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The 3' noncoding region of β-globin mRNA is not essential for in vitro translation

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ABSTRACT

Rabbit β-globin DNA sequence, excised from plasmid pβG1, directs in vitro synthesis of β-globin in a transcription-translation cell-free system, even after specific elimination of the entire 3'-noncoding region. A DNA restriction fragment carrying this 3' noncoding region and hybridized to globin mRNA cannot arrest the cell-free translation of β-globin mRNA.

INTRODUCTION

Eukaryotic mRNAs contain 5'- and 3'- noncoding sequences flanking their coding regions. The 5'-noncoding regions from a variety of mRNAs sequenced thus far do not share common sequences and vary in length from 9 to 94 nucleotides.[2] They usually begin with a cap structure, however.[3] The 3'-noncoding regions all contain the sequence AAUAAA[4], and are generally followed by poly (A) stretches. The patterns of sequence conservation in the untranslated regions of human, rabbit and mouse α- and β-globin mRNAs suggest that these regions have functional importance[2,5]. Unfortunately, nucleotide sequence analysis alone has offered no insights into the nature of these functions.

Several groups have altered mRNA structure and tested the implications of these alterations in cell-free or microinjection systems. The cap structure has been shown to play an important role in the initiation step of protein synthesis[3,6]. The AUG initiation codon is absolutely required for binding of reovirus mRNA to 50S ribosomal subunits, and the nucleotides surrounding the AUG increase this binding[6]. Removal of the poly (A) from a mRNA has little effect on its translatability in cell-free extracts[7,10], but dramatically shortens the half-life of mRNA injected into Xenopus oocytes[11].

If parts of the noncoding regions of mRNA could be specifically eliminated, their involvement in the translation process could be investigated by cell-free translation of the altered mRNA. Unfortunately, the chemical or enzymatic tools that could specifically alter RNA molecules are limited. In contrast, DNA molecules can be specifically cleaved by restriction endonucleases. Therefore the function of specific regions of an mRNA can be evaluated by using restriction fragments of a DNA copy of this mRNA; these fragments can be introduced into a linked transcription-translation cell-free system[12,13] or hybridized to specific regions of mRNA prior to cell-free translation (hybrid-arrested translation)[14]. In this paper we report studies along these lines, using cloned rabbit β-globin DNA, corresponding in sequence to virtually the entire rabbit β-globin mRNA sequence[15,16].

MATERIALS AND METHODS

Enzymes. S1 nuclease was purified by a modification of published procedures[17,18]. The purification scheme is rapid and yields the enzyme in the high concentration necessary for the excision[19] of DNA fragments inserted into bacterial plasmids, by the poly(dA)-poly(dT) tailing method. We have observed that the enzymatic activity always copurifies with a yellow pigment, which can be conveniently followed through the purification steps, making assays unnecessary. Four grams α-amylase (Sigma A6630) were extracted and processed with $(NH_4)_2SO_4$ as described by Vogt[17], except that the heating step was omitted. The first $(NH_4)_2SO_4$ pellet was dissolved in minimal volume of dialyzing buffer (50mM Na acetate, pH 5.0, 1mM $ZnCl_2$) and dialyzed overnight against 2 liters of the same buffer. After removing undissolved material by centrifugation, the dialysate was loaded on a 5ml DEAE-cellulose column, equilibrated with dialysis buffer. The column was washed with 50ml dialysis buffer containing 50mM NaCl, and the enzymatic activity was eluted with dialysis buffer in order to reduce the concentration of NaCl to 50mM, and loaded on a 1ml DEAE-cellulose column, which was washed and developed as described above. One-half ml fractions were collected, the most yellow were pooled, diluted 1:1 with glycerol, and stored at -20°C. In a series of preparations purified by this procedure we were unable to detect activity against double-strated DNA.

One µl of such a concentrated preparation will usually excise the insertion from 10µg hybrid plasmid in a 40µl reaction, under the conditions described by Hofstetter et al.[19]. (45% non-deionized formamide, 0.2M NaCl, 0.03M Na acetate pH 4.5, 1mM $ZnCl_2$), provided that the length of the poly(dA)-poly(dT) tails is of the order of 100 nucleotide pairs. The efficiency of excision drops substantially if the tails are shorter than 50 nucleotide pairs.

