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Cellular basis for the differential response of mouse kallikrein genes to hormonal induction

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The expression of many mouse kallikrein genes in the salivary gland is sexually dimorphic and inducible in females by administration of testosterone or thyroxine. Induction is slow (3-7 days) and is accompanied by the non-uniform differentiation of the cell type expressing these genes from striated duct (SD) cells (female) to granular convoluted tubule (GCT) cells (male). One kallikrein gene, mGK-6, is expressed at an apparently constant total level in male and female and is not induced by either hormone. *In situ* hybridization histochemistry shows that all kallikrein genes analyzed exhibit uniform cellular distribution of expression in the SD cells of the female. The hormonally mediated differentiation of some, but not all, of these cells has different effects on kallikrein gene expression — mGK-6 is repressed while other kallikreins are induced — leading to non-uniform distribution of expression.

Key words: sexual dimorphism/hormone response/kallikrein genes

Introduction

Most studies on regulation of expression of genes from complex, multicellular eukaryotes have been carried out on tissue culture cell lines. These studies suffer from two major problems. Firstly, the cell lines are clonal and do not grow in the environment of their normal whole animal counterpart (e.g. lack cell-cell contact with non-homologous cells). Secondly, analysis of gene expression is usually an average determination of a large number of cells (e.g. nuclear run-off or RNA blot analysis) which need not reflect the situation in individual cells. These studies assume homogeneity of a given cell type. Previous studies using RNA extracted from whole tissues also give results which average-out variations in response that may occur in the different cells present. We have attempted to overcome these problems by analysis of expression in the cells of an experimental animal and by the use of *in situ* hybridization histochemistry techniques. This approach allows for a determination of the relative level of gene expression in individual cells in their 'natural' cellular environment. In conjunction with RNA blot analysis to quantify changes in expression a more complete picture of regulatory control *in vivo* can be gained.

A large number of substances, including proteins encoded by the kallikrein multigene family, have been found to exhibit sexual dimorphism in the mouse salivary gland (Barka, 1980). Various studies have addressed the effect of testosterone or thyroxine on the level of these proteins, and in general, treatment of female mice with either of these hormones raises the level of the proteins to (or beyond) that of the male (Barka, 1980; Chao and Margolius, 1983; Catanzaro *et al.*, 1985). We have reported previously that the expression of some kallikrein genes exhibits this sexual dimorphism in the salivary gland whereas expression of the renal kallikrein gene (mGK-6) is virtually the same in both sexes (van Leeuwen *et al.*, 1986). The level of expression of the kallikrein genes mGK-3 and 4 which encode the γ- and α-subunits of nerve growth factor (NGF), and mGK-13 which encodes an epidermal growth factor (EGF)-associated binding protein, are 30-100 times greater in the male than in the female (van Leeuwen *et al.*, 1986; C.C.Drinkwater, B.A.Evans and R.I.Richards, unpublished data). This difference is similar to that of protein levels of the kallikrein-associated growth factors, EGF and NGF, reported for male and female salivary gland (Barka, 1980).

In this study the cellular distribution of these different genes was further investigated using *in situ* hybridization histochemistry techniques. The duct cells which express kallikrein genes appear to comprise two distinct populations. The first expresses mGK-6 constitutively. Conversely, hormonally responsive cells turn off expression of mGK-6 at a time when expression of the other kallikrein genes is induced. The latter cell type is only found in the submandibular gland, while the former is found in the parotid and sublingual glands in addition to the submandibular gland. This complex distribution of different cellular phenotypes is a major contributor to the differential response of mouse kallikrein genes to hormonal induction.

Results

Differential response to hormone induction

To investigate the difference in expression of kallikrein genes between male and female mice we have treated female mice with either testosterone or thyroxine. The testosterone induction of expression of the kallikrein genes mGK-3, 4, 5 and 6 and the genes encoding NGF and EGF is shown in Figure 1. Dot blots were used to compare more rigorously the total amount of each mRNA expressed in the salivary glands. The integrity of this RNA is apparent from the Northern blot data presented in Figure 2. Although quantitation of polyadenylated RNA by u.v. absorption was the same for each group of mice the hybridization of the thymidine 30-mer [oligo(dT)] to the RNA blot shows that the actual amount of polyadenylated RNA loaded was less for the male mice. The specificity of each probe was determined by Southern blot analysis as described by van Leeuwen *et al.* (1986); no detectable cross-reactivity was observed under the conditions of hybridization used. Total kallikrein gene expression as measured by hybridization to the ubiquitous kallikrein 27-mer (Materials and methods) exhibits testosterone induction of female levels up to male levels. The expression of the growth factors NGF and EGF, and the kallikrein genes mGK-3, 4 and 5 in females is induced by testosterone treatment to levels approach-

