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Injected anti-sense RNAs specifically block messenger RNA translation *in vivo*

(hybrid arrested translation/embryogenesis/gene function)

D. A. MELTON

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT **As a test for a method to analyze gene function in embryogenesis, the translation of a specific mRNA has been blocked *in vivo* by microinjection of complementary (anti-sense) RNA. RNA complementary to globin mRNA was synthesized *in vitro* by transcription of an inverted globin cDNA clone. After injection into frog oocyte cytoplasm, the anti-sense globin RNA forms a hybrid with globin mRNA and selectively prevents its translation. Deletion mapping of the anti-sense RNA shows that the 5′ region of the globin mRNA must be covered in order to block translation. This method may allow one to study the function of many genes for which DNA clones are available by preventing the expression of the endogenous gene as protein.**

In general, mutations in vertebrate genes are not readily obtained nor is it easy to study their effects by conventional genetic analyses. For example, although scores of developmentally interesting genes have been studied in flies and worms, few genes of comparable interest have been identified in frogs, mice, or humans. It is, of course, possible to clone genes that might be important for development from any organism or cell type. Differential screening of cDNA libraries and methods of subtractive hybridization have made it possible to obtain genes that are expressed in only one cell type or at a particular time in development. However, unless one can selectively inactivate these genes and study the resulting mutant phenotypes *in vivo*, it is difficult to ascribe functions to their gene products. This paper describes a method that may allow one to prevent the expression of particular genes in early development. The method is a direct biochemical intervention that involves specifically blocking the translation of a gene's mRNA by RNA·RNA hybridization *in vivo*. The translation block prevents the synthesis of the gene product and, in effect, phenocopies mutations in the gene of interest.

The first demonstration that anti-sense RNAs can be used experimentally to selectively prevent gene expression in eukaryotic cells was provided by Izant and Weintraub's studies on the thymidine kinase gene in injected tissue culture cells (1). They showed that the expression of a thymidine kinase gene injected into mouse L cells is significantly reduced by coinjection of a plasmid that directs the synthesis of anti-sense thymidine kinase RNA (1). Similar experiments using transfected fibroblasts showed that *LacZ* expression is inhibited by cotransfection with plasmids producing anti-sense *LacZ* RNA (2). In *Escherichia coli*, Inouye and co-workers demonstrated that the expression of the outer membrane protein (OmpF) is normally inhibited by the presence of RNA complementary to OmpF mRNA (3), and this led them to construct inducible plasmids that selectively reduce bacterial gene expression by directing the synthesis of specific anti-sense RNAs (4). In retrospect, indications that anti-sense RNAs might be able to experimentally block gene expression come from studies which showed that RNA·RNA or RNA·DNA hybrids can block translation *in vitro* (5–8) and regulate DNA replication (9, 10) and Tn*10* transposition *in vivo* (11).

Despite these very encouraging results, it is not clear that DNA vectors that direct the synthesis of anti-sense RNAs can be used to study gene function during embryogenesis. To produce sufficient quantities of a specific anti-sense RNA, a DNA vector must have an efficient promoter that works in the right cell at the right time, a condition for which there is at present no simple solution. A further problem arises when one is interested in studying the function of genes in cells that are not transcriptionally active. For example, during the early development of most animals, there is little or no transcription. New gene products, many of which may be important for early determinative events, are produced by translation of stored maternal mRNA. With this in mind, an alternative method for supplying anti-sense RNA has been explored. The method involves synthesizing anti-sense RNA *in vitro* with SP6 RNA polymerase and directly injecting the anti-sense RNA into the cell cytoplasm.