Wheat-germ RNA polymerase was prepared by the procedure of Jendrisak and Burgess[20]. Bgl II was a generous gift from Craig Duncan. HhaI and Ava II were purchased from Bethesda Research Laboratories.

DNA Fragments. The DNA fragments used in this study are shown schematically in Figure 1. Fragment A represents rabbit β-globin DNA sequence excised from 500µg plasmid pβG1 using S1 nuclease as described above. The digestion products were extracted with phenol and chloroform (1:1, added sequentially before phase separation) and then ethanol precipitated. They were then dissolved in 200µl 10mM EDTA-Na_2 containing 300µg/ml ethidium bromide and layered on a 12 ml 5% to 20% linear sucrose gradient in 0.1M NaCl, 10mM tris-HCl, pH 7.5 and 1 mM EDTA-Na_2. The gradient was centrifuged for 19 hours at 250,000 x g and the resolved (upper) band of eukaryotic DNA was visualized directly and collected by puncturing the side of the plastic gradient tube with a hypodermic needle. Ethidium bromide was removed by three extractions with an equal volume of isoamyl alcohol and the DNA was ethanol precipitated. (Recently we found convenient to extract ethidium bromide and also concentrate

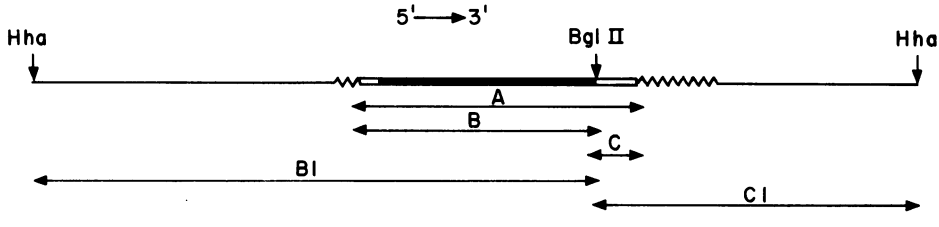

Figure 1. Diagram of the DNA fragments (see Materials and Methods) used in this study. The coding sequence of β-globin DNA (thick line), the noncoding regions of β-globin DNA (open boxes), the poly(dA)-poly(dT) tails (wavy lines) and the pMB9 vector sequences (thin lines), are indicated.

the DNA in one step, by using secondary butanol, as described in Reference 21).

Fragments B and C were prepared from fragment A, by digestion with BglII, which cleaves the sequence once, exactly after the termination codon, followed by gel electrophoresis under non-denaturing conditions in a preparative 6% polyacrylamide slab gel (acrylamide:bis-acrylamide, 30:1). The resolved DNA fragments were visualized by ethidium bromide staining and extracted from the gel as described[22,23]. An aliquot of fragment B was run again on a 6% polyacrylamide gel and was shown not to be contaminated with either fragment A or C (data not shown). Fragments B1 and C1 were prepared by HhaI and BglII double digestion of 150µg pβG1 followed by gel electrophoresis and extraction from the gel, as described above.

Messenger RNA. Rabbit globin mRNA was prepared from salt-washed reticulocyte polysomes (kindly supplied by B. Paterson) by the guanidine-HCl extraction procedure[24] and stored at -80°C. Over a several month period, aliquots of this RNA were thawed, phenol extracted, and enriched by oligo(dT)-cellulose chromatography[25]. We observed that over this several month period, the predominant protein synthesized when mRNA was introduced into the wheat germ cell-free system changed from α- to β-globin.

Rabbit β-globin mRNA was purified by eluting the β-globin mRNA band from a 98% formamide-5% polyacrylamide gel as described previously[23].

Linked Transcription-Translation. Wheat germ RNA polymerase was used in reactions using conditions described previously[12,13]. The only modification introduced was the pre-digestion of wheat germ extract with micrococcal nuclease, as described by Pelham and Jackson for reticulocyte lysate[26].

Hybrid-Arrested Translation was performed as previously described[14].

Analysis of Cell-Free Products. Rabbit α- and β-globin chains were separated on both phosphate-buffered sodium dodecyl sulfate/12% polyacrylamide gels[27] and on tris-glycine-buffered sodium dodecyl sulfate/15-20% gradient polyacrylamide gels[28].

