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The 5′ Untranslated Region of mRNA for Ribosomal Protein S19 Is Involved in Its Translational Regulation during *Xenopus* Development

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During *Xenopus* development, the synthesis of ribosomal proteins is regulated at the translational level. To identify the region of the ribosomal protein mRNAs responsible for their typical translational behavior, we constructed a fused gene in which the upstream sequences (promoter) and the 5′ untranslated sequence (first exon) of the gene coding for *Xenopus* ribosomal protein S19 were joined to the coding portion of the procaryotic chloramphenicol acetyltransferase (CAT) gene deleted of its own 5′ untranslated region. This fused gene was introduced in vivo by microinjection into *Xenopus* fertilized eggs, and its activity was monitored during embryogenesis. By analyzing the pattern of appearance of CAT activity and the distribution of the S19-CAT mRNA between polysomes and messenger ribonucleoproteins, it was concluded that the 35-nucleotide-long 5′ untranslated region of the S19 mRNA is able to confer to the fused S19-CAT mRNA the translational behavior typical of ribosomal proteins during *Xenopus* embryo development.

Previous work has shown that the control of ribosomal protein synthesis in *Xenopus laevis* involves at least two types of regulation (for a review, see reference 2). One works at a posttranscriptional level, modulating transcript processing and stability; the other works at the translational level by changing the efficiency of utilization of the mRNAs specific for ribosomal proteins (rp-mRNA). This translational regulation is clearly observed during *Xenopus* development, when the production and accumulation of rp-mRNA are uncoupled from its utilization (3, 26, 28). In fact, rp-mRNA begins to be synthesized and accumulated at the blastula stage; at this point, many genes are transcriptionally activated at the end of cleavage, but for more than 20 h rp-mRNA is mostly kept unused as light messenger ribonucleoproteins (mRNPs). rp-mRNA is mobilized onto polysomes only at the tail bud stage, when active production of new ribosomes is required, the maternal store being used up. Moreover, translational inactivation of rp-mRNAs has been described during *Xenopus* oocyte maturation (13). We have shown that in contrast to Procaryotes, translational control of rp-mRNA is not due to autogenous regulation by ribosomal proteins (29). Similar translational controls have been shown to be involved in the regulation of ribosomal protein synthesis of other eucaryotic systems such as the mouse (7, 21), *Drosophila melanogaster* (15, 34), and *Dictyostelium discoideum* (35).

The fact that this translational behavior is specific for rp-mRNAs prompted us to look for particular sequences or structures that could differentiate this class of mRNA from others and confer to the mRNAs carrying them their typical translational behavior. At first, we were biased in favor of the 5′ untranslated region (UTR) of the mRNA. In fact, sequence comparisons had revealed the presence of some similarities in the 5′ UTRs of the mRNAs for the few *Xenopus* ribosomal proteins thus far analyzed (19). All of these sequences have quite short 5′ UTRs (35 to 50 nucleotides); they start with a run of 8 to 12 pyrimidines, contain a purine stretch, and have a G+C sequence a few nucleotides upstream of the initiation ATG codon. This fact, together with the observation that all of their genes have a first intron localized exactly at or very close to the initiation ATG codon, seems to support the hypothesis that these untranslated sequences have some important function that, not being codification, could well be regulation of translation. Similar 5′ UTRs have been described for mammalian rp-mRNAs (36).

We describe here experiments that identify the 5′ UTR of the mRNA for *Xenopus* ribosomal protein S19 as a signal responsible for translational control of the mRNA. This result was obtained by substituting this sequence for the 5′ UTR of an unrelated mRNA and reintroducing the fused gene by microinjection in *Xenopus* fertilized eggs. We show that translation of the fused mRNA, like that of rp-mRNAs, is regulated during embryogenesis.

MATERIALS AND METHODS

Construction and screening of an *X. laevis* genomic library. A genomic DNA library was constructed in the vector λEMBL4 as described previously (14). DNA extracted from erythrocytes of a single *X. laevis* adult female was partially digested with *Bam*HI and fractionated on a sucrose gradient. DNA fractions of 15 to 20 kilobase pairs were collected and ligated to the *Bam*HI-prepared arms of λEMBL4. Packaging and plaque hybridization screening were performed essentially as described by Maniatis et al. (17), using a cDNA probe specific for *Xenopus* ribosomal protein S19 that had been previously isolated and characterized (1, 5).

Subcloning and DNA sequence analysis. A restriction map of recombinant bacteriophage λXlrpS19.7 was determined, and the *Bam*HI and *Eco*RI fragments were subcloned in pSP65 according to standard methods (17). DNA sequencing was carried out as described by Maxam and Gilbert (20), with the addition of a T-specific reaction (33). Sequences were analyzed by using the IBI/Pustell package of software.