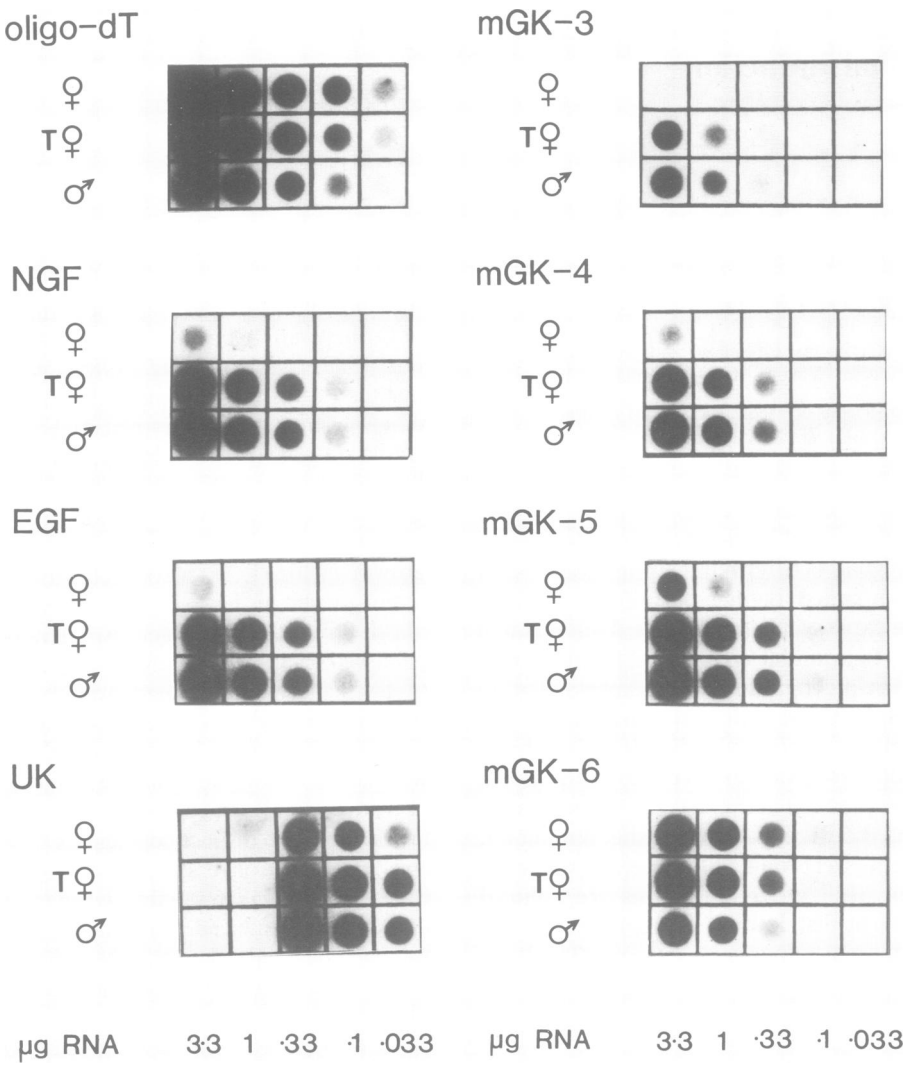

Fig. 1. Testosterone induction of gene expression. Dot blots of polyadenylated RNA from female (♀), testosterone-treated females (T♀) and male (♂) mice. The amount of RNA loaded is shown at the bottom of the figure. Identical blots were hybridized to the oligo(dT) 30-mer, or an 'ubiquitous' 30-mer which hybridizes with all of the kallikrein genes (UK), or 30-mers specific for EGF, NGF or the kallikrein genes mGK-3, 4, 5 or 6. The filter hybridized to the oligo(dT) probe was autoradiographed for only 1 h at −70°C whereas the other filters were exposed to film for 18 h at −70°C.

ing those seen in the male. Figure 1 also shows that mGK-6 levels are higher in the female than levels of other kallikrein genes, that there is little difference in mGK-6 expression between the sexes (allowing for the amount of polyadenylated RNA loaded) and that testosterone had no apparent effect on inducing the expression of this gene.

Figure 2 shows that the administration of thyroxine to female mice has a similar effect on the expression of mGK-3 and preproEGF (and other kallikrein and growth factor genes, data not shown), whereas expression of mGK-6 is again unaffected. This lack of specificity suggests that induction by either of the hormones involves a general and perhaps common mechanism.

Mechanism of hormone induction

Perhaps the simplest explanation for the differential response to hormonal induction by members of the glandular kallikrein gene family might be the presence of hormone responsive elements in the vicinity of hormone responsive genes (e.g. mGK-3) and their absence from non-responsive genes (e.g. mGK-6). This correlation has been previously demonstrated for the differential response of the human metallothionein genes to glucocorticoids (Richards *et al.*, 1984).

Primary transcriptional responses to hormone induction are usually rapid, reaching a maximum in ~12 h (Karin *et al.*, 1980). To ascertain whether testosterone acts directly on the target genes to increase transcription we compared expression of the kallikrein genes mGK-3 and 6, and the growth factor EGF, with levels of tubulin at various times after testosterone treatment. The polyadenylated RNA from salivary glands of male, female and testosterone-treated female mice were subjected to Northern blot analysis.

Figure 3 shows that expression of β-tubulin stays relatively constant during testosterone treatment. The expression of mGK-6 parallels that of tubulin whereas expression of mGK-3 and EGF increases from barely detectable levels in the female up to levels comparable with those measured in the male. Induction of these genes is first apparent ~24 h after administration of testosterone, increasing to male levels between 3 and 7 days. While these experiments do not rule out a role for specific direct hormonal stimulation of transcription, the slow time course, the generality of the response and the accompanying differentiation of the responsive cells suggest that induction is probably not a primary response (Yamamoto and Alberts, 1976).