MATERIALS AND METHODS

Vectors for Synthesizing mRNA and Anti-Sense RNA. All the plasmid DNAs used in this study contain an SP6 promoter, which allows for the efficient synthesis of RNA *in vitro* (12). pSP64-Xβm DNA contains a *Xenopus* β-globin cDNA sequence downstream from the SP6 promoter as described (13). Transcription of *Pst* I-digested pSP64-Xβm DNA with SP6 RNA polymerase produces RNA transcripts that are identical to mature β-globin mRNA except that they contain 12 extra bases at the 5′ end and a stretch of $\approx$30 cytosine residues at the 3′ end following the poly(A) tail.

pAnXβm, anti-*Xenopus* β-globin, also contains a *Xenopus* β-globin cDNA sequence downstream from an SP6 promoter, although in this case the cDNA sequence is inverted relative to the configuration in pSP64-Xβm. Consequently, SP6 RNA polymerase transcribes the coding strand of the cDNA to produce anti-sense that is complementary to mRNA. pAnXβm was constructed by filling in the *Pst* I restriction fragment of pXG8D2, which contains the entire *Xenopus* β-globin cDNA with G·C tails (14), with T4 DNA polymerase and adding *Hin*dIII linkers. This 600-base-pair fragment was inserted into the *Hin*dIII site of pSP62 (12). pAn5′Xβm contains just the 5′ flanking region of the β-globin cDNA inserted in the inverted orientation. This plasmid was constructed by digesting pSP64-Xβm DNA with *Hin*dIII (which cuts upstream of the normal mRNA cap site) and *Bal* I (which cuts 2 bases upstream of the initiation codon) and inserting this 50-base-pair restriction fragment into *Sma* I/*Hin*dIII-digested pSP65 (12). SP6 transcripts of *Hin*dIII-digested pAn5′Xβm are complementary to all but a few bases of the 5′ untranslated region of the β-globin mRNA. pAnXH4, anti-*Xenopus* H4

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histone, contains a *Xenopus* H4 histone DNA sequence downstream from an SP6 promoter such that transcription with SP6 RNA polymerase produces an RNA complementary to H4 histone mRNA. This plasmid was constructed by inserting the *Bam*HI fragment of pcX1H4W1, which contains the entire coding sequence for H4 histone (15), into *Bam*HI digested pSP62.

***In Vitro* RNA Synthesis.** mRNAs and anti-sense RNAs were produced by SP6 *in vitro* transcription (12, 16) of linearized DNA templates as described in detail (12). When necessary, SP6 transcripts were radiolabeled by including [α-^{32}P]UTP or [^{3}H]UTP in the transcription reaction. All transcripts were capped *in vitro* with guanylyltransferase (Bethesda Research Laboratories) as described (13). Denaturing agarose gels containing 7.5% formaldehyde in 1× 3-(*N*-morpholino)propanesulfonic acid (Mops) (17) buffer were used to analyze RNA transcripts as described (12).

Oocyte Injection and Protein Assays. RNAs, stored in ethanol, were collected by centrifugation and dissolved in 0.1× MBSH (18) for injection into stage VI *Xenopus* oocytes. Groups of 5–10 injected oocytes were incubated at 18°C in 1× MBSH containing 0.75–1 mCi of [^{35}S]methionine per ml (1 Ci = 37 GBq). Newly synthesized proteins were examined by homogenizing injected oocytes in 60 mM Tris·HCl, pH 6.8/1 mM phenylmethylsulfonyl fluoride/1% NaDodSO$_4$/100 mM 2-mercaptoethanol using 50 μl per oocyte followed by centrifugation at 10,000 × *g* for 5 min. Total protein from 1/20th of an oocyte (2.5 μl of the homogenate) was fractionated by electrophoresis in NaDodSO$_4$ acrylamide gels (19) using a 17% acrylamide resolving gel with a 5% stacking gel. Gels were fixed with methanol (45%) and acetic acid (10%) and prepared for fluorography (20).

RNA·RNA Hybridization Assay. RNAs allowed to hybridize *in vivo* were extracted from injected oocytes by proteinase K digestion (200 μg/ml) in 50 mM Tris·HCl, pH 7.5/50 mM NaCl/5 mM EDTA/0.5% NaDodSO$_4$, followed by phenol/chloroform extraction and ethanol precipitation. RNA precipitates were dissolved in 300 mM NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA, and digested with RNase T1 (2 μg/ml) and RNase A (40 μg/ml) for 30 min at 30°C (12). Uninjected control RNAs were allowed to hybridize *in vitro* in 80% formamide/40 mM Pipes, pH 6.7/400 mM NaCl/1 mM EDTA for 6 hr at 45°C and then digested with RNases A and T1 as described above. After phenol/chloroform extraction and ethanol precipitation, the RNA digestion products were fractionated on denaturing agarose gels.

