Influence of Duplexes 3′ to the mRNA Initiation Codon on the Efficiency of Monosome Formation†

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ABSTRACT: The structural features of mRNA molecules that determine their relative translational rates are at present poorly defined. An early and potentially rate-limiting step in this process is the assembly of an intact 80S ribosome at the translational initiation codon. It is generally assumed that the efficiency of this reaction is controlled by structures in the 5′ nontranslated region and in the immediate proximity of the AUG initiation codon. In this paper, we present an assay of initial monosome formation and measure the effects of hybridizing mRNA to complementary DNA fragments on the efficiency of this reaction. This hybridization serves to block specific regions of the mRNA from sequence-specific and intramolecular (secondary structure) interactions. We find that cDNAs that block the 5′ nontranslated region, the initiation codon, or regions immediately 3′ to the initiation codon markedly inhibit 80S ribosome attachment. These results are consistent with previous studies by ourselves and others which suggest that the introduction of secondary structures into this region can result in decreased translational efficiency. In addition, however, we note that cDNAs that hybridize to segments of the coding region significant distances (as many as several hundred bases) 3′ to the initiation codon can also inhibit initial ribosome binding. This effect appears to be limited to duplexes within the mRNA coding region since a cDNA hybridizing exclusively within the 3′ nontranslated region does not inhibit, and may actually stimulate, monosome formation. The results of this monosome formation assay therefore suggest that mRNA structures remote from the 5′ terminus and initiation codon may also be important in determining the efficiency of translational initiation.

Gene expression can potentially be regulated at any step from the transcription of a structural gene to the final processing of its protein product. In eukaryotic cells, where transcription and translation are compartmentalized and where mRNAs can be relatively stable, the relative efficiencies with which different mRNAs are translated can significantly influence the pattern of proteins produced. Several examples have now been described in which translational controls significantly affect cell phenotype and function (Babich et al., 1983; Ballinger & Pardue, 1983; Cordell et al., 1982; DiDomenico et al., 1982; Lodish, 1971; Rosenthal et al., 1980); these controls require that the translational system be able to discriminate between different mRNAs and translate them with different efficiencies. Detailed studies in several translational systems suggest that a variety of different mechanisms may allow such discrimination to occur [e.g., during translation of poliovirus (Sonenberg & Lee, 1982) and adenovirus (Reichel et al., 1985) mRNAs and during ribosomal protein synthesis (Warner et al., 1985)]. However, the control mechanisms involved in each of these cases appear to be specific and may not represent the more general mechanisms of translational control.

The structural basis by which a translational system can discriminate between two capped mRNAs and translate them with different efficiencies is not well understood. While the primary structure of an mRNA surrounding an AUG triplet can influence its utilization as an initiation codon (Kozak, 1983), mRNA secondary structure may play a critical role in determining the efficiency of ribosome binding and assembly (Baim et al., 1985; Kozak, 1980, 1986a,b; Pelletier & Sonenberg, 1985). Although most studies concerning the role of mRNA secondary structure in the regulation of translational initiation have focused on the 5′ nontranslated region and sequences immediately 3′ to the AUG, the possibility that sequences in mRNA significantly 3′ to the AUG might also influence ribosome binding efficiency must be considered as well. A contribution of coding region secondary structures to the modulation of ribosome binding activity would expand the potential complexity of translational controls since these structures undergo significant and dynamic changes during the translation process (Kolter & Yanofsky, 1982; Shakin & Liebhaber, 1986b). Such a dynamic state could, for example, serve to differentiate actively translating mRNAs from untranslated or newly initiated mRNA molecules.

In this paper, we establish an assay for initial 80S–mRNA complex formation and use it to evaluate how site-specific perturbations of the secondary and higher order structures of human β-globin mRNA affect the efficiency with which it binds ribosomes. We find that the ribosome binding activity of this mRNA can be substantially altered not only by structural perturbations, which involve regions 5′ to and immediately surrounding the AUG initiation codon, but also by structural perturbations, which involve segments of the coding region remote from the AUG initiation codon. Since the higher order structure of the coding region of an mRNA molecule is dynamically altered during translation, the ribosome binding activity of an mRNA may also vary during its translation, reflecting these dynamic structural changes.

Experimental Procedures

Preparation and Analysis of End-Labeled Human Globin mRNA. Human globin mRNA was isolated from the blood of an adult with sickle cell anemia by phenol/chloroform

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extraction of acid-precipitated reticulocyte ribonucleoproteins as previously described (Liebhaber & Kan, 1982); poly(A+) RNA was isolated from total reticulocyte RNA by column chromatography on oligo(dT)–cellulose (Maniatis et al., 1982). The α- and β-globin mRNAs in poly(A+) reticulocyte RNA were 3′ end labeled with [5′-^{32}P]pCp (cytidine 3′,5′-[5′-^{32}P]-bisphosphate) and T4 RNA ligase and were resolved by electrophoresis on 3.5% acrylamide/8 M urea gels, all as previously described (Shakin & Liebhaber, 1986a,b). Band intensities on gel autoradiographs were quantitated by using a soft laser scanning densitometer with computer integrating capability (Zeineh, Model SL-504-XL, Biomed Instruments, Fullerton, CA). Autoradiographs were obtained at different exposure times so that the intensity of each scanned sample was within the linear range.

