of G_q with receptors and the development of reagents that specifically block the action of these proteins in membranes and whole cells.

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Translational Potentiation of Messenger RNA with Secondary Structure in *Xenopus*

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Differential translation of messenger RNA (mRNA) with stable secondary structure in the 5′ untranslated leader may contribute to the dramatic changes in protein synthetic patterns that occur during oogenesis and early development. Plasmids that contained the bacterial gene chloramphenicol acetyltransferase and which encoded mRNA with (hpCAT) or without (CAT) a stable hairpin secondary structure in the 5′ noncoding region were transcribed in vitro, and the resulting mRNAs were injected into *Xenopus* oocytes, eggs, and early embryos. During early oogenesis, hpCAT mRNA was translated at less than 3 percent of the efficiency of CAT mRNA. The relative translational potential of hpCAT reached 100 percent in the newly fertilized egg and returned to approximately 3 percent after the midblastula transition.

DURING OOGENESIS, THE EGGS OF many animal species accumulate large pools of maternal polyadenylated [poly(A)$^+$] RNA, of which only a variable fraction is recruited onto polysomes (*1*). Although β-actin mRNA is uniformly translated with up to 90% efficiency, the efficiency of translation of core histone, c-*mos*, and certain other mRNAs increases at the time of oocyte maturation (*2*). In addition, during the period of transcriptional quiescence that extends in many species from the time of oocyte maturation to the completion of the cleavage divisions of early embryogenesis, maternal mRNAs such as those encoding histone H1 and fibronectin become translationally activated (*3*). The mechanism by which differential translation of these various mRNAs is achieved remains obscure. It has been speculated that some maternal RNA molecules may be "masked" by binding to cytoplasmic proteins, which effectively removes them from the pool of translationally competent molecules (*4*)—although the nature of such "masking" proteins is not well understood. Alternatively, poly(A)$^+$ RNAs that contain repetitive sequences may hybridize with one another and form complex networks of intermolecular secondary structures that could interfere with oocytic and zygotic translation; the cytoplasmic polyadenylation of certain maternal mRNAs may also be required for their activated translation during oocyte maturation (*5*). We now report that intramolecular mRNA secondary structure can also be important in the regulation of translational activity during this critical phase of development.

A number of mRNA molecules from both eukaryotic viral and cellular sources contain stable palindromic sequences that may form hairpin or stem-loop secondary structures, usually in their 5′ or 3′ untranslated regions. These secondary structures may serve as binding sites for regulatory proteins, such as occurs in the control of ferritin, poliovirus, and human immunodeficiency virus–1 (HIV-1) mRNA translation (*6*), or they may enhance the stability of mRNAs, as is the case with mRNAs that encode histones (*7*), by reducing nuclease digestion. To determine whether such secondary structures may participate in posttranscriptional regulatory mechanisms during oogenesis and

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early development of *Xenopus*, we inserted a highly stable, noncoding palindromic sequence upstream of the AUG translation initiation codon of a bacterial chloramphenicol acetyltransferase reporter gene (Fig. 1). Linearized plasmids were transcribed, capped, and polyadenylated in vitro (*8*) and used to synthesize either unmodified (CAT) transcripts or transcripts with a 25–base pair (bp) hairpin structure in the 5′ untranslated region (hpCAT).

CAT and hpCAT transcripts were microinjected into the animal pole cytoplasm of oocytes, eggs, and embryos of *Xenopus laevis*.

