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Translational Regulation of Human Beta Interferon mRNA: Association of the 3′ AU-Rich Sequence with the Poly(A) Tail Reduces Translation Efficiency In Vitro

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The 3′ AU-rich region of human beta-1 interferon (hu-IFNβ) mRNA was found to act as a translational inhibitory element. The translational regulation of this 3′ AU-rich sequence and the effect of its association with the poly(A) tail were studied in cell-free rabbit reticulocyte lysate. A poly(A)-rich hu-IFNβ mRNA (110 A residues) served as an inefficient template for protein synthesis. However, translational efficiency was considerably improved when the poly(A) tract was shortened (11 A residues) or when the 3′ AU-rich sequence was deleted, indicating that interaction between these two regions was responsible for the reduced translation of the poly(A)-rich hu-IFNβ mRNA. Differences in translational efficiency of the various hu-IFNβ mRNAs correlated well with their polysomal distribution. The poly(A)-rich hu-IFNβ mRNA failed to form large polysomes, while its counterpart bearing a short poly(A) tail was recruited more efficiently into large polysomes. The AU-rich sequence-binding activity was reduced when the RNA probe contained both the 3′ AU-rich sequence and long poly(A) tail, supporting a physical association between these two regions. Further evidence for this interaction was achieved by RNase H protection assay. We suggest that the 3′ AU-rich sequence may regulate the translation of hu-IFNβ mRNA by interacting with the poly(A) tail.

The mRNA of human beta-1 interferon (hu-IFNβ) is very unstable, with a half-life of about 30 min (31). Like other transiently expressed mRNAs, hu-IFNβ mRNA contains an AU-rich motif at its 3′ untranslated region (UTR) (8). This motif was shown to confer instability and to reduce translational efficiency of mRNAs containing it (5, 19, 38). Recently, several proteins which specifically bind to the 3′ AU-rich sequences were identified in a variety of eukaryotic cells and were suggested to play a role in controlling mRNA half-life (4, 6, 13, 24, 42). Although the mechanisms by which the AU-rich sequence exerts its effects on translational efficiency and mRNA stability have not yet been demonstrated, some models have been proposed. Brewer and Ross (7) suggested for c-*myc* mRNA that the poly(A)-binding protein readily migrates from the poly(A) tail to the 3′ AU-rich sequence, leaving the poly(A) tract unprotected from nuclease attack. Another model suggested a possible base pairing between the poly(A) tail and the AU-rich sequence, which may enable nuclease attack at the points of mismatch (45). The 3′ poly(A) tail is a common feature of most of the eukaryotic mRNAs and was proposed to play a major role in mRNA processing, stability, and translation (2, 17, 27).

In this study, we attempted to elucidate the mechanism by which the 3′ AU-rich sequence regulates the translation of hu-IFNβ mRNA. Our results suggest that this translational regulation is achieved by a physical association between the 3′ AU-rich sequence and the poly(A) tail.

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MATERIALS AND METHODS

Oligonucleotides, plasmids, and enzymes. Oligodeoxynucleotides were prepared by using an Applied Biosystems 480B DNA synthesizer. Plasmids pGEM3 and pBluescript KS$^+$ were purchased from Promega Biotec and Stratagene, respectively. The hu-IFNβ clone pBR322/SV40/TK-IFN-G1 was a gift from S. Pestka (Rutgers Medical School, New Brunswick, N.J.). Restriction endonucleases, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA ligase, and T3 and T7 RNA polymerases were purchased from Boehringer Mannheim. Poly(A) was purchased from Sigma.

Subcloning. The *Hae*III-*Bam*HI fragment, corresponding to the full-length hu-IFNβ cDNA (from −94 to +764 according to reference 39) from pBR322/SV40/TK-IFNβ-G1 was subcloned into the *Eco*RV and *Bam*HI sites of pBluescript, downstream from the T3 promoter, to produce pFβ (Fig. 1A). Deletion of the AU-rich sequence from pFβ was performed by using Promega's Erase-a-Base deletion kit according to the manufacturer's protocol. Briefly, pFβ was digested with *Xba*I, adjacent to the 3′ end of hu-IFNβ cDNA, from which point exonuclease III digestion proceeded, and also by *Sac*II to produce a 3′ protruding end which is protected from digestion by exonuclease III. Following blunting of the DNA ends, the deleted constructs were religated. This deletion reaction yielded a plasmid which lacks 79 nucleotides (nt) from the 3′ end of hu-IFNβ. To facilitate further manipulation, the *Hin*dIII-*Sac*I (blunted) fragment from this plasmid was subcloned into *Hin*dIII and *Sma*I sites of pBluescript to produce pβD79 (Fig. 1C). Addition of poly(A) tails was performed by insertion of the *Hin*dIII-*Xba*I fragments from the hu-IFNβ constructs (pFβ and pβD79) into *Hin*dIII and *Xba*I sites of pGA110/11 (Fig. 1B). pGA110 contains a poly(A) tail of 110 residues (in pGEM3), whereas pGA11 is identical to pGA110 except that its poly(A) tail was shortened to 11 residues. The resulting

