Expression of the *c-myc* Proto-Oncogene during Development of *Xenopus laevis*

MICHAEL W. KING, JAMES M. ROBERTS, AND ROBERT N. EISENMAN*

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Received 13 June 1986/Accepted 18 August 1986

We isolated and characterized *Xenopus laevis* *c-myc* cDNAs from an oocyte-specific library. These cDNA clones encompass 2.35 kilobases of the *X. laevis* *c-myc* RNA and contain the entire coding domain of 1,257 nucleotides of the 419-amino acid-long *X. laevis* *c-myc* protein. The 2.7-kilobase *X. laevis* *c-myc* mRNA is expressed in the oocyte, maintained in the egg, and is present throughout the early cleavage stages of embryogenesis. At the time of transcriptional activation in the embryo the *c-myc* RNA levels show a significant decline and then reaccumulate continuously throughout the remainder of premorphogenic development. At the early neurula stage of embryogenesis the pattern of *c-myc* RNA expression is elevated in the mesoderm with respect to the endoderm and ectoderm. In the adult *X. laevis* the *c-myc* mRNA is expressed in some (e.g., skin, muscle) but not all differentiated tissues. The *X. laevis* *c-myc* protein migrates as a doublet of 61,000- and 64,000-dalton species. Both species are phosphorylated in oocytes and somatic cells, exhibit extremely short half-lives of less than 30 min, and are localized to the nuclear fraction of somatic cells. By contrast, the oocyte protein shows both cytoplasmic and germinal vesicle distribution and appears to be stable.

The high degree of evolutionary conservation of individual oncogenes has made it possible to undertake studies on oncogene function in organisms distant from those in which the oncogene had initially been identified. In particular, studies with more primitive eucaryotes have permitted the use of developmental and genetic approaches that are not feasible with mammalian systems. The experiments on *ras* function in yeasts (21, 50) and *src* expression during *Drosophila* development (46) are good examples of both the advantages and the difficulties inherent in this approach.

Another potentially fruitful organism for studying oncogenes is the African clawed toad *Xenopus laevis* which provides an especially useful system for developmental studies. *Xenopus* development is rapid and autonomous, and the embryo is large enough to allow for experimental manipulations such as microinjection and grafting (see reference 20). Recent work has begun to define some of the molecular events associated with important steps in embryogenesis such as oocyte maturation, the midblastula transition, and early tissue specification (for a review, see reference 33). In addition, the *Xenopus* oocyte and egg have provided experimental systems for studying transport of proteins to the nucleus (13, 14, 39), nuclear assembly (19, 35), meiosis (22, 38), RNA localization (43), and transcriptional control (31). The evolutionary proximity of *X. laevis* to mammals also allows a more confident extrapolation of results obtained with oncogenes than may be possible for yeasts or *Drosophila* sp. To date the only homolog of a proto-oncogene that has been identified in *X. laevis* is *c-src* (47).

We sought to identify and analyze the expression of the *c-myc* proto-oncogene products in *X. laevis*. Although little information is available concerning the biochemical function of *c-myc*, there is evidence suggesting a link between *c-myc* and cell proliferation and differentiation (for a review, see R. N. Eisenman and C. B. Thompson, *Cancer Surv.*, in press). The *c-myc* locus in chickens, mice, cats, rodents, and humans has been shown to be subject to a multiplicity of genetic rearrangements which have been associated with

primarily hematopoietic but also nonhematopoietic malignancies (see reference 9 for a comprehensive list of references).

The normal unrearranged *c-myc* locus consists of three exons spanning about 4 to 5 kilobase pairs (kbp) in all species examined thus far. An open reading frame begins at the 5' end of exon 2, extends through exon 2, and terminates nearly two-thirds of the way through exon 3 (7). Transcription from the *c-myc* gene has been found to occur at a low level in most tissues studied (24). Two major mRNAs have been detected with transcriptional initiation sites about 150 nucleotides apart (2, 3). Control of *c-myc* expression has been shown to occur at both transcriptional and posttranscriptional levels (5, 15, 25), with posttranscriptional control occurring through modulation of the turnover of the very unstable *c-myc* mRNAs (5).

c-myc-encoded proteins have been shown to be nuclear phosphoproteins exhibiting short half-lives and apparently nonspecific affinity for DNA (Eisenman and Thompson, in press). Expression of *c-myc* occurs at a constant level throughout the cell cycle (30, 49) although transient increases in expression are observed upon treatment with a variety of mitogenic agents (32; see also reference 11 for a review). Recent evidence suggests that protein kinase C may be involved in mediating *c-myc* responsiveness to mitogens (10). Expression of *c-myc* may also be involved in initial events in differentiation since down-regulation of *c-myc* RNA and protein has been observed upon induction of differentiation in many cell types (15, 23, 26), and constitutive expression of *c-myc* appears to prevent differentiation in mouse erythroleukemia cells (8).

To date there have been no systematic study of *c-myc* expression during embryonic development. As a means of understanding *c-myc* function we identified *c-myc* mRNA and proteins in *X. laevis* and undertook a study of *c-myc* expression during embryogenesis.

MATERIALS AND METHODS

Isolation of *X. laevis* *c-myc* cDNA clones. An *X. laevis* oocyte-specific cDNA library cloned into the *EcoRI* site of

* Corresponding author.

