Pergamon

0960-0760(94)00172-3

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 3, pp. 239–249, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-0760/95 $9.50 + 0.00

Androgen Control of Secretory Component mRNA Levels in the Rat Lacrimal Gland

Jianping Gao,[1,2] Ross W. Lambert,[1,2] L. Alexandra Wickham,[1,2] George Banting[3] and David A. Sullivan[1,2]*

[1]Schepens Eye Research Institute, Boston, MA, [2]Department of Ophthalmology, Harvard Medical School, Boston, MA and [3]Department of Biochemistry, University of Bristol, Bristol, England

The purpose of this investigation was to determine whether the known gender-related differences in, and the endocrine control of, the production of secretory component (SC) by the rat lacrimal gland are associated with alterations in SC mRNA content. Levels of SC mRNA were measured in lacrimal tissues of intact, sham-operated, castrated, hypophysectomized, and testosterone-treated male and female adult rats by Northern blot procedures, which utilized a specific, [alpha-^{32}P]-labelled rat SC cDNA probe. For control purposes, SC mRNA amounts were standardized to the β-actin content in experimental blots. The location of SC mRNA in lacrimal glands was evaluated by *in situ* hybridization techniques, which involved exposure of tissue sections to sense or anti-sense [^{35}S]-labelled SC RNA probes. Our results demonstrate that: (1) lacrimal glands of male rats contain a significantly greater amount of SC mRNA than those of female rats, and that this difference co-exists with distinct, gender-associated variations in the distribution of SC mRNA in lacrimal tissue; (2) orchiectomy or hypophysectomy, but not ovariectomy or sham surgery, leads to a marked decline in the lacrimal SC mRNA content; and (3) testosterone, but not placebo, administration to castrated male and female rats induces a significant increase in the SC mRNA levels in lacrimal tissue. Overall, these findings show that gender, androgens and the hypothalamic–pituitary axis exert a considerable influence on the SC mRNA content in the rat lacrimal gland.

J. Steroid Biochem. Molec. Biol., Vol. 52, No. 3, pp. 239–249, 1995

INTRODUCTION

The functional activity of the secretory immune system of the eye is extremely dependent upon secretory component (SC) [1], the polymeric IgA receptor [2]. This glycoprotein, which is synthesized by acinar and ductal epithelial cells of the lacrimal gland, regulates the transfer of polymeric IgA antibodies from lacrimal tissue to the ocular surface, whereupon IgA protects the cornea and conjunctiva against viral, bacterial, parasitic and toxic agents [1]. Thus, SC plays an essential role in the eye's defense against inflammatory and infectious disease, and thereby helps to preserve anterior segment integrity and maintain visual acuity [1].

Given this critical role of SC in ocular secretory immunity, our research has endeavored to clarify the regulatory processes involved in the production of this lacrimal gland protein. These studies have demonstrated that the synthesis and secretion of SC by rat lacrimal tissue display distinct, gender-related differences, which appear to be due primarily to the influence of androgens [3–6]. Thus, from puberty to senescence, the lacrimal gland output of SC, as well as the corresponding tear SC levels, are significantly greater in males than in females [5]. This sexual dimorphism in ocular SC expression may be erased by orchiectomy, and restored by testosterone administration to castrated rats [3]. In contrast, neither ovariectomy [3], nor treatment with estrogens, progestins, glucocorticoids or mineralocorticoids [3, 6], alters the production of SC by lacrimal tissue. Recently, research has shown that the androgen-induced stimulation of lacrimal gland SC synthesis, which may be elicited by a variety of androgen analogues [7], is mediated through a direct action on acinar epithelial cells [6], and does not seem to require the intracellular conversion of testosterone to dihydrotestosterone (DHT) [6]. Moreover, this androgen modulation of lacrimal SC levels is both time- and dose-dependent [3, 6, 8], and associated with a significant rise in the transport of IgA

*Correspondence to D. A. Sullivan, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114, U.S.A.
Received 19 Aug. 1994; accepted 21 Oct. 1994.

antibodies from lacrimal tissue to tears against an apparent concentration gradient [8]. Of interest, the androgen-related accumulation of SC in tears may be dramatically suppressed by interruption of the hypothalamic–pituitary axis [8, 9].

At present, the cell biological mechanisms underlying the androgen control of SC production by the lacrimal gland remain to be elucidated. Hypothetically, androgen action may be mediated through steroid hormone binding to selective, nuclear receptors in acinar epithelial cells, adherence of these androgen/receptor complexes to genomic acceptor sites and the induction of SC mRNA transcription and eventual translation. In support of this hypothesis, high-affinity and androgen-specific receptors, which associate with DNA, have been identified in lacrimal tissue [10, 11], occur almost entirely within epithelial cell nuclei [12], and are up-regulated by androgen treatment [12]. Furthermore, the androgen enhancement of SC synthesis by lacrimal gland acinar cells may be inhibited by exposure to androgen receptor (i.e. cyproterone acetate), transcription (i.e. actinomycin D) or translation (i.e. cycloheximide) antagonists [4, 13].

The objective of the present investigation was to begin to identify the molecular biological mechanisms involved in the endocrine regulation of lacrimal gland SC. Accordingly, we sought to determine whether the gender-associated differences in, and the androgen control of, acinar cell SC synthesis are mediated through alterations in SC gene expression. In addition, given the impact of the hypothalamic–pituitary axis on SC production [8, 9, 14], we examined whether this hypophyseal influence involves modulation of SC mRNA levels.

MATERIALS AND METHODS

Animals and surgical procedures

Young adult male and female Sprague–Dawley rats (6–8 weeks old) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in temperature-controlled rooms with light and dark intervals of 12 h duration. Orchiectomies, ovariectomies, hypophysectomies and sham-operative procedures were performed by surgeons at Zivic-Miller Laboratories on 6-week-old animals. Rats were permitted to recover for at least 1 week after surgery before experimental treatment. To offset the electrolyte imbalance in rats with pituitary ablation, animals were given a solution containing sodium chloride (2.03 g/l), potassium chloride (0.083 g/l), magnesium chloride (0.017 g/l) and calcium chloride (0.035 g/l) [15]. To verify the success of hypophysectomy, sera were collected from operated rats at the time of sacrifice and thyroxine levels were measured with an RIA kit from ICN Biomedicals, Inc. (Carson, CA). Analysis showed that thyroxine concentrations were within the physiological range in sham-operated animals, but almost completely undetectable in hypophysectomized rats.

Molecular biological procedures

After animal sacrifice by carbon dioxide inhalation, lacrimal glands were removed and immediately processed for various molecular biological procedures. When indicated, the liver, spleen and prostate were also obtained from sacrificed rats, and either fixed for histology (see below) or frozen in liquid nitrogen until experimental use.