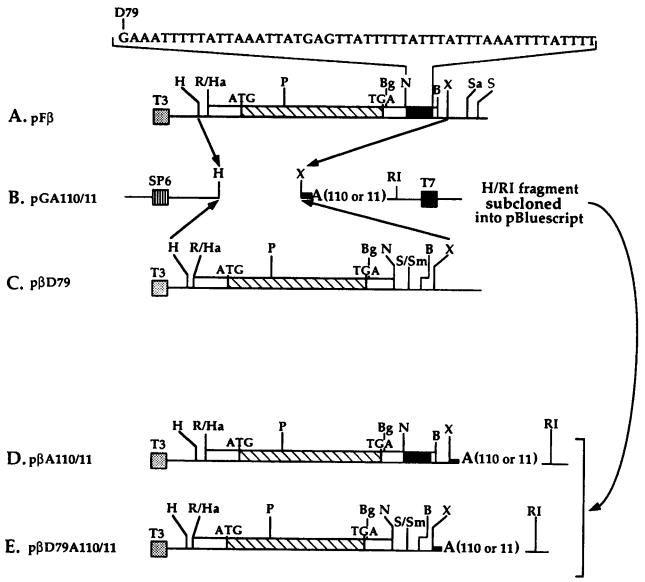

FIG. 1. Construction and schematic representation of the hu-IFNβ constructs used. *Hin*dIII-*Xba*I fragments from either pFβ (A) or pβD79 (C) were subcloned into the *Hin*dIII and *Xba*I sites of the poly(A)-containing vectors pGA110 and pGA11 in pGEM3 (B). The resulting constructs were then digested with *Hin*dIII and *Eco*RI; the DNA fragments were eluted from the gel and subcloned into the *Hin*dIII and *Eco*RI sites of pBluescript downstream from the T3 promoter to produce pβA110/11 (D) or pβD79A110/11 (E). Symbols: ▨, coding region; □, UTR; ■, AU-rich sequence. Restriction enzymes: H, *Hin*dIII; R, *Eco*RV; Ha, *Hae*III; P, *Pst*I; Bg, *Bgl*II; N, *Nde*I; B, *Bam*HI; X, *Xba*I; Sa, *Sac*II; S, *Sac*I; Sm, *Sma*I.

constructs were then digested with *Hin*dIII and *Eco*RI, and the IFNβ fragments were inserted into the same sites of pBluescript, downstream from the T3 promoter, to produce pβA110/11 and pβD79A110/11 (Fig. 1D and E). All subclones were sequenced (in full or at both ends) to ascertain their identity and orientation (36). The *Pst*I-*Bam*HI fragment (β5) from pFβ was subcloned into *Pst*I and *Bam*HI sites of pBluescript to produce pβ5. To construct the plasmids 3′βA110, 3′βA11, and 3′βD79A110, used for studying AU-rich sequence-binding activity, the corresponding plasmids βA110, βA11, and βD79A110 were digested with *Hin*dIII and *Nde*I and filled in with DNA polymerase I (Klenow fragment). The plasmid vectors were eluted from the gel and religated.

In vitro transcription. Plasmids were linearized by cleaving at the *Eco*RI site (leaving a 5′ protruding end [37]) immediately 3′ of the poly(A) tail. pβ5 was linearized by *Bam*HI. The linearized plasmids were treated with 50 μg of proteinase K per ml, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ammonium acetate-ethanol. The DNAs were then subjected to transcription by the appropriate RNA polymerase essentially as described previously (11). For production of mRNAs, the reactions were carried out in the presence of a cap structure [P^1-5′-(7-methyl)-guanosine-P^3-5′-GTP; Boehringer Mannheim]. A tracer amount of [^{3}H]UTP was also added to enable the calculation of the RNA yield. The reactions were terminated by adding RNase-free DNase (1 U/μg of DNA) for 15 min at 37°C and then subjected to extraction with phenol-chloroform and ammonium acetate-ethanol precipitation. The size and integrity of the transcripts were determined on denaturing gels (1.4% agarose, 10 mM CH_3HgOH), by ethidium bromide staining or by Northern (RNA) blot analysis (35). RNA probes were prepared essentially as described above, without adding cap structures, in the presence of 50 μCi of [α-^{32}P]UTP (400 Ci/mmol).

