Structural Features in the 3'-Terminal Region of Polyribosome-bound Rabbit Globin Messenger RNAs*

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(Received for publication, June 21, 1985)

A nuclease S1 mapping procedure was used to identify sites accessible to nucleases in the 3'-noncoding region of the rabbit globin mRNAs. A complex structure was evident in the α-globin species, with one highly accessible single-stranded site, large portions in an accessible double-stranded configuration, and a portion not accessible to any of the nucleases. In the β-globin mRNA, the region was more uniformly accessible to RNase T1 and to a cobra venom enzyme specific for double-stranded RNA, but it had only a single site highly accessible to a bulkier Neurospora endonuclease. The patterns of cleavage were nearly identical in the deproteinized mRNAs and in the mRNAs associated with polyribosomes in reticulocyte extracts. In both species, a zone of secondary structure occurred around the poly(A) junction.

In each species, virtually all the molecules had a poly(A) sequence of at least 20–25 AMP residues. A periodicity in poly(A) size distribution was observed. These results indicate that the beginning of this sequence is well protected against degradation inside the cell and that zones of partial protection occur at measured intervals. In crude extracts, where the poly(A) is covered with proteins, this sequence was protected against nuclease digestion.

The function of mRNA as a template for the assembly of amino acids into polypeptides is strongly modulated in eukaryotic cells. Individual species can differ widely with respect to metabolic stability and efficiency in the translation process. They can also be specific targets for signals leading to changes in stability. The structural basis for this diversity in behavior is not understood. It is possible that it lies in the portions of the mRNA chains that do not code for the amino acid sequences. These noncoding regions can be quite extensive, particularly in the portion of the mRNA that follows the coding segment (Brawerman, 1981; Littauer and Soreq, 1982). The 3' noncoding region is potentially a rich source of information for diversity in mRNA behavior. It varies widely with respect to size as well as nucleotide sequences (Littauer and Soreq, 1982). There is little precise information on the possible role of the 3' noncoding region. Enzymatic removal of much of this region from some mRNA species does not appear to affect their capacity to be translated (Soreq et al., 1981; Kronenberg et al., 1979).

The 3' noncoding region is followed in most known eukaryotic mRNA species by a long poly(A) sequence. The latter is uniform in size in newly synthesized mRNA, but is subject to a degradation process in the cytoplasm that leads to its gradual shortening (Brawerman, 1981). This process appears to be selective (Wilson et al., 1978; Krowczynska et al., 1985). The metabolic processes affecting the poly(A) sequence appear apparently to a diversity in steady-state size distribution of poly(A) segments. This selective poly(A) degradation process may contribute to the information for functional diversity in mRNA. This sequence has been implicated in the control of mRNA stability (Huez et al., 1974; Maniatis et al., 1976), and it is possible that segments below a certain critical size no longer function in that capacity (Nudel et al., 1976). Other studies point to a possible involvement of the poly(A) sequence in the translation process (Doel and Carey, 1976; Palatnick et al., 1984). In view of the possible involvement of the poly(A) sequence in some aspects of mRNA function, the controlled degradation of this sequence may be an important regulatory process.

Some structural features of the mRNA as it exists in the cytoplasm may account for the selectivity of this degradation process. The poly(A) segment is known to be associated with proteins in a manner that leads to protection against nucleases (Kwan and Brawerman, 1972; Soreq et al., 1974; Schwartz and Darnell, 1974; Müller et al., 1978). The poly(A)-protein interaction may be modulated by a neighboring sequence on the mRNA (Bergmann and Brawerman, 1977). Such interactions could account for the diversity in rates of poly(A) loss in individual mRNA species.

The present study was based on the expectation that knowledge of the configuration of the 3' terminal region of the mRNA could lead to understanding of the role of this region. It was also hoped that this knowledge would help explain how poly(A) degradation is controlled. We have examined this configuration in the two adult rabbit globin mRNA species using an S1 mapping technique that permits the identification of sites accessible to nucleases (Albrecht et al., 1984). This technique was used both on deproteinized mRNA and on the mRNA engaged in translation in reticulocyte extracts. The cDNA probes used in this study carry long poly(dT) tails (Okayama and Berg, 1982). This feature provided a means to probe the portion of the poly(A) sequence adjacent to the 3' noncoding region. The results have led to some insight into the manner in which this sequence is protected in the cell during mRNA maturation. We have also observed that base pairing interactions occur at the poly(A) junction and that some portions of the 3' noncoding region are highly exposed in the mRNA associated with polyribosomes in the reticulocyte extract. The proteins known to be associated with mRNA in polyribosomes had only a minimal effect on the configuration of the 3' noncoding region, but caused strong masking of the poly(A) sequence.

