

Translation of mRNA Injected into *Xenopus* Oocytes Is Specifically Inhibited by Antisense RNA

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ABSTRACT The bacteriophage SP6 promoter and RNA polymerase were used to synthesize sense and antisense RNAs coding for the enzymes thymidine kinase (TK) and chloramphenicol acetyl transferase (CAT). Injection of antisense CAT RNA into frog oocytes inhibited expression of sense CAT mRNA. Similarly, antisense TK RNA inhibited expression of sense TK mRNA. Antisense RNAs were stable in oocytes and had no detectable effect on either the expression of endogenous proteins or on the expression of nonhomologous RNA transcripts. CAT activity expressed from a plasmid transcribed in the oocyte nucleus was also inhibited by antisense RNA injected into the oocyte cytoplasm. The data suggest that antisense RNA will be useful in identifying the function of specific mRNA sequences during early development of the frog.

We are trying to identify and characterize genes that participate in normal embryonic development in the frog *Xenopus laevis*. An informative method of studying such genes would be to inhibit their expression in vivo to directly identify and assess the biological functions to which the genes contribute. While *Xenopus* embryos are a favorable system for experimental manipulations and biochemical analyses, the organism is not readily available to classical genetic analysis. One other way that genes may be inhibited is by the introduction of RNA complementary in sequence to the normal message (7). Hybridization between such an antisense RNA and the normal message may prevent translation or processing. It was previously shown (7, 8) that the expression of exogenous genes as well as endogenous cellular genes can be dramatically and specifically inhibited when DNA molecules that express antisense RNA have been introduced into tissue culture cells.

Injection of plasmid DNA directing the production of antisense RNA into *Xenopus* fertilized eggs is possible, however a high concentration of injected DNA is toxic to embryos (4, 19), and the available expression plasmids may not produce large enough amounts of RNA quickly enough to inhibit genes turned on in the rapidly dividing embryo. In contrast, injected RNA is not as toxic and, at least in the case of globin RNA, is very stable in embryos (5).

Recently it has become possible to synthesize large amounts of a specific RNA from a plasmid containing a segment of cloned DNA downstream from the SP6 promoter (3, 14). Using this technology, we have synthesized sense mRNAs coding for the bacterial enzyme chloramphenicol acetyl trans-

ferase (CAT)¹ and herpes simplex virus thymidine kinase (TK), as well as their antisense counterparts. We have first injected antisense, then sense mRNA into oocytes of *Xenopus*, and then extracted proteins for enzyme assay. Using these methods and quantitative measurements of sense and antisense RNA half-lives, we have determined (a) the extent to which expression of these sense mRNAs is inhibited at different concentrations of antisense RNA, and (b) whether an antisense RNA covering the full length of the protein-coding region of mRNA is necessary to effect maximal inhibition. Further, to address the question of whether antisense RNA can inhibit genes transcribed in the cell nucleus, we injected antisense CAT RNA into the oocyte cytoplasm and then tested the expression of a plasmid DNA coding for CAT injected into the oocyte nucleus. Our data complement and support the findings of Melton who has independently obtained similar results using *Xenopus* globin mRNA (13), and the results of Preiss et al. (16) who have shown that antisense RNA complementary to Krüppel mRNA can phenocopy a Krüppel mutation in *Drosophila*.

MATERIALS AND METHODS

Enzymes and Chemicals

SP6 polymerase, radioactive nucleotides, and [¹⁴C]chloramphenicol were obtained from New England Nuclear (Boston, MA); RNase inhibitor, from

¹ Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; TK, thymidine kinase.

Promega Biotec (Madison, WI); RNase-free DNase I and guanylyl transferase, from Bethesda Research Lab. (Bethesda, MD), and, nucleotides and acetyl CoA, from P L Biochemicals Inc. (Milwaukee, WI).

Plasmids

CAT PLASMIDS: CAT coding sequences were excised from pSV2CAT (2) by digestion with Hind III and Sau3A; the ends were filled in with reverse transcriptase and the blunt-ended fragment inserted into the SmaI site of pSP65 (14) to generate pSP65 CAT S (sense) or pSP65 CAT A (anti-sense). Similarly, these sequences were inserted into the SmaI site of a DNA expression vector, employing the murine sarcoma virus LTR promoter and SV40 late polyadenylation sequences.

