Rat Tropoelastin Is Synthesized from a 3.5-Kilobase mRNA*
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A Xgt11 cDNA library was constructed from poly(A)* RNA isolated from aortic tissue of neonatal rats and screened with a human tropoelastin cDNA clone. DNA sequence analysis of several overlapping rat clones confirmed the presence of DNA sequences coding for murine tropoelastin and DNA sequences coding for the 3'-untranslated region of the rat tropoelastin mRNA. Northern blot analysis of total RNA from aortic tissue of neonatal rats using oligonucleotide probes derived from these rat tropoelastin cDNAs demonstrated the presence of a 3.5-kilobase tropoelastin mRNA. The size of this rat tropoelastin mRNA agrees with previous reports for the size of the mRNA coding for tropoelastin in tissue from several vertebrate species but contrasts with several reports suggesting the presence of a higher molecular weight mRNA species responsible for the synthesis of tropoelastin in rodent tissue.

Elastin is a major component of the extracellular matrix of elastic tissue and confers to these tissues the properties of resilience and elastic recoil (1, 2). Highly insoluble and extensively cross-linked elastin is a complex polymer assembled from a soluble precursor, tropoelastin (3-5). Amino acid sequencing of tryptic peptides of tropoelastin (6) and, more recently, polypeptide sequence derived from tropoelastin cDNA, pcHEL-1. (This previously characterized cDNA contains sequences coding for 421 base pairs of the 3'-untranslated region of the rat tropoelastin mRNA. A Xgt11 cDNA library was constructed from poly(A)* RNA isolated from aortic tissue of neonatal rats and screened with a human tropoelastin cDNA clone. DNA sequence analysis of several overlapping rat clones confirmed the presence of DNA sequences coding for murine tropoelastin and DNA sequences coding for the 3'-untranslated region of the rat tropoelastin mRNA. Northern blot analysis of total RNA from aortic tissue of neonatal rats using oligonucleotide probes derived from these rat tropoelastin cDNAs demonstrated the presence of a 3.5-kilobase tropoelastin mRNA. The size of this rat tropoelastin mRNA agrees with previous reports for the size of the mRNA coding for tropoelastin in tissue from several vertebrate species but contrasts with several reports suggesting the presence of a higher molecular weight mRNA species responsible for the synthesis of tropoelastin in rodent tissue.

Materials and Methods

cDNA Library Construction and Screening—Total RNA was extracted from aortic tissue of neonatal rats using a previously described guanidine isothiocyanate extraction procedure (19). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography of total RNA preparations (20). A Xgt11 cDNA library was constructed from neonatal rat aorta poly(A)* RNA by Contech Laboratories (Palo Alto, CA). Recombinant phage populations, transfected into the bacterial host, LE392, were then screened with a radiolabeled human tropoelastin cDNA, pchEL-1. (This previously characterized cDNA contains sequences coding for 421 base pairs of the 3'-untranslated region of the rat tropoelastin mRNA (21).) DNA from plaque-purified autoradiographically positive recombinant phage preparations were digested with EcoRI to release insert-specific cDNA sequences. These EcoRI digests were then subjected to Southern blot analysis using a 32P-labeled pchEL-1. Insert-specific cDNAs, containing sequences cross-hybridizing to pchEL-1, were then isolated by electroelution and subcloned into the M13 vector, mp18 (22).

DNA Sequence Analysis—DNA sequence in single-stranded DNA was determined from mp18 recombinants was carried out using the dyeoxy chain termination procedure, involving a modified T7 DNA polymerase (Sequenase, provided by U.S. Biochemicals, Cleveland, OH) and [35S]dATP (23, 24). Radiolabeled DNA products were size-separated on 100 cm × 0.4 mm nongradient polyacrylamide gels. DNA sequences were determined from autoradiographic exposures of dried gels and analyzed using an IBM-AT personal computer and software program provided by International Biotechnologies Inc. (New Haven, CT).