For the determination of SC mRNA levels in experimental samples by Northern blots, total cellular RNA was isolated from rat lacrimal, liver, splenic and prostatic tissues by utilizing an acid guanidinium–thiocyanate–phenol–chloroform extraction method [16] and poly(A)$^+$RNA was purified from total RNA by using the Micro-Fast Track mRNA Isolation Kit (Invitrogen Corporation, San Diego, CA). The RNA preparations were quantitated by spectrophotometry at 260 nm, fractionated (3–25 μg) on 1.2% agarose gels containing 6.6% formaldehyde, transferred to Immobilon-N (Millipore, Bedford, MA) or Gene-Screen (Dupont/NEN, Boston, MA) membranes by positive pressure with a Posi-Blot (Stratagene, La Jolla, CA), and then fixed by UV crosslinking for 5 min. For comparative purposes, all gels contained an RNA molecular weight ladder (Gibco, Gaithersburg, MD). To permit identification of SC mRNA in blots, a 5′ ~ 950 base pair *Bam*H 1 fragment of the rat SC cDNA [17] was radiolabeled (specific activity $\geqslant 5 \times 10^8$ cpm/μg DNA) with [α^{32}P]dCTP (3000 Ci/mmol; ICN, Irvine, CA) by random priming with an NEN/Dupont Random Primer Extension Kit. This fragment included most of the SC coding region and was obtained from the full length SC cDNA (3083 base pairs, subcloned in the pGEM4 plasmid *Bam*H I site) by excision with the *Bam*H I restriction enzyme and by purification from agarose gels with a GENECLEAN Kit (BIO 101, Inc., La Jolla, CA). Membranes were incubated with the ^{32}P-labelled SC cDNA probe (concentration equalled $1–2 \times 10^6$ cpm/ml) at 65°C for 18–20 h in a hybridization solution containing 5 × SSC (1 × = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution (1 × = 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, and 100 μg/ml salmon sperm DNA. Following hybridization, blots were washed sequentially with 2 × SSC–0.1% SDS and 0.2 × SSC–0.1% SDS for varying times at room temperature and at 65°C. Membranes were then processed for autoradiography (XAR X-ray film [Kodak, Rochester, NY]; conditions: −70°C with one intensifying screen for varying time periods) and analyzed by densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA). To standardize the results, and to confirm that similar amounts of total RNA were analyzed in the various groups, experimental blots were

rehybridized with a ^{32}P-labelled mouse β-actin cDNA probe (cDNA kindly provided by Dr Lan Hu, Dana-Farber Cancer Institute, Boston, MA); this procedure involved an initial stripping of the membrane in 0.1 N sodium hydroxide followed by washing in 0.1 × SSC, 0.5% SDS, 0.2 M Tris (pH 7.4) at 95°C. Statistical analysis of the data was performed with Student's unpaired, two-tailed *t*-test.

To evaluate the distribution of SC mRNA in lacrimal, hepatic and splenic tissues, lacrimal glands were processed for *in situ* hybridization, by using modifications of reported procedures [18, 19]. In brief, tissues were immediately fixed in 4% paraformaldehyde, exposed to varying ethanol and xylene solutions, embedded in paraffin and cut into 6 μm sections, which were placed on slides pretreated with 2% 3-amino propyltriethoxysilane in acetone. Sections were deparaffinized in xylene, rehydrated in a descending ethanol series, refixed in 4% paraformaldehyde, subjected to proteinase K digestion (1 μg/ml at 37°C), acetylated (0.5% acetic anhydride in 0.1 M triethanolamine), immersed in 2 × SSC, and incubated with anti-sense or sense ^{35}S-labelled SC mRNA probes. Labeled riboprobes were prepared by utilizing the linearized (with *Hind*III or *Ava*I) pGEM4 plasmid containing the SC cDNA, [^{35}S]UTP (1350 Ci/mmol, NEN), a riboprobe transcription kit from Promega (Madison, WI), and either SP6 (anti-sense) or T7 (sense; control) RNA polymerases (Promega), followed by treatment with RNase-free DNase I and purification by phenol–chloroform extraction and ethanol precipitation. Radiolabeled probes were denatured by heating at 100°C for 10 min, chilled on ice, mixed in hybridization buffer ($\sim$4.5 × 10^7 cpm/ml, in solution containing 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 5 mM EDTA, 1 × Denhardt's solution, 10 mM DTT, 25 μg yeast tRNA/ml, 0.2 mM UTP/ml and 0.0083 N HCl in 10 mM Tris–HCl, pH 7.6) and applied (30 μl) to sections, which were then coverslipped and incubated for 16–18 h at 37°C. Following hybridization, sections were washed in a series of buffers (50% formamide-2 × SSC-10 mM DTT;

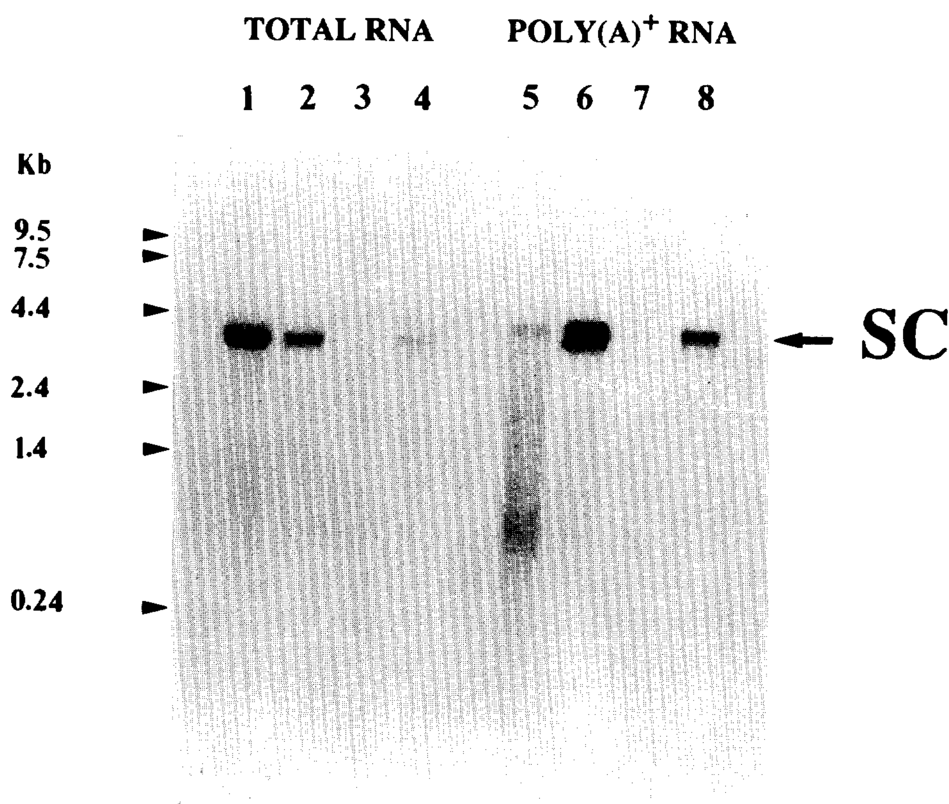

Fig. 1. Analysis and molecular weight characteristics of SC mRNA from the rat lacrimal gland, liver and prostate. Total cellular RNA (25 μg/lane), as well as purified poly (A)$^+$RNA (3 μg/lane), from rat hepatic (lanes 1, 5), lacrimal (lanes 2, 6), splenic (lanes 3, 7; negative control) and prostatic (lanes 4, 8) tissues were resolved on Northern blots, then hybridized to a [^{32}P]-labelled SC cDNA probe, as detailed in the Materials and Methods. The size ($\sim$3.9 kb) of the single SC mRNA band was determined by comparison to known RNA molecular weight standards, which were electrophoresed in an adjacent lane. This hybridization pattern for lacrimal gland SC mRNA was also observed if the radiolabelled SC cDNA probe was replaced with a [^{35}S]-labelled, anti-sense SC riboprobe; no such binding occurred, though, if sense SC riboprobes were utilized.

$4 \times$ SSC-TE [1 mM EDTA, 10 mM Tris–HCl, pH 7.4]) at 37°C, exposed to RNase A (20 μg/ml in $4 \times$ SSC-TE at 37°C for 30 min), rinsed ($4 \times$ SSC-TE at 37°C; 50% formamide-$2 \times$ SSC-10 mM DTT at 65°C), dehydrated, dried at 37°C overnight, dipped in NTB-2 Kodak emulsion (1:1) and exposed for 10–14 days in a tightly sealed, black box at 4°C. After this period, sections were developed with D-19 Kodak developer (1:1) at 19°C, stained with hematoxylin and mounted with permount. The distribution and labeling intensity of grains were then compared between anti-sense and sense exposed sections. Photographs of these sections were obtained by using a Nikon Microflex UFX-II light microscope (original $125 \times$ magnification), equipped with a Nikon FX-35 WA camera and Kodak TMAX 400 film.