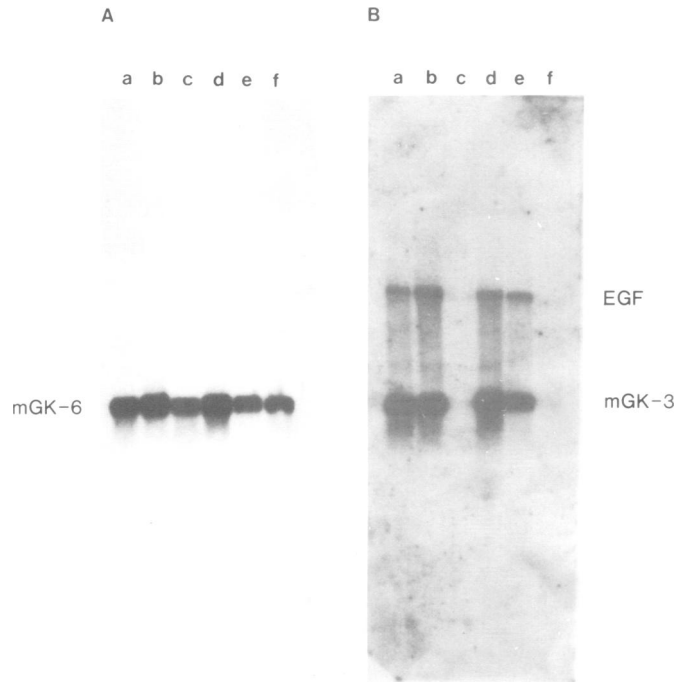

Fig. 2. Northern blot analysis of hormone induction of gene expression. Polyadenylated RNA (10 μg) from male **(lanes a** and **d)**, 7-day testosterone-treated females **(lane b)**, control female **(lanes c** and **f)** and thyroxine-treated female **(lane e)** mice were electrophoresed on 1.5% agarose gels, transferred to nitrocellulose and probed with either the mGK-6-specific 30-mer probe **(A)** or a mixture of the mGK-3-specific 30-mer and the preproEGF-specific probe **(B).**

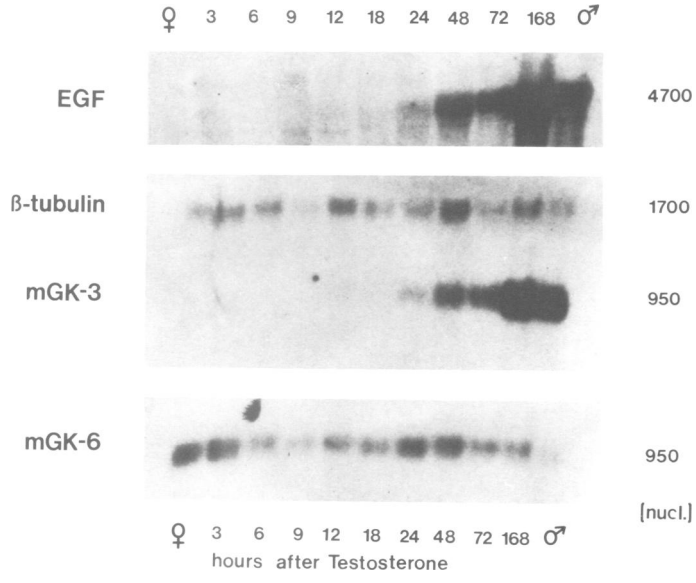

Fig. 3. Northern blot analysis of time course of testosterone induction of gene expression. A Northern blot of salivary gland RNA after hybridization to β-tubulin and the mGK-3 30-mer (18-h exposure shown). The blot was then washed at 70°C for 30 min in 2 × SSC to remove these probes and then hybridized to the pro-EGF 30-mer (18-h exposure shown) and the mGK-6 30-mer (1-h exposure shown). Polyadenylated RNA loaded was from untreated females (♀), testosterone-treated females (3, 6, 9, 12, 18, 24, 48, 72 and 168 h after testosterone treatment), and male (♂) mice. The size of the mRNA transcripts (nucleotides) is shown on the right of the figure.

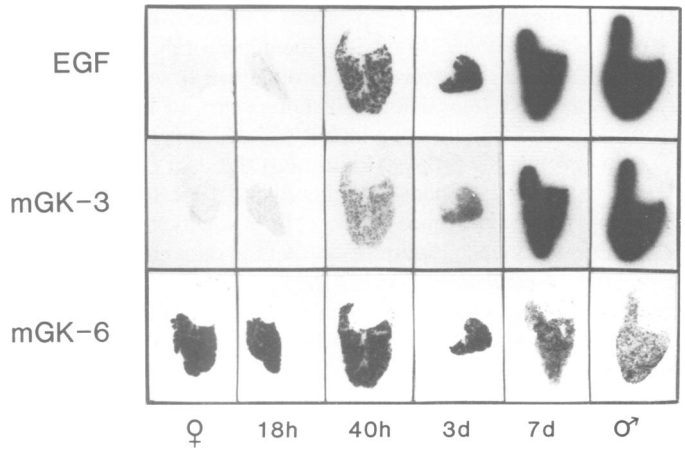

Fig. 4. *In situ* hybridization histochemistry over the time course of testosterone induction. Serial salivary gland sections from untreated female (♀), testosterone-treated females (18 h, 40 h, 3 days and 7 days after testosterone treatment), and male (♂) mice, hybridized to the 30-mer oligodeoxyribonucleotide probes for proEGF, mGK-3 and mGK-6. The sections from male and 7-day testosterone-treated female mice were overexposed in order to visualize hybridization at the early time points of induction.

Serial sections of salivary gland were also hybridized to these 30-mer probes, using *in situ* hybridization histochemistry, to determine whether this induction of expression was a general effect in all cells of the salivary gland or restricted to a limited cell population. Figures 4 and 5 show that the induction of expression appears to occur uniformly over the whole submandibular gland with no detectable expression of the inducible genes in the sublingual or parotid glands. In contrast the level of hybridization to the mGK-6-specific probe decreases slightly over the time course. The apparent discrepancy with dot-blot (Figure 1) and Northern analysis (Figures 2 and 3) may be due to the testosterone induced increase in gland weight (~20%; Chretien, 1977). This increase is largely due to storage granules (i.e. non-cellular material) and therefore the 5-μm section contains a correspondingly smaller percentage of cellular material. The changes seen using hybridization histochemistry are only semi-quantitative as the amount of hybridization depends on the thickness of the section as well as the number and distribution of the different cell types represented in the section (Coghlan *et al.*, 1985).

Figure 5 also shows that there is detectable expression of mGK-6 in the sublingual and parotid glands although at a lower total level than in the submandibular gland. In contrast, sections probed with the mGK-3-specific probe show expression confined to the submandibular gland.

