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Thyroxine and testosterone transcriptionally regulate renin gene expression in the submaxillary gland of normal and transgenic mice carrying extra copies of the *Ren2* gene

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Expression of the mouse renin genes (*Ren*1 and *Ren*2) in the submaxillary gland of female mice has been analyzed following administration of thyroxine (T4) or dihydrotestosterone (DHT). Both hormones appear to act independently on mRNA accumulation which increases about 5 fold over basal level. In vitro transcription assays in isolated nuclei demonstrate that both hormones act at the transcriptional level. The effects of DHT and T4 were also analyzed in transgenic mice obtained by microinjection of the *Ren*2 gene. We show that T4 is as efficient as DHT in promoting renin mRNA accumulation in these transgenic animals, in spite of their low basal level of *Ren*2 mRNA. Structural comparison of the *Ren*1 and *Ren*2 promoters with those of other genes regulated by T4 shows the conservation of two discrete regions.

Hormonal regulation; Transgenic mouse; Thyroxine; Transcriptional regulation; (Submaxillary gland)

1. INTRODUCTION

The mouse renin genes are of particular interest for studying the hormonal control of gene expression. Wild type and most laboratory mouse strains have two renin genes, *Ren*1 and *Ren*2 [1–3]. *Ren*2 expression is at least two orders of magnitude higher than that of *Ren*1 in the SMG, while both genes are expressed at the same level in the kidney [4]. Moreover, these two genes encode distinguishable proteins [5]. Renin activity in the SMG is positively regulated by DHT and T4. These two hormones act independently of each other since SMG renin can be induced by T4 in Tfm/y mice which are resistant to DHT because they lack functional androgen receptors [6]. The induced renin activity in the SMG after the hormonal treatment is correlated with a rapid increase in the level of its mRNA [7,8]. Whether this is the result of an enhanced rate of transcription or a stabilization of the mRNA remains to be established.

Previous experiments on transgenic mice carrying an exogenous *Ren*2 gene showed that transgenic males have levels of *Ren*2 mRNA comparable to that of Swiss male mice (two-renin gene strain). However, transgenic females have a very low level of renin mRNA when compared to Swiss females [9]. To determine whether basal level is controlled by thyroxine, we analyzed the accumulation of the *Ren*2 mRNA in the SMG of transgenic mice in response to this hormone.

We also show that DHT and T4 stimulate the transcription of the *Ren*2 gene and that the *cis*-responsive elements for both hormones are contained in the exogenous DNA fragment carried by transgenic mice.

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Abbreviations: SMG, submaxillary gland; T4, thyroxine; DHT, dihydrotestosterone; Ren, renin; rGH, rat growth hormone

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2. MATERIALS AND METHODS

2.1. *Animals and hormone treatments*

Mouse strains were obtained from the Institut Pasteur and Iffa Credo. Transgenic mice were produced as described [9]. Mice used in all experiments were 8 weeks old. Thyroxine (Sigma) was dissolved in 10 mM NaOH and injected i.p. Mice were treated for 7 days with daily injections of 10 μg of T4 per animal. Testosterone induction was carried out by injection of 12.5 mg of Sterandryl (Roussel). Animals were killed 1 week after hormone injection. For nuclear 'run on' assays, animals were injected i.p. with a single dose of 50 μg of T4 or 10 mg of Sterandryl and killed 2 h after the treatment.

2.2. *RNA analysis*

RNA was prepared from mouse tissues as previously described [9]. Northern blot experiments were performed as described [10] using the renin cDNA [2] as probe. RNA dot-blot experiments were performed as described [11].

2.3. *In vitro transcription in isolated nuclei*

Nuclei isolation was performed essentially as described [12,13]. In vitro transcription was performed for 10 min at 26°C in a 250 μl reaction mixture containing a 100 μl aliquot of nuclei, 120 mM Tris-HCl, pH 8, 1.2 M KCl, 50 mM $MgCl_2$, RNasine (40 U), 2.5 mM GTP/ATP/UTP and 150 μCi of [α-^{32}P]CTP (3000 Ci/mmol). Radiolabelled nuclear RNAs were purified as described [14] and hybridized to a nitrocellulose filter containing 4 μg of the renin cDNA; pUC18 was used as negative control. Under these conditions, the amount of labelled transcripts that hybridize to the cloned cDNA will be proportional to the specific activity of that sequence in the hybridization mixture.