RNase H protection assay. Evidence for interaction between the AU-rich sequence and the poly(A) tail was achieved by RNase H protection assay essentially as described previously (43). Ten femtomoles of ^{32}P-labeled β5 was annealed to 10 ng of the appropriate deoxyoligonucleotide (as a positive control, we used an antisense oligodeoxynucleotide to the sense β5 RNA from +707 to +734, designated dTA28) in a solution containing 25 mM Tris hydrochloride (pH 7.5), 200 mM NaCl, and 5 mM $MgCl_2$. The reaction mixture was heated at 85°C for 2 min, rapidly cooled to 37°C, and then allowed to cool slowly to room temperature. Dithiothreitol was added to a final concentration of 1 mM; the samples were incubated with 0.5 U of RNase H (Amersham) for 30 min at 37°C and then subjected to extraction with phenol-chloroform-isoamyl alcohol (25:24:1). RNA was precipitated with ethanol in the presence of 10 μg of glycogen as carrier (Boehringer Mannheim), resolved on a 5% polyacrylamide gel containing 8 M urea, and subjected to autoradiography.

In vitro translation. Various RNA transcripts (4 μg/ml) served as templates for protein synthesis in a rabbit reticulocyte lysate (Amersham and Promega). The reactions were carried out in the presence of 35 μCi of L-[^{35}S]methionine (>800 Ci/mmol) according to the kit's protocol and with the addition of RNasin (1 U/μl). Samples were taken every 15 min for determination of methionine incorporation. After 60 min, the reaction products were also analyzed by electrophoresis on 12.5 to 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (22) and by autoradiography. For analysis of mRNA stability, samples were taken at various times during the course of incubation, and RNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and analyzed by Northern blotting (36).

Polysome isolation. Equal amounts of ^{3}H- and ^{32}P-labeled mRNAs were mixed and subjected to translation in a rabbit reticulocyte lysate at a concentration of 4 μg/ml. After 20 min of incubation at 30°C, 0.5 ml of ice-cold polysome buffer, containing 0.2 M sucrose, 0.2 M Tris hydrochloride (pH 8.5), 35 mM $MgCl_2$, 25 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 0.1 M KCl, 50 μg of cycloheximide per ml, and 0.5% Nonidet P-40 (12), was added, and the samples were layered on 15 to 50% linear sucrose gradients buffered with 40 mM Tris hydrochloride (pH 8.5), 100 mM KCl, 20 mM $MgCl_2$, and 10 mM EGTA. Polysomes were separated by centrifugation at 175,000 × *g* for 110 min at 4°C in an SW41 rotor. Gradients were fractionated from the top into 0.25-ml aliquots with an ISCO model 640 density gradient fractionator. Purified polysomes (5 A_{260} units) from developing wheat grains were added to some extracts to provide markers for polysome size. Fractions from gradients containing the wheat polysomes were analyzed for A_{254}. Radioactivity in trichloroacetic acid-precipitable RNA from each fraction was measured by scintillation spectroscopy. Controls treated with RNases A (50 μg/ml) and T_1 (1,000 U/ml) at 30°C for 15 min prior to fractionation indicated the presence of a small amount of nonpolysomal material throughout the gradient, ranging from 15 cpm at the bottom to 200 cpm near the monosome fraction (data not shown). To correct for this, the background counts were subtracted accordingly from each fraction. To facilitate comparison of the different RNAs, results were plotted as a percentage of the total (26).

Cell culture and extract preparation. Human fibroblast SV80 cells (41), kindly provided by J. Chebath (Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot, Israel), were grown to 80% confluence in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 5% newborn calf serum at 37°C under 5 to 8% CO_2. The cells were collected, washed twice in phosphate-buffered saline, and lysed by freeze-thawing in 25 mM Tris hydrochloride (pH 7.5)–0.5% Nonidet P-40. The homogenate was centrifuged at 12,000 × *g* for 10 min; the supernatant was collected and stored at −80°C until used.

UV cross-linking and label transfer. Transfer of radioactivity from RNA probes to RNA-binding proteins was carried out essentially as described previously (42). Ten to 15 fmol of ^{32}P-labeled RNAs was incubated with 10 to 15 μg of cytoplasmic proteins extracted from human fibroblast SV80 cells (41) in 20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.9)–60 mM KCl–10% glycerol–1 mM $MgCl_2$ in a total volume of 10 μl. After 10 min of incubation at room temperature, heparin was added to a final concentration of 5 mg/ml (18), and incubation was continued for 10 min at room temperature. The reaction mixtures were then placed in a 96-well culture dish and cross-linked by 254-nm radiation, using a Stratalinker cross-linking apparatus (energy = 240 mJ/cm^2). RNase A (50 μg/ml) and RNase T_1 (1,000 U/ml) were added, and the mixtures were incubated at 37°C for 20 min. An equal volume of 2× SDS loading buffer was added, and samples were heated to 100°C for 5 min and subjected to electrophoresis in 10 to 12% polyacrylamide gels containing 0.1% SDS. The gels were vacuum dried, and ^{32}P-labeled proteins were visualized by autoradiography.

