Identification of a GA-rich Sequence as a Protein-binding Site in the 3’-Untranslated Region of Chicken Elastin mRNA with a Potential Role in the Developmental Regulation of Elastin mRNA Stability*

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Synthesis of aortic elastin peaks in the perinatal period and then is strongly down-regulated with postnatal development and growth. Decreased stability of elastin mRNA contributes to this developmental decrease in chick aortic elastin production. We have previously shown that destabilization of elastin mRNA is correlated with decreased binding of cytosolic protein(s) to a large, GC-rich region of secondary structure in the 3’-untranslated region (3’-UTR) of elastin mRNA. In this study, using gel migration shift assays, deletion constructs, and antisense competition assays, we identify a major protein-binding site in the 3’-UTR of elastin as a GA-rich sequence (UGGGGGGAGGGAGGGAGGGA), which we have designated the G3A motif. This motif is present in the 3’-UTR of elastin from several species. Binding proteins are present in both nuclear and cytoplasmic extracts, and their abundance is associated with tissues producing elastin and correlated with circumstances in which elastin mRNA is stable. These results suggest that the conserved GA-rich sequence of the elastin 3’-UTR is an important element in the regulation of stability of the elastin mRNA.

Elastin is the major connective tissue protein of large arteries such as the aorta, providing these tissues with the properties of extensibility and elastic recoil. Together with collagen, elastin is essential for the structural integrity and physiological function of the arterial blood vessels. Synthesis and accumulation of elastin in the aorta takes place over a relatively short period of time during development and growth, and is essentially complete by early adult life (1, 2). For example, production of elastin in the aorta of the chicken is very rapid around the time of hatching but decreases markedly by 4 weeks and can no longer be detected after 10–12 weeks of age (3). This developmental decrease in elastin synthesis is reflected in a marked reduction in steady-state levels of mRNA for elastin (3, 4).

We have previously shown that decreased stability plays a role in the developmental decline in steady-state levels of elastin mRNA (5). For example, in the 2-day-old chicken, a period at which elastin synthesis is rapid and elastin mRNA is abundant, the half-life of aortic elastin mRNA is approximately 25 h. In contrast, by 8 weeks after hatching when elastin synthesis and steady-state mRNA levels for elastin have fallen to low levels, the half-life of this mRNA has decreased to approximately 7 h. Decreased stability of elastin mRNA has also been reported to be important in developmental regulation of elastin synthesis in rat lung (6), and alterations in elastin mRNA stability have been suggested in at least one form of cutis laxa, a connective tissue disease manifested by defective elastic fiber formation (7). Recently, protein binding to a sequence element in exon 30 of the coding region of elastin has been reported to be important for destabilization of elastin mRNA in adult rat lung fibroblasts, and it has been suggested that increased elastin production induced by treatment of cells with transforming growth factor β may be mediated through this mechanism (8).

In recent years the regulation of mRNA stability has emerged as an important mechanism for controlling gene expression. It is now clear that the stability of different mRNAs can vary greatly in eukaryotes, with half-lives ranging from a few minutes to days. The decay rates of many eukaryotic mRNAs have been shown to be regulated by a variety of developmental or environmental stimuli such as hypoxia (9), hormones (10), and cytokines (11). Most of the mechanisms that control mRNA stability share common features, including specific interactions between trans-acting factors and cis-acting elements in the mRNA (12). These interactions serve to modulate the susceptibility of the mRNA to degradation. Such cis-acting elements might be actual ribonuclease target sites or sites that facilitate or hinder ribonuclease attack elsewhere in the mRNA. Although such cis elements could be present anywhere in the mRNA, many of these sequences are situated in the 3’-untranslated region (3’-UTR). In addition, two common structural elements, the 5’-cap structure and the 3’-poly(A) tail, also appear to have important roles in protecting the mRNA from degradation by ribonucleases.

