Cis-acting elements regulating the placenta-specific promoter of the bovine Cyp19 gene

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**ABSTRACT**

Cyp19 encodes aromatase cytochrome P450, the key enzyme of oestrogen biosynthesis. In the bovine placenta, the majority of Cyp19 transcripts include a 5′ untranslated region which is encoded by exon 1.1; this suggests that its 5′-flanking region is the predominant placental promoter. The aim of the present investigation was to examine the promoter activity of this region and to map cis-acting regulatory elements in order to improve our understanding of the complex regulation of this gene within the placenta. As an initial approach, human JEG-3 choriocarcinoma cells were transiently transfected with reporter-gene constructs consisting of different 5′-flanking sequences of exon 1.1 fused to the luciferase gene as a reporter. To localise and further characterise functional cis-acting elements, targeted point mutations and electrophoretic mobility-shift experiments were used. The data demonstrate, for the first time, (1) that the bovine exon 1.1 5′-flanking sequence is an active promoter, (2) that 404 bp of this region are sufficient for constitutive reporter-gene expression in JEG-3 cells and (3) that the region includes at least two different enhancer elements; the data also suggest (4) that one of these elements consists of the E-box motif CATGTG and that the second enhancer element includes the half-site hexameric sequence AGGTCA and additional nucleotides flanking this element upstream.

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**INTRODUCTION**

Oestrogen biosynthesis is catalysed by the enzyme aromatase cytochrome P450, which is encoded by Cyp19. Most species investigated express Cyp19 in the gonads and the brain (Harada et al. 1992, Lephart et al. 1992, Nitta et al. 1993). However, in rabbits, primates and several ungulate species Cyp19 transcripts were also found in the placenta (Simpson et al. 1994, Corbin et al. 1995, Hinshelwood et al. 1995, Fürbass et al. 1997, Delarue et al. 1998, Vanselow et al. 1999). The expression of Cyp19 is regulated by several promoter regions with tissue-specific preferences. In combination with alternative splicing, this results in the generation of Cyp19 transcript variants with different 5′ untranslated regions (5′UTRs) but identical coding regions (Means et al. 1991, Hinshelwood et al. 1995, Honda et al. 1996, Fürbass et al. 1997, Vanselow et al. 1999). The ovary and the brain express Cyp19 transcripts with different 5′UTRs. However, those tissue-specific transcript variants are conserved among mammalian species, indicating that homologous ovary- and brain-specific promoters are active in various species. In contrast, 5′UTRs of placental Cyp19 transcripts do not show any sequence similarities among species (Harada et al. 1993, Simpson et al. 1994, Hinshelwood et al. 1995, Vanselow & Fürbass 1995). Even species as closely related as cows and sheep express different transcripts in the placenta (Vanselow et al. 1999), suggesting that different, completely unrelated placenta-specific promoters of Cyp19 are used in different species. The locations of these placenta-specific promoters were also found to be very different. Whereas the human and bovine promoters are located considerably distal (40 and 20 kb respectively) from the start of translation (Means et al. 1989, Brunner et al. 1998), the location of the sheep promoter is quite proximal to this start site, almost overlapping ovary-specific promoter 2 (Vanselow...
The data presented here demonstrate that the bovine 5'-flanking region of exon 1.1 is an active promoter in JEG-3 cells. Evidence will be provided to show that promoter activity crucially depends on the E-box element at position −340 bp and on a second enhancing sequence that overlaps the hexameric element at −268 bp.

MATERIALS AND METHODS

Construction of reporter-gene plasmids

Different fragments of bovine Cyp19 promoter 1.1 were designed according to the positions of putative regulatory elements that have been identified using factor software (EMBL Data Library, Heidelberg, Germany), as described by Fürbass et al. (1997), or using MatInspector software (Quandt et al. 1995). Fragments were amplified from bovine genomic DNA using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). Seven different forward primers at positions −1997, −1071, −907, −553, −404, −309 and −170 bp were combined with a reverse primer at position +113 bp (position numbers throughout this paper indicate the 5'-ends of primers or sequence motifs relative to start site 2 of the transcription of exon 1.1; Fürbass et al. (1997)). All primers included a synthetic KpnI site at the 5'-end to allow cloning of the fragments into the KpnI site of the luciferase vector pGL3 Basic (Promega, Heidelberg, Germany).