λ gt10 was kindly provided by Douglas Melton. The bacteriophage were plated out on a single agar dish (24 by 24 cm) generating approximately 200,000 plaques. The phage were then transferred in duplicate to nitrocellulose filters, prehybridized in $5\times$ Denhardt solution ($1\times$ Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) at 65°C for 2 to 3 h, and then hybridized at 65°C with a ^{32}P -labeled probe consisting of a human *c-myc* cDNA clone isolated from the HL-60 cell line (David Bentley, personal communication). The hybridization buffer consisted of $5\times$ SSPE ($1\times$ SSPE is 10 mM NaPO_4 [pH 7.0], 180 mM NaCl, 1 mM EDTA), $5\times$ Denhardt solution, and 0.2% sodium dodecyl sulfate (SDS). The filters were washed at 65°C in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, and positive clones were visualized by autoradiography. All positive clones were plaque purified, and relevant *X. laevis* *c-myc* cDNA fragments isolated from the lambda clones were subcloned into pUC19.

DNA sequencing. Restriction fragments isolated from the two overlapping *c-myc* cDNA clones diagrammed in Fig. 1A were purified by agarose gel electrophoresis and sequenced by chemical degradation (37), or fragments were cloned in M13mp18 and M13mp19 vectors and sequenced by the dideoxy chain termination method (45).

RNA isolation and analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate procedure (6). RNA samples were analyzed for *c-myc* content by agarose gel electrophoresis in 1.2% agarose–2.2 M formaldehyde gels (34). The fractionated RNA was transferred to nitrocellulose and hybridized (48) with ^{32}P -labeled *X. laevis* *c-myc* cDNA probes. RNA bands were then visualized by autoradiography.

Cell culture. The *X. laevis* kidney cell line A6 (42) was cultured in 50% L-15 medium (Gibco) supplemented with glutamine, penicillin-streptomycin, and 10% heat-inactivated fetal calf serum. The cells were incubated at 20°C in tightly sealed 75- cm^2 flasks.

X. laevis oocytes were surgically removed from female toads and dispersed to a single-cell suspension by treatment of the dissected ovary section with 0.2% (wt/vol) collagenase in modified Barth solution (88 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 0.5 mM Na_2HPO_4 , 2 mM NaHCO_3 , 15 mM HEPES [*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.6) containing 50 μg of gentamicin per ml. Oocytes were kept in modified Barth solution at 20 to 25°C and were viable for 1 to 3 days.

Preparation of polyclonal antisera. The coding region of a human *c-myc* cDNA clone (see above) was inserted into the pATH-1 expression vector (kindly provided by T. J. Koerner) which had been modified to remove all but 20 of the *Escherichia coli* *trpE* amino acid sequences and contained two amino acids derived from linker sequences. This vector is termed pEXmyc Δ 22. The predicted protein encompasses the entire *c-myc* coding region including 22 vector-derived amino acids at the amino terminus. Expression of the *myc* protein was induced from the *trpE* promoter by starvation of the host *E. coli* for tryptophan in minimal (M9CA [36]) medium. Induction was carried out at 37°C for 9 h. The expressed *c-myc* protein was purified by preparative gel electrophoresis followed by extraction of the protein from the polyacrylamide gel. Approximately 500 μg of this *E. coli*-expressed protein was mixed with Freund complete adjuvant and injected into a 1-year-old New Zealand White rabbit which had previously been bled to provide preimmune serum. The rabbit was administered booster injections of 500

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *X. laevis* *c-myc*. (A) Schematic representation of the two *Xenopus* *c-myc* cDNA clones which were used for nucleotide sequencing. The stippled box indicates the extent of the major open reading frame present in the sequence. Restriction sites are those which were used for obtaining the nucleotide sequence. The arrows below designate the direction of sequencing. Various fragments were subcloned into M13mp18 or M13mp19 (○) for Sanger sequencing (45) or were end labeled (●) for Maxam and Gilbert sequencing (37). Symbols for restriction enzymes are as follows: □, *Dra*I; ■, *Eco*RI; ●, *Pst*I; ▼, *Pvu*II; ○, *Sau*3AI; △, *Xho*I. bp, Base pairs. (B) Sequence of the *c-myc* RNA from *X. laevis*. The nucleotide sequence is numbered (below the lines) beginning with the first nucleotide of the sequences determined from the cDNA clones. The negative numbers indicate sequences 5' to the putative initiation codon. Nucleotide +1 is the A of the ATG initiator codon. Shown above the nucleotide sequence is the deduced amino acid sequence beginning with the methionine residue which is homologous to that which is found in mammalian and avian *c-myc*. The amino acids are numbered (located to the left of each line) starting with the putative initiator methionine. Shown boxed are the putative initiation methionine, an upstream in-frame methionine that could potentially act as an initiator, and the TAA termination codon.

μg of the *E. coli*-expressed protein every 4 weeks. Immune serum (designated anti- Δ 22-*myc*) was taken by ear bleeding 10 days after each boost.

Immunoprecipitation and gel electrophoresis. A6 cells were labeled 4 to 5 days after passage (1×10^7 to 2×10^7 cells per ml) with either [^{35}S]methionine (600 μCi) or $^{32}\text{P}_i$ (2 mCi) for 40 to 60 min and subsequently processed for immunoprecipitation with the anti- Δ 22-*myc* serum exactly as described previously (29). Oocytes were labeled after collagenase dispersion in 500 μl of modified Barth solution with $^{32}\text{P}_i$ (5 mCi) for 15 h at 20°C . The medium was removed, and the oocytes were washed with ice-cold phosphate-buffered saline, solubilized by homogenization in lysis buffer (29), and centrifuged at $12,000 \times g$ for 30 min at 4°C . The lysate was then subjected to immunoprecipitation with the anti- Δ 22-*myc* serum.

