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Xenopus myc proto-oncogene during development: expression as a stable maternal mRNA uncoupled from cell division

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A *Xenopus* cDNA clone highly homologous to the proto-oncogene c-*myc* has been isolated and used to derive a homologous probe to study *myc* expression during embryonic development. *Myc* RNA is identified as a member of the class of maternal mRNAs expressed before fertilisation. It is highly accumulated from early oogenesis and an unfertilised egg contains 8 pg, about 10^5-fold the *myc* content of proliferative somatic cells. After fertilisation a post-transcriptional regulation of the gene is induced and the accumulated *myc* RNA is degraded ($t_{1/2}$ = 4 h 20 min) to reach a level at gastrula of 10 transcripts per cell; a value maintained during subsequent embryonic development. The *Xenopus myc* protein has also been identified by both *myc*-specific antibodies and hybrid selection experiments. Translation *in vitro* of *Xenopus myc* RNA shows that it encodes a 62-kd protein which is also recognised by *myc* antibodies in oocyte extracts. This protein is accumulated in late oogenesis. The results indicate an unusual uncoupling of *myc* expression and cell proliferation linked to a stabilisation of the RNA product.

Key words: c-*myc* oncogene/c-*myc* protein/maternal RNA/*Xenopus* development

Introduction

One of the most fascinating aspects of animal cell development is the accumulation during oogenesis of maternal mRNA, which is assumed to serve as the predominant template for translation during early embryonic development (Davidson, 1976). One of the best studied examples in this field is the early development of the vertebrate *Xenopus laevis*. In *Xenopus* oogenesis lasts for about two years (Callen *et al.*, 1980) and then, after laying, the unicellular egg is fertilised and transformed into a multicellular complex by a series of extremely rapid mitotic divisions. During this cleavage stage there is no detectable transcription and eggs that are treated with drugs that inhibit RNA synthesis continue cleaving normally (Brachet and Denis, 1963; Newport and Kirschner, 1982). It has been concluded that the cleavage state is under the control of messenger RNA produced during oogenesis and stored in the unfertilised egg. These specific maternal mRNAs are however poorly characterised at present, which has prevented the study of their fate during subsequent development. After 12 cleavages the embryo undergoes an abrupt and concerted developmental change termed the midblastula-transition at which the first new RNA transcripts appear (Brown and Littna, 1964; Bachvarova and Davidson, 1966; Newport and Kirschner, 1982).

Recent analyses of the expression of proto-oncogenes suggest a predominant role of some of these genes in the control of cell proliferation (for reviews see Heldin and Westermark, 1984; Bishop, 1985). Amongst these c-*myc*, the cellular homologue of the avian myelocytomatosis virus oncogene, displays a relatively high increase in the level of its mRNA when G_0 arrested cells are stimulated to divide (Kelly *et al.*, 1983) and the accumulation of c-*myc* RNA in proliferating cells appears to be a general phenomenon.

Cell proliferation is the fundamental event which characterises an early embryo. Therefore, if c-*myc* expression is necessary for proliferation, one could expect its mRNA product to be accumulated as a maternal RNA in oogenesis. In this study we report that *Xenopus myc* RNA is indeed accumulated and has an unusual stability during early oogenesis. This allowed the fate of one specific maternal mRNA to be followed from its early synthesis until late embryonic stages. The *myc* protein synthesised in oocytes is also identified and an unusual uncoupling of *Xenopus myc* expression from cell division during oogenesis and early development is shown.

Results

The proto-oncogene myc is present in the amphibian Xenopus genome

Positive signals were registered when Northern blots as well as slot blots of oocyte poly(A)$^+$ RNAs were hybridised with a labelled *Eco*RI−*Cla*I human c-*myc* exon III probe (Dalla-Favera *et al.*, 1982). An extensive study of *myc* expression in *Xenopus* development necessitated the characterisation of the homologous sequence, both to confirm the identity of this gene in the *Xenopus* genome, and to produce sensitive homologous probes for subsequent studies. We used a cDNA library from defolliculated oocyte poly(A)$^+$ RNA constructed in the λgt10 vector (Materials and methods). The phage library was screened by hybridisation with the human c-*myc* exon III probe described above and 150 of 500 000 colonies gave positive signals. Related cDNA clones were obtained and a DNA sequence spanning the 3′ end of the coding sequence and representing 1045 bp of one of these clones is presented in Figure 1. The *Xenopus* sequence is aligned with chicken c-*myc* (Papas *et al.*, 1984). The presumptive boundary between exons II and III is indicated by analogy with the chicken sequence and gene organisation. The homology between the *Xenopus* sequence and the chicken c-*myc* sequence in the entire exon II−exon III coding region illustrated is 66%. A similar high homology (65%) is found with the human c-*myc* gene (Papas *et al.*, 1984). At the level of the predicted amino acid sequence the homology is 63% with the chicken protein and 62% with the human. A stretch of 421 nucleotides in the presumptive exon III region and one of 138 in the presumptive exon II share even more extensive homology of 75 and 82%, respectively, with the chicken nucleotide sequence; with the amino acid sequence the corresponding homologies are 80 and 84%. The sequence is also homologous to human N-*myc* (Kohl *et al.*, 1986) in these regions, but to a lesser extent than to human c-*myc*. This result

clearly indicates that we have isolated a *Xenopus* coding sequence closely related to c-*myc*, and confirms the conservation of the c-*myc* gene through evolution.

A Southern blot of genomic DNA was hybridised to a single strand labelled probe prepared from the previously characterised 491-bp *Xenopus myc* *Hin*cII−*Sac*I fragment (residues 294−785, Figure 1 and Materials and methods). Two distinct DNA bands were obtained in each of the three restriction digests (Figure 2). An identical result was obtained with a 237-residue single strand *Xenopus probe* (residues 654−891, Figure 1) spanning the 3′ end of the coding sequence of the presumptive exon III. The same procedure was performed in parallel with the chicken erythrocyte genome, which revealed a single DNA band at the position expected for the c-*myc* gene in each digest (Figure 2 and Papas *et al.*, 1984). This result confirms the sequence homology between the *Xenopus myc* and the chicken c-*myc* gene sequence as high stringency was used (Materials and methods). Low-stringency conditions did not reveal additional bands. The *Xenopus* results are consistent with the observation that most genes detected so far in the *Xenopus* genome are present in two copies, and is in accordance with the hypothesis of a total genome duplication during the evolution of *X. laevis* (Bisbee *et al.*, 1977).