Hormone preparations

Placebo (cholesterol, methyl cellulose and lactose; 50 mg)- and testosterone (50 mg)-containing pellets were obtained from Innovative Research of America (Toledo, OH) and subcutaneously implanted in the subscapular region of castrated or hypophysectomized and orchiectomized rats. This method of hormone treatment ensures a continuous exposure to testosterone, and results in the generation of physiological serum testosterone levels (i.e. for an adult male rat).

RESULTS

Evaluation of the methodological approach for the analysis of SC mRNA by Northern blots and in situ hybridization

Prior to assessing the impact of gender and androgen treatment on SC mRNA expression in the lacrimal gland, it was first necessary to optimize, as well as to verify the specificity of, various molecular biological procedures. These methodological studies included the: (1) determination of whether the [^{32}P]-labelled SC cDNA probe binds selectively to a ~3.9 kb species, which is the approximate size of rat SC mRNA [17, 20], in both total RNA and purified poly(A)$^+$RNA preparations from lacrimal tissue; (2) examination of whether the binding pattern of the SC cDNA probe to lacrimal RNA samples is analogous to that found in RNA isolates from SC protein-containing positive (i.e. liver and prostate) or negative (i.e. spleen) tissues; and (3) evaluation of the adherence of sense and anti-sense [^{35}S]-labelled SC RNA probes to sections of rat liver (positive control), as compared to those of spleen (negative control).

Northern blot results showed that the [^{32}P]-labelled SC cDNA probe bound to a single, ~3.9 kb molecular species in both total and purified poly(A)$^+$RNA preparations from the lacrimal gland, liver and prostate, whereas this SC mRNA band was undetectable in splenic samples (Fig. 1). Moreover, studies

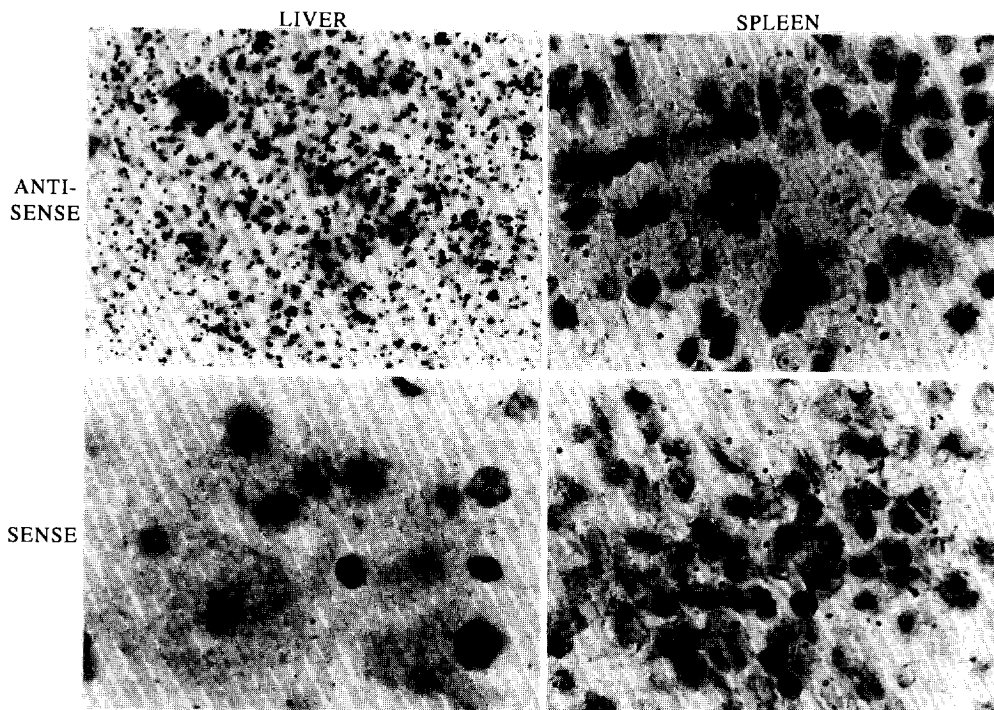

Fig. 2. Examination of the affinity of radiolabelled SC riboprobes to rat hepatic and splenic tissue sections. The liver (positive control) and spleen (negative control) were obtained from a young adult male rat, then processed for *in situ* hybridization procedures. Tissue sections (6 μm) were exposed to anti-sense and sense [^{35}S]-labelled SC RNA probes, stained with hematoxylin and photographed, as described in the Materials and Methods.

demonstrated that replacement of the SC cDNA probe with a [^{35}S]-labelled, anti-sense SC riboprobe resulted in an identical SC mRNA binding profile in Northern blots; in contrast, hybridization with a radiolabelled, sense riboprobe yielded no autoradiographic signals (data not shown). Given these findings, all further Northern blot analyses of lacrimal gland SC mRNA levels were conducted by using total RNA isolates and [^{32}P]-labelled SC cDNA probes. As concerns *in situ* hybridization patterns, the anti-sense, but not sense, [^{35}S]-labelled SC RNA probe bound extensively to SC mRNA in rat liver sections (Fig. 2). However, neither the anti-sense, nor the sense, [^{35}S]-labelled SC riboprobes showed any demonstrable hybridization affinity for the spleen, which served as the negative control tissue (Fig. 2). Overall, these results confirmed the specificity of the SC cDNA and RNA probes for SC mRNA.

Impact of gender on the content and distribution of SC mRNA in the rat lacrimal gland

To determine whether gender influences the amount and distribution of SC mRNA in the lacrimal gland, tissues were obtained from young adult male and female rats ($n = 5$/group) and processed for evaluation by Northern blot and *in situ* hybridization techniques.

As shown in Figs 3 and 4, results demonstrated that gender exerts a profound impact on SC mRNA expression in the lacrimal gland. Thus, SC mRNA levels in lacrimal tissues of males were significantly ($P < 0.005$) greater than those of females (Fig. 3). When these values were standardized to β-actin content, the SC mRNA/β-actin mRNA ratios in male lacrimal glands (1.303 ± 0.078) were 3.7-fold higher ($P < 0.0005$) than corresponding ratios in female tissues (0.353 ± 0.079). These findings could not be explained by possible gender-related variations in total RNA or β-actin mRNA amounts, given that no significant differences in these variables existed between glands of male and female rats.

The effect of gender on SC mRNA levels was also clearly shown by *in situ* hybridization analysis of the cellular density and topographical location of message in lacrimal tissue sections. As demonstrated in Fig. 4, the frequency and distribution of the anti-sense, [^{35}S]-labelled SC riboprobe binding to SC mRNA was far more extensive in glands of males, as compared to those of females. This gender-associated difference in labelling intensity was not duplicated by replacement of the anti-sense probe with a sense riboprobe: under this condition, almost no binding occurred in lacrimal gland sections of male and female rats (Fig. 4).

Influence of castration on the level of SC mRNA in rat lacrimal tissue

To examine whether the gender-related differences in lacrimal gland SC mRNA content were possibly due to the effect of sex steroids, age-matched male and female rats ($n = 5$/treatment group) were subjected to either castration or sham-surgery, and lacrimal tissues were obtained 7 days later to permit measurement of SC mRNA levels by Northern blots.