Plasmid Constructions. The preparation by *Bal*31 digestion of recombinant plasmids containing β-globin cDNAs with graded 5′ deletions has been previously described (Shakin & Liebhaber, 1986b); these plasmids are referred to as p$\beta\Delta X$, where X indicates the 5′ extent of the globin cDNA insert and where +1 refers to the A of the AUG initiation codon. Additional β-globin cDNA subclones were prepared by standard recombinant DNA methodology (Maniatis, et al., 1982) as previously described (Shakin & Liebhaber, 1986b). The region of β-globin mRNA covered by each of these β-globin cDNA subclones is indicated in the plasmid name. The *Mst*II/*Pst*I fragment of β-globin cDNA pβ(180–551) and the *Alu*I/*Pst*I fragment of β-globin cDNA pβ(446–551) were subcloned into the *Sma*I/*Pst*I site of the vector pUC9 and into the *Hin*cII/*Pst*I site of the vector pSP65, respectively. The *Bam*HI/*Pst*I fragment of β-globin cDNA pβ(302–551) was prepared by removing the 5′ *Bam*HI fragment of p$\beta\Delta$+45 and religating the remaining plasmid. The following additional β-globin cDNA fragments, which extended different distances 3′, were also subcloned. pβ(52–179) contains the 5′ β-globin *Pst*I/*Mst*II fragment of p$\beta\Delta$+52 inserted into the *Pst*I/*Sma*I site of pUC9. pβ(119–301) and pβ(146–301) contain the 5′ β-globin *Pst*I/*Bam*HI fragments of p$\beta\Delta$+119 and p$\beta\Delta$+146, respectively, inserted into the *Pst*I/*Sma*I site of pUC9 following blunting of the *Bam*HI sites with Klenow. pβ(180–301) contains the 5′ β-globin *Mst*II/*Bam*HI fragment of pβ(180–551) inserted into the dephosphorylated *Sma*I site of pUC9 following blunting of the *Bam*HI site with Klenow. pβ(302–445) contains the 5′ β-globin *Bam*HI/*Alu*I fragment of pβ-(302–551) inserted into the *Bam*HI/*Hin*cII site of pSP65.

Preparation of mRNA/cDNA Hybrids. mRNA/cDNA hybrids were prepared and analyzed exactly as previously described (Shakin & Liebhaber, 1986b). Briefly, for each hybrid, a β-globin plasmid was digested with the appropriate restriction enzyme(s) to release the cDNA insert, and the DNA was then hybridized to 3′-end-labeled human poly(A+) reticulocyte RNA, which contains α- and β-globin mRNA. In each mRNA/cDNA hybrid, a unique region of the β-globin mRNA was covered by cDNA. Additional nonhomologous terminal DNA bases originating from linker sequences in the vectors were also present at the 5′ and 3′ extents of certain mRNA/cDNA duplexes. Hybrids that contained cDNAs from the 5′ deletion library (i.e., p$\beta\Delta X$) or β-globin cDNAs with 5′ extents at positions +52, +119, and +146 had a tail of 3′ACGTCG5′ at the 5′ extent of the mRNA/cDNA duplex, while hybrids that contained β-globin cDNAs with 5′ extents at position +446 had a tail of 3′GAGATCTCAG5′ at the 5′ extent of the mRNA/cDNA duplex. Hybrids that contained cDNAs from the 5′ deletion library (i.e., p$\beta\Delta X$) or β-globin cDNAs with 3′ extents at position +551 had a tail of 3′CG5′ at the 3′ extent of the mRNA/cDNA duplex; hybrids that contained β-globin cDNAs with 3′ extents at positions +301 and +445 had tails of 3′CCCTTAA5′ and 3′CTGG5′, respectively, at the 3′ end of the mRNA/cDNA duplex. The percentage of the β-globin mRNA in each sample that was hybridized to cDNA was quantitated by digestion an aliquot of the sample with RNase H (Donis-Keller, 1979; Stein & Hausen, 1969); 100% hybridization of the β-globin mRNA to cDNA was confirmed by demonstrating that the hybridized mRNA species (β-globin mRNA) was fully sensitive to RNase H digestion while the α-globin mRNA present in the sample was not. Only samples with 100% hybridization of β-globin mRNA to cDNA were used in the experiments presented in this paper. Mock-hybridized mRNA was prepared in parallel with hybridized mRNA samples under identical conditions except for the exclusion of cDNA from the hybridization reaction. Where indicated, mock-hybridized or hybridized mRNA samples were heat melted at 90 °C for 3 min and then placed on ice prior to their incubation in the ribosome binding assay.

In Vitro Translation Reactions. In vitro translation reactions were carried out in a micrococcal nuclease treated rabbit reticulocyte lysate prepared from New Zealand White rabbits (Pelham & Jackson, 1979) under conditions previously described (Liebhaber et al., 1984). Translation reactions contained 20 ng of 3′-end-labeled human globin mRNA per 15 μL of reaction and were incubated at either 15 or 30 °C for 30 min. Where indicated, the following additions were made: aurintricarboxylic acid (ATA) (Sigma, 0.02 mM final concentration), anisomycin (Sigma, 2 mM final concentration), cap analogue (m^{7}GpppG) (Boehringer Mannheim, 1 mM final concentration), or GTP analogue (GMP-PCP) (Boehringer Mannheim, 8 mM final concentration). At these final concentrations, each of these inhibitors completely blocks the synthesis of [^{35}S]methionine-labeled globin proteins during a 30-min in vitro translation reaction at 30 °C.

Sucrose Gradient Fractionation of in Vitro Translation Reactions. In vitro translation reactions were fractionated on 15–40% sucrose gradients as previously described (Shakin & Liebhaber, 1986a,b). A total of 10 μL of rabbit reticulocyte lysate without nuclease treatment was added to each translation reaction prior to sucrose gradient analysis to enhance the UV absorbance of polysome peaks. The RNA in each gradient fraction was isolated as previously described (Liebhaber et al., 1984) and analyzed by gel electrophoresis and autoradiography as described above.