Construction of the fused S19-CAT gene. To prepare the chloramphenicol acetyltransferase (CAT) fragment, pSV2 CAT was linearized by *Hin*dIII digestion, which cuts upstream of the CAT-coding region, and then treated under

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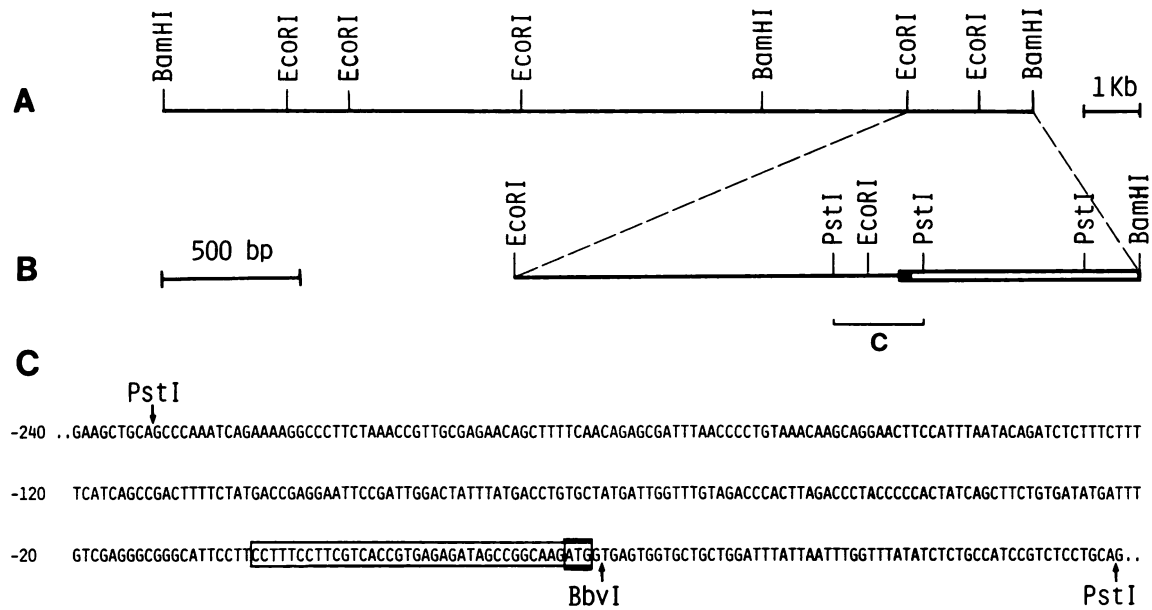

FIG. 1. Structure of the *Xenopus* genomic clone λXlrpS19.7. (A) Restriction map of the whole insert. (B) Region containing the 5′ end of the gene for ribosomal protein S19. Shown are upstream sequences (—), the first exon (■), and part of the first intron (□). (C) Nucleotide sequence of the 5′ end of the S19 gene. The boxed sequence represents the untranslated first exon, and the initiation ATG codon is indicated. The *Bbv*I restriction site used to construct the fused S19-CAT gene is also indicated.

controlled mild conditions with BAL 31 exonuclease to remove the undesired 5′ UTR; after digestion with *Bam*HI, which cuts downstream of the CAT gene, the BAL 31-*Bam*HI fragment was purified. A 290-base-pair (bp) fragment (*Pst*I-*Bbv*I) from λXlrpS19.7, containing upstream sequences to −256 and the 5′ UTR down to the initiation ATG codon (nucleotide +36) of the gene for ribosomal protein S19, was filled in with Klenow enzyme and then mixed with the CAT fragment (BAL 31-*Bam*HI) and with vector pSP65 linearized with *Bam*HI and *Pst*I; ligation was carried out first under conditions optimal for sticky ends and then under conditions optimal for blunt ends. The best clone obtained, which still contained 8 bp of the CAT 5′ UTR, was further modified by oligonucleotide mutagenesis. For this purpose, its *Pst*I-*Bam*HI insert was recloned in the single-strand-producing vector pEMBL8 (8), and the extra nucleotides were removed by oligonucleotide site-directed mutagenesis (10).

Microinjection in fertilized eggs. Eggs were artificially fertilized, microinjected within 1 h with 20 nl of DNA solution, and incubated during development as described previously (27). Staging of embryos was according to Nieuwkoop and Faber (22). For CAT assays and for Southern or Northern (RNA) blot hybridization analysis, pools of 10 to 20 embryos at the appropriate stage were collected, washed, rapidly frozen in dry ice, and stored at −70°C. For polysome analysis, the embryos were washed and used immediately.

