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Developmentally regulated alternative splicing in the *Xenopus laevis* c-Myc gene creates an intron-1 containing c-Myc RNA present only in post-midblastula embryos

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ABSTRACT

Two distinct c-Myc RNA classes have been identified in *Xenopus laevis*, presumably expressed from the duplicated c-Myc locus (1, 6). The major *Xenopus* c-Myc transcripts arise from sites termed P_1 and P_2 similarly to those of the mammalian c-Myc genes. I have used a cloned *Xenopus* c-Myc gene to examine the regulated pattern of expression from this gene during early *Xenopus* embryogenesis. Analysis of the pattern of transcript processing indicates that not only are P_1 and P_2 differentially active during early development but alternatively spliced c-Myc RNAs are generated which contain sequences of the first intron. These intron-1 containing c-Myc RNAs are generated by alternative splicing of transcripts initiated from the major transcription start site, P_2, and are observed only in RNA samples from post-midblastula embryos or *Xenopus* tissue culture cells. *Xenopus* tissue culture cells synthesize two major c-Myc proteins (p61 and p64). *Xenopus* RNAs that do not contain intron-1 sequences synthesize only the p61 species. Two closely spaced ATG codons at the 5′ end of exon-2 are utilized equivalently to generate a p61 doublet. Intron-1 containing RNAs utilize an ATG codon in the intron sequences to synthesize the p64 species as well as the exon-2 ATG codons to synthesize the p61 doublet.

INTRODUCTION

The c-Myc gene has been implicated in the development of a number of human, murine and avian hematopoietic malignancies suggesting that it may be an important regulator of proliferation and /or differentiation of cells of mesodermal origin (2–5). This potential also is supported by evidence that indicates the level of c-Myc expression in early *Xenopus* embryos is highest in the mesodermal germ layer (6).

The expression of the c-Myc gene during early development has been examined in murine (7–9), avian (10,11) and amphibian (1,6,12–15) embryos. In developing avian embryos expression of the c-Myc gene is highest in tissues of mesodermal origin with ectodermal tissues expressing somewhat lower levels and endodermal tissue even lower levels (11). In murine embryos expression of c-Myc is highest in tissues of mesodermal origin while the lowest levels are in ectodermal tissues (8,9). Developing *Xenopus* embryos also exhibit preferential c-Myc expression in mesodermal tissues with ectodermal tissues expressing the next highest levels of c-Myc mRNA (15). The c-Myc proteins can also be detected in post-mitotic avian neurons, in differentiated keratinocytes and in oocytes, demonstrating that expression is not restricted to actively proliferating cells (6,11,16).

The presence of c-Myc in oocytes and early embryos and evidence indicating c-Myc to be a sequence-specific DNA-binding transcription factor (17,18) suggests this protein may be involved in the control of early cellular determination by regulating a specific pattern of gene expression. The regulated patterns of c-Myc expression observed during early vertebrate development suggests that the function of c-Myc may be controlled through temporal restriction of the level of c-Myc RNAs. Evidence presented here indicates that another level of control exists over the expression of *Xenopus* c-Myc RNAs. This control is exerted through developmentally restricted alternative splicing that generates a c-Myc RNA containing intron-1 sequences. Alternative splicing of *Xenopus* c-Myc RNAs suggests that the potential exists to regulate both the temporal and spatial distribution of distinct classes of c-Myc RNA during early embryogenesis. Comparison of the *in vitro* coding capacity of an oocyte c-Myc cDNA lacking intron-1 sequences with a cDNA containing a portion of the sequences of intron-1 indicates that the different c-Myc RNAs possess alternative coding potentials.

MATERIALS AND METHODS

Cell culture

Xenopus tissue culture cells, A6, X58 and XTC were cultured in 50% L-15 media (Gibco) supplemented with glutamine, penicillin-streptomycin and 10% heat-inactivated calf serum. The cells were grown in tightly sealed flasks at 20°C. Total cell RNA was isolated by the guanidinium-isothyocyanate/CsCl procedure (19).

Embryos and oocytes

Oocytes and embryos were collected as previously described (6). Embryos were de-jellied in 2% cysteine-HCl pH 7.9 then incubated in 1/10X Barth's at 20°C. Embryos were removed at various stages of development (20). Total RNA was isolated from oocytes and embryos (19).