80S Ribosome Binding Assay. The ability of the human α- and β-globin mRNAs to bind initial 80S ribosomes (i.e., form monosomes) was assessed by incubating 3′-end-labeled human globin mRNA in a micrococcal nuclease treated rabbit reticulocyte lysate in vitro translation system exactly as described above except that anisomycin was added to a final concentration of 2 mM and the temperature of incubation was reduced to 15 °C. Either 20 ng of labeled human globin mRNA or 10 ng of labeled human globin mRNA hybridized to cDNA was added to each 15-μL reaction. For these assays, the translation reactions containing anisomycin were preequilibrated to 15 °C for 15 min prior to the addition of mRNA. At specific times following the addition of mRNA, 15-μL aliquots were removed from each reaction, placed directly into 135 μL of ice-cold sucrose gradient buffer (Liebhaber et al., 1984) containing 2 units of heparin, and layered directly onto 5-mL 15–40% sucrose gradients. Gradients were centrifuged as previously described (Liebhaber et al., 1984) except that the spin time was extended to 90 min for better

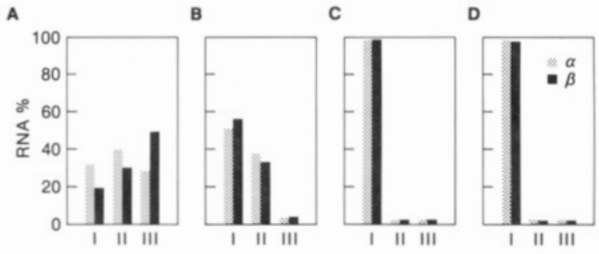

FIGURE 1: Effect of translational inhibitors on the distribution of human globin mRNAs in reticulocyte lysate fractions. ^{32}P-End-labeled human globin mRNA was incubated in the rabbit reticulocyte lysate in vitro translation system in the absence of translational inhibitors (normal translation) (A), in the presence of anisomycin alone (B), or in the presence of anisomycin plus either cap analogue (C) or GTP analogue (D) (see Experimental Procedures). Each reaction was fractionated by sucrose density gradient centrifugation into three fractions, and the amounts of α- and β-globin mRNAs (open and closed bars, respectively) in each of these lysate fractions (I, II, and III) were analyzed by densitometric analysis of gel autoradiographs. Fraction I contains pre-80S RNA, fraction II contains 80S RNA, and fraction III contains polysomal RNA.

resolution of the monosome-containing (80S) fraction. After centrifugation, the gradients were sacrificed, and the RNA in each gradient fraction was extracted and analyzed by gel electrophoresis and autoradiography as described above.

RESULTS

Detection of Initial Monosome Formation. To assay initial 80S ribosome binding, end-labeled reticulocyte mRNA was incubated in in vitro translation reactions in which translational elongation was blocked by the addition of anisomycin (Weber et al., 1979). As an initial control, we demonstrated that during normal translation (i.e., in the absence of any translational inhibitors) α- and β-globin mRNAs were efficiently incorporated into monosomes and polysomes (Figure 1A, fractions II and III, respectively). In contrast, in the presence of anisomycin, these mRNAs were efficiently incorporated into the 80S fraction (Figure 1B, fraction II) but were not incorporated into the polysomes (Figure 1B, fraction III). We further demonstrated that these mRNAs were excluded from both 80S and polysome-containing fractions (fractions II and III, respectively) when translational initiation inhibitors, either cap analogue (Hickey et al., 1979) or GTP analogue (Kozak, 1979), were included in the reaction (parts C and D of Figure 1, respectively). These findings demonstrated that the majority of mRNA incorporated into the 80S fraction in the presence of anisomycin alone (Figure 1B) was present in the form of monosomes and not cosedimenting nonribosomal mRNA complexes.

While globin mRNA was efficiently incorporated into monosomes under the conditions described above (i.e., during incubation at 30 °C in the presence of anisomycin), this incorporation was complete within 1 min of incubation (data not shown). Therefore, these conditions did not permit an analysis of ribosome binding rates. In order to slow the ribosome binding reaction, the temperature of incubation was reduced to 15 °C. During incubation of the human globin mRNAs at 15 °C in the presence of anisomycin, both α- and β-globin mRNAs continued to accumulate in the monosome-containing (80S) fraction for at least 30 min of incubation (see below and Figure 3).

Incorporation of α- and β-Globin mRNAs into Monosomes and Cosedimenting Complexes in the Presence of Anisomycin. During incubation at 30 °C in the presence of anisomycin (Figure 1B), up to 40% of the total mRNA was incorporated into the 80S fraction by the completion of the ribosome binding reaction, almost exclusively in the form of monosomes. While

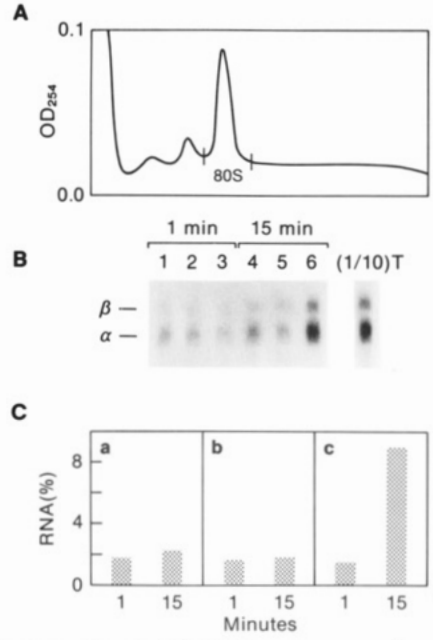

FIGURE 2: Incorporation of mRNA into monosomes and cosedimenting complexes. (A) Absorbance profile at 254-nm OD of an in vitro translation reaction fractionated by sucrose gradient centrifugation with the spin time prolonged for better resolution of the monosome-containing (80S) fraction. Vertical lines indicate the extent of the 80S fraction isolated for analysis of mRNA content. The top of the gradient is to the left. (B) Analysis of ^{32}P-labeled human α- and β-globin mRNA isolated from the 80S fractions of in vitro translation reactions incubated for either 1 or 15 min in the presence of cap analogue plus anisomycin (lanes 1 and 4), ATA plus anisomycin (lanes 2 and 5), or anisomycin alone (lanes 3 and 6). The bands representing α-globin mRNA (bottom) and β-globin mRNA (top) are indicated. Ten percent of the globin mRNA isolated from an unfractionated (total) in vitro translation reaction following 15 min of incubation in the presence of anisomycin alone is shown in the lane labeled (1/10)T. (C) Quantitative analysis of the amount of globin mRNA present in each of the 80S fractions shown in panel B. The amount of globin mRNA (α plus β) in each 80S fraction is plotted as a percentage of the amount of globin mRNA in an unfractionated reaction (100%). Reactions included cap analogue plus anisomycin (a), ATA plus anisomycin (b), and anisomycin alone (c).