As shown in Fig. 5, orchiectomy led to a striking ($P < 0.005$) decline in the absolute amount of SC mRNA, relative to that in lacrimal glands of sham-operated males. Moreover, this castration response was associated with a significant ($P < 0.005$), 5-fold drop in the SC mRNA/β-actin mRNA ratio (sham = 3.042 ± 0.522; orchiectomy = 0.549 ± 0.066) in lacrimal tissue. In contrast, ovariectomy exerted no significant effect on lacrimal SC mRNA content (SC mRNA/β-actin mRNA ratios: sham = 0.065 ± 0.026; ovariectomy = 0.190 ± 0.065) (Fig. 6). In these experiments, neither orchiectomy nor ovariectomy caused any alteration in total RNA or β-actin levels in lacrimal glands, as compared to amounts in tissues of sham-operated controls.

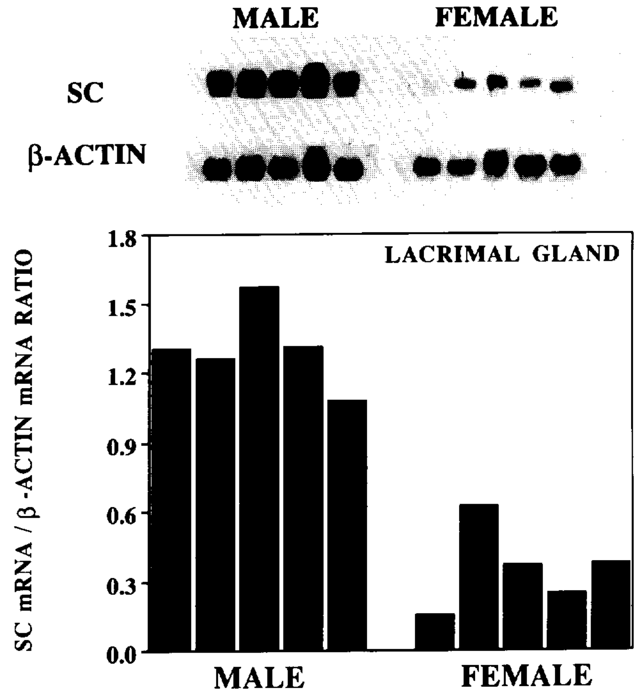

Fig. 3. Influence of gender on the level of SC mRNA in the rat lacrimal gland. Lacrimal tissues were obtained from young adult male and female rats ($n = 5$/gender), processed for the isolation of total RNA, and analyzed (25 μg/lane) for the content of SC mRNA and β-actin mRNA by the use of [^{32}P]-labelled cDNA probes and Northern blot techniques, as explained in the Materials and Methods. Autoradiographic signals were quantitated by densitometry, and the SC mRNA/β-actin mRNA ratios of each sample were then calculated. The absolute amount of SC mRNA (top; $P < 0.005$), as well as the mean SC mRNA/β-actin mRNA ratio (bottom; $P < 0.0005$), were significantly greater in lacrimal glands of males, as compared to those of females.

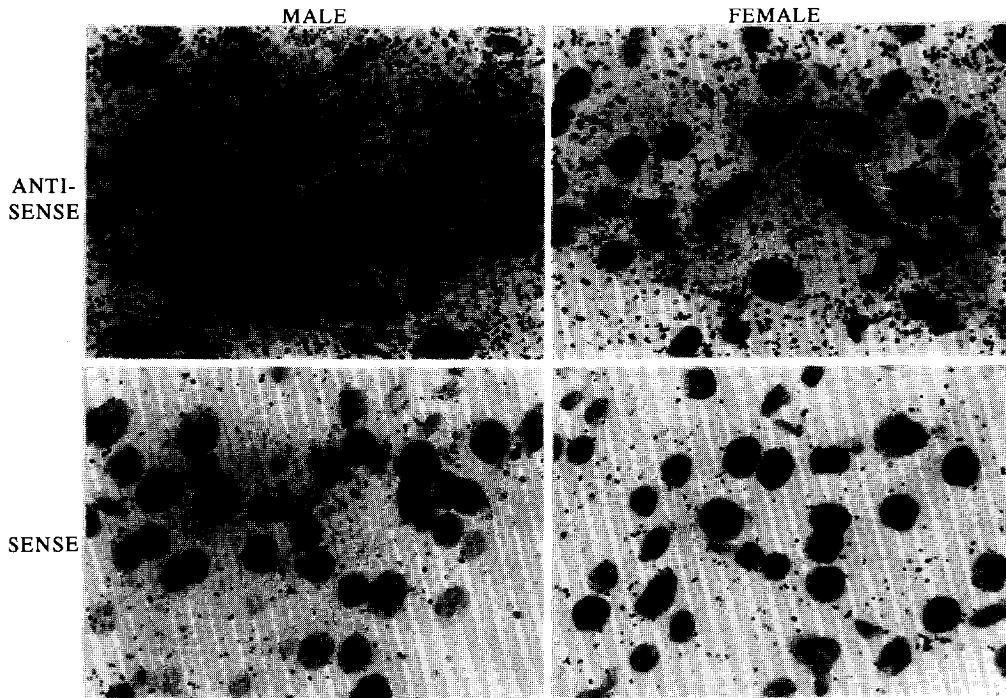

Fig. 4. Impact of gender on the density and distribution of SC mRNA in rat lacrimal tissue. Lacrimal glands from young adult male and female rats were cut into 6 μm sections, and processed for *in situ* hybridization with anti-sense and sense [^{35}S]-labelled SC RNA probes, as explained in the Materials and Methods.

Effect of testosterone treatment on the SC mRNA content in lacrimal glands of orchiectomized and ovariectomized rats

To determine whether the orchiectomy-induced decrease in SC mRNA content in lacrimal tissue might be attributable to the loss of androgens, castrated ale rats (n = 5/group) were treated with placebo compounds or physiological levels of testosterone for 7 days, and then lacrimal glands were processed for SC mRNA quantitation by Northern blots. For comparison, we also assessed the influence of androgen administration on SC mRNA amounts in lacrimal tissues of ovariectomized rats (n = 5/treatment group).

Results showed that testosterone exposure stimulated a significant ($P < 0.01$), 3–5-fold increase in the SC mRNA content in lacrimal glands of both castrated male and female rats, as compared to levels in tissues of placebo-treated controls (Figs 7 and 8). This androgen action was particularly evident by analysis of the corresponding rise ($P < 0.01$) in the SC mRNA/β-actin mRNA ratios in lacrimal samples (males: placebo = 0.148 ± 0.032; testosterone = 0.716 ± 0.050; females: placebo = 0.650 ± 0.130; testosterone = 1.376 ± 0.207) (Figs 7 and 8). In contrast, testosterone administration did not enhance the total RNA levels in lacrimal tissue, and had no effect on the amount of β-actin mRNA in the lacrimal gland.

Impact of hypophysectomy on the total RNA content and the SC mRNA levels in lacrimal glands of male, female and testosterone-treated, orchiectomized rats

Previous research has demonstrated that ablation of the anterior or total pituitary gland in male rats causes a profound decrease in the basal and androgen-induced synthesis and secretion of SC by lacrimal tissue [8, 9, 14]. To begin to explore the mechanism of this effect, we examined whether the hypophyseal control of lacrimal SC production involves modulation of SC mRNA, as well as total RNA, levels. Lacrimal glands were obtained from hypophysectomized, castrated and/or sham-operated male or female rats (n = 4–5/condition), either 16 days after surgery or following an additional 7 day exposure to placebo compounds or testosterone (50 mg implanted pellet). Lacrimal tissues were then processed for the determination of total RNA by spectrophotometry, and SC and β-actin mRNA amounts by Northern blots.

