Functional characterization of the basal promoter of the murine LH receptor gene in immortalized mouse Leydig tumor cells

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ABSTRACT

The nuclear proteins of the LH receptor (LHR) expressing murine Leydig tumor cells (mLTC-1), binding to the LHR primary promoter, were studied by gel retardation assays. Nuclear extracts of HeLa cells, not expressing LHR, were used as control. Protein binding was characterized to the first 173 base pairs (bp) of the LHR 5′-untranslated region, comprising the basal transcriptional promoter activity in mLTC-1 cells, and accounting for the Leydig cell-specific LHR expression. The promoter fragment is GC-rich and contains several Sp1 sites, one activating protein 2 (AP-2) site, and a putative SF-1 binding site. Three subfragments of the 173 bp promoter, I (bases −1 to −55), II (−56 to −102) and III (−103 to −173), were separately analyzed. Fragments II and III formed several complexes with mLTC-1 and HeLa cell nuclear extracts. One complex with fragments II and III, using mLTC-1 and HeLa cell extracts, was similar to that formed with purified Sp1, and it could be removed by an Sp1 oligo and supershifted by an Sp1 antibody. Both fragments formed additional complexes with mLTC-1 cell extracts with no specificity for Sp1. Partly similar, though weaker, complexes were seen with HeLa cell extracts. The most clearcut differences between the protein/DNA complexes formed with LHR expressing mLTC-1 cells and non-expressing (HeLa, COS, HEK 293 and MSC-1) cells were found with fragment I. Extracts of the non-expressing cells formed one prominent protein/DNA complex which was missing in mLTC-1 cells. Purified Sp1 also bound to this fragment. The fragment containing the putative SF-1 binding site did not form any protein/DNA complexes with mLTC-1 cell proteins. In conclusion, the murine LHR primary promoter binds, in addition to the Sp1 and AP-2 transcription factors, several other proteins. The Sp1 protein can bind into at least three different sites in the basal promoter. The other binding proteins differ most clearly between LHR expressing and non-expressing cells in the promoter fragment closest to the translation start site, suggesting a key role for this part of the promoter in cell-specific LHR expression.

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INTRODUCTION

Luteinizing hormone receptor (LHR) expression in the testis shows remarkable selectivity to Leydig cells, whereas in the ovary it is expressed in several cell types, including those of granulosa, luteal, thecal and interstitial cells. In both tissues, various hormones, growth factors and second messenger analogs can modulate the level of LHR expression. In particular, epidermal growth factor, phorbol esters and a high concentration of cAMP have been shown to down-regulate the numbers of LHR in Leydig cells and in differentiated granulosa cells (Ascoli 1981, Rebois & Fishman 1984, Rebois & Patel 1985, Wang et al. 1991a,b). Besides the gonads, low levels of LHR expression have been reported in a number of extragonadal tissues (Ziecik et al. 1986, Reshef et al. 1990, Lei et al. 1993, Reiter et al. 1995, Hämäläinen et al. 1999, Kero et al. 2000). Little is still known about the molecular basis of the restricted high level of LHR expression in specific gonadal cells.

