

Tissue-Specific Control of α_{2u} Globulin Gene Expression: Constitutive Synthesis in the Submaxillary Gland

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Summary

Synthesis of α_{2u} globulin, previously thought to occur only in the male rat liver, has now been demonstrated in the submaxillary salivary gland. Unlike liver, submaxillary synthesis of α_{2u} globulin mRNA is constitutive—that is, independent of the endocrine state, age and sex. Liver and submaxillary α_{2u} globulin mRNAs are of similar size, and their 5' ends map to the same region of the gene. Isoelectric focusing of in vitro translation products revealed that submaxillary mRNA encodes a more acidic subset of α_{2u} globulins than does liver. Salivary α_{2u} globulin mRNA manifests 5% nucleotide divergence, encoding 20 amino acid substitutions, which specifies a more acidic polypeptide than its hepatic counterpart. Thus the liver and submaxillary gland synthesize α_{2u} globulin from different sets of genes that are subject to very different developmental and hormonal control.

Introduction

α_{2u} globulin is a protein (18,700 daltons) synthesized in the liver of mature male rats, secreted into the blood and excreted in the urine (Roy and Neuhaus, 1966). Its hepatic synthesis is under multihormonal control; androgen, glucocorticoid, thyroid hormone, growth hormone and insulin are required to maintain full levels of both α_{2u} globulin synthesis and its hepatic mRNA level (Roy, 1977; Kurtz et al., 1978; Sippel et al., 1975; Kurtz et al., 1976; Roy et al., 1976; Roy et al., 1980; Roy and Dowbenko, 1977; Lynch et al., 1982). α_{2u} globulin is encoded by a multigene family which is composed of 18 to 20 closely related members (Kurtz, 1981; Dolan et al., 1982). Hepatic α_{2u} globulin synthesis begins in male liver at the onset of puberty, and its rate of synthesis increases until the animal reaches sexual maturity. The absence of hepatic α_{2u} globulin synthesis in the adult female rat is due not only to the absence of androgen, but also to suppression of its synthesis by estrogen (Roy et al., 1975). Hepatic synthesis of α_{2u} globulin ceases during hepatocarcinogenesis (P. Feigelson and F. Becker, unpublished), and its functional mRNA is undetectable in hepatomas (Sippel et al., 1976; Feigelson and De Lap, 1978; Nakhasi et al., 1981).

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In addition to its presence in the liver, α_{2u} globulin has been identified immunologically by Roy and Byrd (1976) in extracts of kidney and salivary gland from adult male rats, but not in similar extracts from pre-pubescent or adult female rats. The presence of α_{2u} globulin in the kidney and salivary glands of female rats after intravenous administration led these investigators to conclude that α_{2u} globulin was absorbed from the blood rather than synthesized in situ in the salivary gland.

However, low levels of RNA sequence coding for murine major urinary protein (the murine homolog of α_{2u} globulin) were detected in the mouse submaxillary gland (Hastie et al., 1979). We now report that functional α_{2u} globulin mRNA is also present in the submaxillary gland of the rat. In contrast with the liver, the levels of the salivary gland α_{2u} globulin mRNA are unaffected by hypophysectomy, are independent of the animal's sex and exhibit a different developmental pattern. Furthermore, they are transcribed from a set of genes distinct from that expressed in the liver. We have recently reported the synthesis of α_{2u} globulin in the granular convoluted tubules and the intercalated duct cells of the rat submaxillary gland (Antakly et al., 1982). Some of these duct cells are also known to contain epidermal and nerve growth factor as well as other hormonally regulated polypeptides (Barka, 1980).

Results

Saliva and high speed supernatants (S100) of homogenized submaxillary glands were analyzed by Western blotting. A peptide that comigrated with purified urinary α_{2u} globulin was readily detected by anti- α_{2u} globulin in the saliva and submaxillary gland extracts. Unlike the liver, the salivary gland of immature as well as mature animals contains α_{2u} globulin (Figure 1).

The submaxillary gland was next examined for the presence of α_{2u} globulin mRNA sequences. RNA extracted from the submaxillary glands of immature and mature animals of both sexes was analyzed for α_{2u} globulin mRNA sequences by translation in vitro and by hybridization to hepatic α_{2u} globulin cDNA. Polypeptides synthesized in vitro in response to salivary gland RNAs, and immunoprecipitated with anti- α_{2u} globulin serum, comigrated on SDS-polyacrylamide gels with the single α_{2u} globulin band encoded by adult male liver poly(A)⁺ RNA (Figure 2). α_{2u} globulin was synthesized in response to salivary gland RNA from prepubescent and postpubescent animals regardless of their sex. When translation systems supplemented with pancreatic microsomes were programmed with salivary gland poly(A)⁺ RNA, the immunoprecipitable peptide was again indistinguishable (lanes 2, 4, 6 and 8) from the liver product (lane 10). These results indicate that α_{2u} globulin in the submaxillary gland is synthesized with a leader sequence, as

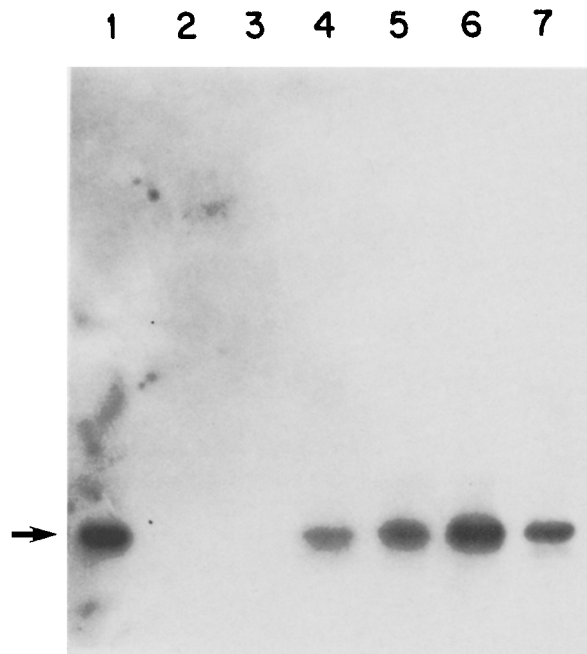


Figure 1. Western Blot of Liver, Submaxillary Gland and Salivary α_{2u} Globulins