RESULTS

The object of these experiments is to determine whether the translation of a particular mRNA can be prevented by injection of anti-sense RNA—i.e., RNA that is complementary to the mRNA. Theoretically, the formation of an RNA·RNA duplex *in vivo* will block translation. A test for the feasibility of this approach is to attempt to block the translation of a mRNA that is efficiently translated in oocytes and one whose protein product is easily detected. Globin mRNA was chosen for these tests because it meets these criteria.

A reliable source of anti-sense RNA is an essential component of this approach to studying gene function. To prepare large amounts of pure anti-sense RNA, cDNA clones corresponding to the mRNA of interest were cloned in transcription vectors that contain an SP6 promoter (Fig. 1; ref. 12). *In vitro* transcription with SP6 RNA polymerase produces microgram quantities of pure RNA (12), either mRNA or anti-RNA depending on the orientation of the cDNA clone in the vector. Synthetic *Xenopus* β-globin mRNA produced by transcription of pSP64-Xβm (Fig. 1) has been previously shown to function as well as authentic β-globin mRNA for

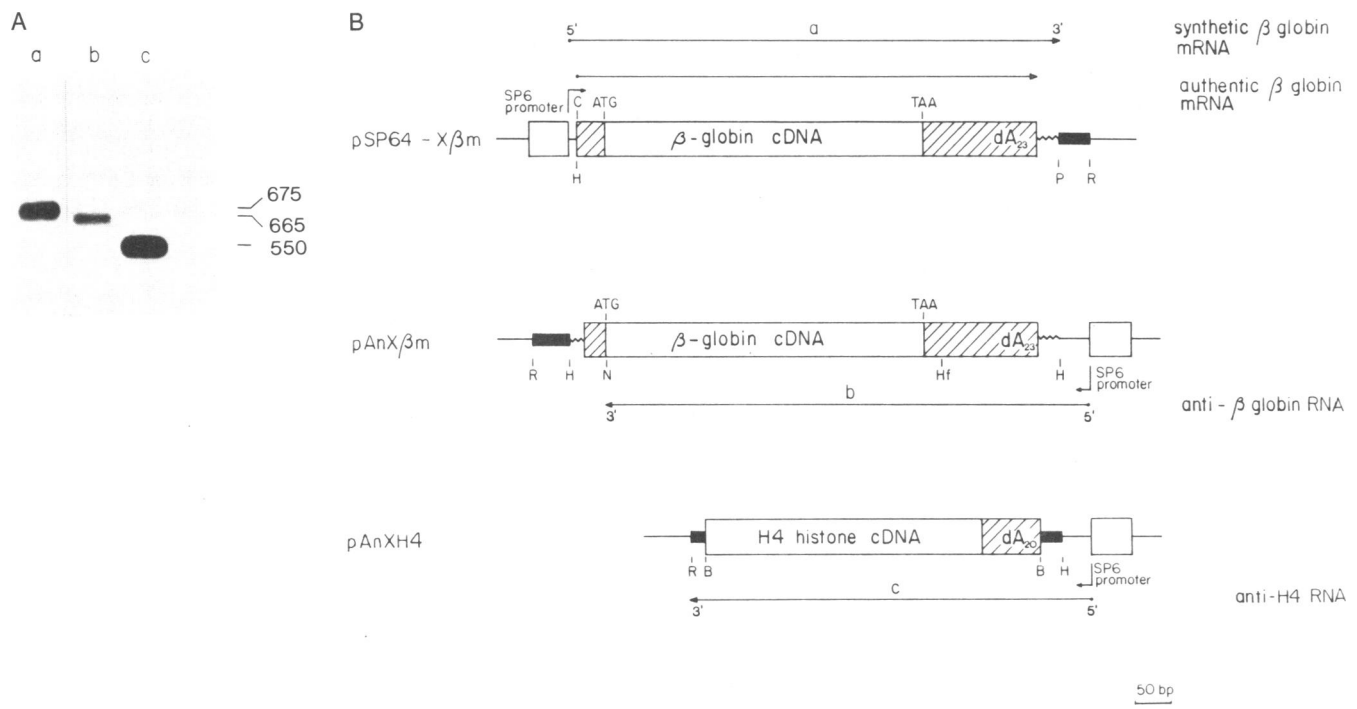

FIG. 1. SP6 plasmids for mRNA and anti-sense RNA synthesis. (*A*) Autoradiogram of RNAs produced by SP6 *in vitro* transcription. Lane a, synthetic β-globin transcribed from *Pst* I-cut pSP64-Xβm. Lane b, anti-sense β-globin RNA transcribed from *Nco* I-cut pAnXβm. Lane c, anti-sense histone H4 RNA transcribed from *Eco*RI-cut pAnXH4. (*B*) Diagram showing relative orientation of the SP6 promoter and cDNA sequences for the DNA templates. SP6 RNA polymerase transcribes only one strand of the double helix (arrow). The 5′ and 3′ untranslated regions are hatched; the poly(A) tail encoded in the cDNA sequence is denoted by dA; G·C tails used for constructing cDNA clones represented by ∿. Portions of the polylinker from pSP64 and pSP65 (12) are represented by ▬. The natural cap site for β-globin mRNA is marked by C. B, *Bam*HI; H, *Hin*dIII; Hf, *Hin*fI; N, *Nco* I; P, *Pst* I; R, *Eco*RI; bp, base pairs.

translation in oocytes (13). The synthetic β-globin mRNA was used for the present study because it is easily obtained and can be readily quantified, unlike natural globin mRNA, which is a mixture of α- and β-globin mRNAs.

