Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1523–1527, April 1975

Two Classes of Translational Control RNA: Their Role in the Regulation of Protein Synthesis

[muscle/reticulocyte lysate/messenger ribonucleoprotein particles/poly(A)/mRNA]

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Communicated by Hewson Swift, January 27, 1975

ABSTRACT **Two classes of translational control RNA (tcRNA) have been isolated from embryonic chick muscle. One of these classes, the tcRNA isolated from messenger ribonucleoprotein particles (mRNP-tcRNA), is effective in inhibiting the translation of mRNP-mRNA while having little if any effect on polysomal mRNA. The other class, polysome-tcRNA, has no effect on mRNP-mRNA while it stimulates the translation of polysomal mRNA. The mRNP-tcRNA contains approximately 50% uridylate residues and forms small but stable hybrids with poly(A), while polysome-tcRNA contains fewer uridylate residues and is much less effective in forming a hybrid with poly(A). A proposed model concerning the role of these two classes of tcRNA in the regulation of protein synthesis is presented.**

In addition to the fact that eukaryotic cells have messenger ribonucleic acids with relatively long half-lives, there is considerable evidence that not all mRNAs are associated with ribosomes (1). These free or stored mRNAs appear to be maintained in an inactive form for a period of time in the cytoplasm. It, therefore, may be necessary for eukaryotic cells to regulate the utilization of long-lived mRNAs as well as to possess a means by which certain mRNAs can be maintained in an inactive form. The translational control of protein synthesis in eukaryotic cells may, therefore, involve both positive [protein specific factors (2–5)] and negative [translational control RNA, tcRNA (3)] control elements.

Translational control RNA (tcRNA) has been isolated from initiation factor preparations and has been demonstrated to specifically inhibit the translation of heterologous mRNAs (3). The inhibition by muscle tcRNA results from the inability of the globin mRNA to bind to ribosomes during the formation of the initiation complex when tcRNA is added to the cell-free system (6). Due to the specific nature of this inhibition, it is likely that the tcRNA interacts directly with mRNA. However, the involvement of initiation factors in this tcRNA-mediated inhibition cannot be excluded.

In order to determine if tcRNA is effective in the regulation of protein synthesis within the cell from which it is derived, we have isolated mRNA from both polysomes and messenger ribonucleoprotein particles (mRNPs) of embryonic chick muscles and tested the ability of muscle tcRNA to inhibit the translation of these mRNAs. We report here that tcRNA may be obtained from either the polysomal or mRNP sources. Both tcRNA preparations inhibit globin synthesis, while only the tcRNA obtained from mRNPs is effective in inhibiting the translation of muscle mRNAs; and, in this case, only the translation of mRNP-mRNA is inhibited. It therefore is likely that at least two classes of tcRNA are present in muscle. It was of particular interest that the tcRNA effective in inhibiting muscle mRNP-mRNA translation contains a poly(U) tract that is absent in the polysomal class of tcRNA. From these results, a model is proposed to explain the manner by which tcRNA specifically regulates the utilization of cytoplasmic mRNAs.

MATERIALS AND METHODS

The preparation of rabbit reticulocyte lysates and the conditions for cell-free protein synthesis were as described (3) except that 50 μM hemin and 15 μg of muscle initiation factor 3 (IF3) were present in the reaction mixture, which had a final volume of 0.1 ml. Incubations were for 30 min at 30°. Twenty-five one hundredth nmol each of 20 amino acids containing 8 μCi of ^{3}H-labeled amino-acid mixture (New England Nuclear Corp.) was added to each incubation mixture. Where indicated, 2 μg of tcRNA and 15 μg of poly(A)-containing mRNA from muscle were added to the cell-free, amino-acid incorporating systems.