RNase protection

Two distinct fragments of the c-Myc gene clone were sub-cloned into pBS+ for production of radiolabeled antisense RNAs. These fragments spanned the exon-1 region of the gene as well as a portion of intron-1. One was a 1200 bp XbaI/PstI fragment that included 224 bp of intron-1. The other was a 520 bp BstNI fragment that included 38 bp of intron-1. Radiolabeled probes were purified on a 5% acrylamide gel and hybridized to 10μg of total RNA for 18 hrs at 42°C in 80% formamide, 400mM NaCl, 1mM EDTA, 40mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) pH 6.4. Following hybridization the non-duplexed RNA was digested in 300mM NaCl, 10mM Tris-HCl pH 7.6, 5mM EDTA, 40μg/ml RNaseA, 2μg/ml RNaseT1 at 30°C for 30 min. The digestion reaction was then extracted with phenol/chloroform, ethanol precipitated and the protected fragments separated on a 6% acrylamide/8M urea sequencing gel and visualized by autoradiography.

PCR amplification

cDNA prepared from total RNA of various embryonic stages and tissue culture cells was amplified by standard PCR. The sequences of the oligonucleotides (oligos) used are indicated by underlining in Figure 3. First strand cDNA was made from various RNAs using either the intron-1 oligo (Figure 3, INT-1) or the exon-2 oligo (Figure 3, EX-2). Following synthesis additional 3' oligo was added along with the 5' oligo (Figure 3, EX-1). The first strand cDNA was then amplified using 35 cycles of PCR. Amplification of DNA from an aliquot of a stage 11 (20) cDNA library also was carried out using the INT-1 or EX-2 oligos and the EX-1 oligo. A 5μl aliquot from each PCR reaction was separated on a 1.5% agarose gel and transferred to nitrocellulose. The filter was hybridized with a probe prepared from a 345 bp PvuII fragment encompassing 127 bp of exon-1 and 218 bp of intron-1 (see Figure 3), washed extensively and exposed to X-ray film.

