The EMBO Journal vol.5 no.13 pp.3571-3577, 1986

c-*myc* expression is dissociated from DNA synthesis and cell division in *Xenopus* oocyte and early embryonic development

François Godeau[1,4], Håkan Persson[2], Harry E.Gray[1,3] and Arthur B.Pardee[1]

[1]Department of Pharmacology, Harvard Medical School and Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, Boston, MA 02115, USA, and [2]Department of Medical Genetics, The Biomedical Center, University of Uppsala, Box 589, Uppsala S-751.23, Sweden

[3]Present address: INSERM U.55, Hôpital Saint-Antoine, 184 rue du Fbg Saint-Antoine, 75012 Paris, France

[4]Present address: Unité de Biologie Moléculaire du Gène, INSERM U.277, CNRS U.A. 535, Institut Pasteur, 25 rue du Dr. Roux, 75724, Paris Cédex 15, France

Communicated by P.Kourilsky

The combined use of a human c-*myc* probe and of an antibody raised against the human c-*myc* gene product demonstrated that the *Xenopus* cells contained a 2.5-kb c-*myc* transcript and synthesized a c-*myc* immunoreactive 65-kd polypeptide. In full-grown oocytes, $p65^{c\text{-}myc}$ was predominantly located in the nucleus. In non-dividing *Xenopus* oocytes c-*myc* mRNA was present at a steady-state level 10^4 times higher than that of growing somatic A_6 cells. This very high level of c-*myc* transcript was reached early in oogenesis and remained constant thereafter. The rate of $p65^{c\text{-}myc}$ synthesis also reached high levels, but only in vitellogenic oocytes, suggesting a post-transcriptional control. Although the cell cycle is resumed at a very fast pace in developing embryos, no further increase in total embryonic content of c-*myc* RNA could be demonstrated up to the swimming tadpole stage. Furthermore, in embryos the rate of synthesis of $p65^{c\text{-}myc}$ decreased to a level markedly lower than that of cell cycle-arrested vitellogenic oocytes. This observation suggests that the function of the c-*myc* gene in the cell cycle may not be implicated directly in sustaining DNA synthesis or mitosis.

Key words: cell cycle/c-*myc*/*Xenopus* development/growth control

Introduction

Oogenesis denotes the complex developmental sequence by which an oogonium differentiates into a mature oocyte able to support animal development as a result of fertilization. In *Xenopus*, this process takes place over an extended period of time (several weeks), during which most of the functions associated with the cell division cycle (i.e. DNA synthesis and mitosis) ceases completely. Oogenesis therefore constitutes a unique example of uncoupled growth, whereby a single cell undergoes a dramatic mass increase without replicative DNA synthesis or cell division (for review, see Gurdon, 1974; Woodland, 1982). During the first 3 days of embryogenesis the developing zygote undergoes a series of rapid cell divisions leading to the formation of a tadpole composed of 10^6 cells (Gurdon, 1974; Woodland, 1982). Thus, *Xenopus* oogenesis is characterized by growth without cell division, and early development is characterized by cell division without growth (Brown and Littna, 1964).

Like other cellular proto-oncogenes, c-*myc* is thought to carry out a crucial function in relation to cellular growth (Bishop, 1985 for review). Its expression is modulated by growth regulators and is correlated with the establishment and maintenance of the 'growing state' in somatic cells (Kelly *et al.*, 1983; Armelin *et al.*, 1984; Campisi *et al.*, 1984). Its precise role in the 'immortalization' of primary cells and in tumor formation remains elusive (Land *et al.*, 1983; Mougneau *et al.*, 1984; Stewart *et al.*, 1984b), but abnormal expression of the c-*myc* gene has been noted in a variety of human and animal tumors (Hayward *et al.*, 1981; Klein and Klein, 1985, for review).

To gain insight into the role of c-*myc* in the control of cellular growth, we have followed the expression of the c-*myc* gene during the sequence of changing growth conditions both preceding and following fertilization. Our results show that c-*myc* is expressed at high levels in 'dormant', non-dividing *Xenopus* oocytes, which contain 10^4 times more copies of c-*myc* transcript than a growing somatic cell in culture. Translation of c-*myc* mRNA into protein is restricted to a specific period in late oogenesis, and rapidly dividing embryos synthesize the *Xenopus* c-*myc* encoded protein at a markedly decreased rate compared with that of their oocyte progenitor, even though the embryonic c-*myc* mRNA content remains unchanged.

Results

A homolog of the mammalian c-myc in Xenopus

To establish the existence of a *Xenopus* gene homologous to the mammalian c-*myc* gene, Southern blot analysis of restricted *Xenopus* liver DNA was performed using a nick-translated probe covering the 3′ exon of the human c-*myc* gene (Battey *et al.*, 1983). Figure 1A shows the pattern of homologous fragments obtained after digestion with *Bam*HI and *Hin*dIII restriction endonucleases. Hybridization was observed even after stringent washing. To determine whether c-*myc* is expressed in *Xenopus* cells, total or poly(A)-containing RNA from *Xenopus* kidney cell line (A_6) was fractionated, transferred to nitrocellulose and hybridized to the human c-*myc* probe. A single 2.5-kb polyadenylated transcript could be detected (Figure 1B).

To further confirm the existence of an amphibian homolog of the mammalian c-*myc* gene and document its expression in *Xenopus* cells, experiments using an antibody directed against the human c-*myc* gene product were carried out. [^{35}S]Methionine-labelled extracts of the *Xenopus* kidney A_6 cell line were immunoprecipitated with an antiserum raised against the N-terminal portion of the human c-*myc* gene product (anti-c-*myc* N-ter I) (Persson *et al.*, 1984, 1985, 1986). As shown in Figure 2, this antibody specifically recognized a 65-kd polypeptide in these extracts.