RESULTS

The 3′ AU-rich sequence of hu-IFNβ mRNA has previously been shown to play a major role in translational regulation. We tested the hypothesis that this effect was due to interaction between the 3′ AU-rich sequence and the poly(A) tail. For this study, four hu-IFNβ constructs were prepared (Fig. 1D and E). Two constructs contained a full-length hu-IFNβ mRNA (Fβ) with a short or long poly(A) tail (pβA11 and pβA110), and two constructs contained a mutated form lacking the last 79 nt of the 3′ UTR (D79; Fig. 1A) with a short or long poly(A) tail (pβD79A11 and pβD79A110). The effects of the 3′ AU-rich sequence and of the poly(A) tail on translational efficiency were studied in a cell-free rabbit reticulocyte lysate system. Linearized templates were transcribed in vitro, and the integrity of the resulting mRNA transcripts was analyzed on a 1.4% agarose–10 mM methylmercuric hydroxide gel either by ethidium bromide staining (Fig. 2, lanes 1 to 4) or by Northern blotting (Fig. 2, lanes 5 to 8). For each of the constructs, a single mRNA species which migrated at the appropriate size for each mRNA transcript was obtained. Equal amounts of each of the four mRNAs were subjected to translation in a rabbit reticulocyte cell-free system. Figure 3 shows that translation of hu-IFNβ mRNA containing a long poly(A) tail (βA110) was significantly lower (threefold) than translation of its counterpart harboring a short poly(A) tail (βA11). In the case of βD79 mRNAs (βD79A110 and βD79A11), in which the AU-rich sequence was deleted, translation was no longer affected by the length of the poly(A) tail (Fig. 3B). Testing the in vitro translation efficiency of a synthetic hu-IFNβ mRNA containing a poly(A) tail of 55 residues (derived from pSP65A [12]), we found the same pattern of inhibition as with 110 A residues (data not shown).

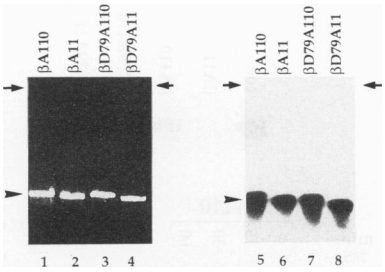

FIG. 2. Analysis of the in vitro-transcribed mRNA transcripts. T3-transcribed RNAs (100 ng of each transcript) were subjected to electrophoresis on a 1.4% agarose–10 mM methylmercuric hydroxide gel and resolved by ethidium bromide staining (lanes 1 to 4) or by Northern blot analysis with the ^{32}P-β5 antisense construct (lanes 5 to 8). The transcripts are identified above the lanes. Arrows indicate the positions of the loading wells, and arrowheads indicate the positions of migration of hu-IFNβ mRNA transcripts.

The possibility existed that the reduced translation of the hu-IFNβ mRNA containing the AU-rich sequence and a long poly(A) tail was due to reduced mRNA stability. This was tested by analyzing the stability of the various mRNAs by Northern blotting at the end of the translation period. Strikingly, following 60 min of incubation in rabbit reticulocyte lysate, only the inefficiently translated mRNA (βA110) remained intact, whereas no intact forms of the more effi-

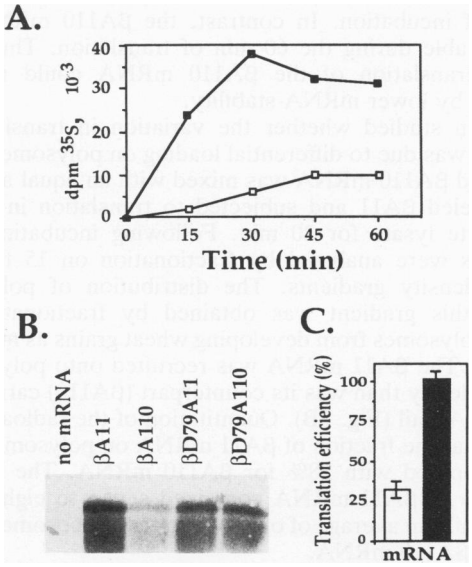

FIG. 3. Translational efficiency of hu-IFNβ transcripts. (A) Rate of [^{35}S]methionine incorporation into proteins of reactions directed by βA11 mRNA (filled squares) or by βA110 mRNA (open squares). (B) Various hu-IFNβ mRNAs (indicated above the lanes) were translated for 60 min in rabbit reticulocyte lysate at a final concentration of 4 μg/ml and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. (C) The relative intensity of the hu-IFNβ protein band translated from either βA11 (filled bar) or βA110 (open bar) mRNA was analyzed by densitometry (average of four different experiments). The average intensity of the translation product of βA11 mRNA was considered 100%. The thin vertical bars represent the standard deviations. The intensity of bands translated by βA11 was significantly different at the 0.05 level as measured by a Duncan test.

