation and DNA was extracted with pronase and SDS. Purified DNA was then centrifuged to equilibrium in CsCl and fractions precipitated with cold TCA and counted. Figure VI shows the radioactivity profiles obtained from both teased out cells and fibrous remains. Of a total of 1720 counts/min incorporated into rDNA, about \( \frac{1}{3} \) are associated with the material remaining after disaggregation. Therefore \( \frac{2}{3} \) of all the amplifying cells have been released during the "teasing-out" procedure. In addition, less than \( \frac{1}{3} \) of the total incorporation into main band DNA is found in the cell suspension.

Having achieved a satisfactory recovery of amplifying cells by mechanical disruption of the ovary, it was necessary to know the condition of the isolated cells. Their viability, as regards amplification and S-phase DNA synthesis, was tested by incubating a preparation derived from 3 ovaries in 2 ml of medium with 100 μCi of (3H)thymidine/ml for 16 hours. After incubation the cells were pelleted in a "bench model" centrifuge, the medium was decanted, and DNA isolated. The radioactivity profile after centrifugation of the DNA in CsCl is shown in Figure IV.2, together with that of a typical whole ovary preparation (3 ovaries incubated for 45 hours with 25 μCi/ml (3H)thymidine). Clearly the released cells are still
FIGURE IV.2. Comparison of distribution of radioactivity between rDNA and main band DNA after (3H)thymidine labelling of whole ovaries (upper gradient) and a teased-out ovarian cell suspension (lower gradient).
viable and incorporate $^{3}H$ thymidine into both DNA peaks. Furthermore, follicle cells have been efficiently removed, so that the main band DNA peak shows fewer counts/min than the rDNA peak. It is likely that much of the remaining radioactive mainband DNA is derived from dividing oogonia and oocytes in premeiotic S phase, follicle cells being now in the minority.

Before further examining the properties of this suspension, it was necessary to discover whether equal volumes were equivalent to one another. Using $^{3}H$ thymidine incorporation into the DNA of each aliquot it was found initially that variation between aliquots could be as high as 50%. However, if several precautions were taken equal volumes gave values for $^{3}H$ thymidine incorporation into DNA within 10% of each other. (Table IV 1(A)).

The following precautions were necessary:

1) When the suspension was divided between various sterile screw-cap tubes, the sterile Pasteur pipette containing the suspension was held nearly horizontal. This prevented aggregates of cells which were able to sink rapidly through the medium from accumulating near the tip of the pipette, and consequently being distributed unevenly between the tubes. In addition the suspension was administered one drop at a time to each tube in a row, passing to the next tube after each drop. In this way any chance variations in the composition of the suspension along the pipette and between pipette loads was maximally distributed.

2) After incubation, tubes containing the cultured cells were placed in ice. A dish of *Xenopus* kidney culture cells which had been treated with trypsin and versene to suspend the cells was
<table>
<thead>
<tr>
<th>Duplicate number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPM $^{3}H$ thymidine incorporated into DNA</td>
<td>873</td>
<td>858</td>
<td>906</td>
<td>874</td>
</tr>
<tr>
<td>CPM $^{3}H$ leucine incorporated into protein</td>
<td>846</td>
<td>891</td>
<td>870</td>
<td>-</td>
</tr>
<tr>
<td>CPM $^{32}P$ rRNA hybridization to saturation</td>
<td>$4.4 \times 10^{3}$</td>
<td>$4.75 \times 10^{3}$</td>
<td>-</td>
<td>-</td>
</tr>
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cooled on ice and 1 drop of this suspension added to each duplicate tube. When the tubes were then centrifuged at low speeds in a "bench" centrifuge in the cold, the added cells helped to bind the ovarian cell pellet together and prevent loss of material when the labelled medium or washings were decanted.

(3) When these cells had been incubated with pronase and SDS for 2 or more hours, 20-50 µg of non-radioactive Xenopus blood DNA or Micrococcus lysodeikticus DNA were added to each tube before shaking with phenol. This DNA, together with that extracted from the Xenopus cultured cells served as carrier to minimize DNA loss during the remainder of the extraction.

Table IV.1 shows that these precautions are effective as equal volumes of the suspension are equivalent within 10% for incorporation of \(^{3}H\)thymidine into DNA, and \(^{3}H\)leucine into protein (Table IV.1 (3)). In an experiment performed as part of a separate project, Xenopus kidney culture cells were labelled with \(^{32}P\)phosphate (10 mCi in 7 mls for 36 hours) and the radioactive RNA hybridised to saturation with DNA from 2 equal volumes of suspended ovarian cells (Hybridisation in 6 x SSC at 65°C for 4 hours). Table IV.1 (C) shows similar hybridisation to the 2 preparations, and this suggests that the amount of rDNA (nearly all amplified) in each sample was closely similar.

Using equivalent aliquots of the ovarian cell suspension it was now possible to estimate the length of time for which these cells remain viable in isolation. 3 ovaries were teased-out in 2 mls of medium and divided dropwise into 4 0.5 ml samples.
0.1 ml of (\(^3\)H)thymidine-containing medium (50 μCi/0.1 ml) was added to one sample at zero time. After 10 hours, DNA was extracted from the labelled tube and (\(^3\)H)thymidine added to the next sample for a further 10 hours. In this way incorporation into ovarian cell DNA could be measured in 4 successive 10 hour periods after teasing-out. Figure IV.3 gives the radioactivity profiles obtained when the DNA from each sample was analysed by CsCl equilibrium centrifugation. The total counts/min in each peak in each 10 hour period are presented in Figure IV.4. It can be seen that incorporation into both peaks between 10 and 20 hours is about ½ the level achieved in the first 10 hours of culture. Between 23 and 33 hours, incorporation falls to less than 10% of the initial values, and by 33 hours rDNA synthesis has effectively ceased. Main band DNA synthesis has increased in the last 10 hour period, however, and this is probably attributable to the outgrowth of fibroblastic cells from some of the follicle cell containing aggregates which are present in the suspension.

A time course of the progress of (\(^3\)H)thymidine incorporation into the DNA of a cell suspension was prepared in order to determine for how long - if at all - the rate of DNA synthesis was linear in isolated cells. A 4-ovary suspension in 3 ml was divided between 6 tubes containing 50 μCi (\(^3\)H)thymidine (dried under vacuum) each. DNA was extracted from one pair of tubes at 2.75 hours, 8.25 hours and 21 hours. 0.2 ml of each 1 ml solution of the purified DNA was precipitated and counted. Figure IV.5 shows that incorporation is linear until 8 hours at least and has fallen off by 21 hours.
FIGURE IV.3. (\(^3\)H)thymidine incorporation into rDNA and main band DNA during 3 almost successive 10 hour labelling periods.
FIGURE IV.4. Schematic representation of figure II.3. Shaded histograms represent rDNA incorporation, unshaded histograms represent main band DNA incorporation.
FIGURE IV.5. Time course of $^{3}H$thymidine incorporation into the total DNA from equivalent aliquots of a cell suspension.
The similarity in counts/min for duplicate samples at each point is clearly demonstrated in this experiment. Precisely why these cells should prove to be of limited viability in isolation is not known. Certainly the extracellular environment in culture is considerably different from that found in the intact ovary. In situ, the oocytes are sealed off from contact with the medium by an outer layer of fibroblastic cells. Also the act of teasing out may physically damage the oocytes. Watson Coggins has observed intercellular connecting filaments between oocytes (Watson Coggins, 1971). These would often be severed during teasing and perhaps a vital function prevented.

Apart from this disadvantage of limited viability, the released ovarian cells have several advantages which can be summarised as follows:

1. They represent a considerable purification of amplifying cells over the whole ovary, making it possible to obtain CsCl gradient profiles in which up to 70% of the incorporated thymidine is in the rDNA peak.

2. Equal volumes of the suspension are very similar in composition. Variation between samples for any of the parameters measured (see Table IV.1) being less than 10%. Thus it is possible to perform (a) reliable control experiments for any treatment of amplifying cells which may be investigated; (b) time course experiments under various conditions - bearing in mind the limited viability of the cells (Figure IV.5); (c) measurements of different parameters simultaneously on separate (but equivalent) aliquots of
FIGURE IV.6. Relationship between (3H)thymidine dose and incorporation into total DNA in an ovarian cell suspension.
a suspension (e.g. RNA synthesis, DNA synthesis and protein synthesis.)

(3) It is likely that any precursor or drug in the medium is more immediately accessible to all the cells in the suspension than in the intact ovary.

Since incorporation of $({}^3\text{H})$thymidine into amplified rDNA is to be a standard assay for amplification in future experiments, the optimal concentration of $({}^3\text{H})$thymidine in the incubation medium was determined. Equivalent aliquots were incubated for 12 hours with various concentrations of $({}^3\text{H})$thymidine (20 Ci/mM), and DNA was extracted and counted.

Incorporation is roughly proportional to concentration up to 50 μCi of this thymidine (Figure IV.6). 100 μCi does not significantly increase the level of incorporation. This may be due to thymidine poisoning of cells. The calibration curve is only applicable to $({}^3\text{H})$thymidine of this specific activity unless the abscissa is converted to mM thymidine/ml.