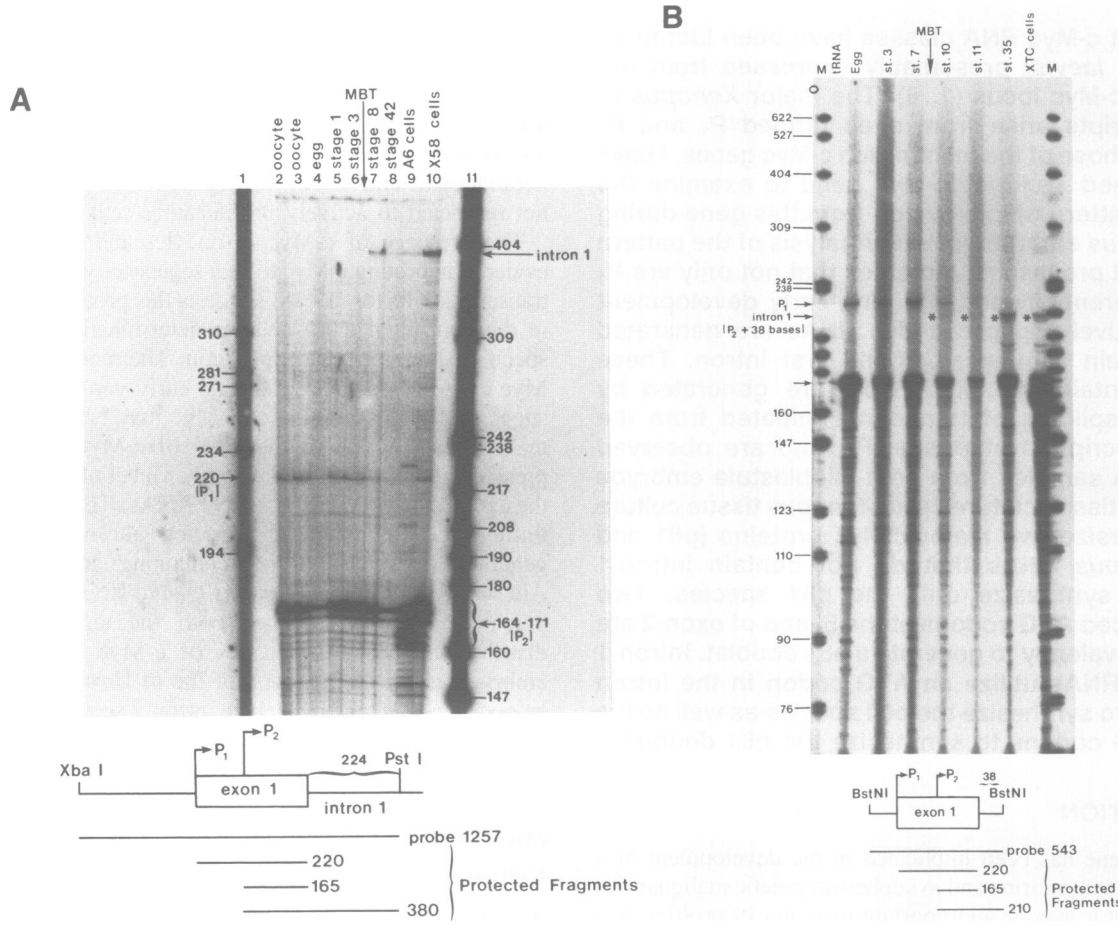

Figure 1. RNase protection analysis. ^{32}P-labeled antisense transcripts synthesized from either the 1.2 kbp XbaI/PstI fragment or the 520 bp BstNI fragment of the *Xenopus* c-Myc gene, diagrammed at the bottom of each panel of Figure 1, were annealed to 10μg of total cellular RNA and analyzed as described in METHODS. **A:** RNase protection results using the 1.2 kbp XbaI/PstI probe. Lane 1, HaeIII markers of ϕX174. The samples used for this analysis are indicated above each lane 2 through 10. Lane 11, MspI markers of pBR322. Arrows on the left and right of the Figure denote the major protected RNA fragments and their sizes. P_1 and P_2 refer to the transcriptional start sites. 'Intron 1' indicates the intron-1 containing fragment. MBT at the top refers to the midblastula transition and indicates that samples to the left are from pre-MBT stages and those to the right are from post-MBT stages. The map below the panel indicates the probe position and the positions of the protected fragments. The numbers associated with the diagram are the relative sizes of the probe, protected fragments and the amount of intron-1 sequences in the probe. The diagram is not to scale. **B:** RNase protection results using the 520 bp BstNI probe. M indicates the pBR322 MspI marker lanes, the sizes of which are indicated. The source of the RNA used is indicated above each lane. MBT indicates the same as for panel A. The arrows indicate the position of the protected fragments. P_1 and P_2 (the unlabeled arrow) are the same as for panel A. The map below the panel diagrams the position of the probe and the protected fragments as well as the amount of intron-1 sequences contained in the probe. 'Intron 1' refers to the protected fragment containing intron-1 sequences. The diagram is not to scale.

Site directed mutagenesis

Mutagenesis was performed on single stranded templates of a pBS+ construct containing *Xenopus* c-Myc cDNA clones. Reactions were carried out exactly as described by the manufacturer (Amersham, Arlington Heights, IL.).

In vitro translations, immunoprecipitations and gel electrophoresis

All *in vitro* translations and immunoprecipitations were carried out as described (6) using a peptide antibody raised against the C-terminal 13 amino acids of the predicted *Xenopus laevis c-myc* protein sequence (6). Immunoprecipitated proteins were analyzed by separation on 10% discontinuous SDS-PAGE followed by fluorography.

RESULTS

To study the developmentally regulated expression of *Xenopus* c-Myc transcripts I have isolated and characterized a *Xenopus laevis* c-Myc gene clone, the sequences of which correspond to the major c-Myc RNA present in oocytes, embryos and tissue culture cells. *Xenopus* c-Myc contains three exons (not shown) as for the mammalian (21) and avian (22) c-Myc genes. In a comparison of the gene sequences with those of my oocyte c-Myc cDNA, I found 15 nucleotide differences in the coding and non-coding regions. Comparison of my gene clone with the sequences of two different c-Myc cDNA clones (1) indicates several nucleotide differences throughout the coding regions and significant differences in the non-coding regions. Of significance is the clear non-identity between the 5′-untranslated sequences of the *mycII* clone and the *mycI* clone. The sequences reported for the *mycI* cDNA most closely resemble those of my cDNA and gene clone. The origin of these sequence differences between three independently isolated *Xenopus* c-Myc cDNAs may reflect allelic differences resulting from the non-inbred nature of *Xenopus laevis* stocks.