S100s from liver or submaxillary gland and saliva containing 200 μ g of protein were subjected to electrophoresis through a 12.5% polyacrylamide–0.1% SDS gel and transferred to nitrocellulose paper. The paper was treated with monospecific anti- α_{2u} globulin serum, and the resulting immune complexes were detected by treatment with ^{125} I-labeled protein A. (Arrow) position of purified (urinary) α_{2u} globulin. (Lane 1) S100 from 74-day-old male rat liver; (lane 2) S100 from 37-day-old male rat liver; (lane 3) S100 from 70-day-old female rat liver; (lanes 4, 5, 6) S100 from submaxillary glands of 84-day-old, 37-day-old and 14-day-old male rats, respectively; (lane 7) saliva from 84-day-old male rat. Radiographic time was 72 hr.

has been demonstrated previously for the hepatic protein (Drickamer et al., 1981).

α_{2u} globulin RNA sequences evident on Northern blots in extracts from submaxillary glands are similar to those of liver. Unlike liver, however, submaxillary α_{2u} globulin RNA sequences are present in prepubescent as well as sexually mature animals regardless of gender (Figure 3). We also estimated the relative abundance of α_{2u} globulin RNA sequences in the submaxillary and liver RNA population by cDNA-excess hybridization to nitrocellulose-bound RNA (dot blot analysis; Figure 4). Spots of approximately equal intensities were obtained by hybridization to 0.05 μ g hepatic RNA (Figure 4, row 5), 0.3 μ g salivary gland RNA from young animals (rows 1 and 3) and 1.0 μ g salivary gland RNA from mature animals (rows 2 and 4). Thus the ratio of α_{2u} globulin mRNA sequences among mature salivary gland, young salivary gland and mature male liver is approximately a 1:3:18. We observe no evident sex-dependent differences in salivary α_{2u} globulin mRNA concentration. These estimates agree well with measurements of functional mRNA levels made by quantitating immunoprecipitates following *in vitro* translation (Table 1).

The endocrine control of salivary gland α_{2u} globulin synthesis is also quite unlike that in the liver. When prepubescent animals of both sexes were hypophysectomized (23 days of age), and their submaxillary RNA populations were examined for α_{2u} globulin sequences two weeks later, the level of these sequences was not substantially diminished relative to the total RNA population (see Figures 3 and 4). The same result was obtained when the salivary gland α_{2u} globulin RNA levels of animals hypophysectomized post-

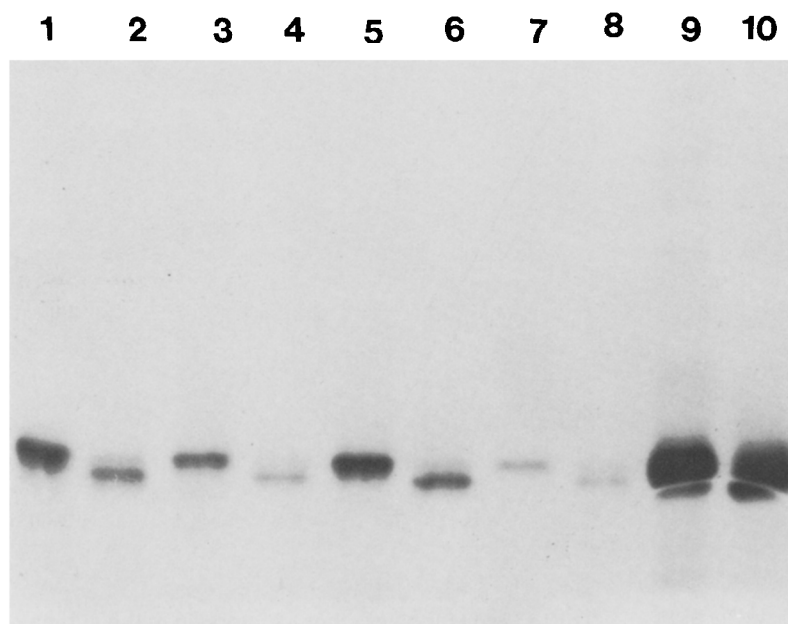


Figure 2. SDS-Polyacrylamide Gel Electrophoresis of α_{2u} globulin Synthesized *In Vitro*

Poly(A)⁺ RNA isolated from submaxillary gland or liver was translated in a reticulocyte-based cell-free system. In each sample, α_{2u} globulin was recovered from the same amount of incorporated radioactivity (150,000 cpm) by immunoprecipitation with anti- α_{2u} globulin. The denatured immunoprecipitates were resolved on a 12.5% polyacrylamide–0.1% SDS gel, after which the gel was prepared for fluorography, dried and exposed to X-ray film at -80°C . The source of the RNA was salivary gland of 21-day-old (lanes 1, 2) or 61-day-old (lanes 3, 4) male rats; salivary gland of 21-day-old (lanes 5, 6) or 61-day-old female rats (lanes 7, 8); liver of 61-day-old male rats (lanes 9, 10). The even-numbered lanes represent α_{2u} globulin synthesized in the presence of dog pancreatic microsomes. Exposure time was 48 hr.

