The 5'-end structure of ovalbumin mRNA in isolated nuclei and polysomes

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Received May 31, 1994; Revised and Accepted September 7, 1994

ABSTRACT

We used the chemical reagents dimethylsulfate and 4'-aminomethyl-4,5',8-trimethylpsoralen and the enzyme T1 ribonuclease to compare the 5'-end structure of ovalbumin mRNA *in situ* in purified hen oviduct nuclei and polysomes with that of the isolated mRNA. The qualitative pattern of structure-dependent base modifications and T1 ribonuclease cleavage sites in intranuclear and polysomal ovalbumin mRNAs was found to be nearly identical to those in isolated ovalbumin mRNA. These structural data are consistent with the presence of a trigonal stem-loop structure at the 5'-end of ovalbumin mRNA (hairpin-1) in nuclei and polysomes. Similar results were obtained for a coding region structure (hairpin-3) in intranuclear ovalbumin mRNA. We have recently shown that hairpin-1 positively affects the rate of ovalbumin mRNA translation *in vitro* and is part of a high affinity binding site for eucaryotic initiation factor-2 (eIF-2). The presence of hairpin-1 in ovalbumin mRNA in both a pre-translation state (nuclei) and active translation state (polysomes) is consistent with its hypothesized biological function as an intracellular initiation signal that facilitates the translation of this mRNA.

INTRODUCTION

We have previously described the secondary structure of the 5' region (nucleotides 1–125) of hen ovalbumin mRNA (1,2; Figure 1), and have recently determined that the hairpin structure at the 5'-end of ovalbumin mRNA (hairpin-1 in Figure 1) positively affects its rate of cell-free translation and is part of a specific, high-affinity binding site for reticulocyte eIF-2 (3). In this paper we examine the 5'-end structure of ovalbumin mRNA in hen oviduct nuclei and polysomes in order to determine if the hairpin-1 structure occurs under biological conditions. Pennequin *et al.* (4) have shown that 99% of cytoplasmic ovalbumin mRNA is actively being translated in monosomes (2%) and polysomes (97%). Studies by Nelson and Winkler (5) on mRNA entry into polysomes suggest that there may be a cooperative interaction between the binding of the first ribosome and subsequent ribosomes, which is mediated by a change in

mRNA structure. These observations suggested that the hairpin-1 initiation signal of ovalbumin mRNA is most likely to occur in the nucleus immediately before mRNA transport into the cytoplasm and the binding of the first ribosome; a condition analogous to adding isolated mRNA to a cell free translation reaction. In the experiments described below we analyzed intranuclear ovalbumin mRNA for the pattern of paired and unpaired bases that we have previously shown to be consistent with the presence of hairpin-1 in isolated ovalbumin mRNA (1,2). This intranuclear analysis was then extended to hairpin-3 (see Figure 1). We also analyzed the 5'-end structure of ovalbumin mRNA in polysomes for changes in the pattern of paired and unpaired bases that would indicate a translation-associated alteration in the structure of hairpin-1. Both nuclei and polysomes turn out to be convenient experimental targets for *in situ* structure-dependent chemical modification and T1 ribonuclease digestion of ovalbumin mRNA.

MATERIALS AND METHODS

Isolation of hen oviduct nuclei and polysomes

Oviducts obtained from White Leghorn hens were rinsed in ice-cold phosphate-buffered saline and immediately frozen in liquid nitrogen. Nuclei (500 mg yield) were prepared from 5 g of frozen oviduct tissue exactly as described by Marzluff and Huang (6). Purified nuclei examined by phase contrast microscopy were found to be intact, smooth and round, and free of whole cells, cell debris and other cytoplasmic material. Northern blot analysis of RNA extracted from purified oviduct nuclei revealed a large amount of mature ovalbumin mRNA that was easily detected by autoradiography (data not presented). Nuclear RNA precursors to ovalbumin mRNA could be seen only by overexposing the Northern blot autoradiogram. Hen oviduct monosomes and polysomes (maximum size of 12–15 ribosomes) were prepared from fresh tissue homogenates by the magnesium precipitation method of Palmiter (7) as described by Clemens (8). This method yields essentially 100% polysome recovery from hen oviduct homogenates (7). Nuclei (170 mg/ml final concentration) or polysomes (176 A₂₆₀ units/ml final concentration) were resuspended in storage buffer [50 mM Tris–HCl, pH 8.0, 5 mM Mg(OAc)₂, 5 mM dithiothreitol, 0.1 mM EDTA, 25% glycerol]