The basal promoter region of the rat (Tsai-Morris et al. 1993) and mouse (El-Hefnawy et al.
1996) LHR genes has been localized to the first 173 nucleotides of the 5'-untranslated region (UTR). This domain induces maximal basal transcription of the LHR gene when transfected into cell types expressing and not expressing the endogenous LHR gene. Regions upstream of this fragment are important in repression of the promoter activity, by silencing the gene expression in non-expressing cell types and by regulating LHR gene expression in gonadal cells (Tsai-Morris et al. 1993, El-Hefnawy et al. 1996). The rat (Tsai-Morris et al. 1991) and mouse (Huhtaniemi et al. 1992) promoter sequences display a high degree of homology (about 75%). The promoter of the human LHR gene is at its 5'-flanking region somewhat less homologous with those of the mouse (66%) and rat (67%). The first 173 bp of the murine LHR promoter contains four potential binding sites for Sp1 (GGGCGG), a general transcription activator (Kadonaga et al. 1986), at nt (−50/−55), (−62/−67), (−79/−84) and (−113/−118). There is also an Sp1 binding site in reverse orientation at nt (−3/−8). However, the exact role of the four Sp1 elements in expression of the LHR gene is still unknown. In the rat basal promoter, two of the four Sp1 binding sites, located at nt (−77/−84) and nt (−135/−154), seem to be important for basal transcriptional activity of the LHR gene (Dufau et al. 1995). In addition to the Sp1 binding sites, the murine LHR primary promoter has the consensus element for activating protein 2 (AP-2) located at nt (−52/−59). The AP-2 binding domain is known to be responsive to cAMP and protein kinase C activation (Imagawa et al. 1987), and in the murine LHR promoter it slightly overlaps with one of the Sp1 binding sites. Interestingly, the LHR basal promoter contains no consensus cAMP or steroid receptor binding sites although LHR is known to be regulated by these substances. On the other hand, a potential SF-1 binding site (CCCAGGTCA), implicated in promoter regions conferring basal and cAMP regulation of several CAMP responsive genes in the gonad and adrenal (Sugawara et al. 1997, Ito et al. 2000), is located at nt (−159/−167).

In a previous paper (El-Hefnawy et al. 1996), we studied the transcriptional activity of various lengths of the murine LHR promoter in transiently transfected mouse Leydig tumor cells (mLTC-1). We also showed that the first 173 nt promoter region binds several nuclear proteins from both LHR expressing (mLTC-1) and non-expressing (HeLa) cells. We have now characterized in more detail the nuclear proteins binding to the primary promoter using electromobility shift assays (EMSA) and shown that, in addition to Sp1 and AP-2 transcription factors, the basal promoter region binds other proteins that are different in LHR expressing and non-expressing cells in the part of the promoter adjacent to the translation start site.

**MATERIALS AND METHODS**

**Plasmid construction and isolation of DNA fragments**

The primary promoter fragment of the 5'-UTR of the murine LHR gene (Huhtaniemi et al. 1992) (the first 173 bp upstream of the translation start site) was released from the pLHR (−173/−1)–luciferase gene construct (El-Hefnawy et al. 1996) using BamHI and XhoI restriction endonucleases (Promega, Madison, WI, USA). This restriction fragment was then divided into three smaller fragments by digesting first with XmaI (Promega) and then isolating the fragments generated by agarose gel electrophoresis, and finally by digesting the remaining larger fragment with GscuI (Sigma, St Louis, MO, USA). The fragments obtained were as follows: I, nt (−1/−55); II, nt (−56/−102); and III, nt (−103/−173) (Fig. 1).

**Cell culture**

The mLTC-1 cell line (Rebois 1982) was cultured in HEPES (20 mmol/l)-buffered Waymouth’s medium (Gibco, Paisley, Strathclyde, UK), supplemented with 9% heat-inactivated horse serum (Gibco) and 4.5% heat-inactivated fetal calf serum (FCS; Biochlear, Wilts, UK) and 50 mg/l gentamycin (Biological Industries, K B Haemek, Israel). The HeLa cells as well as the other LHR non-expressing COS 7 (monkey embryonic kidney), HEK 293 (human embryonic kidney) and MSC-1 (mouse Sertoli) cells were cultured in Dulbecco’s modified Eagles’s medium/F12 (1:1) supplemented with 10% heat-inactivated FCS and 50 mg/l gentamycin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

**Preparation of nuclear extracts and EMSAs**

The nuclear extracts containing transcription factors were prepared from different cells essentially as previously described (Hurst et al. 1990). Proteinase inhibitors (phenylmethysulfonyl fluoride, 0.5 mmol/l; leupeptin, 2 mg/l; soybean trypsin inhibitor, 2 mg/l) and dithiothreitol (DTT; 0.5 mmol/l) were added to all buffers before use. After dilution (to reduce the KCl concentration to 100 mmol/l), the nuclear extracts were frozen in
small aliquots in liquid nitrogen and stored at −70 °C. Protein concentrations of the nuclear extracts were determined by the method of Bradford (1976), using bovine serum albumin as standard.