Indirect immunofluorescence was also used to determine the localization of $p65^{c\text{-}myc}$ on sections of fixed full-grown stage VI oocytes. As shown in Figure 3 (upper panel), anti-c-*myc* N-ter I specifically labelled the nuclei of somatic follicular cells surrounding the oocyte. Examination of the germinal vesicle (the oocyte's nucleus) revealed that $p65^{c\text{-}myc}$ was also predominantly localized within it and was enhanced in the vicinity of the nuclear envelope (Figure 3, middle panel). Some cytoplasmic labelling

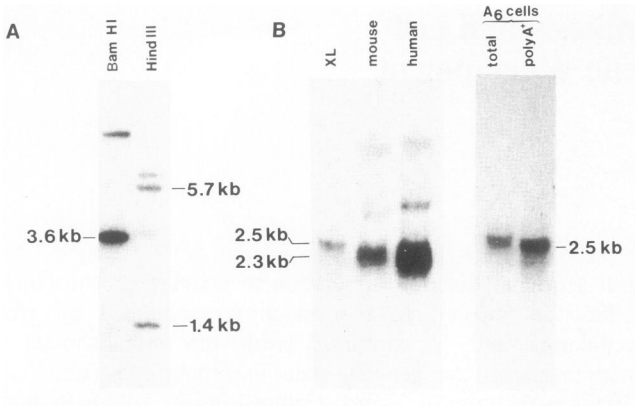

Fig. 1. Detection of sequences related to the human c-*myc* gene in *Xenopus* DNA and RNA. **(A)** Southern blot analysis of *Xenopus* liver DNA. 15 μg of *Xenopus* DNA was digested with the indicated restriction endonucleases, electrophoresed, transferred to nitrocellulose and hybridized to the human c-*myc* 3′ exon probe and stringently washed as described in Materials and methods. **(B)** Northern blot analysis of *Xenopus* RNA. 30 μg of total cellular RNA from *Xenopus* A_6 cells (X.L.), benzopyrene-transformed mouse 3T3 cells (mouse), and T_{24} human bladder carcinoma cells (human) (left panel) or 30 μg of total cellular RNA (total) and 5 μg of poly(A)-containing RNA [poly(A)$^+$] from A_6 *Xenopus* kidney cells (right panel) were analysed simultaneously. After transfer onto nitrocellulose, filters hybridized to human c-*myc* 3′ exon probe and stringently washed as described in Materials and methods.

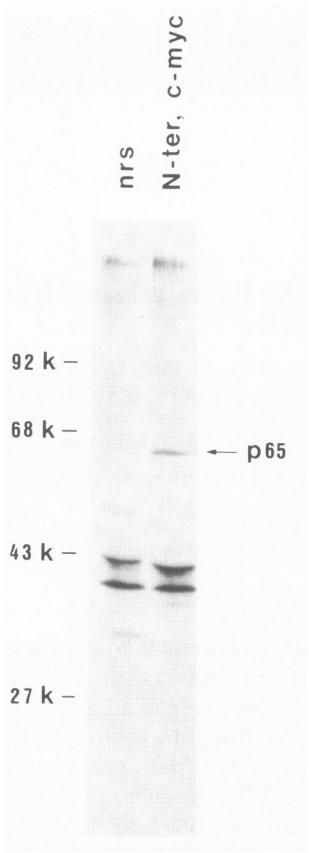

Fig. 2. Immunoprecipitation of [^{35}S]methionine-labelled *Xenopus* cell extracts by antisera directed against the N-terminal region of the human c-*myc* gene product. *Xenopus* A_6 kidney cells were labelled with 50 μCi/ml [^{35}S]methionine and extracts were prepared as described in Materials and methods. Complexes immunoprecipitated by normal rabbit serum (nrs), or with an antiserum raised against the N-terminal portion (N-ter, c-*myc*) of the human c-*myc* gene product were analysed by SDS–polyacrylamide gel electrophoresis and autoradiographed for 4 days.

was also detected in the cytoplasmic spaces in between the yolk platelets, but this was also observed with non-immune serum. (Yolk platelets were also sources of intrinsic non-specific fluorescence.) Furthermore, there was no enhancement of c-*myc* fluorescence in the vicinity of the lampbrush chromosomes (Figure 3, middle and lower panels). The nucleoli were revealed as regions of decreased c-*myc* fluorescence seen against a bright nuclear background. Taken together with the hybridization data, the immunological cross-reactivity between the product of the human c-*myc* gene and a 65-kd nuclear polypeptide strongly suggests that a homolog of the c-*myc* gene is present in *Xenopus* and is expressed in *Xenopus* cultured cells and oocytes.

c-myc is expressed at high levels in non-dividing vitellogenic oocytes

In view of the current notions regarding the role of c-*myc* in the growth-division cycle of cultured cells, the finding that c-*myc* was expressed in non-dividing cells such as oocytes was unexpected. To investigate this possibility further, total cellular RNA was extracted from *Xenopus* ovary and analyzed by Northern blotting. A barely detectable hybridization was observed when total ovarian RNA was analyzed using the human 3′ exon probe, and oligo(dT)–cellulose chromatography led to an enrichment in these sequences (Figure 4A). The ovarian c-*myc* transcript was identical in size (2.5 kb) to that detected in A_6 cells but its abundance was markedly lower.

Since the bulk of the ovarian RNA in *Xenopus* originates from a small number of very large cells, it is possible that individual oocytes contain a large number of copies of c-*myc* transcript. To investigate this possibility and to gain information on the timing of c-*myc* expression in oogenesis, total cellular RNA was extracted from oocytes freed from the surrounding ovarian tissue and follicular cells and separated into individual developmental classes according to the scheme of Dumont (1972): stage I, 200–300 μm in diameter pre-vitellogenic fully transparent, visible nucleus; stage II, 300–350 μm, light yellow, nucleus not visible; stage III, 400–500 μm, yellow brown, starting pigmentation; stage IV, 600 μm, half size; stage V, 800 μm; stage VI, 1–2 mm, fully mature. Transition time from stages II to III, III to IV, IV to V, V to VI requires a minimum of 2, 2, 1 and 1 week, respectively (Scheer, 1973).

