Influence of Androgen on Translatable Renin mRNA in the Mouse Submandibular Gland

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SUMMARY In mature outbred Swiss male mice, submandibular gland renin enzyme activity is 4- and 10-fold higher than in glands of prepubescent males and mature females, respectively. Levels of translatable renin mRNA have been studied in mouse submandibular gland during postnatal development and following administration of testosterone. The $^{[35}S$]methionine-labeled cell-free translation products directed by male glandular mRNA contain a 47 ± 2 kd renin precursor that is not detected in products coded by prepubescent male or female gland mRNA. This cell-free synthesized precursor is detected immunochemically only in the translation products of gland mRNA from males of 33 days or older and from females receiving testosterone administration, a pattern consistent with the measurements of renin enzyme activity. This increase in biologically active renin mRNA is a selective one, since unfractionated male and female mRNAs have similar overall nucleotide sequence complexity corresponding to 1% mouse single copy DNA. The cDNA transcribed from male gland mRNA reacts 5- and 10-fold faster with the template mRNA than with female or prepubescent male gland mRNA, respectively, which indicates that the male gland contains abundant nucleotide sequences that exist at low concentration in the female or prepubescent male. Selective hybrid arrested translation confirms that the levels of renin mRNA are lower in the glands of prepubescent males than in those of the mature males. These data indicate that the regulation of renin enzymatic activity by androgens is mediated by an increase in the levels of translatable renin mRNA both during postnatal development and after testosterone administration. (Hypertension 6: 605-613, 1984)

KEY WORDS • postnatal development • cDNA hybridization • saturation hybridization • hybrid arrested translation

The mouse submandibular gland (SMG) contains an enzyme that resembles renal renin (EC 3.4.99.19) physically, chemically, and immunologically.1 Studies employing DNA recombinants complementary to SMG renin mRNA have demonstrated that extensive structural homology exists between SMG and renal renins2 and that at least two genetically distinct forms of renin exist in mouse SMG.3 The high levels of SMG renin and of its mRNA2,6 relative to those of the kidney and the sexually dimorphic expression of SMG renin and several other enzymes7-10 provide a useful system for investigating renin biosynthesis.2-6 Renin, initially synthesized in SMG in the preprorenin form (Mr 47 ± 2 kd), is processed intracellularly to prorenin (Mr 44 ± 2 kd) and finally to the mature product (Mr 37 ± 2 kd).11 Depending upon the strain of inbred mouse, SMG renin content in females and in castrated males ranges 10- to 100-fold lower than that of the adult male.7-9 Previously,6 we reported that the 47 ± 2 kd renin precursor is an abundant translation product of male SMG mRNA but is not detectable in translations directed by SMG mRNA from females or castrated males and that testosterone injection of female mice for 2 to 3 weeks increases the translatable SMG renin precursor mRNA to the level of that of the male.

In this study, the influence of androgens on the overall pattern of translatable SMG mRNA and on SMG renin biosynthesis has been examined further, particularly during postnatal development. The levels of biologically active renin precursor mRNA are low in prepubescent male SMG, and the ability of SMG mRNA to direct renin precursor synthesis closely parallels the renin enzymatic activity in the gland. The levels of renin precursor mRNA increases to readily detectable levels by Day 33, which precedes the in-
crease in renin enzymatic activity first noted on Day 35. Comparisons of male, female, and prepubescent male SMG mRNA by cDNA hybridization and by saturation hybridization showed that the overall diversity of gland mRNA remained unchanged during the period of renin mRNA accumulation. Taken together, these findings demonstrate that androgenic hormones increase SMG renin activity by inducing an increase in the levels of translatable renin precursor mRNA and that the increase in renin mRNA is relatively selective and part of a developmental pattern in which renin and several other precursor mRNAs accumulate.