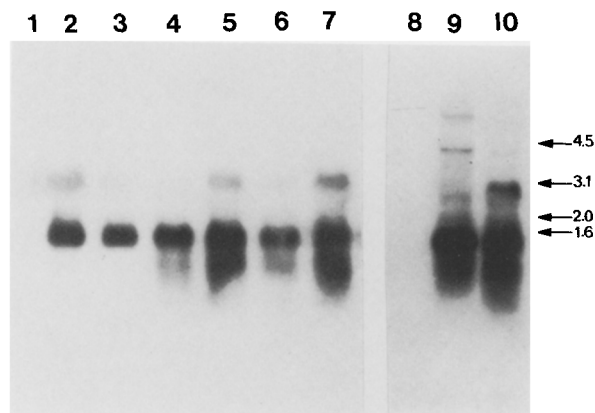


Figure 3. Electrophoretic Properties of Salivary Gland and Liver RNAs (Northern Blot)

Total RNA (30 μ g; lanes 1–7) or poly(A)⁺ RNA (30 μ g; lanes 8–10) was subjected to electrophoresis under denaturing conditions, transferred to nitrocellulose paper, and probed with nick-translated hepatic α_{2u} globulin cDNA as previously described (Lynch et al., 1982). (Lane 1) liver RNA from a hypophysectomized male; (lane 2) salivary gland RNA from hypophysectomized female; (lane 3) salivary gland RNA from hypophysectomized male; (lane 4) salivary gland RNA from mature male; (lane 5) salivary gland RNA from immature male; (lane 6) salivary gland RNA from mature female; (lane 7) salivary gland RNA from immature female. The poly(A)⁺ RNA in lanes 8 (5 μ g) and 9 (5 μ g) was extracted from the livers of mature female and male animals, respectively. The poly(A)⁺ RNA (15 μ g) in lane 10 was from the submaxillary gland of an immature female rat. The 6.6 kb band in lane 9 is visible in lane 10 upon longer exposure. Size markers were a mixture of bacterial and rat ribosomal RNAs concurrently subjected to electrophoresis. Exposure time was 36 hr.

pubescently were measured (data not shown). This situation is in sharp contrast with that observed in the liver, in which α_{2u} globulin RNA sequences disappear following hypophysectomy (Figure 3 and 4; Lynch et al., 1982).

The mRNAs which encode the liver and submaxillary gland forms of α_{2u} globulin can clearly be distinguished by isoelectric focusing of their *in vitro* translation products (Figure 5). This autoradiograph demonstrates that RNA from each tissue directed the synthesis of a different set of α_{2u} globulins. The salivary gland family of peptides is significantly more acidic than that encoded by hepatic mRNA. This observation is compatible with the sequence of a full-length salivary gland α_{2u} globulin cDNA clone (Figure 6). The salivary gland mRNA codes for a protein which, like its liver counterpart, contains 162 amino acids and a 19 amino acid leader sequence. The sequence differs from the major liver α_{2u} globulin mRNA by 5%, coding for the substitution of 20 amino acids, resulting in a net gain of six negative charges in the salivary protein. Seven of these substitutions occur within the relatively small exon III. Comparison of the salivary sequence with a typical α_{2u} globulin gene indicates that salivary and liver splice junctions are identical within the coding region of the RNAs. Acceptor heterogeneity in the removal of intron VI,

however, has been detected in both liver and salivary gland (see bracketed sequence, Figure 6), and is under further study.

To determine whether α_{2u} globulin transcription resembles the tissue-specific expression of salivary and pancreatic α -amylase, whose mRNAs have been reported to be transcribed from different sites on a single gene, the 5' ends of salivary and liver α_{2u} globulin mRNAs were mapped. The data reported in Figure 7 were derived from primer extension and S1 nuclease mapping experiments in which the same site in the DNA (B_{al}I in exon I; Figure 6) was labeled. The primer was prepared by redigesting this labeled DNA with Hae III and isolating the 18 bp fragment. The data demonstrate that the distance from the labeled nucleotide to the 5' end of its exon is identical in both liver and salivary gland (S1 nuclease digestions, lanes 3 and 4), and furthermore, that this distance is the same as the distance from the labeled nucleotide to the 5' end of either RNA template. Therefore, it appears likely that transcription of salivary and liver α_{2u} globulin genes initiates at the same site.

Discussion

The search for an extrahepatic site of α_{2u} globulin synthesis was prompted by our immunocytochemical finding of α_{2u} globulin in kidneys of young and female rats whose livers were devoid of this protein, and whose livers and kidneys were devoid of its mRNA, and by the report of murine major urinary protein RNA sequences in submaxillary glands (Hastie et al., 1979). We have used α_{2u} globulin cDNA to probe for its mRNA in extracts of prostate, testicle, ovary, kidney, spleen, heart, skeletal muscle, pituitary and brain with uniformly negative results. Our contention that the submaxillary gland is a site of α_{2u} globulin synthesis is based on compelling evidence. First, this protein is readily detectable immunochemically in the saliva and in homogenates of the submaxillary gland. This is true regardless of the hepatic level of α_{2u} globulin synthesis. Second, readily detectable levels of functional α_{2u} globulin mRNA are present in the submaxillary gland. Furthermore, these results corroborate our recent immunocytochemical demonstration of α_{2u} globulin within the granular tubular cells of the submaxillary gland (Antakly et al., 1982). α_{2u} globulin is undetectable in the parotid gland, and seems to be largely in the same granulocytic ductal submaxillary cells that are known to produce epidermal and nerve growth factors.