nonribosomal cosedimenting complexes did not represent a significant portion of the total amount of mRNA incorporated into the 80S fraction during incubation at 30 °C, it was not clear whether or not these complexes would represent a greater proportion of the mRNA incorporated into the 80S fraction at earlier points in the ribosome binding reaction when ribosome binding was analyzed at 15 °C. To analyze the relative contributions of monosomes and cosedimenting nonribosomal complexes in the 80S fraction at different times in the ribosome binding reaction, we analyzed the effects of translational initiation inhibitors [either cap analogue (Hickey et al., 1976) or aurintricarboxylic acid (ATA) (Weber et al., 1979)] on the incorporation of mRNA into this fraction following either 1 or 15 min of incubation. These initiation inhibitors were added to the ribosome binding reactions at concentrations that completely blocked the synthesis of [^{35}S]methionine-labeled globin proteins during a 30-min in vitro translation reaction at 30 °C (data not shown). For these studies, each reaction was preequilibrated to 15 °C prior to the addition of mRNA, and the gradient centrifugation time was extended from 45 to 90 min for better resolution of the 80S fraction (Figure 2A). The α- and β-globin mRNAs isolated from the 80S fractions of these reactions following either 1 or 15 min of incubation are shown in Figure 2B. A comparison of the mRNA in samples assayed at 1 min of incubation (lanes 1–3) revealed that approximately the same amount of mRNA was incor-

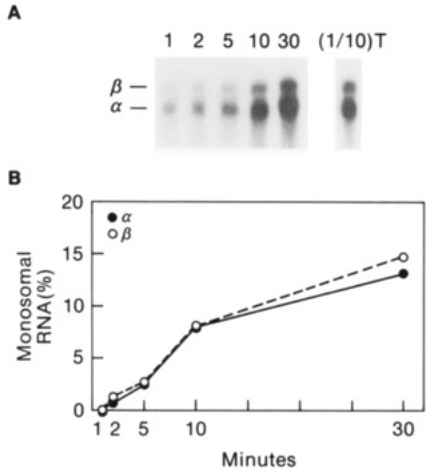

FIGURE 3: Kinetic analysis of α- and β-globin mRNA monosome formation. (A) Analysis of α- and β-globin mRNA incorporated into the 80S fraction of an anisomycin-containing in vitro translation reaction following 1, 2, 5, 10, or 30 min of incubation. The bands representing α-globin mRNA (bottom) and β-globin mRNA (top) are indicated. Ten percent of the globin mRNA isolated from an unfractionated aliquot of the reaction following 30 min of incubation is shown in the lane labeled (1/10)T. (B) Quantitative analysis of the amount of α-globin mRNA (closed circles) and β-globin mRNA (open circles) incorporated into monosomes at each time point of the ribosome binding reaction shown in panel A. The amounts of α-globin mRNA and β-globin mRNA incorporated into monosomes were calculated as described in the text (see Results) and are plotted as percentages of the total amounts of α- and β-globin mRNA, respectively, added to the reaction (100%).

porated into the 80S fraction whether or not translational initiation inhibitors were present. This suggests that the mRNA incorporated into the 80S fraction under these conditions represented nonribosomal mRNA complexes. A comparison of the 1-min results (lanes 1–3) with those obtained following 15 min of incubation (lanes 4–6) revealed that both cap analogue and ATA blocked further accumulation of mRNA into the 80S fraction (lanes 4 and 5, respectively); in contrast, mRNA continued to accumulate during the 15-min incubation in the presence of anisomycin alone (lane 6). The percentage of mRNA in each reaction incorporated into the 80S fraction following either 1 or 15 min of incubation is plotted in Figure 2C. These results suggest that when mRNA is incubated at 15 °C in the presence of anisomycin alone, the mRNA present in the 80S fraction following 1 min of incubation represents a background of nonribosomal mRNA complexes, while the mRNA that subsequently accumulates in this fraction represents monosomes. Therefore, nonribosomal complexes represent a significant background of mRNA in the 80S fraction early in the ribosome binding reaction but become less significant at later time points.

To directly demonstrate the time-dependent incorporation of globin mRNA into monosomes at 15 °C in the presence of anisomycin, 3′ ^{32}P-end-labeled reticulocyte mRNA was incubated in a 15 °C rabbit reticulocyte lysate in vitro translation reaction in the presence of anisomycin, aliquots of the reaction were removed at different times following the addition of mRNA, and the 80S fraction of each aliquot was assayed for α- and β-globin mRNA content. The α- and β-globin mRNAs incorporated into the 80S fractions following 1, 2, 5, 10, and 30 min of incubation are shown in Figure 3A. To determine the amount of α- or β-globin mRNA specifically incorporated into monosomes in each sample, the amount of each of these mRNAs present in the 80S fraction following the first minute of incubation (i.e., nonribosomal background; see above) was subtracted from each subsequent time point.

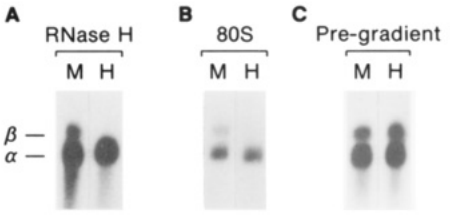

FIGURE 4: Monosome formation of α- and β-globin mRNAs following hybridization to p$\beta\Delta$−50 β-globin cDNA. (A) ^{32}P-Labeled α- and β-globin mRNA digested with RNase H following mock hybridization (M) or hybridization to a β-globin cDNA (H). The cDNA in the hybrid sample p$\beta\Delta$−50 extends from the 5′ terminus of β-globin mRNA to position +551 in the β-globin mRNA 3′ nontranslated region. (B) Analysis of ^{32}P-labeled α- and β-globin mRNAs isolated from the 80S fractions of anisomycin-containing in vitro translation reactions containing the mock-hybridized and hybrid mRNA samples (see panel A) following a 15-min incubation. (C) ^{32}P-Labeled α- and β-globin mRNAs isolated from aliquots of anisomycin-containing in vitro translation reactions containing the mock-hybridized and hybrid mRNA samples (see panel A) following 15 min of incubation and analyzed prior to gradient fractionation.