Protein Sequencing. Both DNA-directed and mRNA-directed protein synthesis were performed as described above, using [^{3}H]-leu-

cine or [^{35}S]-methionine. DNA-directed β-globin chain was eluted from a sodium dodecyl sulfate-polyacrylamide gel, following autoradiographic detection of the band. The protein was electroeluted at 100V for 16 hours into a dialysis bag, together with 100μg rabbit hemoglobin carrier, using the buffer of Laemmli[28] and dialyzed against water for two days. Messenger RNA-directed protein was total protein synthesized in a translation reaction using gel-purified rabbit β-globin mRNA. No detectable α-globin synthesis was stimulated by this mRNA (data not shown). The entire translation reaction was precipitated with 10% trichloroacetic acid, washed with acetone at -20°C, and dried under vacuum. Each protein was then introduced into a Beckman 890 sequenator and subjected to automated Edman degradation[29]. One-third of the material released at each cycle was counted directly, while two-thirds were subjected to thin-layer chromotography, and subsequent counting of the leucine region of the thin-layer plate[29].

RESULTS

Excision of β-globin DNA Sequence from pβG1

Hofstetter et al.[19] showed the β-globin DNA insertion of pβG1 can be specifically excised by S1 nuclease in the presence of 45% formamide, which selectively melts the poly(dA)-poly(dT) tails introduced into the hybrid plasmid during its construction[15].

In preliminary experiments, we titrated our S1 preparation in a series of 40μl reaction mixtures, each containing 10μg pβG1 DNA. We then electrophoresed the digestion products in agarose gels, and selected the amount of nuclease that could produce the most homogenous bands of excised sequence without impairing the vector DNA. For preparative purposes, the volumes of the ingredients of the selected pilot reaction were proportionately scaled up.

To establish the purity of the excised material and determine accurately its length, we end-labeled an aliquot with γ-^{32}P-ATP using T4 polynucleotide kinase[30] and electrophoresed samples on a nondenaturing gel. Figure 2 shows that the excised globin DNA molecules are homogenous and have a length of approximately 555 BP as estimated from the lengths of the markers. This size is in close agreement with the sum of the lengths (165 + 380 = 545 BP) of the two fragments produced by Ava II digestion, which cleaves

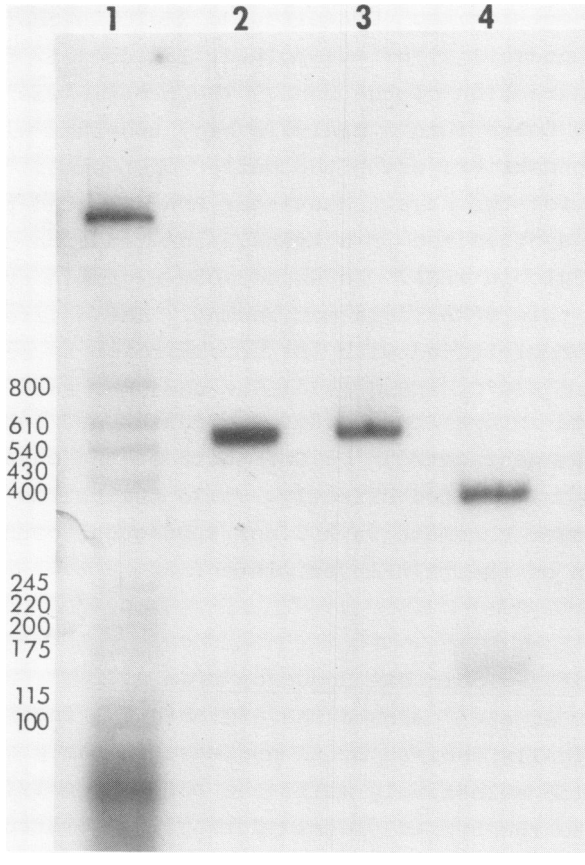

Figure 2. Autoradiogram of ^{32}P-end-labeled globin DNA (excised from pβG1) electrophoresed on a 6% polyacrylamide gel under nondenaturing conditions.
Slot 1: DNA markers (end-labeled pMB9 x HaeIII fragments). The numbers indicate length in BP.
Slot 2 and 3: Excised end-labeled globin DNA.
Slot 4: End-labeled globin DNA digested with AvaII.