Immunoprecipitates collected by incubation with Formalin-fixed *Staphylococcus aureus* cells were separated on discontinuous 10% SDS-polyacrylamide gels and visualized by fluorography as described previously (1). The following prestained molecular weight markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used on each gel: myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α -chymotrypsinogen (25,700).

Immunoblotting. *X. laevis* oocytes were manually dissected into cytoplasmic and germinal vesicle (GV) fractions. Various amounts of total lysate from each fraction were separated by electrophoresis in 10% discontinuous SDS-polyacrylamide gels and transferring to nitrocellulose as described previously (28). The *c-myc* proteins were visual-

B

GGAGAGCGCGAGTAGTAGTGGGAAAGACAACGCTATTGAGACAAGGGAGAGGCAG
 -140 -120 -100
 CCTGTGGATTATATAACGGGACCAACCAAGGAATATTGGACTCGTATTATCCCGGATTAGACGGGAGACCAATCGGATGCGAGGAAAG
 -80 -60 -40 -20 -1

1 MetProLeuAsnAlaAsnPheProSerLysAsnTyrAspTyrAspTyrAspLeuGlnProCysPhePhePheLeuGluGluGluAsnPhe
 ATGCTCTTAATGCCAATTTCCAGCAAGAACTACGACTACGATTATGACTTGCAGCCCTGCTTCTTCTTCTGAGGAGGAGAACTTC
 +1 20 40 60 80

31 TyrHisGlnGlnSerArgLeuGlnProProAlaProSerGluAspIleTrpLysLysPheGluLeuLeuProThrProProLeuSerPro
 TACCACCAGCAAGCCGACTGCAGCCGCCGCCAGTGAGGACATCTGGAAGAAGTTTGAGCTGCTCCCCACCCGCCCTTGTCAACC
 100 120 140 160

61 SerArgArgSerSerGlnSerSerLeuPheProSerThrAlaAspGlnLeuGluMetValThrGluPheLeuGlyGlyAspMetValAsn
 AGCCGCAGATCCAGCCAGTCCAGCCTTTCCCTCCAGCGCTGATCAGCTGGAGATGGTGACGAATTCCTGGAGGGGACATGGTCAAC
 200 220 240 260

91 GlnSerPheIleCysGluAlaAspAspGluAlaLeuLeuLysSerIleValIleGlnAspCysMetTrpSerGlyPheSerAlaAlaAla
 CAGAGCTTTATCTGCCAGGCGGATGACGAAGCCTTGCTGAAGTCCATCGTCATACAGACTGTATGTGGAGTGGATTTTCGGCTGCCGCC
 280 300 320 340

121 LysLeuGluLysValValSerGluLysLeuAlaSerTyrGlnAlaSerArgLysGluSerAlaLeuSerSerSerProCysGlnSer
 AAGCTGGAGAAAGTGGTGTCTGAGAAGCTGGCGTCTTACCAGGCTTCTAGGAAAGAGAGTGCTCTGCTTCTTCTTCTCCGTGTCAGAT
 380 400 420 440

151 GlnProProProSerProLeuLysSerProSerCysHisGlySerLeuSerLeuGlyGlyThrHisArgSerSerHisGlyPheLeuGln
 CAACCACCACCAGCCCGCTTAAGTCTCCCTCGTGTCTGAGAGCTGAGTCTGGGAGGGACCCACAGGAGCAGCCATGGTTTTCTCCAG
 460 480 500 520

181 AspProSerSerAspCysValAspProSerValValPheProTyrProLeuAsnAspSerIleSerAsnAlaSerSerProCysGlnAsp
 GACCCAGCTCGGATTGTGTGTGACCTTCAGTGGTCTTCCATACCCACTGAACGACAGCATTTCCAACGCCAGCTCTCCTTGCCAAGAT
 560 580 600 620

211 LeuIleLeuGluThrProProIleSerSerAsnSerSerSerSerGluSerGluGluGluProGluAspGluAspGluAspCysAspGlu
 CTCATTTTGGAACTCCGCCATCAGCAGTAAACAGCAGCAGCAGTGAATCTGAAGAGGAACAGAAGATGAGGATGAAGACTGCGACGAG
 640 660 680 700

241 GluGluGluIleAspValValThrValGluLysArgGlnSerAlaSerLysArgValGluSerSerHisSerGlnProSerArgPro
 GAAGAGGAGATTGACGTTGTGACAGTAGAAAAAGGCAGTCGGCATCCAGCGGTGAATCCAGTTCTCATTGCGAGCCCTCCCGACCC
 740 760 780 800

271 HisTyrSerProLeuValLeuLysArgCysHisValProIleHisGlnHisAsnTyrAlaAlaSerProSerThrLysValAspTyrVal
 CACTACAGCCCTTTAGTTCTGAAGCGGTGTCAAGTTCCTTCCAAACACAACACTACCGCGGTCTCCCTCGACCAAACTGGACTATGTT
 820 840 860 880