Materials and Methods

Animals

Outbred Swiss (Crl:CD-1 (ICR) BR) female, prepubescent, castrated, and adult (6–8 weeks old) male mice were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts). The sex of animals was confirmed anatomically after removal of SMG. Where indicated, testosterone propionate was administered daily by subcutaneous injection of 1 mg in 0.1 ml polyethylene glycol-400. Animals had free access to food and water and were kept in alternating 12-hour cycles of light and dark.

Preparation and Measurement of Renin

Mice were killed by cervical dislocation, and the excised submandibular glands were disrupted with a Polytron homogenizer (Brinkman Instruments, Inc., Westbury, New York) in 20 volumes of buffer containing 5 mM sodium acetate (pH 5.5), 1% (vol/vol) Triton X-100, 0.5 mM EDTA, 0.5 mM sodium tetrahydrosulfonate (NATT), and 0.2 mM phenylmethylsulfonylfluoride (PMSF). After gentle rocking at 4° C for 30 minutes, homogenates were centrifuged 20 minutes at 4000 g, and the supernatants were assayed for renin activity by using plasma from nephrectomized dogs, and the amount of substrate cleaved during the reaction was >1000 ng angiotensin I (ANG I) equivalent/ml, with no detectable renin activity, as the source of renin activity.

mRNA-Directed Cell-Free Translation

The SMG RNA was deproteinized with guanidine-HCl and by extraction with a phenol/chloroform/isooamy alcohol mixture, and poly(A)-containing (poly(A)+) mRNA was purified by oligo(dT)-cellulose chromatography. Poly(A)+ mRNA dissolved in H2O was quantitated by absorbance at 260 nm (1 A260 unit = 40 μg mRNA) and stored at −20° C at a concentration of 500 μg/ml. Poly(A)+ mRNA was translated in an mRNA-dependent rabbit reticulocyte cell-free lysate purchased from New England Nuclear Corporation (Boston, Massachusetts). One μg of mRNA was translated at 37° C in 25 μl reactions containing the components as specified by the instructions to the New England Nuclear translation system, including 20 μCi [35S]methionine (>1000 Ci/mmol). The mRNA-directed translation products were analyzed in linear 6% to 15% gradient polyacrylamide gels in a discontinuous system. After electrophoresis, gels fixed in 10% acetic acid were stained with Coomassie Brilliant Blue to locate the positions of the molecular weight markers and then soaked in Enhance (New England Nuclear), to increase the sensitivity of autoradiography.

Immunocytochemical Identification of Cell-Free Synthesized Renin Precursor

Cell-free translation products directed by poly(A)+ mRNA from male SMG were diluted 1:4 with immune selection buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1.0 M NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.25 mM NATT, and 0.1 mM PMSF. After an 18-hour incubation at 4° C with renin-specific antiserum (10 μl antiserum per 25 μl translation mixture), immune complexes were isolated by adsorption with S. aureus (IgG Sorb, New England Enzyme Center, Boston, Massachusetts). Nonspecific adsorption of radiolabeled translation products to the heat-killed S. aureus was eliminated by incubation of the bacteria with a 100,000 g supernatant of female gland homogenate (1 ml supernatant per 100 μl packed volume cells). Pretreated S. aureus (1 μl packed cells per μl antiserum) were incubated with the antisera/translation product mixture for 15 minutes at 4° C with gentle agitation, and immune complexes were deposited by centrifugation for 10 seconds in a Brinkman Microfuge. Pellets were washed 3 times with 1 ml volumes of immune selection buffer, and finally washed by centrifugation through 0.2 ml 1 M sucrose in 0.1 M NaCl, 20 mM Tris HCl (pH 7.4) 1 mM EDTA, 1% Triton X-100. Pellets solubilized in 62.5 mM Tris-HCl (pH 6.8), 5% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, and 0.001% bromphenol blue were boiled for 5 minutes and electrophoresed in polyacrylamide gradient slab gels.