The mRNA and tcRNA preparations were prepared from 14-day embryonic chick leg muscle in the following manner. Approximately 45–50 g of muscle was gently homogenized in 0.25 M KCl, 5 mM $MgCl_2$, 20 mM Tris·HCl (pH 7.4) as described (7). The 10,000 × *g* supernatant was layered on six sucrose density gradients [24 ml, 15–40% (w/w) gradient formed over a 3-ml 50% sucrose cushion], containing the same buffer and centrifuged at 25,000 rpm for 3.5 hr in an IEC-SB 110 Rotor. The gradients were subsequently analyzed on a Gilford spectrophotometer by continuous monitoring at 260 nm. A typical absorbance profile is shown in Fig. 1. Fractions A (polysomes) and D (cytoplasmic mRNPs) were collected from each gradient. The polysome (A) and mRNP (D) fractions were then centrifuged for 6 hr at 320,000 × *g*. The pelleted polysomes and mRNPs were subsequently used for preparation of mRNA and tcRNA fractions.

The polysomes and mRNP pellets were resuspended in 2 ml of 50 mM K^+ phosphate buffer (pH 6.8) containing 5 mM EDTA. The samples were dialyzed against 15 volumes of the same buffer for 4 hr at 2°. All glassware was acid-cleaned and baked at 350° for at least 6 hr. The polysomal tcRNA and mRNP tcRNA were prepared by chromatography of the respective dialysates on DEAE-cellulose as described (3). Five to 7 μg of each class of tcRNA was obtained from 45 to 50 g of muscle. Normally, the mRNPs (fraction D, Fig. 1) yielded slightly more tcRNA than did the polysomes.

After dialysis, the polysome and mRNP fractions were extracted with phenol by the method of Aviv and Leder (8) to

Abbreviations: tcRNA, translational control RNA; mRNP, messenger ribonucleoprotein particles.

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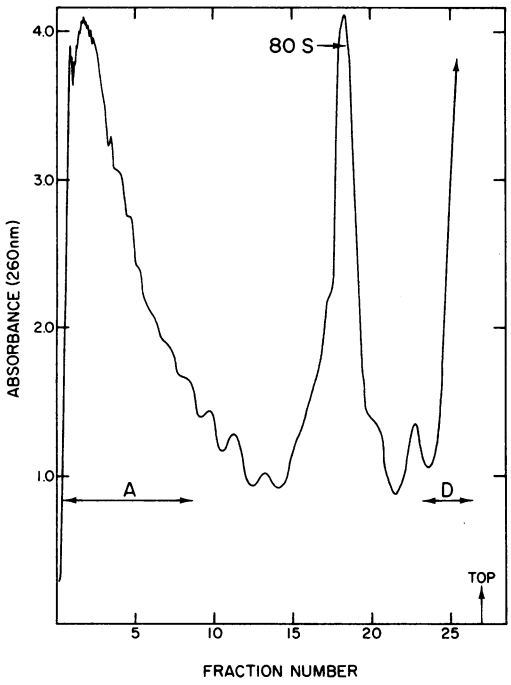

FIG. 1. Sucrose density gradient centrifugation of muscle cytoplasmic fractions. As explained in *Materials and Methods*, six of these gradients were used for preparation of polysomes (A) and mRNP fraction (D). Before analysis of the gradient at 260 nm, the sample layer consisting of 1.5 ml and an additional 0.5 ml were removed from the top of each gradient.

obtain the respective mRNA fractions. After alcohol precipitation of the phenol-extracted RNA, mRNA was isolated by its ability to bind oligo(dT)-cellulose (8). Therefore, all the mRNA subsequently used in the experiments reported here is poly(A)-containing mRNA. In order to remove most of the radioactive globin synthesized during the incubation, we dialyzed the cell-free systems against 55% $(NH_4)_2SO_4$ (pH 7.0) at 5° after completion of the incubation period. The precipitated radioactive proteins were subsequently analyzed by Na dodecyl sulfate-acrylamide gel electrophoresis on 7% gels, as described for myosin (2). The radioactivity of the proteins synthesized was determined by liquid scintillation counting after the acrylamide gels were sliced (2).

