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The Androgen Receptor mRNA is Up-regulated by Testosterone in Both the Harderian Gland and Thumb Pad of the Frog, *Rana esculenta*

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α^{32}P-labelled cDNA probe from plasmid containing rat androgen receptor (rAR) has been tested in hybridization experiments using RNAs from the Harderian gland and thumb pad of the edible frog, *Rana esculenta*. Northern blot analysis has shown a high degree of homology between the rAR cDNA and the frog androgen receptor mRNA (fAR mRNA); this has been supported by both the hybridization conditions (high stringency) and the molecular size of fAR mRNA which is quite similar to those described in mammals (9.4 kb). The role of androgens has been further investigated with respect to the kinetics of expression of fAR mRNA in *in vivo* experiments. In both the Harderian gland and thumb pad, testosterone has increased the levels of fAR mRNA as compared with the untreated groups. The use of cyproterone acetate (CPA) in combination with testosterone has resulted in a loss of the increase in fAR mRNA as compared to testosterone-treated groups, while CPA alone has resembled the control group. In primary cultures of frog Harderian gland and thumb pad cells, the steady-state levels of fAR mRNA have been increased in the cells exposed to testosterone as compared to those not exposed. These findings confirm that, in these androgen target tissues, testosterone exerts an up-regulation on its own receptors, increasing the accumulation of fAR mRNA in the same way as oestrogens up-regulate the expression of their own receptors in *Xenopus* liver and oviduct cells.

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INTRODUCTION

One of the central issues in understanding cellular differentiation is the establishment of tissue specificity by differential gene expression and its hormonal regulation. Among the regulation of gene expression by steroids the lion's share has been made by oestrogens. In this respect there is a considerable amounts of studies in almost all classes of vertebrates, particularly in amphibians [1–3]. In contrast, as far as hormonal regulation of androgens is concerned, the data available in the literature regard mainly mammals [4–10], while no reports are known in lower vertebrates.

In our laboratory we have recently demonstrated the presence of an androgen receptor (AR) in the Harderian gland of the green frog, *Rana esculenta*, [11] and an involvement of testosterone in the regulation of the secretory activity of this orbital gland whose secretory granules are typically seromucoid [12, 13]. In this respect, testosterone induces the incorporation of [^{3}H]uridine into the poly(A)$^+$RNA fraction and also that of [^{35}S]methionine into a newly synthesized protein fraction in primary culture of Harderian gland cells, and the latter effect is prevented by the exposure of the cells to the antiandrogen cyproterone acetate (CPA) [13]. It is noteworthy that the use of CPA, instead of flutamide, is a useful tool to study the effect of testosterone in *Rana esculenta* since it has been used in our laboratory for a long time and we have a considerable amount of information concerning the antiandrogenic effect of CPA in the frog [12–16]. All these findings reveal that the Harderian gland of *Rana esculenta* must be considered a new target tissue for androgens [12]. As far as the presence of diverse androgen-target tissues in the frog *Rana esculenta* is

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concerned, it is worthy to mention a structure such as the thumb pad [17]. The thumb pad is a complex structure well developed during the breeding season, when the plasma testosterone levels are high [18, 19]. Gonadectomy brings about the atrophy of this structure and a decrease in RNA and protein content, while testosterone therapy restores the morphological aspect and the protein and RNA content [14]. Administration of testosterone to castrated animals induces hypertrophy in both epidermal and glandular layers due to the accumulation of PAS-positive secretory granules. This indicates a secretion of mucous type from the glandular cells [14]. Furthermore, an AR has been evidenced and characterized in this secondary sexual character [17].

Since in *Rana esculenta* there are two target tissues for the same hormone and both tissues contain a receptor for this hormone with identical characteristics, this finding appears to be a further model to study tissue specificity in the hormonal regulation of gene expression.

The aim of the present study is to ascertain a possible down- or up-regulation of the AR mRNA in these two tissues which is important in our understanding of the steroid regulation of the gene(s) expressed in the two tissues. This will offer a model comparable with those experienced in *Xenopus laevis* for oestrogen regulated egg protein genes [3, 20].

EXPERIMENTAL

Animals

Adult male frogs, *Rana esculenta*, were captured in the surroundings of Naples (Italy) during May and October. In *in vivo* experiments 10 animals/group were caged and fed *ad libitum*. Adult male rats, Sprague–Dawley strain, obtained by Nossan (Italy) were caged (2/cage) and water and food supplied *ad libitum*.