Northern Blots—RNA preparations were subjected to denaturing formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose, and incubated with either radiolabeled RNA transcripts from a riboprobe recombinant of pchEL-1 or a 32P-labeled oligomer synthesized using a model 381A automated DNA synthesizer supplied by Applied Biosystems (Foster City, CA). Conditions for electrophoresis, transfer, and hybridization of Northern blots with radiolabeled RNA transcripts of pchEL-1 were as previously described (19). Incubation of nitrocellulose filters with radiolabeled oligomers was performed in 6 × SSC, 1 × Denhardt’s, 20 μg/ml tRNA, 0.05% sodium pyrophosphate at 42 °C overnight (25). The oligonucleotide used in these hybridizations was a 24-mer, the reverse complement of this DNA sequence codes for a cross-link domain of rat tropoelastin. The oligomer sequence was as follows: 5'-ATACTTGGCAGCTTTGGCAGCAGC-3'. Post-hybridization wash conditions using this radiolabeled oligomer consisted of a 1-h wash at 37 °C followed by a 10-min wash at room temperature using 6 × SSC, 0.05% sodium pyro-
phosphate. After post-hybridization washing, nitrocellulose filters were exposed to x-ray film at -70 °C for varying periods of time.

RESULTS

DNA Sequence Analysis—Several overlapping cDNA clones were obtained from the neonatal rat aorta Agt11 library that contained sequences homologous to the human tropoelastin cDNA, pcHEL-1. The sequence strategy used to determine DNA sequence from these overlapping cDNAs is presented in Fig. 1. The DNA sequence obtained from these recombinants is presented in Figs. 2 and 3. By comparison to previously described DNA sequences coding for bovine tropoelastin (26, 27) it was clear that these rat cDNA clones contained sequences derived from a tropoelastin mRNA. These include DNA sequences coding for the carboxyl-terminal coding sequence and 3'-untranslated sequence. Peptide sequences derived from rat tropoelastin cDNA clones (Fig. 2) contain considerable sequence homology to the carboxyl-terminal portion of bovine tropoelastin, including a particularly conserved cysteine-containing peptide sequence previously undetected in tryptic peptides of porcine tropoelastin (26). Although the function of this cysteine-containing peptide sequence is unknown, the extent of sequence conservation is similar to the conserved cross-link domain found at the amino-terminal end of this derived peptide sequence. It has been shown previously that these alanine-rich domains in tropoelastin are a necessary prerequisite to the formation of isodesmosine cross-links between individual tropoelastin monomers (2). Although a glycine-rich hydrophobic domain in the derived rat tropoelastin sequence is clearly also conserved within mammalian tropoelastin, there is a striking difference in the aligned chicken tropoelastin sequence in this hydrophobic region, both in terms of the number of amino acid residues and the peptide sequence in this glycine-rich domain (8, 17).

Rat tropoelastin clearly also contains a peptide sequence that is absent in human tropoelastin; Indik et al. (11) have shown that these hydrophobic sequences are missing due to the absence of exons two and three in the human tropoelastin gene. This sequence is present in all other vertebrate tropoelastins analyzed to date, including chicken (8).

The 3'-untranslated region of the rat tropoelastin mRNA contains only a single AAUAAA sequence (Fig. 3). This poly(A) addition signal is located 931 base pairs from the 5'-end of the 3'-untranslated region in a position very similar to the location of a poly(A) addition sequence in the 3'-untranslated region of human and sheep tropoelastin mRNA (11, 28). It has been shown previously that genomic DNA sequences coding for the 3'-untranslated region of human and sheep tropoelastin mRNA contain two poly(A) addition signals (11, 28). Conservation of nucleotide sequence around the AAUAAA sequence in the rat tropoelastin mRNA, together with conservation of the position of this poly(A) addition signal clearly identify this sequence as the poly(A) addition signal most proximal to the 5'-end of the 3'-untranslated region of the rat tropoelastin mRNA.