301 SerSerLysArgAlaLysLeuGluSerAsnIleArgValLeuLysGlnIleSerAsnAsnArgLysCysAlaSerProArgSerSerAsp
 TCTTCCAAAAGGGCGAAACTAGAAAGCAACATCCGGTCTCTAAACAGATCAGCAACAACCGCAAGTGCAGCTCCAGGTCCTCCGGAT
 920 940 960 980

331 SerGluGluAsnAspLysArgLysThrHisAsnValLeuGluArgGlnArgArgAsnGluLeuLysLeuSerPhePheAlaLeuArgAsp
 TCCGAAGAGAACGACAAGAGGAAGACGCAACGTTCTGGAGCGCCAGAGCGGAACGAGCTCAAGTTGAGTTTTTTGCTTGGCGGAT
 1000 1020 1040 1060

361 GlnValProGluValAlaSerAsnGluLysAlaProLysValValIleLeuLysLysAlaThrGluTyrAlaIleSerLeuGlnGluAsp
 CAGGTACCGGAGGTGGCGAGCAACGAGAAGGCCCCCAAGTAGTCATCTCAAAAAGGCAACGGAATACGCCATTTCTCTGCAGGAGGAC
 1100 1120 1140 1160

391 GluArgArgLeuIleArgGluThrGluGlnLeuLysTyrArgLysGluGlnLeuLysGlnArgLeuGlnGlnLeuArgAsnPheVal*
 GAACGGCGGCTCATACGGGAAACAGAAGCTTAAAGTACAGGAAAGAGCAGTTAAACAGAGACTCCAACAGCTGAGGAACTTTGTCTAA
 1180 1200 1220 1240

TTCAAACTCTTATTTAACACTTTATATAAACTGTGACCGTCTATATCAAGTTGTCTCTTCTCCTCGAGACCGACTACAAAAGTGAATTT
 1280 1300 1320 1340

GTAGAACATGGACAACCTGCAGATGATTTCAAACTACACAACCTTGGCATGGTCTCAACAGATTCTGCCAGCACCTTAAAACTGC
 1360 1380 1400 1420

CTCAATACTGGGATTGGGTATTATGGGACTTTGTTTTTTTTTTTCTTGTGCTGAGGGGTATATTGCGCAACTTTTTGTTTGTGTT
 1460 1480 1500 1520

ATTGTTTTTACAACCTTTTGTATTTAAAA
 1540

FIG. 2. Amino acid sequence comparisons among *Xenopus* (X.1.), human, and chicken *c-myc*s. Comparison of the deduced amino acid sequence (in single-letter code) of *X. laevis* *c-myc* with the published sequences for human (51) and chicken (50) *c-myc*s. The dashes in the sequence represent gaps which were introduced in the various *c-myc* sequences to maximize homology. The alignment was obtained by using a combination of visual fit and the GENEPRO sequence analysis software of J. Wallace and J. Brown. All homologous amino acids are boxed. The arrow indicates the position of the boundary between exons 2 and 3 of the human and chicken *c-myc* sequence.

ized by staining with anti- $\Delta 22$ -*myc* serum and ^{125}I -labeled protein A followed by autoradiography.

Subcellular fractionation. A6 cells were labeled with 1 mCi of [^{35}S]methionine for 60 min at 20°C and removed from the flask in STM buffer (0.25 M sucrose, 50 mM Tris hydrochloride [pH 7.4], 5 mM MgCl_2) by scraping. The cells were disrupted by gentle Dounce homogenization. The nuclei were isolated by centrifuging the disrupted cells at 800 $\times g$ for 5 min at 4°C. The nuclear pellet was washed two times with STM buffer and suspended in 1 ml of lysis buffer (29). The solution was sonicated and centrifuged at 12,000 $\times g$ for 10 min to remove debris. The cytoplasmic fraction was adjusted to 0.5% Nonidet P-40, 0.5% deoxycholate, and 0.5% SDS, sonicated, and centrifuged as above to remove debris. Each fraction was subjected to immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) as described above.

RESULTS

Isolation and characterization of *X. laevis* *c-myc* cDNA clones. An *X. laevis* oocyte-specific cDNA library, cloned into the *Eco*RI site of λ gt10, was screened for *c-myc* sequences with a human *c-myc* cDNA as a probe (see Materials and Methods). A total of 12 clones were isolated from the library of approximately 2×10^5 phage. Two of these *X. laevis* *c-myc* clones (designated λ XLmycA and -B) were used for DNA sequence analysis because of their size and overlapping restriction maps (Fig. 1A). Restriction frag-

ments from these cDNA clones were sequenced as described above.

The nucleotide sequence encompassing the region of the *c-myc* coding domain (Fig. 1A) determined from the *c-myc* cDNA clones is presented in Fig. 1B. The open reading frame consists of 1,332 nucleotides, and the deduced amino acid sequence is depicted above the nucleotide sequence. There are 444 amino acids encoded in the open reading frame. There are two potential initiation codons present near the 5' end of the open reading frame (positions -12 and +1, Fig. 1B) that could give rise to two proteins differing by 4 amino acids. We designated the second ATG codon as the initiator of the *X. laevis* *c-myc* protein (generating a 419-amino acid protein) based on the homology of this region to the amino terminus designated for the *c-myc* proteins of humans (53), chickens (52), and mice (3).