Chemicals

Molecular biology grade reagents were used. [α^{32}P]dCTP (3000 Ci/mM) was purchased from Amersham International (Amersham, Italy), testosterone and Tricaine (MS222) from Sigma (St Louis, MO) and CPA Schering (Germany). All the chemicals used for the *in vitro* experiments were cell culture tested.

RNA extraction

All glassware and solutions were sterilized and RNase free. Total RNA was extracted by the procedure of Chomczynski and Sacchi [21] with minor modifications.

In vivo experiment

Male frogs (170) captured during October, were divided into four groups and treated on alternate days as follows: frogs injected with testosterone (10 μg/100 μl/dose); frogs injected with testosterone and CPA (10 μg/100 μl testosterone + 1 mg/100 μl CPA/dose); frogs injected with CPA (1 mg/100 μl/dose); and frogs injected with vehicle (Barth × regular). Animals (n = 10/group) were sacrificed under anaesthesia using MS222 at the beginning (t = 0) and then after 1, 2, 3 and 4 weeks/treatment, respectively. Harderian glands, thumb pads and guts were excised, pooled and stored under liquid nitrogen until extraction of total RNA. At the end of the *in vivo* experiment three rats were sacrificed under anaesthesia and the ventral prostate excised, pooled and used for the extraction of total RNA.

Harderian gland and thumb pad cell culture

Primary cultures of Harderian gland cells were obtained as described previously [13] from tissue pooled by sacrificing 35–40 animals captured during May. Cells were maintained in hormone-free Wolf and Quimby medium supplemented with antibiotics for at least 24 h after plating in order to allow the cells to stick onto dishes and recover from culture shock before treatment with hormones. This pretreatment is important to obtain a reproducible response to the hormone [22]. A similar procedure was performed in obtaining primary cultures of thumb pad cells, in fact the only difference was in the time of enzymatic digestion of the tissue which was carried out for up to 3 h. Testosterone was dissolved in ethanol and added to the cultures after predilution in the culture medium at a final concentration of 10^{-8} M, the ethanol was less than 1%. Control cultures received solvent alone. Exposure or non-exposure to the hormone was carried out for different times (8, 24, 48, and 72 h) after which total RNA was extracted.

Analysis of RNA

Northern blots of RNA prepared from pooled frog Harderian glands, thumb pads, guts and from rat prostate were performed. Total cellular RNA were extracted as described above. RNA (30 μg/sample) was subjected to electrophoresis through 1.2% agarose–formaldehyde gel and transferred to nylon (Nytran, Schleicher & Schuell) membrane. Hybridization was performed with a cDNA probe for rat AR (pGM-3ZrAR) (rAR) [23] oligolabelled with [α^{32}P]dCTP to a specific activity of about 5×10^8 cpm/μg [24] and carried out at 42°C in 50% formamide, 5 × SSC, 0.01 M EDTA, 5 × Denhardt's solution, 0.5% sodium deodecyl sulphate (SDS) and 100 μg/ml of denatured salmon sperm DNA. The filters were washed twice with 2 × SSC at 65°C and once with 0.1 × SSC at 65°C. Dried filters were autoradiographed.

Frog AR mRNA in the in vivo experiment

Total RNA (4 μg) from pooled Harderian glands, thumb pads and rat prostate was respectively bound to nylon (Nytran, Schleicher & Schuell) membranes by UV crosslinking in a slot-blot apparatus (Schleicher &

Schuell) and hybridized to α^{32}P-labelled cDNA probes. Hybridization was performed with plasmid containing rAR (pGM-3ZrAR) and cytoskeletal actin (pXICA1) oligolabelled with [α^{32}P]-dCTP to a specific activity of about 5×10^8 cpm/μg [24] and the conditions were as described above. The relative concentrations of different mRNAs in a constant amount of total RNA were calculated by quantitative densitometry of the autoradiograms based on actin mRNA as an internal control [25].

Frog AR mRNA in cultured Harderian gland and thumb pad cells

Total RNA was extracted from cultured cells. For measuring the relative amounts of frog AR mRNA (fAR mRNA) equal amounts of total RNA (4 or 2 μg) were bound to nylon (Nytran; Schleicher & Schuell) membranes by UV crosslinking and hybridized to α^{32}P-labelled cDNA probes in a slot-blot apparatus as described above. The relative concentrations of different mRNAs in a constant amount of total RNA were calculated as described above.

