Specific hydrolysis of rabbit globin messenger RNA by S1 nuclease

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ABSTRACT

S1 nuclease isolated from Aspergillus oryzae has been used to investigate the secondary structure of rabbit globin messenger RNA (mRNA). The enzyme, which is specific for single stranded nucleotides, digests globin mRNA to a limited extent, with 65-75% of the mRNA nucleotides resistant to digestion under mild conditions. This limited digestion is not due to enzyme inactivation, but rather to the normal activity of the single-strand nuclease. The reaction was studied as a function of temperature, salt and enzyme concentration. Analysis of the products of digestion on 20% acrylamide - 7 M urea slab gels reveals a stable pattern of unique fragments ranging in size from 9 to 71 nucleotides. Separated α and β globin mRNAs show similar, but not identical gel patterns, indicating strong structural similarities between the two species. The high degree of nuclease resistance, along with the fragment patterns seen on polyacrylamide gels, gives evidence to support a model of rabbit globin mRNA which contain specific, rather than random, helical structure.

INTRODUCTION

Recent advances have permitted isolation of a number of eukaryotic messenger RNAs in nearly pure form (1). The availability of these purified mRNAs has led to the discovery that all mRNAs include unique primary structural features in addition to the coding sequence such as the 5' terminal "cap" structure (2,3), the 3'-hydroxyl polyadenylate sequence (1), and the non-coding regions in the 5' and 3' portions of the molecule preceding and following the coding sequence (4). Secondary structure predictions for the 3' non-coding regions of several partially sequenced mRNAs demonstrate the possibility that specific structural homologies may exist (4) among different messengers. Recently, rabbit β-globin messenger RNA has been completely sequenced (5). Computer generated predictions of secondary structure suggest that specific stable structural features exist (6). If so, these regions may have functional significance. Previous workers have made estimates of secondary structure using physical techniques, such as thermal denaturation (7), circular dichroism (8) and
dye binding (9). It has been demonstrated that the amount of helical structure in globin mRNA is greater than that expected for random sequence ribopolymers (7). Further indirect evidence for the existence of specific local secondary structure in rabbit globin mRNA comes from work of Efstratiadis, et. al. (10) on the preparation of cDNA with AMV reverse transcriptase. Partial reverse transcripts of specific length are observed in addition to full length cDNA. It is likely that secondary structure localized at specific points internally along the globin mRNA molecule acts to inhibit movement of reverse transcriptase thus generating premature termination and the partial length products. In the present study, we have utilized SI nuclease isolated from Aspergillus oryzae to investigate the size and extent of helical structure in highly purified rabbit globin mRNA. SI nuclease preferentially degrades single stranded regions in nucleic acids (11) and appears to have no base specificity (12,13). The enzyme has been widely used as a probe for single stranded structure in both DNA and RNA (14-18). The results presented here show that globin mRNA is 65-75% resistant to SI nuclease and that susceptibility to enzymatic digestion changes with conditions known to affect helix stability. The products of digestion appear as discrete bands upon gel electrophoresis in denaturing gels. SI nuclease thus appears to be a useful tool for the study of secondary structure in messenger RNA.

EXPERIMENTAL PROCEDURES

In all cases solutions were prepared using distilled, deionized water, and were sterilized before use.

Purification of rabbit globin mRNA.

Globin mRNA was isolated from reticulocytes of New Zealand female albino rabbits using the method of Nienhuis et.al. (19). The material obtained was further purified by passage over oligo (dT)-cellulose, followed by sucrose gradient centrifugation and a second oligo (dT)-cellulose column (20). The mRNA obtained in this way migrated as one broad band with a midpoint corresponding to 650 nucleotides in a 5% polyacrylamide-98% formamide gel (21). The mRNA was translated in vitro in a wheat germ system (20), and the translation products were α and β globin protein chains only.1

1 N. Vamvakopoulos and G. Thireos, unpublished results
Radioiodination of mRNA.

Globin mRNA was iodinated with Na$^{125}$I (Amersham-Seale) by a modification of the method of Commerford (22) using previously determined optimum Na Sulfite conditions (23). The specific activity was calculated to be at least $3 \times 10^6$ cpm/μg as determined in a triton-toluene scintillation solvent. $^{125}$I-labelled globin mRNA migrated identically to unlabelled material in 5% acrylamide - 95% formamide gels (21), indicating minimal degradation during the iodination procedure.

Separation of α and β globin mRNAs.