Measurement of Submandibular Gland mRNA Diversity and Abundance

Total nucleotide complexity of mouse SMG mRNA was measured by saturation hybridization. Mouse brain single-copy DNA (scDNA) was isolated and la-
beled with [\textsuperscript{3}H]dCTP. \textsuperscript{18, 19} Reaction mixtures (3–5 \( \mu l \)) containing 3000 to 5000 cpm and 12 \( \mu g/\text{ml} \) RNA in 0.4 M Na phosphate (pH 6.8), 5 mM EDTA, and 0.2% sodium dodecyl sulfate were incubated at 67° C for intervals up to 100 hours. The percentage of DNA hybridized was determined by SI nuclease digestion and selection of resistant RNA/DNA duplexes on DEAE-cellulose filters.\textsuperscript{19}

Comparisons of the relative abundance of SMG mRNAs were performed by using cDNA hybridization. The cDNA to male SMG poly(A)+ mRNA was transcribed in reaction mixtures containing 70 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM MgCl\(_2\), 4 mM sodium pyrophosphate, 2.5 mM dithiothreitol, 20 \( \mu g \) oligo (dT\textsubscript{30}), 0.5 mM each of dTTP, dGTP, dATP, 1 mM [\textsuperscript{3}H]-dCTP, and 5 \( \mu g \) of poly(A)+ mRNA.\textsuperscript{20} After 15 minutes at 0° C, 65 units of reverse transcriptase (obtained from Dr. Joseph Beard, Life Sciences, Inc., St. Petersburg, Florida) were added to the reaction, and the reaction was incubated for 15 minutes at 0° C and at 45° C for 45 minutes. The reaction was heated for 5 minutes at 65° C in 0.1 M NaOH, adjusted to 10 mM Tris-HCl (pH 7.4), 0.3 M NaCl, 10 mM EDTA, and 0.2% wt/vol SDS, and deproteinized with phenol/chloroform/isoamyl alcohol. The cDNA was precipitated with 2 volumes of 95% ethanol after chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, New Jersey). The SMG poly(A)+ mRNA from mature and prepubescent male or female mice was hybridized with [\textsuperscript{3}H] cDNA transcribed from submandibular gland poly (A)+ mRNA.\textsuperscript{20} Reaction mixtures containing 0.4 or 0.12 M sodium phosphate (pH 6.8), 1000–5000 cpm [\textsuperscript{3}H]cDNA, and a 10\(^{-2}\) to 10\(^{-1}\) mass excess of poly(A)+ mRNA were incubated at 65° C for intervals up to 100 hours. Reaction mixtures were then diluted to 850 \( \mu l \) in 300 mM NaCl, 50 mM sodium acetate (pH 4.5), 2 mM ZnSO\(_4\), and 10 \( \mu g/\text{ml} \) E. coli DNA. To ensure digestion of unhybridized cDNA, 400 \( \mu l \) aliquots of the diluted mixtures were incubated with SL nuclease at 37° C for 30 minutes. Acid-soluble [\textsuperscript{3}H]cDNA precipitates were collected on nitrocellulose filters (Type DA, Millipore Corporation, Bed ford, Massachusetts) and quantitated by scintillation counting in a toluene-based fluid containing 0.4% wt/vol omnifluor (New England Nuclear).