Differentiation of duct cells

The effect of testosterone and thyroxine to induce differentiation of the striated duct cells in the female salivary gland and the effect of testosterone withdrawal (by castration) to reverse it in the male has been well documented (Chretien, 1977). Essentially these hormones catalyze the transition between two cellular phenotypes: uninduced (striated duct cells) and induced (granular convoluted tubule cells). There is no evidence of cell replication or DNA synthesis during this differentiation and so the alteration in the levels of various proteins seen in these cells must be a consequence of altered transcriptional activity rather than the selection and multiplication of a clonal cell population expressing these inducible proteins. The typical appearance of uninduced duct cells is of centrally located nuclei and very few granules; the mitochondria aligned along the basal membrane of the cells gives them their striated appearance. In contrast the taller and wider induced

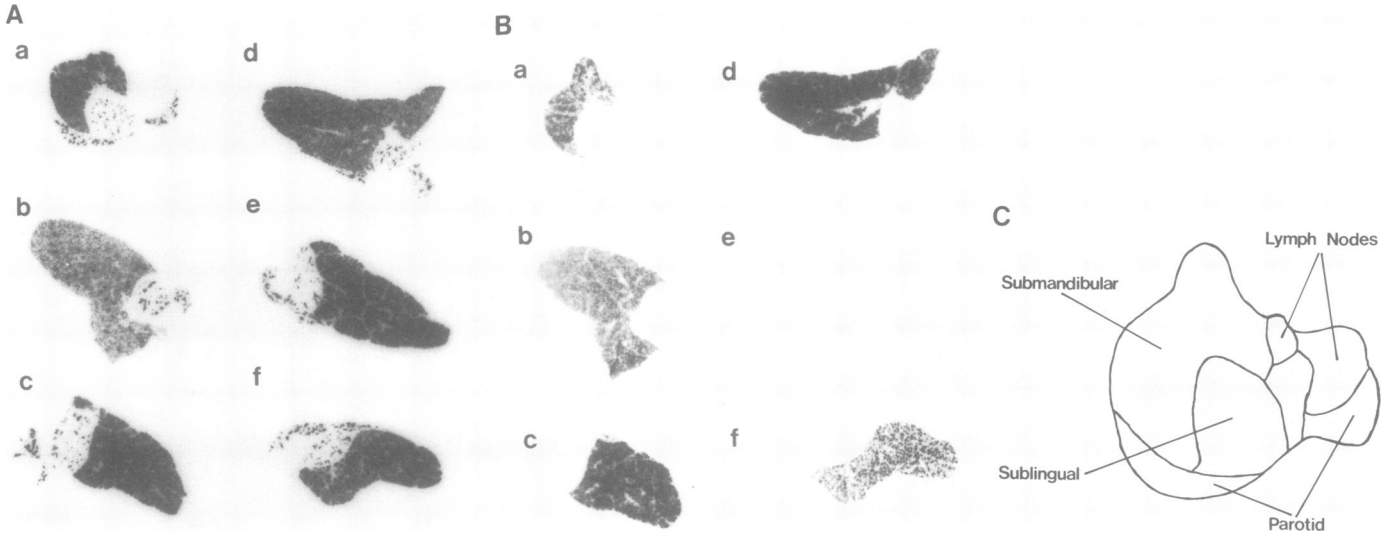

Fig. 5. *In situ* hybridization histochemistry of hormone-induced salivary glands. Serial salivary gland sections of testosterone-treated females (**a** and **d**), male (**b**), thyroxine-treated (**c** and **f**) and control female (**e**) mice probed with either the mGK-6-specific 30-mer **(A)** or the mGK-3-specific 30-mer **(B)**. The component glands from the testosterone-treated female section (a) are illustrated **(C)** in order to identify mGK-6 expression (and the lack of mGK-3 expression) in the sublingual and parotid glands.

cells contain many granules and their nuclei are basally located. The latter cells normally develop out of the distal convoluted portions of the striated ducts during the growth and development of the male mouse (Chretien, 1977).

The morphological changes in the submandibular gland at different time periods after testosterone treatment are shown in Figure 6. In the female (Figure 6a) the nuclei of the striated duct cells have a bead-like appearance around the lumen of the duct and a degree of cellular disorganization occurs 18 h after testosterone treatment (Figure 6b) which is still evident at 40 h (Figure 6c). However, after 3 days (Figure 6d) the duct cells have increased in size and the nuclei are more basally located. At 7 days (Figure 6e) the appearance of the cells is identical to the male submandibular gland (Figure 6f) in that the duct cells have now taken on the typical appearance of the larger granular convoluted tubule cells with the granules looking diffuse and pink in colour. The acinar cells now occupy much less of the total volume of the gland.

Cell-specific expression

Figure 7 shows liquid emulsion autoradiographs of salivary gland sections from male, female and testosterone-treated female mice after hybridization to the probes specific for the kallikrein genes mGK-3 and mGK-6. This figure shows that these two genes are expressed (albeit at different levels) in the same cell types: the granular convoluted tubule cells in the male and the striated duct cells in the female.

In the female the striated duct cells are evenly, but weakly labelled when the mGK-3 probe is used. The exposure time for the female section treated with the mGK-3 probe is ~10 times that of the other sections. The higher level of expression of mGK-3 in males and testosterone-treated females is manifest as an increase in the number of silver grains (mRNA molecules) per cell. The labelling of the granules is relatively evenly distributed over all granular convoluted tubule cells in the sections. Similar to mGK-3 in the female, of all of the striated duct cells label with the mGK-6 probe and the density of labelling is relatively consistent (but higher than mGK-3) over these cells. However, the pattern of localization of mGK-6 transcripts is different in the male and testosterone-treated female mice. The same morphological changes seen in the sections hybridized to the mGK-3 probe are evident, but now not all duct cells have labelled with the mGK-6 probe.

