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Cloning and sequence of several α_{2u}-globulin cDNAs

(amino acid sequence/multiple polyadenylylation sites/glycosylation site/codon usage/signal peptide processing)

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ABSTRACT **We describe a simple cloning procedure for α_{2u}-globulin that requires neither enrichment of mRNA for cloning nor purification of a specific probe for screening recombinant colonies. Total adult male liver poly(A)$^+$RNA was used as template for cloning, and the subsequent recombinant colonies were screened by comparing hybridization to radioactive cDNA probes prepared from hepatic male and female mRNA, respectively. Almost all of the selected "male-specific" clones were later shown to contain α_{2u}-globulin sequences. This cloned α_{2u}-globulin cDNA has been shown to specifically hybridize to male rat liver RNA, which, when isolated and translated *in vitro*, codes for a 21,000-dalton protein (pro-α_{2u}-globulin) immunologically identical to α_{2u}-globulin. When translation occurs in the presence of pancreatic microsomes this *in vitro* synthesized pro-α_{2u}-globulin is processed to the 19,000-dalton mature form of α_{2u}-globulin. The nucleotide sequence of the α_{2u}-globulin cDNA has been determined, thus elucidating the complete amino acid sequence of α_{2u}-globulin and most of the hydrophobic "leader" sequence of pro-α_{2u}-globulin. The amino acid sequence deduced from the cDNA is in agreement with the partial sequence that we previously determined by sequential Edman degradation of the purified protein. α_{2u}-Globulin cDNA clones contain within the 3′-untranslated region one or both of the two putative polyadenylylation/transcription termination sites (A-A-T-A-A-A and A-A-T-T-A-A-A). Either of these can be used, generating α_{2u}-globulin mRNA species of two lengths. A codon usage analysis of the cDNA showed that, although all six leucine codons are used for the 14 leucine residues in mature α_{2u}-globulin, the seven leucines in the partial leader sequence reported are all encoded by the same codon, CTG. The primary amino acid sequence contains a unique Asn-Gly-Ser sequence, likely to be in β-turn conformation, as the probable site of glycosylation for this glycoprotein.**

α_{2u}-Globulin is synthesized in the liver of normal adult male rats, secreted into the bloodstream, and excreted in the urine (1), and represents ≈1% of total hepatic protein synthesis (2). Female liver, male kidney, and several hepatomas do not synthesize α_{2u}-globulin and have no detectable α_{2u}-globulin mRNA sequences (2–4). The biosynthesis of α_{2u}-globulin is also under complex multihormonal control: androgens, glucocorticoids, thyroid hormone, insulin, and growth hormone stimulate and estrogens repress its synthesis (5–9). This regulation *in vivo* has been shown to occur via modulation of the rate of synthesis of hepatic α_{2u}-globulin mRNA (5, 10). The existence of both glycosylated and nonglycosylated forms of α_{2u}-globulin has been reported (11, 12), as well as induction of the glycosylated form by glucocorticoids (13, 14). α_{2u}-Globulin is first synthesized as the 21,000-dalton precursor pro-α_{2u}-globulin, which is then processed to the mature form of the protein (15).

As part of our ongoing research into the regulation of α_{2u}-globulin synthesis, we have cloned the cDNA for this protein. By determining the sequence of this cDNA, we have elucidated the amino acid sequence of α_{2u}-globulin and its precursor and demonstrated the probable site of its glycosylation. Pro-α_{2u}-globulin contains a leucine-rich NH_2-terminal hydrophobic "leader" sequence that uses a single leucine codon. In addition, we have localized two polyadenylylation/transcription termination sites in the cDNA and demonstrated that two distinct sizes of cDNA exist, presumably as a consequence of selective transcription termination or processing at one or another of these sites in the mRNA.