Statistics

Significance of differences was evaluated by using Duncan's test (at $P < 0.05$ and < 0.01) for multigroup comparisons.

RESULTS

Northern blots analysis

We firstly compared the hybridization signals of the AR mRNA in rat prostate (positive control) with the putative AR mRNA in Harderian glands, thumb pads and gut (negative control). Positive signals were obtained in both the Harderian gland and thumb pad although the probe used could have been considered heterolgous, while no signals were obtained for the gut. In all the experiments the signals were evident despite the high stringency conditions. In this regard, it is noteworthy that, the probe used in this study contains both the DNA and the steroid binding domains, whose sequences are usually well conserved. However in the present study there is evidence of a high degree of homology in these two regions, which agrees with previous experiments performed using the two domains separately in which a positive signal in high stringency conditions was obtained. Furthermore the molecular size of fAR mRNA (9.4 kb) (Fig. 1) is similar to that reported for many mammals [4, 7, 26, 27]. Figure 2 shows a typical slot-blot where the RNA from the two frog tissues and the RNA from rat prostate were hybridized with labelled rAR cDNA.

In vivo induction of fAR mRNA in the Harderian gland

Figure 3 shows the kinetics of induction of fAR mRNA in the Harderian gland. In the control group a decline in fAR mRNA was seen throughout the whole experiment. This was due to starvation which impairs the production of androgens [28]. This phenomenon, which usually occurs in *Rana esculenta*, is named physiological castration. In the testosterone injected groups, a time-dependent increase in fAR mRNA was observed, while in the groups treated with testosterone + CPA this increase was less evident, although the levels of fAR mRNA were higher than those observed in the control group. The results obtained in the CPA injected group were similar to those observed in the

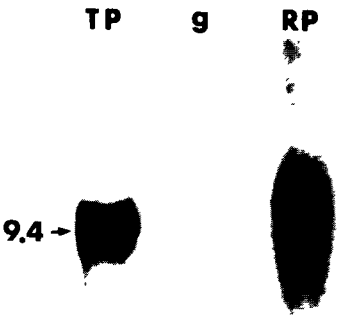

Fig. 1. Representative autoradiograph of Northern blot hybridization analysis of AR mRNA in the thumb pad and gut of the green frog, *Rana esculenta* and rat ventral prostate. A radiolabelled cDNA probe for the rAR was hybridized to 30 μg of total cellular RNA from the thumb pad (TP), gut (g) and rat ventral prostate (RP).

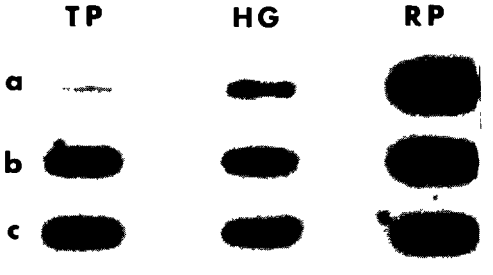

Fig. 2. Representative autoradiograph of a typical slot-blot hybridization analysis of the AR mRNA, after 1 week of hormonal manipulation, in the thumb pad and Harderian gland of the green frog, *Rana esculenta*, and the rat ventral prostate. A radiolabelled cDNA probe for the rAR was hybridized to 4 μg/sample of total cellular RNA from the thumb pad (TP) and Harderian gland (HG) of animals treated as follows: control animals (a); testosterone + CPA treated animals (b); and testosterone-treated animals (c). The autoradiograph shown hybridization to 4 μg (a), 2 μg (b) and 1.5 μg (c) of total cellular RNA from ventral rat prostate (RP).

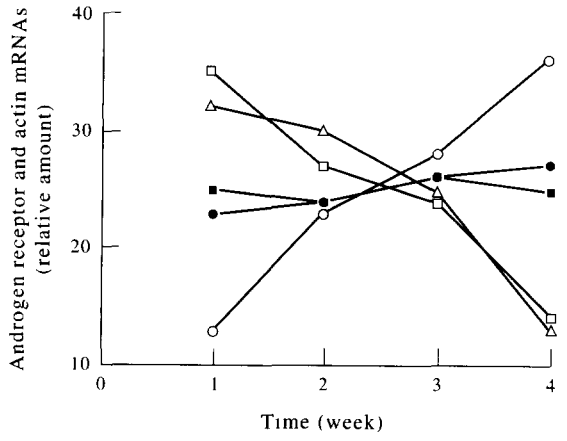

Fig. 3. Steady-state levels of fAR and actin mRNAs in the Harderian gland of the green frog, *Rana esculenta*, as a function of time. The relative concentrations of fAR mRNA have been determined by slot-blot hybridization and expressed as relative densitometric units. (△) control animals; (○) testosterone-treated animals; (●) testosterone + CPA-treated animals; (□) CPA-treated animals. The values of actin mRNA (■) are shown for animals treated with testosterone. Each curve is representative of three different analysis at least. SDs have been obmitted for simplicity.