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and stored in aliquots at -70°C . Nuclei and polysome preparations were analyzed for free ovalbumin mRNA by reverse transcription with the ov125 primer (see below). Extracts of nuclear and polysome pellets (see below) produced full-length ovalbumin cDNA. Corresponding post-nuclear or post-polysomal supernatants produced no ovalbumin cDNA indicating the absence of detectable free ovalbumin mRNA (data not presented).

Structural analysis of nuclear and polysomal ovalbumin mRNA

Reaction of ovalbumin mRNA with dimethylsulfate (DMS; Aldrich Chemical Co.) under 'native' conditions, and UV-catalyzed photoaddition of 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT; HRI Associates) to ovalbumin mRNA were as previously described (1,2). Hen oviduct poly A⁺ RNA (2 μg) in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA was adjusted to 5 mM MgCl_2 and digested (15 min, 37°C) with 0.05 units T1 ribonuclease (Calbiochem). The enzyme was removed by phenol extraction and the mRNA recovered by ethanol precipitation for subsequent analysis by reverse transcription.

Chemical modification reactions and T1 ribonuclease digestions of isolated nuclei or polysomes were performed using the same conditions as for isolated ovalbumin mRNA but empirically optimized for the amount of nuclei or polysomes and reagent per reaction. A fresh aliquot (i.e. not previously thawed) of nuclei or polysomes was thawed on ice for each reaction. For nuclei reactions, nuclei were pelleted by centrifugation and the storage buffer was removed by aspiration. Polysomes were centrifuged briefly and the supernatant (8 μl per reaction) containing soluble polysomes was used for polysome reactions. Each reaction contained 17 mg of nuclei or 1.76 A₂₆₀ units of polysomes in 100 μl final reaction volume. Nuclei or polysomes were: (i) reacted (10 min, 30°C) with 0.25 μl DMS in 'native' RNA reaction buffer (1); (ii) dark pre-incubated (5 min, 4°C) with 17 μM AMT (final concentration) in AMT reaction buffer (2) to allow AMT binding to occur and then irradiated (10 min, 4°C) with 360 nm ultraviolet light; or (iii) digested (30 min, 37°C) with 0.5 units T1 ribonuclease. Following each reaction, nuclei were pelleted by centrifugation. The nuclear pellet was resuspended in lysis buffer (2% sodium dodecylsulfate, 7 M urea, 350 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and deproteinized by extracting twice with phenol-chloroform-isoamyl alcohol (25:24:1 by volume) at room temperature. Polysomes were combined with an equal volume of lysis buffer and similarly extracted. RNA was recovered from the aqueous phase of the phenol extraction by ethanol precipitation.

Methylation of unpaired adenine and cytidine bases, AMT addition to pyrimidine bases (U > C) in double-stranded regions of ovalbumin mRNA and T1 ribonuclease cleavage sites were determined by reverse transcription of ovalbumin mRNA with AMV reverse transcriptase (Life Sciences Inc.) as described previously (1,2). Ovalbumin-specific synthetic oligodeoxynucleotide primers used for reverse transcription were: ov54, a 17mer complementary to ovalbumin mRNA nucleotides 54–70, and ov125, a 20mer complementary to ovalbumin mRNA nucleotides 125–144. Primers were 5'-end radiolabeled by reaction with T4 polynucleotide kinase and [γ - ^{32}P]ATP. Structure-dependent ovalbumin cDNAs were separated by electrophoresis on an 8% sequencing gel and detected by autoradiography (1,2). Each gel also contained four dideoxysequencing reactions performed using the same cDNA primer and untreated ovalbumin mRNA.