MATERIALS AND METHODS

Preparation of Clones. Total poly(A)$^+$RNA from livers of mature male Sprague–Dawley rats was isolated by using guanidine thiocyanate and oligo(dT)cellulose (16, 17) and reverse transcribed by using avian myeloblastosis virus reverse transcriptase (obtained from J. W. Beard) (18) after pretreatment of the RNA with methylmercuric hydroxide (19). Second-strand synthesis was carried out by using DNA polymerase I (Klenow fragment, Boehringer Mannheim) followed by nuclease S1 (Miles) digestion (20). This double-stranded cDNA preparation was partially purified by centrifugation through a neutral sucrose gradient to remove cDNAs smaller than 400 nucleotides. Terminal deoxynucleotidyltransferase (Miles) was used to add homopolymeric deoxycytidine "tails" to the cDNA, and deoxyguanosine tails were added to plasmid pBR322 previously cleaved at its *Pst* I restriction site (21). After hybridization, the chimeric plasmids were used to transform *Escherichia coli* strain HB101 (22). The tetracyline-resistant, ampicillin-sensitive colonies were selected and screened by the method of Grunstein and Hogness (23).

Synthesis of Labeled Probes. Single-strand cDNA probes were synthesized by reverse transcription in the presence of [^{32}P]dNTPs (Amersham, 400 Ci/mM; 1 Ci = 3.7×10^{10} becquerels), using both male and female poly(A)$^+$RNA as template (18, 19), and then used for colony hybridization screening (23). Recombinant plasmid DNA was isolated (24), and the α_{2u}-globulin cDNA insert was excised by using *Pst* I, purified from 1% agarose gels, and labeled with [^{32}P]dNTPs by nick translation (25). This probe was used for blot hybridization and R_0t studies [where R_0t is the initial concentration of RNA (mol of nucleotide per liter) multiplied by time (sec)].

mRNA Selection and Translation. DNA from selected clones was immobilized on nitrocellulose filters (Millipore) and hybridized to total male liver poly(A)$^+$RNA. The filters were

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Abbreviations: kb, kilobase(s); R_0t, (mol of ribonucleotide per liter) multiplied by time (sec); $(R_0t)_{1/2}$, R_0t for half-maximal hybridization to cDNA.

washed free of nonspecific mRNA and the hybridized mRNA was eluted (26, 27) and translated in a wheat germ-based cell-free system containing L-[^{35}S]methionine (28, 29). The translation system was centrifuged at 100,000 × *g*, and an aliquot of the supernatant was immunoprecipitated by using monospecific rabbit anti-α_{2u}-globulin (6). Translation products were analyzed by electrophoresis on 12.5% polyacrylamide/NaDodSO$_4$ slab gels (30) in the presence of 6 M urea. After electrophoresis, gels were fluorographed (31), dried, and exposed to x-ray film. To effect *in vitro* processing of nascent peptide chains (32, 33), 0.5 A_{260} units of a canine pancreatic microsomal membrane preparation (a generous gift from Gunther Blobel) was added to a 100 μl translation system before initiation of translation.

RNA Analysis. RNA was subjected to electrophoresis through 0.8% agarose/6% formaldehyde gels (34), transferred to and immobilized on nitrocellulose paper (35), and hybridized to ^{32}P-labeled α_{2u}-globulin cDNA. The filters were washed (36), dried, and exposed to x-ray film. RNA-excess cDNA solution hybridization (R_0t analysis) was carried out as described (37, 38) using ^{32}P-labeled α_{2u}-globulin cDNA.

Amino Acid Sequence Determination. The partial NH_2-terminal amino acid sequence of urinary α_{2u}-globulin was obtained by the Edman degradation procedure using a Beckman model 890C sequencer with cold trap and a dilute Quadrol program (Beckman 121078).

DNA Sequence Determination. The DNA sequence was determined by the procedures of Maxam and Gilbert (39) with minor modifications. *Pst* I restriction fragments were labeled at their 3′ ends by terminal deoxynucleotidyltransferase addition of cordycepin 5′-[α-^{32}P]triphosphate (Amersham, 3000 Ci/mM). *Pst* I-digested DNA (50 μg, 65 pmol of 3′ termini) was labeled in 100 μl of 140 mM potassium cacodylate/30 mM Tris base, pH 7.2/1 mM $CoCl_2$/0.1 mM dithiothreitol containing 330 μCi of cordycepin 5′-[α-^{32}P]triphosphate and 100 units of terminal transferase at 37°C for 40 min. All other fragments were labeled at their 5′ termini as described by Maxam and Gilbert (39).