The DNA expression vector (EMSV33) was constructed from a 420-bp Hind III-Bgl II fragment of MSV LTR DNA generously provided by B. Graves, Fred Hutchinson Cancer Research Center (deletion mutant B14) and the 237-bp Bam HI to Bcl I fragment of SV40. Both these fragments were first inserted into the polylinker of phage Mp11, then excised using Hind III and Eco RI endonucleases. Religation of the two fragments at their Eco RI sites thus results in an expression DNA fragment containing Sma I, Eco RI, and Bam HI sites for insertion of DNA. The entire fragment is flanked by Hind III sites.

TK PLASMIDS: A sense plasmid was made by digesting the TK 5' deletion mutant $\Delta 5' 0.67$ (12) with Hind III and Eco RI and inserting the coding sequences into Hind III/Eco RI digested pSP64. An antisense TK plasmid was made by digesting the TK 3' deletion mutant $\Delta 3' 1.32$ (12) with Hind III and Eco RI and inserting the coding sequences into Hind III/Eco RI digested pSP64.

Synthesis of SP6 Transcripts

Transcripts were synthesized as described by Green et al. (3). Transcripts were capped with guanylyl transferase as described (3) except that 30 μ M GTP was included in the reaction. Alternatively, RNAs were capped by transcription in the presence of 2.5 mM G(5')ppp(5')G (fivefold excess over GTP) (9). The reactions were maintained at 40°C before and after addition of GpppG and DNA to prevent the precipitation of these components. The biological effectiveness of the latter capping protocol was treated by monitoring the stability and translational efficiency of the synthetic capped RNA. In both respects capping with GpppG was more effective in our experiments; in addition, it involved fewer steps. The amount of transcript made was monitored by incorporation of α -[³²P]GTP or α -[³²P]UTP. Both radioactive and nonradioactive nucleotides were routinely checked by polyethyleneimine-cellulose chromatography (17). For transcription, the SP65 CAT plasmids were linearized with Bam HI, and the SP64 TK plasmids with Eco RI (sense TK) or Pvu II (antisense TK).

Microinjection into Oocytes

RNA was resuspended in diethylpyrocarbonate-treated water for injection. When it was necessary to give oocytes a second injection in a specific location with respect to the first, only oocytes showing a trace of the first injection site were used: the injection site is frequently marked by a concentration of pigment. Groups of 20–60 oocytes were injected and at appropriate times, batches of five were taken for analysis of RNA and enzyme activity. Small groups were taken so as to maximize the chances of seeing variability due to site of injection. In practice such variability was not seen from batches taken from the same group of oocytes.

RNA Analysis

RNA was extracted from injected oocytes (6) and analyzed on formaldehyde agarose gels (1). Gels were fixed in 5% trichloroacetic acid (TCA) and dried for fluorography (10) or direct autoradiography. To measure RNA stability, oocytes were injected with ³²P-labeled RNA and frozen at various times after injection. The counts injected were measured by Cherenkov counting of the oocyte homogenate (the nucleotide from degraded RNA does not appear to leak out of the oocyte). Then the RNA was analyzed as in Fig. 1B by agarose gel electrophoresis, and the optical density of specific autoradiographic bands were determined by microdensitometry. The values were adjusted to take into account variation in the total counts injected from batch to batch and plotted to determine half-life.

Enzyme Assays

Oocytes were frozen in batches of five for enzyme assay. Two sets of five oocytes were used for each determination. TK activity was determined as

described by Merrill et al. (15) except that 20 μ l of extraction buffer was used per oocyte. Homogenate equivalent to four oocytes was used in enzyme assays. CAT activity was determined from the same homogenate as described by Gorman et al. (2) using the equivalent of one tenth to one half an oocyte. For quantitation of CAT activity, reactions were in the linear range (no more than 40% chloramphenicol acetylated). To plot the results shown in Fig. 2 (open squares, solid circles), the enzyme activity for the test RNA was corrected for possible small variations in RNA injected and in variation in expression between oocyte batches. This was done by normalizing activity to the activity produced by the control RNA injected into the same oocytes. For example, in experiments to assay inhibition due to antisense TK, the level of TK activity observed was normalized to the level of activity observed from the control CAT RNA. Activity from the control RNA showed only small variations from a constant activity as seen in Fig. 2. Even without these adjustments, the curves in Fig. 2 are qualitatively the same.