RESULTS AND DISCUSSION

The 5'-end structure of ovalbumin mRNA

As illustrated in Figure 1 and shown previously (2), hen ovalbumin mRNA has a 64 nucleotide non-coding leader (NCL) that contains two stem-loop structures: hairpin-1 (nucleotides 1–35, -9.9 kcal at 30°C) is a trigonal stem structure with two loops, while hairpin-2 (nucleotides 38–60, -4.4 kcal at 30°C) is a simple stem-loop structure with an asymmetrical single nucleotide bulge. Hairpin-3 (nucleotides 66–122, -17.1 kcal at 30°C) is a stem-loop structure that occurs at the beginning of the coding region and includes the AUG initiation codon. Hairpin-3 contains two pairs of symmetrical bulge loops and one asymmetrical single nucleotide bulge.

Figure 1 also shows previously determined sites of adenine and cytidine methylation and of AMT photoaddition to uridines for native (i.e. undenatured) ovalbumin mRNA *in vitro*. Modified bases were detected by reverse transcription from a downstream primer, which produces cDNAs that end at the mRNA nucleotide following (i.e. 3' to) the modified base. Each cDNA is designated by the template mRNA nucleotide at which it terminates, which identifies the preceding (5') mRNA nucleotide as the site of base modification. These structure-dependent chemical modifications are consistent with the secondary structure of ovalbumin mRNA generated by the PC-FOLD (version 4.0) computer program for RNA secondary structure (9) and shown in Figure 1. For native ovalbumin mRNA, methylation occurs primarily at N1 of unpaired adenines and N3 of unpaired cytidines, and is most pronounced for the four adenines in loop-1 of hairpin-1 and the two adenines in the loop of hairpin-3. In native ovalbumin

Figure 1. Secondary structure for nucleotides 1–125 of hen ovalbumin mRNA. This structure was generated by the PC-FOLD (version 4.0) computer modeling program for RNA secondary structure originally developed by Zuker and Stiegler (9). Hairpin-1 (including loop-1 and loop-2), hairpin-2, hairpin-3, the mRNA 5'-end and the initiation codon (boxed AUG) are indicated. Previously determined (1,2) structure-dependent base modifications of native ovalbumin mRNA are also shown. Methylated bases are identified with an asterisk. AMT photoaddition sites are indicated by their relative reactivity as boxed U's (stronger sites) and underlined U's (weaker sites). Strong primary T1 ribonuclease cleavage sites are indicated by large filled arrowheads. Strong secondary T1 cleavages are indicated by filled triangles. Weak T1 cleavages are indicated by open triangles.

mRNA, AMT photoaddition occurs predominantly to uracils in 'loose' helical structures or base stacking arrays (uracils 22, 23, 97, 98 and 102 in Figure 1) that favor intercalation of AMT (1).

Under partial reaction conditions T1 ribonuclease cleaves RNA predominantly at unpaired guanine residues (10). Preliminary experiments established partial digestion conditions (0.001 units T1 ribonuclease/ μ g ovalbumin mRNA) in which the 5' region of hen ovalbumin mRNA was cleaved only at G12 in loop-1 of hairpin-1 and G92 in the loop of hairpin-3 (Figure 1). Secondary cleavages were detected with 0.005 units of T1 and were pronounced with 0.01–0.05 units of T1 (data not presented). Primary T1 cleavages at unpaired guanines G12 and G92 were consistent with the strong methylation of unpaired adenines in the loops of hairpin-1 and hairpin-3 (see above).

AMT photoaddition to unpaired uracils 27–29 in loop-2 of hairpin-1 seems to be at variance with the reaction site requirement for AMT intercalation. However, loop-2 may form an ordered structure capable of reacting with AMT. Highly structured loops that involve base stacking and partial hydrogen bonding by the unpaired nucleotides have been demonstrated by nuclear magnetic resonance in at least two RNA hairpins (11,12) one of which is pyrimidine rich and refractory to reverse transcriptase (11). Additional support for this explanation comes from extensive computer modeling of the ovalbumin mRNA secondary structure. Folding algorithms that require U27–U29 to be base paired produce secondary structures that are largely incompatible with our empirical structural data (data not presented).