Injected Anti-Sense RNA Blocks mRNA Translation in Living Oocytes. Oocytes were injected with anti-sense β-globin RNA (*Nco* I transcript; Fig. 1) and incubated for 5 hr before receiving an injection of β-globin mRNA. In this experiment, a 50-fold excess of anti-sense RNA was injected. All injections were directed into the oocyte cytoplasm. Five hours after injection of the β-globin mRNA, newly synthesized proteins were labeled by incubating the injected oocytes in [^{35}S]methionine. Fig. 2 shows that oocytes injected with anti-sense β-globin RNA are unable to translate β-globin mRNA. A comparison of lanes 2 and 3 in Fig. 2 suggests that the anti-sense β-globin RNA completely blocks translation: globin protein synthesis is undetectable in these oocytes.

Two experiments show that the translation blocking is specific. First, the injection of anti-sense β-globin RNA alone does not cause a general disruption of protein synthesis (Fig. 2, lane 5). Second, the injection of anti-sense H4 histone RNA (Fig. 1) does not prevent the synthesis of β-globin protein in oocytes injected with β-globin mRNA (Fig. 2, lane 4). In confirmation of these results, other experiments have shown that neither anti-sense β-globin RNA nor anti-sense H4 histone RNA blocks the translation of human β-interferon mRNA in injected oocytes (data not shown).

In the experiments described above, the anti-sense RNA was provided to the oocyte before the mRNA was injected. Fig. 2 (lane 7) shows that if the anti-sense β-globin RNA and globin mRNA are coinjected into oocytes, globin protein is not synthesized. Presumably, this is a consequence of the fact that the two complementary RNAs were injected as RNA·RNA hybrids. If the β-globin mRNA is injected first, followed 5 hr later by an injection of the anti-sense β-globin RNA, a very low level of globin protein synthesis is observed (lane 6). Densitometer tracings of the gel show that the amount of globin protein synthesized is reduced by at least a factor of 10 relative to the control where no anti-sense β-globin RNA was injected. If synthetic β-globin mRNA is loaded onto polysomes within the first 5 hr after injection into oocytes, this result suggests that it may be difficult to completely eliminate further protein synthesis from a mRNA that is already being translated.

Tests of the stability of injected anti-sense RNAs show that the RNAs must be capped or they are rapidly degraded. This confirms results previously obtained with capped and uncapped synthetic mRNAs in injected oocytes (13). Capped anti-sense RNAs are very stable in injected oocytes: more than one-half of the injected anti-sense RNA is intact after a 2-day incubation (data not shown).