Although it is now clear that destabilization of mRNA plays a role in the down-regulation of aortic elastin synthesis during development and growth (5, 8), the precise molecular mechanisms contributing to the control of elastin mRNA stability remain to be defined. Previous evidence from our laboratory indicated that cytosolic proteins binding to a large region of stable secondary structure in the 3’-UTR of elastin was asso-

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¶ The abbreviations used are: UTR, untranslated region; nt, nucleotide(s); PCR, polymerase chain reaction; DTT, dithiothreitol; ODN, oligodeoxynucleotide; asODN, antisense oligodeoxynucleotide; hRNP, heterogeneous nuclear ribonucleoprotein; SSPE, saline/sodium phosphate/EDTA; RP4 and RP5, riboprobes corresponding to regions 4 and 5.
ciliated with the stabilization/destabilization of elastin mRNA. Here, using deletion and competition analyses, we map this cis-acting sequence to an approximately 20-nucleotide (nt) purine-rich sequence within this region of secondary structure. This sequence is conserved in the 3′-UTRs of elastins of several species. Furthermore, trans-acting proteins binding to this sequence, apparently present in both cytosolic and nuclear fractions, are enriched in tissues in which elastin is synthesized and are particularly abundant at developmental periods when elastin mRNA is stable.

EXPERIMENTAL PROCEDURES

Synthesis of Full-length 3′-UTR and Deletion Constructs—The cDNA containing the full-length 3′-UTR of elastin was produced by polymerase chain reaction (PCR) synthesis using primers corresponding to the 5′- and 3′-ends of the 3′-UTR. The upper primer contained a 5′ HindIII and the lower primer contained a 3′ XhoI site for subsequent cloning into a pGEM-4Z vector (Promega). PCR solutions included 1× PCR buffer (Qiagen), 5% Me 2SO, 0.2 mM of each dNTPs, 1.0 mM MgCl 2, 0.5 units of Taq polymerase (Qiagen), and either 0.5 μg of genomic DNA or 12.5 ng of pTE2, a cDNA containing the full-length 3′-UTR of chicken elastin mRNA (a gift from Dr. A. T. definition 4, National Defense Medical College, Japan), as template. Each PCR reaction was denatured for 4 min at 94 °C, followed by PCR amplification using a Robocycler 480 (Stratagene) (denaturation, 1 min at 95 °C; annealing, 1 min between 51 °C and 65 °C; extension, 1 min at 72 °C). The full-length elastin 3′-UTR insert was cloned into the pGEM-4Z vector and sequenced.

Deletions of various domains in the elastin 3′-UTR were made using a PCR approach (13) with either chicken heart genomic DNA or pTE2 as the primary template. One PCR reaction used an upper primer containing a 5′ HindIII site, as described above, and a lower primer corresponding to approximately 18 bases of sequence upstream of the deleted sequence together with approximately 18 bases downstream of the deleted sequence. A second PCR reaction used a lower primer containing a 3′ XhoI site as described above, and an upper primer corresponding to approximately 18 bases of sequence upstream of the deleted sequence together with approximately 18 bases downstream of the deleted sequence. The DNA fragments generated in this way were purified using a QiNex II gel extraction kit (Qiagen), mixed in a 1:1 molar ratio and used as a template for primer extension and subsequent PCR amplification using the HindIII upper primer and the XhoI lower primer. This PCR step generated the full-length construct incorporating the desired deletion in the 3′-UTR of elastin, which was subsequently cloned into pGEM-4Z and sequenced. PCR conditions were as described above.