For the EMSA, the BamHI/XhoI digested fragment of the pLHR (−173/−1)–luciferase construct, nt (−1/−173), and the subfragments I, nt (−1/−55), II, nt (−56/−102) and III, nt (−103/−173), were end-labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham International plc, Amersham, Bucks, UK) using the Klenow large fragment of DNA polymerase I (Promega). The reaction mixture contained also 2 µg synthetic oligonucleotides. Oligonucleotides were annealed and the 5′ overhangs were labeled with [α-32P]dCTP using Klenow DNA polymerase. Binding reaction mixture (20 µl) contained 10 µg nuclear extract protein in 12 mmol/l HEPES (pH 7·8), 60 mmol/l KCl, 12% glycerol, 4 mmol/l Tris–Cl, 1 mmol/l EDTA and 1 mmol/l DTT. After preincubation on ice for 15 min, approximately 1 ng of the labeled DNA fragment (about 107 c.p.m./µg) was added to the reaction mixture and the incubation was continued at room temperature for 30 min. The protein-bound DNA complexes were separated from free probe immediately after incubation on a 5% polyacrylamide gel, run in low ionic strength buffer 0·25 × TBE (1 × TBE: 90 mmol/l Tris base, 90 mmol/l boric acid and 2 mmol/l EDTA). After electrophoresis, the gel was dried and subjected to autoradiography on Kodak X-ray film at −50 °C for 48 h.

To study the protein/DNA binding specificity, 1- to 320-fold molar excess of nt (−1/−173) promoter fragment was included in the reaction as a non-labeled competitor. For characterization of bound proteins, the nuclear extracts were incubated in the presence of about 1000-fold molar excess of synthetic oligonucleotide recognition sites for Sp1 or AP-2 transcription factors (Promega), or with polyclonal antibodies to human Sp1 or AP-2 proteins (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For studying Sp1 or AP-2 transcription factor binding, these purified proteins (Promega) were incubated with the labeled promoter fragments in the presence and absence of a 1000-fold molar excess of the corresponding Sp1 or AP-2 oligo recognition sites or polyclonal antibodies respectively.

To explore protein binding to the potential SF-1 binding sequence, complementary oligos for original LHR–SF-1 site (sense: 5′-gggCACAGT CCCAGGTCAGAGGA and antisense: 5′-gggTCC TTGACCTGGGACTGTG) and for that mutated to consensus–SF-1 site (sense: 5′-gggTGGCCACA GTTCAAGGTCAGAGGAGA and antisense: 5′-gggTTCTCTCTAGCTGGAACTGTGGGCCA) were generated (SF-1 consensus binding site is underlined and mutated nucleotide is emboldened). Oligonucleotides were annealed and the 5′-ggg overhanges were labeled with [α-32P]dCTP using Klenow DNA polymerase. Binding reaction, including labeled oligonucleotides and nuclear extracts from mouse Leydig tumor cells, were carried out in the presence of 50- or 200-fold molar excess of unlabeled original or consensus SF-1 oligonucleotides.

**RESULTS**

**Protein binding to the entire 173 bp primary promoter**

In a previous paper, we showed that the 173 bp LHR primary promoter forms several protein/DNA complexes with the LHR expressing mLTC-1 and non-expressing HeLa cell extracts (El-Hefnawy...
et al. 1996), and that these complexes could be completely displaced by an unlabeled 173 bp promoter fragment, indicating specificity of the protein/DNA interaction. The 173 bp promoter fragment bound the purified Sp1 protein and formed with it one major complex with rather low mobility (Fig. 2A). The binding of purified Sp1 protein was prevented by preincubation with a 1000-fold molar excess of Sp1 oligonucleotide or AP-2 polyclonal Ab. The supershifted Sp1/DNA/Ab or AP-2/DNA/Ab complexes are shown by arrows.