Poly(U) was hybridized with [^{3}H]poly(A) (Schwartz/Mann, specific activity 20 mCi/mmol), tcRNA (polysome and mRNP) with [^{3}H]poly(A), and muscle tRNA (2) with [^{3}H]poly(A) in 50 μl of 0.1 M NaCl, 50 mM Tris·HCl (pH 7.2), 2 mM EDTA at 28°. In each case, 10 μg of nucleic acid was added to 35,000 dpm of [^{3}H]poly(A) in the hybridization mixture. After 8 hr, the NaCl concentration was increased to 0.3 M, and 10 units each of T1 RNase and T2 RNase were added. The mixture was allowed to incubate for an additional 30 min. Finally, the RNase-digested hybridization mixture was analyzed by chromatography on a superfine Sephadex G-25 (Pharmacia) column (0.4 × 60 cm) equilibrated with 0.3 M NaCl, 50 mM Tris·HCl (pH 7.2), 2 mM EDTA. The flow rate was kept at 2.0 ml/hr and 0.2-ml fractions were collected. The amount of hybrid formation was determined by radioactivity eluting before the free nucleotides. Base analysis of the hybrid as well as of the two classes of tcRNA were performed by the method of Randerath and Randerath (9) on PEI-cellulose layers with stepwise elution (1.0 M acetic acid, followed by 0.3 M LiCl). The individual compounds were detected by UV light and extracted with 0.7 M $MgCl_2$, 20 mM Tris·HCl (pH 7.4); the absorbance at 260 nm was measured. Molar concentrations were calculated using the molar extinction values of nucleoside monophosphates obtained at 260 nm at neutral pH (9).

TABLE 1. *Amino-acid incorporation in a reticulocyte lysate after addition of different classes of muscle mRNAs and tcRNAs*

mRNA	tcRNA	cpm*
—	—	72,950
mRNP-mRNA	—	73,630
Polysome-mRNA	—	74,920
mRNP-mRNA	Polysome-tcRNA	56,100
mRNP-mRNA	mRNP-tcRNA	39,950
Polysome-mRNA	Polysome-tcRNA	54,850
Polysome-mRNA	mRNP-tcRNA	32,130

* Hot acid-precipitable radioactivity of the reaction mixture (*Materials and Methods*).

RESULTS

If tcRNA is to have a functional role in the cell, it must be shown to effect the utilization of mRNAs isolated from the same source from which the tcRNA is obtained. For this reason we have isolated poly(A)-containing mRNA from muscle polysomes as well as from muscle cytoplasm (mRNPs) (Fig. 1). This should yield two functional classes of mRNA, one being actively translated and one inactive or being stored in the cytoplasm of the cell. In addition, if muscle tcRNA is involved with the utilization of these muscle mRNAs, differences in the tcRNA obtained from these cellular fractions (polysomes and mRNPs) may be expected. As shown in Table 1, upon the addition of either polysomal or mRNP mRNA to the reticulocyte lysate, no significant increase in amino-acid incorporation occurs. As reported previously (3, 6), when muscle tcRNA is added to a cell-free system, a decrease in the total amount of radioactivity is observed. However, the mRNP-tcRNA is more effective than polysomal tcRNA in inhibiting protein synthesis (Table 1), although both classes of tcRNA reproducibly inhibit protein synthesis in this system. Due to the fact that nonpreincubated lysates are used, the decrease in radioactivity incorporated into protein upon the addition of the two classes of muscle tcRNA only reflects the effect on globin synthesis.