Analysis of c-Myc transcript processing by RNase protection

A determination of the position and number of transcriptional initiation sites, identifiable with my gene clone, and the processing of *Xenopus* c-Myc RNAs at the first intron was made by RNase protection assay. Two distinct RNase protection probes were used for this study. The 1.2kbp XbaI/PstI fragment indicated at the bottom of Figure 1A (see also Figure 3) spans the exon-1/intron-1 boundary and contains 224 bases of the 5′ end of intron-1. The 1.2 kb RNase protects a cluster of fragments, seen in all RNA samples analyzed, of approximately 165−170 bases (Figure 1A). The region of transcriptional initiation corresponding to this major cluster of fragments has been termed P_2. A consensus 'TATA-box' resides approximately 25 bp upstream of the predicted position of the P_2 start site (see Figure 3).

Two additional protected fragments, of equal abundance, approximately 220 and 380 bases were detected using the 1.2 kbp probe. These two fragments are 5−10 fold less abundant than the products corresponding to the P_2 initiation site. The level of the 220 base fragment declines in embryos following midblastula, whereas, the 380 fragment is only detectable in RNA from embryos following midblastula (Figure 1A). The site where transcription of the RNA corresponding to the 220 base fragment is predicted to begin is termed P_1 (see Figure 1A). Surprisingly, the region upstream of the P_1 site lacks classical promoter consensus sequences. The relative abundance and size of the P_1 and P_2 initiated RNAs detected by this assay are similar to the *mycI* transcripts detected by primer extension (1).

The protected RNA fragment of approximately 380 bases could correspond to a P_2 initiated transcript that includes the intron-1 sequences contained in the RNase probe (224 bases) or it could represent an RNA from a transcriptional initiation site upstream of P_1 and P_2. To address this question a second RNase probe was prepared from a cloned 520 bp BstNI fragment (see bottom Figure 1B and Figure 3). This probe encompasses 38 bases of the 5′ end of intron-1. The results of this analysis demonstrate that, like the results with the 1.2 kb probe, a small proportion of the *Xenopus* c-Myc transcripts which initiate at the P_2 site (the unlabeled arrow), following midblastula, are alternatively processed such that they contain intron-1 sequences (Figure 1B).