These values were then expressed as percentages of the total amounts of the α- and β-globin mRNAs, respectively, in the unfractionated (total) reaction; these data are plotted in Figure 3B. The percentage of each of these mRNAs incorporated into monosomes during the first 30 min of incubation at 15 °C (approximately 15% of total) does not represent the maximal amount of mRNA in the reaction that could form monosomes, since the ribosome binding reaction does not reach completion by 30 min at this temperature.

Effect of cDNA Hybridization on β-Globin mRNA Monosome Formation. The assay of initial monosome formation was next used to monitor the impact of duplex formation in the mRNA upon 80S binding. As an initial test of the effects of cDNA hybridization on ribosome binding, we compared the ribosome binding activity of mock-hybridized β-globin mRNA to that of β-globin mRNA hybridized to a near-full-length β-globin cDNA, p$\beta\Delta$−50, which extends from the 5′-terminal cap structure of β-globin mRNA to position +551 in the β-globin mRNA 3′ nontranslated region. One hundred percent hybridization of the β-globin mRNA to cDNA in the hybrid sample was confirmed by demonstrating that the β-globin mRNA in this sample was completely sensitive to digestion with the enzyme RNase H (Donis-Keller, 1979), while the β-globin mRNA in the mock-hybridized sample was not sensitive to RNase H digestion (Figure 4A).

To compare the relative abilities of the α- and β-globin mRNAs in the mock-hybridized and hybrid samples to bind an initial 80S ribosome, each of these RNA samples was incubated in a 15 °C translation reaction containing anisomycin. At 15 min following the addition of the mRNA, an aliquot of each reaction was removed, and the 80S fraction of each aliquot was isolated and analyzed for α- and β-globin mRNA content. This analysis (Figure 4B) revealed that the β-globin mRNA in the hybrid sample was specifically excluded from the 80S fraction. To confirm that the exclusion of the β-globin mRNA from this fraction did not simply reflect degradation of the mRNA/cDNA hybrid during incubation in the lysate [i.e., by an RNase H like activity (Minshull & Hunt, 1986)], an aliquot of each reaction (mock and hybrid) was analyzed for RNA content following incubation but prior to gradient fractionation. A comparison of the relative ratios of α- and β-globin mRNAs present in each of these samples (Figure 4C) confirmed that there was no preferential degradation of the β-globin mRNA/cDNA hybrid during the ribosome binding assay. These findings therefore demonstrate that the hybridization of β-globin mRNA to a near-full-length

(−50 to +551) β-globin cDNA results in a specific inhibition of β-globin mRNA monosome formation.

To further map the positional dependence of hybridized cDNAs on the ribosome binding of β-globin mRNA, we compared the ribosome binding activities of a series of β-globin mRNA/cDNA hybrids. Each of the β-globin cDNAs extended from a unique 5′ terminus to a common terminus at position +551, 109 nucleotides within the β-globin mRNA 3′ nontranslated region [Shakin and Liebhaber (1986b) and Experimental Procedures]. In each hybrid sample, the β-globin mRNA was confirmed to be 100% hybridized to cDNA by RNase H assay (see above), while the α-globin mRNA served as an internal control. To determine the ribosome binding activity of each mRNA/cDNA hybrid, samples of mock-hybridized mRNA and hybridized mRNA were incubated in parallel in the ribosome binding assay for 15 min. The RNA in the 80S fraction of each reaction was then isolated and analyzed for α- and β-globin mRNA content. To permit a comparison of the ribosome binding activities of a large number of mRNA/cDNA hybrids, the following approach was used. The ribosome binding activity of each β-globin mRNA/cDNA hybrid was quantitated by dividing the ratio of β- to α-globin mRNA in the 80S fraction of the hybrid sample by the ratio of β- to α-globin mRNA in the 80S fraction of the mock-hybridized sample [(βH/αH)/(βM/αM)]. This approach permitted a quantitative comparison of the abilities of mock-hybridized β-globin mRNA and specific β-globin mRNA/cDNA hybrids to bind an initial 80S ribosome. In each reaction, α-globin mRNA served as an internal control, correcting for any tube-to-tube variability in the activity of the ribosome binding assays.

Figure 5A shows a gel analysis of the 80S fractions of the ribosome binding reactions of several β-globin mRNA/cDNA hybrids. The ribosome binding activities of the full set of mRNA/cDNA hybrids determined in several independent assays are plotted in Figure 5B. In control experiments, we also documented that the ribosome binding activity of the β-globin mRNA in each hybrid sample could be fully restored following heat melting of the hybrid prior to incubation; this confirmed that the alterations in the ribosome binding activity of the hybrids resulted directly from the presence of the duplex in the β-globin mRNA and not simply from the presence of the β-globin cDNA in the ribosome binding reaction. In each case, an aliquot of the reaction was also analyzed prior to gradient fractionation for α- and β-globin content to confirm that there was no selective degradation of the hybridized mRNA species (using the assay shown in Figure 4C). Additional controls (using RNase H) confirmed that there was no dissociation of the mRNA/cDNA hybrid during the ribosome binding reaction. The hybridized mRNA species (α- or β-globin mRNA) isolated from the 80S gradient fraction was also shown to be fully sensitive to RNase H, demonstrating in addition that the mRNA bound to ribosomes was still hybridized to cDNA (data not shown).