the sequence once in the coding region at a site between the nucleotides coding for amino acids 46 and 47. The globin DNA insertion in pβB1 is missing the first thirteen nucleotides corresponding to the 5' terminal region of the mRNA (576 BP, instead of 589), as determined by DNA sequencing[31]. Therefore, if S1 had digested only the tails, the lengths of the two Ava fragments would be 181 and 395 BP. We conclude that under our conditions

S1 "nibbles"[32] 10-15 BP at each end of the excised molecules.

β-globin DNA directs the In Vitro Synthesis of β-globin

When DNA fragments A, B, and C (see Materials and Methods, and Figure 1) were introduced into the linked transcription-translation system, both fragments A and B, but not C, stimulated the incorporation of ^{35}S-methionine into protein (see Table I and Figure 3). Stimulation by fragment A was twenty-fold over background and comparable to the stimulation caused by SV40 form I DNA. Fragment B also stimulated protein synthesis but less than fragment A. This difference might be due to higher amounts of gel impurities contaminating the fragment B preparation. Further, absolute quantitation of these small amounts of DNA is difficult. The

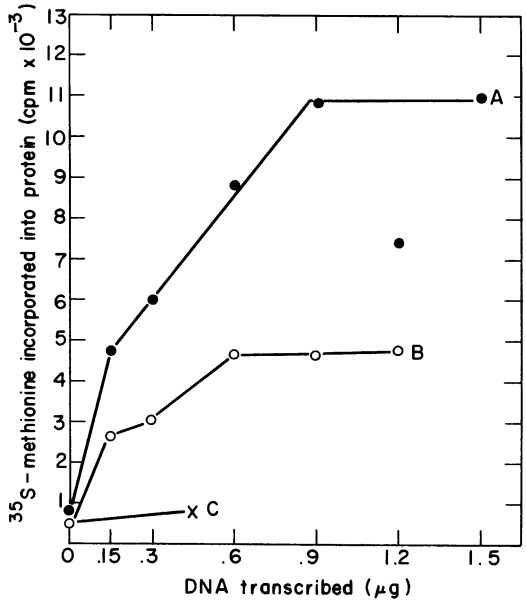

Figure 3. Stimulation of protein synthesis by pβGl fragments introduced into linked transcription-translation system. Transcription-translation were performed as described in Methods and Materials using ^{35}S-methionine. Amounts of DNA used were estimated from their A_{260}, assuming that 1mg/ml of DNA has A_{260} = 20, and also assuming 100% recovery from the preparative gel. 1μl of each 50μl translation was spotted on a Whatman 3MM paper filter, and treated serially with cold 10% trichloroacetic acid (TCA), boiling 5% TCA (10 minutes), cold 5% TCA, and twice with ethanol and ether.

Table 1. ^{35}S-Methionine Incorporation in a Linked Cell-Free Transcription-Translation System[a]

Template	Wheat Germ RNA Polymerase	Incorporation Into TCA Insoluble Material cpm/µl
No DNA	-	515
Globin DNA-fragment A	-	530
Globin DNA-fragment A	+	11,880
Globin DNA-fragment B	+	5,250
Globin DNA-fragment C	+	535
Globin mRNA[b]	-	30,000
SV40 DNA FI	+	36,690

[a]The reaction mixture was incubated for 15 minutes at 37°C under conditions optimal for transcription. This was followed by a further 3 hours incubation at 22°C after the addition of a mixture which yielded final reaction conditions optimal for translation[13]. DNA templates (0.5 to 1µg) were transcribed by 10-15µg of wheat germ RNA polymerase. Aliquots of the reaction mixtures were precipitated with TCA and processed as described in the legend to Figure 3.