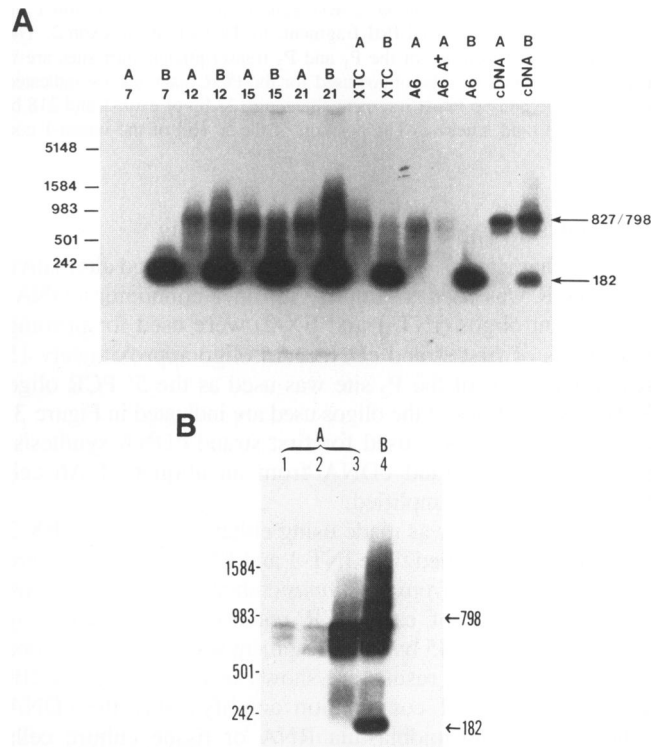

Figure 2. Southern blotting of PCR reaction products. A 10μg aliquot of total RNA (except where indicated) was used for the synthesis of first strand cDNA. The cDNA was then amplified by standard PCR. DNA was extracted from a phage stock of the stage 11 cDNA library for amplification. A 5μl aliquot of each PCR reaction was separated on a 1.5% agarose gel and transferred to nitrocellulose. The filter was hybridized with a radiolabeled probe corresponding to the 345 bp PvuII fragment indicated in Figure 3. **Panel A:** The numbers above each lane indicate the stage of embryonic RNA used to synthesize first strand cDNA. The letters 'A' and 'B' at the top of each lane refer to cDNA generated with INT-1 and EX-2 oligos, respectively. A6 and A6A$^+$ refer to total and poly(A)$^+$ RNA, respectively, isolated from *Xenopus* A6 cells. XTC refers to RNA isolated from *Xenopus* XTC cells. cDNA refers to the stage 11 cDNA library DNA samples used for amplification. **Panel B:** PCR amplification of variously treated samples of A6 cell RNA. Lanes 3 and 4 are total cellular RNA. Lane 1 is total RNA pre-treated, prior to first strand cDNA, with RNase free DNase (Promega, Corp.). Lane 2 is RNA extracted from Mg^{2+} precipitated polysomes. Lanes are marked 'A' and 'B' as for Panel A. The numbers to the left of each panel represent the migration of DNA size markers. The numbers to the right of each panel indicate the sizes of the hybridizing PCR product. The size of the intron-1 containing products generated using INT-1 as the 3′-primer are 798 bp, those using EX-2 as 3′-primer are 827 bp.

Figure 3. Sequences of the *Xenopus* c-Myc gene spanning exon-1, intron-1 and the 5′ end of exon-2. The sequences presented are from the XbaI site, representing the 5′ end of the 1.2 kbp XbaI/PstI fragment, to the PstI site in exon-2. The exon sequences are capitalized, intron and 5′ non-transcribed sequences are small letters. The relative positions of the P_1 and P_2 transcriptional start sites are indicated above the sequence. Representative restriction sites are indicated above the sequence. The positions of the oligos used for the PCR reactions are indicated by underlining with the identifying name above. The probe used to detect the PCR products was the 345 bp PvuII fragment spanning 127 bp of exon-1 and 218 bp of intron-1. The codons altered by site-directed mutagenesis in intron-1 and exon-2 are indicated in bold lettering. The position of the 5′ end of the intron-1 containing cDNA is indicated by the vertical arrow.

PCR Analysis of c-Myc transcript processing

To further characterize the developmentally regulated alternative splicing PCR was used to amplify intron-1 containing cDNA. Two different oligos (INT-1 and EX-2) were used for priming the synthesis of first strand cDNA. An oligo approximately 15 bases downstream of the P_2 site was used as the 5′ PCR oligo (EX-1). The positions of the oligos used are indicated in Figure 3. Total cellular RNA was used for first strand cDNA synthesis. Additionally, first strand cDNA from an aliquot of A6 cell poly(A)$^+$ RNA was amplified.

First strand cDNA was made using either the INT-1 or EX-2 oligos and then amplified. The INT-1 and EX-2 oligos also were used to amplify DNA from a *Xenopus* embryonic stage 11 cDNA library. An aliquot of each PCR reaction was analyzed by Southern blotting. A 345 bp PvuII fragment was used as the probe (see Figure 3). These results are shown in Figure 2A and 2B. The INT-1 and EX-1 combination amplifys only the cDNA produced from post-midblastula RNA or tissue culture cells (Figure 2A, lanes A). Pre-treatment of A6 cell RNA with RNase free DNase does not affect the presence of the intron-1 containing amplification product (Figure 2B, lane 1) indicating that the PCR product is not the result of a potential for genomic DNA contamination of the total RNA preparations. Further evidence, supporting the specificity of the amplification of first strand cDNA containing intron-1 sequences, was obtained by PCR amplification of DNA extracted from a stage 11 cDNA library. The INT-1 and EX-1 combination amplifies, as expected, a DNA fragment from the stage 11 cDNA library identical in size to the PCR products obtained from post-midblastula first strand cDNA.

The EX-2 and EX-1 combination is able to amplify cDNA exhibiting the oocyte c-Myc cDNA type splicing pattern (generating a 182 bp PCR product) from all RNA sources (Figure 2A, lanes B). The EX-2 oligo amplifies an additional fragment only from post-midblastula RNAs that is of similar size to the INT-1 PCR products (827 bp). This is expected since the EX-2 oligo would generate an intron-1 containing cDNA 29 bases longer than the INT-1 oligo (see Figure 3). The EX-2 and EX-1 combination amplifies the 827 bp intron-1 containing product from the stage 11 library DNA and the 182 bp spliced fragment.