Temporal expression of Xenopus myc during embryonic development

Having identified the *Xenopus myc* sequence, an extensive analysis of the expression of this proto-oncogene during embryonic development has been carried out. Analysis was throughout oogenesis, which takes place over two years in the ovaries of this animal (Callen *et al.*, 1980), and from the fertilised eggs up to the feeding tadpole stage. RNA was extracted from a fixed number of defolliculated oocytes or embryos at each stage of development (Nieuwkoop and Faber, 1956) using a procedure tested to give efficient and reproducible RNA recoveries (Materials and methods).

Northern blot analysis on nylon membranes was carried out with the same amount of total RNA (10 μg) for each developmental stage assayed. The blots were probed with the same single strand labelled *Xenopus* *Hin*cII−*Sac*I fragment described above, and the results obtained are shown in Figure 3. In very early oogenesis (stage I) there is a high concentration of *myc* RNA; the major *myc* transcript is 2.7 kb, but minor transcripts at 1.8 and around 4 kb are also present. These two minor transcripts are only detected in early oogenesis and such transcripts have been described in other cell types (Robert-Lezenes *et al.*, 1984;

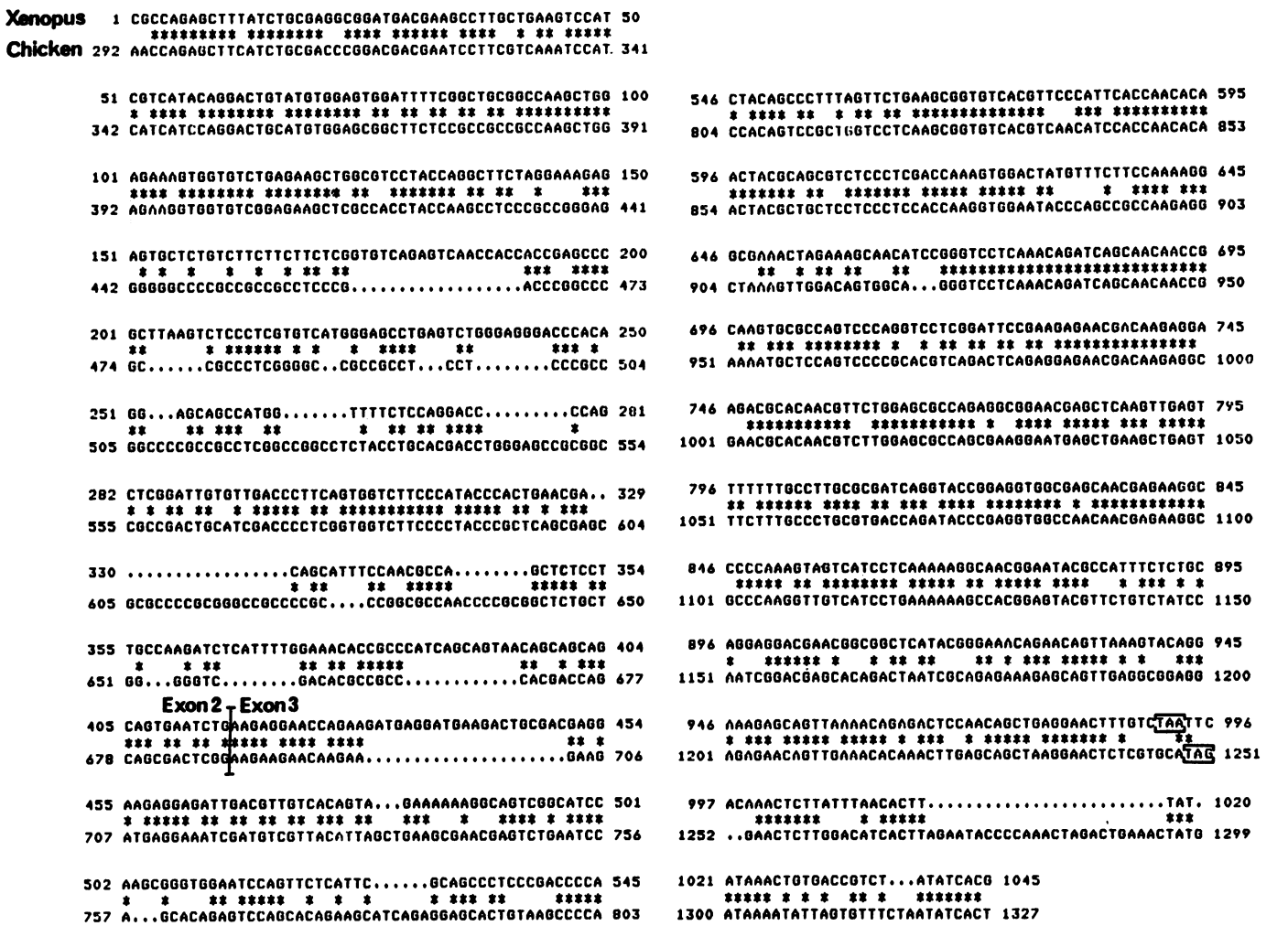

Fig. 1. Sequence of a 1045-bp cDNA clone of *Xenopus myc* and homology with chicken c-*myc*. The *Xenopus myc* nucleotide sequence, obtained on both strands, is shown with the direction of transcription from left to right. The presumptive termination codon (boxed) and exon II−exon III splice junction are indicated. The *Xenopus* sequence is aligned with the chicken c-*myc* DNA sequence using the best-fit alignment analysis of Smith and Waterman (1981).