Results

Influence of Androgen on Submandibular Gland Renin Enzyme Activity

Before examining the influence of androgens on translatable SMG renin precursor mRNA, we measured the SMG renin enzymatic activity during male postnatal sexual development and in females following testosterone administration. Because renin activity in the mouse SMG shows great variation between inbred strains,\textsuperscript{7, 9} it was important to measure renin levels in the outbred Swiss mice used in our studies. In these mice, renin levels of adult male SMG were 10-fold greater than those of female adults (Table 1). Administration of testosterone to female mice daily for 7 days increased SMG renin concentration by 80%, from 0.15 to 0.26 \( \times 10^4 \) ng ANG I/mg protein/hr, which confirmed androgenic regulation of this system. Continued administration of testosterone for 2 additional weeks resulted in the elevation of female SMG renin levels to 1.6 \( \times 10^4 \) ng ANG I/mg protein/hr, a level indistinguishable from that of adult males over 40 days old. Furthermore, within 10 days after castration of adult males, the concentration of SMG renin was reduced to a level only slightly higher than that of females, and by 3 weeks of castration, the levels were indistinguishable from those of females (Table 1). Thus, although genetic background determines the absolute SMG renin levels, the enzyme concentration in these outbred Swiss mice was strongly influenced by androgens.

The influence of testosterone on SMG renin was demonstrated further by measuring SMG renin during postnatal development in male mice at 26 to 42 days old. As shown in Table 2, SMG renin activity was very low in prepubescent mice but, as expected, increased SMG renin concentration by 80%, from 0.15 to 0.9 \( \times 10^4 \) ng ANG I/mg protein/hr, in agreement with previous studies.\textsuperscript{5, 7} To further confirm the presence of renin mRNA, the SMG renin enzymatic activity was measured by resistance to SI nuclease and plotted in cDNA-mRNA against the log equivalent C\(_g\)GT (moles nucleotide/sec/liter).\textsuperscript{21}

Hybrid-Arrested Translation

The cDNA for hybrid-arrested translation was transcribed from submandibular gland poly (A)+ mRNA of 24-day old male mice. The cDNA (0.5 \( \mu g \)) was hybridized at 50° C with 0.5 \( \mu g \) poly (A)+ mRNA in 1.6 \( \mu l \) of 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) (pH 7.4) and 180 mM KCl in sealed capillary tubes. Contents of these hybridization mixtures were added to the translation reactions and incubated as described.\textsuperscript{6, 22} The translational products were analyzed in gradient polyacrylamide gel followed by fluorography as described above. Selected [\textsuperscript{35}S]-labeled proteins located by fluorography were eluted from the polyacrylamide gel using Protosol (New England Nuclear), and quantitated by liquid scintillation counting in a toluene-based fluid containing 0.4% wt/vol omnifluor (New England Nuclear).

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>No. of mice</th>
<th>Renin (( \times 10^4 ) ng ANG I/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8</td>
<td>1.59 ± 0.33</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>Castrated male 10 days postop</td>
<td>2</td>
<td>0.20 ± 0.12</td>
</tr>
<tr>
<td>Castrated male 21 days postop</td>
<td>2</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>Female + testosterone 7 days</td>
<td>4</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Female + testosterone 14 days</td>
<td>4</td>
<td>0.16 ± 0.36</td>
</tr>
</tbody>
</table>

Extracts of submandibular glands were assayed for total protein and renin enzymatic activity. Each animal was assayed in quadruplicate. Values are means ± SEM. ANG = angiotensin.
TABLE 2. Concentration of Submandibular Gland Renin During Male Mouse Development

<table>
<thead>
<tr>
<th>Mouse age (days)</th>
<th>No. of mice</th>
<th>Renin ( \times 10^4 ) ng ANG I/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>5</td>
<td>0.9 ± 0.33</td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>1.2 ± 0.51</td>
</tr>
<tr>
<td>31</td>
<td>7</td>
<td>0.8 ± 0.11</td>
</tr>
<tr>
<td>33</td>
<td>5</td>
<td>0.7 ± 0.04</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
<td>4.9 ± 2.5</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>42</td>
<td>6</td>
<td>3.1 ± 0.97</td>
</tr>
</tbody>
</table>

Submandibular gland extracts from male mice of the indicated ages were assayed for total protein and renin enzymatic activity. Each animal was assayed in quadruplicate. Values are means ± SEM. ANG = angiotensin.

