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Luteinizing Hormone Increases the Abundance of Various Transcripts, Independently of the Androgens, in the Rat Prostate

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Differential display analysis was carried out to find, in the rat prostate, genes that could be regulated by Luteinizing Hormone (LH), independently of the androgens. Hypophysectomized and castrated adult rats were treated with either LH, testosterone or saline. Regulated discrete bands have been eluted and reamplified. After Northern blotting, the levels of mRNA corresponding to 8 PCR fragments were significantly increased by LH treatment. None of these inserts were found to be induced by testosterone. One insert was subcloned, sequenced and identified as the ribosomial protein S 23. A competitive RT-PCR assay was carried out on the full length S 23 cDNA and confirmed that its mRNA levels were stimulated by LH but not by testosterone. These results strongly suggest that the LH membrane receptor, previously shown to be expressed in the rat prostate, has a physiological significance in this organ. Moreover, it appears that the effect of LH on the rat prostate are independent of the androgens.
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Prostatic development and function has long been known to be under androgen control. However, even though essential, androgens alone are not sufficient to induce normal growth and function of the gland (1). Non androgenic hormones must also be considered to explain the proliferation of prostate cancer cells which escape anti-androgen therapy and become androgen unresponsive for growth (2). Following the observation that the involution of the prostate is greater after hypophysectomy and castration than after castration alone, pituitary hormones have been suspected to act on the gland (3). The importance of prolactin action on the prostate has been evidenced by numerous authors during the last 25 years (*e.g.:* 4–8). It is now established that normal rat prostatic cells are equipped with receptors for growth hormone (GH) (9–10) and for luteinizing hormone (Luteinizing hormone/chorionic choriogonadotropin-receptor: LH/CG-R) (11). In addition, a recent study has described the expression of the LH/CG-R in normal human prostates, benign prostatic hyperplasia and prostate carcinomas (12). If the physiological importance of GH for both growth and function of the prostate is already well documented (9–10, 13–14), the available data about the functionality and physiological role of the nongonadal LH/CG-Rs, are still scarce (15): in the rat prostate, LH stimulate cAMP accumulation in explant cultures, but the expression of classical marker genes such as prostatein, probasin and RWB was not modified by this hormone (11).

In the present study, we have used the differential display technique with an experimental model where the indirect effect of LH stimulation of testicular steroidogenesis were impossible (*ie:* the prostate of hypophysectomized and castrated adult rats treated with LH). Negative and positive control animals were treated with a solution of saline and testosterone propionate respectively. The aims of this study were: (i) to determine whether or not LH is able to regulate gene expression in the prostate; (ii) to compare the effects of LH to those mediated by testosterone in the same model; (iii) to identify genes that are susceptible to regulation by LH in non gonadal tissues.

MATERIALS AND METHODS

Animals and hormonal treatments. Normal Wistar rats, hypophysectomized at 1 year of age, were obtained from Charles River (St Aubin Lès Elboeuf, France). These rats were castrated 5 days after hypophysectomy. Treatments were initiated 3 days post-operatively. Animals were randomly divided into three groups and injected daily,

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for 7 days, with either saline (n = 5), 1 mg testosterone propionate (n = 5) or 10 μg hLH (n = 5). The animals were sacrificed 24 h after the last injection. Both the ventral and dorsal lateral prostate were removed and combined. Tissues were immediately stored at −70°C. Highly purified human LH was prepared in our laboratory according to already published method (8 and references therein). The biological potency for the preparation was 81000 IU/mg (International Standard 68/40). Cross contamination of hLH preparation with other pituitary hormones was measured by specific radioimmunoassays and radioreceptorassays (9). These measurements confirmed contamination levels systematically below 0.002% by weight of LH.