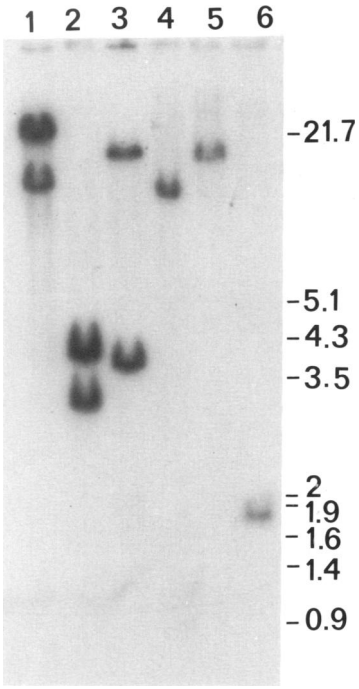

Fig. 2. Genomic Southern blot of *Xenopus myc*. 10 μg *Xenopus* (**1, 2, 3**) or chicken (**4, 5, 6**) nuclear DNA was digested with *Bam*HI (**1, 4**), *Eco*RI (**2, 5**) or *Hin*dIII (**3, 6**) and processed as described in Materials and methods. The blot was hybridised with a single strand labelled probe prepared from the *Hin*cII−*Sac*I *Xenopus myc* fragment (residues 294−785, Figure 1) cloned in M13 mp19. Markers were end-labelled lambda *Eco*RI−*Hin*dIII fragments.

and references cited therein). From mid-oogenesis to the feeding tadpole stage, however, only a single 2.7-kb *myc* band is detected. Neither high-stringency (0.1 × SSPE, 60°C) nor low-stringency (1 × SSPE, 42°C) washes eliminate or reveal any additional band, at both early and late stages of embryogenesis, confirming the relatively simple pattern of expression during *Xenopus* development. This *myc* expression study was controlled internally throughout oogenesis and embryonic development for both the total RNA recovery and specific known transcripts (Figure 3 legend).

The *myc* signal from each developmental stage was precisely quantitated as detailed in Materials and methods and shown in Figure 4A by RNA slot blot hybridisation using the single strand *Xenopus myc Hin*cII−*Sac*I probe described above. The Northern blots (Figure 3) were quantitated by scanner densitometry of the autoradiographs rigorously carried out as described in Materials and methods. This gave the same results for the developmental profile of *myc* expression as the slot blot hybridisation. Figure 4B shows the variation of *myc* RNA expression per oocyte during oogenesis or embryo in early development. A high level of *myc* RNA is registered during early oogenesis. The maximum level of *myc* RNA is observed at mid-oogenesis, during the lampbrush stages, when it reaches 12.7 pg per oocyte. Then a relatively constant level of *myc* RNA is observed up to the stage VI oocyte which contains 7.4 pg *myc* RNA or ~5 × 10^6 *myc* RNA molecules. These values are in good agreement with an independent estimate obtained by the percentage of *myc*-positive recombinants in the oocyte library during its screening which gave an average of 10−18 pg *myc* RNA per oocyte. After fertilisation, a substantial and continuous decrease of *myc* RNA per embryo occurs, reaching a minimum value at the midblastula-gastrula

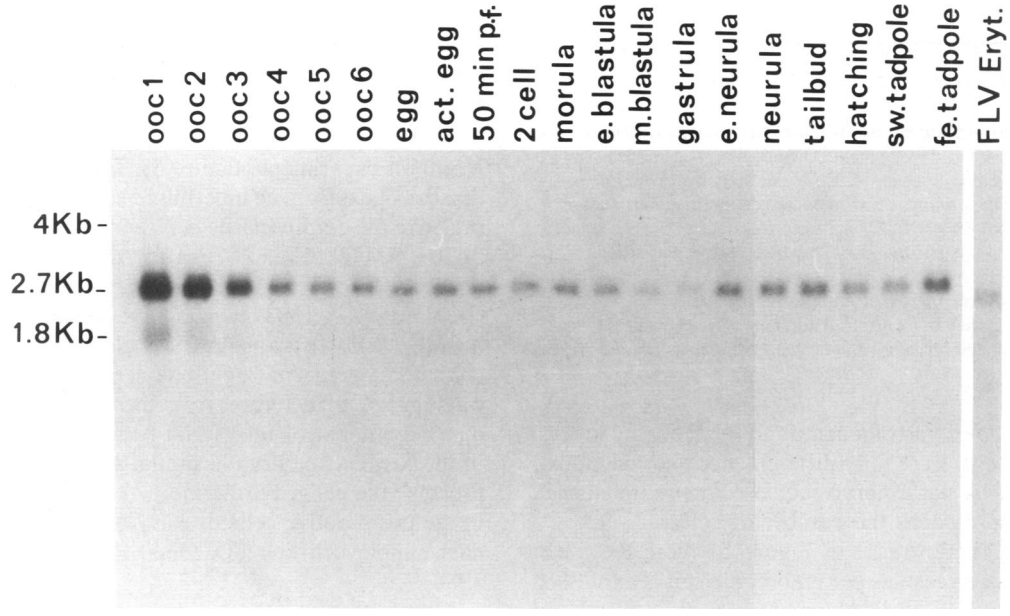

Fig. 3. Expression of *Xenopus myc* during oogenesis and embryonic development. 10 μg total RNA from each stage of oogenesis (except stage I: 7.5 μg) and development analysed was prepared, fractionated on a formaldehyde−agarose gel, and transferred to nylon membranes as described in Materials and methods. Hybridisation was with a labelled single-stranded *Xenopus myc* probe (see Figure 2 legend). 10 μg RNA extracted from activated eggs 50 min after an electric shock (act. eggs) and 5 μg poly(A)$^+$ RNA from Friend leukemia virus induced preleukemic erythroblastic cells (FLV. eryth.) were processed on the same gel. This experiment was controlled internally. The total RNA content increased in early oogenesis, remained approximately constant after fertilisation until the hatching tadpole, and then increased to 9 μg RNA per feeding tadpole (Figure 4B and unpublished results), in accordance with published values (Cabada *et al.*, 1977; Van Dongen *et al.*, 1981; Smith *et al.*, 1984; Brown and Littna, 1964). Similarly, a characteristic profile of ribosomal RNA increasing dramatically from the stage II oocyte and reaching a steady state level at the stage VI oocyte, which is maintained until the hatching tadpole, was observed by ethidium bromide staining (not shown). A further control assaying 5S RNA (Brown and Littna, 1966; Ford, 1971) on the same Northern blots (not shown) also indicated a reproducible RNA recovery.