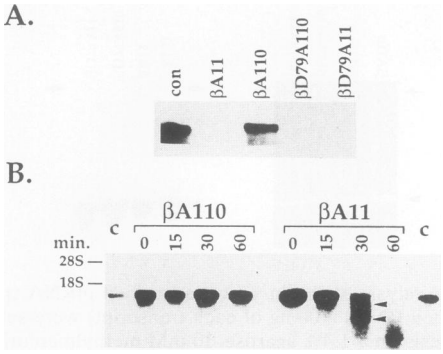

FIG. 4. Stability of hu-IFNβ mRNA transcripts in rabbit reticulocyte lysate. (A) The various hu-IFNβ mRNAs were subjected to translation for 60 min, and mRNAs were then extracted and analyzed by Northern blotting. (B) Stability of the βA110 and βA11 mRNAs during the course of translation. mRNAs and the incubation times are identified above the lanes. C refers to a positive control marker of βA11 mRNA before translation. Every lane in panels A and B was loaded with 2.5 μg of total reticulocyte RNA. The positions of 18S and 28S rRNAs are indicated. Arrowheads indicate degradation products of the βA11 mRNA transcripts.

ciently translated mRNAs could be detected on the autoradiogram (Fig. 4A). Closer analysis (Fig. 4B) revealed that the βA11 mRNA, which was efficiently translated, was degraded during translation. After 30 min of translation, a major part of the βA11 mRNA was either shortened (arrowheads in Fig. 4B indicate two smaller fragments of discrete length) or degraded, and no intact βA11 mRNA could be detected after 60 min of incubation. In contrast, the βA110 mRNA remained stable during the 60 min of translation. Thus, the reduced translation of the βA110 mRNA could not be explained by lower mRNA stability.

We then studied whether the variation in translational efficiency was due to differential loading on polysomes. The ^{32}P-labeled βA110 mRNA was mixed with an equal amount of ^{3}H-labeled βA11 and subjected to translation in rabbit reticulocyte lysate for 20 min. Following incubation, the polysomes were analyzed by fractionation on 15 to 50% sucrose density gradients. The distribution of polysome sizes in this gradient was obtained by fractionation of purified polysomes from developing wheat grains as markers (Fig. 5A). The βA11 mRNA was recruited onto polysomes more efficiently than was its counterpart (βA110) carrying a long poly(A) tail (Fig. 5B). Quantitation of the radioactivity showed that the fraction of βA11 mRNA on polysomes was 42%, compared with 28% for βA110 mRNA. The largest polysomes of βA11 mRNA contained seven to eight ribosomes, while an average of only two to three ribosomes were bound to βA110 mRNA.

The translation study suggested that both the 3′ AU-rich region and the poly(A) tail were essential for the inhibition of βA110 mRNA translation. We hypothesized that a physical association between these two regions led to translation inhibition. We performed an RNA binding assay, using the label transfer procedure (46), to examine the potential interaction between the AU-rich region and the poly(A) tail. A physical association between the AU-rich sequence and the poly(A) tail was expected to mask some of the AU-binding sites and hence to reduce the AU-specific binding activity. As a source for AU-binding proteins, we used a cytoplasmic extract from SV80 human fibroblast cells, which naturally express interferons and were therefore expected to contain