Site-directed mutagenesis was performed using construct −404 (Fig. 1) with the GeneEditor System (Promega). The E-box motif E1 at −56 bp (see Figs 1 and 2) was mutated with the oligonucleotide 5'-CATCCCTCTGGGGCAAGTGGTGAATGCATCAT-3', the E-box motif E2 at −340 bp was mutated with the oligonucleotide 5'-GCTCAAGAGGTCAGTTGCCCCAATCCCTT-3', the hexameric (H) sequence H1 at −268 bp was mutated with the oligonucleotide 5'-AACCCACTGTTTGAATTCAAGTGCCATT-3' and the hexameric sequence H2 at −344 bp was mutated with the oligonucleotide 5'-CCACAGTCATAGGCT-3'. The human JEG-3 choriocarcinoma cell line was used as an in vitro model for trophoblast-specific gene expression.

The CMV/lacZ control plasmid was constructed by first inserting an SpeI/HindIII fragment from pCDM8 (Invitrogen, Groningen, The Netherlands), including the CMV-promoter, into pBluescript II KS (Stratagene, Heidelberg, Germany). Secondly, it was released from pBluescript as a NotI/HindIII fragment and cloned into NotI/HindIII of the lacZ vector described by Vanselow et al. (1994).
The fidelity of all constructs was verified by sequencing the inserted wild-type or mutated promoter fragments from both directions with primers flanking the KpnI cloning site of the pGL3 vector. Sequencing was performed using an automated sequencing system (ABI PRISM 310 Genetic Analyser/ABI PRISM BigDye Kit; PE Biosystems, Weiterstadt, Germany).

Cell culture, transient DNA transfection and determination of reporter-gene activity

Human JEG-3 choriocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in MEM (Eagle’s medium with Earle’s salts and non-essential amino acids) supplemented with 1 mM sodium pyruvate and 10% foetal calf serum (Biochrome, Berlin, Germany).

Cells were transfected by electroporation with the RF-module (Bio-Rad Laboratories, Munich, Germany) using cuvettes with 2 mm electrode gap sizes. The following electrical parameters were determined empirically: field strength, 1.2 kV/cm; modulation, 100%; frequency, 40 kHz; number of pulses, 5; time interval, 1 s; pulse duration, 2 ms. Prior to electroporation, cells were grown to approximately 80% confluence and treated with 0.05% Trypsin/0.02% EDTA solution (Biochrome).
and washed twice in MEM with serum. Usually 4 × 10⁶ trypsinised cells were mixed with 10 µg reporter plasmid (pGL3) in 400 µl buffered sucrose solution (272 mM sucrose, 7 mM sodium phosphate, 1 mM MgCl₂, pH 7.4). The exact amount of plasmids which included promoter fragments was corrected for their molecular weight to provide equal numbers of molecules in all samples; 0.5 µg CMV/lacZ plasmid was co-transfected to normalise for transfection efficiency. All samples were filled up with sheared herring-sperm DNA to a total of 100 µg DNA. Ten minutes after electroporation, the cells were washed twice in MEM with serum and subsequently incubated at 37°C with 5% CO₂.

The activities of reporters (luciferase, β-galactosidase) were measured 48 h after electroporation, using the DualLight Kit from Tropix (PE Biosystems) according to the manufacturer’s instructions. The luminescence signals were measured as relative light units using a luminometer (Lumat LB 9501; Berthold, Wildbad, Germany). In order to correct the measurements for transfection efficiency the luciferase activity was divided by the β-galactosidase measurement from the same sample.

The activities of different promoter fragments (promoter activity; see Fig. 1) were calculated from the corrected luciferase activity relative to the background activity of the promoterless pGL3 vector. All series of experiments were repeated at least three times. One-way ANOVA statistics was performed using the program SIGMA STAT (Jandel Corporation); differences were considered to be significant at P<0.05.

**Electrophoretic mobility-shift assay (EMSA)**

Nuclear extracts were prepared from human JEG-3 choriocarcinoma cells maintained in MEM with serum essentially as described by Andrews & Faller (1991). Double-stranded oligonucleotides containing wild-type sequences were labelled at their 5’-ends using [³²P]ATP and T4 polynucleotide kinase (Sambrook et al. 1989). Binding reactions contained 35 fmol labelled DNA, 10 µg nuclear extract in a total volume of 20 µl binding buffer (20 mM Tris/HCl pH 7.9, 70 mM KCl, 1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 0.01 mg/ml poly d[I-C], 10% glycerol). To prove the specificity of binding, complexes were competed with a 50-fold molar excess of unlabelled double-stranded oligonucleotides with wild-type or mutated binding motifs. After incubation at 20°C for 20 min, samples were subjected to electrophoresis through non-denaturing 6% polyacrylamide 0.5 × TBE (45 mM Trisbase, 45 mM boric acid, 1 mM EDTA) gels. A phosphor imager (STORM 840; Molecular Dynamics, Krefeld, Germany) and IMAGE QUANT software were used for the analysis.