α and β$^{125}$I-labelled globin mRNAs were resolved by electrophoresis in 5% acrylamide-98% formamide gels for 18 hours at 200 V (21). The mRNA bands were detected by autoradiography, excised, and eluted as described by Maniatis et. al. (24).

Isolation and assay of S1 nuclease.

S1 nuclease was purified from crude α-amylase powder by the method of Vogt (12). The single strand specificity was confirmed using the synthetic polynucleotides $^3$H-poly(U) and $^3$H-poly(A), as previously described (25). Enzyme activity was determined according to the method of Vogt (12), using sonicated, heat denatured, calf thymus DNA as substrate. An enzyme unit is defined as the amount of enzyme that solubilizes 10 μg DNA in 10 min at $45^\circ$ in the standard assay (12).

S1 nuclease digestion.

The standard mRNA digestion mixture (100 μl) contained 0.3 M sodium acetate, pH 4.4; 0.002 M ZnCl$_2$; 0.30 M NaCl; 5% glycerol; between 50,000 and 100,000 cpm of purified $^{125}$I-globin mRNA; 1 μg yeast-stripped t-RNA as carrier; and 0-0.5 units of S1 nuclease. Incubations were performed at $22^\circ$ for up to 4 hours. Portions (ranging from 7-10 μl) were withdrawn at various times and analyzed for trichloroacetic acid precipitable radioactivity by precipitation on Whatman 3 mm filter paper discs in omnifluor toluene liquid scintillation mix. The cpm obtained were normalized to zero time counts (enzyme was added after the zero point was obtained) and are presented as percent nuclease resistance. All determinations were performed in duplicate. Variations from these conditions are indicated in the figure legends, as appropriate.

Gel Electrophoresis of S1 digestion products.

S1 nuclease digestion was performed in a similar manner to that described above; except that about 500,000 cpm of $^{125}$I-mRNA and 10 μg carrier t-RNA were added to the reaction mixtures containing enzyme (about
200,000 to the control mixtures without enzyme). SI nuclease concentrations were also increased to maintain the usual enzyme to RNA ratio of 0.125-0.250 U/µg. Reaction volumes were 100µl in all cases. Digestion was stopped by phenol extraction followed by precipitation with 2.5 volumes of ethanol at -70°. After centrifugation for 1 hour at 3600 x g, the pellet was dried, redissolved in 25 µl of deionized formamide, heated at 70° for 3 minutes, then quickly chilled on ice and applied to the gel. Electrophoresis was carried out in 20% acrylamide - 7 M urea slab gels according to Maniatis et al. (21). Estimates of nucleotide lengths from the autoradiograms were determined relative to the marker dyes bromphenol blue (BP) and xylene cylanol (XC), taken as 10 and 29 nucleotides, respectively (21).

RESULTS

Limited Digestion of globin mRNA by SI nuclease.

Incubation of radiiodinated globin mRNA with SI nuclease at 22° produces only a limited digestion (Figure 1). At enzyme concentrations up to 2.4 units per ml, the mRNA is 70-73% resistant to hydrolysis, while raising the enzyme concentration to 5 units per ml makes the molecule more susceptible to enzyme digestion (38% resistance). Thus, conditions can be found (low enzyme concentration, 0.3 M NaCl and 22°C) in which a large portion of globin mRNA is protected from SI nuclease cleavage. The mild digestion conditions described requires prolonged incubation with the SI nuclease, raising the question of loss of enzyme activity over the course of 1-2 hours, thus producing an artifically high resistance pattern.

In order to test for the possibility of enzyme inactivation, the experiment shown in figure 2 was performed. 125I-mRNA was incubated with SI nuclease for 75 minutes, sufficient time for digestion to have nearly halted (see Fig. 1). Addition of fresh iodinated mRNA at this time resulted in another burst of digestion, similar in time course to the initial reaction. These results indicate that loss of available substrate (presumably single stranded ribonucleotides), rather than enzyme inactivation, is the reason for the limited enzyme digestion observed.

Characterization of mRNA digestion by SI nuclease.

In order to determine the effect of the structural integrity of the mRNA on its susceptibility to SI digestion, several parameters known to affect stability of helical structure were examined. We have previously studied the effects of the following on the time course of SI hydrolysis (25): (1) magnesium ion, at concentrations up to 20 mM, has no effect on the extent of hydrolysis, (2) pretreatment of the mRNA with formaldehyde
Figure 1 Digestion of mixed α and β rabbit globin mRNA by SI nuclease at several enzyme concentrations. Reaction mixtures contained 125I-labelled mRNA and carrier t-RNA as described. SI nuclease concentrations were as follows: 0.6 units/ml (Δ), 1.1 units/ml (Ο), 2.4 units/ml (Ο), 5.0 units/ml (X).