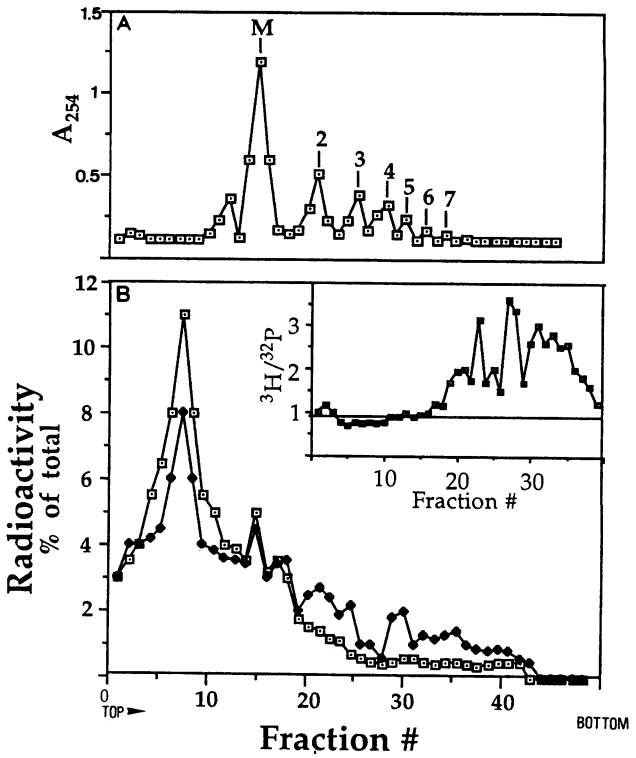

FIG. 5. Polysomal distribution of βA110 and βA11 mRNAs in rabbit reticulocyte lysate. Polysomes were fractionated on a 15 to 50% sucrose gradient, and fractions were collected from the top of the gradient. (A) Fractionation of five A_{260} units of purified polysomes from developing wheat grains, used as markers for polysome size. Fractions were analyzed for A_{254}. M indicates the monosome, and the numbers 2 to 7 indicate the polysome size in ribosome units. (B) Equal amounts of ^{32}P-labeled βA110 mRNA (open squares) and ^{3}H-labeled βA11 mRNA (filled squares) were mixed, subjected to translation at 30°C for 20 min, and fractionated on the sucrose gradients. The trichloroacetic acid-precipitated radioactivity in each fraction is plotted as a percentage of total radioactivity. The inset is the ratio between ^{3}H-βA11 and ^{32}P-βA110 along all gradients.

relatively high levels of AU-binding proteins. The RNA probes used for these experiments corresponded to the 3′ UTR of either AU^{+} (βA110 and βA11) or AU^{-} (βD79A110) mRNA (Fig. 6A). To select specifically for binding to the AU-rich sequence and not to the poly(A) tail, the RNAs were labeled with [α-^{32}P]UTP. The AU^{+} RNA probes (3′βA110 and 3′βA11) transferred their radioactivity to proteins to form three major complexes that migrated between 33- and 50-kDa markers (Fig. 6B, lanes 1 and 2, arrows). Only very faint bands could be detected on the autoradiogram when the extract was incubated with an AU^{-} RNA probe, 3′βD79A110 (Fig. 6B, lane 3). This finding implied that the proteins bound specifically to the AU-rich sequence. Moreover, the AU-binding proteins bound two times less efficiently (as indicated by densitometry analysis; data not shown) to the RNA probe containing the long poly(A) tail (3′βA110; Fig. 6B, lane 1 versus lane 2). The specificity of protein binding to the 3′βA110 RNA was also assessed by competition experiments (Fig. 6B, lanes 4 to 6). A 50-fold molar excess of unlabeled homologous RNAs (3′βA110) clearly reduced the label transfer to the three polypeptides (compare lane 4 with lane 5), while a 100-fold molar excess of unlabeled nonhomologous RNA (βBgl) did not reduce the

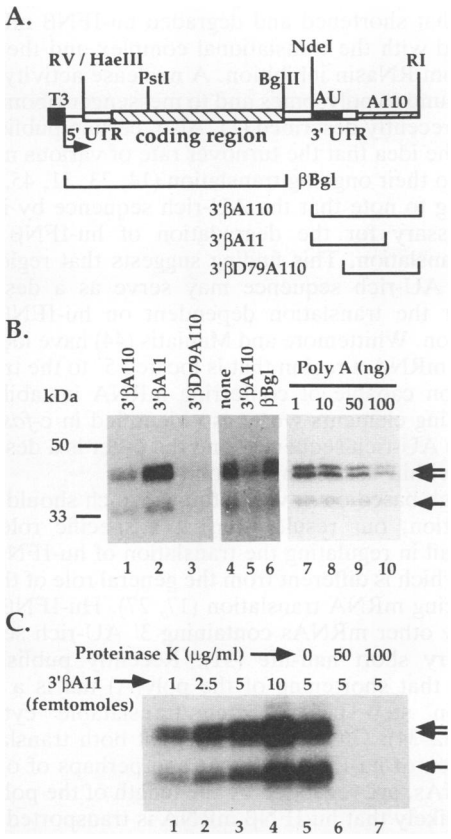

FIG. 6. Evidence that the 3′ poly(A) tail reduces the 3′ AU-rich sequence-binding activity. (A) Schematic representation of the hu-IFNβ gene. RI and RV indicate the sites for *Eco*RI and *Eco*RV, respectively. The locations of the competitor βBgl and of the 3′ RNA probes are depicted below the diagram of the gene. (B) AU-rich sequence-binding activity and competition experiments. [^{32}P]UTP-labeled probes were mixed with 15 μg of SV80 cytoplasmic extract. Following UV cross-linking and RNase treatment, ^{32}P-labeled proteins were analyzed by autoradiography of a 10% polyacrylamide gel containing 0.1% SDS. Lanes: 1 to 3, binding to the RNA probes 3′βA110, 3′βA11, and 3′βD79A110 (10 fmol) without competitors; 4 to 6, binding to the RNA probe 3′βA110 in the presence of no competitor, a 50-fold molar excess of homologous RNA competitor (3′βA110), and a 100-fold molar excess of nonhomologous competitor (βBgl); lanes 7 to 10, samples in which poly(A) was applied to the binding assay at the amounts indicated above the lanes. (C) The cytoplasmic extract was incubated with increasing amounts (indicated above the lanes) of ^{32}P-labeled 3′βA11 RNA (lanes 1 to 4) or preincubated with the indicated concentrations of proteinase K at 37°C for 15 min and then reacted with the RNA probe 3′βA11 (lanes 5 to 7). Specific complexes are indicated by arrows at the right (B and C), and positions of molecular size markers are indicated at left (B).