* This work was supported by National Institutes of Health Grant GM17973 and National Science Foundation Grant PCM8020712. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**Materials and Methods**

Preparation of Cell Extracts and of RNA from Reticulocytes—Reticulocytes were obtained from New Zealand White long-eared rabbits made anemic with phenylhydrazine as described by Pelham and Jackson (1976). The washed cells were lysed with 1 volume of water in the presence of 30 μM hemin. The lysate was cleared by centrifugation and stored at -70°C. RNA was prepared by subjecting samples of the cleared lysate to the alkaline phenol extraction procedure (Geoghegan et al., 1978).

**RNA Fragmentation**—Reticulocyte RNA was incubated at 37°C for 10 min in the presence of 20 mM HEPES at pH 7.0, 80 mM KCl, and 0.5 mM MgCl₂, with indicated amounts of enzyme. The reticulocyte lysate was subjected to the nuclease treatments at 37°C under conditions optimal for exogenous mRNA translation (Cereghini et al., 1979). The lysate was first incubated for 5 min without enzyme and then exposed to the enzyme for the next 4 min. In some experiments, the digestions of the lysate were carried out under the conditions used for deproteinized RNA, with cycloheximide (50 μg/ml) as an additional ingredient to prevent any possible ribosome run-off during the incubation. It was verified that the mRNA in the samples of lysate used for fragmentation was almost entirely in polyribosomes (data not shown). The nucleases used for the fragmentation experiments were RNase T1 (Calbiochem-Behring), a cobra venom nuclease specific for double-stranded RNA, designated as nuclease V1 (P-L Biochemicals), and a Neurospora endonuclease specific for single-stranded RNA (Sigma). After incubation, the mixtures were deproteinized by phenol extraction.

Preparation of Labeled cDNA Probes and S1 Mapping—Recombinant plasmids containing full-length rabbit α- and β-globin cDNA inserts were kindly provided by Dr. Paul Berg (Stanford University). These probes contain long poly(dT) tails complementary to the poly(A) sequence (Okayama and Berg, 1982) and could be used to probe portions of this sequence. The procedures for cleaving the plasmids with EcoRI, labeling the termini with [α-³²P]dATP, hybridization with the fragmented RNA preparations, treatment with nuclease S1 (3–10 units), and separating the fragments were as described previously (Albrecht et al., 1984). Yeast tRNA, however, was omitted from the incubations with nuclease S1.

**Results**

**Distribution of Poly(A) Sizes**—The S1 mapping procedure involved hybridization of fragmented RNA with probes labeled at the 3' end as indicated in Fig. 1 and removal by nuclease S1 digestion of the portions of probe not covered with RNA. The sizes of the resulting probe fragments correspond to the distances between the EcoRI sites in each of the two cloned cDNAs and the 3' terminus of the mRNA fragments. The length of labeled probe complementary to intact α-globin mRNA, up to the poly(A) junction, is 163 nucleotides. The corresponding length of the β-probe is 170 nucleotides (see Fig. 1).

The mapping of intact RNA with the β-probe produced primarily labeled fragments of approximately 200 nucleotides (Fig. 2A). The excess over the 170-nucleotide value is due to T residues that are complementary to the poly(A) sequence (Fig. 1). Previous studies with a β probe containing only 2 such T residues had yielded protected probe fragments of no more than 174 nucleotides in length (Albrecht et al., 1984). The mapping with the present probe also yielded a small population of fragments with sizes greater than 187 nucleotides but smaller than 200 nucleotides, whereas fragments of sizes 171–187 nucleotides were virtually nonexistent. The results indicate that essentially all the β-globin mRNA molecules have a poly(A) sequence containing at least 18 AMP residues and that the great majority of the poly(A) segments are above 30 nucleotides in length. The minor cluster of fragments of 165–168 nucleotides evident in Fig. 2 (band m) corresponds to mRNA chains truncated near the end of the 3'-noncoding region. The occurrence of these molecules with “endogenous” cleavages has been discussed previously (Albrecht et al., 1984).