Protein binding to promoter fragments II and III
The restriction fragments of the 173 bp primary promoter, fragment II, nt (-56/-102) and fragment III, nt (-103/-173) were then analyzed. Fragment II contains two potential Sp1 binding sites and fragment III has one Sp1 binding site (Fig. 1). The AP-2 binding site is destroyed upon digesting the 173 bp fragment with the XmaI restriction enzyme and, for that reason, AP-2/DNA complexes with fragments II and III could not be seen (Figs 3 and 4). Fragments II and III formed some protein/DNA complexes that differed between the mLTC-1 and HeLa cell extracts (Fig. 3A,B and Fig. 4A,B). One of the complexes with both LHR expressing mLTC-1 and non-expressing HeLa cells was similar in mobility to that caused by the Sp1 protein (Fig. 3C and Fig. 4C). This complex could be removed by 1000-fold molar excess of Sp1 ds oligonucleotide and supershifted by Sp1 Ab, in the same way as the complex with the purified Sp1 protein. Fragment II formed with both cell extracts another weaker complex with a somewhat faster mobility, and this could be displaced by the Sp1 oligo but not by the Sp1 Ab (Fig. 3A,B).
II formed also with purified Sp1 protein this weaker complex which now could be removed by the Sp1 oligo and Ab.

After incubating fragment II with the mLTC-1 cell extract, two strong complexes with faster mobility could be seen (Fig. 3), and they could not be removed or supershifted by Sp1 oligo or Sp1 Ab. Much weaker complexes with similar mobility could also be seen with HeLa cells. Fragment III, most distal to the translation start site, also formed with the mLTC-1 cell extract two stronger complexes with quite fast mobility, and some weaker complexes with slower and faster mobilities, as compared with the two stronger complexes. None of these could be removed by Sp1 oligo or Sp1 Ab. In HeLa cells, these complexes were weaker and some of them were missing, or differed in mobility from those of the mLTC-1 cells. As expected, the purified AP-2 protein did not bind to this fragment (Fig. 5D).

Protein binding to promoter fragment I

For separation of fragment I, nt (−1/−55), the 173 bp basal promoter was digested with XmaI which destroyed the AP-2 binding site but preserved the Sp1 binding site, nt (−50/−55), at the end of the fragment. At the 3’-end of fragment I there is an Sp1 binding site, nt (−3/−8), in reverse orientation. With this fragment, the most clearcut differences were seen between the protein/DNA complexes formed by the mLTC-1 and HeLa cell extracts (Fig. 5A,B). These complexes were, however, not removable by Sp1 oligo or Sp1 Ab.

The purified Sp1 protein also formed a complex with this fragment, and Sp1 oligo and Ab could prevent the formation of this complex (Fig. 5C). As expected, the purified AP-2 protein did not bind to this fragment (Fig. 5D).

Because the clearest differences in protein binding profiles between mLTC-1 and HeLa cells were found with fragment I, we found it interesting to compare the protein binding profiles from different LHR non-expressing cells (COS, HEK 293 and MSC-1 Sertoli cells) with that of mLTC-1 cells. The most obvious difference was a strong protein/DNA complex that was present in the cells not expressing LHR, but absent in the mLTC-1 cells (Fig. 6). The distribution of the other complexes was quite similar in the mLTC-1 and control cells. In HEK 293 cells there was one complex with low mobility that was not seen in other cell types, and...
The protein/DNA complex distribution in both testis-derived cell lines (mLTC-1 and MSC-1) was very similar regardless of the above-mentioned strong complex missing in mLTC-1 cells.

The putative SF-1 binding site is not functional on LHR promoter

Specificity of a putative SF-1 binding site located at nt (−159/−167) in the LHR promoter was studied by EMSA. No binding to the original LHR sequence was seen while the LHR sequence mutated to the consensus SF-1 site showed clear binding (Fig. 7). When consensus SF-1 oligonucleotides were used as a competitor this complex disappeared, while no competition was seen with original LHR oligonucleotides.

DISCUSSION

The 5′-flanking region of the mouse LHR gene displays considerable sequence similarity with that...
of the cognate rat gene (Huhtaniemi et al. 1992). Both promoters lack the TATA and CAAT elements, contain the three initiator elements adjacent to the translation initiation codon, and one of the two Sp1 elements (Sp12, $(-79/-84)$) was demonstrated to be functional in the rat promoter (Tsai-Morris et al. 1994). In contrast to the rat, the major transcriptional initiation site of the mouse gene was localized at position $-310$ but also other minor sites were found in that region (Huhtaniemi et al. 1992).