The deduced amino acid sequence of *X. laevis* *c-myc* was compared with the amino acid sequences of human and chicken *c-myc*s (Fig. 2). Identical amino acids are enclosed in boxes in Fig. 2. Many of the nonhomologous amino acids are, however, conservative substitutions. When the number of matching amino acids between *X. laevis* *c-myc* and human and chicken *c-myc*s were assessed for exact identity, we observed 71 and 70% overall homology, respectively.

We sequenced a total of 650 nucleotides further 3' of the sequence indicated in Fig. 1B and found no evidence for other open reading frames in the *c-myc* cDNA (additional sequence available on request). In comparing the 5'- and 3'-untranslated regions of the *X. laevis* *c-myc* cDNA with the

FIG. 3. *X. laevis* *c-myc* RNA expression. Total cellular RNA isolated from various adult tissues or embryos at different stages of development was analyzed for *c-myc* content by Northern blot analysis (see text). The nitrocellulose filters were hybridized with the *c-myc* cDNA probe described in the legend to Fig. 3. Each lane contained 4 μ g of RNA sample unless indicated otherwise. The numbers to the left of panel A and right of panel C represent the migration distances of molecular weight markers. The locations of the *c-myc* and XL-11 RNA species are indicated to the left of panel C. (A) Stage 7, blastula; stage 11, gastrula; stage 16, neurula; stage 49, feeding tadpole. (B) As indicated. (C) Total RNA or poly(A)⁺ RNA was analyzed in this Northern blot. The filter was also blotted with the XL-11 probe (see the legend to Fig. 5B). Each lane contained 5 μ g of RNA.

corresponding regions of the human *c-myc* gene we found limited and scattered homologies. This suggests divergence of these regions between the two species; however, a more careful comparison will need to be made with the entire untranslated regions of the *X. laevis* *c-myc* gene.

We used the 2.0-kbp λ XLmycA probe (Fig. 1A) in Southern blot analysis of *X. laevis* DNA. Preliminary results, after digestion with different restriction endonucleases, indicated a pattern of hybridization more complex than expected for a single-copy gene (data not shown). Such a result could be due to the primordial duplication of the *Xenopus* genome (4) and would be consistent with the duplication found for the *Xenopus src* gene (47). We are currently analyzing *X. laevis* *c-myc* genomic clones to assess the structure and copy number.

Developmental expression of *X. laevis* *c-myc*. The relative levels of *X. laevis* *c-myc* RNA during early development were examined by Northern blotting of RNA from embryos staged by the method of Nieuwkoop and Faber (41). Total embryo RNA was isolated at the developmental stages indicated in Fig. 3A and probed with the 2.0-kbp *Eco*RI fragment of λ XLmycA after electrophoresis and transfer to nitrocellulose. The amount of RNA on each blot was normalized by the quantity of 28S and 18S rRNA. Total RNA was used for all these developmental analyses because it has been shown in other systems that *c-myc* RNA is distributed evenly between the pool of poly(A)⁺ and poly(A)⁻ RNAs (M. Groudine and M. Linial, unpublished observations).

As would be expected, since the cDNA clones were isolated from an oocyte-specific library, the *c-myc* RNA was present in the oocyte as well as the fertilized egg. The size of this *c-myc* RNA species was approximately 2.7 to 2.8 kilobases (kb). This is consistent with the size of the cDNA clones which encompass 2.35 kb of the *c-myc* RNA (Fig. 1A). There was also a minor RNA species of approximately

2.0 kb that hybridized to the λ XLmycA fragment probe (Fig. 3B and C and 4A). This species may represent another *c-myc* RNA generated from a second promoter (2). However, the detection of this smaller RNA species was variable (compare Fig. 3A, B, and C and 4A) and therefore may represent nonspecific hybridization of the probe.

The level of maternal *c-myc* RNA in the oocyte and fertilized egg remained constant throughout the rapid cleavage stages of the blastula (stages 1 to 7) before the midblastula transition when the zygotic genome becomes transcriptionally active (40). At approximately the time of the midblastula transition the steady-state level of the *c-myc* RNA decreased approximately sevenfold between stage 7 and stage 11 (determined by scanning densitometry of Fig. 3A). This disappearance was followed by a gradual increase in steady-state *c-myc* RNA levels relative to rRNA, most probably as a consequence of zygotic *c-myc* RNA expression.

As the developing *X. laevis* embryo progressed from the blastula stage to the gastrula (stage 11), the neurula (stage 16), and to a feeding tadpole (stage 49), the relative increase in the level of the *c-myc* RNA continued. This pattern of *c-myc* RNA accumulation indicates that the *c-myc* gene remains continuously transcriptionally active (when assessed in whole-embryo samples) throughout the major stages of early development after the midblastula transition.