When the same experiment is analyzed, after most of the radioactive globin is removed by $(NH_4)_2SO_4$ precipitation, on Na dodecyl sulfate-acrylamide gel electrophoresis, the effect of the two classes of tcRNA on the translation of both polysomal and mRNP mRNAs can be analyzed. When muscle mRNA is not added to the incubation mixture, only one major radioactive peak is seen migrating where globin would be expected to be found on the gel (Fig. 2A). However, when either polysomal mRNA (Fig. 2B) or mRNP-mRNA (Fig. 2C) is added to the incubation mixture, discrete peaks of radioactivity are observed migrating throughout the gels. The peak observed on both gels at 0.5 cm migrates similarly to myosin heavy chains. This particular peak is always present when polysomal mRNA is present in the cell-free system, but is not reproducibly present when mRNP-mRNA is added. Although some similarities exist between the two gel patterns with these two classes of poly(A)-containing mRNA, there are noticeable

TABLE 2. *Base composition of mRNP-tcRNA and polysome-tcRNA*

	mRNP-tcRNA*		Polysome-tcRNA*	
Bases	nmol	%	nmol	%
AMP	8.0	12	8.6	19
CMP	18.4	28	15.4	34
GMP	7.8	12	8.4	19
UMP	31.2	48	12.8	28

* Ten micrograms of each RNA preparation were analyzed as described in *Materials and Methods*.

differences in the proteins synthesized when the products of the cell-free system are analyzed by Na dodecyl sulfate-acrylamide gel electrophoresis.

If the tcRNA preparation obtained from polysomes is added to the incubation mixture containing polysomal RNA (Fig. 2D), a small but reproducible increase in the radioactivity associated with the proteins synthesized under the direction of the muscle polysomal mRNA is observed. The only notable inhibitory effect is with the proteins migrating where globin is expected to be found on the gel. When the mRNP-tcRNA is added to a similar incubation mixture containing polysomal mRNA, a slight inhibition is observed (Fig. 2C), but not noticeably below that of the control (Fig. 2B). Even this slight inhibitory response of polysomal mRNA is not found reproducibly. Again it can be seem that the major inhibitory effect is on those proteins migrating during electrophoresis with the globin chains synthesized by the reticulocyte lysate.

When the tcRNA isolated from muscle polysomes is added to an incubation mixture containing mRNP-mRNA (Fig. 2E), no effect is seen on those proteins synthesized under the direction of muscle mRNP-mRNA (compare Fig. 2E and C). However, when the tcRNA isolated from mRNPs is added to a similar cell-free system, there is a complete inhibition of protein synthesis directed by these mRNAs (Fig. 2E). The amount of radioactivity incorporated into protein under these conditions (Table 1) is supposedly a result of an incomplete inhibition of globin synthesis. From the results presented in Fig. 2, it can be seen that one class of muscle tcRNA (that class isolated from mRNPs) effectively blocks the utilization of the mRNP-mRNAs, while the other class (polysome-derived) is ineffective in inhibiting the translation of mRNP-mRNA. The addition of polysome-tcRNA actually results in a small stimulatory response on the translation of polysomal mRNA.

In an attempt to explain the differences in behavior of the two classes of tcRNA on the translation of homologous mRNAs, a base analysis was performed. When 10 μg of each tcRNA was analyzed after base hydrolysis, the major difference between the two classes is the uridylate content (Table 2). The mRNP-tcRNA contains almost 50% uridylate, while polysome tcRNA contains approximately one-half that amount. The base composition of the two classes of tcRNA with regard to the other three bases is very similar. Upon analysis, no other bases were detectable by ultraviolet light absorption on the thin-layer chromatography plates.

The fact that mRNP-tcRNA appears to be almost 50% uridylate suggested that a tract of poly(U) may exist on this class of tcRNA which is lacking on the polysomal-tcRNA. In order to test this possibility, we planned to hybridize the