In the following investigations on the effects of cycloheximide and actinomycin D upon amplification, several experiments for which this cell suspension would be suitable have in fact been performed with whole ovaries. This is due to the fact that the cell suspension was only developed quite recently, and work on these projects was quite advanced. Adequate experiments with whole ovaries have not been repeated with the suspension.
CHAPTER V

The relationship between protein synthesis and rDNA amplification

Introduction

It is well established that DNA replication in bacteria is closely linked to protein synthesis (Lark, 1969). In these organisms new protein is required at the initiation site before a round of replication can commence. Once it has begun, however, the process will go to completion in the absence of further protein synthesis. In eucaryotes also, work with synchronised cell lines has shown that inhibition of protein synthesis both before and during S phase prevents further DNA synthesis (Muellar et al., 1962; Taylor, 1965; Terawima and Yasukawa, 1966; Kim et al., 1968; Fujiwara, 1968), though little is known about the role of the necessary proteins in the replication mechanism.

Amplification in *Xenopus* is an unusual departure from S-phase DNA replication as it occurs in dividing cells. Here a single portion of the genome is replicated repeatedly over some 24 days. It was therefore of interest to discover how this special case of DNA synthesis depends on protein synthesis. Cycloheximide was the antibiotic chosen for these experiments and it has been assumed that at the low concentrations used its effect is specifically upon protein synthesis.

Material and methods

Animals were kept and autoradiography carried out as described previously (Chapter II). Both whole and teased-out ovaries were
cultured in vitro in Xenopus medium. \(^3\)H-thymidine, \(^3\)H-leucine and cycloheximide were included in the medium in amounts which varied with the experiment. DNA was extracted by DNA extraction method (2) (see Chapter III - Materials and methods).

A crude protein fraction was prepared from ovaries which had been thoroughly sonicated in 0.1 x SSC. The disrupted cell material was precipitated with 5% TCA at room temperature and the precipitate collected by centrifugation. After a 5% TCA wash, the pellet was dissolved in 0.5 ml of 0.2N NaOH at 37°C for 30 minutes. One half of this solution was precipitated with hot TCA and counted on an Oxoid filter, and the other half was estimated for protein using the Lowry method (Lowry et al., 1951).

Results

In order to find out an effective dose of cycloheximide, one ovary was incubated with \(^3\)H-leucine at each of several cycloheximide concentrations. The specific activity of protein in each ovary was then determined. Figure V.1. shows that 2.5µg/ml of cycloheximide is sufficient to effectively abolish ovarian protein synthesis. An approximate time course of inhibition of protein synthesis is presented in Figure V.2. It is apparent that when the drug was administered inhibition of protein synthesis occurred very rapidly - probably within 1 hour. However, inhibition is reversible, as shown in Figure V.2., at least 4 hours after addition of cycloheximide.

In order to insure that the inhibition of protein synthesis detected biochemically was reflected at the level of the individual amplifying nucleus, autoradiographs of fragments of a single ovary treated
FIGURE V.1. Effect of cycloheximide dosage upon (3H)leucine incorporation into total ovarian protein.
FIGURE V.2. Inhibition of protein synthesis by 2.5 µg/ml cycloheximide. Solid line, control culture showing (3H)leucine incorporation into total ovarian protein with time; broken line, culture treated with cycloheximide at 2 hours; dotted line, culture treated with cycloheximide at 2 hours, but transferred to drug-free medium at 6 hours.
with \( ^3H \) leucine in the presence and absence of cyclohexamide were prepared (Figure V.3.). Fixation in ethanol-acetic acid washes out much of the cytoplasm and it is thought that some nuclear proteins are also lost (Dick and Johns, 1967). However, grain counts over nuclei with well formed caps show a consistent increase over 8 hours, and this can be prevented with cyclohexamide.

The effect upon amplification of cycloheximide was tested biochemically using isopynic centrifugation to separate rDNA from the remainder. Whole ovaries were used initially. They were separated into two, and opposite lobes were cultured with or without cycloheximide in the presence of 50 μCi/ml of \( ^3H \) thymidine for 24 hours. DNA was extracted and centrifuged in CsCl. Radioactivity profiles are shown in Figure V.4. The drug drastically reduces incorporation into both DNA peaks.

It has been demonstrated autoradiographically that amplification occurs much more rapidly in pachytene nuclei than in zygotene (Macgregor, 1968). These biochemical results therefore refer mainly to pachytene amplification as the majority of radioactive rDNA must have come from nuclei at this stage. To test whether amplification in the earlier stages of meiotic amplification was also affected by cycloheximide, autoradiographs of drug-treated and untreated ovaries were prepared after incubation with 25 μCi of \( ^3H \) thymidine for 3 hours. In those ovaries in which protein synthesis had been inhibited, amplification was not detectable in zygotene or pachytene.

The conclusion is therefore that inhibition of protein synthesis by cycloheximide causes rDNA amplification and S phase DNA replication to cease in less than 1 hour.
FIGURE V.3. Effect of cycloheximide upon incorporation of \( ^3\text{H} \)-leucine into amplifying nuclei. Ovary fragments were cultured with 5 μCi\( ^3\text{H} \)-leucine and squash preparations of treated and untreated tissue made at 2 hour intervals. Autoradiographs were exposed for 7 days. Grain numbers over well developed pachytene nuclei were scored. Limits represent the mean + twice the standard error of the mean, i.e., in 95% of samples of this size with this standard deviation, the mean would fall within these limits.
FIGURE V.4. The effect of cycloheximide upon \((^3\text{H})\)thymidine incorporation into total DNA. Open circles, cycloheximide treated; closed circles, control.
In order to quantitate this dependence of rDNA synthesis upon protein synthesis, various doses of cycloheximide were administered to ovarian cells and their incorporation of \( (^{3}H) \)thymidine into "mainband" and rDNA was examined. For these experiments a suspension of ovarian cells was found to be more suitable material than the intact ovary. The suspension was obtained as described in the Methods section of Chapter IV. It is particularly suitable for this experiment as an exact comparison of differently treated samples is required.

A cell suspension from 3 ovaries was divided into 5 0.5ml samples and each was incubated for 18 hours with 5μCi of \( (^{3}H) \)thymidine and doses of cycloheximide ranging from 0 to 2.5 μg/ml. Figure V.5 shows the DNA profiles obtained. When the counts/min above background in each peak are summed and expressed as a percentage of the uninhibited value, these results can be drawn as shown in Figure V.6. It is clear that incorporation into both peaks declines with increasing cycloheximide concentrations. Indeed the level of thymidine incorporation into rDNA shows virtually the same cycloheximide dosage dependencies as does \( (^{3}H) \)leucine incorporation into ovarian protein (Figure V.1) being halved at a concentration of 0.1μg/ml. This suggests that the relationship between rDNA synthesis and protein synthesis is very close and amplification may be limited by the availability of protein. "Mainband" DNA synthesis, however is less sensitive to cycloheximide being reduced by 50% at 0.5μg/ml of the inhibitor.
FIGURE V.5. Incorporation of $^3$H-thymidine into main band and rDNA peaks on CsCl gradients in the presence of various cycloheximide concentrations. Arrows mark the optical density position of Micrococcus Lysodeicticus DNA marker.
FIGURE V.6. Effect of cycloheximide dose upon (3H)thymidine incorporation into main band and rDNA. The values are taken from Figure V.5.
Discussion

The results obtained by analysis of ovarian DNA on CsCl gradients (Figures V.4 and V.5) show negligible thymidine incorporation into rDNA in the absence of protein synthesis. However, it must be born in mind that most of this radioactive rDNA is derived from pachytene cells which are amplifying at the maximum rate. Amplification is first detectable during early zygotene and increases in rate considerably by mid-pachytene. Also, cytological hybridisation (Gall, 1969) has demonstrated multiple copies of the nucleolar DNA in oogonia suggesting slight amplification during early oogenesis. rDNA synthesis in these oogonia and in the early stages of cap formation (zygotene) will contribute relatively little radioactivity to the rDNA peak on CsCl gradients compared to pachytene oocytes. These biochemical observations therefore relate mainly to pachytene cells.

Autoradiography of ovary squashes suggest that this dependence upon protein synthesis is also a property of prepachytene oocytes, as zygotene cells which are visibly amplifying in control squashes show no (3H) thymidine grains over nucleoli after cycloheximide treatment. Little can be said about the production of multiple nucleoli during early oogenesis (if indeed they arise by amplification (Wallace, et al 1971)). As there is no cytologically identifiable stage at which this takes place.

Amplification of nucleolar DNA during meiotic prophase is therefore dependent upon concurrent protein synthesis. This is in contrast to the findings of Darrow and Clever (1970) in their studies on the endoreduplication of polytene chromosomes in Chironimus tetans.
Polytenisation is analogous to amplification in that stretches of the chironimus genome are replicated many times without intervening cell division, yet the prevention of protein synthesis during the intermoult period of the last instar has no effect upon the frequency of replicating nuclei. DNA synthesis is only stopped if cycloheximide is administered at the beginning of the last instar. It would appear that the protein requirements of endoreduplicating nuclei have been fulfilled in advance, whilst amplifying oocytes need concurrent protein synthesis.

Thymidine incorporation into the "main band" DNA peak on CsCl gradients is also depressed in the absence of protein synthesis (Figure V.4 and 5) though less completely than incorporation into rDNA (Figure V.6). The reason for this residual DNA synthesis is not known and the presence of several cell types with possibly different pools and permeabilities contributing to the radioactivity in this peak makes interpretation difficult at this stage.