The endocrine and developmental control of α_{2u} globulin synthesis is radically different in the liver and submaxillary gland. Hepatic levels of α_{2u} globulin mRNA decrease markedly upon deprivation of androgen, glucocorticoid, thyroid hormone, growth hormone or insulin (Roy, 1977; Kurtz et al., 1978; Sippel

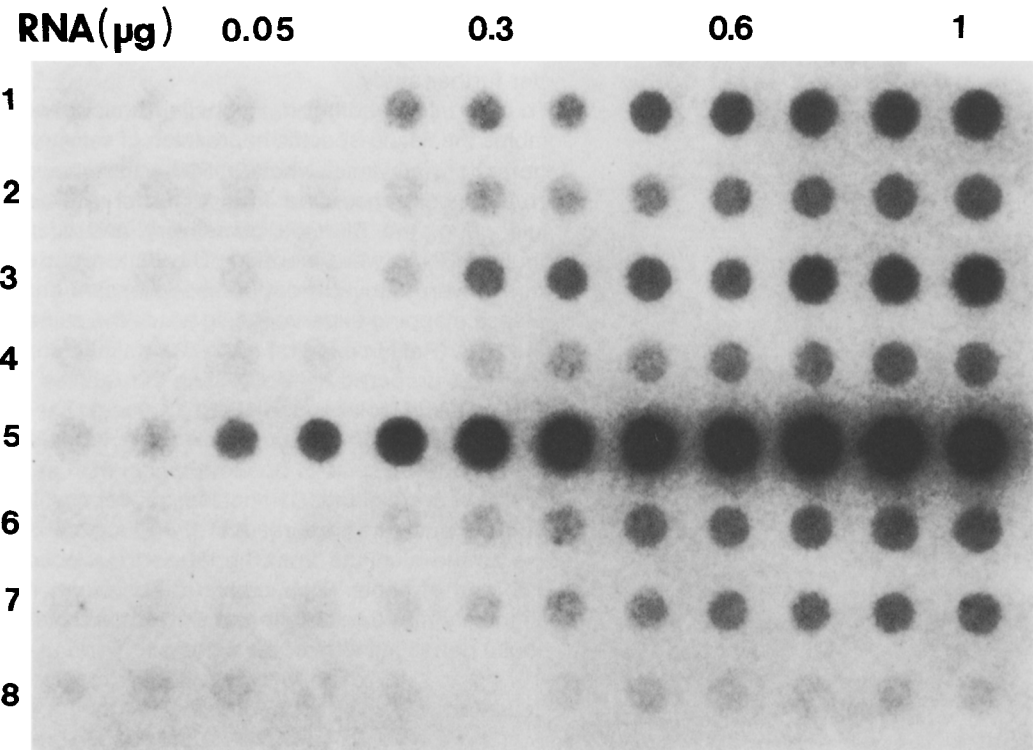


Figure 4. Dot Blot Analysis of α_{2u} Globulin RNA Abundance

Increasing amounts of total RNA (0.01–1.0 μ g) were applied to nitrocellulose paper as described in the Experimental Procedures. The bound RNA was hybridized to an excess of nick-translated α_{2u} globulin cDNA overnight at 42°C, and the blot was washed to remove nonhybridizing probe. The filter was then dried and exposed to X-ray film for 3 days. RNA from salivary gland of 21-day-old (row 1) or 61-day-old (row 2) males; RNA from salivary gland of 21-day-old (row 3) or 61-day-old (row 4) females; RNA from salivary gland of hypophysectomized 41-day-old males (row 6) or females (row 7). Liver RNA from 61-day-old intact males (row 5) or hypophysectomized males (row 8).

Table 1. Relative Abundance of Functional α_{2u} Globulin mRNA in Salivary Gland and Liver

Animal	Tissue	% of Radioactivity Immunoprecipitated As α_{2u} Globulin (Normalized to Adult Male Liver)
21-day-old male	salivary gland	25.2
21-day-old female	salivary gland	27.0
60-day-old male	salivary gland	7.8
60-day-old female	salivary gland	7.6
60-day-old male	liver	100

Counts immunoprecipitated by preimmune serum (about 0.2% of the radioactivity) were subtracted from values obtained with anti- α_{2u} globulin serum and expressed as percent of the total radioactivity incorporated into TCA-precipitable material. These values were then normalized to the adult male liver values. Each value represents the average of triplicate immunoprecipitations of three translations of individual RNAs. A typical translation reaction programmed with liver and salivary poly(A)⁺ RNA resulted in the incorporation of 4.0×10^7 cpm/ml and 1×10^7 cpm/ml into total proteins, respectively. Immunoprecipitation of the liver product with anti- α_{2u} globulin serum resulted in the recovery of 5.5% of this incorporated radioactivity.

et al., 1975; Kurtz et al., 1976; Roy et al., 1976; Roy and Dowbenko, 1977; Roy et al., 1980; Lynch et al., 1982). In addition, estrogen administration or multiple hormone ablations such as hypophysectomy abolish

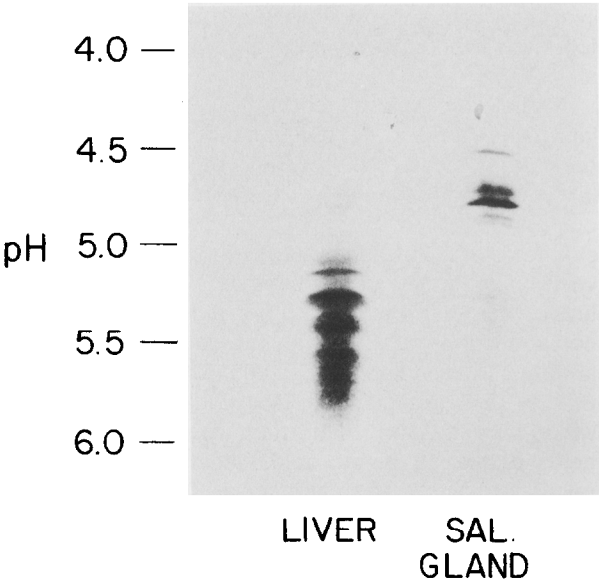


Figure 5. Isoelectric Focusing of Salivary and Hepatic α_{2u} Globulin mRNA Translation Products

Salivary gland RNA from immature male rats or liver RNA from mature male rats was translated in vitro in the presence of dog pancreatic microsomes. α_{2u} globulin peptides were recovered by immunoprecipitation and the denatured immunoprecipitates were applied to a horizontal flatbed isoelectric focusing gel (see Experimental Procedures). Exposure time was 4 days.