The ribosome binding activities of the β-globin mRNA/cDNA hybrids shown in Figure 5 revealed a positional effect of mRNA/cDNA duplexes on the ribosome binding activities of the β-globin mRNA. cDNAs that extended into the 5′ nontranslated region (−20, −50) of β-globin mRNA resulted in a significant suppression of ribosome binding. The mRNA that did incorporate into the 80S fraction during the incubation of these hybrids was considered to represent a background of cosedimenting nonribosomal complexes and not monosomes. This conclusion was based on a kinetic analysis of the incorporation of these hybrids into the 80S fraction, which revealed

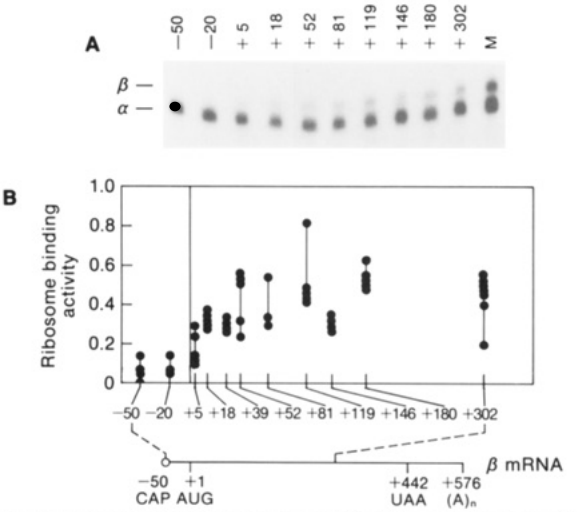

FIGURE 5: Ribosome binding activities of β-globin mRNAs hybridized to cDNAs with 5′-terminal deletions. (A) Gel analysis of β-globin mRNA/cDNA hybrids incorporated into the 80S fractions of anisomycin-containing in vitro translation reactions. The 5′ extent of the cDNA in each mRNA/cDNA hybrid is indicated above each lane, where the position +1 refers to the A of the AUG initiation codon. The bands representing α-globin mRNA (bottom) and β-globin mRNA (top) are indicated. The α- and β-globin mRNAs isolated from the 80S fraction of a reaction containing mock-hybridized mRNA are shown in the lane labeled M. (B) Quantitative comparison of the ribosome binding activities of β-globin mRNA/cDNA hybrids containing cDNAs with 5′-terminal deletions. The ribosome binding activities were calculated as described in the text (see Results). Each data point represents an independent ribosome binding incubation and gradient analysis of a particular hybrid. The data points corresponding to each mRNA/cDNA hybrid are plotted at the position along β-globin mRNA corresponding to the 5′ extent of the cDNA used (indicated along the horizontal axis). A vertical line indicates the position of the A of the AUG initiation codon (+1). The positions of the 5′-terminal cap structure (CAP), initiation codon (AUG), termination codon (UAA), and poly(A) tail [$(A)_n$] along the β-globin mRNAs are indicated below panel B.

that the same percentage of these hybrids seen in the 80S fraction after 15 min of their incubation (1–2% of the hybridized mRNA in the total reaction) was present in this fraction as early as 1 min following the addition of the hybrid to the ribosome binding reaction. The failure of these hybrids to accumulate in the 80S fraction as a function of time paralleled the inability of the unhybridized α- and β-globin mRNAs to accumulate in this fraction as a function of time when monosome formation was blocked by drugs (Figure 2).

Ribosome binding over this background level was first detected in pβΔ+5, which exposes only five bases 3′ to the AUG. The five separate analyses of this hybrid showed values ranging from a background value of 9% to 28% of the unhybridized control (see Discussion). Analysis of the ribosome binding activities of mRNA/cDNA hybrids containing cDNAs that began further 3′ revealed that the ribosome binding activities of mRNA/cDNA hybrids remained depressed even when extensive regions of mRNA 3′ to the AUG were exposed. Each hybrid was assayed in multiple independent experiments, and all data without exclusion are plotted. In some cases (pβΔ+119 and pβΔ+302) there is a single outlying point in an otherwise tightly clustered grouping, while in the case of two other hybrids (pβΔ+5 and pβΔ+52) there is a broad range of data points. The average ribosome binding activities of β-globin mRNA hybridized to cDNAs beginning 18 or more bases 3′ to the AUG were approximately 40% of mock values. There appeared to be some difference in the binding activities of certain of the hybrids by pairwise analysis (the ribosome binding activities of pβΔ+146 and pβΔ+180 are 0.30 and 0.60,

respectively, with nonoverlapping 95% confidence limits), but for most samples tested the means and standard deviations (±2 SD) showed significant overlap.

Comparison of Ribosome Binding Activities of β-Globin mRNA Hybridized to Additional β-Globin cDNA Fragments. Each of the mRNA/cDNA hybrids tested in the previous experiment (Figure 5) had a level of ribosome binding lower than that of the corresponding mock-hybridized mRNA. Because all of the cDNAs tested above covered a common region of the β-globin mRNA sequence (+302 to +551), it was possible that exposure of particular sequences within that region might be required for efficient ribosome binding to occur. Alternatively, it was possible that the presence of an mRNA/cDNA duplex in the mRNA per se (regardless of its position) might have some nonspecific inhibitory effect on ribosome binding. To further define the positional effects of mRNA/cDNA duplexes on ribosome binding and to determine whether any mRNA/cDNA hybrids could bind ribosomes at normal levels, we next measured the ribosome binding activity of β-globin mRNA hybridized to a series of cDNA fragments that covered smaller regions of the β-globin mRNA (Figure 6C). The ribosome binding activities of these hybrids, determined in several independent ribosome binding assays, are plotted in Figure 6B (closed circles). The ribosome binding activities of the β-globin hybrids that contained cDNAs which extended from specific 5′ positions to position +551 in the 3′ nontranslated region (data shown in Figure 5B) are replotted in Figure 6B for comparison (open circles). Each of the five cDNA fragments internal to the coding region [pβ(52–179), pβ(119–301), pβ(146–301), pβ(180–301), and pβ(302–445)] had some inhibitory effect on the ribosome binding activity of β-globin mRNA. The ribosome binding activity of each of these hybrids (Figure 6B, closed circles) was, in general, similar to that of the corresponding hybrid that contained a cDNA fragment extending from the same 5′ terminus to position +551 (Figure 6B, open circles). However, the pairwise comparison of hybrids pβ(52–179) and pβΔ+52 [i.e., pβ(52–551)] suggested that in certain cases the availability of regions in mRNA 3′ to extensive mRNA/cDNA duplexes (and as many as 180 bases 3′ to the AUG) might also influence ribosome binding activity. However, because the 95% confidence limits of these two groups of data overlap, this comparison can only be interpreted in a suggestive manner.