RESULTS AND DISCUSSION

Selection of "Male-Specific" Clones. Eight hundred tetracycline-resistant, ampicillin-sensitive male rat liver cDNA clones were prepared and isolated as described above. Colonies were individually transferred to duplicate nitrocellulose filters, grown overnight, and processed according to Grunstein and Hogness (23). One set of filters was hybridized to ^{32}P-labeled cDNA synthesized by using adult male liver poly(A)$^+$RNA as template, and the second set of filters was hybridized to a corresponding female cDNA probe. The filters were then washed and exposed to x-ray film (36). Comparison of the duplicate autoradiograms enabled selection of 20 clones that contained cDNA sequences complementary to male but not to female rat liver mRNA (Fig. 1).

We wish to stress the simplicity and therefore general applicability of this "plus/minus" technique. When two preparations of similar phenotype (e.g., male and female rat liver) are available, it is far better to use cloning as a technique for purification and amplification than to attempt the arduous task of RNA enrichment.

Selection of α_{2u}-Globulin Clones. DNA from 10 male-specific clones, as well as that from several non-α_{2u}-globulin clones was immobilized on individual nitrocellulose filters and hybridized to total male liver poly(A)$^+$RNA. After washing the filters to remove unhybridized RNA, the specifically hybridized RNA was eluted and translated *in vitro*. Fig. 2*A* shows a fluorogram of a polyacrylamide slab gel used to analyze these translation products, and Fig. 2*B* shows the same samples immunoprecipitated with anti-α_{2u}-globulin. Lanes 1, 2, 4, and 6 each show one major band corresponding to a protein whose mRNA is complementary to the respective cloned cDNA sequence. The protein in lane 1 is derived from a control clone that was not male specific, and the protein in lane 6 (hp22) is derived from a clone (p41) previously shown to contain a male cDNA that is not α_{2u}-globulin (37). Proteins in lanes 2 and 4 were derived from two of the selected male-specific clones. As can be seen in Fig. 2*B*, the proteins in lanes 2 and 4 are immunologically identical to α_{2u}-globulin and those in lanes 1 and 6 are antigenically unrelated to α_{2u}-globulin.

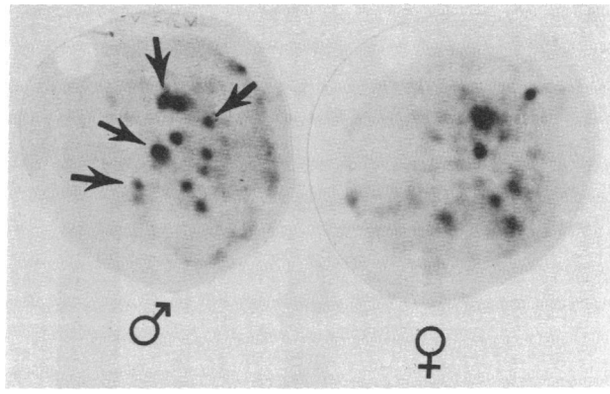

FIG. 1. Comparative colony hybridization. This autoradiogram shows two duplicate colony hybridizations of 60 adult male liver cDNA clones (colony positions are congruent). One filter (♂) was hybridized to ^{32}P-labeled cDNA prepared by reverse transcription of adult male liver poly(A)$^+$RNA, and the other filter (♀) was hybridized to the corresponding female cDNA probe. Arrows indicate male-specific clones—i.e., those that hybridized to the male probe but not to the female probe.