The response of rDNA synthesis to various cycloheximide concentrations follows much the same dosage dependency curve as does protein synthesis (see Figure V.1.). Even slight inhibition of the latter causes a corresponding reduction in (3H)thymidine incorporation, implying that the rate of amplification is normally limited by the rate of protein synthesis in the oocyte. Once again this result mainly reflects the behaviour of pachytene cells and does not necessarily apply to syngotene cells. Indeed protein synthesis is more likely to limit in the late stages of cap formation as it is then that the maximum rate of rDNA replication is reached.

What sorts of protein are limiting is a matter for speculation, but they clearly turn over rapidly either because of instability
or because they are continually drawn upon to associate with newly synthesised DNA. For example, histone has been identified cyto-
chemically in both the rDNA cap (Gall, personal communication) and the rDNA rich "DNA body" found in Acheta (Lima-de-Faria, 1968)
and Tipula (Lima-de-Faria, 1966) oocytes. A continuous supply of these and other protein components of the chromatin cap will be
necessary during amplification, though there may also be unstable proteins more directly involved in the mechanism of rDNA replication
which must be renewed throughout the amplification period.
CHAPTER VI

The relationship between RNA synthesis and rDNA amplification.

Introduction

Amplification is intimately dependent upon a continuous supply of certain proteins. Similarly, the protein pool must depend upon a supply of messenger RNAs which can be translated into the correct amino sequences by the cytoplasmic protein synthesising machinery. The mRNA molecules may be stable and therefore translatable into protein repeatedly over a relatively long period of time, or they may be unstable and therefore rapidly turning over. The experiments to be described were conceived to try and estimate the stability of the mRNA pool. This approach relies upon the immediate inhibition of rDNA synthesis in the absence of protein synthesis, since the length of time for which amplification persists if RNA synthesis is inhibited can then be considered a measure of mRNA stability.

Materials and methods

Actinomycin D is a universal inhibitor of DNA-dependent RNA synthesis and was therefore used in these experiments.

Autoradiography of squash preparations and extraction and ultracentrifugation of DNA were carried out as before. (Chapters II and III "Materials and Methods").

A crude RNA preparation from ovaries was made following incubation with $^3$H)uridine (or $^{32}$P) phosphate by homogenising either whole gonads or teased out cells in 1 ml of 0.15 M NaCl,
0.01 M Tris, pH 7.5 at 0.5% SDS. 100 μg of yeast RNA were then added as carrier and immediately followed by 1 ml of water saturated phenol. After a 30 minute, rapid shake at room temperature, the aqueous and phenolic phases were separated in a bench centrifuge. The aqueous layer was reshaken with phenol and respun. The final aqueous phase was shaken with ether several times to remove phenol and finally bubbled with air to evaporate the remaining ether. This solution was precipitated with TCA in the cold and the precipitate trapped on an Oxoid filter to permit scintillation counting.

Results

Actinomycin D has been shown to inhibit transcription in many eucaryotic and procaryotic systems. Its effect upon RNA synthesis in the ovary was roughly determined by incubating ovaries of similar size with 25 μCi/ml of \(^{3}H\)uridine for 14 hours in the presence of various actinomycin D concentrations. Figure VI.1 shows that 10 μg/ml is sufficient to inhibit nearly all ovarian RNA synthesis.

A time course of inhibition was performed to estimate the rapidity with which inhibition took place after actinomycin D was added to the medium. Fragments of ovary were used in this experiment and they were standardised by preincubating for 14 hours with 0.02 mCi/ml \(^{32}P\)phosphate (see Chapter IV). The 6 fragments were then transferred to 2 sterile plastic dishes containing 2 ml of Xenopus medium and 25 μCi/ml \(^{3}H\)uridine. After 2 hours incubation RNA was extracted from 1 fragment from each dish, and 20 μg of actinomycin D were added to 1 dish making it 10 μg/ml. At 5 hours and again
FIGURE VI.1. Calibration of effect of actinomycin dosage on whole ovary RNA synthesis. Total RNA was extracted from 1 ovary after 14 hours at each concentration.

FIGURE VI.2. Time course of inhibition of RNA synthesis by 10 µg/ml actinomycin.
at 8 hours a fragment was removed from each dish and RNA extracted. RNA samples were precipitated and counted in both $^{3}H$ and $^{32}P$ channels. Each $^{3}H$ value was divided by the $^{32}P$ counts/min for the same sample and the results plotted in Figure VI.2. It can be seen that inhibition has taken effect within 3 hours and probably inside 1 hour.

As amplifying cells are in the minority in the ovary, it was possible that the complete inhibition of RNA synthesis observed biochemically was not felt in pachytene oocytes. This was tested by autoradiography of ovaries which had been incubated with $^{3}H$uridine. Preliminary experiments showed that incorporation of $^{3}H$uridine into the chromosomes of pachytene nuclei is extensive. (For a detailed autoradiographic study of RNA synthesis in the ovary, see Chapter III). However, when 10 µg/ml actinomycin were included in the incubation medium, follicle cells showed negligible incorporation, but pachytene and diplotene nuclei still showed considerable labelling. At concentrations of actinomycin as high as 40 µg/ml, this incorporation was not significantly reduced. If, however, ovaries were preincubated for 12 hours with 10µg/ml of inhibitor before addition of $^{3}H$uridine, little incorporation was detectable over any ovarian nuclei.

This delayed effect of the drug upon RNA synthesis in pachytene cells is quantitated in Table VI.1. In this experiment fragments of ovary were cultured with 10 µg/ml actinomycin for various periods of time before a 1 hour pulse with 12µCi/ml of $^{3}H$uridine. The fragments were fixed, squashed and coated with stripping film. Grain counts over pachytene nuclei in developed autoradiographs show that incorporation into control fragments
<table>
<thead>
<tr>
<th>Time (hrs) before 1 hr 3H-uridine pulse</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average grain no. per pachytene nucleus in the absence of Act.D.</td>
<td>77</td>
<td>163</td>
<td>226</td>
<td>202</td>
</tr>
<tr>
<td>Average grain no. per pachytene nucleus in the presence of Act.D.</td>
<td>78</td>
<td>68</td>
<td>31</td>
<td>4</td>
</tr>
</tbody>
</table>
during the 1 hour pulses increases for the first 6 hours of culture and remains roughly constant thereafter. This may be because increased RNA synthesis is required in adapting to culture conditions, or it may be that the uridine pools in the tissue gradually become saturated with exogenous uridine. In actinomycin treated cells, no increase is observed, though in the first hour of culture the grain counts are similar to the initial control value. Instead, uridine incorporation in successive 1 hour pulses declines steadily until, by 12 hours exposure to actinomycin, little incorporation above background is detectable in any ovarian cells. Thus cap cells are affected by actinomycin within 3 hours of being exposed to it, but the inhibition is gradual and is almost complete by 12 hours.

The synthesis of RNA in the presence of actinomycin could be detected biochemically in teased out ovarian cells. Here follicle cells have been largely removed, and oocytes form a significant proportion of the cells which remain. A cell suspension was made from 1 ovary and divided between 4 tubes containing 50 μCi of (³H)uridine and differing concentrations of actinomycin. After 14 hours incubation RNA was extracted from the cells in each sample. Each RNA solution was divided into 2 portions; one half was precipitated and counted immediately and the other half was hydrolysed at 37°C in 1N NaOH for 1 hour before precipitation. RNA is hydrolysed to nucleotides in alkali and therefore all radioactivity should disappear after hydrolysis. Any which remains is present in some alkali stable macromolecule e.g. DNA. Unhydrolysable counts/min were subtracted from total counts/min for each RNA
sample to obtain a true value for \( (^{3}H) \)uridine incorporation. Figure VI.3 is the calibration curve obtained. Inhibition is maximal at 10 \( \mu g/ml \) (as shown in Figure VI.1) but is not complete. 7% of RNA synthesis persists in the presence of high concentrations of the drug. This residual 7% presumably reflects oocyte RNA synthesis which slowly declines during the first 12 hours of incubation with actinomycin. Oogonia and the few remaining follicle cells are inhibited rapidly.

In an effort to determine whether a reduction in RNA synthesis in amplifying cells of whole ovary has any effect on the rate of amplification, opposite halves of 10 ovaries were separated and cultured with and without 10 \( \mu g/ml \) of actinomycin. After 6 hours preincubation, 50 \( \mu Ci/ml \) \( (^{3}H) \)thymidine were added to each culture and incubation continued for another 12 hours. DNA was finally extracted from each group of half-ovaries and analysed by ultracentrifugation in CsCl (Figure VI.4). The 2 profiles are not strictly comparable as opposite halves of ovaries are often dissimilar, but it is apparent that after 6 hours pretreatment with actinomycin, the rates of amplification and S phase DNA synthesis in the whole ovary are reduced, though apparently much less so in the ribosomal peak than in the main band peak. This implies that DNA synthesis of both kinds is to some extent dependent upon RNA synthesis.

It was now necessary to find out how close was this dependence. The autoradiographic results suggest that RNA synthesis in pachytene cells is negligible after 12 hours with actinomycin. An experiment was therefore designed to compare rDNA synthesis in
FIGURE VI.3. Calibration of effect of actinomycin dosage upon \( ^3\text{H} \) uridine incorporation into RNA of equivalent aliquots of an ovarian cell suspension.
FIGURE VI.4. Effect of 10 μg/ml actinomycin upon DNA synthesis in half ovaries. Treated halves were preincubated with actinomycin for 6 hours before addition of 50 μCi/ml (³²P)thymidine. Control halves were incubated identically in the absence of actinomycin.