Each of the cDNAs tested above inhibited the ribosome binding of β-globin mRNA to some degree. Since each of these cDNAs also covered a portion of the mRNA coding region, we next measured the ribosome binding activity of a β-globin hybrid that contained a cDNA fragment entirely internal to the β-globin mRNA 3′ nontranslated region, pβ-(446–551) (Figure 6C). In contrast to all of the other cDNAs tested, this cDNA did not inhibit, and actually enhanced to a small degree, the ribosome binding activity of the β-globin mRNA.

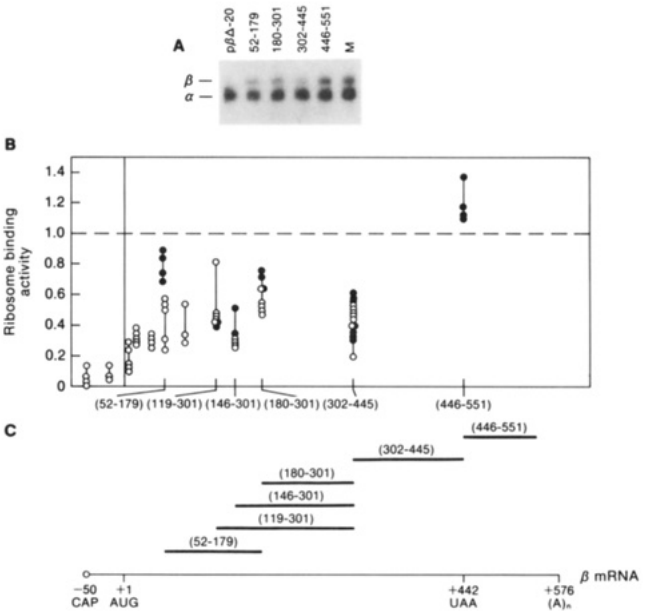

FIGURE 6: Ribosome binding activities of additional β-globin mRNA/cDNA hybrids. (A) Gel analysis of β-globin mRNA/cDNA hybrids incorporated into the 80S gradient fractions of anisomycin-containing in vitro translation reactions. The region of β-globin mRNA covered by the cDNA in each hybrid is indicated above each lane, where the position +1 refers to the A of the AUG initiation codon; the cDNA in the hybrid pβΔ−20 extends from position −20 to position +551. The bands representing α-globin mRNA (bottom) and β-globin mRNA (top) are indicated. The α- and β-globin mRNAs isolated from the 80S fraction of a reaction containing mock-hybridized mRNA are shown in the lane labeled M. (B) Ribosome binding activities of β-globin mRNA/cDNA hybrids. The data points corresponding to each mRNA/cDNA hybrid are plotted at the position along β-globin mRNA corresponding to the 5′ extent of the cDNA used. The ribosome binding activities of hybrids containing the cDNAs shown in panel C are shown in closed circles, and the region of β-globin mRNA covered by each of these cDNAs is indicated below each set of data points. The ribosome binding activities of β-globin mRNA/cDNA hybrids containing cDNAs with 5′-terminal deletions and extending to position +551 (data shown in Figure 5B) are also shown in open circles for comparison. A vertical line indicates the position of the A of the AUG initiation codon (+1). (C) Regions of β-globin mRNA covered by β-globin cDNA fragments. The positions of the 5′-terminal cap structure (CAP), initiation codon (AUG), termination codon (UAA), and poly(A) tail [$(A)_n$] of β-globin mRNA are indicated.

Discussion

To investigate the role of early steps in translation in overall translational control, we have established an in vitro assay that directly measures monosome formation on a specific mRNA species. We have used reticulocyte RNA as the test system since over 90% of its mRNA is composed of two mRNA species: α- and β-globin. The simplicity of this system allows us to directly compare in the same reaction tube the monosome formation rates of two mRNAs, one of which (β) has been structurally altered and the other of which (α) serves as a control. By modifying the structure of the β-globin mRNA using a series of blocking cDNAs, we can begin to determine in a controlled fashion the importance of various regions of the β-globin mRNA in its efficiency of monosome formation.

A comparison of the monosome formation activities of a variety of β-globin mRNA/cDNA hybrids yielded both expected and novel results. As expected from previous work (Hastie & Held, 1978; Miller et al., 1980; Peterson et al., 1977; Liebhaber et al., 1984; Shakin & Liebhaber, 1986b), cDNAs that covered the initiation codon (pβΔ−50 and pβΔ−20) completely inhibited ribosome binding, while cDNAs that were closely juxtaposed 3′ to the AUG (e.g., pβΔ+5) appeared to depress but not completely inhibit this reaction. Both of these results may reflect a direct steric inhibition of the attachment to and migration of the 40S ribosomal subunit along the 5′ nontranslated region, as well as the stable assembly of the 80S ribosome at the initiation codon. The decreased binding of 80S ribosomes resulting from hybridization of pβΔ+5 cDNA correlates with the study of Baim et al. (1985), which demonstrated that the introduction of a stable secondary structure into the yeast iso-1-cytochrome *c* mRNA at a position beginning 18 bases 3′ to the AUG resulted in a significant reduction in the ability of this mRNA to load ribosomes during active translation; while the authors of that study suggest that

this reduction in ribosome loading might reflect a block at the level of translational elongation, our findings suggest the presence of a stable secondary structure within the 5′ proximal coding region could also decrease ribosome loading during translation by decreasing the efficiency of ribosome binding. The exact proximity of critically important secondary structures to the AUG may vary from mRNA to mRNA. While the β-globin mRNA hybridized to the pβΔ+18 cDNA demonstrated significant monosome formation, α-globin mRNA hybridized to a corresponding α-globin cDNA truncated at the +18 position (pαΔ+18) assayed in the same way had a marked supressive effect on the ribosome binding activity of human α-globin mRNA (unpublished data). The number of bases 3′ to the AUG that must be available for stable 80S assembly may therefore vary between mRNAs, possibly reflecting differences in their unique higher order structures in this region.