Processing of Pro-α_{2u}-Globulin. The α_{2u}-globulin band (Fig. 2*A*, lane 2) migrates with an apparent M_r of 21,000 or ≈2000 larger than urinary α_{2u}-globulin. This fact, in addition to the secretory nature of α_{2u}-globulin, suggests that it is derived from a larger precursor (pro-α_{2u}-globulin), as has been demonstrated with a number of other secretory proteins (32, 33). Fig. 2*A*, lane 3, shows the translation product derived when canine pancreatic microsomal membranes are added to the lane 2 translation reaction before mRNA addition. This protein is also immunologically identical to α_{2u}-globulin (Fig. 2*B*, lane 3′), but it has an apparent M_r of 19,000 and comigrates with purified urinary α_{2u}-globulin (arrow). This M_r differential of 2000 predicts a leader sequence for pro-α_{2u}-globulin of ≈18 amino acid residues. Addition of the microsomal preparation after the cessation of translation resulted in the unprocessed translation product of M_r 21,000 (unpublished data). These findings and our earlier results (15) demonstrate the precursor–product relationship between pro- and mature α_{2u}-globulin by showing that only the pro-α_{2u}-globulin form is encoded by mRNA complementary to α_{2u}-globulin cDNA (Fig. 2*A*, lanes 2 and 4). When the same RNA is translated in the presence of pancreatic microsomes, mature α_{2u}-globulin is isolated (Fig. 2*A*, lane 3). That processing *in vivo* is rapid is indicated by the fact that only the mature form of α_{2u}-globulin is isolated from rat liver after a 15-min pulse amino acid incorporation (15).

Hybridization Studies Using Cloned α_{2u}-Globulin cDNA. α_{2u}-Globulin cDNA isolated from the recombinant clone corresponding to Fig. 2*A*, lane 4, was ^{32}P labeled by nicktranslation. This radioactive α_{2u}-globulin probe was then hybridized to a nitrocellulose filter (23) that contained all 20 of the original male-specific recombinant colonies. Nineteen of these clones hybridized to the α_{2u}-globulin probe. These data suggest that