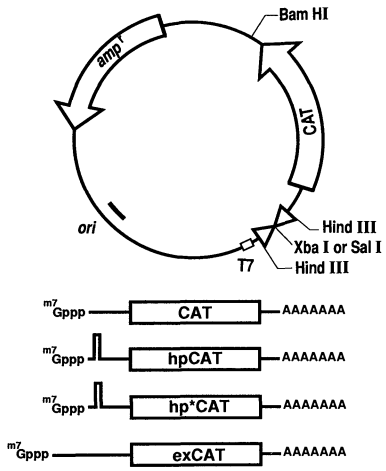

Fig. 1. Construction of the hpCAT plasmid. The CAT gene was obtained by digestion of pSV2-CAT (*9*) with Hind III and Sau 3A and was inserted into pGEM-4 (Promega). The resulting plasmid was linearized and transcribed in vitro (*8*) from the T7 promoter (box) to generate 5′-capped CAT transcripts that lack hairpin structures. Polyadenylation reactions were performed separately (*8*). The hpCAT plasmid was generated by digesting the pUC12 plasmid (*28*) with Hind III and Xba I, to release a portion of the multiple cloning sequence, and religating the resulting fragments. The ligation products were digested with Hind III to generate an inverted nucleotide sequence from Hind III to Xba I to Hind III (50 bp total). This inverted sequence (opposed arrowheads) was then inserted into the CAT plasmid at the Hind III site (*29*). The structure of the resulting hpCAT transcript after in vitro synthesis is shown below the plasmid. The 25-bp hairpin structure has a GC content of 60% and is located 6 nucleotides downstream of the mRNA cap site and 30 nucleotides upstream of the AUG codon. A second hairpin sequence, generated from the Hind III–Sal I fragment of the multiple cloning sequence of pGEM-9Zf(−) (Promega), was also inserted into the Hind III site of the CAT plasmid and used to make hp*CAT transcripts. The 28-bp hairpin has a GC content of 46% and the predicted free-energy change ($\Delta G°$) for the unfolding of each hairpin is −70 kcal/mol (*30*). A 55-bp nonpalindromic multiple cloning sequence from the Hind III to Eco RI sites of pUC12 was placed upstream of the CAT gene and used to make CAT transcripts with a 5′ extension (exCAT). Abbreviations: *ori*, origin of replication; *amp*^{r}, gene conferring ampicillin resistance.

Cytoplasmic extracts were prepared 2 hours after injection and assayed for CAT enzyme activity (*9*). At each of these developmental stages, enzyme activity was uniformly high when samples were microinjected with control CAT mRNA. Samples injected with hpCAT mRNA, however, showed large variations in enzyme activity at the various developmental stages (Fig. 2A). Enzyme activity in hpCAT-injected samples was <3% of control values in stage 4 oocytes and increased to 100% of control values in fertilized eggs and in cleaving embryos up to at least the eight-cell stage (Fig. 2B). To analyze the activity of the microinjected hpCAT mRNA at later developmental stages (when embryos contain many cells and cannot be uniformly microinjected), we used coenocytic embryos, in which the cleavage divisions are artificially suppressed (*10*). Enzyme activity recovered from coenocytic embryos injected with hpCAT mRNA declined again to 3% of control values soon after the midblastula transition.

To establish whether the variations in enzyme activity were specific to the secondary structure present on hpCAT mRNA, we constructed two additional plasmids (Fig. 1) that encoded either CAT mRNA with a different hairpin sequence (hp*CAT) or CAT mRNA with a 5′ extended sequence (exCAT) of similar length but without secondary structure. We found no difference in enzyme activity when samples were injected with CAT or exCAT mRNA, but samples injected with hp*CAT showed changes in activity among the developmental stages that were essentially identical to those observed with hpCAT mRNA (Fig. 2C). The variations in enzyme activity were therefore associated neither with a simple increase in length of the 5′ untranslated leader sequence nor with a particular sequence within the hairpin, but rather depended on the presence or absence of a stable hairpin structure within that domain.

It was possible that the variable expression of CAT enzyme activity arose from differential stability of the CAT mRNA with or without the hairpin; for example, a double strand–specific ribonuclease could preferentially attack the hpCAT mRNA in early stage oocytes or late blastula embryos, thereby preventing its translation. To test this, we injected CAT or hpCAT mRNA at the various developmental stages, recovered total RNA either immediately or 1 to 4 hours after injection, and analyzed the recovered RNA by Northern blot hybridization to a ^{32}P-labeled antisense CAT probe. No evidence of enhanced instability of hpCAT mRNA at any developmental stage was found (Fig. 3). Thus, the differences in the amounts of CAT enzyme activity expressed from CAT mRNA with and without the hairpin structure could not be caused by differential mRNA stability and therefore must be caused instead by developmentally regulated translation of the modified RNA structure.

According to the "scanning" model for eukaryotic ribosomal activity (*11*), the 40*S* ribosomal subunit and initiation factors (eIFs) first attach at the 5′ cap site of the mRNA and then progress in the 3′ direction until an AUG codon is encountered, at which point assembly of the 80*S* ribosome is completed and translation begins. An assumption of the scanning model is that the ribosomal precursor migrates through and unwinds any secondary structure it encounters before initiating protein synthesis. Evidence has suggested that the mature 80*S* ribosomal complex can unwind extremely stable hairpins [free-energy change ($\Delta G°$) > −70 kcal/mol] located downstream of the AUG codon (*12*). In the hpCAT mRNA, however, the secondary structure is located upstream of the AUG codon, and the activity that unwinds the hairpin therefore cannot be due to a mature 80*S* ribosomal complex. Alternatively, it is possible that the developmentally regulated increase in translation of hpCAT is associated either with internal attachment and assembly of the 80*S* ribosomal complex (that is, downstream of the hairpin), as occurs with certain uncapped viral RNAs (*13*), or with a "jump" by the ribosome across the hairpin structure, as occurs with bacteriophage T4 gene *60* mRNA (*14*). Whereas either mechanism could obviate the need for movement of the ribosomal precursor through the hairpin, there is no evidence at present that these are common translational events in eukaryotic cells. We favor instead an increased abundance or activity of a factor that would potentiate translation during these early developmental stages by unwinding the intrastrand duplex regions in hpCAT mRNA and allowing the unimpeded progression of the ribosomal precursor from the 5′ cap site to the initiation codon.