Translation Blocking Is Accomplished by RNA·RNA Hybridization *in Vivo*. To test whether the observed translation blocking is the result of RNA·RNA duplex formation, RNAs injected into oocytes were extracted and digested with single-strand specific ribonucleases. When ^{32}P-labeled globin mRNA is extracted from injected oocytes and digested with RNase A and RNase T1, all of the RNA is degraded (Fig. 3). In sharp contrast, the ^{32}P-labeled globin mRNA is not completely digested by RNase A and RNase T1 if it is isolated 5 hr after injection into oocytes that had been previously injected with anti-sense β-globin RNA. This protected RNA, indicative of an anti-sense RNA·mRNA duplex, is not observed if the mRNA is injected into oocytes containing anti-sense RNA and immediately extracted and processed for RNase digestion along with the anti-sense RNA. Thus, the anti-sense RNA·mRNA duplex is only observed if the injected mRNA is incubated in oocytes containing anti-sense RNA and must, therefore, be the result of hybridization *in vivo*.

The anti-sense RNA·mRNA duplex formed in injected oocytes is ≈220 base pairs long, the same size as the protected anti-sense RNA·mRNA duplex observed after hybridization *in vitro* (Fig. 3). The anti-sense RNA used for this experiment, a transcript of *Nco* I-digested pAnXβm (Fig. 1),

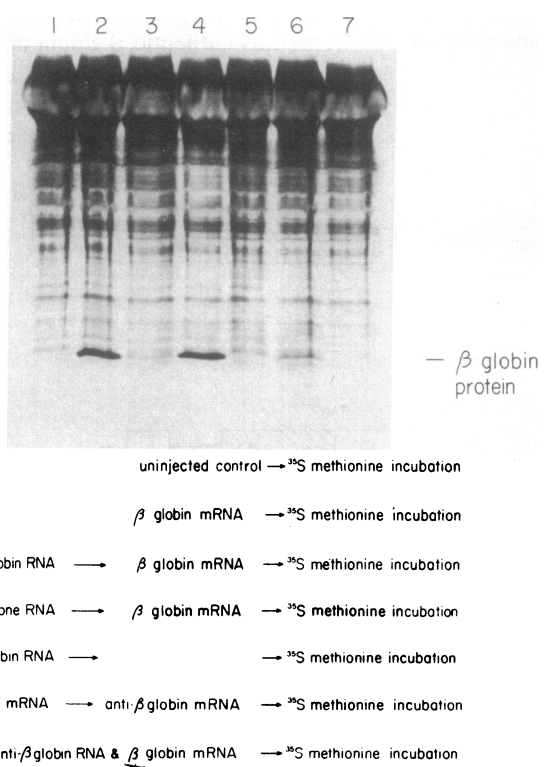

FIG. 2. Translation blocking by injection of anti-sense RNA. Oocytes were injected with antisense β-globin RNA (see Fig. 1; 25 ng or 0.1 pmol per oocyte) and/or β-globin mRNA (see Fig. 1; 0.5 pg or 2 fmol per oocyte) according to the schedule shown at the bottom. Arrows indicate a 5-hr incubation period. Newly synthesized proteins were labeled by incubation in [^{35}S]methionine for 15 hr. The autoradiogram of $NaDodSO_4$ acrylamide gel shows the proteins from 1/20th of an oocyte. Lane numbers correspond to the injection schedule at the bottom.