3. RESULTS

3.1. *Transcription of* Ren*2 gene in isolated nuclei*

To determine whether the accumulation of SMG renin mRNA after DHT and T4 treatment is due to an increased rate of transcription or to a post-transcriptional event, we carried out in vitro transcription assays in isolated nuclei extracted from the SMG of hormone-treated and untreated females. In the experiment shown in fig.1, four females received a single injection of either DHT (12.5 mg) or T4 (50 μg). Nuclei isolated from the SMG of female mice 2 h after administration of the hormone and nuclei isolated from the SMG of untreated females were brought to the same concentration, then in vitro transcription was performed as described in section 2. The same amount of labelled RNA (10^7 cpm) purified from SMG nuclei of hormone-treated and untreated females was hybridized onto replica filters containing the renin cDNA. The results of this analysis showed

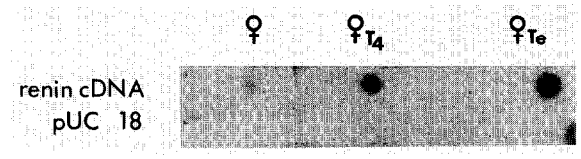

Fig.1. Induction of transcription in isolated nuclei of SMG renin mRNA by thyroxine and testosterone. 4 μg plasmid containing the renin cDNA or pUC18 were spotted onto nitrocellulose filters as described in section 2. Equal counts of nascent-labelled nuclear RNA from SMG of T4-treated (♀T4), DHT-treated (♀Te) or untreated females (♀) were hybridized to replicate filters.

that females treated with DHT or T4 have higher levels of renin mRNA when compared to untreated controls (fig.1). DHT causes a 4-fold induction compared to 2-fold with T4 under these experimental conditions. Plasmid pUC18 was used as control DNA to monitor for non-specific binding of RNA. We conclude that the hormonal induction of the *Ren*2 gene is achieved mainly at a transcriptional level.

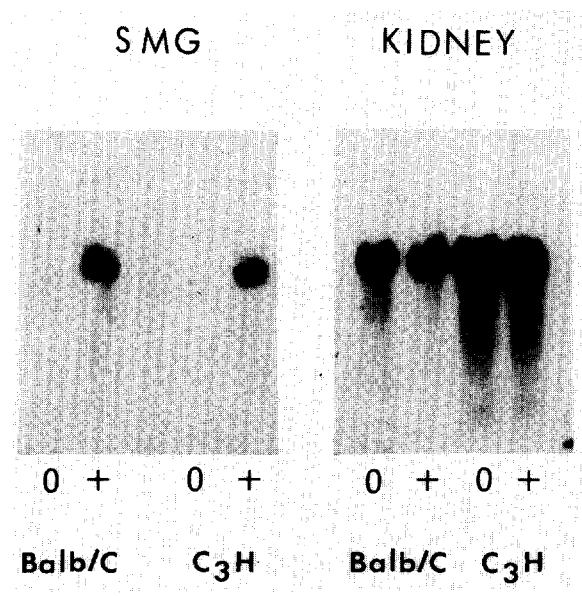

Fig.2. Northern blot analysis of SMG and kidney poly(A)$^+$ RNA extracted from normal and treated mice. 10 μg poly(A)$^+$ RNA was electrophoresed through a 1.1% agarose-formaldehyde gel, transferred to nitrocellulose and hybridized to the renin probe. Balb/C and C_3H mice were treated with 10 μg of thyroxine (+) daily as described in section 2. Untreated mice (0) of each strain were used as controls.

3.2. *Stimulation of mRNA* Ren*1 accumulation by thyroxine and testosterone*

To examine whether *Ren*1 is also regulated by DHT and T4 in the SMG, despite the different level of *Ren*1 mRNA accumulation in this tissue when compared to *Ren*2 mRNA, DHT and T4 have been administered to females of two different mouse strains possessing only the *Ren*1 gene (Balb/C and C_3H). 10 μg of poly(A^+) RNA prepared from SMG, kidney and liver of control or treated females were analyzed by Northern blot hybridization, using the renin cDNA [2] as probe. The results presented in fig.2 show that, in the SMG, *Ren*1 mRNA accumulation increases about 5- to 10-fold over basal level after T4 treatment. Similar results are obtained after administration of testosterone to females (not shown). Although these hormones are known to exert effects on various types of kidney cells, administration of DHT or T4 had no effect on the amount of *Ren*1 mRNA in the kidney (fig.2). Renin mRNA was not detected in the liver of mice following DHT or T4 treatments (not shown). We conclude that these hormones increase the levels of *Ren*1 mRNA in a tissue-specific manner.