Likely candidates for this translational potentiator include members of a diverse family of RNA helicases (*15*) that were first identified in mammalian cells as eukaryotic eIFs. Members of this family behave as RNA-dependent adenosine triphosphatases (ATPases) and unwind mRNA secondary structures in either direction (*16*). The enzyme responsible for the recently identified *Xenopus* double-stranded RNA unwinding-modification activity (*17*) might function as an RNA helicase, although its normal role is presently unknown. The *Xenopus* enzyme (i) can unwind sense-antisense intermolecular RNA duplexes; (ii) deaminates adenosine to

inosine, thereby destabilizing or preventing the reformation of secondary structure; and (iii) is found in the nuclei of oocytes and somatic cells and in the cytoplasm of eggs and embryos (*18*). Although the molecule responsible for the activity detected in our experiments appears to differ from the unwinding-modification enzyme in that it can unwind, or permit ribosomes to bypass, intramolecular secondary structure and is detected in the cytoplasm of stages 5 and 6 oocytes, we cannot exclude a role for the unwinding-modification enzyme in this process. If this enzyme were to act on the hairpin in the 5′ untranslated region of hpCAT mRNA, the modification of adenosines to inosines might permanently destabilize the secondary structure of the mRNA and thereby explain its increased translational potential.

Variable helicase and translational activities may be important in the regulation of cell behavior. For example, NIH 3T3 cells that overproduce eIF-4E display a transformed phenotype (*19*), possibly because of an increased translational potential of proto-oncogene transcripts. Oncogenic viral products such as the large T antigen of simian virus 40 also show RNA helicase activity (*20*), and transcripts of a germ cell–specific RNA helicase have been detected during mouse spermatogenesis (*21*). Products of the maternally transcribed *Drosophila* gene *vasa* are also candidate RNA helicases. The *vasa* gene product is apparently required for normal development, because mutants for *vasa* are sterile and lack embryonic pole cells and polar granules (*22*). These results suggest that unique RNA helicases are both present and necessary during certain critical periods of gametogenesis, early development, and tumorigenic progression.

Two-dimensional gel electrophoretic analyses have revealed that several proteins are newly synthesized between stages 4 and 6 of oogenesis in *Xenopus*, and the rate of synthesis of many more increases during this period (*4*)—observations that are consistent with translational regulation of some mRNAs. Although most of the proteins that behave in this way remain unidentified, the synthesis of the proto-oncogene product c-Myc shows precise posttranscriptional regulation in that the protein is synthesized later in oogenesis than is the mRNA (*23*); the kinetics of accumulation of Myc protein correspond closely to those we observe for the translational potential of hpCAT mRNA. Although the *Xenopus* c-*myc* genes are not yet fully characterized, they encode several distinct mRNAs with 5′ untranslated regions that vary from 111 to 361 bases in length (*24*). As with the corresponding rodent and human c-*myc* genes, multiple transcription initiation sites are used to generate transcripts with the potential to form extensive 5′ secondary structures (*25*) that may interfere with translational activity. For example, when the 5′ untranslated domain of the murine c-*myc* gene is fused to the CAT gene and the resulting hybrid transcripts are microinjected into *Xenopus* oocytes, translational efficiency is approximately one-fortieth of that of CAT RNA lacking the 5′ domain (*26*). Cytoplasmic proteins that bind specifically to the 5′ region of c-*myc* mRNA (*27*) may contribute to the control of c-*myc* expression. However, the translational potentiator observed here, which appears to lack sequence specificity of action (Fig. 2), may also be important in this posttranscriptional regulation. That is, the natural targets for the activity of the potentiator may be molecules with extensive 5′