- actinomycin treated x-----x control.
the first 12 hours of exposure to the drug with rDNA synthesis in the second 12 hours. In addition, RNA synthesis in the presence of actinomycin was analysed during both periods so that the extent of inhibition might be monitored biochemically. Uninhibited controls for DNA synthesis and RNA synthesis during both periods were performed. All these incubations were run simultaneously on equal volumes of a teased-out cell suspension derived from 4 ovaries. DNA and RNA were extracted after each 12 hour labelling period. DNA was analysed on CsCl gradients and the radioactivity profiles are shown in Figure VI.5. The comparisons are more easily made in Figure VI.6 as the total counts/min in each peak are represented in a histogram together with a similar representation of the RNA counts/min. Half of each RNA sample was again hydrolysed to establish the proportion of radioactivity not present in RNA. The RNA counts/min were corrected accordingly.

RNA synthesis falls to about \( \frac{1}{3} \) of its uninhibited level in the first 12 hours of actinomycin treatment. Bearing in mind Figure VI.2, which shows that most RNA synthesis in whole ovary ceases immediately upon treatment with actinomycin, it is likely that this RNA synthesis is largely derived from the oocytes in the suspension which, autoradiography has shown, do not immediately cease uridine incorporation. The fact that uridine incorporation is as much as \( \frac{1}{3} \) of the control level after 12 hours with actinomycin, compared with the 7% observed in Figure VI.3, suggests that this preparation is relatively rich in oocytes. This is supported by the distribution of radioactivity between the 2 DNA peaks of
FIGURE VI.5. The effect of actinomycin on DNA synthesis in ovarian cells during the first and second 12 hours of treatment. For details see text.
FIGURE VI.6. Effect of actinomycin upon RNA, rDNA and mainband DNA synthesis during the first and second 12 hour periods of a 24 hour treatment. Shaded histograms show incorporation in the presence of actinomycin. Unshaded histograms show control levels.
controls after CsCl centrifugation (Figure VI.5). Just over half of all the DNA synthesised in the suspension is rDNA.

If the "resistant" RNA synthesis in the first 12 hours is indeed attributable to oocytes, then oocyte RNA synthesis between 12 and 24 hours with the inhibitor falls below 1% of its 0-12 hour value. This is less than 3% of the total uninhibited RNA synthesis.

In the first 12 hours with actinomycin, DNA synthesis in the main band is 45% of control, and in the rDNA peak it is 95% of control. Thus only main band DNA synthesis is significantly affected by actinomycin at this stage. In the second 12 hours the amount of label in both rDNA and main band peaks has decreased compared to the uninhibited controls. rDNA incorporation is now about 50% of control, and main band is about 33% of control.

Clearly rDNA synthesis does not feel the effects of actinomycin until at least 12 hours after the beginning of treatment. On the other hand DNA replication during S phase (main band DNA synthesis) is halved within the first 12 hours, and is still further reduced in the next 12 hours.

Discussion

Evidence from the autoradiography of \(^3\)H)uridine-treated ovaries, supported by the biochemical results, suggests that, in the presence of actinomycin D, RNA synthesis in amplifying cells declines steadily over several hours, and is by 12 hours, negligible. By contrast amplification shows no significant decrease during this first 12 hour period. In the course of the second 12 hours with actinomycin, when little or no RNA synthesis is taking place in the
ovary, total incorporation into rDNA is about half of its inhibited level.

The fact that main band DNA synthesis is reduced several hours earlier than rDNA synthesis in the presence of actinomycin correlates strongly with the observed rapid cessation of RNA synthesis in oogonia compared to the resistance of amplifying cells.

In the light of what has been learnt about the dependence of amplification on protein synthesis, it is likely that RNA transcription is important to the amplifying cell because it supplies the messenger RNAs upon which the proteins required for rDNA synthesis are made. The most obvious hypothesis consistent with these results is that the messengers are stable for several hours and code for proteins during this time. As "old" messengers are broken down they would normally be replaced by new ones synthesized in the nucleus. In the presence of actinomycin the supply of new messenger RNA is gradually cut off in amplifying cells until, by 12 hours, very few new messengers are being made at all. Amplification, which is absolutely dependent upon de novo protein synthesis, could therefore last only as long as the dwindling messenger RNA pool was capable of supporting protein synthesis. This hypothesis explains the continuation of rDNA synthesis for a limited period after RNA synthesis has ceased.

There are, however, several limitations inherent in the data which allow alternative explanations for the results.

Firstly, only total RNA synthesis has been measured either autoradiographically or biochemically, and therefore RNA synthesis
has been considered completely inhibited when the bulk of detectable synthesis has ceased. Synthesis of some RNA species is known to be more sensitive to actinomycin than synthesis of others. For example, 28S and 18S rRNA production can be completely stopped at concentrations of the drug which have no effect upon 4S and 5S rRNA synthesis. It is quite possible, therefore, that these "amplification" messengers can continue to accumulate indetectably for some time after the majority of pachytene RNA synthesis has ceased. This would allow the possibility that amplification is dependent upon concurrent messenger RNA synthesis. It is equally likely that messenger RNA synthesis in cap nuclei stops rapidly after treatment with actinomycin and that the extensive RNA synthesis which is observed in the hours after exposure to the inhibitor represents synthesis of RNA whose production is not easily susceptible to the drug, but which does not affect amplification.

A further possibility is that the eventual decline of rDNA synthesis in the oocyte may not be due to the primary effect of actinomycin upon RNA production. It may conceivably be that at these high concentrations of the inhibitor, secondary toxic effects become important and amplification declines merely as a result of unhealthiness in the cell.

In addition to these considerations, an entirely different explanation for the results is necessary if RNA is involved in amplification more directly than as a messenger for essential proteins. Such a possibility follows directly from the hypothesis of Tocchini-Valentini and Crippa (1971) that amplification occurs through an RNA-dependent DNA polymerase. Details of the mechanism
for this process have not yet been suggested, but it is possible that in the absence of RNA synthesis the supply of RNA transcripts of the ribosomal genes may become used up and amplification prevented in this way.
CHAPTER VII

RNA-Dependent DNA synthesis as a possible mechanism for amplification

Introduction

Temin and Mitzutani (1970) and Baltimore (1970) independently reported the discovery in 4 animal tumour viruses of an enzyme which was capable of using endogenous viral RNA or an RNA-DNA hybrid as a template for the synthesis of DNA. Since that time every RNA tumour virus has been shown to possess the enzyme. In addition, some normal cells have been found to possess a similar ability to convert artificial RNA-DNA hybrids into DNA (Ross, et al., 1971). It has not yet proved possible to characterise the enzyme responsible, and it is not clear that it reflects a normal cellular process rather than an artefact induced by the assay conditions.

Tocchini-Valentine and Crippa (1971) have suggested that an RNA-dependent DNA polymerase is responsible for the amplification of rDNA in Xenopus oocytes. They propose that RNA copies of the rDNA repeating unit (Birnstiel et al., 1968) are transcribed by DNA-dependent RNA polymerase and then "copied" by RNA-dependent DNA polymerase to produce a final double stranded rDNA molecule. Repeating units would then be strung together by a ligase to produce the polycistronic DNA strands which are found in post-amplification oocytes.

Evidence in favour of the hypothesis is suggestive, but is not conclusive proof of the existence of such a mechanism. Because
of the interesting nature of the proposal, I undertook to investigate some predictions of this theory.

Materials and Methods

Culture conditions, DNA extraction and isopycnic centrifugation were carried out as before.

RNA for acrylamide gel electrophoresis was prepared as follows: both whole ovaries and teased out cells were homogenised in 1 ml of fresh Kirby's medium (Tri-isopropyl naphthalene sulphonate, 1 g; Phenol/cresol, 6 ml; 4 amino salicylate, 6 g; NaCl, 1 g; water up to 100 ml). 1 ml of phenol/cresol was then added and the suspension thoroughly mixed. A room temperature spin at 3,000 revs/min for 5' separated the phases. The aqueous layer was removed and made 0.5 M NaCl. It was then mixed with a second equal volume of phenol/cresol and respun. The aqueous phase was reprecipitated with 2 volumes of ethanol at 20°C for at least 5 hours, and the precipitate collected at 3,000 revs/min for 10 minutes at 0°C and dissolved in 0.1 ml of SDS-acetate (0.15 M sodium acetate, 0.5% SDS, pH 6) and precipitated with 2 ml ethanol. The nucleic acids were pelleted, and washed with 90% ethanol to remove SDS.

DNasing was carried out in 0.2 mls of RNase-free DNase solution in M.E.S. buffer (0.05 M 2-(N-morpholino) ethane sulphonic acid, 0.0025 M magnesium acetate, pH 7) for 20 minutes on ice.

At this stage 20 µg of unradioactive Xenopus kidney culture cell RNA was added in 0.1 mls of SDS-acetate to allow optical scanning of the gel for the major RNA species. All RNA was then
precipitated with 2 volumes of ethanol, pelleted, and dissolved in half-strength "running-buffer" containing 10-15% sucrose. This solution was layered on top of a 2.2% acrylamide gel and run at 7 volts/cm length and 11 mA for 5 hours.