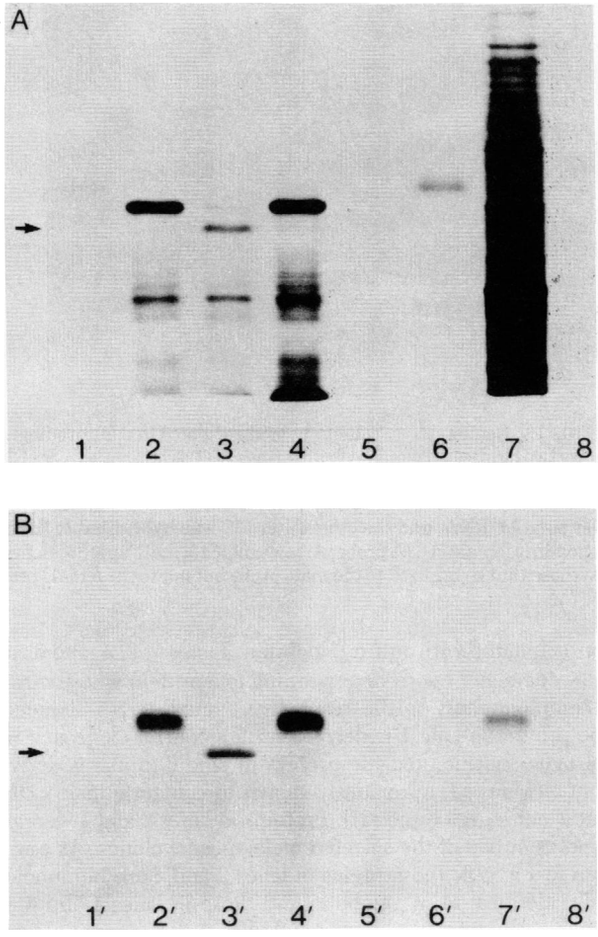

FIG. 2. Translation of mRNAs complementary to cloned cDNAs. (*A*) Gel profile showing the translation products of rat liver mRNAs complementary to several cloned cDNAs. Lanes 2 and 4, proteins derived from two of the selected male-specific clones; lane 1, protein derived from a control clone that was not male specific; lane 6, protein derived from clone p41, previously shown to contain a male cDNA that is not α_{2u}-globulin (37); lane 3, translation product derived when canine pancreatic microsomes are included in the lane 2 translation reaction; lane 5, control demonstrating the lack of specific hybridization to the plasmid vector pBR322; lane 7, total translation products from poly(A)$^+$ male liver RNA; lane 8, endogenous activity of the wheat germ system. Arrow indicates the mobility of purified urinary α_{2u}-globulin. (*B*) Lanes 1′–8′, the corresponding peptide products, if any, immunoprecipitable with anti-α_{2u}-globulin. Those in lanes 2′–4′ are immunologically identical to α_{2u}-globulin, and those in lanes 1′ and 6′ are antigenically unrelated to α_{2u}-globulin.

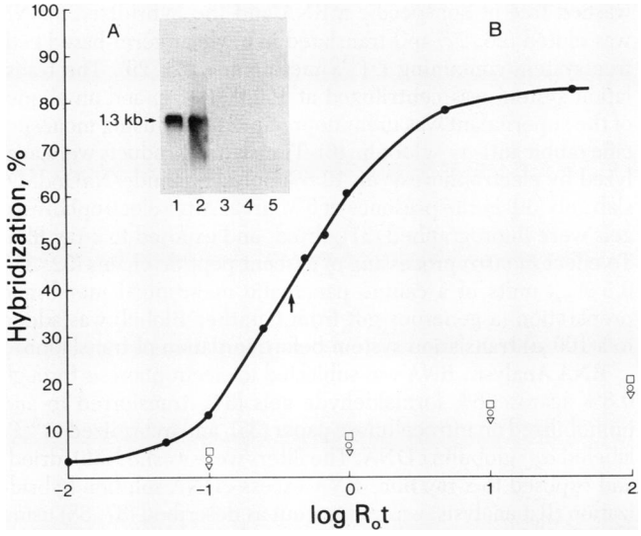

FIG. 3. (*A*) Autoradiogram of blot hybridization. RNA from five sources was subjected to electrophoresis through a 0.8% agarose/6% formaldehyde gel, immobilized on a nitrocellulose filter, and hybridized to ^{32}P-labeled α_{2u}-globulin cDNA. Lane 1, total adult male liver RNA (23 μg); lane 2, adult male liver poly(A)$^+$RNA (10 μg); lane 3, adult female liver poly(A)$^+$RNA (10 μg); lane 4, adult male kidney poly(A)$^+$RNA (10 μg); lane 5, hepatoma 7793 poly(A)$^+$RNA (10 μg). The size of mature α_{2u}-globulin mRNA is $\approx$1.3 kb (lanes 1 and 2). In addition, three α_{2u}-globulin RNA precursors are visible in lane 2, with apparent sizes of 6.6, 4.2, and 2.1 kb. (*B*) RNA excess-cDNA hybridization (R_0t analysis). Total poly(A)$^+$RNA from adult male liver (●), female liver (□), hepatoma 7793 (○), and male kidney (▽) was hybridized to ^{32}P-labeled α_{2u}-globulin cDNA. The RNA–cDNA hybrids were digested with nuclease S1, and acid-precipitable material was assayed. Arrow indicates $(R_0t)_{1/2}$. Hybridization is normalized to 100% of theoretical.

α_{2u}-globulin mRNA is the predominant male-specific mRNA present in rat liver.

This same α_{2u}-globulin probe was hybridized in solution to poly(A)$^+$RNA from male liver, female liver, male kidney, and hepatoma 7793 to a final R_0t of 200 (Fig. 3*B*). Under these conditions, only RNA isolated from male liver showed detectable hybridization. The R_0t for half-maximal hybridization to cDNA [$(R_0t)_{1/2}$] with male RNA indicates an approximate abundance of 1–2%, assuming α_{2u}-globulin is of average complexity. This abundance is consistent with that shown by *in vitro* translation (9) and the finding that, of the 800 original recombinant clones, 19 contain α_{2u}-globulin sequences.