All oligodeoxynucleotides were synthesized at the Center for Applied Genomics, Hospital for Sick Children, Toronto, Canada. Primer sequences used were as follows: Upper HindIII, CCGAAGCTTGCCGCACTCACCGCACTXhoI (XhoI site after the deletion), GGCCTTAGACGTTAAAAACCTGCTCCT; domain B deletion (5′-fragment, lower), GGCGTCCGGTGGGGAATTTACATT; domain B deletion (3′-fragment, upper), AATGTAAATCCCAACAGGTCCGCGGAGACC; domain D deletion (5′-fragment, lower), CACCCGGGACCTCTTTTATCCCTGAGTGCCGGCC; domain D deletion (3′-fragment, upper), GGCGGCGGACCTGAGTTGAAACGCCGCGGAGACC; domain E deletion (5′-fragment, lower), AAAAGGTGATACCATGTAGACCCCCGCGGAGTGC; domain E deletion (3′-fragment, upper), GCAGCTGCGCCGGCGGTACTGTTACCTACCTTGG; region 3 deletion (5′-fragment, lower), TCCCCGCCGGGGACGGTGGGGTGGCCTCGGGG; region 3 deletion (3′-fragment, upper), CGCGGGGTACGACGGCAAGGAGGGGCCGCGGACGCGG; domain F deletion (5′-fragment, lower), AGCCGAGGAGAAGACCATGGATCTCTGTTTMTT.

A construct containing the elastin 3′-UTR, lacking the sequence from domains B through E (3′-E), was prepared as described previously (4). Synthesis of Constructs Containing Region 4 and Region 5 in the Elastin 3′-UTR—Constructs containing sequence from either region 4 or region 5 of the elastin 3′-UTR were prepared by cloning a double stranded oligodeoxynucleotide into the multiple cloning region of the pGEM-4Z vector. The sense strand sequence of the oligodeoxynucleotide used to synthesize the region 4 construct was TGGGGGGGAGGGGACGACGGGCAGATCGCGCGCGGGGCGGGCGGCGG, with additional 5′ XhoI and 3′ HindIII sites. The sense strand sequence of the oligodeoxynucleotide used to synthesize the region 5 construct was TGGGGGGGAGGGGACGACGGGCAGATCGCGCGCGGGGCGGGCGGCGG, with additional 5′ EcoRI and 3′ HindIII sites. The sense strand sequence of the oligodeoxynucleotide used to synthesize the region 5 construct was TGGGGGGGAGGGGACGACGGGCAGATCGCGCGCGGGGCGGGCGGCGG, with additional 5′ XhoI and 3′ HindIII sites.
**RESULTS**

Sequence comparisons of the 3' UTRs of chicken, rat, human, and bovine elastins are shown in Fig. 1A. Chicken elastin has the shortest of these UTRs, with a total of 491 nucleotides from the first nucleotide beyond the stop codon to the polyadenylation signal (4), compared with the longer bovine (965 nt) (16), human (1181 nt) (17), and rat (1168 nt) (18) untranslated sequences. The chicken elastin 3' UTR contains two polyadenylation signals, although the first of these does not appear to be used. All of these 3' UTRs are notably GC-rich, especially the chicken 3' UTR.

**DISCUSSION**

The 3' UTRs of elastin mRNAs from various species have been shown to be important for regulating mRNA stability. The chicken 3' UTR contains two 3' UTR binding sequences important for cytosolic polyadenylation (4) and cytosolic mRNA stability (17, 18). The sequence comparison of the 3' UTRs of chicken, rat, human, and bovine elastins reveals several conserved regions, indicating that these sequences might be important for regulating mRNA stability across species. The exact mechanism by which these conserved sequences affect mRNA stability remains to be determined.

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deletions in the 3′-UTR on RNA-protein complex formation. Details of the methodology are described under “Experimental Procedures.” Cytosolic extracts (2.5 μg) from 2-day chicken aorta were incubated with radiolabeled riboprobe. After RNase digestion, the complex was resolved by native polyacrylamide gel electrophoresis. Riboprobes ΔB, ΔD, and ΔE (panel A) are identical to the full-length 3′-UTR riboprobe except for deletions of domains B, D, and E, respectively (Fig. 1). Δ3 and Δ4 (panel B) are identical to the full-length 3′-UTR riboprobe except for deletions of sequence regions 3 and 4, respectively (Fig. 1B).