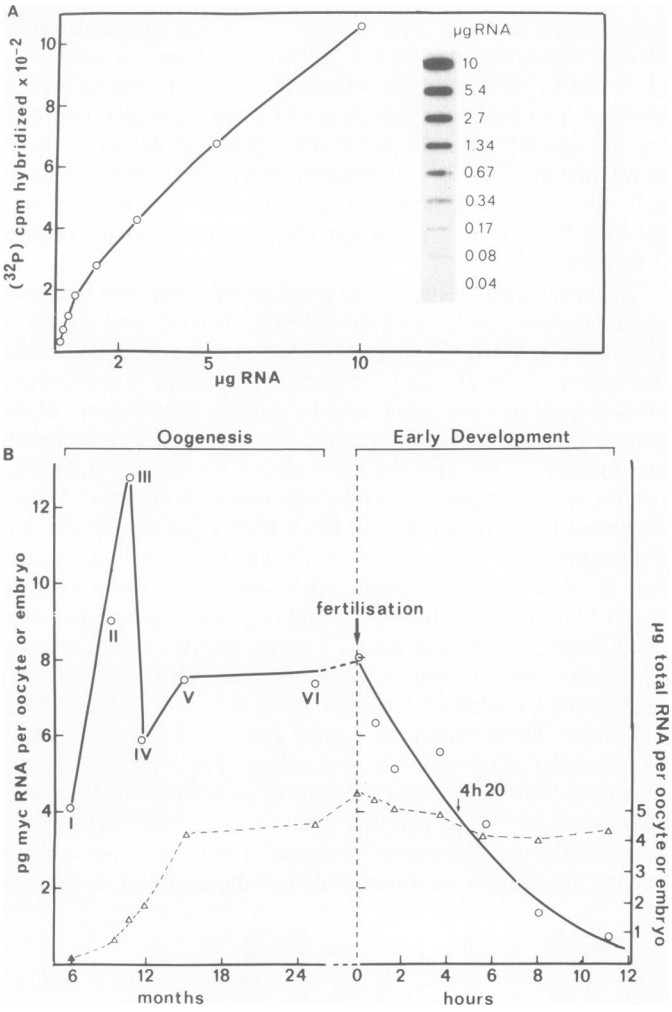

Fig. 4. Variations in the amount of *myc* mRNA per *Xenopus* oocyte or embryo during oogenesis and development. Quantitation was carried out over the range 0–10 μg oocyte stage VI RNA per slot, hybridised to labelled *Xenopus myc* DNA as indicated in Materials and methods (**panel A**). After autoradiography of the filter, the slots were cut out and counted in a liquid scintillation counter (panel A). This quantitation and scan densitometry of the Northern blot (Figure 3) were used to plot the variation in the level of *myc* RNA per oocyte or embryo during *Xenopus* development (○—○), **panel B**. In panel B the total RNA content recovered per oocyte or embryo is also indicated (△—△), as are the stages of oogenesis (I–VI).

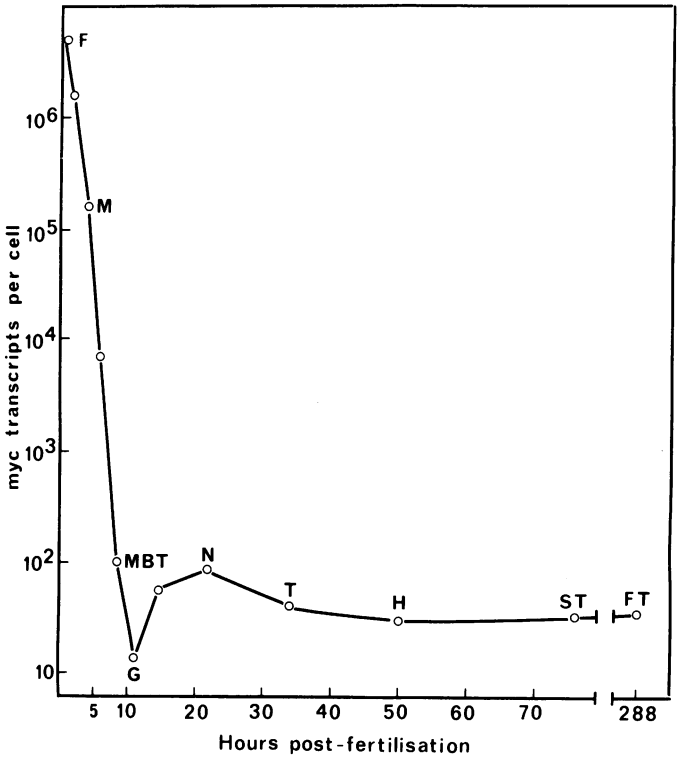

Fig. 5. Mean number of molecules of *myc* RNA per cell during embryonic development. The level of *myc* RNA was determined by quantitation of both Northern blot hybridisations and RNA slot blot hybridisations as described in Materials and methods. The same pattern was observed in each case. Since only one band of 2.7-kb RNA is observed after fertilisation the number of molecules can be estimated from the amount of *myc* RNA detected. The number of cells per embryo at each different stage was taken from Dawid (1965). F, fertilisation; M, morula; MBT, midblastula transition; G, gastrula; N, neurula; T, tailbud; H, hatching tadpole; ST, swimming tadpole; FT feeding tadpole.

stage (Figure 4B). After gastrula and up to the feeding tadpole, when a number of organs are fully differentiated and functional, the level of *myc* RNA per embryo increases approximately in the same proportion as does the number of cells.

This observation is illustrated in Figure 5 where the mean number of *myc* RNA molecules per embryonic cell is expressed as a function of the time of development. An unfertilised egg contains 8 pg of *myc* RNA, or 5×10^6 transcripts. After fertilisation the number of *myc* RNA molecules progressively decreases to ~10 per cell at gastrula and subsequently is maintained at a mean value of ~50 transcripts per cell up to the feeding tadpole stage. The mean quantity of *myc* RNA per cell at gastrula and in subsequent stages is similar to that value obtained with highly proliferative preleukemic cells. In this case, 5 μg poly(A)$^+$ RNA representing 10^6 Friend virus induced preleukemic mouse erythroblastic cells (Materials and methods and Robert-Lezenes *et al.*, 1984) was processed in parallel in the same Northern experiment (Figure 3). From densitometric quantitation the signal from an unfertilised egg was equivalent to 5×10^6 preleukemic erythroblastic cells and that from a gastrula embryo of 30–60 000 cells to 5×10^5. Although the mouse c-*myc* sequence, which shares 70% homology with the *Xenopus myc* probe (Bernard *et al.*, 1983), would have been less efficiently detected than the *Xenopus* sequence, it is clear that the probe can detect the heterologous chicken c-*myc* sequence even at high stringency (Figure 2). Therefore, from these overall results we conclude that the amount of *myc* RNA per cell from the gastrula stage of the *Xenopus* embryo is similar to that in highly proliferative preleukemic cells. Furthermore, it is also similar to that found in the proliferative cells of early human placenta or the malignant tumour cell line COLO320 HSR (Pfeiffer-Ohlsson *et al.*, 1984).