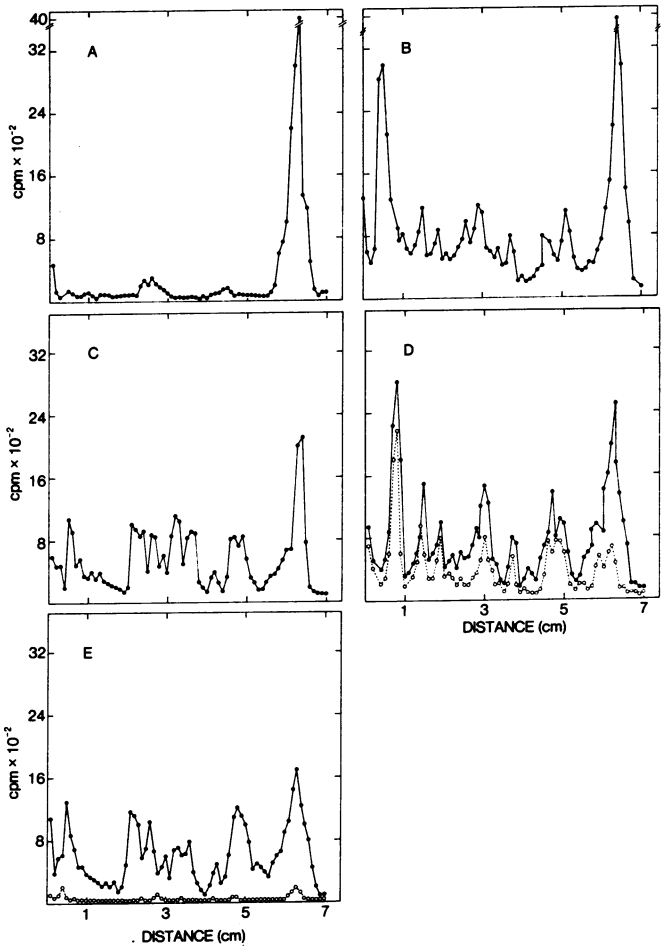

FIG. 2. Electrophoretogram of the products of the cell-free system under the direction of muscle polysome-mRNA and muscle mRNP-mRNA and the effect of two classes of tcRNA on their translation. After incubation, the reaction mixtures were treated with 55% $(NH_4)_2SO_4$ and the precipitates analyzed by acrylamide gel electrophoresis (*Materials and Methods*). (A) Reticulocyte lysate with no added mRNA or tcRNA, globin chains migrate to 6–6.7 cm under the conditions used here; (B) plus polysomal mRNA; (C) plus mRNP-mRNA; (D) ●, polysomal mRNA and polysome-tcRNA added; ○, polysomal mRNA and mRNP-tcRNA added; (E) ●, mRNP-mRNA and polysome-tcRNA added; ○, mRNP-mRNA and mRNP-tcRNA added to reaction mixture.

two tcRNA classes with [^{3}H]poly(A). However, a system had to be devised to detect the presence of small hybrids (possibly 30 nucleotides or less in length). When poly(U) is hybridized to [^{3}H]poly(A) and subsequently digested with T1 and T2 ribonucleases, a major radioactive peak elutes from the Sephadex G-25 column in the excluded volume (Fig. 3A). Following this is a broad spectrum of hybrids eluting in different size ranges until single nucleotides (fractions 47–57) are eluted. This column elution pattern suggested that hybrids of both small and large size could be detected by this means. In order to ascertain that complete digestion of the [^{3}H]poly(A) occurs after addition of T1 and T2 ribonucleases, [^{3}H]poly(A), treated in the same manner as the poly(U)·[^{3}H]-poly(A) hybrids, was chromatographed on the column. All of the radioactivity eluted as free nucleotides (Fig. 3A). In order to assure that nonspecific hybrids would not be formed,