After electrophoresis, gels were extruded from the tubes, washed in water overnight and scanned in a Joyce Loeb U.V. scanner. To line up the O.D. and radioactivity accurately in the middle of the gel, a needle with a trace of Indian ink was used to inject a marker and the gel rescanned. The gel was then frozen in dry-ice and sliced on a Mickle gel slicer. Slices were placed in scintillation vials with 0.5 ml of 1 N ammonia and warmed and dried. 0.5 ml of water were added to swell the gel slices, followed by 10 ml of scintillator.

Samples of AF/ABDMP (2,5-dimethul-4-N-benzyl derietyl rifampicin) were gifts from Subak-Sharpe (Glasgow University), and Tocchini-Valentini (Naples). Samples were dissolved in dimethyl sulphoxide (DMSO) at 5 mg/0.1 ml and stored at -20°C. When required the solution was gently thawed and a small amount added with a 10 µl micro syringe to cold ‘Xenopus’ medium with constant mixing. This was always done just before the start of the experiment to minimise decomposition.

AF/AP (3-piperazinoiminomethyl rifampicin SV) was a gift from Sylvestri (Gruppo Lepetit S.p.A). It was handled in the same manner as AF/ABDMP.

Results

Ribosomal DNA is composed of many tandem repeats of a single
unit (Birnstiel et al., 1968). Each unit is made up of a 28S and an 18S rRNA cistron and about 50% "spacer" DNA. Amplified DNA is structurally identical to the somatic variety in this respect (Dawid et al., 1970). Only part of this unit is transcribed during the synthesis of rRNA, the majority of the spacer DNA remaining genetically inactive. However, it follows directly from the RNA-dependent mechanism that an RNA copy of the entire repeating unit must exist and probably be synthesised in amplifying cells.

Acrylamide gel electrophoresis has proved to be a highly sensitive technique for the resolution of RNA mixtures and accurate characterisation of the components (Loening, 1967). It was therefore decided to search by this method for a labelled RNA transcript of the ribosomal genes amongst the RNA of (3H)uridine treated ovaries. The smallest possible transcript would be a copy of a single rDNA unit.

The molecular weight of the rDNA repeating unit is 9 x 10^6 daltons (Dawid et al., 1970). An RNA copy of this stretch of DNA would be approximately 4 x 10^6 daltons in molecular weight and have a sedimentation constant of about 47S. On acrylamide gels, therefore, its appearance is to be expected in the usually unoccupied region between the 40S rRNA precursor and high molecular weight "nuclear heterogeneous" RNA which does not normally move into a 2.2% gel.

RNA was extracted from 5 whole ovaries after incubation with 100 μCi/ml (3H)uridine for 24 hours. Purification did not include a DNase step. The final RNA solution was applied to a 2.2%
acrylamide gel together with 20 µg of total *Xenopus* kidney culture cell RNA as carrier. Figure VII.1 shows the optical density scan obtained after 5½ hours electrophoresis, and the counts/min in 0.25 mm. slices. Apart from the normal 40S, 28S and 18S rRNA peaks, there is, in the high molecular weight region, a conspicuous peak of incorporation. However, it coincides exactly with the optical density peak of contaminating DNA. To find out whether the radioactivity was contained in an RNA species whose mobility was identical with DNA, or in the DNA itself, the experiment was repeated, but including a DNase step in the purification. Counts/min in each slice are plotted in Figure VII.2. There is no significant labelling above background in the 47S region in 24 hours, and this implies that the radioactivity previously present in this region was associated with the DNA peak.

If 47S RNA is indetectable because of its instability, then a more likely means of detecting it would be by a short (³H)-uridine pulse. This was tried, but the shortest pulse to give significant incorporation into 5 ovaries was 3 hours, and the level of labelling in this time was low. Using 5 teased-out ovaries in 1 ml of medium containing 100 µCi (³H)uridine for 3 hours, the acrylamide gel pattern shown in Figure VII.3 was obtained. Counts/min in the 40S peak are very low, 31S rRNA (Loening, 1969) is more heavily labelled than the 40S precursor, and little label has reached 28S RNA in this time. There is no significant peak of incorporation present in the 47S region of the gel, though it is not possible to detect the presence of a peak less than 50% of the size of the 40S peak at this level of labelling.
FIGURE VII.1. Acrylamide gel electrophoresis of 24 hour \(^{3}H\)uridine labelled RNA from 5 whole ovaries. The 22\% gel was U.V. scanned (dotted line) after electrophoresis, and 0.25mm slices were scintillation counted. The U.V.-detectable RNA is mostly carrier, but the DNA peak is from the ovaries. Approximate positions of major RNA species and DNA are indicated. The high molecular weight region of the gel has been redrawn on an expanded scale to show more detail.
FIGURE VII.2. Acrylamide gel electrophoresis of DNAsed uridine labelled RNA from 5 ovaries.
FIGURE VII, 3. Acrylamide gel electrophoresis of RNA from teased out cells labelled for 3 hours with (³H)uridine.
It follows from these experiments that any 47S RNA is either
(1) turning over too rapidly to accumulate sufficiently to allow
detection, or (2) synthesised at a very low rate over the 24 hour
\(^{3}H\)uridine labelling period; or (3) synthesised in advance and
therefore not acquiring \(^{3}H\)uridine label at the time of amplifi-
cation.

A further prediction, which must be fulfilled if the RNA-
dependent mechanism is occurring, is that - as in the case of RNA
virus directed DNA synthesis (Manly et al., 1971) - an RNA-DNA
hybrid is intermediate in the amplification process. In the
extreme case, where RNA-dependent DNA polymerase is totally res-
ponsible for amplification, large numbers of hybrid molecules are
likely to exist in amplifying cells. It has been shown that
rRNA-xDNA hybrids band at a higher density on CsCl gradients than
dNA-DNA duplexes with the same sequence (Wallace and Birnstiel,
1966). This means that after a short \(^{3}H\)thymidine pulse, some
labelled DNA might be extracted in the hybrid replicative form
and detected in the high density region of a CsCl gradient.

To this end, 5 ovaries were teased out (to facilitate rapid
penetration of \(^{3}H\)thymidine) and labelled with 100 μCi/ml
\(^{3}H\)thymidine for 3 hours. DNA was then extracted as usual,
including RNAsing. It has been assumed that the routine extract-
ion method does not discriminate against RNA-DNA hybrids, and in
particular, that any hybrids are resistant to RNase in the same
manner as artificially formed RNA-DNA molecules. Figure VII.4 is
the radioactivity distribution after centrifugation in a CsCl
gradient of average density 1.722 g/cm\(^3\). No counts are detectable.
FIGURE VI-4. Incorporation of $^3$H-thymidine into the DNA of an ovarian cell suspension during a 3 hour incubation. RNA/DNA hybrid would be expected to band in the heavy region of the gradient.
in the high density region of the gradient.

An alternative method of detecting a hybrid intermediate is to label the RNA component. 2 whole ovaries were therefore incubated with 2.5 μCi/ml $^{14}$C thymidine (520 mCi/mM) and 100 μCi/ml $^3$H uridine for 20 hours. DNA was then isolated, and to remove excess uridine labelled RNA, treated for 1 hour at 37°C with 330 units/ml RNase T1 and 100 μg/ml RNase A. The CsCl gradient profile from these ovaries is shown in Figure VII.6. Figure VII.6 shows the result of a similar experiment using teased-out ovaries, and low specific activity $^{14}$C thymidine (60.5 mCi/mM). The distribution of $^{14}$C thymidine counts is typical for each type of cell preparation, but in both gradients considerable $^3$H uridine label is associated with the DNA peaks.

How much, if any, of the uridine incorporation is present in RNA was determined by hydrolysing 1 half of a DNA preparation isolated from 5 ovaries after a 14 hour incubation with 50 μCi/ml $^3$H uridine. After halving the DNA solution each portion was centrifuged to equilibrium in CsCl. The gradients were dripped as usual, and one set of fractions was hydrolysed for 1 hour in 1 N NaOH at 37°C. Both gradients were then precipitated and counted. It is apparent from Figure VII.7, that, within the limits of the experiment, all $^3$H uridine incorporation into each peak is alkali stable and therefore is not present in RNA. Either $^3$H uridine has been incorporated into DNA directly, or it has been converted to a DNA precursor and then incorporated. To make sure that no DNA precursor was present in this uridine initially, unradioactive pure uridine was included in 100-fold
FIGURE W65. Incorporation of (3H)uridine and (14C)thymidine counts into the DNA of whole ovaries.
FIGURE VI.6. Incorporation of $\text{H}_2\text{Uridine}$ and $\text{C}^{14}\text{Thymidine}$ into the DNA of a teased out ovarian cell suspension.
FIGURE VII. The effect of alkali hydrolysis upon (\(^{3}\)H)uridine counts/min. associated with ovarian DNA. Each gradient contains 1 half of a single DNA preparation.
excess of the labelled uridine. Compared with control incorporation, counts/min in the cold uridine DNA sample were about 1/100 of the uninhibited value, thus ruling out the possible presence of a radioactive DNA precursor.