After fertilisation there is no detectable RNA synthesis during the first 12 synchronous divisions that preceed the mid-blastula transition, which occurs after ~9 h (Brown and Littna, 1964; Bachvarova and Davidson, 1966; Newport and Kirschner, 1982). Therefore, the observed decrease in the amount of *myc* RNA per embryo during this period (Figures 4B and 5) is likely to correspond to the degradation of the *myc* RNA population previously stored in the egg. The half-life of this process was measured to be 4 h 20 min, which strikingly corresponds to half the time to reach the mid-blastula transition. We cannot formally exclude a specific and minimum expression of the *myc* gene

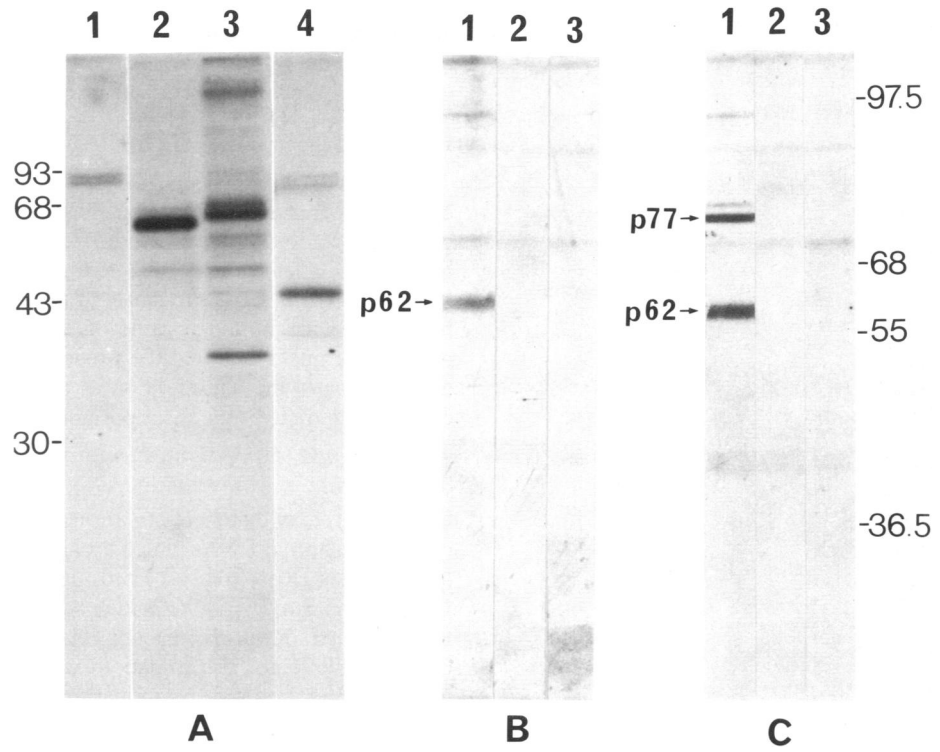

Fig. 6. Detection of *myc* protein in *Xenopus* oocytes by immunoblot. (**A**) The human c-*myc* antiserum specificity was assayed by immunoprecipitation of the *myc* proteins from human COLO320 HSR cells (**lanes 1, 2** and **4**) and mouse Rec2A fibroblasts (**lane 3**) as described in Materials and methods. Bound antigens were fractionated on a SDS 12.5% polyacrylamide gel and visualised by autoradiography. **Lane 1**, preimmune rabbit serum; **lanes 2** and **3**, human c-*myc* antipeptide serum; **lane 4**, W6/32 anti-HLA monoclonal antibody, as a control in this experiment, recognising the 45-kd HLA heavy chain. (**B** and **C**) Immunoblots from SDS 10% polyacrylamide gels of stage VI oocyte unlabelled proteins were probed with two different human c-*myc* antibodies (**B lane 1** and **C lane 1**). In **lanes 2** and **3** of B and C the antisera have been preincubated with 1 or 2 μg respectively of the 32 amino acid C-terminal *myc* polypeptide (Materials and methods). The molecular weights indicated were estimated from mol. wt. markers (Amersham and Boehringer) processed in parallel.

during this period, but note that transcription has never been registered at this stage of development, even when very sensitive methods were applied (Newport and Kirschner, 1982). Moreover, this would not affect the observed half-life of the *myc* RNA substantially, due to the time needed for accumulating the high level of the original *myc* RNA population during oogenesis (see Discussion).

In summary, we have observed an unusual accumulation of the *Xenopus myc* gene transcript during oogenesis, which allows the storage of a high amount of *myc* RNA (8 pg/egg) before fertilisation. During the early cleavage stage, characterised by transcriptional quiescence, the amount of this RNA per embryo steadily decreases until gastrula; in subsequent stages of development a constitutive amount of *myc* RNA per cell is maintained, presumably a consequence of new transcription.

The Xenopus myc protein is present in the oocyte

Analysis of the *myc* protein was carried out using a series of specific c-*myc* antibodies raised against a 32 amino acid C-terminal polypeptide of the human c-*myc* protein (Materials and methods). To detect unambiguously the protein present, we performed immunoblots of the total proteins of full grown oocytes (stage VI), rather than more classical immunoprecipitations of labelled proteins, which limit the analysis to those proteins synthesised during the labelling period (Materials and methods). These antibodies recognise both human p62 and mouse p64 c-*myc* proteins (Figure 6A). All five antibodies assayed recognised a 62-kd protein in the *Xenopus* oocyte extract and two of them also recognised a 77-kd protein. In all cases the detection of the two bands was abolished by pre-incubation of the serum with the C-terminal polypeptide. Figure 6B and C are results obtained with two of these antibodies, one (panel B) recognising the 62-kd protein and the other (panel C) recognising both the 62-kd and 77-kd proteins. The disappearance of these specific bands after pre-incubation with the C-terminal polypeptide provides good evidence that these two proteins are *myc*-related.

To identify more clearly the protein encoded by *myc* RNA, a hybrid selection experiment was performed. To hybrid-select *Xenopus myc* RNA, M13 single strand DNA containing the 1045 nucleotide *myc* fragment (shown in Figure 1) was bound to nitrocellulose filters and hybridised to oocyte poly(A)$^+$ RNA. The RNA hybridised was eluted from the DNA and its product identified by translation *in vitro* (Materials and methods). As shown in Figure 7, the hybrid-selected RNA encodes a single 62-kd protein. Taken together these data provide strong support for the hypothesis that the 62-kd protein is a *Xenopus myc* protein. A 65-kd band has been detected previously as one of several proteins in *Xenopus* oocytes immunoprecipitated by an antibody raised against the N terminus of the human *myc* protein (Persson *et al.*, 1984). The 77-kd protein, although undetected in the hybrid selection experiment, reproducibly cross-hybridises with some *myc* antibodies and therefore was studied in parallel with the 62-kd *myc* protein using an antibody recognising both proteins (Figure 6C).