When a fixed amount of total cellular RNA was hybridized to the human c-*myc* probe (Figure 4A), a 2.5-kb transcript could be detected in oocytes from stages II through V. The intensity of the hybridization signal was maximal in RNA from the earliest oocytes (stage II), decreased with oocyte development, and became undetectable in stage VI oocytes. However, a c-*myc* transcript was clearly present in stage VI oocytes when poly(A)-containing RNA from these cells was analyzed (data not shown). It must be emphasized that the steady-state level of polyadenylated RNA per oocyte attained at stage II is maintained throughout oogenesis (Golden *et al.*, 1980) while other RNA species (transcribed primarily by RNA polymerase I) continue to accumulate (Ford, 1971; Scheer, 1973). Thus, the relative abundance of any specific polyadenylated transcript as a fraction of total cellular RNA decreases steadily with oocyte development. Therefore, we determined the content of c-*myc* oocyte per oocyte at each stage of oogenesis by densitometric quantitation of the autoradiogram. On a per cell basis, no differences in the c-*myc* RNA content of oocytes from the different developmental stages could be demonstrated (Figure 4C). Moreover, when the content of c-*myc* transcript in oocytes was compared with

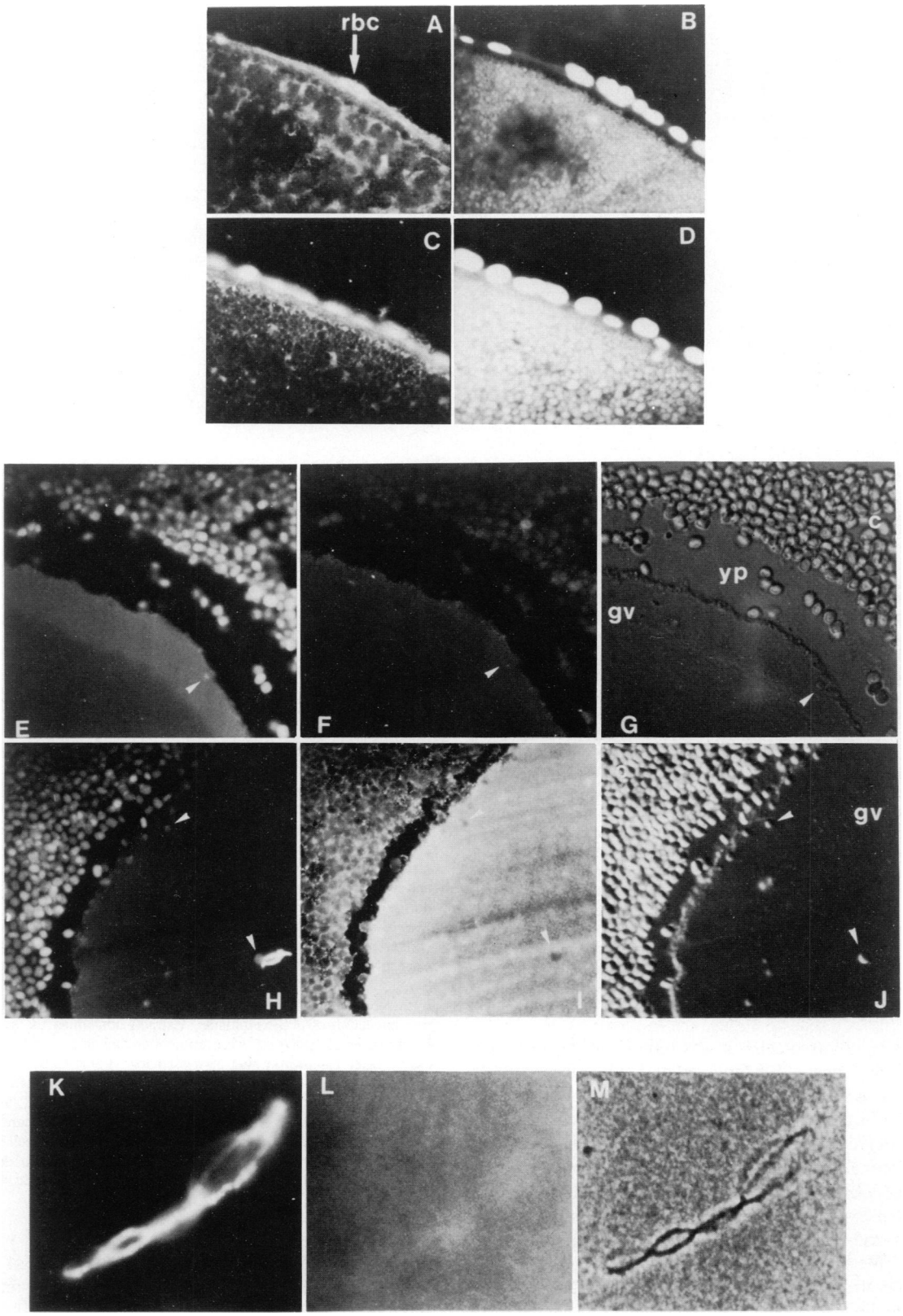

Fig. 3. Localization of $p65^{c\text{-}myc}$ by indirect immunofluorescence in sections of *Xenopus* stage VI oocytes using anti-c-*myc* N-ter I antiserum. Fixed, rehydrated ovarian sections (9 μm) were treated with a 1:40 dilution of either non-immune rabbit serum or anti-c-*myc* N-ter I antiserum (anti-c-*myc* N-ter I) in PBS containing 0.2% gelatin and 0.5 μg/ml of bisbenzimine H 33342 fluorochrome. Sections were then processed for indirect immunofluorescence using rhodamine-conjugated goat anti-rabbit immunoglobulins as described in Materials and methods. **Upper panel:** cortex of stage VI oocytes incubated with **(A,B)** non-immune rabbit serum or **(C,D)** anti-c-*myc* N-ter I antiserum. **(A,C)** Rhodamine fluorescence; and **(B,D)** DNA-specific Hoechst 33342 fluorescence. rbc: red blood cell. (×400). **Middle panel:** germinal vesicles of stage VI oocytes incubated with **(E,F,G)** non-immune rabbit serum or **(H,I,J)** anti-c-*myc* N-ter I antiserum. **(E,H)** DNA-specific Hoechst 33342 fluorescence; **(F,I)** rhodamine fluorescence; **(G,J)** phase contrast. Arrowhead indicates nucleoli. c = cytoplasm; gv = germinal vesicle; yp = yolk platelet. (×400). **Lower panel:** high magnification of a lampbrush chromosome present in a section of a germinal vesicle incubated with anti-c-*myc* N-ter I. **(K)** DNA-specific Hoechst 33342 fluorescence; **(L)** rhodamine fluorescence; **(M)** phase contrast. (×2400).