Extraction and analysis of DNA and RNA. Each group of embryos, still frozen, was homogenized in 100 μl of 0.25 M Tris hydrochloride (pH 7.5). A 30-μl sample was rapidly withdrawn for CAT assays (see below), and 200 μl of the sodium dodecyl sulfate-containing buffer was immediately added to the remaining material for nucleic acid extraction by the proteinase K-phenol-chloroform method (30). Samples of total nucleic acids were used for DNA analysis by Southern blot, either undigested or after digestion with a restriction enzyme. Other samples of total nucleic acids were used for RNA analysis by Northern blot. For S1 mapping analysis of the 5′ end of S19-CAT mRNA, the 350-bp *Eco*RI fragment of pS19CAT, including upstream sequences and the first exon of the S19 gene and part of the CAT-coding sequence, was end labeled with T4 polynucleotide kinase and strand separated on a polyacrylamide gel. The appropriate single-stranded probe was annealed to total RNA from 15 injected embryos, treated with S1 nuclease, and analyzed by polyacrylamide gel electrophoresis as previously described (6).

CAT assays. Homogenates (see above) were spun in an Eppendorf microfuge in the cold for 10 min, and the yolk-free supernatant was stored at −70°C. Enzyme assays were performed as described previously (11) and analyzed by thin-layer chromatography.

Polysome-mRNP distribution of mRNA. Cytoplasmic extracts from 20 to 50 embryos were prepared, loaded on 15 to 50% sucrose gradients, and centrifuged in a Beckman ultracentrifuge with an SW41 rotor for 100 min at 37,000 rpm as previously described (28). Optical density at 260 nm was monitored while gradient fractions were collected; these were precipitated with 3 vol of ethanol overnight at −20°C, and RNA was extracted from precipitates with proteinase K-phenol-chloroform (30). Total RNA from gradient fractions was analyzed by Northern blot hybridization as previously described (26).

Quantification of autoradiography. X-ray films of Southern and Northern blots and of CAT assays were quantified by analysis with an LKB Ultroscan XL laser densitometer.

RESULTS

Isolation and characterization of the 5′ region of the gene for ribosomal protein S19. An *X. laevis* genomic library was constructed in the phage vector λEMBL4 and screened with a cDNA probe specific for *Xenopus* ribosomal protein S19 that had been previously isolated and characterized (1, 5). One of the positive recombinants, λXlrpS19.7 (Fig. 1A), was found to contain the first exon of the gene for ribosomal