The mapping with the α-probe yielded a more complex distribution of fragments covering the poly(A) sequence (Fig. 2A). Two major clusters were evident. One corresponds to sizes ranging from about 190 to 205 nucleotides and the other to sizes from about 220 to 230 nucleotides. The distribution of fragments corresponding to poly(A) segments, obtained by densitometry scanning of the autoradiogram in Fig. 2A, is shown in Fig. 3A. A different cloned α-globin cDNA, with a

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1 The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
longer poly(dT) sequence, yielded the distribution of poly(A) termini shown in Fig. 3B. These results show that the great majority of α-globin mRNA chains have poly(A) segments with at least 24 AMP residues. They also indicate that at least two major poly(A) size classes occur in this mRNA species. One consists of segments with about 25-40 nucleotides, and the other of segments with about 55-70 nucleotides.

It was verified that the poly(dT) tail in the α-probe is homogeneous in size, and therefore appropriate for the analysis of poly(A) size distribution. This was determined by cutting the end-labeled EcoRI probe at a BamHI restriction site located in the vector close to the poly(dT) sequence. Electrophoresis of the digested material yielded a single sharp band (data not shown). A minor cluster of probe fragments with 167-170 nucleotides (band n) can also be seen in Figure 2. This band, however, is very faint. It tended to be almost nonexistent in some experiments and was more intense when excessive amounts of nuclease S1 were used for the removal of unreacted probe fragments.

Most of the material capable of annealing to the poly(dT) portion of the probes was removed by adsorption of the RNA preparations on oligo(dT)-cellulose. Under the binding conditions used in this study, the adsorbent failed to bind a small population of α-globin mRNA chains with poly(A) segments up to about 20 nucleotides in length (Fig. 2B). A small portion of the β-globin chains with poly(A) segments up to 30 nucleotides in length also failed to bind to the oligo(dT)-cellulose.

Sites Accessible to Nucleases in the 3'-Noncoding Region—Exposed sites in this region were analyzed using two nucleases specific for single-stranded RNA, RNase T1, and a Neurospora endonuclease. An enzyme from cobra venom specific for double-stranded RNA, nuclease V1, was also used in order to identify accessible sites not cleaved by the first two enzymes because of their double-stranded configuration. Nuclease V1 caused extensive cleavage of the 3'-noncoding region of both mRNA species, but was not active when applied to the crude reticulocyte extract, even at levels 10 times higher than those effective on deproteinized RNA. The cleavage patterns revealed by S1 mapping are shown in Figs. 4 and 5, and the locations of the major sites of cleavage are indicated in Figs. 6 and 7. The relation between band size and position along the mRNA chain is also indicated in the latter two figures.

The deproteinized α-globin mRNA contained few sites that could be cleaved by the enzymes specific for single-stranded RNA. One site was highly sensitive to these two enzymes (Figs. 4 and 5, band a). This site is located at positions 529-531 (Fig. 6). Adjacent to it was a long segment readily cleaved by nuclease V1 (Fig. 4, lane c; Fig. 5, lane d). This segment is represented by the large cluster of fragments of sizes 93-142. This pattern of cleavages indicates that a large portion of the noncoding region, extending from positions 480 to 532 (see Fig. 6), is readily accessible to macromolecules. Most of it is in double-stranded configuration, and it is terminated by a short, highly exposed, single-stranded segment (band a). A stem-loop configuration could account for the high degree of exposure of the latter site, and the segments surrounding this site (positions 522-529 and 534-541) are complementary to each other (see Fig. 6). One of these segments is sensitive to nuclease V1, as indicated by the strong bands of sizes 134-141 in Fig. 4 (lane c) and in Fig. 5 (lane d). The complementary segment, however, is not sensitive to V1, as indicated by the
Globin mRNA Configuration

FIG. 5. Cleavages caused by Neurospora endonuclease in deproteinized RNA and in reticulocyte lysate. Samples of reticulocyte RNA and of lysate were incubated under identical conditions, except for the presence of 50 μg/ml cycloheximide in the lysate samples. RNA samples were incubated without enzyme (lane a) and with 2 × 10^-2 unit of Neurospora endonuclease (lane b). Samples of lysate were incubated without enzyme (lane c) and with 1.4 × 10^-2 unit of *Neurospora* endonuclease (lane f). RNA cleavage patterns caused by 3 × 10^-4 units of RNase T1 (lane c) and by 3 × 10^-2 unit of nuclease V1 (lane d) are included for comparison. Arrowheads indicate position corresponding to poly(A) junction.