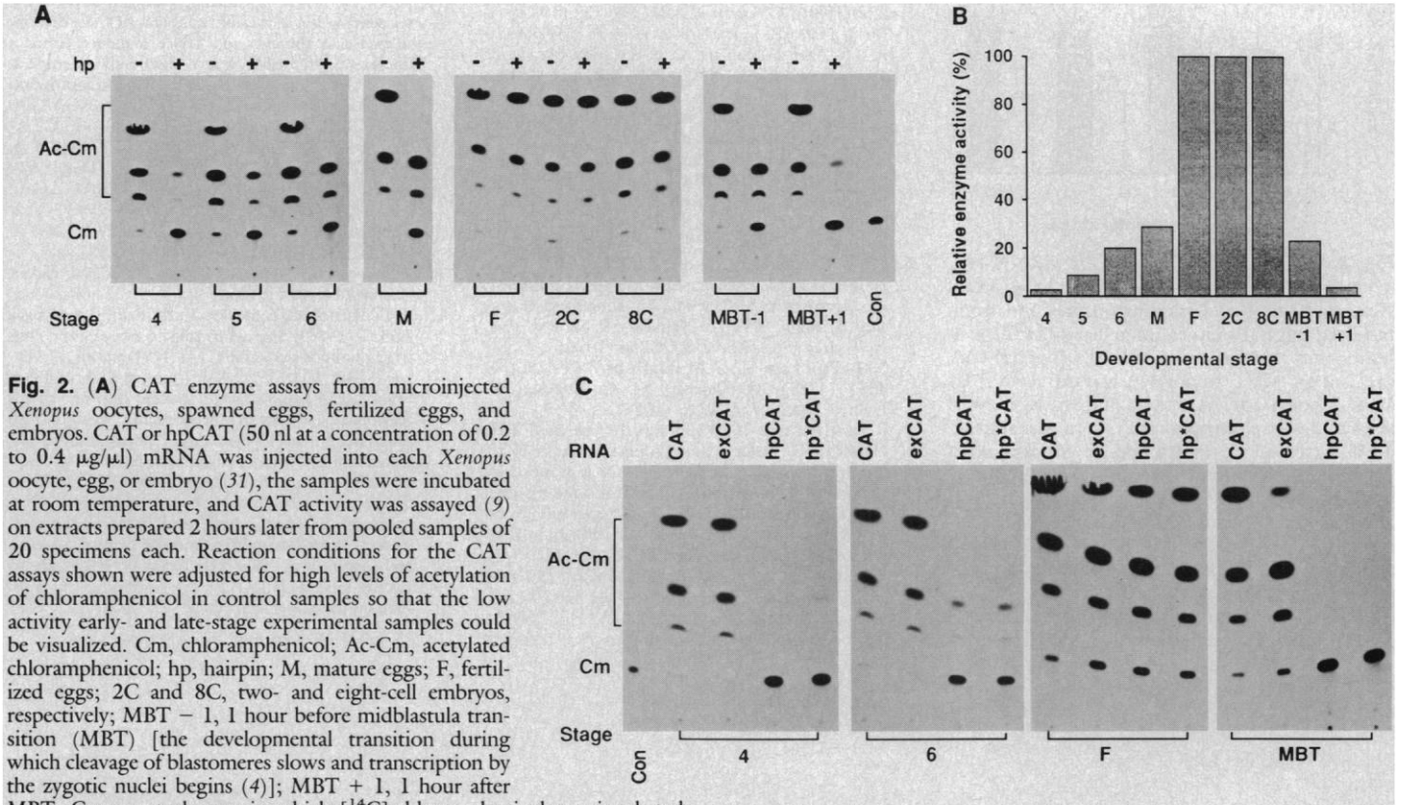

Fig. 2. (**A**) CAT enzyme assays from microinjected *Xenopus* oocytes, spawned eggs, fertilized eggs, and embryos. CAT or hpCAT (50 nl at a concentration of 0.2 to 0.4 μg/μl) mRNA was injected into each *Xenopus* oocyte, egg, or embryo (*31*), the samples were incubated at room temperature, and CAT activity was assayed (*9*) on extracts prepared 2 hours later from pooled samples of 20 specimens each. Reaction conditions for the CAT assays shown were adjusted for high levels of acetylation of chloramphenicol in control samples so that the low activity early- and late-stage experimental samples could be visualized. Cm, chloramphenicol; Ac-Cm, acetylated chloramphenicol; hp, hairpin; M, mature eggs; F, fertilized eggs; 2C and 8C, two- and eight-cell embryos, respectively; MBT − 1, 1 hour before midblastula transition (MBT) [the developmental transition during which cleavage of blastomeres slows and transcription by the zygotic nuclei begins (*4*)]; MBT + 1, 1 hour after MBT; Con, control assay in which [^{14}C]-chloramphenicol was incubated with uninjected *Xenopus* extract. (**B**) Quantitation of hpCAT mRNA translation. Autoradiograms were scanned with an LKB Ultroscan XL densitometer, and CAT enzyme activities in hpCAT mRNA–injected samples were calculated as percentages of activities in control CAT mRNA injected samples, with correction for substrate depletion. In repeated experiments, the relative translational efficiency of hpCAT mRNA in stage 4 oocytes and in post-MBT embryos varied from 1 to 3%. (**C**) Specificity of translational potentiation. Stage 4 or stage 6 oocytes, fertilized eggs, and embryos at the MBT were injected with CAT, exCAT, hpCAT, or hp*CAT mRNA, and the resulting CAT enzyme activity was assayed as in (A). CAT and exCAT mRNAs were translated with equal efficiency at each developmental stage, whereas both hpCAT and hp*CAT mRNAs were inhibited in translation at early and late developmental stages and fully translated at the time of fertilization.