As shown in Table 1, hypophysectomy led to a striking, 60% reduction in the total RNA content in lacrimal glands of male and female rats, as compared to those of sham-operated controls. This response appears to have been due to the precipitous ($P < 0.0005$) decline in lacrimal gland weight following surgery, given that no significant difference existed between the total RNA/lacrimal gland weight ratios of control and hypophysectomized rats. With regard to SC mRNA, pituitary extirpation resulted in a further, significant

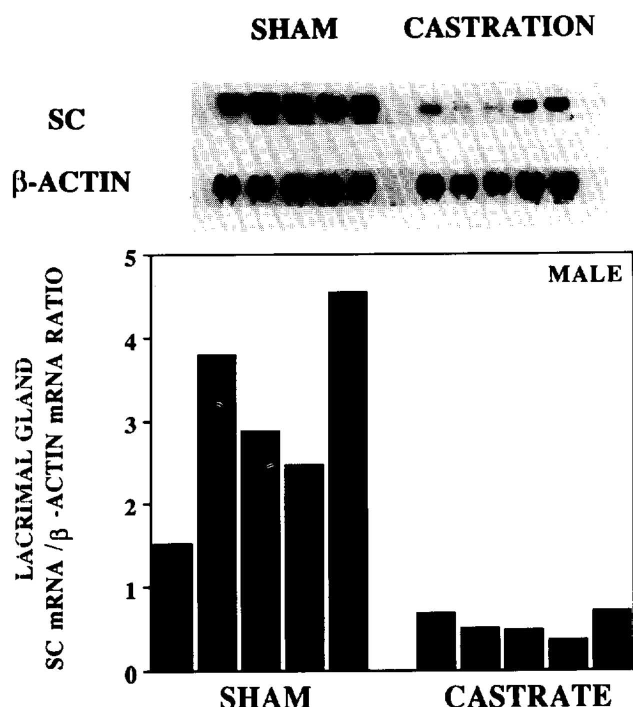

Fig. 5. Effect of orchiectomy on the content of SC mRNA in the rat lacrimal gland. Lacrimal tissues were collected from male rats ($n = 5$/group), 7 days after these animals had undergone either sham-surgery or castration. Glands were then processed for the preparation of total RNA samples, which (25 μg/lane) were examined for SC and β-actin mRNA expression by Northern blots, as outlined in the legend to Fig. 3. The SC mRNA levels (top), and the mean SC mRNA/β-actin mRNA ratios (bottom), were significantly ($P < 0.005$) decreased by orchiectomy, relative to amounts in sham-operated controls.

($P < 0.01$) diminution in the absolute levels of this message, but not that of β-actin, in male rats. Consequently, the SC mRNA/β-actin mRNA ratios were 2.5-fold less ($P < 0.01$) in lacrimal tissues of hypophysectomized, compared to control, male animals (Table 1). In contrast, pituitary removal did not appear to influence the relative amount (i.e. per 15 μg RNA sample) or SC or β-actin mRNA in lacrimal glands of female rats: the lacrimal SC mRNA/β-actin mRNA ratios were not significantly different between hypophysectomized and sham-operated animals (Table 1).

Of interest, interruption of the hypothalamic–pituitary axis did not seem to interfere with the androgen-related impact on lacrimal gland SC mRNA content. Thus, administration of testosterone for 7 days to hypophysectomized and castrated male rats ($n = 5$/treatment group) induced a significant ($P < 0.01$), 2-fold rise in the absolute level of SC mRNA in lacrimal tissue, as compared to that in glands of placebo-exposed controls (data not shown).

DISCUSSION

Our past research has demonstrated that androgens directly stimulate the synthesis and secretion of SC by lacrimal gland acinar epithelial cells [6, 13, 21, 22], significantly enhance the transfer of SC from lacrimal tissue to tears [8] and elicit a marked increase in the tear SC levels of rats [3, 4, 7, 8]. These hormone actions require the presence of an intact, hypothalamic–pituitary axis [8, 9] and appear to account for the distinct, gender-related differences in SC production by the rat lacrimal gland [3–6]. To extend these results, the current investigation was designed to examine the molecular biological mechanisms underlying these androgen effects. Our findings provide evidence that the gender-associated variations in, and the androgen regulation of, lacrimal SC output involve pronounced changes in SC gene expression. Thus, SC mRNA content in lacrimal glands of male rats is significantly greater than that in tissues of females, and this sexual dimorphism seems to be due to the influence of androgens. Moreover, our results show that the pituitary gland exerts a considerable impact on SC mRNA levels in the lacrimal gland.

Our observation that the amount of a specific mRNA in the lacrimal gland varies according to gender is not surprising. During the past 5 decades, numerous, gender-related differences have been identified in the structure and function of lacrimal tissue, including variations in the morphology of acinar cells, the density

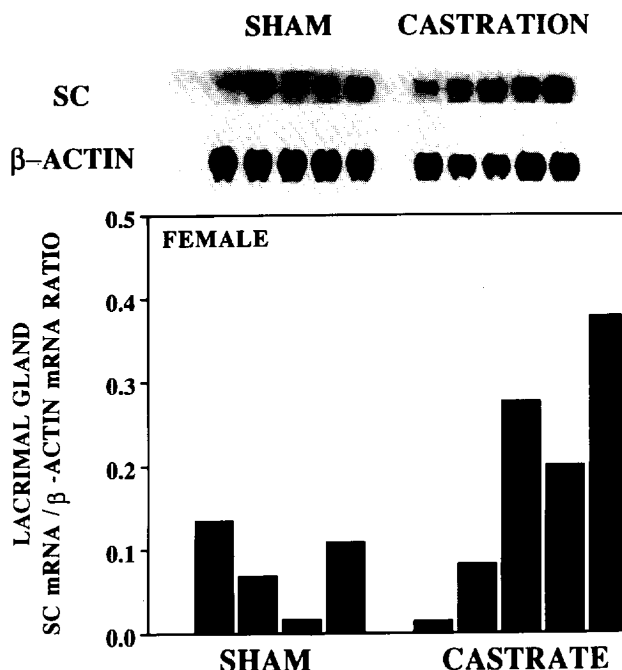

Fig. 6. Influence of ovariectomy on the level of SC mRNA in rat lacrimal tissue. Young adult female rats ($n = 5$/group) were subjected to either sham-surgery or castration, then allowed to recover for 7 days. Following this time interval, lacrimal glands were obtained and processed for the measurement of SC mRNA and β-actin mRNA content by Northern blots, as explained in the legend to Fig. 3. Results show that ovariectomy exerted no significant impact on SC or β-actin mRNA amounts in lacrimal tissue, as compared to message levels in glands of sham-operated controls.

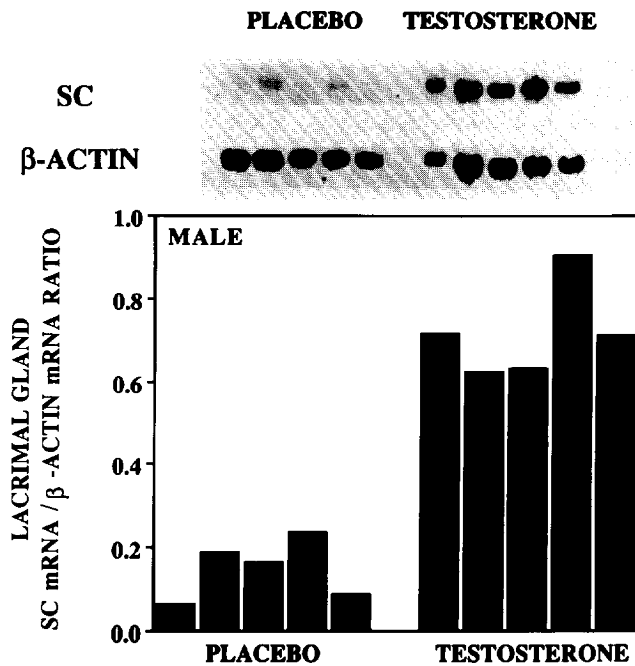

Fig. 7. Androgen-induced increase in SC mRNA content in lacrimal tissue of orchiectomized rats. Lacrimal glands were collected from castrated male rats ($n = 5$/group) following 7 days of treatment with placebo or testosterone (50 mg pellet) and processed for the measurement of SC and β-actin mRNA levels by Northern blots, according to procedures outlined in the Materials and Methods. The total amounts of SC mRNA (top; $P < 0.001$), as well as the SC mRNA/β-actin mRNA ratios (bottom; $P < 0.0005$), were significantly augmented in lacrimal glands by exposure to androgens, as compared to placebo compounds.