Differential display of RNA. The method that we have used was adapted from the original protocol of Liang and Pardee (16−17). Total RNA was extracted from tissue samples by the single-step guanidium-phenol-chloroform method (18). Aliquots of total RNA (50 μg) were incubated for 30 min at 37°C with 20 units of ribonuclease inhibitor (Promega, Madison, WI), 120 units of DNAse I (Boehringer, Mannheim, Germany) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. RNA samples were extracted with phenol/chloroform (3:1), ethanol precipitated in the presence of 0.3 M NaOAc, and were redissolved in diethyl pyrocarbonate-treated water. Reverse transcriptions were preformed at 37°C for 1 hour in the presence of: 0.3 μg of total RNA, 30 units of ribonuclease inhibitor (Promega), 20 μM dNTPs, 10mM DTT, 2.5 μM of one of the downstream primers (Tandil S.A., Paris, France; table 1) and 300 units of MoMuLV reverse transcriptase (GIBCO-BRL Europe, Ghent, Belgium), in a total volume of 30 μL. One μL of each cDNA preparation was then subjected to 40 cycles of polymerase chain reaction (PCR) with 30 sec at 94°C, 1 min at 40°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. The PCR mixture was as follows: 0.5 μM upstream primer (arbitrary decamer, Tandil; table 1), 2.5 μM downstream primer, 1.5 μM dNTPs, 0.1 μCi [α-^{33}P]dATP (NEN, Boston, MA), 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), and 1.5 mM $MgCl_2$ buffer. One-third of the final reaction volume was subjected to electrophoresis on a 6% nondenaturing polyacrylamide gel followed by drying of the gel and by autoradiography for 48 hours.

Isolation of differentially amplified products. Autoradiographs were aligned to their gels, and slices containing differentially amplified bands were cut from the gel. The slice was then soaked in 100 μL of water, boiled for 10 min and spun to remove debris. One third of this solution was re-amplified by PCR using the same primers and conditions as described above, except that 0.2 μM of both primers and 50 μM dNTPs were used with 2.5 units of AmpliTaq DNA polymerase. After visualisation on agarose gel electrophoresis, an aliquot (0.5 μL) of the amplified fragment was labeled by PCR amplification (20 cycles) in 10 μL of the mixture already described for the re-amplification, but with 30 μCi of [α-^{32}P]dATP (NEN) instead of unlabeled dATP.

Northern blot analysis. RNAs were denatured at 65°C for 15 min in buffer containing 45% formamide, 5.4% formaldehyde, and MOPS (morpholino-propane sulfonic acid) 1X, pH 7.0. The material was then separated through a 1% agarose gel containing 1.9% formaldehyde and transferred by means of a VacugeneTM apparatus (Pharmacia, Uppsala, Sweden) to a gene screen plus filter (NEN). The 0.24-9.5 RNA ladder (GIBCO-BRL) as well as 18S and 28S ribosomal RNAs were used as size markers. After baking at 80°C for 2 hours, the filters were prehybridized and hybridized at 42°C using the manufacturer's recommended mixtures. The hybridized blots were washed in 0.1 × SSC, 0.1% SDS at 52°C.

Autoradiography was carried out at −70°C with Hyperfilm β max and an intensifying screen (Amersham, Aylesbury, UK). The relative abundance of the various transcripts was quantified by scanning the autoradiograms (Ultrogel-Scan, LKB). The variability of both loading and transfer was monitored by ethidium bromide staining of the gel.

Sequence analysis. The PCR inserts which gave LH regulated transcripts after Northern blot analysis, were cloned into pCR II by TA cloning (Invitrogen, San Diego, CA). The obtained clones were screened again by Northern blotting. A clone corresponding to PCR insert n°6 and giving the same signal by Northern blotting was sequenced using an automatic DNA sequencer 373A (Applied Biosystem, Foster City, CA) with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystem) and the T7 or the SP6 primers. This sequence was compared to the GenBank and EMBL Bank using the Fasta program.

Competitive semi-quantitative RT-PCR. Five μg aliquots of total RNA were reverse transcribed into single strand cDNA using superscript reverse transcriptase (GIBCO-BRL Europe, Ghent, Belgium) and the oligo dT 12-18 (Pharmacia). The relative efficiencies of the reverse transcription steps were compared between the samples by amplifying a 411 bps β-actin insert (primer sequence see table 1) with an increasing number of cycles, followed by agarose gel electrophoresis. The reverse transcribed first strand cDNAs were amplified using the primers designed to correspond to the rat S 23 cDNA sequence 1 → 21 at the 5′-end and 456 → 430 at the 3′-end (18, table 1). One hundred microliters of PCR reaction mixture contained 1 pM of each oligonucleotide primer and 2.5 U Taq polymerase (Boehringer, Mannheim, Germany). The reaction was started at 96°C for 2 min and ran for 30 cycles (1 min at 96°C, 1 min at 54°C and 1 min at 72°C). The last extension was for 10 min at 72°C.