Analysis of Protein Expression

Newly synthesized proteins were analyzed as described by Laskey et al. (11) using [³⁵S]methionine. Radioactivity from CAT protein was prominent in fluorographs when 0.5 ng CAT mRNA was injected.

RESULTS

Synthesis and Stability of Injected RNA

Coding sequences for the enzymes CAT and TK were inserted into plasmids containing an SP6 promoter. Transcripts were synthesized from plasmid DNA (diagrammed in Fig. 1A) and these transcripts were capped either enzymatically (using vaccinia virus guanylyl transferase) or during transcription (using diguanosyl 5' triphosphate [GpppG] as substrate). All transcripts described in this paper were fairly stable after injection into oocyte cytoplasm. Both sense and antisense RNAs had half-lives of between 8 and 12 h (see Fig. 1). In our experiments, capping with GpppG resulted in a higher proportion of RNA molecules that were stable presumably because it was more quantitative than capping using guanylyl transferase. In confirmation of the results of Green et al. (3), uncapped transcripts had a half-life of <1 h.

Inhibition of Translation of mRNA

Either antisense CAT or antisense TK RNA was injected into the cytoplasm of oocytes, and a mixture of CAT and TK sense mRNA was injected 5–6 h later to assure random diffusion of the previously injected antisense RNAs. After overnight incubation, the oocytes were analyzed for TK and CAT activity. As shown in Fig. 2, antisense CAT RNA inhibits translation of CAT mRNA, and antisense TK RNA inhibits translation of TK mRNA. The amount of inhibition observed varied between experiments; Fig. 2 shows the experiment in which the least inhibition was observed. In two experiments CAT activity was inhibited 60–80-fold (e.g., Fig. 4), whereas in another two the results were closer to those presented in Fig. 1 (8–10-fold, e.g., Fig. 3).

We have excluded the possibility that there is nonspecific inhibition of translation by antisense RNA. In the experiments presented here, the oocytes were injected with both CAT and TK mRNAs. We were thus able to show that in the oocytes where CAT expression is inhibited by antisense CAT RNA, there is no inhibition of TK activity. The same specificity was true for TK, where antisense TK RNA inhibited expression of TK but not CAT mRNA (Fig. 2). In addition, the injection of 20 ng of antisense RNA into oocyte cytoplasm affected neither the amount of endogenous protein synthesis nor the profile of newly synthesized proteins detected by SDS polyacrylamide gel analysis (data not shown). This result is in