Xenopus myc protein synthesis during oogenesis

Since substantial transcription of the *Xenopus myc* gene occurs during oogenesis the appearance of the *myc* protein during that long period was followed. Figure 8 shows that both the 62-kd *myc* protein and the 77-kd protein were first detectable during

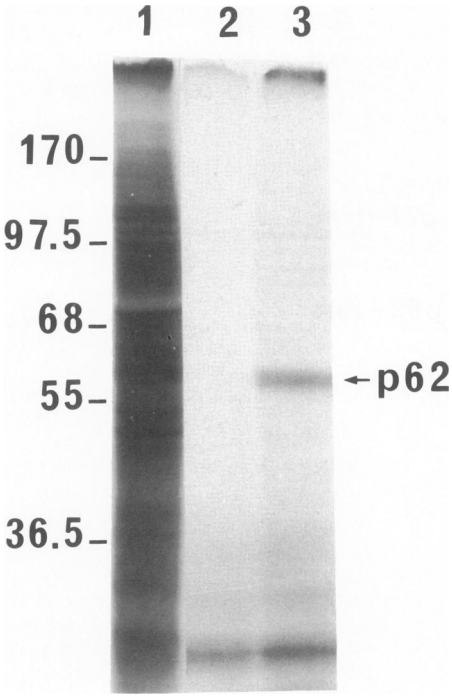

Fig. 7. Translation *in vitro* of hybrid-selected *myc* mRNA. Oocyte poly(A)$^+$ was hybrid selected with a *Xenopus* single strand *myc* DNA, eluted, and translated in a reticulocyte cell free system as described in Materials and methods. Translation products were analysed on 10% SDS–polyacrylamide gels followed by fluorography. **Lane 1**, translation products of total poly(A)$^+$ oocyte RNA; **lane 2**, endogenous synthesis in cell-free system without exogenous RNA; **lane 3**, translation products of hybrid selected *myc* RNA.

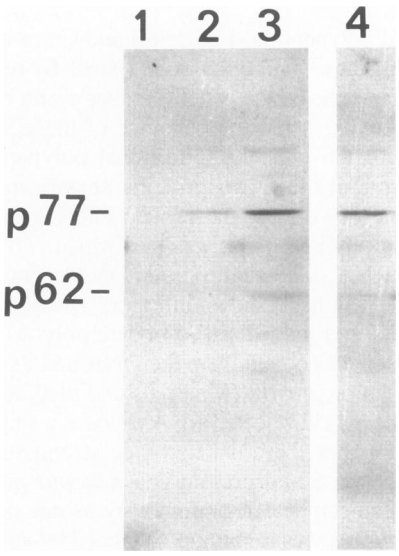

Fig. 8. Expression of *Xenopus myc* protein during oogenesis. 40 μg proteins from stage I–II early oocytes (**1**), stage III–IV mid-growth oocytes (**2**), stage V–VI late oocytes (**3**) or from two-cell embryos (**4**) were fractionated by SDS–PAGE and analysed by immunoblotting as described in Materials and methods.

mid-oogenesis and accumulated to a maximum level in late oocytes; moreover, the two proteins are also present in the two-cell embryo. The first appearance of these proteins correlates with the lampbrush stages of oogenesis, that is they are only detected long after the *myc* transcripts are apparent in early oogenesis. We do not know if they arise from translation of the mRNA synthesised during the lampbrush stage or from mRNA synthesised during early oogenesis, but untranslated before mid-oogenesis.

Discussion

The *Xenopus myc* gene is the first amphibian nuclear proto-oncogene identified at the nucleotide sequence level. Two distinct genes appear to be present in the genome, but it has yet to be determined whether both are active throughout development or if their products have the same function. The high degree of homology with the c-*myc* gene of chicken, mouse and human of both the nucleotide and the predicted amino acid sequences again indicates the conservation of this proto-oncogene during evolution and suggests an important common function.

Xenopus myc expression during oogenesis and embyronic development

Our results show that the *Xenopus myc* RNA belongs to the class of stable maternal RNAs which are accumulated from very early oogenesis (Rosbash and Ford, 1974; Cabada *et al.*, 1977; Dolecki and Smith, 1979; Golden *et al.*, 1980). The maximum level of *myc* RNA is reached later around the lampbrush chromosome stage. The amount of *myc* RNA is unchanged after ovulation and therefore hormonal maturation of the oocyte and the progression through the meiotic cycle has no long-term effect on the steady-state level of this RNA. Two proteins were detected by anti-*myc* antibodies in the oocyte. One of them, the 62-kd protein, is clearly encoded by the *Xenopus myc* RNA and the second, the 77-kd protein, appears to be related to *myc* as it can be detected by cross-reactivity with two out of five different *myc* antibodies tested. The analysis during oogenesis shows that the maximum *myc* protein level is reached in the full-grown oocyte long after the maximal RNA accumulation, suggesting a delayed translation of *myc* RNA.

After fertilisation the egg undergoes 12 rapid cleavages during which there is no detectable RNA synthesis. Within 1 h of cleavage 12, new small transcripts are synthesised (Newport and Kirschner, 1982); soon after the embryo enters the gastrula stage and new poly(A)$^+$ RNAs begin to accumulate. Fertilisation is the start of a progressive degradation of the stably accumulated *myc* RNA stored in the egg (5×10^6 copies) until gastrula (about 30 000 cells) when a minimum level is reached (10 copies/cell). This level is similar to the c-*myc* RNA per cell in several highly proliferative cell-types (see Results). During the subsequent stages of development the *myc* RNA per cell remains approximately constant, presumably due to the synthesis of new transcripts, although there is an apparent small increase at neurula. It will be of interest to quantitate the *myc* protein in the rapid cleavage phase of the embryo when the *myc* RNA is declining. One hypothesis is that the protein will be maintained at a constitutive level; this may or may not require prior stock-piling.

The accumulation of enzymes and structural proteins involved in cell proliferation has been well documented during oogenesis (Davidson, 1976; Laskey, 1979; Woodland *et al.*, 1979; Van Dongen *et al.*, 1981). Our study shows such a phenomenon at the level of a specific messenger RNA, implicated in the regulation of cell proliferation.