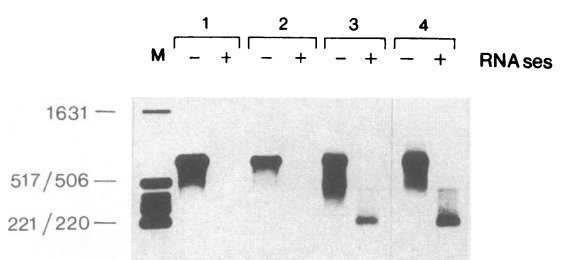

FIG. 3. Anti-sense RNA·mRNA hybrids are formed *in vivo*. ^{3}H-labeled anti-sense β-globin RNA (0.5 Ci/mmol) was injected into oocytes (25 ng per oocyte) and followed 5 hr later by the injection of ^{32}P-labeled globin mRNA (0.5 ng per oocyte; 100 Ci/mmol). The injected RNAs were extracted immediately (lane 2) or after a further 5-hr incubation (lane 3). One-half of the sample was digested with RNases A and T1 (+) and the other half (−) was loaded directly onto a 2% denaturing agarose gel. Lane 1 is a control of uninjected ^{32}P-labeled globin mRNA. Lane 4 is a control in which ^{32}P-labeled globin RNA was hybridized with ^{3}H-labeled anti-sense β-globin RNA *in vitro* for 6 hr and subsequently treated with RNases A and T1. The autoradiogram only shows the ^{32}P-labeled RNAs, derived from the β-globin mRNA; the anti-sense RNA was purposely labeled at a low specific activity with ^{3}H and is not visible. M, DNA markers with lengths noted in nucleotides.

should protect 625 bases of the mRNA. The reason why a much shorter RNA duplex is protected is not known, but it may be due to the formation of strong intramolecular hairpins (21).

An Anti-Sense RNA Must Cover the 5′ End of the mRNA to Block Translation. To determine the regions of a mRNA that must be hybridized or covered in order to block translation, different length anti-sense RNAs were prepared as shown in Fig. 4. Four anti-sense β-globin RNAs having the same 5′ end but different 3′ ends can be prepared by linearizing the DNA template pAnXβm at different sites with various restriction enzymes. While all four anti-sense RNAs can hybridize to the 3′ flanking region of the mRNA, each covers different amounts of the mRNA's protein coding and 5′ flanking region. Injection results show that anti-sense RNAs that hybridize to only the 3′ half of the protein coding region and/or the 3′ nontranslated flanking sequences are unable to block globin protein synthesis (Fig. 4). Anti-sense globin transcripts from *Bam*HI- or *Hin*fI-digested pAnXβm have no demonstrable effect on globin mRNA translation. In contrast, anti-sense globin transcripts from *Eco*RI- or *Nco* I-digested pAnXβm entirely eliminate globin protein synthesis.

These results suggest that the 5′ region of the message, where ribosomes bind and initiate translation, must be covered by anti-sense RNA in order to block translation. To further define the effective site of action for anti-sense RNAs, transcripts from *Hin*dIII-digested pAn5′Xβm were tested for translation blocking. This anti-5′ globin RNA is complementary to the 5′ end of the mRNA from the natural cap site to 2 bases upstream of the AUG codon. Although this anti-sense RNA does not hybridize to any of the protein coding region and covers only 45 bases in the 5′ flanking region, it is nevertheless able to prevent globin mRNA translation (Fig. 4, lane 5). Thus, anti-sense RNAs that hybridize to the mRNA region encoding the amino-terminal residues of the protein, including the initiation codon (anti-globin RNA *Nco* I and anti-globin RNA *Eco*RI; Fig. 4) and anti-sense RNA that hybridizes to the 5′ untranslated region (anti-5′ globin RNA) are equally effective at blocking translation. It is, therefore, concluded that the critical region for blocking mRNA translation with an anti-sense RNA is the 5′ region of the mRNA. All together, the data presented in Fig. 4 indicate that anti-sense RNAs are effective at blocking initiation but not elongation of protein synthesis.