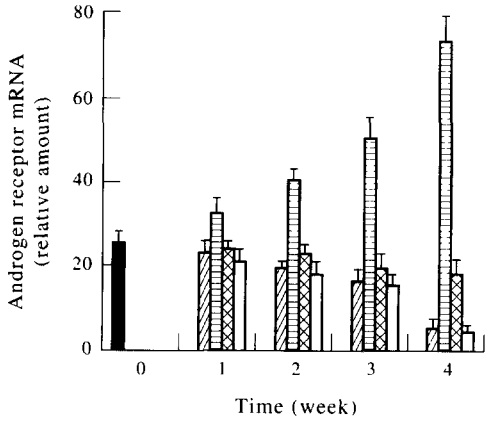

Fig. 5. Time-point analysis of fAR mRNA in the Harderian gland of the green frog, *Rana esculenta*. The figure shows the differences, between the diverse hormonal manipulated groups, in the relative concentrations of fAR mRNA determined by slot-blot hybridization. (■) Control animals $t = 0$; (▨) control animals; (▤) testosterone-treated animals; (■) testosterone + CPA-treated animals; (□) CPA-treated animals. Values are mean ± SDs of at least three different hybridizations.

control group. No differences were seen between the groups concerning the expression of actin mRNA.

In vivo induction of fAR mRNA in the thumb pad

In the thumb pad (Fig. 4) similar findings were observed to those seen in the Harderian gland, although the absolute values were different (see Fig. 2). In fact in this experiment the decrease in fAR mRNA in both the control and CPA-treated groups was also seen. Replacement therapy with testosterone induced a time-dependent increase in fAR mRNA that was prevented by the use of CPA in combination with testosterone. As far as the analysis of actin mRNA is concerned, the results showed that this gene was expressed at the same rate during the experiment.

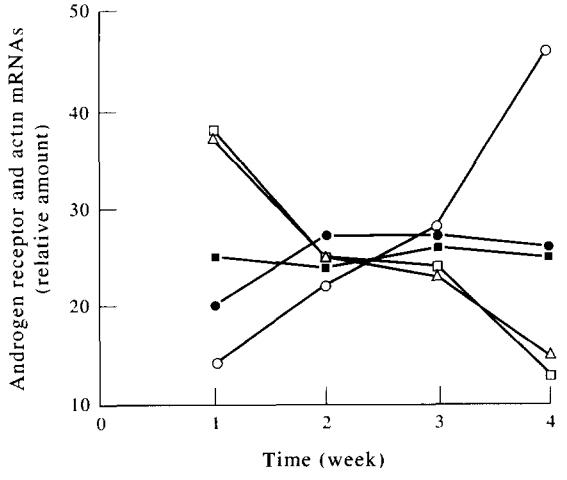

Fig. 4. Steady-state levels of fAR and actin mRNAs in the thumb pad of the frog, *Rana esculenta*, as a function of time. The relative concentrations of fAR mRNA have been determined by slot-blot hybridization and expressed as relative densitometric units. (△) control animals; (○) testosterone-treated animals: (●) testosterone + CPA-treated animals; (□) CPA-treated animals. The values of actin mRNA (■) are shown for animals treated with testosterone. Each curve is representative of three different analysis at least. SDs have been obmitted for simplicity.

It must be noted that a down-regulation seemed to occur during the first week in the testosterone-treated group. In fact the analysis of differences during the experimental-time concerned only differences within the group and not between groups, whilst in studying the levels of fAR mRNA between groups the results obtained showed an up-regulation of fAR mRNA, starting from the first week (see following section and Figs 5 and 6).

Time-point analysis of fAR mRNA in the Harderian gland

In comparing the fAR mRNA levels between the experimental groups during the whole experiment (Fig. 5) it has been possible to note a significant increase ($P < 0.01$) in fAR mRNA in the group treated with testosterone with respect to the control group and that this difference increases starting from the first week. The use of testosterone + CPA results in a lesser increase of fAR mRNA as revealed by comparison with the testosterone-treated group ($P < 0.05$ testosterone treated group vs testosterone + CPA-treated group), although the difference with the control group is still significant ($P < 0.05$ testosterone + CPA-treated group vs control group), particularly after the third and fourth week of treatment. Lastly the values obtained from the CPA-injected group were similar to those of the control group showing no significant differences.