FIG. 6. Location of nuclease-sensitive sites in 3'-noncoding region of α-globin mRNA. Sequence data are from Heindell et al. (1978). Numbers indicate positions of residues in mRNA sequence, and numbers in parentheses indicate sizes of probe fragments that would correspond to cleavages at these positions. Arrows represent major RNase T1 cleavage sites; dashed lines, segments susceptible to nuclease V1; and dotted lines, segments susceptible to *Neurospora* endonuclease. Highly exposed sites are emphasized by double arrows and double arrowhead; sites discussed in text are identified by letters. The termination codon is at positions 463–465.

The absence of bands of sizes 146–153. It is not sure, therefore, that this structure occurs. Another portion of the noncoding region, extending from the translation termination site (position indicated by letter t in Fig. 4) to position 480 (band size 92), was resistant to all three nuclease and must be buried within a complex structure. The cleavages close to the poly(A) junction are discussed below.

The 3'-noncoding region of the β-globin mRNA was more uniformly sensitive to nuclease. Nearly all the G residues in this region have been shown to be cleaved by RNase T1 (Albrecht et al., 1984). Exposure to the *Neurospora* endonuclease showed the occurrence of one highly sensitive site (Fig. 5, band p in lane b). This site corresponds to positions 515–519 on the mRNA (Fig. 7). Some of the sites readily cleaved by RNase T1 showed little sensitivity to the *Neurospora* enzyme (see Fig. 7). RNase T1 is a small protein (Egami et al., 1964) and could perhaps reach sites not easily accessible to the bulkier *Neurospora* nuclease (Rabin and Fraser, 1970). Exposure to nuclease V1 indicated the occurrence of many regions of secondary structure (Fig. 4, lanes c and d). One cluster of 4 G residues resistant to RNase T1 (G"—G") was cleaved by nuclease V1, as indicated by the band cluster of sizes 105–109 in Fig. 4, lane c. It is located near a cluster of 4 C residues, also sensitive to V1 (bands 122–126), and a short stem-loop structure involving these two complementary segments is possible (see Fig. 7). Two additional stem-loop structures could be constructed using segments sensitive to nuclease V1 (see Fig. 7). However, one of the complementary segments in each of these two stems was also sensitive to the *Neurospora* endonuclease specific for single-stranded RNA (bands p and q in Fig. 5). These stems, which consist of only A-U base pairs, must be rather unstable under the conditions used for the enzyme treatments. This could explain why these segments could have both a double-stranded and single-stranded character. Only the segments bearing AMP residues were cleaved by the *Neurospora* enzyme (see Fig. 7).

The distribution of exposed single-stranded sites was nearly identical in deproteinized mRNA and in the mRNA as it occurs in the reticulocyte lysate (Fig. 4, lanes e and f; Fig. 5, lanes b and f). One site relatively resistant to cleavage by the *Neurospora* enzyme in deproteinized β-globin mRNA was far more exposed in the reticulocyte lysate (Fig. 5, compare band s in lanes b and f).

The secondary structure of the mRNAs was examined after melting and quick cooling of deproteinized RNA preparations. The nuclease V1 cleavage patterns obtained with RNA before and after melting were essentially unchanged (Fig. 4, compare lanes b and c). Both the sizes and relative intensities of the bands corresponding to cleavages in the noncoding region were the same. The melting, however, made the α-globin mRNA more sensitive to degradation by nuclease V1, as indicated by the lower intensity of the bands corresponding to intact RNA from preparations digested after melting (compare lanes b and c). The destabilizing effect was not observed in the β-globin mRNA.