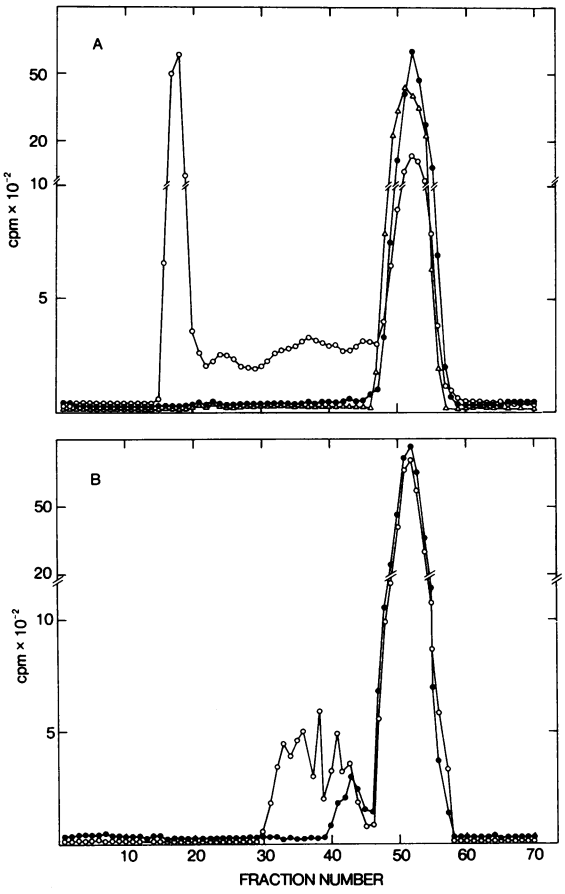

FIG. 3. (A) Chromatographic analysis of hybrids formed with poly(U) and [^{3}H]poly(A). Hybridization was performed as described in *Materials and Methods*. After T1 and T2 ribonuclease treatment, the hybrids formed were analyzed by chromatography on Sephadex G-25. After fractions were collected, the radioactivity was determined by scintillation counting. O, hybrids formed between poly(U) and [^{3}H]poly(A); ●, [^{3}H]poly(A) treated in similar manner and analyzed for resistance to T1 and T2 RNase digestion; △, muscle tRNA reacted with [^{3}H]poly(A) and analyzed for nonspecific hybrid formation. (B) Chromatographic analysis of hybrids formed between [^{3}H]poly(A) and mRNP-tcRNA (O), and polysome-tcRNA (●). Hybrid formation and analysis are as described in *Materials and Methods* and legend of panel A.

muscle tRNA was treated in a similar manner [allowed to react with [^{3}H]poly(A) under the conditions of poly(U)·[^{3}H]-poly(A) hybridization]. No detectable nonspecific hybrids were observed (Fig. 3A).

When the two classes of tcRNA were tested for poly(U) tracts by hybridization with [^{3}H]poly(A) and subsequent digestion with T1 and T2 ribonucleases, elution profiles of the radioactivity indicated that hybrids were formed using mRNP-tcRNA consisting of from 10 to 20 bases, while polysome-tcRNA showed only the presence of the smallest detectable hybrids (Fig. 3B). The size of the hybrids is estimated from vitamin B12 and cytochrome *c* elution properties. The base composition of the mRNP-tcRNA·[^{3}H]poly(A) hybrids shown in Fig. 3B after base hydrolysis was 10.2 nmol of AMP and 9.6 nmol of UMP. These results indicate that in order for tcRNA to be an effective inhibitor of protein synthesis, it must contain a poly(U) tract. Of course, the rest of the molecule must also be functionally important, for the inhibition of protein synthesis brought about by this molecule has been shown here and previously (3, 6) to be specific.

DISCUSSION

We have shown here that two functionally distinct classes of tcRNA are present in muscle. One of these specifically inhibits the translation of poly(A)-containing mRNA found in mRNPs, while having little or no effect on the translation of poly(A)-containing polysomal mRNA. This mRNP-tcRNA is also more effective in inhibiting globin synthesis in the reticulocyte lysate than the polysome-tcRNA. The polysome-tcRNA, in turn, has little, if any, inhibitory effect on the translation of either polysomal mRNA or mRNP-mRNA from muscle. In fact, it is found to stimulate the synthesis of proteins coded for by the polysomal mRNA. Bogdanousky *et al.* (10) have reported a small RNA isolated from reticulocyte ribosomal wash that stimulates α-globin synthesis. Therefore, it is likely that the polysome-tcRNA we observe in muscle may be of a similar class of tcRNA as that observed by Bogdanousky *et al.* (10) and different from the mRNP-tcRNA that inhibits the translation of specific mRNAs. Nevertheless, the finding that muscle tcRNA is active in regulating the utilization of muscle mRNAs lends support to a physiological role of this RNA species. The reason for the inhibition of globin synthesis in the heterologous cell-free system by muscle tcRNA is unclear (3, 6).