[b]The data from the cell-free translation of rabbit globin mRNA (1µg) included in this Table are from a different but similar experiment and are shown for purposes of comparison.

protein synthesized was electrophoresed through SDS-polyacrylamide gels using both the phosphate buffer of Weber and Osborn, which easily separates rabbit α- and β-globin[27], and the tris-glycine system of Laemmli. In both systems, fragments A and B stimulated the synthesis of protein that co-migrated with the rabbit β-globin made from rabbit globin mRNA in the wheat germ translational system (Figure 4). Moreover, in preliminary experiments, one of three peaks eluted from a carboxymethyl-cellulose column co-migrated with carrier rabbit β-globin (data not shown).

To further identify the protein whose synthesis was directed by globin DNA, we used ^{3}H-leucine in protein synthesis reactions and then compared the N-terminal sequences of DNA-directed and mRNA-directed globin, using the automated Edman degradation pro-

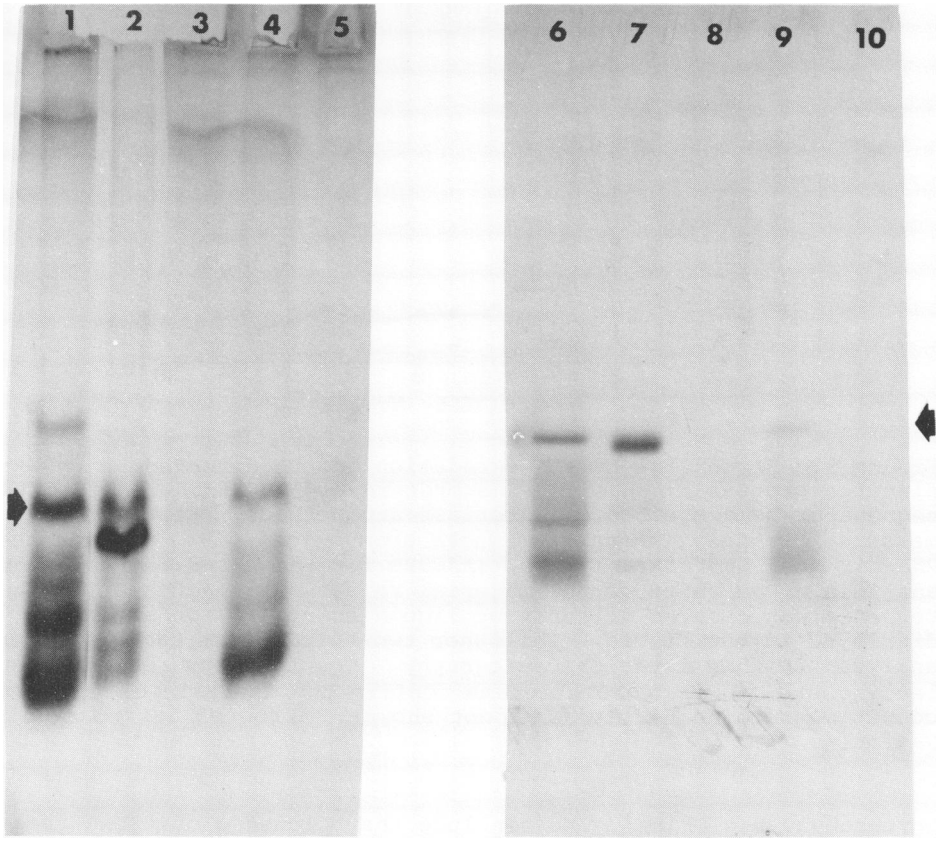

Figure 4. SDS-polyacrylamide gel electrophoresis of proteins synthesized in linked transcription-translation system. Proteins were synthesized as described in Materials and Methods using ^{35}S-methionine. 5µl of each 50µl reaction were then treated with DNAse I and pancreatic RNAse, 0.2 mgµml each, for 10 minutes at 37°C. They were then electrophoresed through a 12% polyacrylamide -SDS gel using the tris-glycine discontinuous system of Laemmli. For comparison, a wheat germ translation, using globin 9S RNA isolated from rabbit reticulocyte polysomes was treated identically. The gels were fluorographed[37] for 3 days using Kodak XR-5 X-ray film, except for channels 2 and 7, which were fluorographed for one day. Channels 1-5, phosphate gel: 1, fragment A; 2, globin mRNA; 3, fragment C; 4, fragment B; 5 , no DNA added. Channels 6-10, tris-glycine gel: 6, fragment A; 7, globin mRNA; 8, fragment C; 9, fragment B; 10, no DNA added. Arrows point to rabbit β-globin.