Two distinct alternatives could have given rise to this pattern of expression. Either a small population of striated duct cells has failed to undergo differentiation to granular convoluted tubule cells, or differentiation was complete and is accompanied by the inactivation of the mGK-6 gene in most but not all cells. The morphology of the cell types in the male and treated-female ducts appears to be consistent. However, the sublingual and parotid glands contain mGK-6 expressing duct cells which are refractory to hormone induction (Figure 8). In this figure it is evident that there are two types of duct present in the male and testosterone-induced female. Striated ducts containing only non-responsive cells are in the minority in the submandibular gland but comprise all secretory ducts seen in the sublingual and parotid (not shown) glands, while granular convoluted tubules comprise mainly responsive cells and are more predominant in the submandibular gland. This suggests that the striated duct cells of the female mouse salivary gland comprise a mixture of hormone responsive and non-responsive cell types. Cells that respond to testosterone or thyroxine undergo differentiation which is accompanied by induction of responsive genes (such as EGF, NGF and certain kallikreins, e.g. mGK-3 and mGK-4) and inactivation of the kallikrein gene mGK-6. Cells that do not undergo hormone-induced differentiation continue to express mGK-6 and may in fact increase the level of expression of this gene since the total glandular level of mGK-6 expression does not appear to alter as dramatically as the decrease in the number of cells expressing this gene.

Discussion

Administration of testosterone or thyroxine to female mice causes an increase in salivary gland levels of biologically active proteins, including members of the glandular kallikrein family (Barka, 1980). We have previously demonstrated, by quantitative dot-blot analysis, that one kallikrein gene, mGK-6, does not exhibit

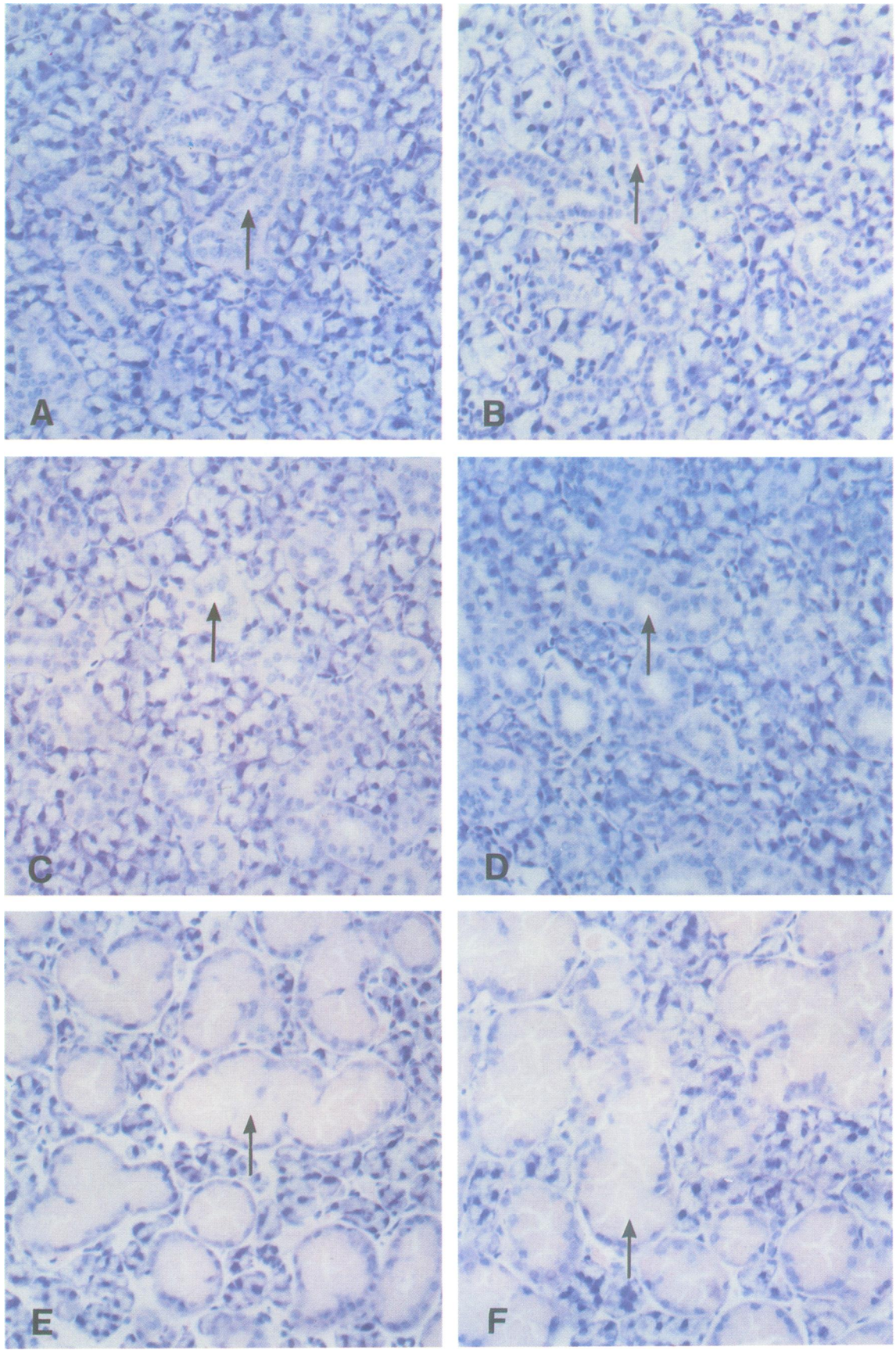

Fig. 6. Morphological changes induced by testosterone. Four-micron paraffin sections showing morphology of submandibular gland from **(A)** untreated female, **(B–E)** females treated with testosterone for 18, 40, 72 (3 days) and 168 h (7 days) respectively, and **(F)** male mice. The lumen of a single duct in each field is arrowed. Magnification ×200.