39. This increase coincided with the onset of puberty, the increase in plasma testosterone, the development of external genitalia, and the growth of the SMG. These observations in outbred Swiss mice agree with the pattern of renin enzyme accumulation reported in other strains of mice and establish the temporal pattern of SMG renin accumulation during the androgen-induced growth and development in the mouse.

Translatable Renin Precursor mRNA in Mouse Submandibular Gland

Cell-free translation products of mouse SMG mRNA from males or testosterone-treated females had been previously shown to contain at least three to four abundant polypeptides that are not detectable in translations directed by untreated female or castrated male SMG mRNA. One of these is a 47 ± 2 kd polypeptide that was identified as a renin precursor (preprorenin) on the basis of precipitation with renin-specific antiserum. This polypeptide and other abundant male "specific" polypeptides were not visible in the \[^{35}\text{S}\]methionine-labeled products directed by SMG mRNA from the females (Figure 1 left) or 24-day-old prepubescent males (Figure 1 right). In males less than 33 days old, renin precursor was not readily detected in total translation products (Figure 2) or among in vitro synthesized polypeptides selectively immune-precipitated with renin-specific antiserum (Figure 3). However, preprorenin was detected readily in the translation products coded by glandular mRNA from all males 33 days or older. This was associated with a parallel increase in SMG renin levels (Table 2). The slight discrepancy between the timing of the increase in renin mRNA levels (Day 33) and the increase in renin enzymatic activity (Day 35) was probably due to biological variability between animals and/or variability in the experiments, since the renin assays and the mRNA isolation were performed on separate sets of animals. Thus, SMG renin precursor mRNA levels not only changed in adult female and castrated male mice receiving male sex hormone, as previously reported, but also changed during the period of androgenic stimula-

FIGURE 1. Rabbit reticulocyte cell-free translation products directed by submandibular gland (SMG) mRNA from adult male, female, and prepubescent male mice. The \[^{35}\text{S}\]methionine-labeled polypeptides coded by SMG poly(A)\(^+\) mRNA were analyzed in linear 6% to 15% gradient polyacrylamide gels, and radiolabeled proteins were visualized by fluorography.

Left Panel: Products of adult male SMG (Lane A), female SMG (Lane B), or endogenous reticulocyte lysate mRNA (Lane C). Right Panel: Translation products of adult (Lane A), or prepubescent male (Lane B) SMG mRNA. Bars at left indicate the position of the molecular weight markers in descending order: phosphorylase A (92 kd), ovalbumin (45 kd), and trypsinogen (24.5 kd). The bars on the right indicate the position of the 47 ± 2 kd renin precursor (upper bar) and the position of the female specific proteins (lower bars in Figure 1 A).
low concentrations or not at all in the glands of mature males (Figure 2). Whether the accumulation of these mRNAs was stimulated by estrogen or inhibited by testosterone is unknown. The sexual dimorphic expression of this mRNA further indicated the complexity of regulatory process in the SMG.

**mRNA Diversity in Mouse Submandibular Gland**

To delineate further the molecular events associated with androgen-regulated renin accumulation, we analyzed total SMG mRNA in populations of adult male, adult female, and developing male mice by nucleic acid hybridization. Measurements of total nucleotide complexity by saturation hybridization provided estimates of overall mRNA diversity or of the number of different mRNA sequences in each SMG mRNA population. When subpopulations of male and female SMG mRNA were hybridized to saturation with single-copy mouse brain DNA (scDNA), the total nucleotide complexities of total cytoplasmic RNA, total cytoplasmic poly(A) + mRNA and polysomal poly(A) + mRNA were judged to be similar in that all samples hybridized to a total of 0.9% to 1% of scDNA irrespective of sex (Table 3). Since each cytoplasmic RNA fraction derived from the same fraction of scDNA (within experimental error), the following conclusions may be inferred. First, the SMG does not contain a measureable quantity of unique, nonpolyadenylated mRNA, since total cytoplasmic RNA and poly(A) + RNA have the same complexity. Second, the SMG does not contain a large pool of unique, nontranslated mRNA, because total cytoplasmic RNA and polysomal RNA also exhibit similar nucleotide complexities. Third, since in each instance RNA from male and female SMG contain the same number of different mRNAs, androgenic stimulation probably does not induce a large number of new mRNA molecules. Nevertheless, saturation hybridization is of limited sensitiv-