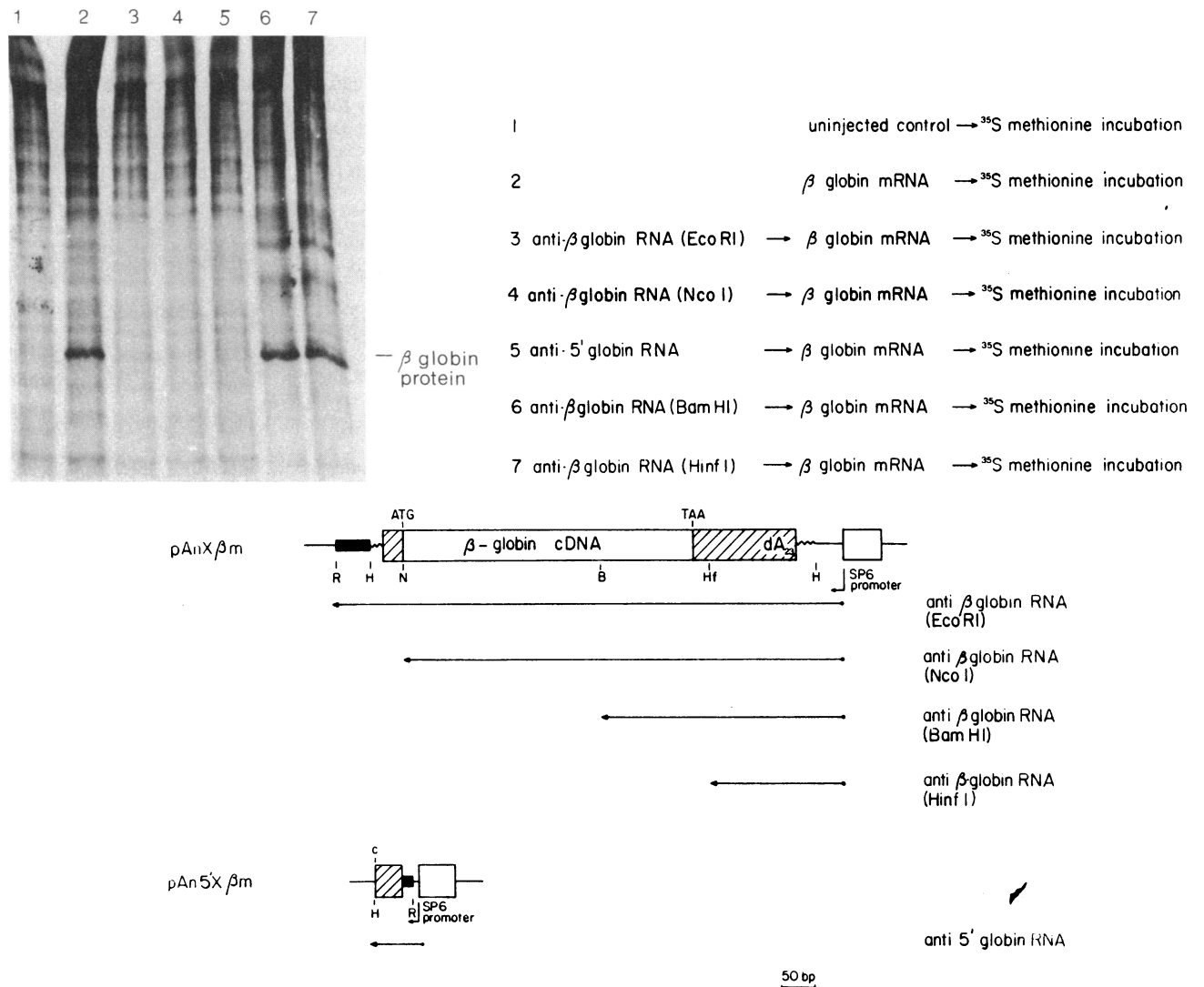

FIG. 4. Translation blocking by anti-sense RNAs that hybridize to portions of the mRNA. Different anti-sense globin RNAs were synthesized using the DNA templates shown at the bottom. Oocytes were injected with each individual anti-sense RNA (20 ng per oocyte) and subsequently with mRNA (0.5 ng per oocyte) according to the schedule at the right. Arrows indicate a 5-hr incubation period. Newly synthesized proteins were labeled for 17 hr by incubation in [^{35}S]methionine. The autoradiogram of a $NaDodSO_4$ acrylamide gel shows the proteins from 1/20th of an oocyte. Lane numbers correspond to the injection schedule at the right; other abbreviations are as in Fig. 1.

DISCUSSION

The results presented here show that RNA complementary to a mRNA can completely prevent translation of the mRNA if the two RNAs are present in the same cell. The block to translation is the result of RNA·RNA duplex formation *in vivo* and is consequently very specific. These and other (1, 2, 4) results suggest that any biological process that requires RNA base pairing, perhaps including RNA processing events, might be interrupted by the introduction of anti-sense RNA.