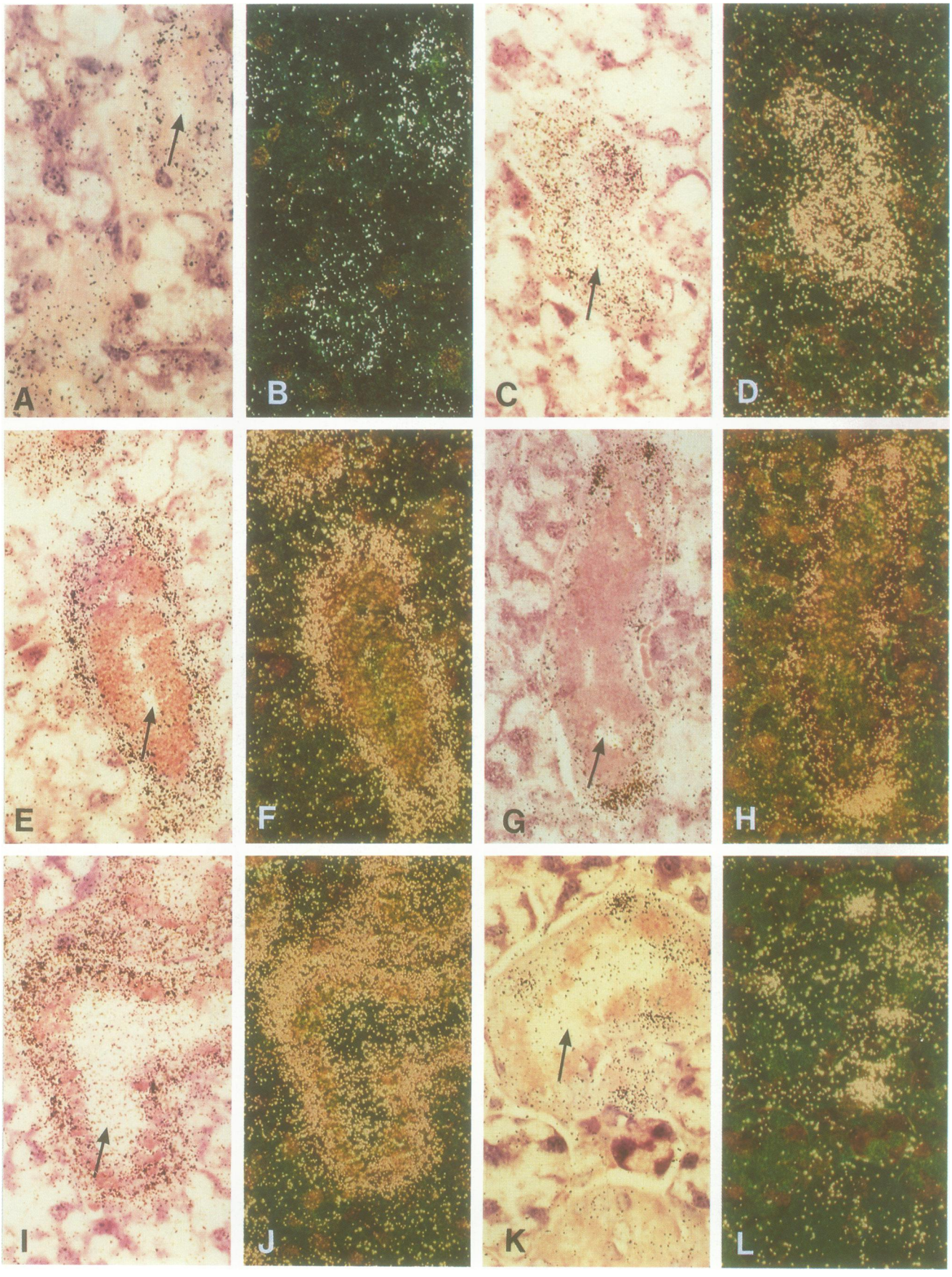

Fig. 7. Cellular location of kallikrein gene expression. Autoradiographs of 5-μm sections from mouse submandibular glands hybridized with ^{32}P-labelled oligodeoxyribonucleotide probes for mGK-3 **(A, B; E, F; I, J)** or mGK-6 **(C, D: G, H: K, L)**. Paired photographs by bright-field and dark-field microscopy of a single field are from female **(A, B, C, D)**, 7-day testosterone-treated female **(E, F, G, H)** and male **(I, J, K, L)**. The lumen of striated ducts in the female and granular convoluted tubules in the treated female and male sections is arrowed. Magnification ×600.