deletion of these domains on the ability of riboprobes (Fig. 1B) to bind to cytosolic proteins extracted from 2-day-old chicken aorta was investigated, concentrating particularly on domains B, D, and E, because these were contained within the region of secondary structure, which we had previously shown to be required for cytosolic protein binding (4). Gel migration shift assays using riboprobes made from these deletion constructs are shown in Fig. 2A. Deletions of domains B and E had no effect on protein binding. In contrast, deletion of domain D consistently decreased cytosolic protein binding. Deletion of domain A also had no effect on the formation of RNA-protein complexes in these assays (data not shown). The importance of domain D for protein binding was supported by the fact that deletion of a larger region of the 3′-UTR, which included domain D, designated region 3 in Fig. 1B, also strongly diminished protein binding (Fig. 2B). In contrast, deletion of a region of the 3′-UTR not containing domain D, designated region 4 in Fig. 1B, had no effect on protein binding (Fig. 2B).

Identification of domain D as the site of protein binding was further confirmed by gel migration shift assays using a 42-nucleotide riboprobe corresponding to region 5 in Fig. 1B. This riboprobe included the entire domain D as well as 12 additional nucleotides on the 3′-side of domain D. Protein binding to this riboprobe was strong with cytosolic extracts from aortic tissues of 2-day-old chickens but significantly diminished with extracts from 15-week-old chicken aorta (Fig. 3A, left panel). This was consistent with data published previously from our laboratory using full-length 3′-UTR riboprobes (4). Unlabeled RP5 effectively competed for this labeled riboprobe (RP5, Fig. 3A, right panel), but protein binding was not diminished by competition with another riboprobe (RP4) corresponding to a region of 3′-UTR not containing domain D. Differences in position of the labeled band and the presence of double bands in some lanes are related to the amounts of cytosolic protein used in the gel shift assays, with the upper band predominating when increased amounts of protein are used.

Mapping of sequences important for protein binding also utilized competition experiments in which antisense oligodeoxynucleotides were annealed to the radiolabeled riboprobe before the addition of protein extracts (19). For these experiments, three antisense oligodeoxynucleotides were made corresponding to regions 3a, 3b, and 3c in the 3′-UTR (Fig. 1B). These were designated asODN3a, asODN3b, and asODN3c. Using a 1000-fold molar excess of these antisense oligonucleotides, effective inhibition of RNA-protein complex formation was seen only with asODN3b (Fig. 3B).

These data indicated that the major protein-binding site mapped to an approximately 20-nt purine-rich sequence (UGGGGGGAGGGAGGGAGGGA) corresponding to region 3b (Fig. 3C), and containing at least a 4-fold repeat of a GGGA motif. This region was therefore designated as the G3A site. Consistent with the GA-rich nature of the identified binding site, unlabeled poly(G) and poly(GA) RNA polymers could compete with the radioactively labeled elastin riboprobe for cytosolic protein binding in gel shift experiments, but neither poly(A) nor poly(C) RNA polymers were effective competitors (data not shown).

To characterize proteins binding to the G3A site, radioactively labeled riboprobes were incubated with cytosolic extracts followed by UV cross-linking. After RNase digestion, the resulting RNA-protein complex was electrophoresed on SDS-polyacrylamide gels (Fig. 4). Using the 3′-UTR as a riboprobe, major protein bands corresponding to molecular masses of approximately 36 and 62 kDa could be detected. No protein bands were seen when cytosolic extracts were incubated with a riboprobe (ΔB–E) lacking the region of secondary structure in the 3′-UTR of elastin. This was consistent with earlier results showing that protein binding required the presence of this region of stable secondary structure (4). In addition, consistent with earlier gel migration shift data both shown here (Fig. 3) and published previously (4), protein binding was significantly stronger in cytosolic extracts from aortic tissue of 2-day-old chickens as compared with similar extracts from 15-week-old animals. Furthermore, protein binding activity was stronger in nuclear extracts as compared with cytosolic extracts (Fig. 4B), although the patterns of protein bands in nuclear and cytosolic extracts were essentially identical. Age differences in protein binding activity similar to those seen in cytosolic extracts were also observed for nuclear extracts (data not shown).