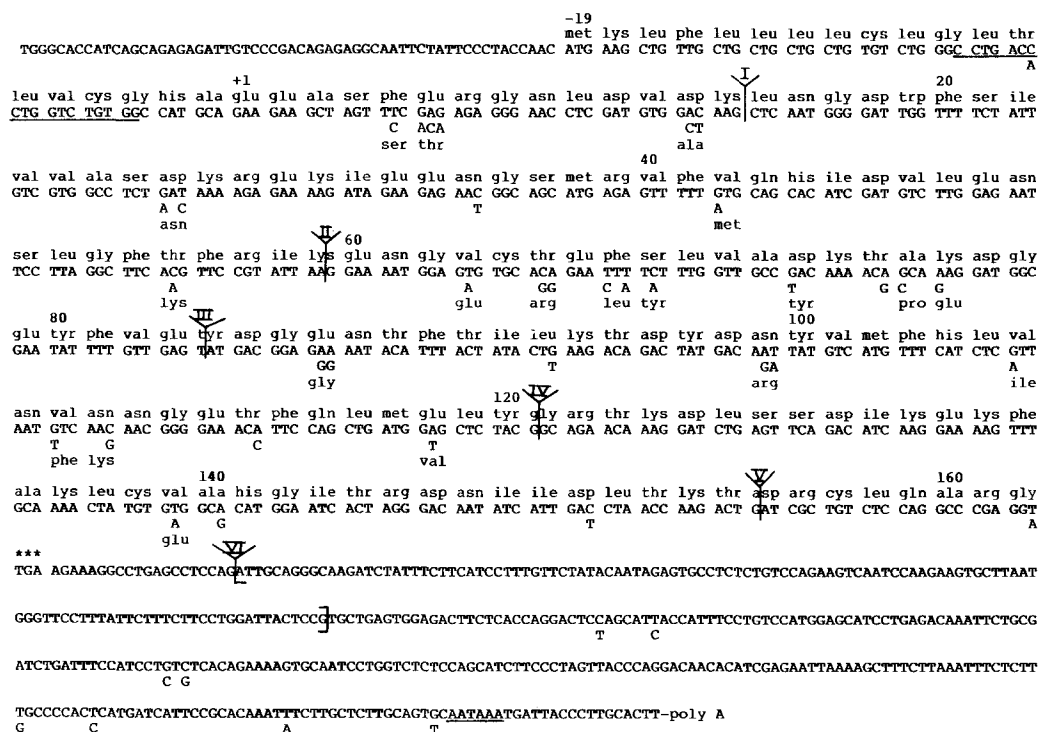


Figure 6. Sequence of a Submaxillary Gland α_{2u} Globulin cDNA

The sequence of submaxillary gland α_{2u} globulin cDNA and the predicted amino acid sequence are shown. The sequence presented is of salivary origin; divergence from the typical liver cDNA-protein sequence (Unterman et al., 1981) is indicated below the character string. Amino acids -19 through -1 indicate the leader sequence (Unterman et al., 1981; Drickamer et al., 1981). The amino acid designated +1 is the amino terminus of the mature protein. The underlined sequence 5'-CCTGACCCCTGGTCTGTGG-3' indicates the complement of the primer used in determining the 5' end of the mRNA (see Figure 7). The 121 bp nucleotide sequence in the 3' noncoding region (in brackets) is not present in a typical hepatic α_{2u} globulin gene cDNA (Unterman et al., 1981). This sequence actually corresponds to the 3' region of the last intervening sequence in the α_{2u} globulin gene. Preliminary evidence indicates that this heterogeneity in the 3' noncoding region of α_{2u} globulin mRNA is the result of alternate splicing pathways of some α_{2u} globulin transcripts. The termination codon (TGA) is starred and the putative polyadenylation signal 5'-AATAAA-3' is underlined. The positions of the exon:intron junction are shown, for example, intron I, ∇ (Dolan et al., 1982).

hepatic α_{2u} globulin synthesis. In contrast, submaxillary α_{2u} globulin RNA levels are essentially unaffected by any of these endocrine manipulations. This lack of response to sex steroids may explain why no gender-specific differences are observed in submaxillary α_{2u} globulin synthesis. It is in regard to the lack of endocrine modulation that we characterize salivary gland α_{2u} globulin synthesis as "constitutive." It is noteworthy that the lack of hormonal modulation of salivary gland α_{2u} globulin synthesis is not simply due to the lack of hormone responsiveness of this tissue. Submaxillary α_{2u} globulin has been localized solely in the duct cells of the submaxillary gland (Antakly et al., 1982); these cells are the site of synthesis of androgen-responsive proteins such as epidermal growth factor (Barka, 1980). In addition, this cell type has been demonstrated to be glucocorticoid- and thyroid-hormone-responsive (Barka, 1980).

The ontogeny of α_{2u} globulin production is also very different in the liver and salivary gland. While the hepatic level of α_{2u} globulin mRNA increases in males throughout puberty to a constant adult level, submaxillary α_{2u} globulin mRNA levels compose a somewhat smaller percentage of the total RNA in the adult gland

than in the prepubescent gland. Since the measured decreased of the α_{2u} globulin RNA during development is roughly inversely proportional to the increasing size of the gland, the actual amount of this protein produced as the animal matures is approximately constant. It is also in this sense that α_{2u} globulin synthesis may be considered constitutive.