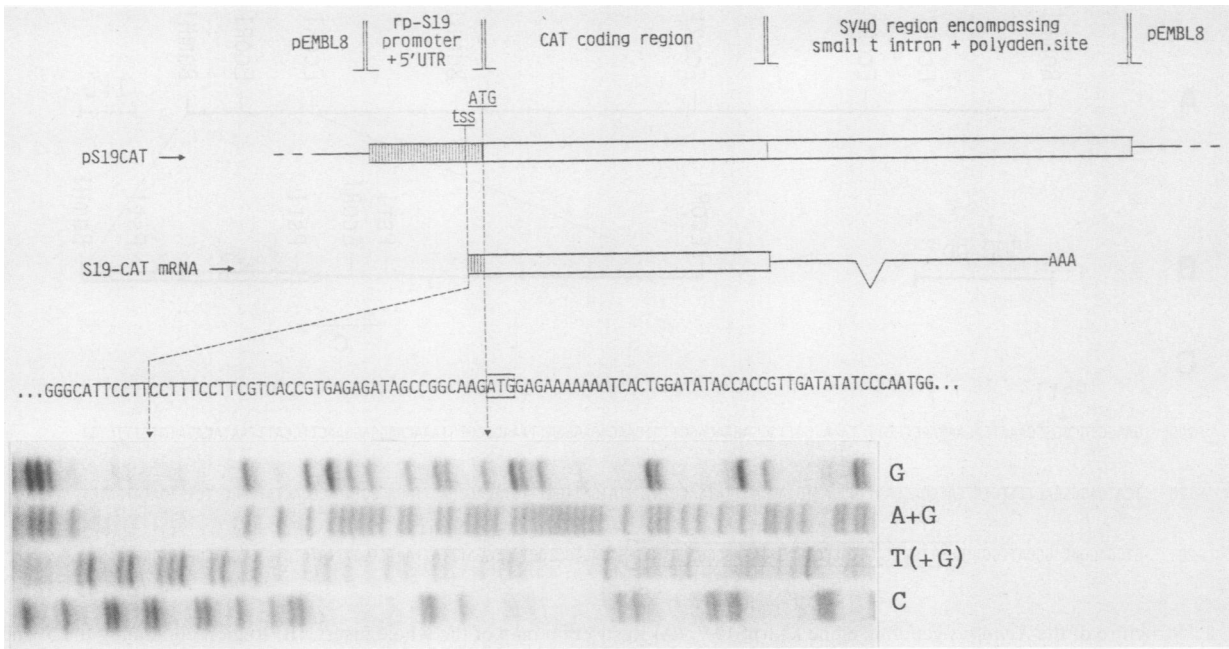

FIG. 2. Structure of the fused gene construct pS19CAT and of the corresponding mRNA. The transcription start site (tss) of S19-CAT mRNA and the translation start codon (ATG) of the CAT-coding region are indicated. The nucleotide sequence encompassing these two sites is shown. The T reaction (33) was not completely specific with respect to G.

protein S19, its upstream sequences, and part of the first intron (Fig. 1B). The nucleotide sequence of the region relevant to this work is shown in Fig. 1C. The transcription start site was identified by comparison with four independent S19 cDNA clones derived from two different full-length cDNA banks; all of them start at the same nucleotide (1; unpublished results). Moreover, comparison with the mRNAs for several different ribosomal proteins of vertebrates confirmed this identification. In fact, the 35-bp-long first exon of the S19 gene initiates in the middle of a 16-bp-long run of pyrimidines. It is preceded by a noncanonical TATA box and a G+C-rich sequence and terminates exactly after the initiation ATG codon, being followed by a typical donor consensus sequence for splicing (Fig. 1C). This gene organization is typical of ribosomal protein genes so far analyzed in *X. laevis* (4, 16) and mammals (9, 31, 36, 37).

Construction of the fused gene. A fragment of the S19 gene comprising upstream sequences to −256 and the 5′ UTR down to the initiation ATG codon was fused to the CAT gene deleted of its promoter and, in contrast to standard use of the CAT reporter gene, also of its 5′ UTR. This CAT gene, being derived from pSV2CAT, has a eucaryotic 3′ end with the polyadenylation-termination region of the simian virus 40 small-t-antigen gene (11). Construction of this fused gene was complicated by the fact that although the S19 gene has a restriction site in a good position at the end of the untranslated region, no usable site is present in or close to the corresponding position of the CAT gene. For this reason, the 5′ end was trimmed with BAL 31, and the construct was modified by oligonucleotide site-directed mutagenesis as described in Materials and Methods. The final construct, pS19CAT (Fig. 2), has exactly the desired structure, with the S19 5′ UTR and the CAT-coding sequence precisely joined at the ATG initiation codon.

Persistence during embryogenesis of the fused S19-CAT gene injected in fertilized eggs. Previous experiments in which plasmid DNA was injected into *Xenopus* fertilized eggs showed varying degrees of persistence and replication of the injected DNA during development. Different DNA molecules behave differently, as if certain sequences may increase the efficiency of replication of the plasmids; in some cases, the sizes of the molecules and their topological structures, circular or linear, are also important factors affecting persistence and amplification (for an example and for references, see reference 18). Considering this variability, and to find the best injection conditions for our purpose, we performed some preliminary experiments to determine the behavior in this respect of our plasmid pS19CAT and of pSV2CAT, to be used as control. In this study, amplification of the injected material was not required; rather, a reproducible persistence of the injected DNA for the period under study (up to stages 30 to 33) was necessary. Different amounts of the two plasmids were microinjected (0.1 to 2 ng per egg), and circular or linear forms were also tested (not shown). It was concluded that injection of 300 to 400 pg of circular DNA per embryo (Fig. 3) was appropriate. Under these conditions, plasmids pS19CAT and pSV2CAT behave similarly. They are not amplified but are quite stable, persisting during embryogenesis with a continuous slow decrease up to stages 32 to 33.

The fused S19-CAT gene is properly transcribed and translated when injected in *Xenopus* fertilized eggs. Upon injection in fertilized eggs, pS19CAT and the control pSV2CAT were both transcriptionally silent during cleavage and started to be transcribed after the blastula stage (not shown). For the purpose of this work, it was important to ensure that transcription of the injected pS19CAT would initiate at the correct transcription start site. This was verified by an S1 protection experiment (Fig. 4). It appeared that transcription started in the middle of the 12-pyrimidine stretch, as expected.