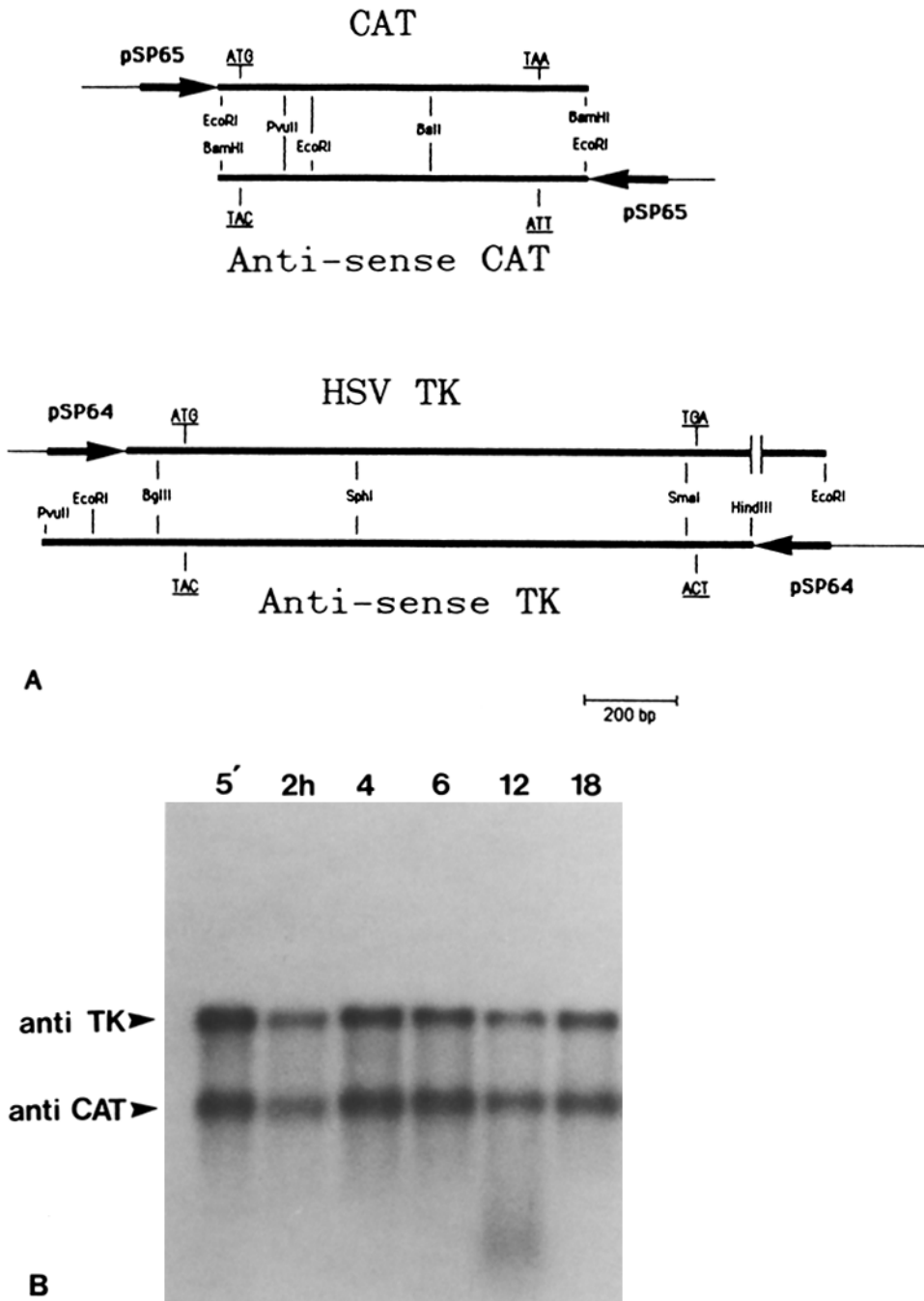


FIGURE 1 (A) Schematic representation of linearized plasmids used in transcription reactions. The heavy arrow marks the SP6 promoters. The SP6 CAT plasmids give an 800 nucleotide transcript. The position of the translation initiation codon and its complement are marked. To generate anti-sense RNA that did not cover the AUG translation initiation codon, the plasmid was linearized with Pvu II. The TK transcripts were longer; TK sense was 2,200 nucleotides, and antisense was 1,600 nucleotides. To generate TK antisense that did not cover the AUG, the plasmid was linearized with Sph I. (B) Stability of antisense RNA in oocytes. Fluorograph of radioactive transcripts of antisense TK and CAT plasmids. RNA was trace-labeled with α -[32 P]-GTP during synthesis. Approximately 10 ng of each RNA were injected per oocyte. After injection RNAs were extracted and separated on a denaturing agarose gel (1).

contrast to results obtained when purified translatable mRNAs are injected into oocytes: Such RNAs do compete for translation either of endogenous or injected mRNAs (11). Presumably these antisense RNAs do not compete for translation because they are themselves inefficiently translated.