Hybridization of the labeled α_{2u}-globulin cDNA to RNA immobilized on nitrocellulose paper (Fig. 3*A*) indicates that the mature α_{2u}-globulin mRNA is $\approx$1.3 kilobases (kb) (Fig. 3*A*, major band in lanes 1 and 2). In addition, apparent precursors of 6.6, 4.2, and 2.1 kb can be seen (Fig. 3*A*, lane 2) suggesting a minimum of three intervening sequences in the primary transcript. There is no detectable hybridization to RNA from female liver, male kidney, and hepatoma 7793 (Fig. 3*A*, lanes 3–5, respectively). Finally, use of this cloned cDNA as a sequence probe has facilitated our analysis of the control of α_{2u}-globulin RNA levels by hormones and during development. In addition we have used this probe to isolate a series of α_{2u}-globulin genomic clones from a λ phage library (unpublished results).

Nucleotide Sequence of α_{2u}-Globulin cDNA. Our sequence-analysis strategy is outlined in Fig. 4. The sequences were largely determined either in both directions or from the two complementary strands. The sequence reported in Fig. 5 is a

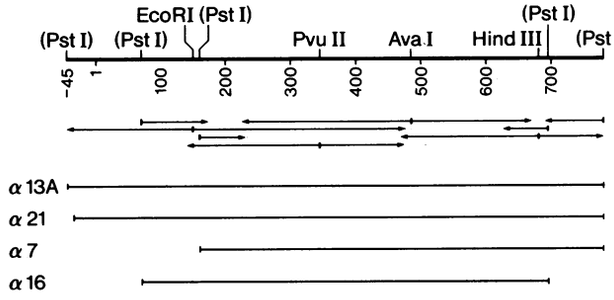

FIG. 4. Sequence-analysis strategy for cloned α_{2u}-globulin cDNA. The direction and extent of each sequencing reaction is indicated by the horizontal arrows, originating at the restriction sites shown. The *Eco*RI, *Pvu* II, *Ava* I, and *Hind*III sites are internal to the α_{2u}-globulin cDNA sequence, whereas the *Pst* I sites are terminal constructs generated by G-C tailing and insertion at the *Pst* I site of pBR322.

FIG. 5. α_{2u}-Globulin cDNA and amino acid sequences. The nucleotide sequences as determined by the procedures of Maxam and Gilbert (39) and the deduced amino acid sequence are shown. Position 1 indicates the NH_2-terminal amino acid of mature α_{2u}-globulin. Positions −1 through −15 define the amino acids of the hydrophobic signal peptide. Amino acid residues that were independently determined by sequential Edman degradation of the purified protein are underlined. The probable site of glycosylation (Asn-Gly-Ser) is indicated by a dot (●). The two alternative polyadenylylation/transcription termination sites (A-A-T-A-A-A and A-A-T-T-A-A-A) are underlined. The termination codon (TGA) is starred.

composite derived from four overlapping clones, although one of these clones does contain the complete cDNA sequence (Fig. 4, α13A). In the overlapping regions, there is 100% homology among all four clones where sequenced. The nucleotide sequence contains only one long open reading frame; the other two reading frames manifest multiple termination codons throughout this region. The coding sequence has two in-phase terminators nine nucleotides apart.

The amino acid sequence deduced from the nucleotide sequence (Fig. 5) is in full agreement with the partial NH_2-terminal amino acid sequence that we derived by sequential Edman degradation of the purified protein (Fig. 5, underlined amino acids). In addition, the deduced amino acid composition is consistent with our amino acid composition data (unpublished), as well as with that of Lane and Neuhaus (40). Authentic α_{2u}-globulin has a M_r of 18,709 and contains 162 amino acid residues. A comparison of the first 43 residues of α_{2u}-globulin with a library of amino acid sequences was conducted by the National Biomedical Research Foundation (Georgetown University Medical Center, Washington, DC). This comparison was extended by a computerized alignment procedure of the complete protein sequence, as outlined by Schwartz and Dayhoff (41), and modified and applied by us (42). These analyses indicate that α_{2u}-globulin bears a surprisingly close evolutionary relationship to the 162 amino acid protein bovine lactoglobulin, with an alignment score of 10.3 ± 0.4, equivalent to a $P < 10^{-23}$.