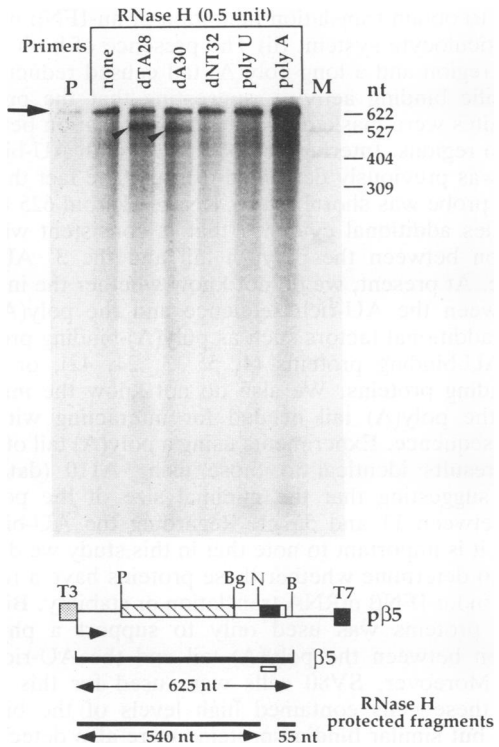

FIG. 7. Interaction of the polydeoxyadenosine within the AU-rich region. RNase H protection assays were performed in which various polydeoxynucleotides and polyribonucleotides (indicated above the lanes) were first annealed with 10 fmol of ^{32}P-labeled β5 RNA and subjected to digestion with 0.5 U of RNase H at 37°C for 30 min. The protected fragments were analyzed on a 5% polyacrylamide gel containing 8 M urea and by autoradiography. M indicates the size markers of the ^{32}P-labeled *Msp*I fragments of pBR322. dTA28 is the positive control; the arrow indicates the position of the β5 RNA probe, and arrowheads indicate the RNase H-protected fragments. A diagram of the plasmid used (pβ5) is shown schematically at the bottom. The lengths of the probe (β5) and its expected protected fragment are shown. The horizontal open bar indicates the oligodeoxynucleotides annealed to the AU-rich region (filled box) of the β5 RNA probe.

binding activity (compare lane 4 with lane 6). Addition of exogenous poly(A) to the binding reaction mixture containing the 3′βA11 RNA probe (10 fmol) also reduced the AU-specific binding activity (Fig. 6B, lanes 7 to 10). Figure 6C shows that the AU-binding activity increased linearly with increasing amounts of the probe (3′βA11) from 1 to 10 fmol (lanes 1 to 4). Moreover, the binding activity involved protein factors inasmuch as no complexes were formed when the extract was preincubated with proteinase K (Fig. 6C, lanes 5 to 7).

The interaction between the poly(A) tail and the AU-rich sequence was also addressed by RNase H protection assay. ^{32}P-labeled β5 RNA was annealed to various deoxyoligonucleotides and polyribonucleotides and subjected to digestion with RNase H. Figure 7 shows that the presence of the positive control dTA28 and the polydeoxyadenosine (dA30) in the annealing mixture caused the shortening of the β5 RNA probe from 625 nt to about 540 nt (Fig. 7, arrowheads), indicating that the polydeoxyadenosine indeed interacted within the AU-rich region. No shortening was evident when the RNA probe was annealed with dNT32 or with polyribonucleotides [poly(A) and poly(U)].

DISCUSSION

Previous reports demonstrated that the 3′ AU-rich consensus sequence (UUAUUUAU) of hu-IFNβ mRNA inhibited its translation (19–21). In the present report, we showed that the translation of hu-IFNβ mRNA was affected by both the 3′ AU-rich region and the poly(A) tail. Several lines of evidence suggest that interaction between the AU-rich region and the poly(A) tail may lead to translation inhibition. (i) Both the 3′ AU-rich sequence and the poly(A) tail were