of lymphocytes, the synthesis of certain glycoproteins, immunoglobulins, enzymes, receptors and hormones, and the content of nucleic acids [23, 24]. Furthermore, significant, gender-associated variations have been shown to exist in the lacrimal concentration of α 2u globulin, major urinary protein and cystatin-related protein mRNAs [25–27]. These sexual differences in the anatomy, physiology, immunology and genetics of the lacrimal gland occur in a variety of species and almost invariably reflect androgen activity [23, 24].

Of particular interest, the sexual dimorphism in, and the hormonal control of, lacrimal SC mRNA and protein expression may represent a phenomenon unique to the ocular secretory immune system. Thus, the levels of SC mRNA and the synthesis of SC protein [4] are significantly higher in lacrimal tissues of males, as compared to those of females. In contrast, no analogous gender differences in SC production are apparent in other mucosal immune sites, including the salivary gland, lung and intestine [5; D. A. Sullivan, unpublished data). Similarly, the nature of the androgen regulation of SC dynamics may be unique to the lacrimal gland: androgens have no effect on SC content in saliva, respiratory secretions, intestinal fluids and urine [7], and may actually decrease SC elaboration by mammary gland epithelial cells [28]. Recently, investigators have reported that androgens may also stimulate the accumulation of SC in the rat prostate [29] and human prostatic LNCaP cells [30]. However, the steroidal specificity and extent of this hormone action may be unlike that found in lacrimal tissue. For example, the steroid control of prostatic SC is not limited to androgens: estrogens also enhance SC levels in this tissue [29], yet these hormones have no impact on lacrimal gland SC content [3, 6]. Furthermore, the androgen-induced rise in SC concentration within LNCaP cells [30] is not apparently paralleled by an increase in SC secretion [31]; such a secretory response to androgens, though, is expressed by acinar epithelial cells of lacrimal tissue [6]. Consequently, the ability of androgens, and not other steroids, to augment SC mRNA levels, SC synthesis and/or SC output may be restricted to the lacrimal gland. For comparison, estrogens, progestins or glucocorticoids modulate SC protein production in the salivary gland, mammary gland, liver, small intestine, uterus, cervix and/or vagina [30, 32–37]. This differential regulation of SC underscores the site-selectivity in the hormonal control of the secretory immune system [1, 24].

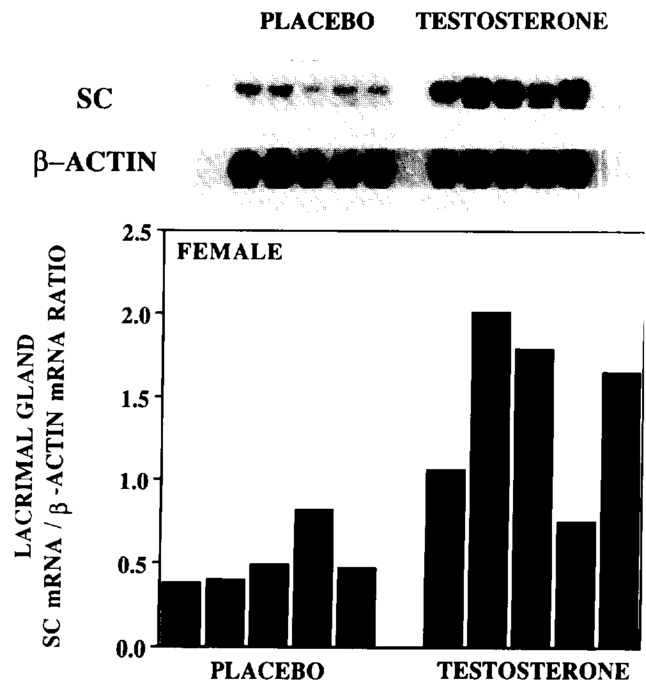

Fig. 8. Effect of testosterone administration on the SC mRNA levels in lacrimal glands of castrated, female rats. Lacrimal tissues were obtained from ovariectomized rats ($n = 5$/group) after 7 days of placebo or testosterone (50 mg pellet) administration. Glands were then processed for the Northern blot analysis of SC mRNA and β-actin mRNA content, as detailed in the Materials and Methods. The absolute SC mRNA level (top) and the SC mRNA/β-actin mRNA ratio (bottom), were significantly ($P < 0.01$) greater in lacrimal tissues of androgen-treated rats, as compared to those of placebo-exposed controls.

Table 1. Influence of hypophysectomy on the total RNA content and SC mRNA/β-actin mRNA ratio in lacrimal glands of male and female rats

Gender/surgery	Lacrimal gland weight (mg)	Total RNA (mg)	Total RNA (mg)/ LGW (mg)—%	SC mRNA/ β-actin mRNA ratio
Male				
Sham-surgery	238 ± 17	2.21 ± 0.16	0.94 ± 0.05	17.9 ± 3.1
Hypophysectomy	110 ± 7*	0.86 ± 0.14*	0.77 ± 0.09	7.1 ± 0.9†
Female				
Sham-surgery	228 ± 19	2.34 ± 0.51	1.03 ± 0.23	6.7 ± 1.1§
Hypophysectomy	106 ± 6*	0.95 ± 0.16‡	0.88 ± 0.11	13.9 ± 4.0

Lacrimal glands were obtained from sham-operated or hypophysectomized male and female rats (n = 4–5/treatment group) 16 days following surgery. After recording the combined weight (LGW) of both glands, tissues were processed for the determination of total glandular RNA content by spectrophotometry at 260 nm, and the measurement of SC mRNA and β-actin mRNA levels by Northern blots (15 μg RNA per lane) with appropriate [^{32}P]-labelled cDNA probes, as described in the Materials and Methods. *$P < 0.0005$; †$P < 0.01$; ‡$P < 0.05$ significantly less than value of sham-operated control group; §$P < 0.05$ significantly less than value of sham-operated males.

The mechanism by which androgens regulate SC mRNA levels and SC protein synthesis in the lacrimal gland may well involve a receptor-mediated induction of SC gene transcription and consequent translation. Classically, androgens bind to specific, high-affinity receptors in the nucleus, and the resulting monomeric and activated hormone–receptor complex associates with an androgen response element in the control region of a given target gene. The hormone complex then dimerizes with another androgen-bound receptor and, in combination with appropriate silencers, tissue-specific and basal promoter elements, regulates gene transcription [38–41]. This androgen activity often leads to an increase in mRNA production and eventually protein synthesis [e.g. 38, 42–44]. In support of this hypothesis, specific, high-affinity binding sites for androgens exist in lacrimal tissue [10, 11], and these receptors, which are located in epithelial cell nuclei [12], adhere to DNA [11]. Moreover, the androgen stimulation of SC synthesis may be prevented by cellular exposure to androgen receptor antagonists [13] and to inhibitors of transcription and translation [4, 13]. However, it is possible that other processes may also be involved in, or mediate, androgen action on lacrimal SC. For instance, androgen control of SC gene transcription might require the induction of intermediate protein synthesis [e.g. 45]. In addition, the androgen modulation of SC protein might reflect hormone-induced alterations in the stability and/or translational efficiency of SC mRNA [e.g. 38, 46, 47]. Further research is necessary to distinguish between these possibilities.