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Regulation of translatable mRNA during postnatal development of male mouse submandibular gland (SMG). The [35S]methionine-labeled cell-free translation products directed by mRNA purified from SMG of mice at different stages of postnatal sexual development were analyzed as in Figure 1. The white arrows in the fifth and sixth lanes (33 and 36) denote the cell-free synthesized renin precursor. The black arrows denote the female-specific mRNA translation products. The numbers at the top of each lane refer to the ages of the mice in weeks.

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Appearance of the renin precursor mRNA during postnatal development of mouse submandibular gland (SMG). The [35S]methionine-labeled translation products were reacted with renin-specific antiserum to selectively precipitate cell-free synthesized renin precursor. Immune-selected translation products were analyzed by polyacrylamide gel electrophoresis followed by fluorography. Immune-selected translation products were coded by mRNA from retired breeder males (Lane A); for retired breeder females (Lane B); and for males aged 18 days (Lane C), 19 days (Lane D), 22 days (Lane E), 24 days (Lane F), 33 days (Lane G), 36 days (Lane H), 40 days (Lane I), 46 days (Lane J), and 48 days. As in Figure 1, bars at the left denote molecular weight markers; the bar at the right shows the position of the renin precursor. Note the increase in renin precursor at and after 33 days. The constant band (MW ~35 kd) present in all the lanes is due to nonspecific binding of a [35S]labeled protein to S. aureus, and its intensity is constant in all lanes. In contrast, preprorenin exhibits age-related changes paralleling the changes in renin activities (Table 2).
with a similar fraction of input cDNA. These findings confirmed results of scDNA saturation hybridization in that all RNAs reacted with the cDNA to the same extent. However, the rate of cDNA hybridization was 5- to 10-fold greater with male poly(A)⁺ mRNA than with mRNA from prepubescent male SMG (Figure 4 left) or female SMG (Figure 4 right), which suggests that adult male SMG contains abundant polynucleotides that occur at much lower frequency in the female or prepubescent male gland and, thus, react more slowly with male SMG cDNA. The cDNA hybridization results are consistent with the translational analysis of these mRNAs (Figures 1 and 2 right), which showed abundant in vitro-synthesized polypeptides in male SMG mRNA translation products but not in those of prepubescent male or female mRNA. Within the limitations of these techniques, the cell-free translation and molecular hybridization experiments showed that the male, female, and prepubescent male SMG contain similarly diverse mRNA populations but that levels of certain sequences, including renin, are higher in the adult male gland. Thus, androgenic stimulation exerts selective influences on some mRNAs but does not generally enhance accumulation of all mRNAs.

Analysis of Renin mRNA by Hydrid-Arrested Translation

Hybrid-arrested translation confirmed that renin mRNA was abundant in the mature male gland but not in that of the prepubescent male. This analysis was based on the observation that after hybridization of a complex mixture of cDNAs with a population of mRNAs, only mRNAs lacking a complement will translate, but those mRNAs in RNA:DNA duplexes

![Graph showing cDNA hybridization with submandibular gland (SMG) poly(A)⁺ mRNA.](http://hyper.ahajournals.org/fig1-4e.png)