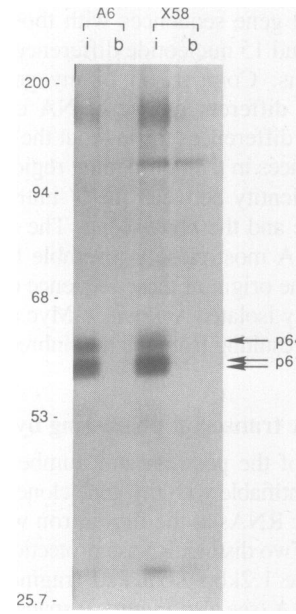

Figure 4. Immunoprecipitation of c-Myc proteins from ^{32}P-labeled A6 and X58 cells. Lanes 1 and 2, immunoprecipitations from A6 cells. Lanes 3 and 4, immunoprecipitations from X58 cells. The letter *i* represents immunoprecipitates and *b* immunoprecipitates using antibody pre-blocked by peptide. Numbers to the left of the figure represent the migration of molecular weight standards ($\times 10^{-3}$) 200=myosin heavy chain; 94=phosphorylase B; 68=BSA; 43=ovalbumin; 25.7=carbonic anhydrase. Arrows denote the positions of the p61 doublet and the p64 species.

The presence of the intron-1 containing DNA in an oligo(dT) primed cDNA library was expected since PCR amplification of first strand cDNA from A6 poly(A$^+$) RNA yielded the inton-1 product (Figure 2A). In order to determine if the intron-1 RNA exhibited a functional role in translation of c-Myc proteins RNA

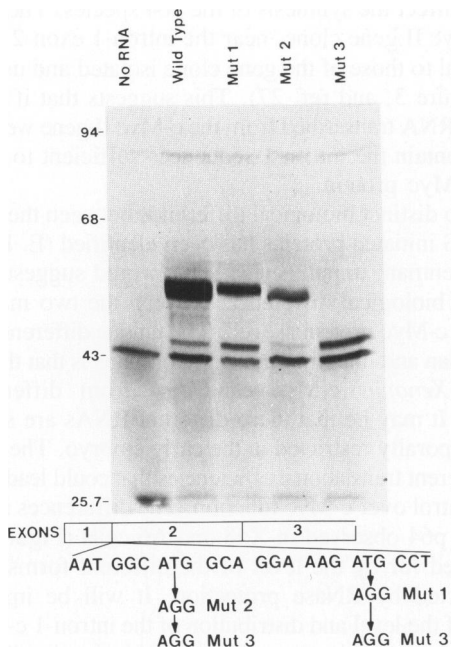

Figure 5. Analysis of *in vitro* synthesized c-Myc proteins. RNA was transcribed from the oocyte c-Myc cDNA clones. The RNA molecules were of four types. The wild type contains both 5′ exon-2 AUG codons intact. The mutants which were generated consisted of AUG conversion to AGG. Mut 1 changed the second exon-2 AUG, Mut 2 changed the first exon-2 AUG and Mut 3 changed both of these AUG codons. The *in vitro* transcripts were translated in a reticulocyte lysate and the proteins analyzed by SDS-PAGE. Lane 1, no RNA added to lysate. Lane 2, Wild type RNA. Lane 3, Mut 1 RNA. Lane 4, Mut 2 RNA. Lane 5, Mut 3 RNA. The sequences below the figure represent the region of the oocyte cDNA clone surrounding the ATG codons at the 5′ end of exon-2. Numbers to the left represent molecular weight markers as for Figure 4.

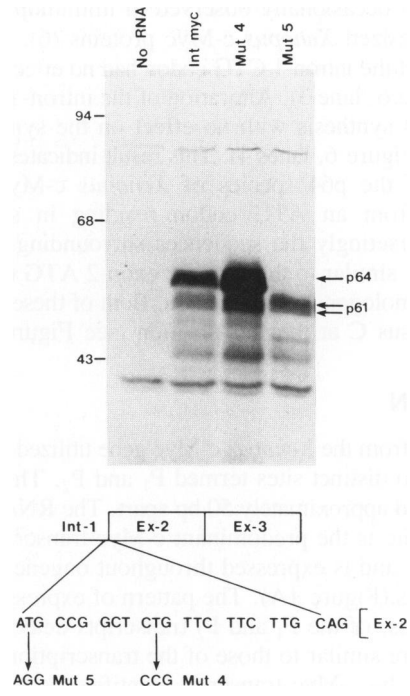

Figure 6. Analysis of *in vitro* synthesized c-Myc proteins. RNA was transcribed from the intron-1 containing cDNA clone. Two mutant clones of the intron-1 cDNA were also used. Mut 4 changed the CTG to a CCG codon and Mut 5 changed the ATG to an AGG codon. Translation products were analyzed as for Figure 5. Lane 1, no RNA. Lane 2, intron-1 RNA. Lane 3, Mut 4. Lane 4, Mut 5. Numbers to the left are markers as in Figure 5. The sequences below the Figure show the position of the mutated codons.