An alternative version of the structure in Figure 1 (not generated by computer modeling) is a 'classical' tRNA-like

pseudoknot (13) formed by base pairing between the 5'-CUUU-3' sequence (nucleotides 26–29) and the 5'-AAAG-3' sequence (nucleotides 44–47) in the loop of hairpin-2. This structure preserves stem-loop-1 of hairpin-1, hairpin-2 and hairpin-3 but eliminates stem-loop-2 of hairpin-1. A loop consisting of unpaired nucleotides 30–37 would link the pseudoknot stem to the stem of hairpin-2. Continuous linear stacking of these two stem regions would give this structure added stability. The stems of hairpin-1 and/or hairpin-3 may also stack with this structure. This pseudoknot would require adenines 44–46 to base pair, in contrast with our previous results (2).

In the experiments described below, DMS and AMT were used to probe ovalbumin mRNA structure in isolated nuclei and polysomes because of their ability to enter and modify RNA within cellular compartments (14,15). T1 ribonuclease was selected as a structural probe because it may enter the nucleus by virtue of its small size (11 kDa) and because it has been used to probe the structure of globin mRNA in polysomes (16).

The 5'-end structure of ovalbumin mRNA in isolated nuclei

Hairpin-1. Figure 2 shows the detection by reverse transcription of paired and unpaired bases in nucleotides 1–35 of isolated ovalbumin RNA (Lanes 1, 3, 5, 7) and of intranuclear ovalbumin mRNA (Lanes 2, 4, 6, 8). Untreated mRNA (lane 1) and nuclei (lane 2) gave common cDNAs at nucleotides 19, 20, 21 and 27. These background cDNAs are consistent with previously reported termination by reverse transcriptase at dinucleotides CA, CU and UA in the mRNA (probably the result of endogenous ribonuclease H activity in AMV reverse transcriptase; 2,10), and are uninformative about mRNA structure unless significantly enhanced relative to controls in a structure-dependent reaction.

The pattern of AMT photoaddition to intranuclear ovalbumin mRNA (Figure 2, lane 4) was identical to that of isolated ovalbumin mRNA (Figure 2, lane 3). Common prominent cDNAs at nucleotides 23, 24, 28, 29 and 30 resulted from AMT photoaddition to uridines 22, 23, 27, 28 and 29 respectively. As explained above, these modified uracils occur in the stem and loop-2 of hairpin-1 (Figure 1 and refs 1,2). It should be noted that a background cDNA at nucleotide 30 appears in the untreated nuclei control (Figure 2, lane 2) but not the mRNA control (Figure 2, lane 1). The same cDNA is clearly AMT-specific for isolated mRNA (compare lanes 1 and 3 in Figure 2) and appears to be sufficiently enhanced in the intranuclear mRNA (Figure 2, lane 4) to consider it an AMT-generated cDNA. A similar but very weak AMT photoaddition to U10 in the mRNA (Figure 2, lane 3, cDNA 11) probably results from a continuous stacking array with the stem produced by partial base pairing (constrained by the size of the loop) of U10 with A15 in loop-1 of hairpin-1.

Methylation of adenines 11, 13, 14 and 15 in both isolated and intranuclear ovalbumin mRNAs produced common cDNAs at nucleotides 12, 14, 15 and 16 respectively (Figure 2, lanes 5 and 6). These adenine methylations are consistent with their assignment as unpaired bases in loop-1 of hairpin-1 (Figure 1 and refs 1,2). Likewise, A30 methylation produced a common cDNA at nucleotide 31, which is consistent with its assignment as an unpaired base in loop-2 of hairpin-1 (Figure 1). Methylation of base-paired C32 (and to a lesser extent A33) produced the cDNA at nucleotide 33. C32 methylation was not observed previously (2).

The cDNAs derived from methylation of intranuclear ovalbumin mRNA were qualitatively identical to but present in

Figure 2. Autoradiogram of sequencing gel showing cDNAs produced by reverse transcription of isolated ovalbumin mRNA (odd numbered lanes) and oviduct nuclear RNA (even numbered lanes) with the ov54 primer (see Materials and Methods). Isolated mRNA and purified nuclei were treated as follows: untreated controls (lanes 1 and 2); AMT photoaddition (lanes 3 and 4); methylation with DMS (lanes 5 and 6); digestion with T1 ribonuclease (lanes 7 and 8). Numbered cDNA bands are shown between the pair of lanes that contain the reaction that generated the cDNAs. Each cDNA is numbered with the mRNA (template) nucleotide at which it terminates. Lanes containing the dideoxysequencing reactions ddA, ddC, ddT and ddG are labeled with the complementary mRNA (template) nucleotide U, G, A and C respectively so that the mRNA sequence can be read directly from the autoradiogram.