A novel Northern blotting strategy involving probing of the RNA-protein complex with radiolabeled antisense ODNs was also used to confirm the G3A sequence as the binding site. Conventional RNA gel shift assays were carried out using non-radiolabeled riboprobes and cytosolic extracts from 2-day-old and 15-week-old chicken aortas. After RNase digestion and electrophoresis on native gels, the RNA-protein complexes were transferred to nylon membranes and subsequently probed with radioactively labeled antisense ODNs. Results of these experiments are shown in Fig. 5A. A single band was detected using either asODN3 or asODN3b as radiolabeled probes to hybridize to the protein-protected, RNase-resistant region. No signal was detected when asODN2, asODN3a, or asODN4 (data not shown) were used as radiolabeled probes for Northern blotting. A faint band corresponding to the RNA-protein complex was detected using asODN3c after longer exposure time.

Gel migration shift data (Fig. 3), UV cross-linking data (Fig. 4), and previous reports from our laboratory (4) had shown that the RNA-protein complex detected in gel shift assays was more prominent in cytosolic extracts from aortic tissue of 2-day-old chicken, when elastin mRNA is stable, as compared with similar extracts from 15-week-old chicken in which elastin mRNA is relatively unstable. Consistent with these results, the RNA-protein complex detected using a radiolabeled asODN3b probe was also more prominent in aortic extracts from 2-day-old as compared with 15-week-old chickens (Fig. 5A). Furthermore, the RNA-protein complex detected with asODN3b was also more prominent in extracts of fresh aortic tissue from 2-day-old chickens as compared with similar tissue, which had been subjected to organ culture for 16 h (Fig. 5B). This result was consistent with earlier evidence from our laboratory for destabilization of elastin mRNA during organ culture (5). In the absence of unlabeled riboprobe, no band could be detected with radiolabeled asODN3b, indicating that this labeled anti-
sense oligodeoxynucleotide was not binding directly to the cytosolic protein. Furthermore, preincubation of the cytosolic extract with proteinase K before incubation with the unlabeled riboprobe abolished detection of RNA-protein complexes with asODN3b (Fig. 5B). Although the protein or proteins binding to the G3A sequence was present in cytosolic extracts of aortic tissues, consistent with earlier data (Fig. 4) a comparison of nuclear and cytosolic extracts demonstrated that this protein was clearly enriched in nuclear extracts (Fig. 5C).

Sequences corresponding to the G3A sequence, identified as the major protein-binding site in the 3'-UTR of chicken elastin mRNA, are conserved in the 3'-UTRs of human, bovine, and rat elastin mRNAs (Fig. 3C). Such cross-species sequence conservation is consistent with a biological significance for this cis-acting sequence. Because of the occurrence of G3A-like sequences in 3'-UTRs of other elastin mRNAs, the tissue and species specificities of the G3A binding site were assessed. Because protein binding could be detected in both cytosolic and nuclear fractions, for convenience these experiments utilized total tissue extracts of various tissues from 2-day-old chickens, riboprobe RP5 was incubated with 5.0 μg of cytosolic extract from 2-day chicken aorta in the presence and absence of RNA competitors consisting of unlabeled RP5 or unlabeled RP4, a riboprobe corresponding to region 4 of the elastin 3'-UTR (Fig. 1B). B, mapping of the protein-binding site using competing antisense oligodeoxynucleotides (asODNs). Unlabeled antisense ODNs corresponding to sequences 3a, 3b, or 3c in the 3'-UTR of chicken elastin (Fig. 1B) were preincubated at 1000-fold molar excess with radioactively labeled full-length riboprobe. After subsequent incubation with cytosolic extracts (10 μg) from 2-day-old chicken aorta and RNase digestion, the complex was resolved by native polyacrylamide gel electrophoresis. C, comparison of the identified protein-binding region of the 3'-UTR of chicken elastin with corresponding sequences in the 3'-UTRs of rat, human, and bovine elastins. The protein-binding region (G3A sequence) in chicken elastin corresponds to ODN 3b (UGGGGG-GAGGGAGGGAGGGA). The sequences corresponding to riboprobe RP5 and ODNs 3a, 3b, and 3c are indicated.