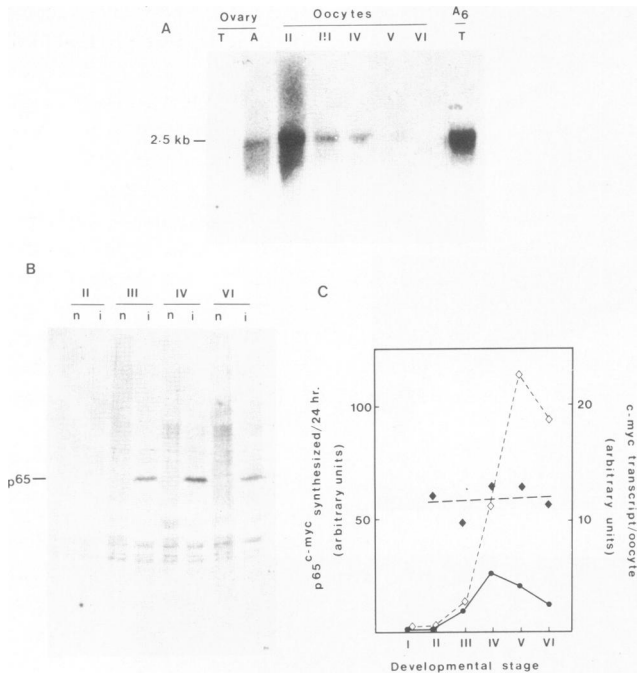

Fig. 4. Expression of the *Xenopus* c-*myc* gene during oogenesis. **(A)** Northern blot analysis of oocyte RNA. 30 μg of total cellular (T) or 5 μg of poly(A)-containing (A) RNA from an unfractionated *Xenopus* ovary were analysed (ovary), and 30 μg of total cellular RNA from isolated defolliculated oocytes sorted into stages (II−VI) as indicated were analysed (oocytes). Right lane: 30 μg of total cellular RNA from A_6 *Xenopus* kidney cells. The filter was hybridized to the human c-*myc* 3′ exon probe and washed stringently as described in Materials and methods. Exposure was for 48 h. **(B)** Immunoprecipitation of [^{35}S]methionine-labelled extracts of defolliculated, sorted *Xenopus* oocytes with the anti-human c-*myc* antiserum. Oocytes were isolated as described in Materials and methods and staged as described by Dumont (1972) and labelled for 24 h with [^{35}S]methionine. For each developmental stage, constant amounts of incorporated radioactivity were incubated with either a normal non-immune rabbit serum (n), or the immune serum anti-human c-*myc* N-ter I (i). Washed complexes were electrophoresed on SDS gels, dried, and exposed for 6 days. **(C)** Temporal pattern of *Xenopus* c-*myc* gene expression in oogenesis. Densitometric quantitation of $p65^{c\text{-}myc}$ immunoprecipitated as in **B** from constant amounts of radioactivity (closed circles) or from a fixed number of [^{35}S]methionine-labelled oocytes (open symbols) (◇ --- ◇). Densitometric quantitation of the abundance of c-*myc* transcript per oocyte after Northern blot analysis (◆ --- ◆).

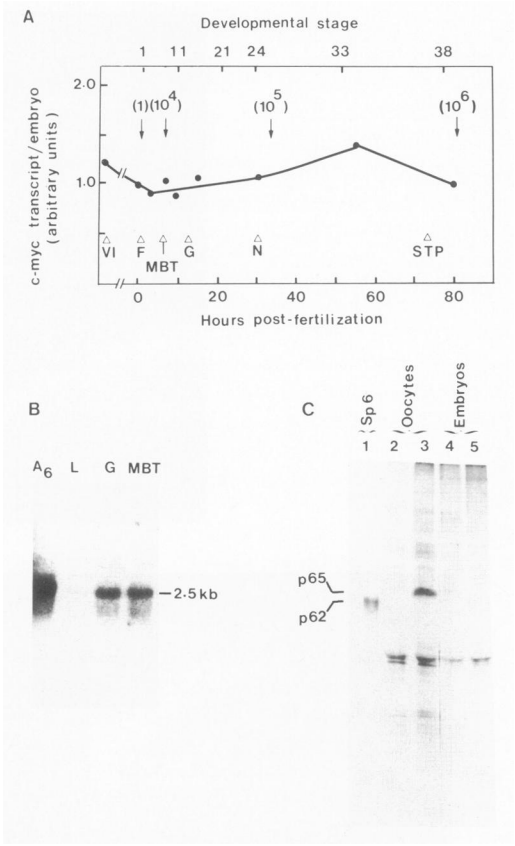

Fig. 5. Expression of the *Xenopus* c-*myc* gene during embryonic development. **(A)** Embryonic content of c-*myc* transcript. 30 μg of total RNA from each developmental stage was analysed by Northern blotting, hybridized to the human c-*myc* 3′ exon probe and quantified by densitometry. The level of c-*myc* transcript in unfertilized eggs was arbitrarily set at 1.0. Stage VI oocyte RNA (VI) was electrophoresed on the same gel. Upper arrow indicates the number of cells, and lower open arrowheads indicate the developmental stages: stage VI oocyte (VI), fertilization (F), mid-blastula transition (MBT), gastrula (G), neurula (N) and swimming tadpole (STP). **(B)** Northern blot analysis of *Xenopus* RNA. 30 μg of unfractionated cellular RNA [not poly(A)-selected] from A_6 *Xenopus* kidney cells (A6) was analysed together with 5 μg of poly(A)-containing RNA from adult *Xenopus* liver (L), gastrula (G) and mid-blastula stage of *Xenopus* embryos. RNA was analysed and hybridized as in **A**. **(C)** Synthesis of $p65^{c\text{-}myc}$ in *Xenopus* development. Isolated oocytes were labelled by incubation with [^{35}S]methionine. Embryos were labelled by microinjection of [^{35}S]methionine. Extracts containing the same amount of incorporated radioactivity were immunoprecipitated with the anti-human c-*myc* N-ter I antiserum and processed for autoradiography. **Lane 1** (Sp6) contained the *in vitro* translation product of an *in vitro*-transcribed mouse c-*myc* cDNA prepared as described (Persson *et al.*, 1985) which was used as a control. **Lane 2**, pre-vitellogenic stage II oocytes. **Lane 3**, stage V vitellogenic oocytes. **Lane 4**, embryos labelled at the 1-cell stage and homogenized after 16 h of development. **Lane 5**, embryos injected with [^{35}S]methionine 16 h after fertilization and homogenized at 48 h after fertilization.

that in somatic A_6 cultured cells, it was found that, on a per cell basis, oocytes contained ~10^4 times more c-*myc* transcript than exponentially growing A_6 cells. When normalized with respect to cell volume, however, the concentration of c-*myc* RNA in oocytes was roughly similar to that of somatic cells.