**Figure 4.** Graphs showing cDNA hybridization with submandibular gland (SMG) poly(A)⁺ mRNA. Left Panel: Hybridization of cDNA with poly(A)⁺ mRNA from adult male (●) or prepubescent male (○) SMG. Right Panel: Hybridization of cDNA with SMG poly(A)⁺ mRNA from adult male (●) or adult female (○) mice. The curves were drawn by inspection, but differences between the curves were confirmed by computer analysis.
will not translate because of steric constraints. In this study, total cytoplasmic RNA from mature or immature males was hybridized to cDNA transcribed from prepubescent male SMG mRNA (cDNAp). We assumed that if prepubescent male SMG contained a low concentration of renin mRNA relative to adult males, the renin cDNA levels, as a result of transcription of prepubescent male mRNA, would also be low. Hence, the inhibition of adult SMG renin mRNA translation as a result of hybridization would be negligible. Indeed, the results (Figure 5 left) indicated that although the translation of the mRNA sequences common to both the adult and prepubescent glands were inhibited due to mRNA/cDNA hybridization (for example, Bands 1, 4, 5, and 6, Lanes E, F, G), the translation of the adult SMG renin precursor mRNA (Band 2) persisted even after 1 hour of hybridization with cDNAp. Densitometric tracings of the fluorogram provided a quantitative evaluation of the extent to which the synthesis of the proteins designated was inhibited (Figure 5 right). Band 3 is an endogenous reticulocyte protein, which was labeled independent of translation and thus can serve as an invariant internal marker. Quantitation of the radioactivity in each band shown in Figure 5 left and determination of the areas under the peaks of the densitometric scans (not shown) indicated that the incorporation into the renin precursor (Band 2) was inhibited by 30%, while incorporation into the other proteins was inhibited by up to 60% (Table 4). We believe that the differences in inhibition between the renin precursor and other protein translation was significant and not casual. The 30% decrease in renin mRNA translation was probably due in part to the well-known nonspecific inhibitory effect of DNA itself on mRNA translation. In addition, it must be remembered that renin mRNA is present in juvenile male

<table>
<thead>
<tr>
<th>Band</th>
<th>0 minutes hybridization (cpm)</th>
<th>60 minutes hybridization (cpm)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5804</td>
<td>3408</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>2409</td>
<td>1886</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>4532</td>
<td>5082</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1309</td>
<td>772</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>1081</td>
<td>496</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>2381</td>
<td>1380</td>
<td>50</td>
</tr>
</tbody>
</table>

The bands designated in Figure 5 left were excised, and the radioactivity in each band was determined. Radioactivity in Bands 1, 2, 4, 5, and 6 represents the difference between the cpm of the individual bands and the corresponding legion of Lane A (no-mRNA). Band 2 is the renin precursor. Band 3 is a reticulocyte protein whose labeling occurs independent of translation. Therefore, hybridization with cDNA will not inhibit the labeling of this protein. With the radioactivity in this band used as an internal standard, the radioactivity in all lanes was normalized, and the percentage of inhibition was calculated.

![Figure 5](https://hyper.ahajournals.org/content/611./f5)

**Figure 5.** Selective hybrid-arrested translation of submandibular gland (SMG) mRNA. Total cytoplasmic RNA from prepubescent male (Lanes B–D) or adult male (Lanes E–G) mice was hybridized to cDNA transcribed from prepubescent SMG mRNA for 0 minutes (B, E), 30 minutes (C, F), or 60 minutes (D, G) prior to addition to translation mixture. The resulting translation products were analyzed as in Figure 1. Left Panel: Fluorogram of resulting gel. Right Panel: Densitometric tracing of fluorogram.
mice, albeit at low levels. Thus, cDNA transcribed from juvenile gland mRNA will cause some inhibition of the translation of adult male renin mRNA.

Thus, as previous analyses of the female SMG mRNA population have shown, the renin mRNA was present in very low concentrations in the prepubescent gland, which indicated that testosterone alters the cytoplasmic levels of renin mRNA.