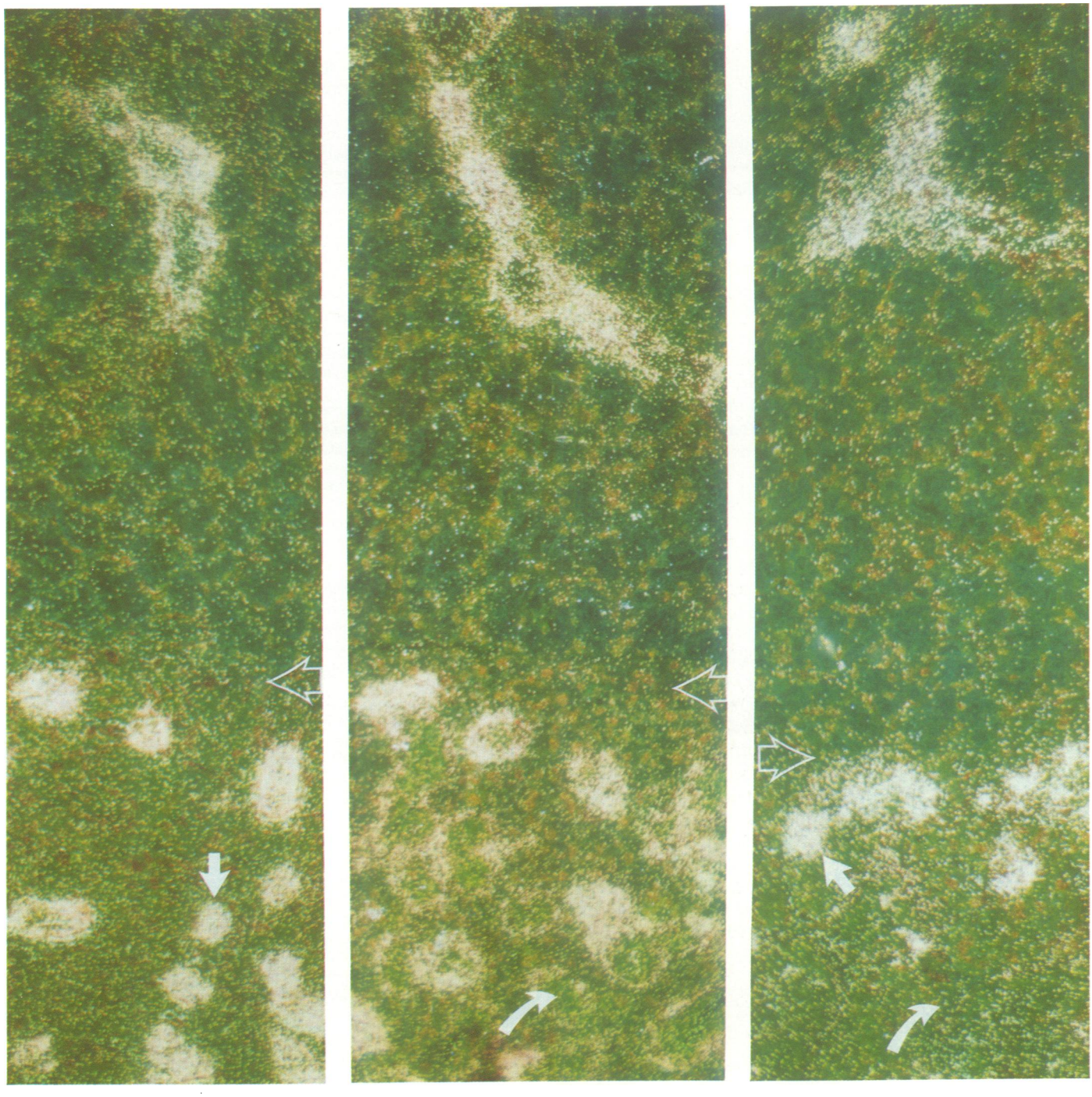

Fig. 8. Expression of the mGK-6 gene in the duct cells of the submandibular and sublingual glands. Autoradiographs of 5-μm sections from mouse salivary glands hybridized with a ^{32}P-labelled oligodeoxyribonucleotide probe specific for mGK-6. The sections show a portion of both sublingual (upper) and submandibular (lower) glands, delineated by open arrows. Striated ducts and granular convoluted tubules are indicated by solid, straight and curved arrows respectively. Dark-field photomicrographs show a single field of (left panel) female, (centre panel) 7-day testosterone-treated female and (right panel) male. Magnification × 250.

sexual dimorphism of expression. We have used the hormone induction regimen in order to study the difference in regulation of the closely linked and highly homologous genes mGK-3, 4, 5 and 6. Differences in expression of these genes could have been due to a number of possible factors including differences in the sites of expression or hormone-responsiveness of gene transcription. The use of *in situ* hybridization histochemistry showed that all of these genes are expressed (albeit at different levels) in the same cell type in the female but that the hormone-dependent differentiation of some, but not all of these cells affects the pattern of expression of these genes.

Testosterone induction of gene expression

In order to ascertain whether the hormones were exerting their effect directly on the responsive genes via receptor−DNA interactions (Karin *et al.*, 1984) we first analysed the time course of induction. Primary induction is usually rapid (8−18 h) while both testosterone and thyroxine induction was only maximal after

7 days and was associated with a change in morphology of the cells expressing the responsive genes. This change in morphology involved differentiation of the cells rather than selective proliferation of a particular cell type. In addition the induction kinetics of the responsive genes, including two non-kallikrein genes, appeared to be identical. Taken together these results imply that these hormones are not necessarily acting directly on the responsive genes and that the induction may involve a fairly general increase in transcription from a quiescent (SD) to an active (GCT) state.

Expression of mGK-6

The major sites of expression of mGK-6 are the kidney, pancreas and both male and female salivary glands, whereas the major site of other kallikrein gene expression is the male salivary gland (van Leeuwen *et al.*, 1986). In addition, we have shown here that mGK-6 expression is not induced in female mice by administration of testosterone or thyroxine as are the other genes. In fact in certain cells, in which expression of many kallikrein genes is induced, the expression of mGK-6 is reduced. These results suggest that a molecular mechanism exists to regulate the expression of mGK-6 in the salivary gland in a different manner to that of the other members of this multigene family.

The differential response of the kallikrein genes to hormones may reflect their diverse functions: namely, kinin generation by mGK-6 and biosynthesis of growth factors by mGK-3 and 4 (as discussed in van Leeuwen *et al.*, 1986). Recent experiments by Tsutsumi *et al.* (1986) indicate a permissive role for salivary gland EGF in spermiogenesis. The regulation of EGF biosynthesis (pro-EGF and EGF-binding proteins) by testosterone suggests that EGF may mediate some of the stimulatory effects of this steroid on spermiogenesis. The sexual dimorphism of expression of a large variety of substances in salivary glands (including other kallikrein-like proteins) may similarly contribute to further anatomical and physiological differences between the sexes.