Due to the prevalence of post-transcriptional controls, levels of a cognate poly(A)-containing transcript do not faithfully reflect the variations in expression of a given gene in *Xenopus* development (Bienz and Gurdon, 1982; Lee *et al.*, 1984; Rebagliati *et al.*, 1985). We have therefore used the anti-c-*myc* N-ter I antiserum to determine the timing of $p65^{c\text{-}myc}$ synthesis during oogenesis. Defolliculated oocytes separated into distinct developmental classes were labelled with [^{35}S]methionine and extracts containing equal amounts of incorporated radioactivity were immunoprecipitated with the anti-human c-*myc* N-ter I antiserum (Figure 4B). Pre-vitellogenic oocytes (stage II) synthesized virtually no c-*myc* protein, whereas vitellogenic oocytes (stages III and IV) synthesized this polypeptide at a maximally high rate. In stage VI oocytes, a lower rate of synthesis of $p65^{c\text{-}myc}$ was reproducibly observed. Figure 4C illustrates the results of a similar experiment in which defolliculated oocytes were separated into a larger number of stages. The synthesis of $p65^{c\text{-}myc}$ was undetectable in oocytes younger than stage III, reached a maximum in stage IV and decreased in stage V and VI oocytes. When this analysis was carried out on a per cell basis (instead of as a fraction of total protein synthesis in each individual group), the profile obtained was similar, but stage V oocytes were found to synthesize $p65^{c\text{-}myc}$ at the highest rate (Figure 4C). Although methionine pools may differ among oocytes of various stages, therefore preventing the determination of absolute rates, large oocytes reaching the last phase of oogenesis accumulated the c-*myc* protein at a 100-fold higher rate than did pre-vitellogenic

oocytes, despite an unchanged c-*myc* RNA content. In spite of differing labelling conditions for somatic cells and oocytes, a crude comparison of the rate of $p65^{c\text{-}myc}$ synthesis in oocytes with that of somatic A_6 cells revealed that, on a per cell basis, stage VI oocytes synthesized $p65^{c\text{-}myc}$ at rates ~4 orders of magnitude higher. These data confirm that c-*myc* is expressed at high levels in non-dividing oocytes and indicate that its expression is confined to specific periods of late oogenesis by a post-transcriptional regulatory mechanism.

c-myc expression decreases in cleaving and early embryos

Next, we examined c-*myc* expression in developing embryos in which the cell cycle has resumed and which undergo a large number of cell divisions and synthesize large amounts of DNA in a very short time (Newport and Kirschner, 1982a,b). Thus the fast, synchronous cleavage divisions led to the formation of 10^4 cells in 6 h (Newport and Kirschner, 1982a). After the mid-blastula transition, the cell cycle lengthens, but the pace of proliferation remains high enough to yield 100-fold more cells in <3 days (Newport and Kirschner, 1982a,b). Figure 5A depicts the embryonic content of c-*myc* transcript determined by Northern blot analysis at different stages of early development. No difference could be demonstrated between unfertilized eggs and stage VI oocytes. Similarly, the onset of cleavage divisions did not bring about any change in the embryonic content of c-*myc* mRNA. Even after the mid-blastula transition, which marks the onset of new transcription (Newport and Kirschner, 1982b), this level remained unchanged. Later, no further changes were noted in the gastrula, neurula and even the tail-bud stage of *Xenopus* development. Thus, the dramatic increase in cell number characterizing early development was accomplished without any increase in the total embryonic content of c-*myc* mRNA. It is noteworthy that, as a result of this increase in cell number, the c-*myc* mRNA content per average individual cell decreased proportionally during this period. The abundance of c-*myc* mRNA of total *Xenopus* embryo RNA remained consistently lower than that of *Xenopus* cultured A_6 cells, but was always higher than that of non-growing liver tissue. Hybridizable c-*myc* transcript in unfractionated (total) RNA from A_6 cells was much higher than in polyadenylated liver RNA and embryonic c-*myc* mRNA was intermediate in abundance (Figure 5B).

To examine the possibility that a fixed number of c-*myc* transcripts could be translated with changing efficiency during the course of development, embryos were labelled by injection with [^{35}S]methionine at various times after fertilization, and the cell extracts were immunoprecipitated with the anti-c-*myc* N-ter I serum. As shown in Figure 5C, the accumulation of labelled $p65^{c\text{-}myc}$ polypeptide during the first 16 h of development was markedly decreased when compared with stage V (and even stage VI) oocytes, assuming that the stability of the c-*myc* gene product is not strongly affected by fertilization. Furthermore, the synthesis of $p65^{c\text{-}myc}$ became undetectable in embryos labelled from the 16th to the 48th hour after fertilization. Thus, the data suggest that the accumulation of newly synthesized c-*myc* protein is not required to sustain the dramatic rate of DNA synthesis and cell division characterizing early development.

Discussion

The results reported here demonstrate the existence of a gene in *Xenopus* homologous to the mammalian c-*myc* gene. In addition to the nucleotide sequence relatedness revealed by nucleic acid hybridization, homology at the protein level can be inferred from the immunological evidence. The antibody used in this study recognizes c-*myc* gene products in cells from a variety of species, and v-*myc* and c-*myc* related polypeptides in cells containing copies of the cognate resident gene (Persson *et al.*, 1984, 1985, 1986; and unpublished observations). In *Xenopus* somatic cells and oocytes, this antibody recognizes a 65-kd protein localized predominantly in the nucleus, as expected for a c-*myc* gene-encoded product (Persson and Leder, 1984; Eisenman *et al.*, 1985). Furthermore, c-*myc* mRNA is more abundant in exponentially growing cells of an established *Xenopus* line than in an essentially non-growing tissue such as adult liver. This differential expression has also been noted for the mammalian c-*myc* gene (Stewart *et al.*, 1984a). Although nucleotide and protein sequence data are still lacking, the convergence of these structural, immunological and physiological observations indicates that *Xenopus* carries a homolog of the mammalian c-*myc* gene.