Aliquots of 10 μl were resolved on 2% agarose gels. The size of the DNA species generated was determined using a Hind III digest of

TABLE 1

Primers Used in the Study

Upstream primers	Downstream primers
A. Differential display	
N° 1 : 5′ **TACAACGAGG** 3′	N° 1 : 5′ **TTTTTTTTTTTAA 3′**
N° 2 : 5′ **GATCAAGTCC** 3′	N° 2 : 5′ **TTTTTTTTTTAC** 3′
N° 3 : 5′ **GATCTCAGAC** 3′	N° 3 : 5′ **TTTTTTTTTTAG** 3′
N° 4 : 5′ **GGTACTAAGG** 3′	N° 4 : 5′ **TTTTTTTTTTCA** 3′
N° 5 : 5′ **GATCACGTAC** 3′	N° 5 : 5′ **TTTTTTTTTTCC** 3′
N° 6 : 5′ **CTTTCTACCC** 3′	N° 6 : 5′ **TTTTTTTTTTCG** 3′
B. S 23	
5′ **ATGGGTAAGTGTCGTGGTCT** 3′	5′ **TATTCTGTTTCCATTGTCAAAATTTT** 3′
C. β-Actin	
5′ **CGTGAAAAGATGACCCAGAT** 3′	5′ **ATTGCCGATAGTGATGACCT** 3′

phage λ and a 1 kb DNA ladder (Gibco-BRL). The identity of the amplified product was controlled by restriction analysis with Alu 1 which generate 65, 180 and 250 bps fragments.

To generate a competitor to serve as the internal standard for S 23, a previously described lower stringency PCR method was used (20). Briefly, RT-PCR was carried out at a lower annealing temperature (35°C) to decrease the stringency of priming. This yielded multiple non specific products. As a competitor for the native S 23 message, a ± 200 bps fragment was selected, excised from the gel and reamplified with high stringency conditions for primer annealing (*ie:* 54°C). Because this competitor was generated using the S 23 primers, the sequence of the priming sites is identical to that of native S 23 message. Therefore, the ± 200 bps fragment is able to compete and serve as an internal standard for quantitation.

RESULTS AND DISCUSSION

In our animal model, hypophysectomized and castrated rats were treated with either LH, testosterone or saline in order to differentiate between the direct and indirect effects of LH on the prostate. When the differential display technique was performed using total prostatic RNA from these rats, it was seen that LH alone appeared to regulate certain genes.

Six upstream primers were combined to 6 downstream primers (table 1). Considering that a single differential display reaction generate about 150 different cDNA fragments (17), our screening theoretically involved 5400 different gene products (150 × 6 × 6). Among more than 250 cDNA fragments which appeared to be differentially regulated by LH, 48 were arbitrarily chosen for further studies. They were excised, reamplified in the presence of [α-^{32}P] dATP and individually analysed by Northern blotting. In most of the cases, no signal was observed, probably because of the low sensitivity of the Northern blotting when compared to PCR-based RNA analysis techniques.

Eight of these cDNA fragments displayed mean transcript levels which were significantly higher in the LH treated group than in the saline controls (figure 1, table 2). This demonstrate that the LH/CG-Rs expressed in rat prostate are functionally coupled to effector pathway(s). Curiously, none of the eight LH sensitive fragments was significantly regulated by testosterone suplementation (figure 1, table 2). It should be noted that, in these experiments, we have obtained cDNAs fragments which were regulated by testosterone, but not by LH (data not shown).

These data suggest that distinct signaling pathways are involved in the response of prostate cells to LH on the one hand and to androgens on the other hand. It has been shown that, in the gonads, the LH/CG-R is mostly coupled to the cAMP pathway (19). Therefore it is possible that the promotors driving the expression of genes corresponding to the transcripts regulated by LH, are equipped with response elements for cAMP (CRE), but not for androgens (ARE). An analysis of these clone's 5′ regulatory regions should be carried out to confirm or to reject this hypothesis.