Preliminary experiments (not shown) indicated that *Xenopus* embryos (and oocytes) microinjected with pS19CAT produced CAT activity. The level of expression of the fused

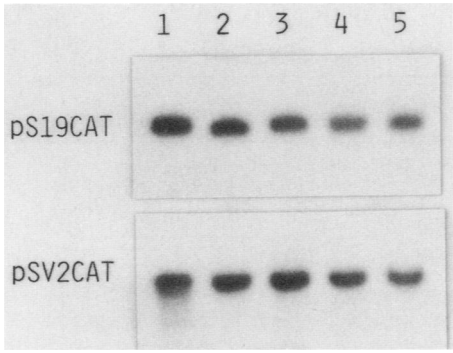

FIG. 3. Persistence in developing embryos of pS19CAT and pSV2CAT injected into fertilized eggs. A 400-pg sample of plasmid DNA was injected into each egg within 1 h of fertilization; at various developmental stages, DNA was extracted and analyzed by Southern blot hybridization using a CAT probe. Each lane was loaded with the DNA equivalent to one embryo, linearized by digestion with *Pst*I. Lanes: 1, before injection (400 pg of plasmid DNA); 2, soon after injection; 3 to 5, stages 8, 18, and 32.

gene was of approximately the same magnitude as that obtained by parallel microinjections of pSV2CAT; generally, the amount of extract corresponding to one injected embryo or oocyte was sufficient for the assay. Nevertheless, for the experiments described below we always prepared extracts from pools of at least 10 embryos to minimize the effect of injection variability.

Pattern of appearance of CAT activity during embryogenesis after injection of the fused S19-CAT gene. *Xenopus* fertilized eggs were injected with 400 pg of pS19CAT and of pSV2CAT as a control. At different stages of development,

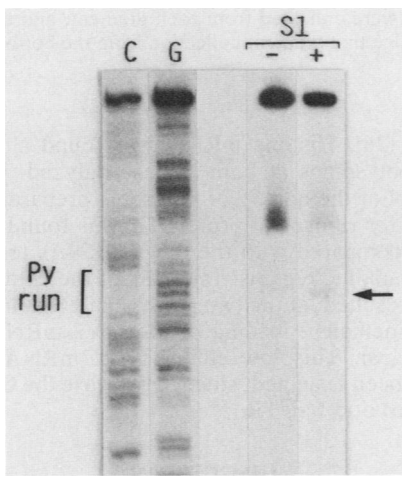

FIG. 4. S1 nuclease mapping of the 5′ end of S19-CAT mRNA. Total RNA prepared from 15 embryos at stage 30, previously injected with 400 pg of pS19CAT, was annealed to an end-labeled single-stranded probe (350 nucleotides long) encompassing the 5′ end of the fused gene and digested with S1 nuclease as described in Materials and Methods. The extracted DNA was run on a sequencing gel (+) in parallel with the untreated probe (−) and with C and G sequencing samples (20) of the same fragment. The arrow points to the protected fragment, 250 nucleotides long, whose 5′ is located in the middle of the pyrimidine (Py) run (G+A in this complementary strand). The band migrating between the probe and the protected fragment, which is present also in the S1-untreated control sample, is probably due to secondary structures.

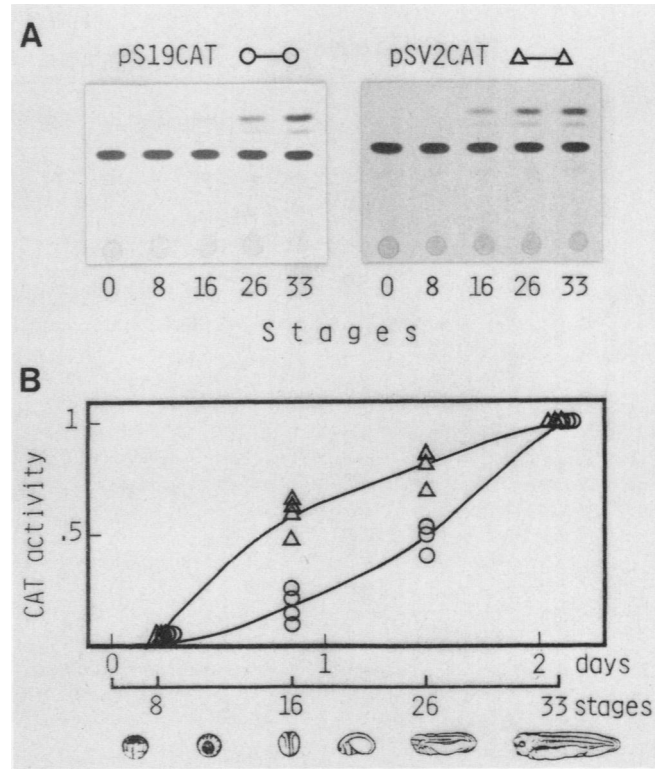

FIG. 5. CAT activity during embryogenesis after injection of pS19CAT and pSV2CAT. Fertilized eggs were injected with 400 pg of plasmid DNA, and CAT activity was analyzed on extracts prepared from embryos at different stages of development. (A) Representative results; (B) combined quantified data of several experiments, each normalized with respect to the last point.