**Discussion**

The renin-angiotensin system is central to cardiovascular homeostasis, and the activity of this system is regulated by many hormones including steroids. For example, estrogens and glucocorticoids increase the hepatic synthesis and release of angiotensinogen. Estrogens also induce uterine renin synthesis, since uterine renin activity increases during estrogen administration, puberty, estrus, and pregnancy. The concentration of uterine renin increases 40-fold in pregnancy and exceeds circulating and renal renin levels. On the other hand, the concentration of the mouse SMG renin in male mice is 10- to 100-fold higher than in those of female mice or castrated male mice.

The regulation of gene expression by testosterone has been shown in many systems to be mediated, in part, by a selective increase in the cytoplasmic levels of specific mRNAs. For example, mouse liver, mouse kidney, rat prostate gland, and rat seminal vesicle contain one or more specific mRNAs that accumulate to abundant levels under constant exposure to testosterone. Expression of kidney-abundant protein and major urinary protein in the mouse kidney and liver, respectively, occurs in females but at lower levels than in males. In this regard, androgenic regulation of SMG renin during postnatal sexual development and after exposure of female and castrated male mice to testosterone follows a similar pattern. This conclusion is drawn from measurement of biologically active renin precursor mRNA in cell-free translation products, from testosterone induction of female SMG renin levels to those of adult males, and from nucleic acid hybridization studies. Hybrid-arrested translation experiments suggest that renin-mRNA is specifically abundant in SMG of adult males. It should be pointed out, however, that the hybrid arrested translation assay may be limited as a quantitative method to use in a complex RNA population since it is subjected to a number of variables such as length of cDNA and abundance of the mRNA. Nevertheless, these experiments provide an insight into the relative levels of SMG renin mRNA. Thus, it appears that the androgen regulation of SMG renin mRNA is mediated by an alteration in the rate of accumulation of this mRNA in the cytoplasm and that this induction is accompanied by a complex pattern of developmental changes in which certain other specific mRNAs also accumulate during the transition to sexual maturity.

Nucleotide complexity determinations (Table 3) revealed that the mRNA diversity of the submandibular gland is low (1.1% of scDNA) relative to that of other mouse organs. In kidney and liver, for example, the nucleotide complexities of polysomal mRNAs are twice those of the SMG mRNAs equaling ~2.2% of single copy DNA. However, similar analysis of cellular RNA from SMG and kidney showed hybridization to 6.5% of single copy DNA, or ~13% of genomic DNA assuming asymmetric transcription, in each instance. Since the differences between total cellular RNA and cytoplasmic RNA are nuclear sequences, these results demonstrate that transcriptional diversity is equivalent in SMG and kidney even though the SMG cytoplasm contains 50% fewer different mRNAs. The differences in nucleotide complexity exhibited by these organs may reflect differences in the number of different functions performed by each organ, in the number of different cell types of each organ, or in the efficiency of processing nuclear transcripts into functional cytoplasmic mRNA.

Cytoplasmic renin precursor mRNA accumulation after exposure to testosterone may be mediated by an increase in the rate of appearance of the mRNA in the cytoplasm or by increased mRNA stability. Consistent with the former mechanism, it has been shown that radiolabeled testosterone accumulate in the nuclei of submandibular gland cells as well as in those of the kidney and seminal vesicles. More recent experiments with cDNA recombinants have shown that levels of translatable renin precursor mRNA and of renin mRNA sequence correlate closely, which implies transcriptional regulation as a major control mechanism. Nevertheless, the regulation of mRNA stability remains to be elucidated during androgen induction. The recent availability of renin cDNA and a SMG cDNA library in our laboratories should enable investigators to address these questions as well as other issues pertaining to the molecular regulation of renin biosynthesis.

**Addendum**

Since the acceptance of this manuscript, we have confirmed the androgenic regulation of translatable SMG renin mRNA by RNA blot hybridization analysis using the renin cDNA clone. PSM479 (kindly provided by Dr. K.W. Gross). Similarly, we have also confirmed the appearance and accumulation of SMG renin mRNA during postnatal development of male mice.

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