Antisense RNA Which Does Not Hybridize to the 5' End of mRNA Inhibits Translation, but Less Well

To test whether antisense RNA needs to hybridize to the entire length of mRNA, and in particular, needs to form a duplex with the ribosome binding site, we synthesized a truncated antisense transcript that would only hybridize to protein coding and 3' untranslated portions of mRNAs. A transcript of CAT antisense RNA truncated at the Pvu II site (Fig. 1) leaves 154 bases of 5' sense mRNA exposed, including

the AUG codon and 37 codons of protein coding sequence. As shown in Fig. 3 (open square), this RNA does inhibit CAT expression, but only by about threefold, compared to the 10-fold for full length antisense CAT observed in this experiment. A similar threefold level of inhibition was obtained in two additional experiments with the 3' CAT antisense probe and two experiments with a 3' antisense TK probe (not shown). Since we hope to use the antisense methodology to inhibit translation of mRNAs in normal *Xenopus* development, we are encouraged by these results because they suggest that cDNA clones that are not full-length may be used to inhibit a gene, albeit less efficiently.

Inhibition by Antisense RNA, Diffusion, and Hybridization Kinetics

For both CAT and TK, the curves in Fig. 2 show that a

small fraction (5–10%) of the enzyme activity is not inhibited by a large excess of antisense RNA. The antisense RNA is in vast excess and if hybridization occurred in the same way as in solution, we can calculate using Rot analysis that saturation should occur within minutes under the conditions used (e.g., 0.5 ng sense RNA, 20 ng antisense RNA, 1 μ l oocyte volume). It is important to know how the resistant fraction of activity arises since it may be critical that complete inhibition of activity is achieved when we try to mimic mutations by injection of antisense RNA directed against developmentally controlled transcripts. There are three main possibilities for the discrepancy between the predictions of solution hybridization and the observed inhibition in vivo: (a) the viscosity of the cytoplasm may put limits on diffusion of RNA; (b) a fraction of the mRNA may exist in an inaccessible compartment (for example, in polysomes or other ribonucleoprotein particles); and (c) duplexes formed in vivo might melt or be denatured by the translational machinery.

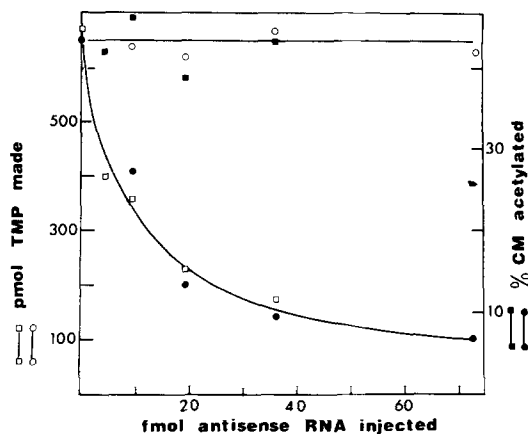


FIGURE 2 Antisense RNA inhibits expression only of homologous mRNA. Oocytes were injected with increasing doses of antisense CAT (○, ●) or antisense TK (□, ■) RNA. 5 h later, the oocytes were injected with a mixture of sense CAT and TK mRNA (each at 0.5 ng per oocyte). After overnight incubation, the oocytes were assayed for both CAT and TK activities.

To investigate whether diffusion limited the rate of hybridization in vivo, we quantitated CAT activity 5 and 18 h after injection (Fig. 3). For both time points, the inhibition was the same, approximately 10-fold. Thus, inhibition takes place quickly, but nevertheless is not complete. To check further that diffusion does not limit the rate of hybridization, we injected oocytes with antisense RNA and 3 h later injected sense mRNA either into the same place, or the opposite side of the cell. Both sets of oocytes showed the same 10-fold level of inhibition compared with controls. We conclude from this experiment that RNA is freely diffusible in the cytoplasm, and therefore that diffusion should not limit the inhibition. A second possibility is that there is an putative compartment; however, if this were true then this compartment must have access to the translation machinery since our assay depends on expression of protein. It is unlikely that the inaccessible