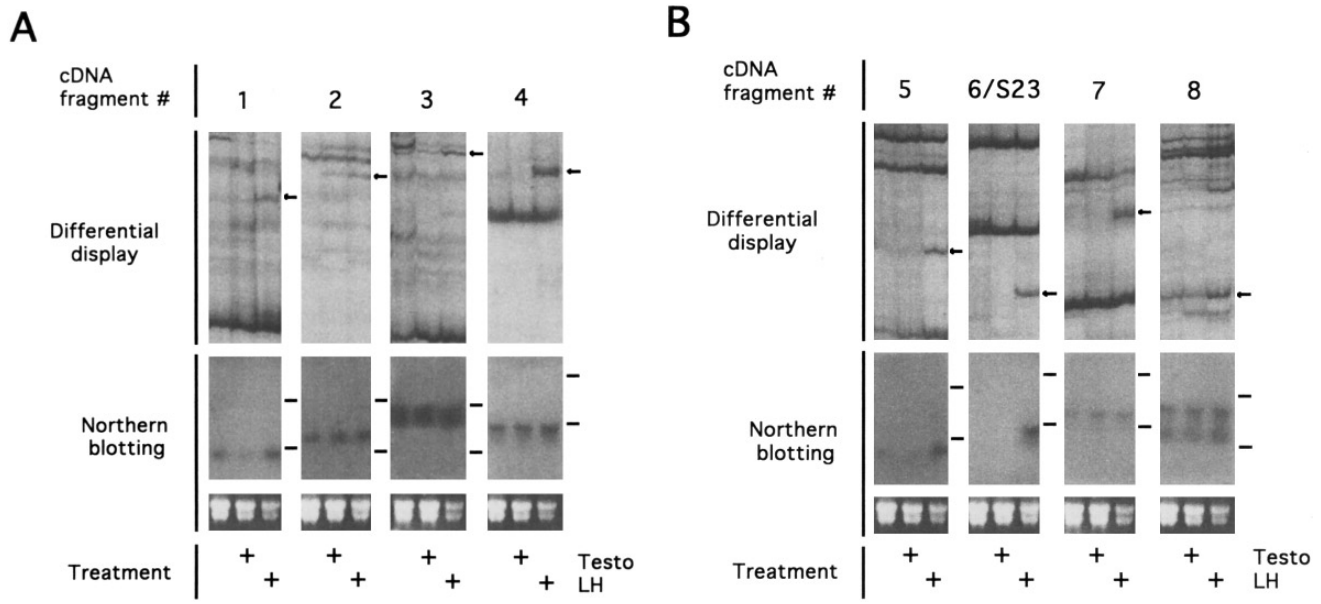

FIG. 1. Differential display and Northern blot analyses showing the 8 regulated cDNAs fragments in the prostate of saline, testosterone or LH treated rats. (A) cDNAs fragments N° 1, 2, 3 and 4 are indicated by the arrows on the differential display gels. The PCR amplification reactions were performed in the presence of [α-^{33}P] dATP. Samples were run on a 6% PAGE, under non denaturing conditions, then autoradiographed for 24 hours. Northern blots were carried out using the eluted differential display cDNA fragment and are shown below each differential display gel. 10 μg of total RNA, as estimated by spectroscopy, was loaded on each lane. The amounts of RNA loaded was confirmed by ethidium bromide staining of the gels. Positions of the 18 and 28 S rRNA are indicated by dashes on the right of each blot. (B) Differential display and Northern blot analyses of the cDNAs fragments N° 5, 6, 7 and 8.

The cDNA fragment n° 6 has been subcloned and partially sequenced. After comparison of the obtained sequence to existing data banks, it was found that this fragment displayed a 97% homology with 120 bps located in the 5′ region of the ribosomal protein S 23 cDNA (20).

In order to confirm that the transcript corresponding to the S 23 gene was positively regulated by LH in the rat prostate, we have cloned its full length cDNA, selecting PCR primers on both sides of the published coding sequence (20). The specificity of the amplification was assessed on the basis of the size of the amplify fragment and by restriction analysis (data not shown). This S 23 probe, when labeled by random priming and used in Northern blotting, displayed strong cross-hybridizations with 18 and 28 S rRNAs. For that reason, we have developed a competitive RT-PCR assay. The competitor was obtained by low stringency primer annealing as previously described (21–22).