embryos were collected, washed, and frozen. Each pool of embryos was homogenized, and a sample of the extract was withdrawn for the CAT assay. The remaining material was used for DNA (and sometimes RNA) extraction to be analyzed by Southern (and Northern) blot hybridization with a CAT-specific probe; these analysis were performed to check the persistence and transcription of injected DNA in the various samples and could be used to normalize CAT data for those samples that showed abnormally high or low persistence of the injected DNA. Figure 5A shows an example of the CAT assay; Fig. 5B graphically summarizes the results of this and other experiments. CAT activity was absent up to stage 8 (blastula), according to the known fact that a general activation of transcription occurs only at this stage. At that time CAT activity appeared in both pSV2CAT- and pS19CAT-injected embryos but reproducibly with different patterns. In embryos injected with pSV2CAT, activity increased rather quickly after the blastula stage, whereas in those injected with pS19CAT it increased rather slowly at this stage but rose later. This behavior mimics the pattern of synthesis of ribosomal proteins in normal *Xenopus* development (28) and suggests that the fused S19-CAT mRNA behaves translationally like an rp-mRNA.

Polysome-mRNP distribution of S19-CAT mRNA. The pattern of appearance of CAT activity described above suggests but does not prove that the S19-CAT mRNA is translationally controlled like a ribosomal protein. However, it seemed possible to prove this notion definitively by analyzing the distribution of the fused S19-CAT mRNA between poly-