was isolated from polysomes, extracted from A6 cells by magnesium precipitation, and used for first strand cDNA synthesis and PCR amplified. The results of this analysis indicate that the intron-1 containing RNA species is actively translated in *Xenopus* cells.

Analysis of *Xenopus* c-Myc translation products

The primary translation products of the c-Myc genes of human, murine and avian origin are two highly related proteins which differ only by a short N-terminal extension. *Xenopus laevis* tissue culture cells also synthesized two c-Myc phosphoproteins of apparent molecular weight p61/p64 (6, and Figure 4). The human c-Myc proteins are synthesized from a single RNA species. A CUG codon is used to initiate synthesis of the p67 species and an AUG codon is used for p64 (23, 25).

Xenopus c-Myc sequences do not contain any CTG or ATG codons in exon-1 upstream of, and in-frame with, the c-Myc coding region in exon-2 and -3 (1, see Figure 3). The cDNA and gene clone sequences indicate the presence of two in frame ATG codons spaced 4 codons apart at the 5′ end of exon-2 (see bottom of Figure 5A for sequences at the 5′ end of exon-2). The sequences of intron-1 found in the alternatively spliced RNAs contain both an ATG and CTG in-frame with the exon-2 and -3 coding region.

To examine whether one or both of the exon-2 ATG codons and intron-1 ATG or CTG codons direct the synthesis of *Xenopus* c-Myc proteins, *in vitro* translation of *in vitro* synthesized RNAs was carried out. The *in vitro* synthesized proteins (Figure 5) were compared to the proteins synthesized by A6 and X58 cells (Figure 4). The antibody used in this study was raised against a peptide corresponding to the C-terminal 13 amino acids of the predicted *Xenopus* c-Myc coding region. Both of the cell lines synthesize p61 and p64 as determined by immunoprecipitation from ^{32}P-labeled cells (Figure 4). The p61 species actually comprises two closely spaced polypeptide bands while p64 is a single band.

In the reticulocyte lysate, RNA from the oocyte cDNA synthesizes only the p61 doublet species (Figure 5, lane 2), whereas, RNA from the intron-1 containing cDNA synthesizes both the p61 doublet and p64 (Figure 6, lane 2). To determine the codons utilized to synthesize these different species of c-Myc protein, site-directed mutagenesis was employed to alter the two ATG codons at the 5′ end of exon-2 and the ATG and CTG codons in intron-1. The codons altered are indicated in Figure 3 as well as the bottom of Figures 5 and 6. RNA was then synthesized from the wild type and mutant cDNAs and used to direct protein synthesis in a rabbit reticulocyte lysate system.

The two exon-2 ATG codons were altered only on the oocyte cDNA. When the second exon-2 AUG codon was mutated, *in vitro* synthesis of the lower band of the p61 doublet was no longer seen (Figure 5A, lane 3). The converse result was obtained by mutagenizing the first exon-2 AUG codon (Figure 5A, lane 4). When both of these AUG codons were altered on the same construct, the p61 doublet was not produced (Figure 5A, lane 5). The smaller protein seen migrating at approximately 48 kDa represents a protein initiated at a downstream exon-2 ATG. The

p48 species is occasionally observed in immunoprecipitates of *in vivo* synthesized *Xenopus* c-Myc proteins (6).

Alteration of the intron-1 CTG codon had no effect on synthesis of p64 (Figure 6, lane 3). Alteration of the intron-1 ATG codon abolished p64 synthesis with no effect on the synthesis of the p61 doublet (Figure 6, lanes 4). This result indicates that, at least a portion, of the p64 species of *Xenopus* c-Myc protein is synthesized from an ATG codon residing in sequences of intron-1. Intersetingly the sequences surrounding the intron-1 ATG are very similar to those of the exon-2 ATG that precedes the c-Myc homologous coding region. Both of these codons have a non-consensus C at the +4 position (see Figures 3 and 5).