Although the hormonal control and developmental course of α_{2u} globulin synthesis in these two tissues could hardly be more different, we were unable to distinguish between the α_{2u} globulin RNAs by hybridization to cDNA or by in vitro translation followed by SDS-polyacrylamide gel electrophoresis. The high degree of sequence homology which apparently exists among the members of the α_{2u} globulin gene family (Dolan et al., 1982) probably precludes the development of gene-specific probes. We are able, however, to distinguish between hepatic and submaxillary gland α_{2u} globulin mRNAs by isoelectric focusing of the in vitro translation products. Since posttranslational processing does not occur in a reticulocyte lysate-based system, the different isoelectric forms are assumed to reflect differences in mRNA sequences. Such differences could be due to alternate processing

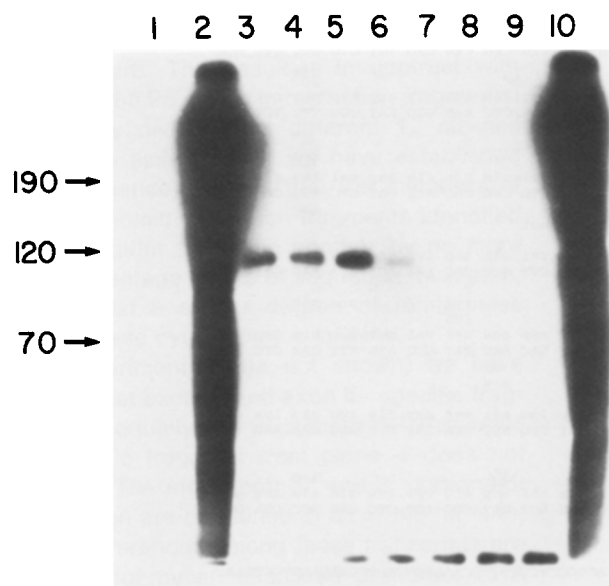


Figure 7. Mapping of the 5' End of the Salivary Gland and Liver mRNAs

Liver α_{2u} genomic clone 207-4 was digested with restriction endonuclease Bal I. This generates a 1.8 kb fragment which extends from the Bal I site in exon I (the 3' end of the sequence underlined in Figure 6) to a Bal I site in the 5' flanking region of the gene (Dolan et al., 1982). This DNA was end-labeled by the action of T4 polynucleotide kinase, and an aliquot was hybridized to liver or submaxillary gland mRNA. The resultant hybrids were then treated with S1 nuclease, and the size of the protected DNA fragment was determined by comparison with known standards after electrophoresis through a denaturing 8% acrylamide gel. (Lane 3) the DNA fragment protected from digestion by liver RNA; (lane 4) that protected by salivary gland RNA; (lanes 2 and 10) hybrids prior to S1 nuclease digestion; (lane 1) a control hybridization without hybridization to RNA.

Another aliquot of the ^{32}P -labeled Bal I-digested DNA was further digested with restriction endonuclease Hae III to yield a labeled 18 bp fragment (the underlined sequence in Figure 6), which was then purified from a denaturing 20% acrylamide gel. This fragment was hybridized to liver or submaxillary gland poly(A)⁺ RNA and extended by the action of reverse transcriptase. The length of the newly synthesized DNA was estimated after electrophoresis through a denaturing 8% polyacrylamide gel. (Lane 5) the DNA fragment synthesized on a liver RNA template; (lane 6) a salivary gland template; (lanes 7 and 8) prior to the action of reverse transcriptase; (lane 9) without hybridization to RNA. The bands present at the bottom of lanes 5 through 9 are the primer. Size markers (arrows) were ^{32}P -labeled ϕX174 DNA which was digested with Hae III. All the DNA fragments were subjected to electrophoresis on the same gel.

of the same gene transcripts or to the accumulation of different sets of α_{2u} globulin gene transcripts. The facts that the translation products are of the same size and that mutations occur throughout the length of the cDNA rather than as apparent substitution of blocks of sequence strongly support the latter hypothesis. Sequencing a number of liver cDNAs has revealed that they differ by less than 2% over their full length (Dolan et al., 1982). The sequence of this submaxillary gland cDNA reveals a divergence of 5%. While the mutations responsible for this divergence are scattered throughout the cDNA, the divergence is signif-

icantly greater in exon III, 13.5% compared to 4.3% in the other exons. This may indicate a domain that is important for salivary and/or hepatic α_{2u} function. We have not yet determined whether different salivary gland cDNAs are as closely related (that is, less than 2% divergence), but the isoelectric focusing patterns suggest that this may be so.

A situation somewhat analogous to α_{2u} globulin with regard to tissue-specific gene expression is the major mouse amylase gene. The major α -amylase mRNAs from these two tissues are transcribed from a single gene, but the starting point of transcription has been shown to differ in liver and in submaxillary gland (Young et al., 1981). The resulting mRNAs are of slightly different lengths and their 5' noncoding regions are different, although the body of the messenger is the same. Pursuing our interest in tissue-specific control of gene expression, we determined whether α_{2u} globulin was similarly controlled. This does not seem to be the case, since our primer extension and S1 nuclease studies map the 5' end of α_{2u} globulin mRNA from either tissue to the same locus on the α_{2u} globulin gene, indicating that the transcription start site is the same in both tissues.

The discovery of α_{2u} globulin in the submaxillary gland has forced us to revise our concepts of α_{2u} globulin expression. We currently envisage the α_{2u} globulin gene family as consisting of two broad classes; the members of one class, or subset, are more closely related to one another (that is, about 2% divergence) than to a given member of the other subset (that is, about 5% divergence). One subset of genes is expressed predominantly in the liver, is under endocrine control and has peptide products that appear in the urine of adult male animals. The other is expressed predominantly in the submaxillary gland, is not under endocrine control and has peptide products, which are more acidic than their liver counterparts, that appear in the saliva of immature and mature rats of both sexes. The salivary gland subset is "on," the liver subset is "regulated" and the entire set is "off" in nonexpressing tissues. We are currently studying representative members of each class of genes in an attempt to understand the basis of the "on" versus "regulated" modes of control.