It has been reported that the only amino sugar in the glycosylated form of α_{2u}-globulin is *N*-acetylglucosamine (11, 12). All glycoproteins that use *N*-acetylglucosamine have their glycosidic linkage with an asparagine residue. These glycoproteins also show a requirement that the asparagine be within one of two amino acid sequences, Asn-X-Ser or Asn-X-Thr (43). Therefore, the probable glycosylation site for α_{2u}-globulin is asparagine residue 35, in the unique Asn-Gly-Ser sequence (Fig. 5, black dot). A single base change at this site in another expressed α_{2u}-globulin gene would explain the existence of both glycosylated and nonglycosylated forms of this protein (11, 12). An analysis of this region of the amino acid chain by the predictive scheme of Chou and Fasman (44) indicates that this asparagine residue is contained in a sequence (residues 34–37) that exhibits a strong potential to exist in β-turn conformation (45). This may facilitate accessibility and interaction of this sequence with the glycosylation enzyme system.

The longest clone sequence extends 45 nucleotides 5′ of the codon for the NH_2-terminal glutamic acid of the mature protein. These 45 nucleotides encode an exceptionally hydrophobic amino acid sequence that contains seven leucine residues. A codon usage analysis of the total protein shows no significant divergence from the average codon usage of other vertebrate genes, including the use of all six leucine codons (46). In the leader sequence of pro-α_{2u}-globulin, however, all seven of the leucine residues are encoded by the same codon, CTG. Whether this represents an opportunity for translational control of this secreted protein is uncertain.

Essentially all known eukaryotic mRNA sequences contain a specific hexanucleotide (A-A-U-A-A-A) 15–20 nucleotides 5′ of the poly(A) tail. There are two reported cases where a variant heptanucleotide (A-A-U-U-A-A-A) is found (47, 48). In the case of anglerfish pancreatic somatostatin II mRNA, both of these sequences are present, centered 17 and 37 nucleotides from the poly(A) (48). It has been proposed that these sequences may be signals for either polyadenylylation or transcription termination (49). Setzer *et al.* have observed at least four species of dihydrofolate reductase mRNA that appear to differ only in the length of their 3′-untranslated regions. The longest of these mRNAs contains the sequence A-A-U-A-A-A near its poly(A), the shortest contains only an A-A-U-A near its poly(A), and the other two have yet to be sequenced near their poly(A) sites (50).

The α_{2u}-globulin cDNA sequence contains both the A-A-T-A-A-A and A-A-T-T-A-A-A oligonucleotides near its 3′ end (Fig. 5, underlined). Of the four clones whose 3′ sequences have been determined, three have a poly(A) sequence 20 nucleotides 3′ of the A-A-T-A-A-A site. However, the fourth clone (Fig. 4,

α16) is 74 nucleotides shorter than the other three, although it contains a poly(A) sequence 20 nucleotides 3′ of the A-A-T-T-A-A-A site. This A-A-T-T-A-A-A sequence is present in the first three clones at precisely this location, yet it is not used as the polyadenylylation/transcription termination signal in these cases. All four of these clones have 100% sequence homology where their sequencing overlapped (a minimum of 300 nucleotides), which supports our belief that they were not transcribed from different expressed genes. We therefore consider that these cDNAs represent two classes of mRNAs, which were generated by differential site-specific polyadenylylation or transcription termination. Although only four clones have been sequenced thus far, it would appear that, in α_{2u}-globulin mRNA, A-A-U-A-A-A is the preferred polyadenylylation/transcription termination signal, an observation consistent with the comparative frequency of usage of these two oligonucleotide sequences in other eukaryotic mRNAs.

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