Upon base analysis of the two classes of tcRNA, it was observed that the mRNP-tcRNA contains a high percentage of uridine. This uridine appears to exist in a poly(U) tract in the molecule, for it forms a small but stable hybrid with [^{3}H]-poly(A). The polysome tcRNA differs from the mRNP-tcRNA in this regard, for it contains less uridine and is considerably less capable of forming a hybrid with [^{3}H]poly(A). The functional differences in the two classes of tcRNA noted above may therefore result from the presence or absence of a uridylate-rich region within the molecule.

Of interest to these observations is the finding by Molloy *et al.* (11) concerning the occurrence of uridylate-rich regions in heterogeneous nuclear RNA of HeLa cells. These regions, consisting of approximately 30 nucleotides, appear to be absent in mRNA and are not located at the 3′ end of heterogeneous nuclear RNA. The suggestion that this poly(U) region, in itself, functions in protein synthesis by forming a hybrid with the poly(A) on mRNA has been shown not to be the case, for: (*a*) the removal of poly(A) from mRNA does not appear to affect the translation of the mRNA (12), and (*b*) complexing poly(U) with the poly(A) on mRNA also has no effect on the translation of the mRNA (13). However, the mRNP-tcRNA is composed of only one-half uridine and still forms stable hybrids with [^{3}H]poly(A). Also, mRNP-tcRNA appears to be messenger specific. Therefore, the complete molecule may be functional in the recognition of both the 5′ and 3′ ends of mRNA.

On the basis of these observations, we propose the model shown in Fig. 4 as a working hypothesis concerning the synthesis and function of tcRNA. Heterogeneous nuclear RNA (HnRNA) is transcribed, containing a poly(U) tract located distal from the 3′ end. At the 3′ end, the mRNA is located which is subsequently adenylylated. This aspect of the model is based on the findings of Molloy *et al.* (11). The size of the poly(U) tract reported by Molloy *et al.* (11), is within size