Experimental Procedures

Animals

Sprague-Dawley rats (Charles River Co.) of both sexes were maintained on a 12 hr light-dark cycle and fed commercial chow ad libitum. Hypophysectomized rats (Charles River Co.) were kept under the same conditions, except that their drinking water contained 6% glucose. Animals were hypophysectomized at 22 days of age and rested a further 19 days before being killed. Any hypophysectomized animal which gained weight was eliminated from the experiments. Animals were killed by decapitation, and their livers and submaxillary glands were immediately excised, frozen in liquid nitrogen and stored at -80°C . Saliva was collected from lightly anesthetized (phenobarbital) rats after subcutaneous injection of epinephrine.

Chemicals

^{35}S -methionine (1300 Ci/mmole), gamma ^{32}P -ATP (2000 Ci/mmole), α - ^{32}P -labeled dATP, dCTP, dGTP, TTP (410 Ci/mmole) and ^{125}I -labeled protein A were obtained from Amersham Co. Reticulocyte lysate was obtained from Amersham Co. and used as directed. All enzymes were purchased from Bethesda Research Laboratories and used in the buffers recommended by the supplier.

Western Blotting

Detergent-treated post polysomal supernatants (S100) were prepared from fresh submaxillary glands or liver (Kurtz et al., 1976) and subjected to electrophoresis through 12.5% polyacrylamide-0.1% SDS gels (Laemmli, 1970). Following electrophoresis, proteins were transferred to nitrocellulose paper (Schleicher and Schuell Co. (S&S) BA 85) and incubated with anti- α_{2u} globulin sera and ^{125}I -labeled protein A with modifications of the procedures of Symington et al. (1981) and Towbin et al. (1979) suggested by Barinaga et al. (1981).

Hybridization Analysis of RNA

RNA was extracted (Chirgwin et al., 1979), and the poly(A)⁺ RNA was selected as described (Aviv and Leder, 1972). Northern blot analysis of RNA was performed as described previously (Lynch et al., 1982), with the nick-translated cDNA insert from α_{2u} globulin clone $\alpha 7$ (Unterman et al., 1981) as a probe. Relative amounts of α_{2u} globulin RNA sequences were measured by applying 0.01 to 1.0 μg of total RNA to nitrocellulose paper with a dot blot template (Minifold, S & S). Each RNA sample, mixed with 25 μg yeast RNA in $2 \times \text{SSC}$, was transferred onto nitrocellulose paper (BA 85, S & S), which had been soaked overnight at 37°C in $20 \times \text{SSC}$. After washing with four successive additions of 0.5 ml of $6 \times \text{SSC}$, the nitrocellulose paper was dried in vacuo at 80°C , hybridized to an excess of nick-translated α_{2u} globulin cDNA according to the procedure of Wahl et al. (1979), and exposed to X-ray film.

RNA Translation

Translation of poly(A)⁺ RNA, immunoprecipitation of the translation product by anti- α_{2u} globulin serum, quantitation of α_{2u} globulin synthesis and denaturing acrylamide gel electrophoresis of the immunoprecipitated translation products were performed as previously described (Lynch et al., 1982). To process the nascent α_{2u} globulin polypeptide chains in vitro, we added 0.5 A_{280} units of a pancreatic microsomal preparation (a gift from G. Blobel) to a 100 μl lysate mixture before translation was initiated by addition of RNA.

Isoelectric Focusing

Isoelectric focusing of immunoprecipitated translation products was performed on a horizontal slab gel. The gel was made by mixing 14 ml of freshly deionized 10 M urea, 4.6 ml acrylamide:bisacrylamide (29.1:0.9), 0.4 ml Nonidet-P40, 1% ampholytes (Ampholine, LKB; pH range 3.5–10), 0.5% ampholytes (pH range 4.0–6.0) and 0.5% ampholytes (pH range 6.0–8.0). This solution was degassed for 2 min, after which 0.04 ml 10% ammonium persulfate and 0.028 ml TEMED were added. This solution was quickly poured into a $0.75 \times 160 \times 180$ mm mold. Following polymerization, the gel was transferred to a 22×12.5 cm sheet of thin plastic. The trimmed gel was then placed on a water-cooled flatbed gel apparatus (Multiphor, LKB), and paper electrode strips soaked with 1 M NaOH (anode) or 1 M H_3PO_4 (cathode) were placed on the edges of the gel surface. The radioactive immunoprecipitates were suspended in the O'Farrell (1975) lysis buffer containing 1% glycine and 0.3 mg/ml of purified urinary α_{2u} globulin (Kurtz et al., 1976) and heated at 37°C for 15 min. After centrifugation to remove bacterial debris, the supernatant (approximately 12 μl) was loaded directly onto the surface of the gel 3 cm from the anode. Electrophoresis was carried out at a constant power of 15 watts for 90 min. The gel containing the focused proteins was fixed, stained with Coomassie blue, destained, dried and exposed to X-ray film.

Cloning and Sequencing of Salivary Gland α_{2u} Globulin cDNA

Poly(A)⁺ RNA was isolated from the submaxillary glands of 25-day-old female Sprague-Dawley rats. To generate a cDNA clone bank, we

inserted cDNA reverse-transcribed from this RNA into the Pst I site of pBR322 by the dG:dC homopolymeric tailing technique, as described previously for hepatic α_{2u} globulin cDNA (Unterman et al., 1981). Six of 1600 salivary clones were identified as containing α_{2u} globulin sequences by screening these clones with the PST I-excised nick-translated hepatic α_{2u} globulin cDNA by the procedure of Grunstein and Hogness (1975). The sequence of the longest cDNA insert (1.0 kb) was determined along the entire length of both strands by the method of Maxam and Gilbert (1977).

Mapping and Comparison of the 5' Termini of Submaxillary Gland and Hepatic α_{2u} Globulin mRNA

The location of the 5' end of α_{2u} globulin mRNA was determined both by primer extension and S1 nuclease mapping of a mRNA-DNA hybrid. The methodology was identical with that used to determine the 5' end of hepatic α_{2u} globulin mRNA (Dolan et al., 1982).