Here we have demonstrated that total c-*myc* mRNA levels are identical in *Xenopus* oocytes and embryos and that they remain constant during the developmental processes of oogenesis and embryogenesis. In spite of these invariant c-*myc* mRNA levels, the rate of synthesis of the c-*myc*-encoded protein undergoes dramatic changes during this period, being highest in the second phase of oogenesis. To account for this discrepancy, we postulate a translational control of the c-*myc* mRNA. In *Xenopus* development, such mechanisms have been invoked for the expression of heat-shock (Bienz and Gurdon, 1982), histone (Ruderman *et al.*, 1979), fibronectin (Lee *et al.*, 1984) and ribosomal protein genes (Pierandrei-Amaldi *et al.*, 1982). This putative regulation of c-*myc* expression operates both in oogenesis to trigger the translation of a pre-formed transcript and probably again in early embryonic development to suppress the rate of $p65^{c\text{-}myc}$ synthesis.

c-*myc* expression is tightly coupled to the cellular growth state and may play a role in the establishment and maintenance of the growing state in cultured cells (Kelly *et al.*, 1984; Campisi *et al.*, 1984). We attempted therefore to relate the observed developmental pattern of c-*myc* expression to the contrasted growth conditions of oocytes and embryos (Gurdon, 1974; Newport and Kirschner, 1982a,b). No coincidence could be found between the time of occurrence of c-*myc* expression and that of major cell cycle events such as DNA synthesis and mitosis. The dissociation of c-*myc* mRNA levels from cell division is apparent since, on a per cell basis, mRNA levels are highest in non-dividing oocytes and decrease exponentially, on average, in embryonic cells in which the cell cycle has resumed. This lack of correlation becomes more pronounced when the rate of $p65^{c\text{-}myc}$ is considered. Therefore, in *Xenopus* oocyte and embryonic development, c-*myc* gene expression does not coincide with the occurrence of DNA synthesis and cell division.

Two interpretations of these data are possible. (i) The c-*myc* gene product could be devoid of function in the oocyte itself and be simply stored therein to sustain cell division in the developing embryo. Such accumulation in the oocyte has been observed for several gene products such as nucleoplasmin (Mills *et al.*, 1980), RNA polymerase (Roeder, 1974) and histones (Woodland and Adamson, 1977). This possibility cannot be ruled out by our experiments, since we have only measured rates of synthesis and not total cellular content of the c-*myc* gene product. (ii) Alternatively, the product of the c-*myc* gene could participate in the growth-associated function active in oogenesis but repressed in early development. Thus the lack of coincidence of c-*myc* expression and cell division would result from the unique uncoupling of the different major growth-associated functions observed in development.

Ribosomal RNA synthesis takes place primarily in mid and late vitellogenic oocytes, which are known to accumulate ribosomes on a large scale so as to reach an rRNA content equivalent to that of 10^6 somatic cells (Brown and Littna, 1964; Rosbash and Ford, 1974). As a result of this accumulation, the developing embryo is endowed with a number of ribosomes sufficient to sustain embryogenesis in the virtual absence of rRNA synthesis (Brown and Gurdon, 1964) and ribosomal protein synthesis (Pierandrei-Amaldi *et al.*, 1982). Thus, in complete contrast with DNA synthesis and mitosis, rRNA synthesis is a major growth-associated function that is maximally active in mid to late oogenesis and that undergoes a reduction in early development. Hence, based on the present observations of c-*myc* gene expression in *Xenopus* development, it is conceivable that the c-*myc* gene product may participate in the regulation of rRNA synthesis in oocytes. Furthermore, in addition to the temporal coincidence, we have found a close correlation between $p65^{c\text{-}myc}$ rate of synthesis and the corresponding rRNA content growing in oocytes from stage II to V ($r = 0.97$).

Even though these correlations can only be suggestive of a functional relation, it is worthy of note that rRNA synthesis and c-*myc* gene expression share two common characters in their respective cell cycle-dependent variation. (i) Both rRNA synthesis (Mauck and Green, 1973; Eliott and McLaughlin, 1978) and c-*myc* expression (Kelly *et al.*, 1983; Campisi *et al.*, 1984) rapidly respond to proliferative stimuli. (ii) Continuous expression throughout the cell cycle of growing cells has been reported for the expression gene (Hann *et al.*, 1985; Persson *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985) as well as for ribosomal genes (Scharff and Robbins, 1965). It is conceivable that cells in which the expression of ribosomal genes expression escapes the normal controls exerted by the hormonal or nutritional factors present in the environment and becomes 'constitutively' expressed, might gain a growth advantage over their normal counterparts.

Materials and methods

Biological materials

Male and female *X. laevis* frogs were maintained in the laboratory at 19°C. A_6 *Xenopus* kidney cells (Rafferty, 1969) were obtained as frozen ampoules from the American Type Culture Collection.

Chemicals and radiochemicals

Collagenase (type I) and human chorionic gonadotropin were purchased from Sigma Chemical Co. (St. Louis, MO). Cysteine hydrochloride and guanidine isothiocyanate were purchased from Fluka (Switzerland) and oligo(dT)–cellulose was from Collaborative Research. Restriction endonucleases were purchased from New England Biolabs. All other chemicals were of reagent grade from different suppliers. [^{32}P]dCTP (3000 Ci/mmol) and [^{35}S]methionine (800 Ci/mmol) and the reticulocyte lysate *in vitro* translation system were obtained from New England Nuclear. The SP6 *in vitro* transcription system was purchased from Promega Biotec.

Isolation, handling and culture of Xenopus oocytes and embryos

After immersion-anesthesia of the animals in 0.25% tricaine methane sulfonate (Sandoz, Switzerland), ovarian lobes were surgically removed, washed with modified Barth's saline Hepes (MBSH) (Gurdon and Wickens, 1983) and dissociated by overnight incubation at 20°C in calcium-free MBSH containing 2 mg/ml collagenase. Crude separation of pre-vitellogenic and vitellogenic oocytes was obtained by differential sedimentation, and oocytes were further sorted manually under a dissecting microscope into the developmental classes described by Dumont (1972).