Figure 2 Addition of 125I-rabbit globin mRNA during SI nuclease digestion. A standard SI nuclease digestion reaction (22°C, 2.4 units/ml enzyme) contained 87,600 cpm of 125I-mRNA. An equal amount of mRNA was added after 75 minutes of reaction. Calculations of % nuclease resistance following the addition of mRNA at 75 minutes were corrected for the amount of radioactivity estimated to represent the initial mRNA present, using the appropriate data of figure 1.
at elevated temperatures, in order to disrupt hydrogen binding, greatly lowers the nuclease resistance. (3) removal of the 3' terminal poly(A) portion of the mRNA using Ribonuclease H (26) does not significantly change the time course or extent of hydrolysis as compared to intact globin mRNA. When SI nuclease cleavage of globin mRNA was studied as a function of temperature, the results shown in figure 3 were obtained. These experiments were performed using 5 units per ml SI nuclease, resulting in more extensive digestion than at 2.4 units per ml. The degree of digestion is sensitive to temperature. This would be expected given that thermal melting of helical structure occurs. At 0°C, digestion is very limited, while at 42°C, the mRNA is nearly completely degraded. The small amount of nuclease resistance, at 42°C is interesting in that it may represent well matched, exceptionally stable helical structure.

The effect of sodium chloride on the SI digestion is shown in Fig. 4. Increasing salt concentration stabilizes helical structure in nucleic acids and is known to increase the thermal melting transition for globin mRNA (27). The salt dependency of mRNA hydrolysis by SI nuclease is consistent with the salt stabilizing effect. As the salt concentration is lowered, nuclease resistance is greatly decreased, while at the highest NaCl concentration tested (0.5 M) the mRNA is nearly completely protected. This salt effect cannot be accounted for by NaCl influencing enzyme activity, since SI nuclease is completely active at salt concentrations up to 0.3 M, and is only slightly less active at 0.5 M in digesting poly(rU) (data not shown, 28).

Analysis of the products of digestion on gels.

It is of interest to characterize the SI digestion products in order to determine whether the secondary structure within globin mRNA is random or consists of unique helical regions. If the helices are indeed specific structural features, SI digestion under the mild conditions used might be expected to produce, consistently, a family of double-stranded oligo-ribonucleotides of varying lengths, and these should be separable on denaturing polyacrylamide gels. Figure 5 is an autoradiogram of a 7 M urea - 20% acrylamide slab gel which shows the SI digestion products formed at various incubation times at 22°C. This gel resolves oligo-ribonucleotide fragments up to about 80 nucleotides in length. In the absence of enzyme (slot 5), all 125I-labelled mRNA remains at the origin, indicating that no cleavage has occurred as a result of the incubation conditions. A distinct pattern of discrete length fragments, ranging in size from 17 to 52 nucleotides, is generated, by 2 hours which is unchanged at 4 hours.
Figure 3  SI digestion of $^{125}$I-labelled globin mRNA as a function of temperature. Reaction mixtures contained 5.0 units/ml enzyme in all cases.

Figure 4  Effect of NaCl on digestion of globin mRNA by SI nuclease. $^{125}$I-labelled rabbit globin mRNA was incubated with SI nuclease at 2.4 units per ml and 22° as described except that the final concentrations of NaCl were varied as follows: no NaCl (O), 0.3 M (△) and 0.5 M (□).
Figure 5 Autoradiogram of S1 digestion products of $^{125}$I-labelled rabbit globin mRNA obtained at several incubation times. Samples were applied to a 20 x 20 cm 20% acrylamide - 7 M urea slab gel, and were run at 200 volts for 20.5 hours. Slot 1: 30 minutes; slot 2: one hour; slot 3: two hours; slot 4: four hours; slot 5: no enzyme, four hours.

The resistance of the fragments to further digestion over a period of hours suggests that a limit digest has been achieved, with production of fragments having double stranded character. A similar study, not shown, in which temperatures of incubation was varied produced gel patterns similar to the 4 hour result in figure 5 at 22°C. However, at 42°C, no large fragments were seen. These gel patterns were found to be in excellent agreement with the temperature dependency seen in figure 3, in which digestion at 0° and 22° is limited, while at 42°, most of the mRNA is degraded. The results in figure 5 and the temperature study demonstrate that discrete length fragments can be generated from globin mRNA using a single strand specific nuclease.
Figure 6 Autoradiogram of S1 nuclease digested rabbit α and β globin RNAs. mRNA was digested with S1 nuclease at 2.4 units/ml and 22°C for 2 hours. Samples were applied to a 20% acrylamide - 7 M urea gel and were run at 200 volts for 16 hours. Slot 1: α globin mRNA, no enzyme; slot 2: α globin mRNA, + enzyme; slot 3: β globin mRNA, no enzyme; slot 4: β globin mRNA, + enzyme.
**Digestion of separated α and β globin mRNA.**