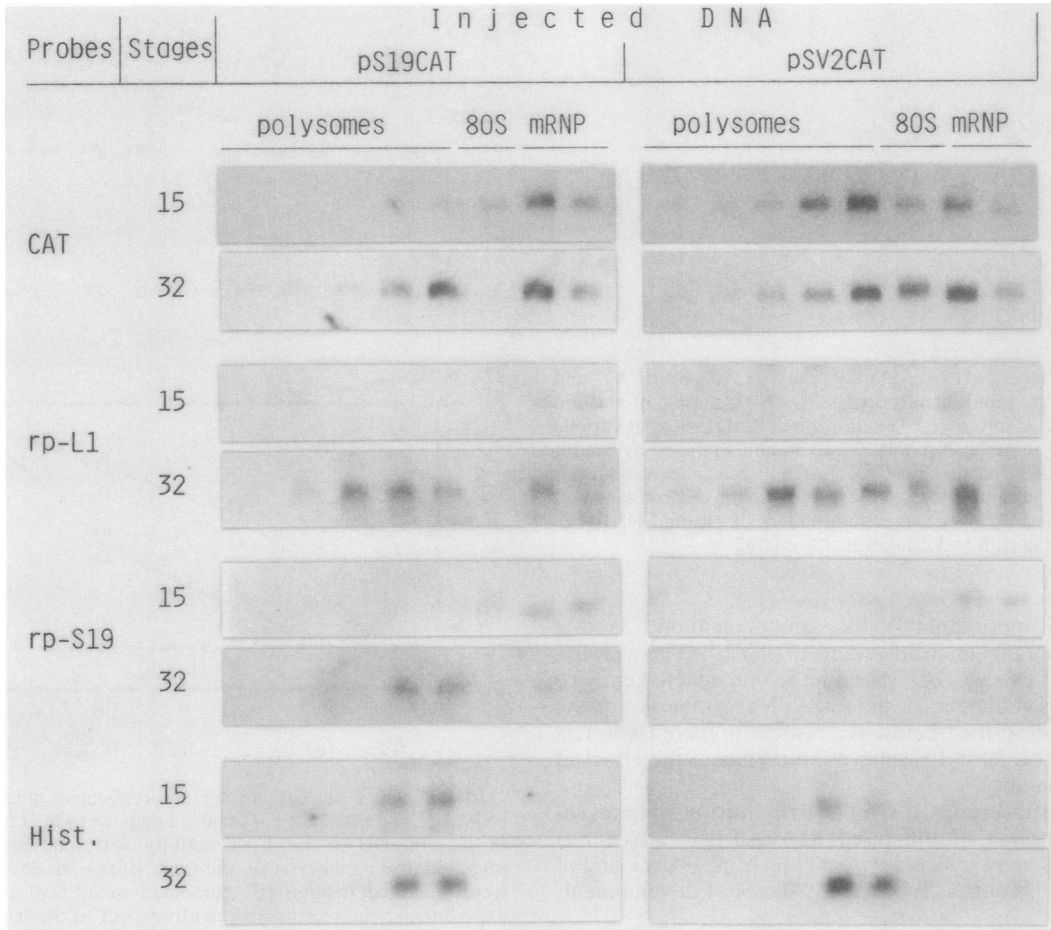

FIG. 6. Polysome-mRNP distribution of mRNAs in pS19CAT- and pSV2CAT-injected embryos. Cytoplasmic extracts from injected embryos of stages 15 and 32 were fractionated on sucrose gradients, eight fractions were collected from each gradient, and the RNA was extracted and analyzed by Northern blot hybridization with the indicated probes. Notice that gradient collection from the bottom resulted in volume variability of the last upper fraction.

somes and mRNPs in injected embryos. It is known that the translational control of rp-mRNA observed in developing *Xenopus* embryos operates by changing the distribution of this class of mRNA between polysomes and mRNPs (3, 26, 28). Groups of embryos injected with pS19CAT and groups injected with pSV2CAT were collected around stage 15, when in normal embryogenesis the majority of rp-mRNA is on mRNPs, or later at stages 32 to 33, when 60 to 80% of rp-mRNA is on polysomes. Nuclei were removed, and the cytoplasmic extracts were centrifuged on sucrose gradients. Each gradient was loaded with material corresponding to 30 embryos. Eight fractions were collected from each gradient while the optical density at 260 nm was monitored. The RNA was extracted from each fraction and analyzed by Northern blot hybridization. Figure 6 shows the results obtained with different probes: a CAT-specific sequence, ribosomal protein S19 and L1 sequences to check the endogenous rp-mRNA, and a histone probe as an unrelated control. Autoradiograms of this and another experiment were quantified by densitometry. S19-CAT mRNA recruitment on polysomes appeared to increase from about 20% to more than 50% between the two stages analyzed; this loading pattern was similar to that of mRNA for ribosomal protein S19 as well as for L1, whose recruitment increased from about 15 to 20% to 60 to 70%; these values agree with previous data for rp-mRNAs (26). Histone mRNA was found almost exclusively on polysomes at both stages analyzed, providing a good control of the quality of polysome preparations. Also, the mRNA for ribosomal protein L1 was found on heavier polysomes compared with the other mRNAs tested, which had shorter coding regions. Injection of the control plasmid pSV2CAT resulted, at the two stages analyzed, in a constant but rather inefficient loading of the CAT mRNA on polysomes (Fig. 6). This low efficiency of mRNA utilization might have been expected, since in this case the CAT mRNA had a procaryotic 5′ UTR.

DISCUSSION

Regulation of gene activity can occur at multiple steps of the transfer of genetic information from DNA to proteins. Transcriptional regulatory mechanisms have been the most widely studied during these last years, partly because differentiation genes are in general regulated at this level. Other types of controls that operate at several posttranscriptional steps have also been described; of these, translational regulation is recently attracting some attention.

In both procaryotes and eucaryotes, the synthesis of ribosomal proteins is regulated, although not exclusively, at the translational level. In *Escherichia coli*, the translation of

rp-mRNA can be blocked by some regulatory ribosomal proteins that bind to the 5′ end of their mRNAs, thus feedback regulating their own production (23). In *X. laevis*, translation has been shown to be one of the two levels at which the production of ribosomal proteins is controlled during development (the other is the processing and stability of transcripts). However, in contrast to findings for procaryotes, evidence has been obtained which rules out the possibility of a feedback action by ribosomal proteins at this level (26, 29). An observation exemplifying this translational control consists in the uncoupling of the accumulation and utilization of rp-mRNA during *Xenopus* development (3, 28). The mRNAs specific for ribosomal proteins start to be synthesized around the blastula stage, when transcription by the three RNA polymerases resumes after the transcriptionally silent cleavage period of embryogenesis, but they remain mostly sequestered in mRNPs. Only at stages 26 to 30 is a large fraction of the rp-mRNAs found on polysomes and actively translated. A translational inactivation of rp-mRNAs has been described in oocytes, in which ribosomal protein synthesis ceases upon hormone-induced maturation (13). This cessation results from the dissociation of rp-mRNA from polysomes and is accompanied by its deadenylation.