In addition to nucleotide sequence conservation around the poly(A) addition signal, nucleotide sequence homology was found throughout the untranslated region of mRNAs coding for rat, human, and bovine tropoelastin (Fig. 4). Limited sequence homology was observed between nucleotide sequences only at the 5'-end of the 3'-untranslated region of rat and chicken tropoelastin mRNA (29). The 3'-untranslated region of chicken tropoelastin mRNA contains two poly(A) addition signals within an untranslated domain that is only 40% of the size of the 3'-untranslated domain found in the bovine or human tropoelastin gene; no sequence conservation is evident around these consensus sequences. Further, the relative positions of these AAUAAA sequences differ according to the position of these sequences in the 3'-untranslated region of mammalian tropoelastin mRNAs.

Northern Blot Analysis—Northern blot analysis, using RNA transcripts of pcHEL-1, of total RNA isolated from aortic tissue obtained from rats of different ages demonstrated the presence of two cross-hybridizing RNA species (Fig. 5). The sizes of these RNAs are 3.5 and 4.8 kb. The 3.5-kb RNA is abundant in RNA preparations from aortic tissue obtained from neonatal rats; a 3.5-kb RNA is undetectable in total RNA isolated from aortic tissue of 10-week-old rats. The 4.8-kb RNA species, in contrast, continues to be present in abundance in adult aortic tissue. In order to unambiguously determine from which RNA species the rat tropoelastin cDNAs were derived, an oligonucleotide was synthesized using cDNA sequence coding for rat tropoelastin. Incubation of a radiolabeled 24-mer to RNA preparations obtained from rat aortic tissue clearly indicated hybridization only to 3.5-kb mRNA sequences. Northern blot analysis with this oligonucleotide probe is specific not only for a 3.5-kb tropoelastin mRNA but is also specific for mRNA sequences coding for rat tropoelastin; the 24-mer does not detect a 3.5-kb tropoelastin mRNA in fetal bovine nuchal ligament RNA. The analogous sequence in bovine tropoelastin mRNA differs from the rat tropoelastin mRNA sequence by 4 nucleotides (27). This sequence difference is sufficient to ensure that a short oligonucleotide derived from rat tropoelastin mRNA will not

Fig. 1. Strategy for DNA sequence analysis of overlapping rat tropoelastin cDNA clones. DNA sequence was obtained from six overlapping cDNA clones obtained from a neonatal rat aorta cDNA library. The orientation of these clones (number 1-6) relative to the 3'-end of the rat tropoelastin mRNA (dashed line) is indicated. (1) identifies the position of the termination codon. Overlapping DNA sequence was obtained from this cDNA population as indicated by the parallel arrows. Arrows with a solid line indicate DNA sequence obtained using the Messing universal primer. Arrows with a dashed line indicate DNA sequence obtained using synthetic oligonucleotides complementary in sequence to DNA sequences present within the cDNA sequences. The intervals on the scale are 200 base pairs.
FIG. 2. DNA sequence coding for the carboxyl-terminal end of rat tropoelastin. Line 1, nucleotide sequences obtained from several overlapping rat cDNAs; line 2, amino acid sequence derived from rat tropoelastin cDNAs; line 3, aligned amino acid sequences obtained from the corresponding region of human tropoelastin (9); line 4, aligned amino acid sequences obtained from bovine tropoelastin (27). Aligned amino acid sequences that differ from the derived amino acid sequences from the rat tropoelastin cDNAs are indicated. I indicates additional amino acids found in the human or bovine tropoelastin sequence. [...] indicates a dipeptide sequence missing in bovine tropoelastin. The larger square brackets are explained by the asterisk included in the figure. The termination codon is underlined.

FIG. 3. DNA sequence coding for the 3'-untranslated region of the rat tropoelastin mRNA. This nucleotide sequence commences immediately 3' of the termination codon (underlined). The poly(A) addition signal is boxed.