lower yields than those derived from the isolated mRNA. Reduced nuclear cDNA yields could be due to partial masking of the mRNA by nuclear proteins and/or reduced methylation resulting from competition for DMS by other DNA and RNA substrates in the nucleus. Interaction with nuclear proteins may also alter the accessibility of some mRNA sites relative to others with or without changing mRNA structure. The relatively reduced yield of DMS cDNAs (Figure 2, lane 6) suggests that nuclear proteins may decrease the accessibility of hairpin loop-1 to DMS. The qualitative and nearly quantitative identity of the AMT cDNAs (Figure 2, lanes 3 and 4) suggests that the stem of hairpin-1 is conserved and readily accessible in the nucleus. The previously undetected C32 methylation is more pronounced in isolated mRNA than in nuclear mRNA. The apparent requirement for at least one unpaired base to stabilize an RNA trigonal structure (17) may make C32 more susceptible to methylation in the isolated mRNA. Masking or stabilization of base-pairing by nuclear proteins may explain the diminished C32 methylation in nuclei as compared to the isolated mRNA. Methylation of C32 and A33 is also compatible with the pseudoknot structure described above.

T1 ribonuclease cleavage of isolated and intranuclear ovalbumin mRNAs gave common cDNAs at nucleotides 13, 25 and 32 (Figure 2, lanes 7 and 8), although the cDNAs were barely visible in the nuclear experiment. In isolated mRNA (Figure 2, lane 7), the relatively high T1 ribonuclease:mRNA ratio (see Materials and Methods) produced a strong primary cleavage at G12 (cDNA 13), and secondary cleavages at base-paired guanines G16, G24, G31, G34 (cDNAs 17, 25, 32 and 35 respectively)

and at A33 (cDNA 34). Strong secondary cleavages at G31 and G34 probably result from the disruption of hairpin-1 by primary T1 cleavage at G12 (10). Weak secondary cleavages at G16 and G24 reflect their location in the stem of hairpin-1 in the few mRNA molecules that remain uncut at G12. Most ovalbumin mRNA molecules are cleaved by T1 ribonuclease as indicated by the barely visible band of full-length cDNA in the mRNA experiment (Figure 2, lane 7). T1 cleavage at A33 occurs because excess T1 ribonuclease secondarily cuts RNA 3' to CA and UA sequences (18). Strong T1 cleavages at G31, G34 and A33 also are consistent with the pseudoknot structure described above in which these bases are unpaired.

Like the AMT modification of uridines 27–29, the background cDNAs at nucleotide 27 in lanes 1–6 of Figure 2 suggest that loop-2 of hairpin-1 may be an ordered structure that poses a barrier to reverse transcriptase. The oligomeric dissociation of mRNA nucleotides 1–12 following T1 cleavage at G12 would most likely destabilize the stem-loop-2 structure of hairpin-1, which would be consistent with the exclusive absence of cDNAs at nucleotide 27 in the T1 experiment.

In the nuclear experiment (Figure 2, lane 8), faint T1-generated cDNAs were visible at nucleotides 13, 25 and 32. Low cDNA yields could result from under or over digestion of the intranuclear mRNA by the T1 enzyme. Underdigestion resulting from poor nuclear penetration by the T1 enzyme or mRNA masking by nuclear proteins should still produce an amount of full length cDNA similar to that observed in the nuclear RNA control (Figure 2, lane 2). This is clearly not the case. Overdigestion resulting from a high intranuclear T1:mRNA ratio is suggested by the similar intensity of primary (cDNA 13) and secondary (cDNAs 25 and 32) T1 cleavages in the nuclear experiment. While non-specific degradation of intranuclear ovalbumin mRNA

Figure 3. Autoradiogram of sequencing gel showing cDNAs produced by reverse transcription of isolated ovalbumin mRNA (odd numbered lanes) and oviduct nuclear RNA (even numbered lanes) with the ov125 primer (see Materials and Methods). This autoradiogram is labeled as described in Figure 2.