**Fig. 3.** A, gel migration shift assays using riboprobe RP5, which contains domain D. For comparisons between cytosolic extracts from aortic tissues of 2-day-old and 15-week-old chickens, riboprobe RP5 was incubated with 5.0 μg of cytosolic extracts (left). For competition studies (right), riboprobe RP5 was incubated with 2.0 μg of cytosolic extracts from 2-day chicken aorta in the presence and absence of RNA competitors consisting of unlabeled RP5 or unlabeled RP4, a riboprobe corresponding to region 4 of the elastin 3'-UTR (Fig. 1B). B, mapping of the protein-binding site using competing antisense oligodeoxynucleotides (asODNs). Unlabeled antisense ODNs corresponding to sequences 3a, 3b, or 3c in the 3'-UTR of chicken elastin (Fig. 1B) were preincubated at 1000-fold molar excess with radioactively labeled full-length riboprobe. After subsequent incubation with cytosolic extracts (10 μg) from 2-day chicken aorta and RNase digestion, the complex was resolved by native polyacrylamide gel electrophoresis. C, comparison of the identified protein-binding region of the 3'-UTR of chicken elastin with corresponding sequences in the 3'-UTRs of rat, human, and bovine elastins. The protein-binding region (G3A sequence) in chicken elastin corresponds to ODN 3b (UGGGGG-GAGGGAGGGAGGGA). The sequences corresponding to riboprobe RP5 and ODNs 3a, 3b, and 3c are indicated.

**Fig. 4.** UV cross-linking analysis of proteins binding to the 3'-UTR of chicken elastin mRNA. A, cytosolic extracts (20 μg) from aortic tissues of 2-day or 15-week chickens were incubated with radioactively labeled riboprobes. The RNA-protein complex was then cross-linked by UV irradiation, treated with RNase to digest unprotected RNA, and subjected to SDS-polyacrylamide gel electrophoresis. Riboprobe ΔB-E lacks the region of stable secondary structure previously identified in the 3'-UTR of chicken elastin mRNA (4). Migration positions of molecular mass standards (left lane) are indicated. B, comparison of protein-binding activity detected by UV cross-linking analysis in nuclear extracts (N) as compared with cytosolic extracts (C) from aortic tissue of 2-day-old chickens. Details of the methods for preparation of nuclear and cytosolic extracts are described under “Experimental Procedures.” Migration positions of molecular mass standards (left lane) are as indicated in A.
Here we have extended these investigations, mapping the site of binding of the developmentally regulated protein or proteins to a purine-rich sequence of approximately 20 nucleotides located within the region of secondary structure previously implicated in regulation of elastin message stability (4). This sequence (UGGGGGAGGGAGGGAGGGA) has been designated as the G3A site. Data from deletion constructs, competition with antisense oligodeoxynucleotides, and competition with RNA nucleotides are all consistent with protein binding at the G3A site. Furthermore, this site has also been specifically identified by Northern blotting of RNA-protein complexes using short, radioactively labeled antisense probes.

The G3A site includes four tandem repeats of a GGGA sequence. Similar GGGA repeats are also present at approximately the same site in human, bovine, and rat elastin 3'-UTRs, and we have shown that tissue extracts from ovine aorta and rat aorta and lung tissues appear to bind to this chicken G3A sequence in gel shift experiments. Although only partial sequence is available for the 3'-UTR of ovine elastin mRNA, the G3A region of this 3'-UTR is identical to that of bovine elastin (20). Conservation of this G3A site, particularly between the more phylogenetically separated avian and mammalian species, and the particular presence of the binding protein or proteins in tissues actively producing elastin, suggest that there is a significant elastin-related biological role for this sequence. Although we would predict that this role is generally related to developmental regulation of elastin mRNA stability, at present such a correlation has been made only for chicken elastin.