Recently, studies have also demonstrated that the androgen control of SC production by lacrimal gland acinar cells may be enhanced or suppressed by neurotransmitters, cytokines and secretagogues [13, 21, 22]. Thus, the androgen stimulation of SC synthesis may be augmented by vasoactive intestinal peptide, β-adrenergic agonists, interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2), and curtailed by cholinergic agonists (e.g. carbachol choline) [21]. The impact of these non-steroidal agents on SC output may be mediated through changes in intracellular adenylate cyclase and cAMP activity [21]. Consistent with this hypothesis is the finding that acinar cell treatment with cyclic AMP analogues (e.g. 8-bromoadenosine 3′:5′-cyclic monophosphate), cyclic AMP inducers (e.g. cholera toxin) or phosphodiesterase inhibitors (e.g. 3-isobutyl-1-methylxanthine) elicits heightened SC production [13, 21, 22]. In addition to these compounds, insulin, extracellular calcium, high-density lipoprotein, and factors from the thyroid and adrenal glands may also modify androgen action on SC synthesis by lacrimal tissue [14, 22]. Whether these neural, endocrine, immune and metabolic agents directly control, or possibly alter the androgen modulation of, SC mRNA levels in the lacrimal gland remains to be determined.

Our research examining the influence of the hypothalamic–pituitary axis on basal and androgen-induced SC mRNA levels in lacrimal tissue was prompted by several previous observations, including: (1) pituitary ablation causes a significant atrophy of acinar epithelial cells [48], a dramatic reduction in lacrimal fluid and protein secretion [14, 15], and a striking decrease in tear SC levels [14] of male rats; and (2) interruption of this axis in male rats leads to a marked diminution in the density of androgen receptors in acinar cell nuclei [12], a precipitous decline in the lacrimal SC response to androgens *in vivo* [8, 9], and a significant attenuation in the acinar cell capacity to synthesize SC following androgen exposure *in vitro* [6]. Our present results indicate that the hypothalamic–pituitary axis may regulate SC mRNA levels in the rat lacrimal gland and that this control may be influenced by gender. Thus, hypophysectomy caused a significant decrease in the relative proportion of SC mRNA in glands of male, but not female, rats. This effect appeared unrelated to the overall decline in total tissue RNA levels, given that this latter reduction occurred in both sexes. Rather, the

selective impact on SC mRNA content in males may have been due to the fact that pituitary extirpation represents a functional castration, and thereby removes the stimulatory effect of androgens. In addition, gender differences apparently exist in the lacrimal gland susceptibility to pituitary hormones [49], which could theoretically play a role in the nature of the SC mRNA response to hypophysectomy. In contrast, an explanation for the other, multiple changes in lacrimal gland structure and function, which follow after pituitary extirpation but not castration [24], has yet to be advanced.

Of interest, our findings also demonstrated that testosterone treatment of hypophysectomized and orchiectomized rats induces an increase in the absolute level of SC mRNA in the lacrimal gland. Consequently, given that these surgical procedures curtail the androgen-induced production of SC by lacrimal tissue *in vivo* [8, 9], it would appear that this interference may be primarily post-transcriptional.

In summary, this study shows that gender, androgens and the hypothalamic–pituitary axis significantly influence SC mRNA content in the lacrimal gland. Moreover, these results begin to explain the molecular biological mechanisms underlying the endocrine control of lacrimal SC synthesis. Our ongoing research is designed to identify the nature of this hormonal modulation of SC mRNA (e.g. transcriptional rate vs stability), as well as to determine whether the associated androgen regulation of lacrimal gland IgA production [8] also involves control of IgA mRNA expression.

Acknowledgements—The authors express their appreciation to Dr Louane E. Hann and Dr Robin S. Kelleher for their excellent technical advice, and to Dr Lan Hu for generously providing the mouse β-actin cDNA. This research was supported by NIH grants EY05612 (DAS), EY02882 (DAS) and EY07074 (RWL), a grant from the Massachusetts Lions' Research Fund (DAS), and grants from the Medical Research Council (GB) and Agriculture and Food Research Council (GB) of the United Kingdom.

REFERENCES

1. Sullivan D. A.: Ocular mucosal immunity. In *Handbook of Mucosal Immunology* (Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. McGhee and J. Bienenstock). Academic Press, Orlando, FL (1994) pp. 569–597.
2. Casanova J. E.: Transepithelial transport of polymeric immunoglobulins. *Ann. N. Y. Acad. Sci.* 664 (1992) 27–38.
3. Sullivan D. A., Bloch K. J. and Allansmith M. R.: Hormonal influence on the secretory immune system of the eye: androgen regulation of secretory component levels in rat tears. *J. Immunol.* 132 (1984) 1130–1135.
4. Sullivan D. A., Bloch K. J. and Allansmith M. R.: Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. *Immunology* 52 (1984) 239–246.
5. Sullivan D. A. and Allansmith M. R.: The effect of aging on the secretory immune system of the eye. *Immunology* 63 (1988) 403–410.
6. Sullivan D. A., Kelleher R. S., Vaerman J. P. and Hann L. E.: Androgen regulation of secretory component synthesis by lacrimal gland acinar cells *in vitro*. *J. Immunol.* 145 (1990) 4238–4244.
7. Sullivan D. A., Hann L. E. and Vaerman J. P.: Selectivity specificity and kinetics of the androgen regulation of the ocular secretory immune system. *Immun. Invest.* 17 (1988) 183–194.
8. Sullivan D. A. and Hann L. E.: Hormonal influence on the secretory immune system of the eye: endocrine impact on the lacrimal gland accumulation and secretion of IgA and IgG. *J. Steroid Biochem.* 34 (1989) 253–262.
9. Sullivan D. A.: Influence of the hypothalamic–pituitary axis on the androgen regulation of the ocular secretory immune system. *J. Steroid Biochem.* 30 (1988) 429–433.
10. Edwards J. A., Kelleher R. S. and Sullivan D. A.: Identification of dihydrotestosterone binding sites in the rat lacrimal gland. *Invest. Ophthalmol. Vis. Sci. Suppl.* 31 (1990) 541.
11. Ota M., Kyakumoto S. and Nemoto T.: Demonstration and characterization of cytosol androgen receptor in rat exorbital lacrimal gland. *Biochem. Int.* 10 (1985) 129–135.
12. Rocha F. J., Wickham L. A., Pena J. D. O., Gao J., Ono M., Lambert R. W., Kelleher R. S. and Sullivan D. A.: Influence of gender and the endocrine environment on the distribution of androgen receptors in the lacrimal gland. *J. Steroid Biochem. Molec. Biol.* 46 (1993) 737–749.
13. Lambert R. W., Kelleher R. S., Wickham L. A., Vaerman J. P. and Sullivan D. A.: Neuroendocrinimmune modulation of secretory component production by rat lacrimal, salivary and intestinal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 35 (1994) 1192–1201.
14. Sullivan D. A. and Allansmith M. R.: Hormonal influence on the secretory immune system of the eye: endocrine interactions in the control of IgA and secretory component levels in tears of rats. *Immunology* 60 (1987) 337–343.
15. Sullivan D. A. and Allansmith M. R.: Hormonal modulation of tear volume in the rat. *Exp. Eye Res.* 42 (1986) 131–139.
16. Chomczynski P. and Sacchi N.: Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* 162 (1987) 156–159.
17. Banting G., Brake B., Braghetta P., Luzio J. P. and Stanley K. K.: Intracellular targeting signals of polymeric immunoglobulin receptors are highly conserved between species. *FEBS Lett.* 254 (1989) 177–183.
18. Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Smith J. A., Seidman J. G. and Struhl K. (Eds): *Current Protocols in Molecular Biology*. John Wiley & Sons, NY (1991) pp. 4.10.1–4.10.9.
19. Takeda H. and Chang C.: Immunohistochemical and in-situ hybridization analysis of androgen receptor expression during development of the mouse prostate gland. *J. Endocr.* 129 (1991) 83–89.
20. Huling S., Fournier G. R., Feren A., Chuntharapai A. and Jones A. L. Ontogeny of the secretory immune system: maturation of a functional polymeric immunoglobulin receptor regulated by gene expression. *Proc. Natn. Acad. Sci.* 89 (1992) 4260–4264.
21. Kelleher R. S., Hann L. E., Edwards J. A. and Sullivan D. A.: Endocrine, neural and immune control of secretory component output by lacrimal gland acinar cells. *J. Immunol.* 146 (1991) 3405–3412.
22. Hann L. E., Kelleher R. S. and Sullivan D. A.: Influence of culture conditions on the androgen control of secretory component production by acinar cells from the lacrimal gland. *Invest. Ophthalmol. Vis. Sci.* 32 (1991) 2610–2621.
23. Sullivan D. A. and Sato E. H.: Immunology of the lacrimal gland. In *Principles and Practice of Ophthalmology* (Edited by D. M. Albert and F. A. Jakobiec). W. B. Saunders Company, Philadelphia, PA (1993), pp. 479–486.
24. Sullivan D. A.: Hormonal influence on the secretory immune system of the eye. In *The Neuroendocrine-Immune Network* (Edited by S. Freier). CRC Press, Boca Raton, FL (1990) pp. 199–238.
25. Shaw P. H., Held W. A. and Hastie N. D.: The gene family for major urinary proteins: expression in several secretory tissues of the mouse. *Cell* 32 (1983) 755–761.
26. Gubits R. M., Lynch K. R., Kulkarni A. B., Dolan K. P., Gresik E. W., Hollander P. and Feigelson P.: Differential regulation of α 2u globulin gene expression in liver, lachrymal gland, and salivary gland. *J. Biol. Chem.* 259 (1984) 12,803–12,809.
27. Winderickx J., Vercaeren I., Verhoeven G. and Heyns W.: Androgen-dependent expression of cystatin-related protein