3.3. *Thyroxine treatment induces* Ren*2 mRNA accumulation in transgenic females*

Because thyroxine induction of endogenous renin mRNA is associated with an enhanced rate of renin gene transcription (see section 3.2), we have examined whether the exogenous *Ren*2 gene carried by transgenic female mice is induced after the administration of thyroxine. 8-week-old females of three transgenic lines were injected with T4, DHT or both hormones as described in section 2. Swiss females of the same age and subjected to the same hormonal treatment, were used as controls. Total RNA (1 μg) prepared from SMG of these animals was analyzed by Northern blot hybridization and renin mRNA accumulation was quantified by dot blot hybridization. Since the amount of renin mRNA found in the SMG of two-renin gene strains (like Swiss mice) is two orders of magnitude higher than in one-renin gene strains (like Balb/c), we used in these experiments amounts of RNA that allowed one to discriminate between renin transcripts coming from the endogenous *Ren*1 gene and the transgene *Ren*2.

Fig.3 shows that T4 and DHT induce SMG renin mRNA to the same extent in transgenic females

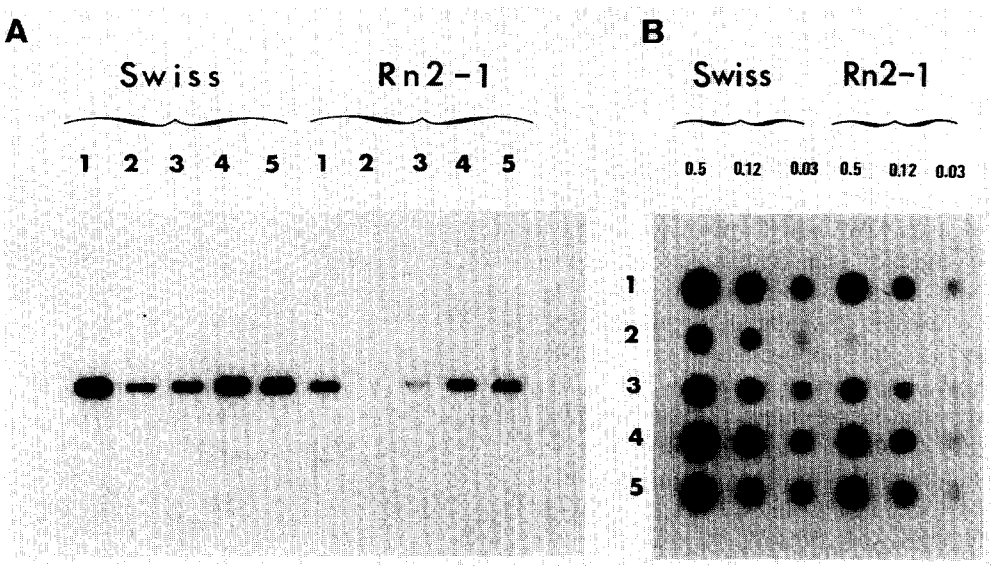

Fig.3. Analysis of SMG renin mRNA extracted from normal and transgenic females in response to testosterone and thyroxine. (A) Northern blot of total RNA (1 μg) prepared from SMG of Swiss and transgenic female mice (Rn2-1). Lanes: 1, males; 2, females; 3, T4-treated females; 4, DHT and T4-treated females; 5, DHT-treated females. (B) Dot-blot analysis of hormone-treated transgenic females compared to Swiss female mice. Total SMG RNA of males (1), females (2), T4-treated females (3), DHT- and T4-treated females (4), DHT-treated females (5) were spotted onto nitrocellulose and hybridized to the renin probe. The amount of RNA spotted was 0.5 μg and subsequent serial dilutions were 1:4.