Expression in adult tissues. The distribution and level of expression of the *c-myc* in different adult tissues of *X. laevis* was assessed by Northern blotting with the λ XLmycA 2.0-kbp probe. These results are shown in Fig. 3B and C. The level of *c-myc* RNA expression in the adult tissues was compared with the level of expression in the oocyte. The skin was the only tissue examined which demonstrated a level of expression equivalent to that of the oocyte. Interestingly, the established kidney cell line, A6, possessed the

FIG. 4. *X. laevis* germ layer *c-myc* expression. Germ layers were dissected from the ventral halves of stage 13 embryos, and total cellular RNA was isolated as described in the text. The *c-myc* RNA content of the germ layers was then analyzed by Northern blot analysis. Each lane contained 4 μ g of total RNA. (A) Hybridization with the 2.0-kbp fragment of λ XLmycA. Arrows indicate the positions of the 2.7-kb *c-myc* RNA (top) and the 2.0-kb RNA detected with the *c-myc* probe (bottom). (B) Hybridization with the 2.0-kbp fragment of λ XLmycA as well as a probe (XL-11) which exhibits equivalent germ layer distribution of a 1.05-kb RNA and endoderm-specific hybridization to a 1.6-kb RNA (J. Roberts, unpublished data). The positions of the *c-myc* and XL-11 RNA species are indicated.

highest level of *c-myc* RNA expression of any sample examined. The liver, skin, and kidney had demonstrable levels of *c-myc* RNA expression, whereas muscle, heart, and intestine had very low levels. Erythrocytes, assayed with poly(A)-selected RNA, were virtually devoid of any *c-myc* RNA.

The elevated level of expression of *c-myc* in the A6 cell line is not just solely a consequence of elevated expression of all RNA species. Another *X. laevis* RNA species (XL-11) which demonstrates an equally abundant level of expression in all tissues excluding eggs (data not shown) had equal abundance in A6 cells and in kidney (Fig. 3C). The *X. laevis* *c-src* RNA has also been detected at high levels in this cell line (Robert Steele, personal communication).

Germ layer *c-myc* distribution. One feature of the developmental expression of *X. laevis c-myc* is demonstrated in Fig. 4A. The ventral halves of embryos at stage 13 (early neurula) of development were dissected into their individual germ layers. Total RNA extracted from the resultant samples was analyzed for *c-myc* RNA content by Northern blotting. The amount of RNA in each lane was equalized by the content of 28S and 18S RNA. The level of the *c-myc* RNA in the ventral mesoderm tissue was 10-fold higher than in the ectoderm and 3-fold higher than in the endoderm as determined by densitometric scanning of the autoradiogram shown in Fig. 4A.

The differential distribution of *X. laevis c-myc* RNA in the germ layers was verified by using the XL-11 cDNA which hybridizes to an endoderm-specific RNA species of 1.6 kb as well as an abundant RNA species of 1.05 kb which is equivalently represented in all three germ layers. This result (Fig. 4B) demonstrates that the *c-myc* RNA content of the

ventral mesoderm is indeed elevated with respect to that of the ectoderm and endoderm.

***X. laevis c-myc* protein.** For our initial characterization of the *Xenopus c-myc* protein we used a broadly cross-reactive rabbit antiserum (anti- Δ 22-*myc*) prepared against the human *c-myc* protein expressed in *E. coli*. Bacterial expression of *c-myc* was obtained with a plasmid vector containing the *trpE* promoter (see Materials and Methods). The antisera prepared against the bacterially expressed protein immunoprecipitates human, avian, and murine *c-myc* proteins (data not shown).

A6 kidney cells were labeled with either [35 S]methionine or 32 P_i followed by immunoprecipitation of the labeled *c-myc* proteins as described in Materials and Methods. Analysis of the immunoprecipitated [35 S]methionine-labeled proteins from these cells by SDS-PAGE revealed two major proteins of apparent molecular weights of 61,000 and 64,000 (Fig. 5) which were recognized by the anti- Δ 22-*myc* serum (lane 1) but not by the preimmune serum (lane 2). Immunoprecipitation of these two proteins was blocked by prior incubation of the anti- Δ 22-*myc* serum with the purified *E. coli*-expressed *c-myc* protein (Fig. 5, lane 4, blocked). In addition, proteins having the identical electrophoretic mobility as p61/64 were specifically immunoprecipitated by affinity-purified antibodies against a synthetic peptide corresponding to the 12 C-terminal amino acids of *X. laevis c-myc* (data not shown). Immunoprecipitation of 32 P_i-labeled A6 cells clearly revealed

FIG. 5. Immunoprecipitation of labeled *X. laevis c-myc* protein from A6 cells. A6 cells were labeled with [35 S]methionine (500 μ Ci) or 32 P_i (2 mCi) for 30 min, lysed, and subjected to immunoprecipitation followed by SDS-PAGE as described in the text. Lanes 2 and 5, Preimmune serum. Lanes 1, 3, and 6, Anti- Δ 22-*myc* serum. Lane 4, Anti- Δ 22-*myc* serum blocked with *E. coli*-expressed *c-myc* protein. The arrows to the right indicate the migration of p61/64 *c-myc*. The numbers to the left represent protein molecular weight markers (10^3).

the *X. laevis* c-myc proteins at 61,000 and 64,000 daltons also exist as phosphoproteins (Fig. 5, lane 6). The protein migrating at 46,000 daltons (in Fig. 5 and 8) does not represent a c-myc species. It was observed only variably in specific and nonspecific immunoprecipitates and does not possess the properties of known c-myc proteins (i.e., nuclear localization, phosphorylation, rapid turnover, precipitation by *Xenopus* anti-myc peptide serum, or block with expression vector protein [see Fig. 5, 6, and 8] [data not shown]).

To assess the relative distribution of the *X. laevis* c-myc protein within the cell, A6 cells were labeled with [³⁵S]methionine, separated into cytoplasmic and nuclear fractions, immunoprecipitated with anti-Δ22-myc serum, and analyzed by SDS-PAGE. The c-myc proteins appeared to be associated almost exclusively with the nuclear pellet (Fig. 6).