Time-point analysis of fAR mRNA in the thumb pad

Figure 6 shows the results obtained in the thumb pad. The levels of fAR mRNA decrease significantly ($P < 0.01$) in both the control and CPA-treated groups during the 4 weeks of treatment. Testosterone therapy increases the levels of fAR mRNA in a time-dependent manner, resulting in a highly significant value as revealed by comparison with all the experimental groups ($P < 0.01$ testosterone-treated animals vs control animals; $P < 0.01$ testosterone-treated animals vs CPA-treated animals). The latter effect was partially prevented by the use of CPA in combination with testosterone ($P < 0.05$ testosterone-treated group vs testosterone + CPA-treated animals).

Induction of fAR mRNA in cultured cells

Figure 7 shows the kinetics of induction by testosterone of fAR mRNA in (a) the Harderian gland and (b) the thumb pad cells. In both primary cultures during the experiment a decline in fAR mRNA in the control cultures was observed. Addition of testosterone at a concentration of 10^{-8} M reverses this profile and causes an accumulation of about 10-fold of fAR mRNA by 48 h. Actin mRNA was constantly expressed in both the control (not shown) and testosterone exposed groups, either in the Harderian gland, or in the thumb pad cells. As far as the apparent initial down-regulation of fAR mRNA in the cells exposed to testosterone with respect to the control cells is concerned, it is noteworthy, that the differences must be considered within the group and not between groups, while the differences between the groups resemble those observed in the analysis of the fAR mRNA in the *in vivo* experiment (not shown).

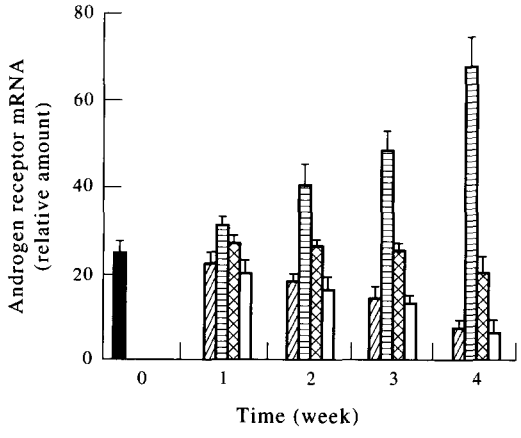

Fig. 6. Time-point analysis of fAR mRNA in the thumb pad of the green frog, *Rana esculenta*. The figure shows the relative difference, between the diverse hormonal-manipulated groups, in the relative concentrations of fAR mRNA determined by slot-blot hybridization. (■) Control animals $t = 0$; (▨) control animals; (▤) testosterone-treated animals; (■) testosterone + CPA-treated animals; (□) CPA-treated animals. The values are means ± SD of at least three different hybridizations.

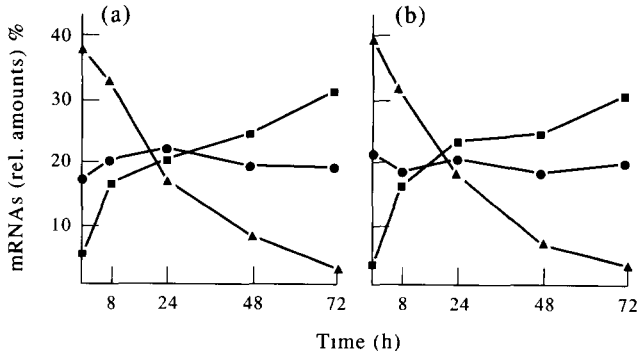

Fig. 7. Steady-state levels of fAR and actin mRNAs in primary cultures of the Harderian gland (a) and thumb pad (b) cells as a function of time following the addition or not of testosterone 10^{-8} M. After 24 h in culture, cells were exposed or not to the hormone for different times, following which total RNA was extracted. The concentrations of fAR mRNA and actin mRNA were determined by slot-blot hybridization and expressed as relative densitometric units for equal numbers of cells. (■) Testosterone-exposed cells; (▲) unexposed cells. The values of actin mRNA (●) are shown for cells treated with testosterone. Each curve is representative of at least three different analysis. SDs have been obmitted for simplicity.