cedure. Figure 5 shows that both proteins had identical patterns with leucine peaks at positions 3 and 14 just as are found in

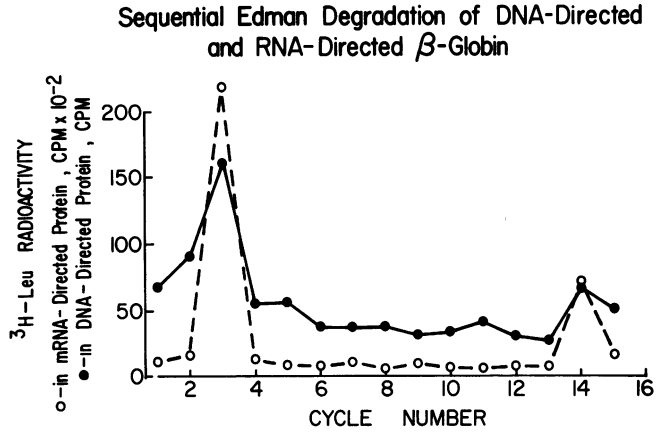

Figure 5. Sequential Edman degradation of DNA-directed and mRNA-directed protein synthesis. The proteins were introduced into the sequenator as described in Materials and Methods. Repetitive yield, measured by analyzing carrier whale apomyoglobin, was 96% for the RNA-directed protein analysis, and 92% for the DNA-direction protein analysis. One third of the material released at each step in the sequence was counted directly by liquid scintillation and are displayed in the figure. The other two-thirds from each step were analyzed by thin layer chromatography. Counts recovered from the leucine spot of the chromatograms confirm the analysis of total counts measured directly (data not shown). Arrow points to rabbit β-globin.

erythrocyte β-globin[33]. We conclude that cloned globin DNA fragment can direct β-globin synthesis which is accurately initiated in vitro.

Translation of β-globin mRNA Arrested by Hybridization to β-globin DNA Fragments

Paterson et al. hybridized rabbit globin RNA with fragment A under conditions[14] that favor DNA:RNA hybridization. This hybridized nucleic acid was subsequently introduced into a wheat germ translational system and the synthesis of β-globin was specifically arrested. Since mRNA sequences hybridized to DNA are unavailable for protein synthesis in this system, we tested the effect of hybridizing DNA fragment C1, carrying sequences corresponding to the 3'-untranslated region of β-globin mRNA to rabbit globin mRNA. Figure 6 demonstrates that translation of β-globin mRNA was unaffected by approximately 5-fold excess fragment C1. In contrast, as expected, fragment B1, which includes sequences