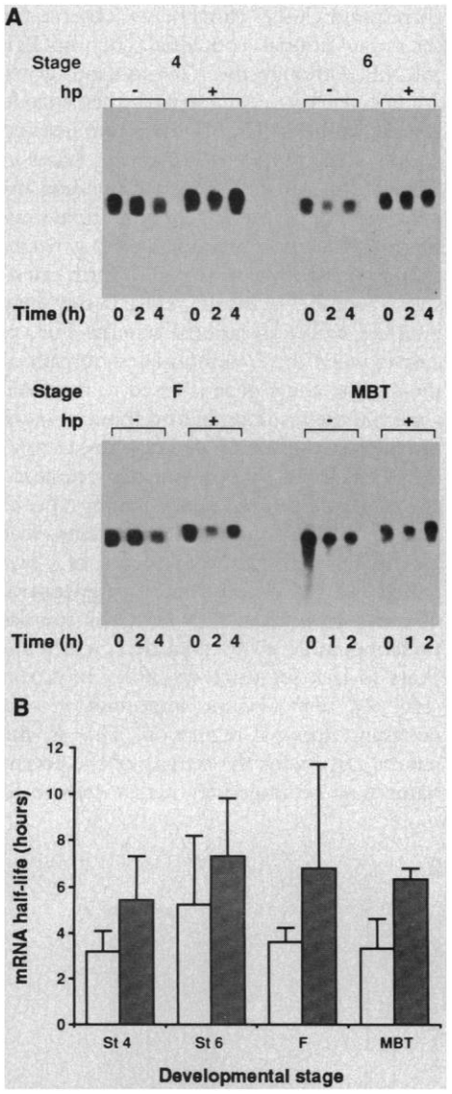

Fig. 3. Stability of CAT and hpCAT mRNA. (**A**) Total RNA was extracted as described (*32*) from *Xenopus* stage 4 and stage 6 oocytes and from fertilized eggs (F) either immediately or 2 or 4 hours after microinjection. With coenocytic embryos at the MBT, total RNA was extracted at 0, 1, or 2 hours after injection. Purified RNA was separated by electrophoresis on a denaturing 1.5% agarose gel, transferred to nitrocellulose paper, and hybridized to a ^{32}P-labeled antisense CAT mRNA probe at 42°C; hp, hairpin. (**B**) Autoradiograms were quantitated by densitometric scanning, and the half-life of CAT (open bars) and hpCAT (solid bars) mRNA at each developmental stage was calculated with an assumption of exponential decay kinetics. Bars show the mean ±SD values for three measurements in each case.

secondary structure, such as c-*myc* mRNA, and the potentiator may thereby permit the accelerated accumulation of translation product in a developmentally controlled manner.

Oocytes of *Xenopus* and many other species are rich in masked mRNAs, the controlled translation of which is likely to be of critical importance to oogenesis and early development. It is possible that differential recruitment of transcripts with 5′ secondary structure by a developmentally regulated translational potentiator may be an important component in this activation (*19*). Furthermore, the efficient translation in somatic cells of c-*myc* and other transcripts with extensive 5′ secondary structure may require the activation of a translational potentiator during the stimulation of cell proliferation (*18*). If such a potentiator were activated inappropriately or in excess in somatic cells, it could lead to the increased translation of transcripts such as c-*myc*, thereby contributing to oncogenic progression (*25*).

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