DISCUSSION

It is becoming increasingly clear that the number of hormone receptors, which are important to our understanding of hormone action, can be up- or down-regulated by the ligand or by another hormone [29]. Although down-regulation of receptors by their respective hormonal ligand is a well-studied phenomenon, relatively less is known about autoup-regulation of receptors. However, an increasing number of observations on the phenomenon of up-regulation of receptor number have now been reported [30]. A typical example of the up-regulation of receptors is provided by the activation *de novo* by oestrogens of silent vitellogenin genes in male *Xenopus* hepatocyte primary cultures and the induction of frog oviduct specific protein-1 gene in primary cultures of *Xenopus* oviduct cells which are both tightly coupled to a considerable up-regulation of oestrogen receptor protein and its transcripts [2, 20, 31]. Autoinduction of other steroid receptors has also been reported for androgen in the rat prostate [26], rat phallus [32], rat sertoli and peritubular cells [7] and human prostate [5]. Down-regulation of receptor mRNA by its own ligand has been reported in tumor-derived cell lines [10, 33, 34].

In this study we report, for the first time, the presence and the regulation of an AR mRNA in a non-mammalian vertebrate. In fact, although the probe used in our experiments is heterolgous a high degree of homology between the rAR and the frog can be postulated because the probe contains mainly the DNA and steroid binding domains. This finding is strongly suggested by either the hybridization conditions or the

molecular size of fAR mRNA which is similar to those reported for other vertebrates [4, 7, 26, 27].

The importance of up-regulation of the receptor mRNA lies in the possibility of understanding the amplification of a given hormonal response. That this phenomenon could be important for the physiology of both the Harderian gland and thumb pad is suggested by the analysis of the annual modifications of either biochemical or morphological parameters of these two tissues [12, 14]. Thus, the presence of high levels of both androgens and ARs is required for the normal function of the thumb pad, while castration brings about a regress of both glandular and epidermal layers. This effect is completely reversed by testosterone replacement therapy [14]. Similarly, in the Harderian gland a high accumulation of the secretory proteins follows the presence of high levels of ARs in the gland [11]. Castration modifies, either the electrophoretic pattern of proteins [15], or the ultrastructural architecture of the glandular cells [35].

Our results on the profiles of fAR mRNA show an up-regulation of fAR mRNA by its own ligand (Figs 3 and 4). This phenomenon is well correlated with the biochemical and morphological parameters of these structures. In fact in *in vivo* experiments an accumulation of fAR mRNA has been shown in animals under testosterone therapy, while a loss of fAR mRNA occurs in the control and CPA-treated animals. In these experiments, the use of testosterone, in combination with CPA, as an antiandrogen, causes a lower but significant increase in fAR mRNA with respect to the testosterone injected animals. It is important to note that the use of CPA in combination with testosterone, leads to constant fAR mRNA levels during the whole experiment, competing with testosterone in the induction and deinduction of fAR mRNA. In fact in these groups any increase in fAR mRNA levels was seen in both the Harderian gland and the thumb pad. Although there are other antiandrogens that must be considered much more effective, the use of CPA as an antiandrogen in the frog has been supported by several observations in our laboratory [13, 15, 16]. This finding strongly suggests the up-regulatory action of testosterone itself.

Further evidence of the up-regulatory effect of testosterone has been provided by experiments carried out in primary cultures. In both cell types, an accumulation of fAR mRNA has been found starting from 8 h of exposure to the hormone, while in the control cultures a $t_{1/2}$ of approx. 24 h has been seen [see Fig. 7(a and b)]. In this case, the disappearance of fAR mRNA reflects the inhibition of both transcription and stabilization of fAR mRNA.

Taken together, all these findings strongly support the up-regulation of fAR mRNA in the Harderian gland and thumb pad of the green frog, *Rana esculenta* and that this up-regulation could be considered an autoinduction since, the effects seen in the present paper, particularly those obtained in the cell culture study, lead us to hypothesize that testosterone itself induces the synthesis of the mRNA for its own receptor.

Lastly, considering both the morphological and the biochemical differences between the Harderian gland and the thumb pad, it is conceivable that the ARs have different functions in these two tissues. The fact that similar processes underlie the activation of different gene(s) by the same hormone, operating via the same receptor, means that their comparisons offer a further model to study the tissue specificity in the hormonal regulation of gene expression in lower vertebrates.

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