Although the G3A sequence appears to be the major protein-binding site in the 3'-UTR associated with developmental regulation of elastin mRNA stability in chicken, other minor protein-binding sites may also be present. Indeed, the fact that deletion of domain D or region 3 did not totally abolish protein binding supports the presence of such accessory sites. Some affinity of the G3A binding protein for single GGGA motifs, seven of which occur outside domain D and region 3 (Fig. 1A), may account for the weak binding remaining after deletion of these regions. On the other hand, the presence of other proteins binding to non-G3A sites but with similar mobility on gel shift experiments cannot be totally ruled out.

The ability of short, radioactively labeled antisense oligonucleotide probes to hybridize specifically to RNAse-protected RNA-protein complexes appears to be unusual and has not, to our knowledge, been previously reported. This hybridization was not the result of direct binding of antisense oligonucleotide probes to proteins, because no signal was apparent with cytosolic proteins alone. Nor was such binding the result of dissociation of the RNA from the complex during transfer to nylon membranes, because RNA-protein complexes UV cross-linked before transfer could still be detected in this way (data not shown). Whatever the mechanism, the specificity of the hybridization, despite the presence of protein, indicates that sufficient RNA nucleotide sequence must remain available to interact with the antisense probe.

The G3A site does not resemble other known cis-acting 3'-UTR sequences affecting mRNA stability, including AU-rich elements of c-myc or c-fos mRNAs (21), iron-responsive elements of transferrin receptor mRNA (22), or the C-rich element of a-globin mRNA (23). Although the G3A sequence is well-conserved within elastin 3'-UTRs, few occurrences of this sequence in 3'-UTRs of other messages could be identified by data base searches. Although the sequence match is strong in human acid phosphatase 5 (GGGGAGGAGGGAGGGAGGA, GenBank™ accession number NM001611), this sequence is not as well-conserved at similar sites in rat (GenBank™ accession

**DISCUSSION**

Earlier data from our laboratory demonstrated that one factor contributing to the decline in steady-state levels of elastin mRNA with postnatal development and growth of the chicken aorta was a progressive destabilization of elastin mRNA (5). These data showed that the half-life of the message decreased from about 25 h in the 2-day-old chicken when elastin mRNA and synthesis is abundant, to approximately 7 h in the 8-week-old chicken when elastin production and mRNA levels are in rapid decline. Subsequently, we identified a large GC-rich region of stable secondary structure in the 3'-UTR of elastin mRNA and correlated binding of cytosolic protein or proteins to this region with developmental stages when the elastin message was stable (4).

To confirm that this binding was to the G3A site, these RNA-protein complexes prepared using unlabeled riboprobes were transferred to nylon membranes and probed with radioactively labeled asODN3b (Fig. 6A), as described for Fig. 5. Again, the RNA-protein complex detected in this way was prominent in extracts of aortic tissues from all species examined and in lung tissues of chicken and rat. In contrast, extracts of liver tissue from chicken and rat showed little or no binding. These results demonstrated clearly that proteins binding to the chicken G3A sequence were present in tissues of other species and that these proteins were particularly prominent in tissues producing elastin.