The base composition of amplified rDNA has been studied (Dawid et al., 1970) and no uridine was reported. It therefore seems likely that conversion to a DNA precursor is occurring. This cannot be thymidine as uridine carries the \(^3\text{H}\) atom in the 5' position and this would have to be replaced in substituting a methyl group at this position. A clue to the problem is provided by the distribution of the 2 types of radioactivity between rDNA and mainband DNA peaks in Figures VII.5 and VII.6. The ratio of \(^{14}\text{C}\) counts/min in rDNA to \(^{14}\text{C}\) counts/min in main band DNA is usually about half the same ratio for \(^3\text{H}\) counts/min. In shorthand,

\[
\frac{\text{(}^{3}\text{H) rDNA}}{\text{(}^{3}\text{H) mainband DNA}} = 2 \times \frac{\text{(}^{14}\text{C) rDNA}}{\text{(}^{14}\text{C) mainband DNA}}
\]

Assuming that all precursor pools are in equilibrium with the medium, then this ratio reflects the base composition of the 2 peaks of DNA. It is known from base composition studies that,

\[
\frac{\text{(G + C)} \text{rDNA}}{\text{(G + C)} \text{mainband DNA}} = 2 \times \frac{\text{(A + T)} \text{rDNA}}{\text{(A + D)} \text{mainband DNA}}
\]

Since \(^{14}\text{C}\) is known to be present in thymidine, then it is likely that \(^3\text{H}\) is present in guanine or cytosine. The fact that uracil is a pyrimidine makes cytosine (also a pyrimidine) the
most likely candidate, and this is supported by consultation of the metabolic pathway. Deoxycytidine triphosphate (dCTP), the presumptive DNA precursor, is closely related to uridine triphosphate (UTP), conversion of the RNA precursor to the DNA precursor occurring in the following steps:

<table>
<thead>
<tr>
<th>UTP</th>
<th>CTP</th>
<th>CDP</th>
<th>dCDP</th>
<th>dCTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>ADP</td>
<td>ADP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
</tbody>
</table>

If this conversion is occurring in the ovary, then unradioactive deoxycytidine should compete out (³H)uridine counts from the DNA. Figure VII.8 shows that 50 µg/ml of unradioactive deoxycytidine will reduce the incorporation of 50 µCi/ml (³H)uridine into both DNA peaks over 20 hours by just over 70%. In this experiment 1 µCi/ml (¹⁴C)thymidine of low specific activity (¹⁴C) thymidine (60.5 Ci/mM) was included in the incubation medium to insure that all DNA synthesis was not reduced in excess deoxycytidine. The low (¹⁴C) incorporation is not noticably different in the 2 samples.

Having failed to detect either a 47S RNA molecule which would be capable of coding for the amplified rDNA, or an RNA-DNA intermediate for the reaction, it seemed appropriate to perform the experiment which first suggested that a mechanism of this kind might be occurring in amplifying oocytes (Tocchini-Valentine and Crippa, 1971). This experiment involves the use of the rifampicin derivative AF/ASDMP.

Some rifampicin derivatives are capable of inhibiting the DNA polymerase activity of RNA tumour viruses (Gurgo et al., 1971).
FIGURE VII8. The effect of 50 µg/ml deoxycytidine. HCl upon incorporation of (3H)uridine into DNA of an ovarian cell suspension.
In particular AP/ABDJ4P at 100 µg/ml has been found to inhibit completely the virus directed incorporation of radioactive thymidine into DNA in 1 hour. The same concentration had no effect upon the activity of DNA polymerase isolated from an established human cell line, nor upon E.coli DNA polymerase.

Using AP/ABDMP on whole ovaries, Tocchini-Valentini and Crippa found that the batch of ovaries treated with the drug showed no incorporation of (³H)thymidine into the rDNA peak on CsCl gradients, whereas those not treated showed normal rDNA labelling. Mainband incorporation is the same in both treated and untreated batches, and from this fact they deduce that main band DNA synthesis is unaffected by the drug. Such a conclusion is, however, not justified as different ovaries are not subject to quantitative comparison in this way.

If any conclusions supporting a distinct mechanism for rDNA amplification are to be drawn from the use of AP/ABDMP, then its specificity for rDNA synthesis must be demonstrated. For this purpose the effect of 100 µg/ml AP/ABDMP upon amplification and S-phase DNA replication was investigated using equivalent aliquots of an ovarian cell suspension. 2 such aliquots of a suspension derived from 3 ovaries were incubated with and without AP/ABDMP for 24 hours at 25°C in the present of 100 µCi/ml (³H)-thymidine. Figure VII.9 gives the CsCl gradient profiles of radioactive DNA isolated from these cells. In the control, incorporation into rDNA exceeds that into main band DNA. Treated cells, however, have ceased making rDNA, and incorporation into main band DNA is cut to $\frac{1}{3}$ of its control value. It is apparent from these results that rDNA amplification is highly susceptible
FIGURE VII9. Incorporation of (3H)thymidine into main band and rDNA in the presence of 100 μg/ml AF/ABDMP. The asymmetrically distributed high background is due to contamination of a 2 year old (3H)thymidine sample. It probably represents incorporation into RNA and/or proteins. The arrows mark the optical density position of Micrococcus Lysodeicticus DNA in these gradients.
to AF/ABDMP. Main band DNA synthesis is also inhibited, but incompletely. This latter effect would not be expected if the drug was specifically inhibiting RNA-dependent DNA synthesis.

Another rifampicin derivate which is known to inhibit the DNA polymerase activity of RNA viruses is AF/AP (Sylvestri, 1971). Using this compound at 100 µg/ml under the same conditions as before, 2 identical radioactivity profiles (Figure VII. 10) were obtained. AF/AP clearly has no effect upon DNA synthesis in either peak. The experiment also shows that the solvent (DMSO) is not toxic in the amounts used (2 µl/ml).

In view of the toxicity of AF/ABDMP to ovarian DNA synthesis the possibility that decomposition to toxic products had occurred during the incubation was investigated. It was learned at this time that AF/ABDMP loses $\frac{1}{3}$ of its in vitro activity overnight at 0°C (Szilagyi, personal communication). In the original studies on the effect of these derivates upon "reverse transcriptase", Gurgo et al., (1971) used 1 hour assay periods at 37°C. Ovarian cells were therefore incubated for 2 hours at 25°C with 50 µCi/ml ($^3$H)thymidine in the presence and absence of AF/ABDMP. A third equivalent aliquot of the same cell suspension was similarly incubated in the presence of 100 µg/ml AF/ABDMP which had been preincubated for 4 hours at 25°C. DNA from each batch of cells was centrifuged to equilibrium in CsCl, and fractions were precipitated and counted. The 3 gradients are shown in Figure VII.11. Both AF/ABDMP treated samples show no RNA incorporation. Main band incorporation is 15% of control in the "unpreincubated" sample and 7% of control in the "preincubated" sample.
FIGURE VII.10. The effect of AF/AP upon (\(^3\)H)thymidine incorporation into the DNA of an ovarian cell suspension.
FIGURE VIII. The effect of fresh and preincubated AF/ABDMP upon incorporation of $^{3}H$thymidine into DNA of an ovarian cell suspension.
The fact that AF/ABDMP which had been held at 25°C for 4 hours proved twice as toxic to S phase cells as the "fresh" compound suggests, firstly, that the substance alters substantially in 4 hours at this temperature, and secondly, that the products of this alteration are toxic to DNA synthesis in the ovary.

There is also good reason to believe that AF/ABDMP decomposes at -20°C in DMSO solution. Thus the sample of AF/ABDMP used in the experiment shown in Figure VII.9 proved more toxic to S phase DNA synthesis in later experiments, and was eventually completely toxic. A new batch was therefore obtained (Tocchini-Valentini, Napoli). This batch was used in Figure V.11 and it prevented most main band incorporation of thymidine even in 2 hours, suggesting that either an impurity was initially present, or decomposition had occurred in the solid before dissolving in DMSO. The toxicity of this batch is demonstrated by its effect upon DNA synthesis in whole ovaries over 24 hours (Figure VII.12). Thymidine incorporation is negligible compared with the control level.

The considerable instability of this compound over a few hours at 25°C, and the apparent toxic nature of the decomposition products, is a serious impediment to the use of AF/ABDMP on living tissue. It is difficult to rigorously distinguish between specific inhibition by the native drug, and generalised toxicity after its breakdown.

One further experiment is pertinent to the problem of RNA-dependent amplification. Hydroxyurea (HU) is known to be a specific inhibitor of DNA synthesis in all systems so far studied (Rozenkranz and Levy, 1965). It is also known not to affect repair
FIGURE VII 13. The effect of $10^{-3}$ M Hydroxyurea (HU) upon amplification and S-phase DNA synthesis in the ovary.
DNA synthesis (Stern et al., 1971) by which nucleotides in one strand of a DNA duplex are replaced. When 2 aliquots of a cell suspension from 1 ovary were incubated at 50 μCi/ml (3H)thymidine, with and without 75 μg/ml (10^{-3} M) HU, the incorporation into the DNA of each preparation followed the pattern shown in Figure VII.13. Both peaks are reduced to the same extent with HU. In the absence of any studies upon the effect of HU upon RNA-dependent DNA synthesis, it can merely be stated that an inhibitor which has so far shown unusually high specificity in vivo for chromosomal DNA replication inhibits both amplification and S-phase DNA synthesis to the same extent.