FIG. 4. Comparison of nucleotide sequence homology within the 3'-untranslated region of rat, human, bovine, and chicken tropoelastin mRNA. A forward hash matrix homology search was carried out at an 80% homology using a Pustell sequence analysis program with a range setting of 10. The rat 3'-untranslated sequence presented in Fig. 3 was compared to the 3'-untranslated sequence present in the bovine (7), human (11), and chicken tropoelastin mRNA (29). b.p., base pair(s).

form a stable hybrid with the closely related sequence in bovine tropoelastin mRNA. Similarly, the absence of any hybridization of this oligomer to the 4.8-kb mRNA species suggests that these mRNAs lack the exact or almost exact sequences complementary to the rat tropoelastin mRNA-specific oligonucleotide.

Similar results were obtained with a 38-mer oligonucleotide coding for the last 36 translated nucleotides of elastin and a 17-mer coding for a hydrophobic sequence of elastin, suggesting that fortuitous alternate splicing of a single exon did not result in the abolishment of hybridization of any one oligomer.

FIG. 5. Northern blot hybridization analysis of total RNA isolated from rat aorta. Lane A, total RNA isolated from aortic tissue of 3-day-old neonatal rats; lane B, total RNA isolated from the aorta of 10-week-old rats; lane C, total poly(A)+ RNA isolated from fetal bovine mucosal ligament. Panel 1, autoradiographic exposure of lanes A–C following hybridization with a radiolabeled 24-mer. The sequence of this oligonucleotide is presented under “Materials and Methods.” Panel 2, autoradiographic exposure of nitrocellulose filters containing lanes A–C following hybridization with a human tropoelastin cDNA, pHEL-1. The indicated sizes (kb) were determined from the migration rates of 28 S rRNA (detected, following Northern transfer of total RNA, by hybridization with a 28 S rDNA clone, pAA (33) and an RNA molecular weight ladder (Bethesda Research Laboratories) detected by hybridization to radiolabeled λ DNA).
DISCUSSION

The cDNA sequences presented in this paper were clearly derived from a rat tropoelastin mRNA. The Northern blot hybridization data have also established that the size of the rat tropoelastin mRNA is 3.5 kb. This 3.5-kb mRNA species was detected in neonatal rat tissue and undetected in aortic tissue from adult rats. A decrease in steady state tropoelastin mRNA levels during the development of aortic tissue in rats is consistent with previous studies demonstrating that tropoelastin synthesis in several elastic tissues is maximal in late prenatal and early postnatal vertebrate life (30-32).

The detection of a 3.5-kb tropoelastin mRNA in rat tissue is consistent with the size of the mRNA coding for tropoelastin detected in tissue obtained from several vertebrate species derived from a rat tropoelastin mRNA. The Northern blot hybridization data have also established that the size of the mRNA species hybridization data have also established that the size of the mRNA coding for tropoelastin (14, 15). Earlier studies have suggested the presence of a higher molecular weight mRNA species coding for rat tropoelastin. The Northern blot hybridization results presented in this paper also document the presence of a higher molecular weight RNA species detected using a human tropoelastin cDNA clone. In adult rat tissue particularly, where no 3.5-kb mRNA could be detected, only a higher molecular weight 4.8-kb RNA was evident. A previous report describing the size of rat tropoelastin mRNA was carried out using RNA extracted from rat smooth muscle cells in culture (14); a similar higher molecular RNA was also detected by Raghow and co-workers (15) in total RNA isolated from lung tissue of adult hamsters. It is very likely therefore that the higher molecular weight RNA species detected in these studies is analogous to the 4.8-kb mRNA shown to be present in total RNA from rat tissue in this paper.

From the results presented in this paper, however, it is clear that the 4.8-kb mRNA does not code for rat tropoelastin. The precise identity of the 4.8-kb RNA species is unclear. Deak and co-workers have found a similar high molecular weight RNA species of human origin; these authors have described the size of rat tropoelastin mRNA was carried out using homologous sequences in ribosomal RNA. While it is clear that the 4.8-kb mRNA does not code for rat tropoelastin.

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