Synchronously cleaving embryos were obtained by *in vitro* fertilization essentially as described by Newport and Kirschner (1982a,b). Briefly, testes were removed from male animals and kept in MMR (100 mM NaCl, 2 mM KCl, 1.0 mg $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA, pH 7.8) containing 10% fetal bovine serum at 4°C. Unfertilized eggs were obtained by injecting females with 1500 IU of human chorionic gonadotropin 12 h before use and were collected by squeezing the female in a Petri dish containing MMR. The egg layer was rubbed with a testis freshly teased open. Sperm activation was triggered by addition of distilled water, marking the time of fertilization. Embryos were transferred into 1/20 MMR and dejellied 1–3 h post-fertilization by treatment with 2% cysteine-HCl brought to pH 8.0 with KOH. After jelly coat dissolution, embryos were transferred back into 1/20 MMR and allowed to develop. Stages were determined according to Nieuwkoop and Faber (1956).

Cell culture

A_6 *Xenopus* kidney cells were cultured in Leibowitz L_{15} medium diluted with distilled water 60:40 (v/v) and supplemented with 10 mM Hepes pH 7.35, 10 μM hypoxanthine (Sigma), 4 mM glutamine and 10% fetal bovine serum (Gibco) at 20°C. Cultures were equilibrated with air and kept in the dark. T_{24} human bladder epithelioma cells and benzopyrene-transformed BALB/c 3T3 mouse fibroblasts (BP A31) were cultivated in the Dulbecco's modification of Eagle's minimum medium (DME) containing 10% fetal bovine serum and 10% fetal calf serum, respectively, as described (Persson *et al.*, 1985).

Cell labelling and immunoprecipitation

Xenopus A_6 cell monolayers were washed once in medium lacking methionine and incubated for 4 h at 20°C in the same medium containing 10% fetal bovine serum and 50 μCi/ml [^{35}S]methionine. Cell extracts were made in 1 ml of high salt RIPA buffer (50 mM Tris–HCl, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonylfluoride, pH 7.5).

Oocytes were incubated in 100 μl MBSH containing 0.5 mCi/ml [^{35}S]methionine for 24 h at 20°C, washed with unlabelled MBSH, and extracted with 1 ml of high salt RIPA buffer as described above. Extracts were briefly sonicated, and clarified by centrifugation at 4°C (12 000 *g* for 10 min).

Embryos were injected, while in MMR containing 5% Ficoll, with 50 nl of [^{35}S]methionine (5–10 μCi) either at the single cell stage or into the blastocele for older embryos. After the desired incubation time in 1/20 MMR, embryos were extracted in high salt RIPA buffer as described for *Xenopus* oocytes. Extracts were immunoprecipitated as described previously (Persson *et al.*, 1984). Washed immune complexes were analysed on 10% SDS–polyacrylamide gels, and processed for fluorography and densitometry as described previously (Laemmli, 1970).

Extraction and analysis of cellular RNA and Xenopus DNA

Total cellular RNA was extracted with 4 M guanidine isothiocyanate and purified through a 5.7 M CsCl cushion essentially as described (Chirgwin *et al.*, 1979). Poly(A)-containing RNA was purified by oligo(dT)–cellulose chromatography (Aviv and Leder, 1972). Formaldehyde-denatured RNA samples were electrophoresed in 1% agarose gels containing 0.7% formaldehyde, soaked briefly in 20 × SSC and transferred to nitrocellulose in the same solution (Persson *et al.*, 1985). High mol. wt DNA was extracted from adult *Xenopus* females after disruption of the tissue in liquid N_2 and digestion with proteinase K essentially as described (Blin and Stafford, 1976). After repeated phenol extractions, DNA was further purified by banding in a CsCl density gradient (Maniatis *et al.*, 1982). DNA was digested with restriction endonuclease and fragments were electrophoresed on 1% agarose gels, and transferred to nitrocellulose.

DNA probes and hybridization

The 1.3-kb *ClaI–EcoRI* restriction fragment containing the 3′ exon region of the human c-*myc* gene (Battey *et al.*, 1983) was labelled by nick-translation to a specific activity of ~10^6 c.p.m./ng. Filters were hybridized for 18 h to the labelled probe as described (Battey *et al.*, 1983) and washed at 54°C in 15 mM NaCl, 1.5 mM sodium citrate (0.1 × SSC) containing 0.1% SDS and exposed to Kodak XAR5 films at −80°C with intensifying screens.

Indirect immunofluorescence

Pieces of ovary were fixed for 3 h in a solution containing 25 ml of saturated $HgCl_2$, 5 ml of 5% trichloroacetic acid and 15 ml of formaldehyde (Analar) (Hausen *et al.*, 1985) and then transferred in absolute ethanol at −20°C for 1 week. Fixed oocytes were incubated in dimethoxy-propane for 1 h and embedded in polyester wax (Dreyer *et al.*, 1982). Sections (9 μm) were rehydrated and incubated for 30 min first in PBS containing 0.2% gelatin and then in a 1:40 dilution of anti-c-*myc* N-ter I antiserum containing 0.5 μg/ml bisbenzimide H 33342 fluorochrome in PBS 0.2% gelatin. Sections were washed three times in PBS 0.2% gelatin and treated with a rhodamine-conjugated goat anti-rabbit IgG (Nordic Tilburg, Netherlands) as described (Moya *et al.*, 1985), mounted in PBS 90% glycerol, examined and photographed under an epifluorescence microscope.

Acknowledgements

We thank Rose Kindy and Robert Curtin for excellent technical assistance. We are grateful to Dr P.Gounon for his help with ovarian sections. During this research, F.G. was supported in part by INSERM (France), by a BRSG grant to the Dana-Farber Cancer Institute, and by a short-term EMBO fellowship. H.E.G. was supported in part by a Tumor Biology Training grant to the Dana-Farber Cancer Institute, by a grant from the Swedish National Science Foundation to H.P., and by an INSERM (France) fellowship (poste orange). This research was supported by a Public Health Service grant GM CA 24571 from the National

Institutes of Health to A.B.P. and by grants from the Swedish National Science Foundation and the Swedish Medical Research Council to H.P.