Attempts to detect the oocyte-specific c-myc proteins by immunoprecipitation of lysates from oocytes labeled with [³⁵S]methionine were unsuccessful. This was not due to an inability to incorporate the [³⁵S]methionine into total oocyte protein since a range of proteins (10 to 300 kilodaltons) could be labeled (data not shown), and the c-myc proteins could not be detected in an equivalent amount of trichloroacetic acid-precipitable oocyte lysate as was used for analysis of A6 cells.

To determine whether c-myc proteins were present at all in oocytes, we analyzed steady-state protein levels by immunoblotting. Oocytes were dissected to separate the GV from the cytoplasmic fraction. Immunoblotting and detection of relative c-myc proteins was carried out as described

FIG. 6. Subcellular localization of *X. laevis* (XL) c-myc protein. A6 cells were labeled with [³⁵S]methionine (³⁵S-Met) (1 mCi) for 45 min, and the nuclear (N) and cytoplasmic (C) fractions were separated (see text). Equivalent quantities of each fraction (representing equal numbers of cells) were then immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography (see text). The arrows to the right indicate the migration of p61/64 c-myc. The numbers to the left represent protein molecular weight markers (10³).

FIG. 7. Analysis of the c-myc proteins in the *X. laevis* (XL) oocyte. (A) Oocyte immunoblot. Oocyte GV and cytoplasmic fractions (see text) were analyzed for c-myc protein content by immunoblotting as described in Materials and Methods. The nitrocellulose filters were incubated with either preimmune serum (lanes 1 to 5) or anti-Δ22-myc serum (lanes 6 to 10) followed by ¹²⁵I-labeled protein A and visualization by autoradiography. Lanes 1 and 6 contain approximately 90 μg of total GV protein. Lanes 2 and 7 contain 90 μg of cytoplasmic protein. Lanes 3 and 8 contain twofold more total cytoplasmic protein. Lanes 4 and 9 contain fourfold more total cytoplasmic protein. Lanes 5 and 10 contain approximately 20 ng of affinity-purified *E. coli*-expressed human c-myc. (B) Immunoprecipitation of ³²P-labeled oocyte c-myc. Oocytes were labeled with ³²P_i (5 mCi), lysed, and subjected to immunoprecipitation followed by SDS-PAGE as outlined in the text. Immune lane is anti-Δ22-myc serum. The arrows to the right indicate the migration of p61/64 c-myc. The numbers to the left represent protein molecular weight markers (10³).

in Materials and Methods. These results are depicted in Fig. 7A. A single species of approximately 64,000 daltons was detected by this analysis migrating just below the bacterially expressed 68,000-dalton c-myc protein (Fig. 7A, lanes 6 to 9). Increasing amounts of the cytoplasmic fraction were analyzed to confirm the presence of this 64,000-dalton species in this fraction. Both GV and cytoplasmic fractions contained nearly equivalent levels of c-myc protein (per milligram of total protein) (Fig. 7A, lanes 6 and 7). It is not clear at this time why only one of the two c-myc species (i.e., the 64,000-dalton species) is detected by this immunoblotting technique. This phenomenon has been observed for human c-myc proteins of HL-60 and HeLa cells analyzed in a similar manner with anti-Δ22-myc serum (data not shown). To determine whether the oocyte c-myc proteins are phosphorylated, oocytes were labeled in the presence of ³²P_i for 15 to 18 h and then subjected to immunoprecipitation (Fig. 7B) with preimmune serum or anti-Δ22-myc serum (immune). This result indicates that the same p61/64 c-myc species seen in the A6 cell line are present in the oocyte and exist as phosphoproteins. The incorporation of ³²P_i into the oocyte c-myc proteins may be the result of the phosphate exchange on preexisting molecules of these proteins.

Stability of *X. laevis* c-myc proteins. One characteristic feature of the c-myc proteins from mammalian and avian cells is their extremely rapid turnover. These c-myc proteins

FIG. 8. Stability of *X. laevis* (XL) *c-myc* protein. The relative rate of metabolism of the *X. laevis* *c-myc* protein was determined with the A6 kidney cell line. The cells were pulse-labeled for 45 min with [35 S]methionine (1 mCi) (35 S-Met) followed by a chase with medium lacking label for the indicated times. At each time point the cells were lysed, immunoprecipitated, and analyzed by SDS-PAGE as described in the text. The arrows to the right indicate the migration of p61/64 *c-myc*. The numbers to the left represent protein molecular weight markers (10^3).

have been shown to have a half-life of between 20 and 40 min at 37°C (29). To assess the rate of *X. laevis* *c-myc* turnover, A6 cells were pulse-labeled for 40 min with [35 S]methionine followed by a 2-h chase in the absence of label. At various times during the chase the cells were lysed, and the lysates were subjected to immunoprecipitation with anti- $\Delta 22$ -*myc* serum. These results are shown in Fig. 8. It is clear that the *X. laevis* p61/64 *c-myc* proteins turn over very rapidly, with a half-life of approximately 30 min. This is similar to the human and avian *c-myc* proteins even though the A6 cells are cultured at 20°C as opposed to 37°C for most mammalian and avian cell cultures. When HeLa cells are pulse-labeled at 37°C and then chased at 20°C there is no significant turnover of the *c-myc* proteins even after 120 min (data not shown).