**FIG. 5. Northern blotting of the RNA-protein complex using radioactively labeled antisense oligodeoxynucleotides.** Details of the method are described under “Experimental Procedures.” A, cytosolic extracts from aortas of 2-day or 15-week chickens (8 µg) were incubated with unlabeled full-length riboprobes, digested with RNAse, and separated by native polyacrylamide gel electrophoresis. RNA-protein complexes were transferred to HyBond N membranes, and the membranes were probed with radioactively labeled antisense oligodeoxynucleotides (asODNs) corresponding to regions 2, 3, 3a, and 3b (Figs. 1B and 3C) of the 3'-UTR of chicken elastin mRNA. Lanes using asODN 3c as a radioactively labeled probe were exposed for 10 days. All other lanes were exposed for 3 days. B, Northern blotting of the RNA-protein complex as described in A, using asODN 3b as a radioactively labeled probe, comparing cytosolic extracts (8 µg) of freshly dissected aortic tissues from 2-day-old chickens (2d) with aortic tissues that had been extracted after 16 h of organ culture (2d inc). The Northern blotting procedure was also carried out in the absence of unlabeled full-length riboprobe (2d no RNA) and after pretreatment of the protein extract with 1 mg/ml proteinase K at 37 °C for 30 min. C, Northern blotting of the RNA-protein complex as described in A, using asODN 3b as a radioactively labeled probe, comparing nuclear (nuc) and cytosolic (cyt) extracts (8 µg) of 2-day-old chicken aortic tissues.
Tissue extracts were prepared as described under “Experimental Procedures.” Aortic, lung, liver, and heart tissues of 2-day-old chickens, from aortic, lung, and liver tissues of 9-day-old rats, and from aortic tissues of a 100-day gestation fetal sheep were incubated with radiolabeled riboprobe corresponding to the full-length 3′-UTR of elastin. After RNase digestion, the complex was resolved by native polyacrylamide gel electrophoresis. B, tissue extracts (each containing 10 μg of protein) from aortic, lung, liver, and heart tissues of 2-day-old chickens, from aortic, lung, and liver tissues of 9-day-old rats, and from aortic tissues of a 100-day gestation fetal sheep were incubated with unlabeled riboprobe corresponding to the full-length 3′-UTR of elastin, digested with RNase, and the incubation mixture was resolved by native polyacrylamide gel electrophoresis. RNA-protein complexes were then transferred to HyBond N membranes, and the membranes were probed with a radioactively labeled antisense oligodeoxynucleotide (asODN3b) corresponding to the G3A motif.

Our data indicate that the aortic G3A binding protein is enriched in nuclear as compared with cytoplasmic fractions, suggesting that interaction of protein with the G3A sequence may take place before transport of elastin mRNA from the nucleus to the cytoplasm. The presence of the protein in the cytosolic fraction may therefore be due to its carriage into the cytoplasm with the elastin mRNA. However, we cannot rule out the possibility that the protein is exclusively nuclear and that the detection of smaller amounts of binding protein in the cytosolic fraction may simply be accounted for by contamination from the nuclear fraction during the fractionation procedure.

At least three members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins, hnRNP A1 (24), hnRNP F (25), and hnRNP H′ (DSEF-1) (26, 27), are known to bind to G-rich RNA sequences, including, in most cases, at least one GGGA motif. However, any relationship between the G3A binding protein and known G-binding hnRNPs remains speculative, because these hnRNPs have usually been implicated in regulation of message splicing or in pre-RNA cleavage and polyadenylation rather than mRNA stability (28).

Sequences containing a single GGGA motif have also been identified as particularly effective targets for antisense oligonucleotide-induced degradation of a variety of mRNAs by RNase H (29). The basis for the prevalence of a TCCC sequence in the most potent antisense oligonucleotides is not understood, although it has led to the suggestion that the GGGA motif may be a preferred site for RNase digestion (29). If this is the case, it is tempting to speculate that the presence of a tandemly repeated GGGA sequence in elastin mRNA may make this message particularly susceptible to RNase attack when the G3A site is not protected by protein binding. However, it is not clear how regulation of turnover of elastin mRNA could be related to a process thought to involve degradation of DNA-RNA hybrids by RNase H.

The data presented here provide further insights into the regulation of elastin mRNA stability during aortic development and growth. Although the evidence for the role of this cis-acting sequence is correlative, the phylogenetically conserved nature of the G3A motif, the prevalence of the binding protein in tissues producing elastin, and the correlation of levels of binding protein with circumstances of stable elastin mRNA all suggest that this motif plays an important role in determining turnover rates of the elastin message. Although the mechanism by which interaction of proteins with the G3A sequence might affect message stability remains a matter of speculation, the fact that this sequence is located within a large region of stable secondary structure in the 3′-UTR of the elastin mRNA (30) suggests the possibility that RNA/protein interactions at this site may alter the stability of this secondary structure, perhaps affecting the accessibility of endogenous RNases to the mRNA. However, detailed understanding of the mechanism of this process awaits further characterization of the nature of binding protein and the consequences of its interaction with the G3A motif in elastin mRNA.

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