References

Armelin,H.A., Armelin,M.C.S., Kelly,K., Stewart,T., Leder,P., Cochran,B.H. and Stiles,C.D. (1984) *Nature*, **310**, 665–660.

Aviv,H. and Leder,P. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1408–1412.

Battey,J., Moulding,C., Taub,R., Murphy,W., Stewart,T., Potter,H., Lenoir,G. and Leder,P. (1983) *Cell*, **34**, 779–787.

Bienz,M. and Gurdon,J.B. (1982) *Cell*, **29**, 811–819.

Bishop,J.M. (1985) *Cell*, **42**, 23–38.

Blin,N. and Stafford,D.W. (1976) *Nucleic Acids Res.*, **3**, 2303–2315.

Brown,D.D. and Gurdon,J.B. (1964) *Proc. Natl. Acad. Sci. USA*, **51**, 139–146.

Brown,D.D. and Littna,E. (1964) *J. Mol. Biol.*, **8**, 688–695.

Campisi,J., Gray,H.E., Pardee,A.B., Dean,M. and Sonenshein,G.E. (1984) *Cell*, **36**, 241–247.

Chirgwin,J., Aeyble,A., McDonald,R. and Rutter,W. (1979) *Biochemistry*, **18**, 5294–5299.

Dreyer,C., Scholtz,E. and Hausen,P. (1982) *Wilhelm Roux's Arch.*, **190**, 228–233.

Dumont,J.N. (1972) *J. Morphol.*, **136**, 153–180.

Eisenman,R.N., Tachibana,C.Y., Abrams,H.D. and Hann,S.R. (1985) *Mol. Cell. Biol.*, **5**, 114–126.

Eliott,S.C. and McLaughlin,G.S. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4384–4388.

Ford,P.J. (1971) *Nature*, **233**, 561–564.

Golden,L., Schafer,U. and Rosbach,M. (1980) *Cell*, **22**, 835–844.

Gurdon,J.B. (1974) *The Control of Gene Expression in Animal Development*. Harvard University Press, Cambridge.

Gurdon,J.B. and Wickens,M.P. (1983) *Methods Enzymol.*, **101**, 370–386.

Hann,S.R., Thompson,C.B. and Eisenmann,R.N. (1985) *Nature*, **314**, 366–369.

Hausen,P., Wang,Y., Dreyer,Y. and Stick,R. (1985) *J. Embryol. Exp. Morphol.*, **89**, suppl., 17–34.

Hayward,W.S., Neel,B.G. and Astrin,S.M. (1981) *Nature*, **290**, 475–480.

Kelly,K., Cochran,B.H., Stiles,C.D. and Leder,P. (1983) *Cell*, **35**, 603–610.

Klein,G. and Klein,E. (1985) *Nature*, **315**, 190–195.

Laemmli,U.K. (1970) *Nature*, **227**, 680–684.

Land,H., Parada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.

Lee,G., Hynes,R. and Kirschner,M. (1984) *Cell*, **36**, 729–740.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1983) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.

Mauck,J.C. and Green,H. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 2819–2822.

Mills,A.D., Laskey,R.A., Black,P. and De Robertis,E.M. (1980) *J. Mol. Biol.*, **139**, 561–568.

Mougneau,E., Lemieux,L., Rassoulzadegan,M. and Cuzin,F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5758–5762.

Moya,M., Dautry-Varsat,A., Goud,B., Louvard,D. and Boquet,P. (1985) *J. Cell. Biol.*, **101**, 548–559.

Newport,J. and Kirschner,M. (1982a) *Cell*, **30**, 675–685.

Newport,J. and Kirschner,M. (1982b) *Cell*, **30**, 687–696.

Nieuwkoop,P.D. and Faber,J. (1956) *Normal Tables of Xenopus laevis (daudin)*. Elsevier, North-Holland, Amsterdam.

Persson,H. and Leder,P. (1984) *Science*, **225**, 718–721.

Persson,H., Hennighausen,L., Taub,R., DeGrado,W. and Leder,P. (1984) *Science*, **225**, 687–693.

Persson,H., Gray,H.E. and Godeau,F. (1985) *Mol. Cell. Biol.*, **5**, 2903–2912.

Persson,H., Gray,H.E., Godeau,F., Braunhut,S. and Bellvé,A.R. (1986) *Mol. Cell. Biol.*, **6**, 942–949.

Pierandrei-Amaldi,P., Campioni,N., Beccari,E., Bozzoni,I. and Amaldi,F. (1982) *Cell*, **30**, 163–171.

Rabbitts,P.H., Watson,J.V., Lamond,A., Forster,A., Stinson,M.A., Evan,G., Fisher,W., Atherton,E., Sheppard,R. and Rabbitts,T.H. (1985) *EMBO J.*, **4**, 2009–2015.

Rafferty,K.A. (1969) In Mizell,M. (ed.), *Biology of Amphibian Tumors*. Springer-Verlag, Berlin, pp. 52–81.

Rebagliati,M.R., Weeks,P.L., Hurvey,R.P. and Melton,D.A. (1985) *Cell*, **42**, 769–777.

Roeder,R.G. (1974) *J. Biol. Chem.*, **249**, 249–255.

Rosbash,M. and Ford,P.J. (1974) *J. Mol. Biol.*, **85**, 87–101.

Ruderman,J.V., Woodland,H.R. and Sturgess,E.A. (1979) *Dev. Biol.*, **71**, 72–82.

Scharff,M.D. and Robbins,E. (1965) *Nature*, **208**, 464–468.

Scheer,U. (1973) *Dev. Biol.*, **30**, 13–28.

Stewart,T.A., Bellvé,A.R. and Leder,P. (1984a) *Science*, **276**, 707–710.

Stewart,T.A., Pattengale,P.K. and Leder,P. (1984b) *Cell*, **38**, 627–637.

Thompson,C.B., Challoner,P.B., Neiman,P.E. and Groudine,M. (1985) *Nature*, **314**, 363–366.

Woodland,H.R. (1982) *Biosci. Rep.*, **2**, 474–491.

Woodland,H.R. and Adamson,E.D. (1977) *Dev. Biol.*, **57**, 117–135.

Received on 12 August 1986; revised on 8 October 1986