(Rn2-1) as in Swiss female mice. Similar results were obtained with the other two transgenic lines (not shown). Each hormone acts independently to stimulate *Ren*2 transcription. In both cases, the hormonal treatment increased renin mRNA in the SMG of female mice to a level equivalent to that of males. Because of the difference in basal levels, renin mRNA was increased around 20-fold in transgenic mice, versus 4–5-fold in normal two-gene strains. The finding that induction by DHT is more efficient than that with T4 was also observed in isolated SMG nuclei.

4. DISCUSSION

In vitro transcription assays on SMG nuclei provide direct evidence that T4 and DHT induce the level of *Ren*2 mRNA by increasing the rate of *Ren*2 transcription, the effect being observed as early as 2 h after hormone administration. However, this does not in itself prove that the hormones act directly on the *Ren*2 gene. It is conceivable, though unlikely, that another protein is very rapidly induced and transcriptionally activates the *Ren*2 gene.

The results presented here have shown that administration of T4 or DHT increases the accumulation of *Ren*1 mRNA in the SMG of mice possessing only one renin gene. When an exogenous *Ren*2 gene with its flanking regions is microinjected in fertilized mouse eggs carrying only the *Ren*1 gene, the transgene is regulated by T4 and DHT in the same manner as the *Ren*2 gene of wild type two-gene strains. Indeed, although the same renin probe is used to detect *Ren*1 or *Ren*2 mRNA, the use of very low amounts of RNA allows one to distinguish between transcripts coming from the transgene (*Ren*2) or from the endogenous gene (*Ren*1). Thus, transgenic female mice are sensitive to T4 induction suggesting that this hormone is not involved in the regulation of the renin basal level. The fact that *Ren*1 and *Ren*2 expression is regulated by T4 and that thyroxine *cis*-responsive elements are present in the DNA fragment carried by transgenic mice led us to compare the promoter sequences of the two renin genes with those of other T4 regulated genes. Most of the data concerning the identification of *cis*-acting elements which mediate T4 response have come from studies of rGH regulation. We compared the 5′-flanking region of the rGH gene to the 5′-flanking regions of the *Ren*1 and *Ren*2 genes. Two regions of striking similarity within two discrete T4 responsive elements described by Wight et al. [15] were found. The decanucleotide motif, TGGGTGGTCT, present in the gene coding for

Table 1

Presence of conserved motifs in the 5′-flanking sequences of thyroxine responsive genes

rGH	TGGGTGGTCT		GAAAACAGGTAG	
	−257	−248	−45	−34
*Ren*2	TGGGTGGTCT		GAAAAGAGGTAG	
	−675	−666	−569	−558
*Ren*1	TGGGTGGTCT		GAAAAGAGGTAG	
	−464	−455	−357	−346
hGH	TGGGTGGTAT		GGAAAGGGATAG	
	−238	−225	−383	−372
Angiotensinogen	GCGGTGCTCT		CAAAACAGCGGC	
	−638	−629	−613	−602
αNGF	TTGGGGGTCA		GCAAGCAGGAAG	
	−334	−325	−222	−210

5′-flanking regions of six thyroxine-responsive genes were compared for common sequence motifs. Genes compared are from rat growth hormone (rGH), mouse renin genes (*Ren*1 and *Ren*2), human growth hormone (hGH), angiotensinogen and the α-subunit of mouse nerve growth factor (αNGF). The positions relative to the principal transcriptional start point have been indicated

the rGH (positions −248 to −257 relative to the transcription start point [16]), and in the two mouse renin genes (positions −455 to −464 for *Ren*1; −666 to −675 for *Ren*2 [17]). The second conserved element which is the dodecanucleotide GAAAACAGGTAG is found in the three genes with only one mismatch (the C is replaced by a G in the two renin genes). This motif is located at positions −34 to −45 for rGH; −346 to −357 for *Ren*1 and −558 to −569 for *Ren*2 (table 1). These two regions are also found in the gene coding for the hGH [18,19]. The genes for angiotensinogen and the α-subunit of the mouse nerve growth factor are regulated by T4 [20–23] and contain two sequences resembling those described above, although similarity does not exceed 70% for the first region and 75% for the second one. We are currently attempting to establish whether these sequences in the two renin genes bind the thyroxine-receptor complex.

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