The studies described so far were performed with total rabbit globin mRNA, which is a mixture of α and β globin mRNAs (28,29). The S1 fragments are derived from both species. Separation of the α and β mRNAs can be achieved by electrophoresis of the mixed globin mRNA for an extended period on a low percentage denaturing gel (5% acrylamide, 95% formamide), and elution of the α (faster) and β (slower) migrating bands (29-31). When purified α and β globin mRNAs, separated in this manner, were subjected to S1 digestion (Fig. 6), the gel patterns which resulted were somewhat similar. These results indicate that the two molecular species both probably assume relatively stable secondary structures that are protected from S1 nuclease.

**DISCUSSION**

Highly purified rabbit globin mRNA was subjected to digestion with the single stranded nuclease S1 from Aspergillus oryzae. Globin mRNA is largely resistant to S1 nuclease S1 under appropriate conditions (room temperature, high salt, low enzyme concentration). This limited digestion was shown to be due to resistance of the mRNA itself to enzyme action, rather than to enzyme inactivation during incubation. The percentage of nucleotides protected from the single strand specific nuclease is rather high and varies depending on the S1 concentration. Similarly, high estimates of helical structure in globin mRNA were obtained at pH 4.5 by thermal denaturation (25). S1 nuclease activity is maximal at pH 4.0-4.3, with almost no activity above pH 6.0 (12), necessitating the use of an acidic condition for these studies. Estimates of secondary structure at neutral pH range from 52-70% (25,27,32). The larger amount of nuclease resistance that we find is not unreasonable, since helical structure in globin mRNA increases as the pH is lowered (25).

The degree of nuclease resistance in globin mRNA is influenced by enzyme concentration, temperature and salt. At enzyme concentrations up to 2.4 units per ml, hydrolysis occurs to the same limited extent, while at 5 units per ml, much greater digestion occurs. Similar results were obtained with S1 digestion of SV40 DNA (15). The increased digestion at higher enzyme levels may be due to slight contamination by T1 or other nucleases; or to a lack of absolute specificity for single stranded RNA (33). However, the presence of small amounts of contaminating enzyme does not alter the conclusions of this paper in any way. Also, tertiary interactions in the mRNA resulting from weak base pairing might be disrupted at high enzyme concentrations, unfolding the molecule to ex-
pose previously inaccessible single stranded bases.

The influence of salt concentration and temperature on the SI digestion is consistent with changes in helical content in globin mRNA observed using optical methods (25,27), and is entirely analogous to results obtained with single strand nuclease digestions of SV40 DNA (14, 15), MS2 RNA (13) and tRNA (17,34). The data reported here, along with our previous observations (25), show that agents known to disrupt helical structure (high temperature, low salt, formaldehyde) increase SI susceptibility. The results indicate that SI nuclease can be used as a probe for specific double stranded structure in mRNA.

Gel analysis of the SI digestion products consistently produces discrete oligonucleotide fragments with lengths of approximately 9 to 71 nucleotides. The broadness of the bands shown is probably due to length heterogeneity within each band. Formation of discrete length fragments from the limited SI digestion is a strong indication that specific helical structure exists in globin mRNAs. Furthermore, the similarity between the separated α and β gel patterns points toward a high degree of conservation of helical regions within the two mRNA species. Sequencing data obtained for the non-coding regions adjacent to the 3'-terminal poly(A) sequence of the α and β globin mRNAs of rabbit, among others (4,5,35) display striking homology, leading the authors to argue for an evolutionary stability and a functional role for the conserved sequence (3). Although these studies are limited to relatively small regions of the molecules, they support the concept of specific secondary structural features in globin mRNA, since homologous hairpin structures can be predicted from several of the sequences (4). Recent computer generated secondary structure predictions comparing the 3' non-coding regions of rabbit and human β-globin mRNAs indicate that homologous structures exist that may become stabilized during evolution (6).

Nuclease SI digestion of human globin mRNA (kindly supplied by B. Forget and S. Weissman) produces discrete fragments on denaturing gels (results not shown). Experiments are in progress to further characterize and map these fragments within the known sequences of both the rabbit and human mRNAs.

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