It is important to emphasize that the translation blocking experiments presented here were performed with exogenous RNAs injected into oocytes. The abolition of protein synthesis is observed if the injected anti-sense RNA is already in the oocytes when the mRNA is injected. While this is clearly not equivalent to blocking translation of endogenous mRNAs, this experiment may provide a good test for situations in which an untranslated maternal mRNA is recruited to polysomes for translation. In this regard, it is worth noting that these experiments show that it is possible to prevent translation of as much as 500 pg of globin mRNA or the equivalent of 1% of the total mRNA in an oocyte. This encourages one to try blocking translation of specific endogenous mRNAs that are likely to be much less abundant. In other instances, the order of RNA injections was reversed so that the anti-sense RNA was injected into oocytes that were previously injected with globin mRNA. This may mimic situations in which one is trying to block translation of mRNA that is already active in translation. In this test performed here, globin protein synthesis was severely reduced (a factor of > 10), but not abolished, somewhat analogous to a "down" mutation.

It is well known that mRNAs (and it is presumed that anti-sense RNAs) are not present as naked polynucleotides in the cell, but rather are assembled into ribonucleoprotein structures (22). While the state of the injected RNAs with respect to ribonucleoprotein formation has not been examined, the results suggest that at least some portion of the mRNA is available for hybridization. Most of the parameters affecting hybridization reactions *in vitro* have not been examined in oocytes, but preliminary findings suggest that 10 ng of anti-sense RNA per oocyte is sufficient to block the translation of 500 pg of mRNA per oocyte. Moreover, since no effort was made to deposit the anti-sense RNA and mRNA in the same region of the oocyte, the results suggest that the RNAs diffuse freely within the cell.

Hybridization with cloned DNA molecules can block translation of specific mRNAs *in vitro* (23). These so-called hybrid-arrested translations have shown that it is not necessary to form a duplex with the entire mRNA; hybrids formed with the 5′ untranslated region prevent translation (5). The data presented here are consistent with these studies in that an anti-sense RNA complementary to the 5′ untranslated region of the β-globin mRNA blocks translation. Moreover, anti-sense RNAs complementary to only the 3′ part of the globin mRNA are unable to block translation (Fig. 4). Perhaps these latter anti-sense RNAs are unable to form hybrids with the mRNA *in vivo* and, therefore, cannot affect translation. Alternatively, it may be the case that once ribosomes bind and initiate translation, the presence of an RNA·RNA duplex downstream has little or no effect, as if the ribosome can peel away the anti-sense RNA. In any case, a practical conclusion from these studies is that anti-sense RNA that covers the initiation codon and some of the 5′ untranslated region should be used for translation blocking experiments.

The use of anti-sense RNAs to block gene expression has the potential to help study viral and cellular gene functions in cells that are not amenable to conventional genetic analyses (1). For cells that are transcriptionally active, it is possible to supply a specific anti-sense RNA via a suitably constructed DNA plasmid and achieve a significant reduction in the expression of particular genes (1, 2, 4). Although further studies are required to determine whether protein synthesis can be entirely eliminated using this approach, the administration of specific anti-sense RNAs via DNA plasmids has certain attractions. For example, it may be possible to target the site and time at which anti-sense RNA is supplied by using tissue-specific or inducible promoters to control the expression of anti-sense RNA. In other circumstances, it may be preferable to directly inject *in vitro*-synthesized anti-sense RNAs into cells as described here. In particular, when the cells of interest are not transcriptionally active, such as during early frog development, the injection of anti-sense RNA into the cytoplasm may allow one to eliminate or substantially reduce the expression of specific genes. The stability of the RNA·RNA duplexes and consequent translation blocking observed in injected oocytes encourages us to try this approach in studying embryonic determination in frog development.

Note Added in Proof. R. Harland (personal communication) has recently found, in experiments similar to those reported here, that the translation of TK and chloramphenicol acetyltransferase mRNAs can be inhibited in oocytes by injection of anti-sense RNA.

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