In the rat LHR gene, the transcriptional activity is dependent on two of the four Sp1 binding domains, Sp11 nt $(-77/-84)$ and Sp14 nt $(-135/-154)$, each contributing equally to the transcriptional activity (Tsai-Morris et al. 1993, 1994, Dufau et al. 1995). In addition, the rat LHR promoter has been shown to be regulated by at least three other domains, C-box bp $(-42/-73)$, M1 bp $(-24/-42)$ and R bp $(-1266/-1307)$, which bind multiple factors in a tissue-specific manner (Tsai-Morris et al. 1994, Dufau et al. 1995).

The LHR gene in mLTC-1 cells appears to be constitutively repressed and requires specific activation (Tsai-Morris et al. 1994). The LHR is induced by cAMP (LaPolt et al. 1990, Segaloff et al. 1990) but no consensus cAMP response elements (upstream of the transcription start sites) can be found in the promoter. Instead, the rat LHR primary promoter has an AP-2 response element, which is known to be responsive to both cAMP and protein kinase C (Imagawa et al. 1987). Evidence for AP-2 regulation of the rat LHR basal promoter was provided by Nelson et al. (1994) who showed, in gel shift assay, the binding of purified AP-2 protein to the rat LHR primary promoter and, in DNAse I footprinting assay, protein extracts from MA-10 Leydig cells and MSC-1 Sertoli cells that protected a region $(-68$ to $-50)$ that encompasses the AP-2 site.

Deletion studies have identified a minimal fragment $(-40$ to $-70)$ in the rat LHR 5'-flanking region that responds to cAMP with decreased transcription (Nelson et al. 1994). This fragment contains an AP-2 binding site, which also could mediate the cAMP response. Functional data, however, indicated that the effect of cAMP was not mediated by the AP-2 site (Nelson et al. 1994). We have shown that the purified AP-2 protein binds to the basal promoter, forming two complexes with slightly different electrophoretic mobilities. These complexes could be removed by AP-2 oligonucleotide and supershifted by AP-2 antibody. This indicates that the AP-2 protein specifically binds to its recognition sequences, as was corroborated by the lack of AP-2 binding to fragments II and III after the AP-2 binding sequence was destroyed by XmaI digestion. Thus, one of the cell extract proteins binding to the 173 bp promoter is likely AP-2.

The first 173 nt of the mouse LHR promoter contain, in addition to an AP-2 binding site, four potential Sp1 binding domains (GGGCGG) located at nt $(-50/-55)$, $(-62/-67)$, $(-79/-84)$ and $(-113/-118)$, as well as an Sp1 binding domain in reverse orientation at nt $(-3/-8)$ (Fig. 1). Also a potential SF-1 binding site (CCCCAGTCTCA) conferring basal and cAMP regulation of several cAMP responsive genes in gonad and adrenal (Sugawara et al. 1997, Ito et al. 2000) is located at nt $(-159/-167)$. In gel shift assays with the whole 173 bp fragment, there were several shifted protein/DNA complexes by both LHR expressing and non-expressing cells. The most probable binding factor is undoubtedly the transcription factor Sp1. Surprisingly, none of the several complexes with mLTC-1 or HeLa cell extracts using the entire 173 bp fragment could be displaced by the Sp1 oligo even in a 1000-fold molar excess, or displaced or supershifted with the Sp1 Ab (results not shown).