Secondary Structure around the Poly(A) Junction—Nuclease V1, an enzyme specific for double-stranded RNA, caused cleavages at the beginning of the poly(A) sequence in both mRNA species (Fig. 4, see bands n and v). Sites near the end of the 3'-noncoding region were also sensitive to nuclease...
V1 (Fig. 4, see bands below n and v in lanes c). The cleavages at the latter sites were observed consistently in the experiments with the a-probe, but not in all experiments with the b-probe. The sensitive site at the beginning of the poly(A) sequence appears to be highly accessible in the a-globin mRNA, as indicated by the fact that it was cleaved preferentially when the RNA was exposed to a lower level of enzyme (Fig. 4, band n in lane d). The results suggest the occurrence of a double-stranded structure around the poly(A) junction. The fact that this structure remains after melting and quick cooling of the RNA suggests that the interaction is with another portion of the same mRNA chain. There is no obvious sequence complementarity between the sites around the poly(A) junction and any other portion of the 3'-noncoding regions (see Figs. 6 and 7).

*Masking of the Poly(A) Sequence in the Reticulocyte Extract—*It is well established that the poly(A) sequence in polyribosomes is associated with proteins that protect it against digestion by nucleases (Brawerman, 1981; Littauer and Soreq, 1982). The effect of the Neurospora endonuclease on the poly(A) was examined in order to determine whether the protection is uniform along the entire sequence, or whether particular regions are protected to different extents. Digestion of deproteinized RNA caused apparently random cleavages in this sequence, as shown by the accumulation of chains with short poly(A) segments (Fig. 5, lanes b). It can also be seen in this figure that the poly(A) sequence was far more sensitive to the enzyme than the adjacent portion of the 3'-noncoding region. In the reticulocyte extract, the entire poly(A) region seemed well protected (Fig. 5, lanes f). The size distribution of poly(A) segments in the a-globin mRNA, analyzed as in Fig. 3, was essentially the same before and after exposure to the Neurospora enzyme (data not shown).

**Relation of Highly Accessible Sites to Endogenous Cleavage Sites**—We had observed previously that our RNA preparations contained some truncated mRNA chains generated by cleavages at specific sites, particularly in the noncoding regions (Albrecht et al., 1984). These cleavage appear to have occurred in the intact cells, as indicated by the fact that incubation of the reticulocyte extracts at 37 °C did not cause further digestion at these sites. The major endogenous cleavage site in the 3'-noncoding region of the a-globin mRNA corresponds to the site highly sensitive to the Neurospora nuclease and to RNase T1 (Fig. 5, compare band o in lanes a–c and f). It can also be seen in Fig. 5 that this site is not subject to cleavage in the crude extract incubated without nuclease (compare lanes a and e). Some of the endogenous cleavage sites in b-globin mRNA also correspond to sites sensitive to the Neurospora enzyme. In this case, however, the sites that were most exposed in the extract did not correspond to endogenous sites (compare bands p and s in lanes a and f). These findings suggest that some of the sites on mRNA that are highly exposed in reticulocyte extracts may also be susceptible to cleavage inside the cells.

**DISCUSSION**

The findings in this study indicate the occurrence of a complex configuration at the 3' end of the globin mRNAs. It appears from the analysis of poly(A) sizes that the first 20–25 AMP residues in this sequence are well protected from the degradation process that causes poly(A) shortening in the cells. More zones of protection are seen in the a-globin mRNA about 40–50 and 70–86 nucleotides away from the poly(A) junction. There may be additional protected zones, but the limiting length of the poly(DT) segments in the cDNA probes did not permit the analysis of poly(A) segments longer than 85 nucleotides in the a-globin mRNA and 30 nucleotides in the b-globin species. Evidence for the occurrence of discrete poly(A) size classes has been available for quite some time (Mansbridge et al., 1974; Jeffery and Brawerman, 1974). This discontinuous distribution of zones of cleavage could conceivably be determined by a special organization of the proteins associated with the poly(A) sequence. These proteins would have to be anchored in a specific fashion at the beginning of this sequence in order to ensure the retention of a minimum of 20–25 AMP residues on the mRNA chains and to provide partial protection at measured distances along the sequences.