Figure 4. Autoradiogram of sequencing gel showing cDNAs produced by reverse transcription of isolated ovalbumin mRNA (odd numbered lanes) and oviduct polysomal RNA (even numbered lanes) with the ov54 primer (see Materials and Methods). Isolated mRNA and isolated polysomes were treated as described in Figure 2 and this autoradiogram is labeled as described in Figure 2.

cannot be ruled out, the barely perceptible full-length cDNA band in the nuclear experiment (Figure 2, lane 8), like that observed for the isolated mRNA (see above), suggests that this result is T1-dependent.

Hairpin-3. The data in Figure 2 support the conclusion that hairpin-1 of ovalbumin mRNA or some closely related version is conserved in isolated nuclei. In order to determine if any more of the *in vitro* structure of ovalbumin mRNA occurred in intranuclear ovalbumin mRNA, we analyzed a mRNA coding sequence that is part of hairpin-3. Figure 3 shows a comparison of structural data for hairpin-3 in isolated ovalbumin mRNA (lanes 1, 3, 5, 7) and nuclei (lanes 2, 4, 6, 8). Both isolated and intranuclear ovalbumin mRNAs produced common cDNAs at nucleotides 99, 103, 104, 111, and 112 (Figure 3, lanes 3 and 4) that were the result of AMT photoaddition to uracils 98, 102, 103, 110 and 111 respectively. AMT photoaddition to these specific uracils is consistent with their location in the stem of hairpin-3 (Figure 1 and ref. 2). Common AMT photoadditions to uracils 95, 96, 97, 100 and 101 (cDNAs 96, 97, 98, 101 and 102 respectively) were also observed but are not labeled in Figure 3 in the interest of clarity.

Both isolated and intranuclear ovalbumin mRNAs exhibited strong methylation at A93 and A94 (Figure 3, lanes 5 and 6, cDNAs 94 and 95 respectively). These adenine methylations are consistent with their assignment as unpaired bases in the loop of hairpin-3 (Figure 1 and ref. 2). Likewise, common methylations at A74, C76, A105, C112, A113 and A114 (Figure 3, cDNAs 75, 77, 106, 113, 114 and 115 respectively) were consistent with their assignment as unpaired bases within the stem of hairpin-3 (Figure 1 and Ref. 2). The location of A85 opposite a bulge (U99) in a U-rich stem region of hairpin-3 probably makes it accessible to methylation resulting in the cDNA at nucleotide 86. The yield of methylation-dependent cDNAs is similar in mRNA and nuclei (Figure 3, lanes 5 and 6) suggesting that in nuclei hairpin-3 is more accessible than hairpin-1 to the DMS reagent (compare with Figure 2). This effect may be due to the absence of masking by nuclear proteins or to protein-enhanced accessibility that preserves the loop and adjacent stem structure of hairpin-3.

Cleavage by T1 ribonuclease at G92, G99, G104 and G107 produced common cDNAs at nucleotides 93, 100, 105 and 108 respectively in isolated and intranuclear ovalbumin mRNAs (Figure 3, lanes 7 and 8). Weak cleavage at G77, G78 and G83 also can be seen the mRNA experiment (Figure 3, lane 7, cDNAs 78, 79 and 84 respectively). Guanines 83 and 92 are unpaired while guanines 77, 78, 99, 104 and 107 are in metastable regions of secondary structure (GU base pairs, bulge loops) that would make them susceptible to T1 cleavage. The strong cleavage at G92 in isolated mRNA is consistent with its location as an unpaired base in the loop of hairpin-3 and with the methylation data for A93 and A94. G92 appears to be a primary T1 cleavage site. Poor cDNA yields for G cleavages preceding G92 suggest that most mRNA molecules were cleaved at G92. Secondary cleavages (see discussion above) produced by disruption of the hairpin-3 structure following cleavage at G92 would likewise explain the strong cDNAs at nucleotides 100, 105 and 108 in isolated mRNA (Figure 3, lane 7). The lower yield of nuclear T1-specific cDNAs is discussed above.