DISCUSSION

Two experiments by Tocchini-Valentini and Crippa have suggested that 47S RNA molecules (the presumed templates for rDNA synthesis) are being synthesised in amplifying cells. (1) (3H)-uridine labelled RNA was isolated from amplifying ovaries and analysed by sedimentation through a sucrose gradient. Different regions of the gradient were hybridized to denatured rDNA which had been immobilised on filters. In addition to the normal hybridization peaks at 18S, 28S and 40S, some radioactivity in the 47S region of the gradient proved complementary to rDNA. No peak of radioactivity is detectable at 47S before hybridization. (2) Ovaries were labelled with (3H)uridine and DNA isolated and banded on CsCl. Label was found in a peak close in density to the rDNA. This label was thought to be present in the RNA component of RNA–DNA hybrids – intermediates in the synthesis of rDNA. The
material has been isolated from large numbers of ovaries and a 47S rRNA melted from it.

I have been unable to detect the 47S rRNA species by acrylamide gel electrophoresis, despite the high resolving power which it affords. It is, of course, possible that the molecule is labelled in such small amounts that it is not visible above background.

Unlike Tocchini-Valentini and Crippa, I was not able to detect an RNA-DNA hybrid by labelling with \((^{3}\text{H})\text{uridine}\). The \((^{3}\text{H})\) counts in both rDNA and main band DNA are alkali stable and appear to be the result of a conversion of \((^{3}\text{H})\text{uridine}\) to \((^{3}\text{H})\text{deoxy-cytidine}\). Unless our DNA extraction methods discriminate differently between some DNA containing fractions, then I cannot account for the negligible \((^{3}\text{H})\text{uridine}\) incorporation into the main band which these workers have found, nor for their ability to isolate a 47S rRNA from this putative rRNA - rDNA hybrid.

The disadvantages inherent in the use of AF/ABDMP have been mentioned above, but there are several other relevant points which may be considered.

(1) Tocchini-Valentini (unpublished observation) has observed that AF/ABDMP has an inhibitory effect upon RNA polymerase. If so, then this argues against a specific target enzyme for the drug.

(2) Recent experiments by Szilágyi and Pennington (1971) suggest that AF/ABDMP is not a specific inhibitor of RNA-dependent DNA synthesis even within the RNA viruses. Rapid inhibition of in vitro bacterial and viral DNA-dependent RNA synthesis takes place in the presence of the drug. In the bacterial system this
inhibition requires a concentration of AF/ABDMP more than 100 fold less than that required to inhibit viral "reverse transcription".

(3) Ficq and Bracnet (in press) have performed autoradiography upon ovaries incubated with 100 µg/ml AF/ABDMP and 100 µCi/ml (³H) thymidine. Their incubations were conducted at 8°C, presumably to prevent decomposition of the drug over the incubation period. At this temperature inhibition of incorporation into pachytene caps is slight after 24 hours, but is marked after 48 hours and complete by 60 hours. In view of the steady decomposition of AF/ABDMP which is occurring even at this temperature (¼ of AF/ABDMP activity in vitro is lost overnight at 0°C), it remains a possibility that these findings are the result of a non-specific inhibitory effect. Furthermore, Ficq and Bracnet state that S phase cells are unaffected by the drug. Unfortunately they do not offer evidence for this point. Indeed, in the experiment which they describe it would not be possible to assess S-phase inhibition as thymidine incorporation into S phase nuclei is not progressive and therefore grain counting is not useful.

From my own results and from the findings mentioned above, it is reasonable to conclude that AF/ABDMP has not provided unequivocal evidence for RNA-dependent DNA amplification.

The possibility that RNA-dependent DNA polymerase is present in the cells of multicellular organisms was originally proposed to explain some aspects of cell differentiation (Tocchini-Valentini,
and Crippa, 1971). By the action of this enzyme, selected regions of the genome which are to be transcribed extensively in the final differentiation state of the cell, might be copied by an RNA polymerase and amplified independently of chromosomal DNA through RNA-dependent DNA synthesis. The amplifying oocytes of Xenopus are a natural choice for the initial search for RNA-dependent amplification of this kind. There is, however, a weakness in the logic behind the search. Namely, that multiple rDNA copies already exist in the oocyte before the start of meiotic amplification. Thus the major advantage of this theory, which is to allow excision of rDNA from the chromosomes without the problems of specific DNA breakage and reunion is therefore not necessary as the problem has been solved previously. Even if the original extrachromosomal rDNA copies were obtained in this way, it is questionable whether the synthesis of an RNA copy of the repeating unit, formation of rDNA using that copy as a template and subsequent ligation of the fragments into polycistronic DNA fibres is "more favourable" to the cell than the DNA-dependent DNA polymerisation used by S phase cells. There are more than 2 million rDNA repeating units in the diplotene oocyte. RNA-dependent amplification on a 47S RNA would therefore have to synthesise and ligate, on average, at least 3,000 separate units each hour to complete the process in 24 days.

If amplification does in fact proceed in this manner, then RNA-dependent DNA synthesis must offer an advantage over DNA-dependent replication which is at the moment obscure.
A general discussion of the mechanism of amplification

Since experimental results have been fully discussed in a section at the end of each chapter, this discussion will consider the unifying theme which runs through all the investigations reported here - the molecular mechanism of amplification.

DNA synthesis is not well understood even in microorganisms, and in eucaryotes the situation is less clear. The replication of a well-studied, reiterated DNA during amplification in *Xenopus* oocytes may have much to offer as a system for the study not only of amplification itself, but also of the process by which DNA is replicated in higher organisms.

Two main questions must be answered. Firstly, what is the origin of the DNA which is to be amplified; and secondly what are the molecular details of the process by which amplification proceeds and is terminated?

In answer to the first question Brown and Blackler (in the press) have convincingly argued that amplified DNA originates at the nucleolus organizer in each generation (see Chapter I). The occurrence of oogonial multiple nucleoli implies that the first copies are released from the nucleolus organizers before the onset of the wave of oogonial mitoses which precedes meiosis.

Answers to the second question are still at the hypothetical stage. They are, in the main, variations upon the two extremes proposed by Miller (1966); namely the chromosome-based mechanism, and the extrachromosomal mechanism. Circumstantial evidence favours the extra-chromosomal alternative. The first such
evidence derived from an autoradiographic study ($^3\text{H}$)thymidine incorporation into pachytene caps (Macgregor, 1968). An approximate correlation between the mass of amplified DNA in the cap and the amount of ($^3\text{H}$)thymidine incorporation was noted. It could be most easily explained if extrachromosomal DNA replication was progressively making more DNA templates available for DNA replication. A chromosome-based mechanism would be expected to maintain a constant rate of incorporation since a fixed number of templates is operating.

The increase in thymidine incorporation is considerably less than exponential, however, and this permits alternative interpretation of this result. For example, the rate of chromosomal RNA synthesis also shows a large increase in rate from zygotene to late pachytene (i.e. the amplification period). It is possible that the supply of messenger RNAs for the synthesis of "limiting proteins" (see Chapter V) increases over this period. In other words the rate of amplification may not be limited by the availability of template DNA but by the supply of necessary proteins and perhaps other parameters. It is worth noting that, if a "cascade" mechanism is operating, the supply of essential proteins may prevent a truly exponential increase in rate, since sufficient protein may not be present to allow all available rDNA to replicate at the same time.

Recent autoradiographic studies have pointed to extrachromosomal rDNA replication for a different reason. In ultrathin sections a nucleolar structure is visible within the pachytene cap. E.M.
autoradiography shows that this structure, which is probably derived from the leptotene nucleolus, does not incorporate $^{3}H$-thymidine, whereas label is found in the surrounding cap material (Watson Coggins, 1971). It remains possible in this experiment that radioactive DNA is synthesised at the nucleolus and then rapidly transferred to the cap. That this is not the case is demonstrated by autoradiographs of squash preparations after a 15 minute pulse with $^{3}H$thymidine (Chapter II). They show that DNA synthesis is occurring in all parts of the cap simultaneously. Furthermore, in one nucleus incorporation was absent over a pale staining region in the cap, a region which has been shown to incorporate $^{3}H$uridine extensively during amplification, and which probably corresponds to the nucleolar structure seen by Watson Coggins (1971). Taken as a whole, the evidence suggests that the leptotene nucleolus passes intact into the pachytene cap and becomes active in rDNA synthesis, whilst all around, the cap is engaged in rDNA replication.

Although it is possible to construct alternative explanations, these events strongly suggest that, during pachytene, the nucleolus does not participate in the amplification process. Interpretation is hindered by ignorance of the nature of the nucleolar structure at this time. The leptotene nucleolus contains multiple copies of the nucleolar DNA, probably derived from those seen in oogonia, but it is not known whether the chromosomal nucleolus organiser DNA also contributes to its make-up. In this connection it is of interest that, by pachytene, the chromosomes are so arranged that the secondary constriction at which the rDNA is situated (Rafferty and Sherwin, 1969), is physically distant from the cap.
This is inferred from the facts that the constriction is close to one end of chromosome 12, and that in the pachytene chromosome bouquet configuration, chromosome ends are distal to the cap. It is therefore possible that both the nucleolus and the cap DNA at this stage contain only extrachromosomal DNA.

Assuming that replication is indeed extrachromosomal, the molecular details of the process remain a mystery. The current possibilities will be considered.

Since circular DNA molecules can be isolated from multiple nucleoli in the lampbrush stage, it is tempting to see an analogy between amplification and circular DNA replication in microorganisms. Such replicating circles have been found in E. coli (Cairns, 1963) polyoma virus (Bourguax et al., 1971) and mitochondria (Kasamatsu et al., 1971). Similar structures have not been looked for in amplifying cells.