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References

- Antakly, T., Laperche, Y. and Feigelson, P. (1982). Synthesis and immunocytochemical localization of α_{2u} globulin in the duct cells of the rat submaxillary gland. *J. Histochem. Cytochem.* 30, 1293–1296.
- Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Nat. Acad. Sci. USA* 69, 1408–1412.
- Barinaga, M., France, R., Meinkoth, J., Ong, E. and Wahl, G. M. (1981). Methods for the transfer of DNA, RNA and protein to nitrocellulose and diazotized paper solid supports. (Keene, New Hampshire: Schleicher and Schuell Co.)
- Barka, T. (1980). Biologically active polypeptides in submandibular glands. *J. Histochem. Cytochem.* 28, 836–859.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* 18, 5294–5299.
- Dolan, K. P., Unterman, R., McLaughlin, M., Nakhasi, H. L., Lynch, K. R. and Feigelson, P. (1982). The structure and expression of very closely related members of the α_{2u} globulin gene family. *J. Biol. Chem.* 257, 13527–13534.
- Drickamer, K., Kwok, T. J. and Kurtz, D. T. (1981). Amino acid sequence of the precursor of rat liver α_{2u} globulin. *J. Biol. Chem.* 256, 3634–3636.
- Feigelson, P. and De Lap, L. W. (1978). Control of specific messenger RNA species in liver and hepatomas. In *Advances in Experimental Medicine and Biology* 92, H. P. Morris and W. E. Criss, eds. (New York and London: Plenum Press), pp. 307–330.
- Grunstein, M. and Hogness, D. S. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Nat. Acad. Sci. USA* 72, 3961–3965.
- Hastie, N. D., Held, W. A. and Toole, J. J. (1979). Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. *Cell* 17, 449–457.
- Kurtz, D. T. (1981). Rat α_{2u} globulin is encoded by a multigene family.

- J. Mol. Appl. Genet. 1, 29–38.
- Kurtz, D. T., Sippel, A. E. and Feigelson, P. (1976). Effect of thyroid hormone on the level of the hepatic mRNA for α_{2u} globulin. *Biochemistry* 15, 1031–1036.
- Kurtz, D. T., Chan, K.-M. and Feigelson, P. (1978). Glucocorticoid induction of hepatic α_{2u} globulin synthesis and messenger RNA level in castrated male rats in vivo. *J. Biol. Chem.* 253, 7886–7890.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lynch, K. R., Dolan, K. P., Nakhasi, H. L., Unterman, R. and Feigelson, P. (1982). The role of growth hormone in α_{2u} globulin synthesis: a reexamination. *Cell* 28, 185–189.
- Maxam, A. M. and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Nat. Acad. USA* 74, 560–564.
- Nakhasi, H. L., Lynch, K. R., Dolan, K. P., Unterman, R., Antakly, T. and Feigelson, P. (1981). Modifications in α_{2u} globulin gene structure, transcription; and mRNA translation in hepatomas. *J. Biol. Chem.* 257, 2726–2729.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007–4021.
- Roy, A. K. (1977). Early events in the steroidal regulation of α_{2u} globulin in rat liver. *Eur. J. Biochem.* 73, 537–543.
- Roy, A. K. and Byrd, J. G. (1976). Evidence for the concentration of α_{2u} globulin by salivary glands. *J. Endocrinol.* 71, 265–266.
- Roy, A. K. and Dowbenko, D. J. (1977). Role of growth hormone in the multihormonal regulation of messenger RNA for α_{2u} globulin in the liver of hypophysectomized rats. *Biochemistry* 16, 3918–3822.
- Roy, A. K. and Neuhaus, O. W. (1966). Proof of the hepatic synthesis of a sex-dependent protein in the rat. *Biochim. Biophys. Acta* 127, 82–87.
- Roy, A. K., Chatterjee, B., Prasad, M. S. K. and Unaker, N. J. (1980). Role of insulin in the regulation of the hepatic messenger RNA for α_{2u} globulin in diabetic rats. *J. Biol. Chem.* 255, 11614–11618.
- Roy, A. K., McMinn, D. M. and Biswas, N. M. (1975). Estrogenic inhibition of the hepatic synthesis of α_{2u} globulin in the rat. *Endocrinology* 97, 1501–1508.
- Roy, A. K., Schiop, M. J. and Dowbenko, D. J. (1976). The role of thyroxine in the regulation of translatable messenger RNA for α_{2u} globulin in the rat liver. *FEBS Lett.* 64, 396–399.
- Sippel, A. E., Feigelson, P. and Roy, A. K. (1975). Hormonal regulation of the hepatic messenger RNA levels for α_{2u} globulin. *Biochemistry* 14, 825–829.
- Sippel, A. E., Kurtz, D. T., Morris, H. P. and Feigelson, P. (1976). Comparison of in vivo translation rates and messenger RNA levels of α_{2u} globulin in rat liver and Morris hepatoma 5123D. *Cancer Res.* 36, 3588–3593.
- Symington, J., Green, M. and Brackmann, K. (1981). Immunoautoradiographic detection of proteins after electrophoretic transfer from gels to diazo-paper: analysis of adenovirus-encoded proteins. *Proc. Nat. Acad. Sci. USA* 78, 177–181.
- Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* 76, 4350–4354.
- Unterman, R. D., Lynch, K. R., Nakhasi, H. L., Dolan, K. P., Hamilton, J. W., Cohn, D. V. and Feigelson, P. (1981). Cloning and sequence of several α_{2u} globulin cDNAs. *Proc. Nat. Acad. Sci. USA* 78, 3478–3482.
- Wahl, G. M., Stern, M. and Stark, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. *Proc. Nat. Acad. Sci. USA* 76, 3683–3687.
- Young, R. A., Hagenbüchle, O. and Schibler, U. (1981). A single mouse α -amylase gene specifies two different tissue-specific mRNAs. *Cell* 23, 451–458.