essential to obtain translation inhibition of hu-IFNβ mRNA in the reticulocyte system. (ii) The presence of both the 3′ AU-rich region and a long poly(A) tail caused reduction in AU-specific binding activity, suggesting that the protein-binding sites were masked by physical association between these two regions. Interference of poly(A) with AU-binding activity was previously described (6). (iii) The fact that the β5 RNA probe was shortened by RNase H from 625 to 540 nt provides additional evidence that is consistent with an interaction between the poly(A) tail and the 3′ AU-rich sequence. At present, we do not know whether the interaction between the AU-rich sequence and the poly(A) tail requires additional factors such as poly(A)-binding proteins (2, 3), AU-binding proteins (4, 5, 13, 24, 42), or other RNA-binding proteins. We also do not know the minimal size of the poly(A) tail needed for interacting with the AU-rich sequence. Experiments using a poly(A) tail of 55 nt yielded results identical to those using A110 (data not shown), suggesting that the minimal size of the poly(A) ranges between 11 and 55 nt. Regarding the AU-binding proteins, it is important to note that in this study we did not attempt to determine whether these proteins have a regulatory role in hu-IFNβ mRNA translation or stability. Binding of these proteins was used only to support a physical interaction between the poly(A) tail and the AU-rich sequence. Moreover, SV80 cells were used for this study because these cells contained high levels of the binding proteins, but similar binding proteins were also detected in the reticulocyte lysate, although at a much reduced level (data not shown).

The mechanism by which the association between the AU-rich sequence and the poly(A) tail regulates the translation of hu-IFNβ mRNA is not known. One way to explain the translational inhibition is via the formation of double-stranded RNA followed by activation of the double-stranded RNA-dependent protein kinase (dsI [10, 16]). This possibility is of a particular interest inasmuch as some of the antiviral mechanisms elicited by interferons involve the regulation of enzymes, such as dsI and 2′-5′ oligo(A) synthetase, by double-stranded RNA (30). However, we could not detect any activation of dsI in rabbit reticulocyte lysate upon addition of the βA110 mRNA (data not shown). Another likely explanation is that at least part of the translational inhibition was due to masking of the poly(A) tail. Previous studies showed that poly(A) tails have a central role in enhancing mRNA translation in rabbit reticulocyte lysate (9, 15, 26, 27) and in a variety of other organisms (17, 25, 27, 28, 33). The poly(A) tail was also suggested to play an important role in the pattern of loading of the mRNA on ribosomes (9, 12, 26). It has been demonstrated in various systems that deadenylated mRNAs are recruited less efficiently onto polysomes than are their counterparts carrying poly(A) tails (9, 12, 17, 26, 27). Strikingly, the pattern of loading of hu-IFNβ mRNA carrying a long poly(A) tail on ribosomes was very similar to that of deadenylated mRNAs. Thus, we suggest that the wild-type hu-IFNβ mRNA was translated as a deadenylated mRNA as a result of masking of its poly(A) tail.

The hu-IFNβ mRNAs that were translated more efficiently in the reticulocyte lysate were also less stable. These data suggest that degradation of the hu-IFNβ mRNA was linked to its ongoing translation. The degradation in the reticulocyte lysate is of a particular interest, since this system is known to lack free RNases (29), and in addition, our reaction mixture also contained an RNase inhibitor, RNasin (35). It therefore seems likely that the nucleolytic activity that shortened and degraded hu-IFNβ mRNA was associated with the translational complex and thereby protected from RNasin inhibition. A nuclease activity which is tightly bound to polysomes and to messenger ribonucleoprotein was recently described (1). Also, certain published data support the idea that the turnover rate of various mRNAs is coupled to their ongoing translation (14, 23, 31, 45, 47). It is interesting to note that the AU-rich sequence by itself was not necessary for the degradation of hu-IFNβ mRNAs during translation. This finding suggests that regions other than the AU-rich sequence may serve as a destabilizing signal for the translation dependent on hu-IFNβ mRNA degradation. Whittemore and Maniatis (44) have identified in hu-IFNβ mRNA a region that is located 5′ to the translation stop codon capable of conferring mRNA instability. Two destabilizing elements were also identified in c-*fos* mRNA; one is the AU-rich sequence, and the other is a destabilizing element within the coding region (40).

Although based on in vitro studies which should be taken with caution, our results suggest a specific role for the poly(A) tail in regulating the translation of hu-IFNβ mRNA in vivo, which is different from the general role of this region in enhancing mRNA translation (17, 27). Hu-IFNβ mRNA, like many other mRNAs containing 3′ AU-rich sequences, has a very short half-life (31). Recently published data indicated that shortening of the poly(A) tail is a potential maturation step in preparing translatable cytoplasmic mRNA (32–34). Our data suggest that both translation and degradation of hu-IFNβ mRNA, and perhaps of other AU-rich mRNAs, are regulated by the length of the poly(A) tail. It seems likely that hu-IFNβ mRNA is transported from the nucleus to the cytoplasm as a translation-incompetent poly(A)-rich stable mRNA, which may become translationally active and at the same time also very labile upon shortening of its poly(A) tail. This may ensure an efficient regulation of hu-IFNβ synthesis.

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