DISCUSSION

We isolated *X. laevis* *c-myc* cDNA clones encompassing the entire coding region of the *c-myc* protein. These cDNA clones were used to assess the distribution of *c-myc* RNA sequences during early *X. laevis* development as well as adult tissue-specific distributions of this RNA. In addition we used a polyclonal *c-myc* antiserum, derived by immunizing a rabbit with bacterially expressed human *c-myc* protein, to characterize the *X. laevis* *c-myc* protein.

The *c-myc* amino acid sequences previously determined for both the mouse (3) and the human (53) proteins have

demonstrated the very high degree of conservation (93% identity) of the *c-myc* gene product in mammalian development. When the comparison is made between the mammalian and avian *c-myc* proteins (52) it can be seen that there is 70% identity. We demonstrate here that the *c-myc* protein from amphibians is highly related to both the avian (70% identity) and mammalian (71% identity) *c-myc* proteins (Fig. 2). This result demonstrates a conserved gene product spanning some 380 to 400 million years. *X. laevis* represents the most primitive organism from which *c-myc* has thus far been characterized.

Our primary goal in cloning the *X. laevis* *c-myc* RNA was to obtain homologous probes with which to examine the spatial and temporal regulation of the expression of the *c-myc* proto-oncogene during the period of early *X. laevis* embryogenesis. In this report we describe our initial characterization of the temporal pattern of *c-myc* RNA expression in early *X. laevis* embryos.

The mature *X. laevis* oocyte (Dumont [16a] stage VI) contains a store of maternally derived *c-myc* RNA and protein (Fig. 3A). We do not as yet know whether the *c-myc* RNA detected in the stage VI oocyte is translationally active but suspect it is inactive since we cannot detect de novo synthesis of *myc* protein. Thus, *c-myc* RNA molecules may be part of the maternal store of mRNA which is only utilized at later stages of development (12) or possibly during hormone-induced meiotic maturation of the oocyte (44, 51).

Our results indicate that the *c-myc* protein in the oocyte may be much more stable than in somatic cells (Fig. 8), possibly allowing it to be distributed to the blastomeres of the early cleavage-stage embryo (see below). Although *c-myc* protein could be detected in the oocyte by Western blotting, the stage VI oocyte *c-myc* protein could not be labeled with [35 S]methionine. Thus, we could not assess their half-life, but since oocytes can remain at stage VI for many months we assume the *c-myc* proteins are stable. This contrasts with the extreme instability of *c-myc* protein observed in somatic cells (Fig. 8). Indeed, there have been no previously reported examples of a lengthened half-life for the *c-myc* proteins.

We were surprised to detect a significant portion of the *X. laevis* *c-myc* protein in the oocyte cytoplasm (Fig. 7A, lanes 7 to 9) given that in *X. laevis* somatic cells the protein was found associated with the nuclear compartment (Fig. 6). The *c-myc* proteins in somatic cells of other species are also found associated with the nucleus (27) as is the viral cognate (1). It has been previously shown that proteins associated with the GV of the oocyte become distributed to the animal cytoplasm after maturation (16). However, we used oocytes before hormone-induced GV breakdown. One possibility is that *c-myc* protein represents a nuclear component stored in the cytoplasm for future use. It has been shown previously that injection of DNA into oocyte cytoplasm leads to the formation of apparently complete nuclei, suggesting that the cytoplasm is rich in nuclear structural elements (19), and *c-myc* protein may be one of these (17).

Many *X. laevis* oocyte nuclear antigens are found to be highly enriched in the nuclei of specific cell types later in development (16). If sequestering of the *c-myc* protein is similarly restricted to specific compartments it might allow regions of the embryo to respond differently to a *c-myc*-induced signal. In support of this contention is the finding that the ventral mesoderm of the early neurula stage embryo (stage 13) contains a higher level of *c-myc* RNA than is present in the ectoderm and endoderm cells (Fig. 4A and B). It is interesting that the hematopoietic system, among oth-

ers, is derived from the ventral mesoderm and that most *myc* alterations in higher eucaryotes have been found in hematopoietic cells.

We also analyzed the expression pattern of the *c-myc* proto-oncogene in a number of *X. laevis* adult tissues (Fig. 3B and C). The expression of this gene in the adult tissues of *X. laevis* exhibits a more generalized pattern of expression than would be predicted based solely on the proliferative capacity of a given differentiated tissue. The *c-myc* gene is expressed, as expected, in tissues with proliferative cell types such as the skin, liver, and intestine. However, the *c-myc* RNA is expressed in heart and skeletal muscle (albeit to a low level) which are tissue types with limited proliferative capacity. We do not know to what extent these adult tissues may be contaminated with proliferating cells. The postmitotic erythrocyte, however, contains vanishingly low levels of the *c-myc* RNA since only traces could be detected in poly(A)-enriched RNA samples from erythrocytes (Fig. 3C).

It has previously been shown for chicken tissues (24) and very recently been reported (54) that tissues of the adult mouse exhibit a pattern of *c-myc* expression very much the same as shown here for *X. laevis*. These data support the notion that *c-myc* expression is compatible with the maintenance, if not the establishment, of the differentiated state (11). Indeed, a recent report has shown that *c-myc* expression is not incompatible with the differentiated state of rat myogenic cells (18).

The ease with which the *X. laevis* embryo can be experimentally manipulated should now allow us to assess the involvement of the *c-myc* proto-oncogene during development by interfering with *c-myc* expression at specific stages of embryogenesis. Such experiments are currently in progress.

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