DISCUSSION

Transcription from the *Xenopus* c-Myc gene utilized in this report initiates at two distinct sites termed P_1 and P_2. These initiation sites are spaced approximately 50 bp apart. The RNA transcribed from the P_2 site is the predominant c-Myc transcript identified with this gene and is expressed throughout oogenesis and early embryogenesis (Figure 1A). The pattern of expression, and the size differences, of the P_1 and P_2 transcripts detected with this c-Myc gene are similar to those of the transcriptional start sites described for the c-Myc transcript identified as *myc*I (1). The localization of the P_1 and P_2 sites identified in this report are essentially identical to those recently reported by Principaud and Spohr (27). The *myc*II transcript is substantially different in the 5′ non-translated regions when compared to this c-Myc gene clone and would not be detected with the RNase probes utilized in this study (1, 27).

Analysis of the processing of the *Xenopus* c-Myc transcripts synthesized from this gene clone has reveals a pattern of developmentally regulated alternative splicing. The alternative splicing is only observed in transcripts arising from transcription of the zygotic genome. The transcripts produced during oogenesis from the maternal genome are not alternative spliced. As would be expected, detection of the intron-1 containing *Xenopus* c-Myc RNA species is only possible when the RNase protection probes incorporate portions of the intron-1 sequences. Recent analysis of *Xenopus* c-Myc transcription patterns from the 2 c-Myc genes utilized probes that did not contain intron-1 sequences and, therefore, did not detect the alternative splicing product (27).

Xenopus c-Myc RNAs synthesized from a cDNA clone that do not contain sequences of intron-1 are capable of synthesizing only the p61 doublet species (Figure 5, lanes 3−5). However, *Xenopus* c-Myc RNAs synthesized from a cDNA clone that does contain intron-1 sequences direct the synthesis of both the p61 doublet and the p64 species. The results presented in this report indicate that the intron-1 containing RNA is capable of synthesizing both the p64 and p61 species of *Xenopus* c-Myc protein. However, the ability to utilize the exon-2 ATG codons in the intron-1 containing cDNA appears greatly diminished compared to the oocyte-type cDNA. The reduced initiation at the exon-2 ATG codons is unchanged even when the intron-1 ATG is removed (Figure 6, compare lane 4 with 2 and 3). This is in contrast to experiments done with human c-Myc in which the exon-1 CTG codon was changed to an ATG codon. In this context both exon-1 and exon-2 ATG codons initiated *in vitro* c-Myc protein synthesis equivalently (not shown).

The sequences of the second *Xenopus* c-Myc gene, c-Myc II (27), as well as those of the *mycII* cDNA (1, 27), also do not contain either ATG or CTG codons in the sequences of exon-1 that could direct the synthesis of the p64 species. The sequences of the c-Myc II gene clone, near the intron-1 exon-2 boundary, are identical to those of the gene clone isolated and used for this report (Figure 3, and ref. 27). This suggests that if alternative splicing of RNA transcribed from the c-Myc II gene were to occur it would contain the intron-1 sequences sufficient to synthesize the p64 c-Myc protein.

As yet no distinct biological difference between the human C-TG or ATG initiated proteins has been identified (E. Blackwood and R. Eisenman, unpublished). This would suggest that there may be no biological difference between the two major forms of *Xenopus* c-Myc protein as well. The unique difference between the amphibian and mammalian c-Myc proteins is that the different forms of *Xenopus* c-Myc can arise from different RNA molecules. It may be that these different RNAs are spatially as well as temporally restricted in the early embryo. They may also exhibit different translational efficiencies that could lead to another level of control over c-Myc function. The differences in the level of p61 and p64 observed in X58 and A6 cells (Figure 4) could be accounted for by the level of the different forms of c-Myc RNA detected by RNase protection. It will be important to determine if the level and distribution of the intron-1 c-Myc RNA in early *Xenopus* embryos correlates with the distribution and level of p64 c-Myc.

The observed pattern of alternative splicing of *Xenopus* c-Myc RNAs is similar to the processing of L-myc transcripts. Alternative processing of L-myc RNAs generates an intron-1 containing L-myc RNA that utilizes a CTG codon in the intron-1 sequences to direct the synthesis of a 66 kDa L-myc protein (24,26). The CTG initiated p66 form of L-myc protein exhibits transforming activity like the ATG initiated p60 species (26) suggesting that, like c-Myc, no distinct biological difference exists between the different forms of L-myc proteins.

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