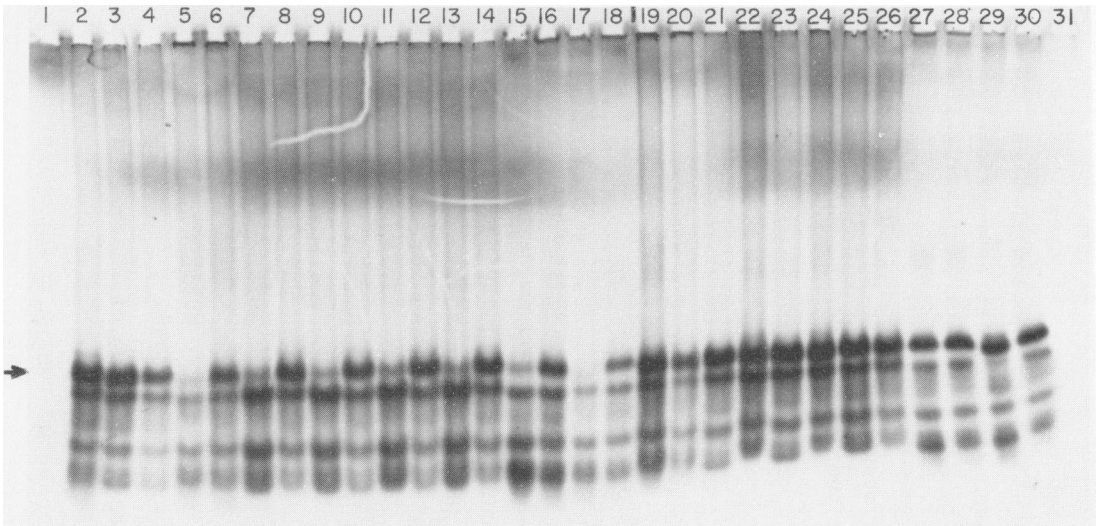

Figure 6. Hybrid-arrested translation of globin mRNA hybridized with plasmid fragments B1 and C1. The products of hybrid-arrested translation labeled with [^{35}S]-methionine, were displayed on a phosphate-buffered sodium dodecyl sulfate/12% polyacrylamide slab gel. Except for wells 1, 5, 17, 18 and 31, where total counts were too low, 20,000 cpm from each reaction were loaded in each well. The gel was fluorographed and developed using Kodak XR-5 film for six hours. Samples were as follows: 1 and 31, no added mRNA or DNA. Reactions 2-30 contained 500 ng rabbit globin mRNA and the following amounts of DNA: 2, none; 3, none, carried through hybrid-arrest procedure; 4, none, same as 3 but heat-melted after hybridization; 5, 2µg fragment B1; 6, heat-melt of 5; 7, 0.2 µg fragment B 1; 8, heat melt of 7; 9, 0.4µg fragment B1; 10, heat melt of 9; 11, 0.8µg fragment B1; 12, heat melt of 11; 13, 1.2µg fragment B1; 14, heat melt of 13; 15, 1.6µg fragment B1; 16, heat melt of 15; 17, 2.4 µg fragment B1; 18, heat melt of 17; 19, 0.4 µg fragment of C1; 20, heat melt of 19; 21, 0.6 µg fragment C1; 22, heat melt of 21; 23, 0.8 µg fragment C1; 24, heat melt of 23; 25, 1 µg fragment C1; 26, heat melt of 25; 27, 1.4 µg fragment C1; 28, heat melt of 27; 29, 1.6 µg fragment C1; 30, heat melt of 29. Arrow points to rabbit β-globin.

corresponding to the β-globin structural information, was able to arrest specifically the translation of β-globin. Melting of these hybrids returned β-globin synthesis to normal. Except at the highest two concentrations of DNA, total protein synthesis was unaffected. We demonstrated that fragment C1 does in fact bind to β-globin mRNA under the conditions of hybridization, by treating the hybrid with calf thymus ribonuclease H[34,35] and showing that the ^{32}P-end-labelled β-globin mRNA decreased its mobility appropriately on a 98% formamide-5% polyacrylamide gel after the ribonuclease H treatment (data not shown). Thus we conclude that β-globin can be made in the wheat germ cell-free system even when the entire 3'-untranslated region of the mRNA has been hybridized to DNA.

DISCUSSION

Our results demonstrate that neither the first 23 to 28 nucleotides of the 5'-noncoding region nor the entire 3'-noncoding region (95 nucleotides before the poly-A tail) of the β-globin mRNA are essential for protein synthesis in the wheat germ cell-free translational system. These results are consistent with the findings of Kozak and Shatkin[6], who could find no part of the 5'-untranslation regions of reovirus mRNAs uniquely necessary for ribosomal binding. We similarly have shown that the first 23 to 28 bases of rabbit β-globin mRNA are not needed for protein synthesis *in vitro*. Our results cannot answer questions concerning the involvement of the cap structure in *in vitro* translation under our conditions, because we do not know whether the RNA synthesized in the linked transcription-translation system becomes capped by the wheat germ extract. Capping activity has been found in wheat germ extracts (B.M. Paterson and M. Rosenberg, personal communication), but is probably present in very low amounts.

Baralle[36], noting a potential region of base-pairing between the 5'- and 3'-untranslated regions of rabbit β-globin mRNA, pointed out that the 3'-untranslated region could be involved in the initiation of protein synthesis. Though our results cannot exclude the possibility that the 3'-untranslated region facilitates or inhibits translation by such a base-pairing or some other mechanism they clearly show that this region is not an absolute requirement for *in vitro* translation.

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