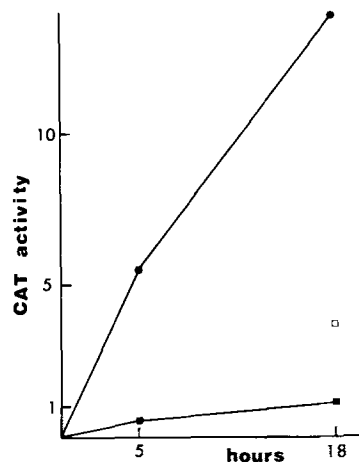


FIGURE 3 Time course of CAT expression in the presence of antisense TK RNA (●) or full length antisense CAT RNA (■). Inhibition is rapid but not complete. □, CAT activity in the presence of truncated CAT antisense RNA (which does not cover the AUG translation initiation codon). CAT activity is expressed as % CAT acetylated in the standard reaction using extract equivalent to one tenth of an oocyte.

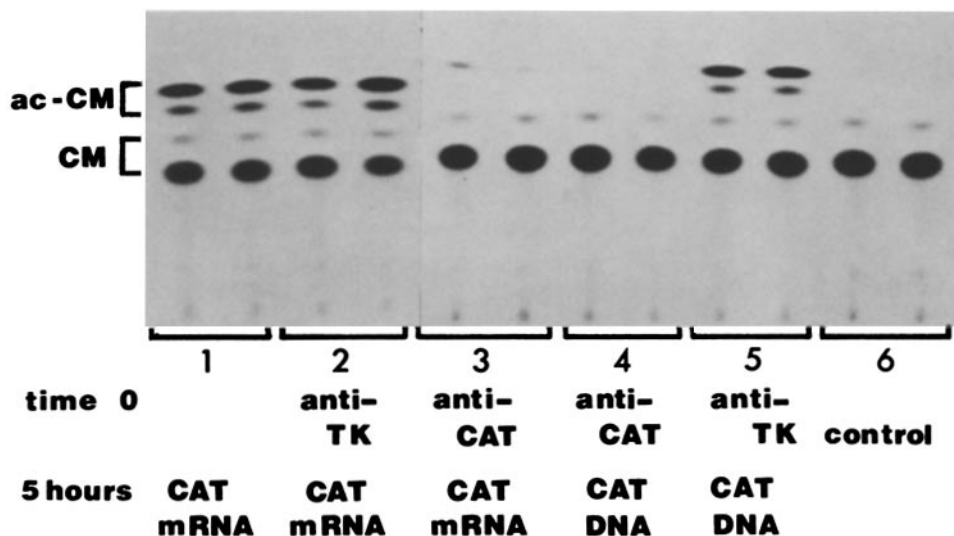


FIGURE 4 CAT expression from either injected mRNA or injected plasmid DNA is specifically inhibited by CAT antisense RNA. Antisense CAT RNA (10 ng per oocyte) or antisense TK RNA (20 ng per oocyte) were injected as indicated. 5 h later, either CAT mRNA was injected into the cytoplasm or an expression plasmid coding for CAT was injected into the nucleus. After 15 h of incubation, two batches of five oocytes were assayed for CAT activity. The figure shows an autoradiograph of thin layer chromatography plates used to analyze the products of the reaction. The starting material, chloramphenicol (CM), contains a minor contaminant which is not a product of CAT

activity (e.g., 6; control incubation with extract from uninjected oocytes). The migration position of acetylated chloramphenicol is indicated (ac-CM). CAT antisense RNA inhibits expression either of CAT mRNA (compare 2 and 3) or of CAT DNA (compare 4 and 5).

compartment is simply the polyribosome, since Melton (13) has shown that β -globin mRNA already loaded onto polyosomes is still accessible to inhibition by antisense globin RNA, although less efficiently than if antisense RNA is introduced before globin mRNA. A third possibility is that the translation machinery has a finite, but very limited probability of denaturing RNA duplexes. Presumably, this occurs all of the time with RNA duplexes present in mRNA. This might predict that antisense RNA that perfectly overlaps the 5' end of the mRNA might be more efficient in inhibiting translation since it would limit the access of ribosomes at the 5' end. Indeed, recent work (8) has shown that the expression of a TK gene can be reduced to zero by a plasmid that produces antisense RNA overlapping only the 5' noncoding region. In contrast, the antisense RNAs used in this work did not have perfect complementarity overlapping the 5' end of the mRNA; instead, 25 bases of the CAT mRNA and 14 bases of TK mRNA (encoded by the SP6 plasmid before the insert) would be single stranded.