The purpose of this study was to identify the sequence or structure responsible for the translational behavior of this class of mRNAs during embryogenesis. We have fused the putative regulatory portion of the gene for ribosomal protein S19 to the coding region of a reporter gene (CAT), introduced the construct in vivo by microinjection in fertilized eggs, and analyzed the utilization of the fused mRNA in comparison with the typical translational behavior of rp-mRNA during embryogenesis.

To allow unequivocal interpretation of the results, we took care to construct a precisely designed fused gene in which the 5′ UTR of the gene for *Xenopus* ribosomal protein S19 was joined exactly at the initiation ATG codon with the coding sequence of the CAT gene deleted of its own 5′ UTR. At the 3′ end, the CAT-coding sequence was joined to a eucaryotic polyadenylation-termination signal derived from the simian virus 40 gene for small t antigen. The mRNA produced by this construct is identical to that produced by pSV2CAT, used as a control, except for the first 35 untranslated nucleotides.

Upon introduction in vivo by microinjection in fertilized eggs, both the fused S19-CAT gene and the control pSV2CAT are transcriptionally activated after the blastula stage, as also happens for many endogenous genes. CAT activity appears after this stage, but the pattern of increase is delayed in pS19CAT in comparison with pSV2CAT, resembling the pattern of appearance in the embryo of newly synthesized ribosomal proteins (28). That this delay is indeed due to regulation of mRNA utilization has been unequivocally demonstrated by analyzing the distribution between polysomes and mRNPs of the S19CAT mRNA and comparing it with the distribution of endogenous mRNAs for ribosomal and for unrelated proteins.

Thus, we can conclude that the 5′ UTR of the mRNA for ribosomal protein S19 confers to an unrelated mRNA a translational regulation property similar to that of rp-mRNA during *Xenopus* embryogenesis. The structural similarity of this 5′ UTR to those of other rp-mRNAs (19) suggests that our conclusion is of more general validity for the translationally controlled rp-mRNAs. This 5′ UTR can thus be viewed, by analogy with transcriptionally regulated systems, as a *cis*-acting element for the regulation of mRNA utilization, as has already been demonstrated for a few other unrelated mRNAs such as ferritin (12), c-*myc* (24), and poliovirus (25) mRNAs.

At present, we are not in a position to formalize a model describing the translational regulation of ribosomal protein synthesis at the molecular level. However, besides the involvement demonstrated here of the 5′ UTR of rp-mRNA, other significant clues are available.

(i) The distribution pattern of rp-mRNA along polysome gradients is clearly bimodal, with the recruited fraction of rp-mRNA fully loaded with ribosomes, as observed in mammals (21) and *X. laevis* (26). Thus, the rp-mRNAs are subject to a particular form of translational control in which they are either translationally inactive or fully active. In other words, each mRNA molecule either is or is not available for translation, rather than its utilization being regulated by modulating the efficiency of an initiation step, which would result in a shift of an rp-mRNA on more or less heavy polysomes.

(ii) In any given situation, the percentage, rather than the amount, of rp-mRNA to be loaded onto polysomes appears to be regulated; also, when the amount of mRNA for ribosomal protein L1 was increased 10-fold by microinjection of the L1 gene in embryos, it was distributed between polysomes and mRNPs in the same relative proportion observed in control embryos of the same stage (27). A possible explanation is that the utilization efficiency of rp-mRNA is dictated directly by some affinity rule not involving any specific positive or negative factor. Alternatively, a regulatory factor exists that affects positively or negatively the availability of rp-mRNA for translation and is present in very large excess with respect to the L1 mRNA, suggesting that it is shared with other similarly regulated rp-mRNAs. Interestingly, studies on the translational control of human ferritin expression have revealed a similar mRNA dosage independence of the proportion of the mRNA that is translated (32).

(iii) Finally, this translational regulation of rp-mRNA appears to respond to the needs of the cell for new ribosomes as if affected directly or indirectly by the amount of available unused ribosomes; a control of this type, which would provide a regulatory loop highly significant for the physiology of the cell, was at first suggested on the basis of some circumstantial observations (26) and has been now tested experimentally (P. Pierandrei-Amaldi et al., submitted for publication).

Although difficult to reconcile, all of these facts provide important clues for future attempts to understand the molecular mechanisms underlying the translational control of ribosomal protein synthesis in *Xenopus* development. The fused S19-CAT gene can now be used for precise identification, by site-directed mutagenesis of the 5′ UTR, of the sequence element responsible of translational control. Measurements of CAT activity at stages 15 and 30 in embryos injected with the mutated plasmids can provide a relatively easy assay. One can also examine possible interactions of this 5′ UTR sequence with other components (factors, ribosomes, etc.) that might have a regulatory role in the synthesis of ribosomal proteins.

ACKNOWLEDGMENTS

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