Through analysis of the cellular distribution of expression of kallikrein genes in the mouse salivary glands we have observed a previously undescribed complexity which can largely account for the differential response of these genes to testosterone induction. It will be important to establish the generality of this phenomenon since models for the control of gene expression are based on studies in clonal cell lines and may therefore not reflect the diversity which is apparent *in vivo*.

Materials and methods

RNA preparation

Salivary glands were surgically removed from Balb/c mice and homogenized in 5 M guanidinium thiocyanate. RNA was extracted by centrifugation through a CsCl cushion (Chirgwin *et al.*, 1979) and polyadenylated RNA was isolated by passage of total RNA over an oligo(dT)−cellulose column (Aviv and Leder, 1972).

Oligodeoxyribonucleotide probes

In order to measure total kallikrein mRNA (from all family members) an oligodeoxyribonucleotide complementary to a highly conserved part of kallikrein genes (from nucleotide number 1556 to 1582 of mGK-6) was chemically synthesized as described by van Leeuwen *et al.* (1986). This ubiquitous 27-mer hybridizes to 24 of the 25 members of the mouse kallikrein gene family (C.C.Drinkwater, B.A.Evans and R.I.Richards, unpublished results). Gene-specific 30-mers for members of the kallikrein gene family were synthesized and tested as previously described (van Leeuwen *et al.*, 1986). Specific 30-mers complementary to nucleotides 264−293 of mouse NGF (Scott *et al.*, 1983a) and nucleotides 399−428 of mouse EGF (Scott *et al.*, 1983b) were used to detect the respective mRNA sequences (NGF ~1300, EGF ~4700 bases). The oligodeoxyribonucleotides were radioactively labelled to a specific activity of $\sim 10^8$ c.p.m./μg with [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham) and T_4 polynucleotide kinase (Amersham) as previously described (van Leeuwen *et al.*, 1986).

Dot-blot analysis

Polyadenylated RNA was denatured with formaldehyde for 10 min at 60°C by a modification of the method of White and Bancroft (1982) as previously described (van Leeuwen *et al.*, 1986). Serial dilutions in 15 × SSC (1 × SSC is 0.15 M NaCl, 0.15 M Na citrate) were dotted onto nitrocellulose using a Schleicher and Schuell Minifold apparatus.

Northern blot analysis

Polyadenylated salivary gland RNA (10 μg) from mice killed 3, 6, 9, 12, 18, 24, 48, 72 and 144 h after testosterone treatment or after 7 days thyroxine treatment was loaded onto 1% agarose gel in 2.2 M formaldehyde. After electrophoresis the gel was blotted onto nitrocellulose filters by the method of Thomas (1980).

Filter hybridization

After baking at 80°C for 2 h under vacuum, the nitrocellulose filters were prehybridized for 3 h at 42°C in buffer containing 5 × SSC, 50 mM sodium phosphate, pH 6.5, 0.02% bovine serum albumin, 0.02% Ficoll, 0.05% polyvinylpyrrolidone, 30 μg/ml denatured calf thymus DNA, 50% formamide. Hybridization to the labelled 30-mer oligodeoxyribonucleotides was performed for 48 h at 4°C, blots were washed in 2 × SSC at room temperature (2 × 15 min) and then at 60°C (30 min). Filters were exposed for autoradiography at −80°C with intensifying screens.

To enable more accurate comparison of RNA loaded onto the dot blot from different groups of animals, an identical blot was hybridized to a thymidine 30-mer [oligo(dT)] probe. Because of the lower hybridization affinity of the oligo(dT) 30-mer, this hybridization was allowed to proceed for at least 72 h at 4°C, and the filters washed at room temperature only.

β-tubulin cDNA was used as an internal standard on the Northern blot and was labelled by a modification of the random-primed method (van Leeuwen *et al.*, 1986).

Hormone treatment

For testosterone treatment adult female Balb/c mice were injected s.c. with 0.1 ml testosterone enanthate (Primoteston Depot, Schering P/L), 5 mg/ml in sesame oil. Mice (n = 10) were given one injection 7 days prior to sacrifice and the salivary gland RNA prepared for dot blots. For the time course of testosterone induction, mice were injected 3, 6, 9, 12, 18, 24, 48, 72 (3 days) and 168 h (7 days) prior to sacrifice.

For thyroxine treatment adult female Balb/c mice (n = 6) were injected daily i.p. with 50 μl thyroxine (Oroxine, Wellcome Australia Ltd) 0.2 mg/ml in normal saline for a period of 7 days.

Groups of untreated female and male mice were killed at the same time as the hormone-treated mice and their salivary glands processed identically. As the thyroxine-treated experiment was performed at a later date a new set of untreated mice was included for this group as control.

In situ hybridization histochemistry

Mouse salivary glands were excised, frozen in liquid propane and freeze-dried at −45°C and 5 × 10^{-2} Torr for 72 h. Tissues were warmed to 20°C, removed from vacuum, fixed in paraformaldehyde vapour at 37°C for 2 h, vacuum infiltrated with paraplast (Lancer, St Louis) for 30 min at 60°C and embedded in paraplast. Sections cut at 5 μm were floated onto submerged slides (coated with 1% gelatine hardened with 0.25% formaldehyde), allowed to dry at 40°C and stored at −20°C. After dewaxing and three rinses in absolute ethanol, hybridization and subsequent procedures were as described by Coghlan *et al.* (1985).

Tissue morphology

Mouse salivary glands were fixed at room temperature in 4% formaldehyde in 0.1 M phosphate, pH 7.2, with 9 g/l NaCl. Tissues were dehydrated and embedded in paraffin by conventional procedures. Four-micron sections were stained with hematoxylin and eosin.

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