The stability of myc RNA during oogenesis and early development

The half-life of c-*myc* RNA in various proliferative cell-types, both normal as well as transformed, has been measured to be ~15 min (Dani *et al.*, 1984). In contrast, the accumulation of *Xenopus myc* RNA to a high level in early oogenesis and the

maintenance of this level during the whole process of oocyte growth indicates that this RNA is very stable in oogenesis. Indeed it has been shown that at least 400 days are required to accumulate the more abundant oocyte poly(A)$^+$ sequences (Perlman and Rosbash, 1978). The same calculation shows that at least 280 days are needed to accumulate the *myc* transcripts during oogenesis. The stability of *myc* mRNA appears to be an important property as recent studies in proliferative cells have shown that c-*myc* gene expression may be regulated both at the transcriptional level (Kelly *et al.*, 1983; Greenberg and Ziff, 1984) and at the level of message stability (Dani *et al.*, 1984; Blanchard *et al.*, 1985). The stability of *Xenopus myc* RNA during oogenesis may partly be due to masking proteins which prevent translation of the mRNA in young oocytes (Smith *et al.*, 1984). We note that the observed delayed appearance of the *myc* protein (Figures 4B and 8) could be explained by a decrease in these masking proteins as oogenesis progresses (Smith *et al.*, 1984).

A degradation of *Xenopus myc* RNA is induced at fertilisation, but its relatively long half-life (4 h 20 min) observed during the successive cleavages of the embryo is also unusual. One possible explanation is that this half-life is a consequence of an 'abnormal' accumulation of *myc* RNA in the egg before fertilisation, which saturates the degradative potential of the cleaving cell. Therefore, the relative stability of *myc* RNA after fertilisation may not be due to specific structural properties of the RNA, but to a stoichiometric regulation at the post-transcriptional level. The *myc* expression registered after the mid-blastula transition could be indicative of a more classical somatic regulation of this gene with degradation matched by new transcription.

Uncoupling of myc transcription and cell division

The correlation between cell proliferation and c-*myc* RNA expression has been shown by numerous investigators (Kelly *et al.*, 1983; Blanchard *et al.*, 1985). However, we have shown that an unfertilised egg, a cell which does not and clearly must not divide, expresses *myc* RNA to a level at least 10^5-fold that in a variety of proliferative cells. Additionally, we have shown that the *myc* protein is also accumulated in oogenesis. Therefore in the unfertilised *Xenopus* egg the expression of *myc*, both at the level of RNA and protein, is uncoupled from DNA synthesis and cell division. Clearly *myc* expression *per se* is not sufficient for these processes. Moreover, if the *myc* protein regulates cell division there must be a control mechanism regulated at fertilisaton that allows it to function in this role during embryonic cleavage, but not in the mature, unfertilised egg.

After fertilisation, in a period of extremely active cell division, when no RNA synthesis is detected even by very sensitive techniques (Newport and Kirschner, 1982), the *myc* RNA is progressively degraded; nevertheless its level per cell remains greater than that in the highly proliferative cells cited above. It appears, therefore, that in the cleaving *Xenopus* embryo *myc* transcription itself may again be uncoupled from cell division. The overall results do not exclude that the *myc* gene product has a regulatory role on transcription, as has been suggested (Kingston *et al.*, 1985), but any effect of it on cell proliferation in *Xenopus* early cleavage cannot be through the stimulation of transcription, as the early embryo is transcriptionally quiescent.

This study is a rare example of an identified maternal mRNA transcript that has been analysed through oogenesis and early embryonic development, and is the first of a gene implicated in a regulatory role. It also shows a major uncoupling of *Xenopus myc* RNA expression from cell proliferation. We suggest that the accumulation of stable *myc* RNA in oogenesis is a specialised adaptation necessary to support the active cell division linked to transcriptional quiescence following fertilisation. Furthermore, this study illustrates novel aspects of the stability and regulation of the mRNA and protein products of the *myc* proto-oncogene in both embryonic development and cell proliferation.

Materials and methods

Collection of oocytes, eggs and embryos

Xenopus animals imported from South Africa (South African Farms, Fish Hoek) were accommodated and fed as described by Gurdon (1967) and Gurdon *et al.*, (1984) except that silence was maintained in the animal room.

Oocytes were collected and defolliculated as described by Gurdon and Wickens (1983), sized under the microscope according to the six stages describe by Dumont (1972) and either processed immediately or frozen at −80°C. The absence of follicule cells was checked by microscopic examinations of Hoechst 33258 (Sigma) stained oocytes. Eggs were obtained as previously described (Méchali and Kearsey, 1984), and fertilisation *in vitro* was carried out as described by Gurdon (1967). Embryos were harvested at different developmental stages (Nieuwkoop and Faber, 1956), dejellied in 2% cysteine in sterile 0.1 × Modified Barth Solution pH 7.9 (Gurdon and Wickens, 1983), and frozen at −80°C.

Molecular cloning and sequencing

Xenopus myc cDNA was isolated from a cDNA library prepared from defolliculated oocyte poly(A)$^+$ RNA and kindly supplied to us by D.Melton (Rebagliati *et al.*, 1985). Screening of the recombinant library was with a human c-*myc* exon III *Eco*RI−*Cla*I labelled probe (Dalla-Favera *et al.*, 1982) as described by Maniatis *et al.* (1982). Subcloning of the positive clones was either in pUC or M13 vectors as described by Messing (1983). Sequencing was determined on M13 mp18 and mp19 recombinant vectors by the method of Sanger *et al.* (1977). Subclones were obtained from defined restriction fragments or by progressive digestion of a fragment by exonuclease III (Henikoff, 1984).

DNA and RNA preparations

Blood was collected by cardiac puncture of the anaesthetised animal, and high mol. wt DNA was purified from isolated erythrocyte nuclei by slight modifications of the method described in Maniatis *et al.* (1982). Chicken erythrocyte DNA was a gift from J.C.Weil (Institut Jacques Monod).

For large RNA preparations, homogenised oocytes were treated with 250 μg/ml proteinase K in 10 mM Tris acetate pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 0.2% SDS (30 min, 0°C). RNA was extracted by slight modifications of a hot phenol procedure (Maniatis *et al.*, 1982), followed by precipitation with 2.5 M LiCl. Poly(A)$^+$ RNA was isolated by chromatography on poly(U)−Sepharose (Pharmacia) successively performed twice. The integrity of the final RNA populations was analysed by formaldehyde agarose gel electrophoresis and by *in vitro* translation assays in rabbit reticulocyte lysates (Maniatis *et al.*, 1982).