It has been shown that the poly(A)-protein interaction in cytoplasmic extracts of mouse erythroleukemia cells leads to a periodicity in the distribution of nuclease-sensitive sites along this sequence, with a 27-nucleotide spacing between these sites (Baer and Kornberg, 1980). An interaction of this kind, if it were to occur in the cell, could be responsible for the distribution of poly(A) sizes observed in the present study. Our experiments with Neurospora endonuclease, however, did not provide any clear indication of periodicity in the distribution of masking agents. The poly(A) sequence appeared to be uniformly protected against the endonuclease, both in poly(A)-protein complexes and reticulocyte extracts. The present experiments deal with protection of poly(A) segments still attached to the mRNA, whereas the study of Baer and Kornberg (1980) apparently dealt with protection in poly(A)-protein complexes only. It has been shown previously that the degree of protection is reduced when the poly(A)-protein complex is severed from the rest of the mRNA (Bergmann and Brawerman, 1977). It could be that the process leading to the controlled degradation of the poly(A) sequence on mRNA involves transient alterations in the poly(A)-protein interactions that cause the unmasking of specific sites in this sequence.

The use of the cobra venom nuclease V1, an enzyme that is specific for double-stranded segments in RNA, provided information on double-stranded structure in the 3'-noncoding region. The results show that this region contains a large proportion of double-stranded segments. One of these segments appears to span the poly(A) junction. Several A residues at the beginning of the poly(A) sequence may be base paired with U residues, as judged by their sensitivity to RNase T1. The polyadenylate segment complementary to the poly(A) junction appears to be part of the same RNA chain. This is indicated by the rapid reconstitution of the double-stranded structure after melting of the RNA. We were not able to identify such a sequence within the 3'-noncoding region of either mRNA species.

The experiments with RNase T1 and the Neurospora endonuclease provided an analysis of accessible sites not engaged in complementary base pairing. Sites not cleaved by these enzymes were deemed to be either in double-stranded regions or in regions shielded by tertiary folding of the RNA. For instance, the cluster of G residues at positions 525–528 in the b-globin mRNA, which was not cleaved by RNase T1, was found to be in an accessible double-stranded region sensitive to nuclease V1. Some portions of the 3'-noncoding region of a-globin mRNA appear to lie within a complex structure inaccessible to macromolecules, as indicated by the fact that they were not cleaved by any of the enzymes. Our results also show that individual single-stranded sites differ widely with respect to degree of accessibility. This is indicated by the large differences in band intensities in the S1 mapping patterns. It is also suggested by the fact that some sites accessible to the small RNase T1 were not cleaved by the far bulkier Neurospora endonuclease.

The patterns of cleavage in the a-globin mRNA observed...
in this study resemble to some extent those described previously for this species (Vary and Vournakis, 1984). One major difference is the sensitivity of the sites next to the poly(A) to nuclease V1. In the β-globin species, there is little resemblance between the present cleavage patterns and those described previously. In the latter study, the mRNA species were modified by removal of the poly(A) sequence. It would be tempting to attribute the discrepancies to the presence of this sequence, but the results are probably not comparable because of differences in the conditions used for enzymatic digestion and in the mRNA isolation procedures.

The comparison of cleavage patterns in deproteinized mRNA and in the mRNA as it occurs in crude reticulocyte extracts showed that the removal of proteins and of ribosomes had little effect on the configuration of the 3'-noncoding region. Eukaryotic mRNA is known to be associated with large amounts of proteins (Freobrazhensky and Spirin, 1978), but our results provided no clear indication of direct interaction between proteins and the 3'-noncoding region. In contrast, strong interactions between the poly(A) sequence and proteins were evident. The results also indicate that the presence of ribosomes on the coding region does not affect substantially the configuration of the 3'-noncoding region. It appears from our results that the configuration of the mRNA in its functional state is determined primarily by RNA-RNA interactions confined to this region, although interactions with the 5'-noncoding region remain a possibility. It was possible to construct stem-loop structures in the β-globin mRNA that account for most of the sites sensitive to nuclease V1 (Fig. 7). In the α-globin mRNA, however, most of the V1-sensitive sites could not be fitted into credible base paired structures. Nuclease V1 can recognize interactions other than those in the classical Watson-Crick base pairs and also bases engaged in tertiary folding (Lockard and Kumar, 1981). It could be that the structures recognized by this enzyme in the present study involve a variety of interactions, not limited to the conventional hydrogen bonding in the A-U and G-C base pairs.

Acknowledgments—We thank Dr. Paul Berg for the gift of globin cDNA probes and Ann Boyle for technical assistance. We also wish to thank Dr. John Coffin for helpful discussions.

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