(CRP) in the exorbital lacrimal gland of the rat. *J. Steroid Biochem. Molec. Biol.* 48 (1994) 165–170.
28. Weisz-Carrington P., Emancipator S. and Lamm M. E.: Binding and uptake of immunoglobulins by mouse mammary gland epithelial cells in hormone-treated cultures. *J. Reprod. Immunol.* 6 (1984) 63–75.
29. Stern J. E., Gardner S., Quirk D. and Wira C. R.: Secretory immune system of the male reproductive tract: effects of dihydrotestosterone and estradiol on IgA and secretory component levels. *J. Reprod. Immunol.* 22 (1992) 73–85.
30. Weisz-Carrington P., Farraj M., Asadi F., Sharifi R., Keleman P. R., Hwang L. and Buschmann R. J.: Expression of secretory component on DU-145 and LN-CAP prostatic carcinoma cell lines is hormone dependent. *FASEB J.* 4 (1990) A1709.
31. Lambert R. W., Kelleher R. S., Wickham L. A., Gao J. and Sullivan D. A.: Neural-endocrine control of secretory component synthesis by lacrimal gland acinar cells: specificity, temporal characteristics and molecular basis. *Adv. Exp. Med. Biol.* 350 (1994) 175–180.
32. Wira D. R. and Rossoll R. M.: Glucocorticoid regulation of the humoral immune system. Dexamethasone stimulation of secretory component in serum, saliva and bile. *Endocrinology* 128 (1991) 835–842.
33. Wira C. R. and Colby E. M.: Regulation of secretory component by glucocorticoids in primary cultures of rat hepatocytes. *J. Immunol.* 134 (1985) 1744–1748.
34. Buts J. P., Vaerman J. P. and Delacroix D. L.: Ontogenic changes in secretory component expression by villous and crypt cells of rat small intestine. *Immunology* 54 (1985) 181–187.
35. Sullivan D. A., Underdown B. J. and Wira C. R.: Steroid hormone regulation of free secretory component in the rat uterus. *Immunology* 49 (1983) 379–386.
36. Wira C. R. and Sullivan D. A.: Estradiol and progesterone regulation of immunoglobulin A and G and secretory component in cervicovaginal secretions of the rat. *Biol. Reprod.* 32 (1985) 90–95.
37. Murdoch A. J. M., Buckley C. H. and Fox H.: Hormonal control of the secretory immune system of the human uterine cervix. *J. Reprod. Immunol.* 4 (1982) 23–30.
38. Clark J. H., Schrader W. T. and O'Malley B. W.: Mechanisms of action of steroid hormones. In *Williams Textbook of Endocrinology* (Edited by J. D. Wilson and D. W. Foster). W. B. Saunders, Philadelphia, PA (1992) pp. 35–90.
39. Trapman J., Ris-Stalpers C., van der Korput J. A. G. M., Kuiper G. G. J. M., Faber P. W., Romijn J. C., Mulder E. and Brinkmann A. O.: The androgen receptor: functional structure and expression in transplanted human prostate tumors and prostate tumor cell lines. *J. Steroid Biochem. Molec. Biol.* 37 (1990) 837–842.
40. Simental J. A., Sar M., Lane M. V., French F. S. and Wilson E. M.: Trancriptional activation and nuclear targeting signals of the human androgen receptor. *J. Biol. Chem.* 266 (1991) 510–518.
41. Rundlett S. E., Wu X-P. and Miesfeld R. L.: Functional characterizations of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. *Molec. Endocr.* 4 (1990) 708–714.
42. Mooradian A. D., Morley J. E. and Korenman S. G.: Biological actions of androgens. *Endocrine Rev.* 8 (1987) 1–28.
43. Myal Y., Robinso D. B., Iwasiow B., Tsuyuki D., Wong P. and Shiu R. P. C.: The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. *Molec. Cell Endocr.* 80 (1991) 165–175.
44. Zietler P., Argente J., Chowen-Breed J. A., Clifton D. K. and Steiner R. A.: Growth hormone-releasing messenger ribonucleic acid in the hypothalamus of the adult male rat is increased by testosterone. *Endocrinology* 127 (1990) 1362–1368.
45. Winderickx J., Hemschoote K., De Clercq N., Van Dijck P., Rombauts W., Verhoeven G. and Heyns W.: Tissue specific expression and androgen regulation of different genes encoding rat prostatic 22-kilodalton glycoproteins homologous to human and rat cystatin. *Molec. Endocr.* 4 (1990) 657–667.
46. Rosewicz S., McDonald A. R., Maddux B. A., Goldfine I. D., Miesfeld R. L. and Logsdon C. D.: Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. *J. Biol. Chem.* 263 (1988) 2581–2584.
47. Dong Y., Poellinger L., Gustafsson J. A. and Okret S.: Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. *Molec. Endocr.* 2 (1988) 1256–1264.
48. Martinazzi M.: Effetti dell'ipofisectomia sulla ghiandola lacrimale extraorbitale del ratto. *Folia Endocr.* 150 (1962) 120–129.
49. Martinazzi M.: La ghiandola lacrimale extraorbitale o ghiandola di Loewenthal: sua morfologie e controllo ormonale. *Recent Progr. Med. (Roma)* 34 (1963) 491–514.