These data generally support the presence of hairpin-3 in intranuclear ovalbumin mRNA. However, the stem region of

hairpin-3 closest to the loop may be partially destabilized in nuclei since the adenine and cytidine bases immediately preceding and following A85 (Figure 3, cDNA 86) appear to be methylated in nuclei (Figure 3, lane 6) but not mRNA (Figure 3, lane 5). In contrast, this same stem region in intranuclear mRNA reacts strongly with AMT (see above). We interpret these results to mean that, in nuclei, the stem region of ovalbumin hairpin-3 adjacent to the loop is sufficiently metastable to allow some base methylation to occur but not to produce strong T1 cleavage sites within the same stem sequence (Figure 1, nucleotides 81–89). AMT intercalation shifts the structural equilibrium towards a base-paired stem structure and results in the uracil modifications described above.

Analysis of hen oviduct polysomes for the presence of hairpin-1 of ovalbumin mRNA

Our analysis strongly indicates the presence of hairpin-1 at the 5'-end of intranuclear ovalbumin mRNA. This result suggests that hairpin-1 is a conserved feature of ovalbumin mRNA in the pre-translation state and therefore could be available to facilitate the initiation of translation as the mRNA enters the cytoplasm. If hairpin-1 is an initiation signal for repeated rounds of initiation then it also should be conserved on ovalbumin mRNAs in the act of translation. We therefore investigated hen oviduct polysomes for the presence of ovalbumin mRNA hairpin-1 using the same battery of RNA structural probes.

Figure 4 shows the comparison between isolated ovalbumin mRNA (lanes 1, 3, 5, 7) and polysomal ovalbumin mRNA (lanes 2, 4, 6, 8). Isolated and polysomal ovalbumin mRNAs have the following features in common: (i) background cDNAs at nucleotides 19, 20 and 21 (Figure 4, lanes 1 and 2); (ii) AMT photoadditions to uracils 22, 23, 27, 28 and 29 (Figure 4, lanes 3 and 4, cDNAs 23, 24, 28, 29 and 30); (iii) methylations at A11, A13, A14, A15, A30 and C32 (Figure 4, lanes 5 and 6, cDNAs 12, 14, 15, 16, 31 and 33); and (iv) T1 ribonuclease cleavages at guanines 12, 16, 24, 31 and 34 (Figure 4, lanes 7 and 8, cDNAs 13, 17, 25, 32 and 35). These results are nearly identical to those in Figure 2 and the structural significance of these cDNAs has been discussed above. In each experiment the polysomes give identical cDNAs to isolated mRNA but at lower yields. (Some bands are partially obscured by the lane background and therefore did not reproduce well in the photograph.) One major difference between nuclei and polysomes is the accessibility of G12 to cleavage by T1 ribonuclease (compare lanes 8 in Figures 2 and 4). The presence of equivalently strong cDNA signals at nucleotide 13 (Figure 4, lanes 7 and 8) indicates that G12 in polysomal mRNA is as accessible to T1 digestion as G12 in isolated mRNA. We interpret this result to mean that stem-loop-1 of hairpin-1 remains accessible to the translation apparatus while the mRNA is being translated and therefore could function as a specific signal for each initiation event. Changes in the structure of hairpin-1 associated with the initiation of protein synthesis (e.g. melting of secondary structure) would necessarily be transient and difficult to detect. Studies of globin mRNA in reticulocyte lysates indicate that its 5'-end is more accessible to T1 ribonuclease in the presence of cytoplasmic 'factors' (16). Finally, polysomal ovalbumin mRNA was refractory to analysis of hairpin-3 by any of our structural probes. No structure-dependent cDNAs were detected by reverse transcription with the ov125 primer indicating that this region of the mRNA was probably masked by a ribosome (data not presented).

In conclusion, we have demonstrated that a pattern of paired and unpaired bases, which is consistent with the hairpin-1 structure of isolated ovalbumin mRNA, is also present in both intranuclear and polysomal ovalbumin mRNA. A pattern of paired and unpaired bases consistent with a metastable version of hairpin-3 was also found in intranuclear ovalbumin mRNA. These results, combined with our finding that hairpin-1 positively affects the rate of ovalbumin mRNA translation *in vitro* and forms part of a high affinity binding site for eIF-2, has led us to hypothesize that hairpin-1 is an intracellular structural feature of ovalbumin mRNA that facilitates its translation probably through its specific interaction with eIF-2.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant GM41257 to C.D.L. and by a Bristol-Myers Squibb Company Award of Research Corporation to R.A.K.

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