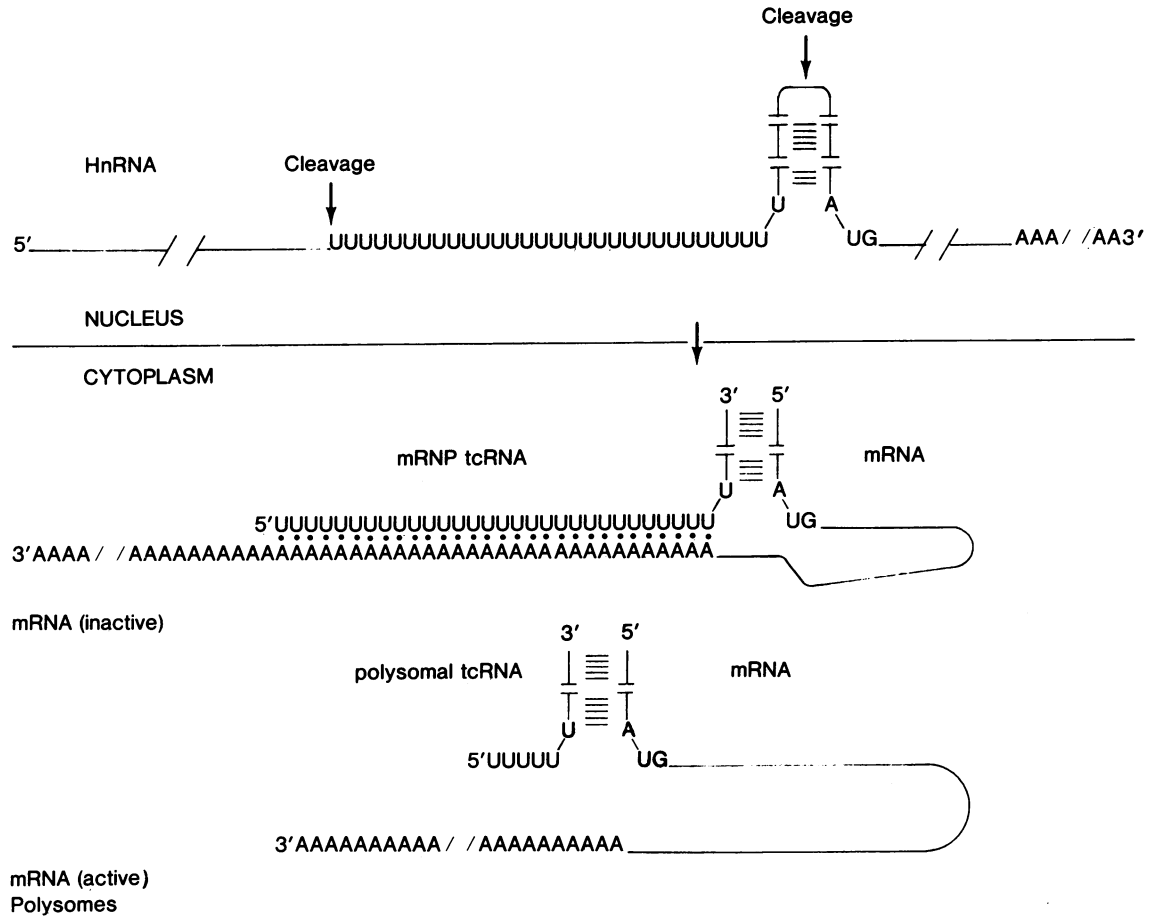

FIG. 4. A proposed model for the functional role of tcRNA in the regulation of protein synthesis. Hyphens have been omitted for brevity. HnRNA, heterogeneous nuclear RNA.

range of the poly(U)·[^{3}H]poly(A) hybrids we report from the mRNP-tcRNA. The proposed model, suggests that on the 3′ end of the poly(U) tract a sequence of nucleotides exists that will specifically recognize a sequence of nucleotides at the 5′ end of the mRNA. During the processing of the HnRNA, the portion of the molecule consisting of the poly(U) tract and adjacent nucleotides specific for the mRNA is maintained intact and becomes the tcRNA. This molecule, by virtue of its ability to hybridize with both ends of the mRNA, and to do so specifically, circularizes the mRNA, thereby making it inactive for protein synthesis. This is the mRNP (mRNA-tcRNA-protein) complex found in the cytoplasm. In the model, the poly(U) tract found on mRNP-tcRNA is hydrolyzed, opening up of the mRNA–tcRNA complex, thereby making it available for translation. In support of this aspect of the model is the similar base composition of polysome-tcRNA and mRNP–tcRNA with regard to adenosine, guanosine, and cytidine.

Although the model shown in Fig. 4 is only presented as a working hypothesis to aid in describing the results reported here, it also offers a direct link between transcription and translational control of protein synthesis in eukaryotic cells. In addition, it proposes a functional role of the poly(A) segment found on most eukaryotic mRNAs, although certainly a size discrepancy exists between the poly(A) and poly(U) segments of the different molecules. Additional evidence in support of the model showing (*i*) the requirement of the poly-(A) segment of mRNA for the tcRNA inhibition, (*ii*) the isolation of myosin specific tcRNA, and (*iii*) the expected size on acrylamide gels of polysomal and mRNP-tcRNA, is to be published elsewhere.

This work was supported by NIH Grant no. HD 03316. S.M.H. is a recipient of an NIH Career Development Award. The work was also supported by NCI CA 14733.

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