The purified human Sp1 protein bound to the 173 bp promoter fragment and formed a clear complex which was displaced by Sp1 oligo and supershifted by Sp1 Ab. When this 173 bp fragment was divided into three subfragments, the purified Sp1 protein was found to bind to all of them. Also, one of the protein/DNA complexes, formed with fragments II and III using mLTC-1 and HeLa cell extracts, could be displaced by the Sp1 oligo and supershifted by the Sp1 Ab. Fragments I–III also formed several other protein/DNA complexes, not displaced by the Sp1 oligo or Ab, indicating that additional protein(s) can bind to various sites in the LHR primary promoter. As shown by the rat LHR promoter, the Sp1 protein only binds to the Sp1 and Sp14 sites (Tsai-Morris et al. 1994). In the mouse promoter, there is an Sp1 binding site at nt $(-79/-84)$ corresponding to the rat Sp12 site at nt $(-77/-84)$. This is located in fragment II, which binds to the purified Sp1 protein forming a strong protein/DNA complex, also displaced by Sp1 oligo and supershifted by Sp1 Ab. Fragment II contains another Sp1 binding site at nt $(-62/-67)$ and we cannot exclude the possibility that it also binds Sp1. The other Sp1 binding site in the rat promoter, Sp14 at nt $(-135/-154)$, cannot be found in the mouse promoter. The mouse 173 bp promoter contains the most upstream Sp1 binding site at nt $(-113/-118)$. This is located in fragment III (nt $-103$ to $-173$) which also formed a clear complex with the Sp1 protein. Moreover, fragment III contains the potential SF-1 binding site.
CCCAGGTCA at nt (–159/–167) with only a single nucleotide (underlined) difference from the consensus SF-1 binding sequence (PyCAAGGPyCPu) (Morohashi et al. 1992). However, by using a fragment containing the SF-1 binding site as probe we could not see any mLTC-1 cell protein binding (Fig. 7). By mutating C to A in the SF-1 core sequence we generated a consensus SF-1 site which had the ability to form protein complex. It seems therefore that the SF-1 core sequence CAAGG is crucial for SF-1 binding.

The clearest differences in protein/DNA complexes between the mLTC-1 and HeLa cell extracts were found in the most proximal fragment I, nt (–1/–55), which seems to bind unknown proteins in both LHR expressing and non-expressing cells. Interestingly, with this fragment all the LHR non-expressing cells formed one very clear protein/DNA complex, which was lacking in LHR expressing mLTC-1 cells (Fig. 6). The protein(s) involved in this complex may be related to the absence of LHR expression in these cells, and they may be related to the repression of LHR expression. Fragment I contains an Sp1 recognition sequence at nt (–50/–55) at the end of the fragment and another one in reverse orientation at nt (–3/–8). This fragment also formed a complex with purified Sp1 protein, and it could be displaced by Sp1 oligo and recognized by Sp1 Ab. Since most transcription factors function in an orientation-independent manner, the complementary sequence also represents a specific binding site (Faisst & Meyer 1992). Fragment I formed several protein/DNA complexes with the mLTC-1 and also HeLa cell extracts. However, none of these complexes could be recognized by Sp1 oligo or Ab.

Transcription factor AP-2, the molecular weight of which is about 50 kDa, binds as a dimer to a palindromic binding site and it has also been shown to bind to the Sp1 (and nuclear factor–1 and similar virus–40) binding site (Faisst & Meyer 1992). We found that AP-2 was unable to bind to any of the three promoter subfragments (I–III). Thus it seems that in the LHR primary promoter AP-2 does not bind to Sp1 sites but specifically to an AP-2 binding domain, as we showed that purified AP-2 binds to the entire 173 bp fragment but not to the three subfragments after its binding site was destroyed by XmaI digestion.

Also several other transcription factors, such as BTEB (Imataka et al. 1992, Sogawa et al. 1993), ETP (Kageyama et al. 1989), GCP-1 (Kageyama & Pastan 1989) and LSF (Huang et al. 1990), have been reported to bind to GC boxes. The Sp1 protein can be distinguished from GC-binding non-Sp1 proteins with Sp1 Ab treatment and thus by the formation of immunological supershifts or disappearance of protein/DNA complexes. In this study, the supershift formation of protein/DNA complexes with mLTC-1 and HeLa cells was seen with fragments II and III but not with fragment I which, nevertheless, bound the purified Sp1 protein. Thus it seems that at least the Sp1 binding sites in fragments II and III bind Sp1 protein both in LHR expressing and non-expressing cells. The binding of the non-Sp1 proteins to some of the LHR primary promoter Sp1 binding sites, or their competition with Sp1 for these recognition sequences, and hence their possible role in cell-specific expression of the LHR gene, cannot be ruled out.

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