While the findings described above demonstrate that mRNA/cDNA duplexes near the initiation codon of the β-globin mRNA can influence its ribosome binding activity, other findings in this study reveal a less expected result: duplexes in β-globin mRNA far 3′ from the initiation codon can affect its ribosome binding efficiency as well. Even coding region duplexes that began several hundred bases 3′ to the AUG (e.g., pβΔ+302) significantly inhibited the ribosome binding activity of the β-globin mRNA. In addition to the general ability of coding region cDNAs to inhibit ribosome binding, the data also suggest that the exact 5′ extent of a cDNA may influence the degree to which it inhibits ribosome binding [for example, compare pβΔ+146 and pβΔ+180 (Figure 5B)]. In addition, cDNAs, such as pβ(52–180) and pβΔ+52 (Figure 6B), which shared the same 5′ extent but extended different distances 3′, appeared to inhibit the ribosome binding activity of β-globin mRNA to different degrees. This result suggests that the availability of regions in mRNA significantly 3′ to the AUG can be important in determining ribosome binding activity, even when these regions are separated from the AUG by extensive mRNA/cDNA duplexes.

In previous studies (Liebhaber et al., 1984; Shakin & Liebhaber, 1986b), we demonstrated that the elongating 80S ribosome can efficiently dissociate cDNA fragments that are hybridized to mRNA coding regions. These mRNA/cDNA hybrids are translated with full efficiency. This contrasts with this study in which we demonstrate an adverse effect of these same cDNAs upon initial 80S binding. This difference in the effect of the hybrids upon initial 80S binding and upon active translation probably reflects the removal of the hybridized cDNA from the mRNA during translational elongation (Shakin & Liebhaber, 1986b). In an actively translating system, the effect of the hybrid would therefore be lost as soon as the mRNA is traversed by the first ribosome. In contrast to this experimental situation, short-range secondary structures in the coding region of native mRNA might re-form after each ribosome passes. In that case these short-range structures would be expected to exert a continuing effect upon ribosome assembly and subsequent overall efficiency of steady-state translation. By introducing site-specific secondary structures in synthetic mRNAs at positions suggested to be of interest by this study, it may now be possible to confirm and extend these findings with less artificial approaches.

The mechanism by which the coding region cDNAs suppress monosome formation is not clear. Since translational elongation is chemically blocked in this ribosome binding assay and because these duplexes are remote from the AUG, the ribosomes bound to the mRNA in these hybrids do not interact directly with the coding region mRNA/cDNA duplexes; the effects of these duplexes on ribosome binding must therefore be exerted in an indirect fashion. The inhibitory effects of these cDNAs on ribosome binding appear to be both specific and positionally dependent since the ribosome binding activity of the hybrid that contained the pβ(446–551) cDNA was not depressed. Since several relatively small nonoverlapping cDNA fragments each inhibit β-globin mRNA ribosome binding, it is unlikely that efficient ribosome binding of β-globin mRNA requires the sole exposure of a unique area of the β-globin coding region. Instead, maximally efficient ribosome binding of the β-globin mRNA appears to require the availability of the entire β-globin mRNA coding region as well as the 5′ nontranslated region. This suggests that ribosome binding activity is determined by complex higher order structures that involve extensive regions of the β-globin mRNA molecule.

It is interesting to note that the only cDNA tested that did not inhibit ribosome binding, pβ(446–551), was also the only cDNA completely internal to the 3′ nontranslated region. This finding suggests that mRNA sequences in the 3′ nontranslated region are not critical to efficient ribosome binding. This conclusion is consistent with the observation that β-globin mRNA was still translated efficiently when the 3′ nontranslated region was removed (Kronenberg et al., 1979) and with other studies which suggest that the 3′ nontranslated regions of mRNAs may exist as independent structural domains that are specifically protected from disruption of secondary structure during translation (Shakin & Liebhaber, 1986b). Comparisons of the functional effects of duplexes in the coding region and 3′ nontranslated region of other mRNAs might reveal whether these findings reflect a general difference between the importance of mRNA structures in these two regions in translational initiation.

The findings described above directly demonstrate that duplexes in an mRNA can significantly alter its ribosome binding activity in a positionally dependent manner. These findings are consistent with those of other studies, which suggest that mRNA secondary structure may play a critical role in the regulation of translational initiation (Baim et al., 1985; Kozak, 1980, 1986a,b; Pelletier & Sonenberg, 1985). Our finding that duplexes far 3′ from the initiation codon can influence ribosome binding further suggests that extensive regions of mRNA molecules may directly or indirectly contribute to the formation of the structures that determine their ribosome binding rates. This suggests that structural perturbations which occur at any site along an mRNA molecule could potentially alter its ribosome binding activity. Several studies demonstrate that such perturbations in mRNA secondary structure occur as a direct result of the translation process. Certain translational factors, including the cap binding protein (Lawson et al., 1986; Sonenberg & Lee, 1982) and the 80S ribosome itself (Liebhaber et al., 1984; Shakin & Liebhaber, 1986b), have been shown to contain helix-destabilizing activities, which can unfold duplexes in mRNA. In addition, studies of attenuation in prokaryotic biosynthetic operons have demonstrated specific and functionally critical changes in mRNA secondary structure in response to site-specific positioning of ribosomes along the mRNA coding region (Kolter & Yanofsky, 1982). On the basis of these studies and the findings in this paper, it seems likely that the secondary structure of an mRNA and its ribosome binding activity are not fixed but instead vary in a dynamic fashion depending on the translational state of the mRNA and on the exact regions of the mRNA that are involved at any moment

in interactions with ribosomes and other factors.

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