Figure 2 clearly shows that the three different reverse transcription reactions had comparable yields (fig. 2A) and that the amount of S 23 cDNA was much higher in the LH group than in the saline and testosterone control groups (fig. 2B).

It is interesting to note that certain other ribosomal proteins have been implicated in cellular regulation *eg:* L7 has been shown to act as a transactivating factor and L5 forms part of P53/mdm2 complex (23–25). Therefore, the possibility that S 23 has a regulatory function, in addition to its role in the ribosomal machinery, should be investigated.

Although it has been shown that LH/CG-Rs are expressed in many nongonadal tissues, including normal human prostate, benign prostatic hyperplasia and prostate carcinomas; the functional roles of these extragonadal receptors have not yet been elucidated (12,15). S 23 could be an interesting marker of LH action in these newly identified target organs.

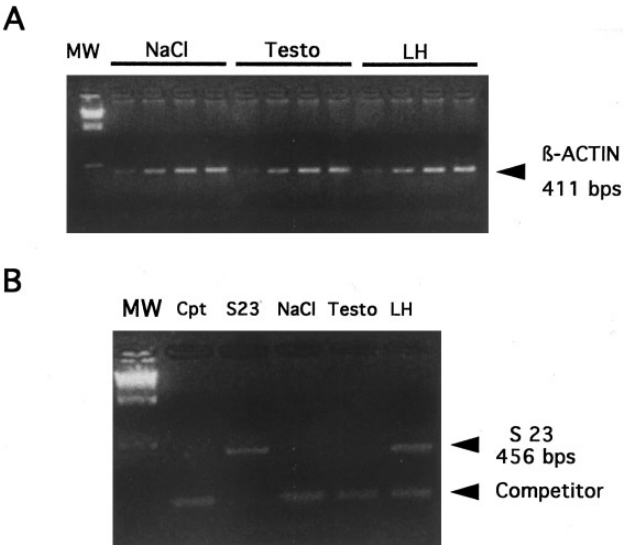

FIG. 2. Competitive RT-PCR to measure the effect of saline, testosterone or LH on S 23 mean transcrit level in the rat prostate. (A) Comparison of the reverse transcription efficiency and RNA integrity for the saline, the testosterone and the LH groups. A 411 bps β-actin insert was amplified, from 1 μl of the different reverse transcription reactions with an increasing number of cycles, followed by agarose gel electrophoresis. (B) 5 μl of each reverse transcription reaction were subjected to competitive RT-PCR. The competitor (Cpt) was generated by low stringency PCR carried out with the specific S 23 primers, as described in the materials and methods section. Subsequently, the same quantity (*ie:* about 10 pg) of the competitor were added to each reaction. The products were separated by 1% agarose gel and visualized by ethidium bromide staining of the gel. MW, Molecular weight marker was λ phage DNA digested with HindIII.

TABLE 2

Relative Abundance of mRNAs for the Differentially Regulated cDNA Fragments

Treatment	NaCl	Testo	LH
Clone N°			
1	1.0	0.7 ± 0.3	3.9 ± 1.0*
2	1.0	0.9 ± 0.2	2.8 ± 0.6*
3	1.0	1.4 ± 0.3	2.8 ± 0.9*
4	1.0	0.8 ± 0.4	3.2 ± 0.8*
5	1.0	0	8.5 ± 1.6*
6 = S23	1.0	0	9.5 ± 0.8*
7	1.0	1.0 ± 0.4	3.1 ± 0.5*
8	1.0	1.3 ± 0.4	2.5 ± 0.4*

* Northern blots shown in fig. 1 were quantified by densitometry. The values obtained were corrected according to the amount of 18S and 28S rRNAs. Data are presented in arbitrary units and represent means ± SE of 2 individual experiments (the results for NaCl-treated rats were arbitrarily taken as 1.0). Asterisk marked values were significantly different from the corresponding saline-treated control at the 5% level (Scheffe's test).

In conclusion, the increase in the abundance of various gene products after treatments of hypophysectomized and castrated rats with exogenous LH strongly suggests a physiologically significant role for the prostate LH receptor. Moreover, these effects of LH are, at least in part, distinct to those mediated by the androgens. The potential implication of LH in prostatic pathologies should therefore be investigated further.

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