A more specific suggestion for the amplification mechanism has been offered by Brown et al (in the press) They suggest that the "rolling circle" model of Gilbert and Dressler (1968) might operate. This process involves continuous replication of a length of DNA in such a manner that the replicated DNA adds to the length of the original molecule. In the absence of scission a long molecule made up of replicas of the original DNA joined end to end would be produced.

Finally there is the RNA-dependent mechanism dealt with in Chapter VII. The experiments reported there do not favour a mechanism of this kind, but neither do they disprove its existence.
Any proposal for the amplification mechanism must attempt to explain the huge variation in the size of DNA molecules associated with nucleolar cores from fully grown oocytes. In particular it must explain the occurrence of DNA stretches much larger than the organizer itself (Miller and Beatty, 1969). Precise replication of the nucleolus organizer region, or a copy of it would be expected to generate DNA of identical length in a manner comparable with DNA replication in microorganisms. Both the "rolling circle" model and the RNA-dependent mechanism can easily cope with this dilemma. In the former, a long molecule composed of rDNA repeating units would be randomly chopped by exonucleases into pieces whose length would vary; and in the latter, repeating units would be strung together by ligase, again into units of varying length.

As already mentioned, the microorganism-type of replication should give rise to molecules of uniform size. There is, however, a possible way round this objection if the phenomenon of crossing over (recombination) is invoked. In a highly repetitive DNA such as rDNA it is quite likely that crossing over occurs readily, given the necessary nicking and repairing enzymes. Synapsis is known to take place at zygotene, and in the lily the requisite enzymes have been isolated from zygotene cells (Stern, 1971). In the replicating cap structure, where DNA is perhaps nicked as part of the process of DNA synthesis, crossing over within the high concentration of repetitive DNA may be easier to tolerate than to prevent.
The diagrams below show (a) how crossing over might release circular rDNA from the nucleolus organiser (b) and how it might also give rise to rDNA circles larger than the original nucleolus organiser.

The reason why amplification is terminated after the synthesis of roughly 30 μg of rDNA poses yet another problem. Each oocyte must be capable of measuring some aspect of the process. Apart from direct measurements of the mass of amplified DNA or the number of replications, more indirect controls are possible. For example, the synthesis of those proteins upon which amplification is absolutely dependent may cease as the cell enters diplotene.

Several experimental approaches to the problems posed by amplification are possible.

In considering the various hypotheses, the RNA-dependent mechanism stands apart, since it is based on a fundamentally different form of DNA polymerisation. A test of this theory should be feasible by looking for the characteristics of RNA-dependent DNA synthesis, as distinct from the orthodox DNA dependent reaction, in
amplifying ovaries. The experiments presented in Chapter VII constitute preliminary attempts to do this, and on the whole suggest that an RNA dependent mechanism is not operating. More crucial test experiments have since been devised. The most promising of these involves an inhibitor of DNA synthesis, mitomycin C. This substance specifically inhibits DNA replication by covalently crosslinking thymidine residues in the replicating DNA duplex. Since an RNA-DNA hybrid intermediate contains uridine, and not thymidine, in the RNA strand, then it may be that cross-linking cannot occur. If this is the case, then RNA-dependent synthesis would be resistant to mitomycin C and the two mechanisms could be distinguished by its use.

Much of what is known about DNA synthesis is based on the visualisation of replicating DNA. This can be achieved in two main ways: (1) electron microscopy of replicating DNA after spreading (e.g. Kasamatsu et al, 1971). (2) autoradiography of DNA fibres from (3H)thymidine pulsed cells (Huberman and Riggs, 1966; Callan, unpublished).

In adopting the first of these techniques for the investigation of DNA amplification, it must be born in mind that it has previously been used to study DNA synthesis in microorganisms with low molecular weight genomes. This kind of molecule can readily be isolated in bulk without fear of breakage. The average *Xenopus* nucleolus organiser contains about 2-3 mm of rDNA and would therefore be considerably more fragile during extraction and centrifugation. A possible way round this problem would be to adopt the gentlest possible DNA isolation procedure. For example,
high molecular weight DNA is yielded when tissue is incubated with "sarcosyl" - a mild detergent - and pronase for several hours and then gently mixed with saturated CsCl solution prior to centrifugation (Gall et al, 1971). Just how high in molecular weight this DNA would prove to be is not known, but it may be possible to spread it suitably for electron microscopy. In this way the structural features of rDNA at various stages of duplication could be observed.

The more indirect technique of DNA fibre autoradiography provides quite different information about DNA synthesis. Only the length of DNA synthesised during incubation with \(^3H\)thymidine is visible. The DNA is trailed across a nitrocellulose filter after very gentle isolation and then the filter is coated with stripping film. Thus all DNA strands are similarly orientated and grain "tracks" on the same molecule are in line. Their distance apart gives the length of replicating units on the DNA, and the length of individual tracks after various pulse lengths gives the replication rate.

Using this method it will be possible to discover whether amplifying DNA is divided into replicating units, or whether each stretch of nucleolar DNA is replicated from a single site in the same manner as a bacterial chromosome. The presence of tandem replicating units would also disprove the RNA dependent mechanism, as this process requires segments of DNA (each about 4 µ in length) to be synthesised prior to ligation. Similarly, replicating units would not be consistent with a "rolling circle" form of DNA synthesis (Brown and Blackler, in the press). Such a mechanism would be
expected to produce one growth point per molecule.

Fibre autoradiography again requires very high molecular weight DNA. The isolation technique outlined above may prove adequate, though it would also be possible to use a pure preparation of amplifying oocytes. This is possible as the technique only detects replicating DNA and, once premeiotic S-phase is completed, only rDNA is synthesised in oocytes. Also it would not be necessary to purify the rDNA by centrifugation and risk DNA strand breakage.

An interesting possibility, which would replace the need to purify either the amplifying rDNA or the oocytes before autoradiography, arises if the cells are labelled for a relatively long period of time. Under these conditions some rDNA would pass through more than one replication "generation", and hence acquire label in both strands of the double helic, whilst main band DNA, if only one S-phase is experienced during the pulse, would label in only one strand. (The pulse would have to be shorter than the smallest inter-S-phase period so that no chromosomal DNA could label both strands by entering a second S-phase). Provided that tracks made by DNA labelled in both strands can be visually distinguished from single strand labelled tracks, then it should be possible to unequivocally identify rDNA amongst the various labelled strands on an autoradiograph. rDNA replicating units, if they occur, may now be recognised as stretches of double label along single strand labelled DNA.

It is worth noting that the RNA-dependent mechanism would immediately produce double-strand labelled DNA, since replication is not semi-conservative.
A technique which has not yet been adapted to the visualisation of amplifying DNA, is electron microscopy of deoxyribonucleoprotein (DNP) which has been sedimented onto a grid. Miller and Beatty (1969) developed this technique to observe transcription in multiple oocyte nucleoli, and have since used it successfully in studying lambrush chromosomes and bacterial RNA synthesis. Since DNA is not purified as part of this technique, any proteins and RNA remain attached to the DNP. Thus the position of polymerases or an unusual arrangement of DNP components may be detected.

In addition to these visualisation methods, there exist biochemical techniques for the study of DNA synthesis which can be adapted to the oocyte system. One experiment, devised by Pardue, offers hope of a final solution to the problem of whether amplification is chromosomal, or "cascade".

The experiment can be explained as follows. If an amplifying ovary is exposed to the density label bromodeoxyuridine (BUDR), some rDNA will become heavy in both strands (HIH) after more than one replication "generation" has elapsed. This will occur whether the chromosomal or the cascade mechanism is operating. The difference lies in the fact that in the former mechanism replicated DNA would be stored in the cap, whilst in the latter it would be repeated re-replicated.

By now removing the BUDR and exposing the ovary to a radioactive DNA label (e.g. \(^{3}H\)thymidine) these two alternatives can be distinguished. The "cascade" mechanism should incorporate label into replicating HIH rDNA causing a radioactive band to appear in a
density gradient close to the bouyant density of (heavy-light) HL rDNA. On the other hand a chromosomal mechanism would not utilise HH rDNA synthesised during the BUdR pulse, as this will have moved away from the chromosomal organiser into the cap for storage. In this case radioactive rDNA will appear at, or near, the bouyant density of LL rDNA.

The possibility that both labels arrive in the same DNA strand by elongation during radioactive labelling of a chain whose replication began during the BUdR pulse does not arise, as only the fate of HH rDNA is monitored. HH rDNA must be fully substituted with BUdR in both strands and therefore completely replicated prior to the second pulse.

It is possible that the presence of BUdR in both strands prevents further DNA replication. To counteract this it would be necessary to reverse the order of labelling. Thymidine labelled DNA would now become heavy with BUdR, but the advantage of following only HH rDNA would be lost. A chase period between labels, sufficiently long to complete synthesis of partially replicated rDNA molecules would be a less elegant alternative.

Another biochemical technique which may prove useful is neutral or alkaline sucrose gradient centrifugation of gently isolated amplifying rDNA. This may give the sedimentation constant, and hence the approximate molecular weight of each replicated rDNA strand.

By adopting these experimental approaches to the amplifying oocyte it should be possible to gain an insight into the intricacies of the process by which this DNA is exclusively amplified. The
results may provide information of use in solving the more formidable problems of chromosomal DNA replication.
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