The analysis of *myc* expression at different developmental stages was on RNA extracted by a urea−LiCl method (Auffray and Rougeon, 1980) using frozen oocytes or embryos homogenised at a constant RNA/LiCl−urea ratio determined in pilot experiments. No difference between the RNA extracted by the phenol−LiCl and LiCl−urea procedures was detected by either the electrophoresis or translation assays.

Poly(A)$^+$ RNA from Friend leukemic virus preleukemic cells was from F.Moreau-Gachelin (Robert-Lezenes *et al.*, 1984). Total RNA was extracted from 4.4 g mouse spleen (10^6 cells per mg), and the poly(A)$^+$ RNA population was purified by chromatography on oligo(dT)−cellulose successively performed twice.

Hybridisations

Restricted *Xenopus* genomic DNA was fractionated by agarose gel electrophoresis and transferred to nylon Zeta-Probe membranes (Biorad) (Reed and Mann, 1985). Hybridisation was with *Xenopus myc* single-stranded probes prepared according to Messing (1983); the radioactive strand was isolated by electrophoresis and electroelution. Hybridisation was at 42°C in 50% formamide as in Maniatis *et al.* (1982), after which the blots were washed twice in 2 × SSC, 0.1% SDS (42°C, 30 min), once in 1 × SSC, 0.1% SDS (42°C, 30 min), once in 0.5 × SSC, 0.1% SDS (55°C, 30 min) and once in 0.1 × SSC, 0.1% SDS (55°C, 30 min). Northern blots were on Hybond N nylon membranes (Amersham) and hybridisations were with the *Xenopus myc* probe as described above. The blots were washed at 42°C twice in 5 × SSPE, once in 1 × SSPE, 0.1% SDS (30 min), once in 0.5 × SSPE, 0.1% SDS (30 min), and once in 0.2 × SSPE, 0.1% SDS (30 min).

Autoradiography and scanning densitometry measurements

Autoradiography was carried out with Fuji RX films under the conditions described by Laskey (1984). For precise quantitation the films were flashed to an OD_{450} of 0.15 and exposed at −80°C using an intensifying screen (Dupont Cronex LP). Different exposures were taken to control the linearity of the film response. Scanner densitometry was performed on a Shimadzu Dual Wavelength scanner CS-230.

Quantitation of the myc RNA concentration

RNA samples were adjusted to 4.6 M formaldehyde, 7.5 × SSC in 400 μl, incubated for 15 min at 65°C, chilled, filtered on nitrocellulose in a slot blot apparatus (Schleicher and Schuell), washed with 400 μl 10 × SSC and fixed (80°C, vacuum). A single strand DNA probe, the *Xenopus myc* HincII−SacI fragment, was used instead of a nick-translated probe to avoid probe auto-hybridisation in solution. Labelling was according to Messing (1983) in a reaction medium of known specific activity. The radioactive strand was isolated and hybridisation at 42°C with an excess of probe was carried out as described above. After autoradiography of the filter the slots were cut out and the radioactivity counted. From the specific activity and length of the probe the c.p.m. value obtained was converted into RNA amounts. Although this procedure may suffer from incomplete retention of RNA on nitrocellulose thus underestimating the RNA content, the values obtained are proportional to the quantity of RNA loaded (Figure 4A) and are in agreement with an independent measurement obtained by determining the percentage of recombinants in the oocyte library.

Protein extraction and SDS−PAGE

Total oocytes were extracted by homogenisation in 1% SDS, 0.5% NP40, 0.5% sodium deoxycholate, 20 mM EDTA, 5 mM DTT, 1 μM pepstatin, 1 μg/ml Leupeptin, 1 mM PMSF in 50 mM Tris−HCl pH 7.5. Yolk proteins were selectively removed by controlled freon extraction (Gurdon and Wickens, 1983) and the remaining proteins were separated on SDS 10% polyacrylamide gels (Maniatis *et al.*, 1982).

Hybrid-selected translation

Hybrid selection of *myc* RNA was essentially as described by Ricciardi *et al.* (1979), with M13 single strand *myc* cDNA fixed on nitrocellulose filters, boiled twice in 0.5 ml H_2O for 1 min to remove DNA not tightly bound, and hybridized using oocyte poly(A)$^+$ RNA. The filters were washed and the hybridised RNA was eluted by boiling for 1 min in 300 μl H_2O containing 15 μg calf-liver tRNA. RNA was recovered by ethanol precipitation, treated by RNAse-free DNase (Miles) and translated in a rabbit reticulocyte lysate (Promega Biotec). The [^{35}S]-methionine-labelled translation products were characterised by polyacrylamide gel electrophoresis as described above.

Antibodies, immuno-blotting and immunoprecipitation

The anti-human c-*myc* sera were raised in rabbits against a 32 amino acid long peptide from the carboxy terminus (residues 408−439) of the predicted human c-*myc* protein (Evan and Hancock, 1985). The peptide was conjugated to the purified protein derivative (PPD) of mycoplasma tuberculosis (molar ratio 2 peptide:1 PPD) by addition of 0.05% glutaraldehyde in borate buffer pH 8 (final peptide concentration 2 mg/ml) for 3 h at room temperature. Coupling was stopped by 0.1 M glycine ethylester pH 8, the complex precipitated, solubilized in phosphate buffered saline and mixed with Freund's incomplete adjuvant (1:1). The mixture was administered subcutaneously to rabbits at multiple sites at 3-week intervals, and the serum prepared after five immunisations.

Extracted proteins were separated by SDS gel electrophoresis and electrophoretically transferred to nitrocellulose (Schleicher and Schuell BA85) in a transblot cell (Biorad). After washing for 1 h in 10 mM sodium phosphate buffer pH 7.5, 145 mM NaCl, 0.2% Tween 20 and 1% non-fat dried milk, blots were incubated with the antibody overnight at 4°C and then for 5 h with horseradish peroxidase conjugated goat anti-rabbit IgG (Miles). Peroxidase activity was detected by chloronaphthol. Human COLO320HSR and mouse SEWA Rec2A cell lines were labelled with [^{35}S]methionine, extracted, immunoprecipitated and analysed as described by Evan and Hancock (1985).

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