An observation that argues against the interpretation that RNA duplexes can be denatured by the ribosome comes from experiments in which sense and antisense RNAs were mixed before injection (500 μ g/ml antisense, 10 μ g/ml sense). In three such experiments, no CAT activity was detected above background (>1,000-fold inhibition). Although this experiment shows that hybrids formed *in vitro* are stable, it is nevertheless possible that hybrids formed *in vivo* are different and less stable.

We conclude that while the bulk of the antisense inhibition is compatible with simple hybridization and diffusion predictions, there is residual activity that is not readily explained. This may reflect the capacity of the translation machinery to denature and translate an RNA molecule. If this is true and general, then this may place certain restrictions on the use of antisense RNA when trying to phenocopy gene mutations. It also emphasizes the need to understand as many parameters of the inhibition process as possible. On the other hand, it is also possible that completely null phenotypes may be hard to interpret and indeed, that it is advantageous to have leaky mutations induced by antisense RNA.

Inhibition of Expression from DNA

To test whether endogenous genes may be inhibited by injection of exogenous antisense RNA, we used a model system that more closely mimics the expression of normal genes. We achieved this by injecting an expression plasmid coding for CAT into the nucleus of oocytes. Oocytes were first injected with antisense TK or CAT RNA into the cytoplasm, and then with CAT DNA into the nucleus. After overnight incubation the oocytes were assayed for enzyme activity. When compared to the control antisense TK RNA, antisense CAT RNA inhibited expression of enzyme from the expression plasmid (Fig. 4). In four experiments, inhibition was 5-, 8-, 30-, and 140-fold. We have no explanation for this variability. We assume that the mechanism of inhibition in this case is at the level of translation blocking, but we have not ruled out the possibility that antisense CAT RNA gets into the nucleus and inhibits some steps of RNA processing.

DISCUSSION

The primary goal of this study is to demonstrate that antisense

RNA inhibition is a general phenomenon that can also occur in frog oocytes. In this we have confirmed the results of Melton (13) and extended them to other genes. These demonstrations are crucial to our long-term goal of using this technique to identify genes important for early frog development. A second goal of this study is to establish a system where we can readily study the detailed mechanism of inhibition. The frog oocyte is ideal for this purpose since defined quantities of specific RNA can be easily introduced and the levels of RNA assayed during the course of the reaction. The use of mRNA coding for enzymes allows quantitation of expression. Our results demonstrate that antisense inhibition is specific and requires about a 10-fold excess of antisense RNA. In contrast to previous work using a DNA template to transcribe antisense RNA *in vivo* (7, 18) here we use a capped antisense RNA made *in vitro* with the SP6 RNA polymerase. This gives good inhibition in this system and is reasonably stable. Antisense RNA directed against the 3' end of an mRNA is capable of inhibition, but is less effective than the entire antisense RNA. Finally, the inhibition process to a first approximation seems to follow simple hybridization theory; however, a small fraction of sense RNA seems to be resistant to inhibition in this system. We do not understand the basis for this residual activity. Finally, we have shown that it is possible to inhibit the expression of a gene transcribed in the nucleus by injection of antisense RNA into the cytoplasm.

We thank Pei Feng Cheng for excellent technical assistance and Helen Devitt for typing the manuscript. We also thank J. G. Izant and D. Bentley for their comments on the manuscript. We thank Barbara Graves, Steve McKnight, Bob Kingsbury, Dan Moran, and Paul Krieg for gifts of plasmids, and Doug Melton for communicating results before publication.

This work was supported by a European Molecular Biology Organization Fellowship to Dr. Harland and a grant from the National Institutes of Health to Dr. Weintraub.

Received for publication 5 April 1985, and in revised form 1 June 1985.

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