**RESULTS**

**Deletion analysis of the exon 1.1 5’-flanking region**

A deletion series was generated by a PCR in order to map the positions of active regulatory elements. Different forward primers that had been designed...
according to the locations of putative regulatory elements (Fig. 1) were used in combination with a reverse primer, providing that the fragments included all the transcription start sites of promoter 1.1 (Fürbass et al. 1997). The fragments were cloned into pGL3 upstream from the luciferase reporter gene. All constructs of the deletion series were transiently transfected into JEG-3 cells together with the CMV/lacZ control plasmid by electroporation. Constructs including promoter fragments −1071, −1071, −907, −553 and −404 showed 16- to 24-fold enhanced activity relative to that obtained with the promoterless pGL3 vector (Fig. 1). The differences between these constructs were not significant. In contrast, the promoter activity of fragment −309 was significantly reduced to 9, whereas that of fragment −170 was barely above background. This demonstrates that the sequence from −170 to −404 bp of the promoter 1.1 region is necessary for high-level constitutive expression of reporter-gene constructs in JEG-3 cells and that this region contains at least two different cis-acting elements which are destroyed or removed by deleting the sequences upstream from positions −170 and −309 bp respectively. As a control for directed promoter activity, fragment −1071 was tested in both orientations. In its complementary orientation (−1071c) no activity was detectable, whereas in its natural orientation fragment −1071 showed the highest activity relative to all other constructs. In order to screen for serum effects on reporter-gene expression, JEG-3 cells transfected with construct −1071 were incubated at different serum concentrations (0, 1, 3 or 10%) 24 h after electroporation. None of these concentrations changed promoter activity greatly (data not shown).

Mutagenesis of putative regulatory elements

Construct −404 bp, which proved to be sufficient for strong constitutive expression, was mutated within the consensus sequences of different putative regulatory elements in order to determine which of these elements mediates enhancing activity and thus plays a functional role in promoter activity. Four different constructs were generated with targeted mutations of the E-box elements E1 at −56 bp and E2 at −340 bp and of the half-site hexameric sequences H1 at −268 bp and H2 at −334 bp. All constructs were transiently transfected into JEG-3 cells and their expression evaluated 48 h after electroporation. The luciferase measurement for each sample was corrected for β-galactosidase activity. As shown in Fig. 2, the mutation of elements H2 and E1 did not change the reporter-gene expression, whereas alterations to consensus sequences E2 and H1 significantly reduced the promoter activity to approximately 50%.

Binding of nuclear factors to the exon 1.1 5’-flanking region

Reporter-gene experiments suggested that both the E-box element E2 and the half-site hexameric element H1 might mediate enhancing activity. Therefore the protein-binding capacity of both elements was analysed in competition EMSA experiments. Radiolabelled double-stranded oligonucleotides including potential binding motifs were incubated with nuclear proteins from JEG-3 cells in the presence of unlabelled double-stranded competitor oligonucleotides including either wild-type or mutated binding motifs and were then subjected to polyacrylamide gel electrophoresis. With oligonucleotides including the overlapping H2 and E2 motifs at positions −334 and −340 bp, a strong protein/DNA complex was observed (Fig. 3a, lane 2) which could be removed by competition with an excess amount of identical, but unlabelled, oligonucleotides (Fig. 3a, lane 3). Competition was also very efficient with an unrelated oligonucleotide that included the E1 motif of position −56 bp (Fig. 3a, lane 5). This indicates that these almost completely unrelated oligonucleotides which share only the same E-box motif bind the same protein(s) and thus are perhaps able to form the same protein/DNA complex. In contrast, the complex was not removed by oligonucleotides that are identical to the labelled oligonucleotide probes except for the completely altered E2 binding motif, indicating that the protein-binding capacity of these oligonucleotides was virtually eliminated by the mutation (Fig. 3a, lane 4). The mutation of only the central nucleotides of the E2 motif, however, which was sufficient to considerably reduce promoter activity in reporter-gene assays (E2 mut see Fig. 2), did not significantly alter protein-binding capacity (not shown).

Oligonucleotides that included the hexameric H1 motif as a probe also produced a strong complex with JEG-3 nuclear proteins (Fig. 3b, lane 2) which could be competed with identical but unlabelled oligonucleotides (Fig. 3b, lane 3). However, the complex was still efficiently reduced even when the H1 motif was entirely mutated (Fig. 3b, lane 4), indicating that this sequence still competes for the same DNA-binding proteins. This is consistent with the observation that oligonucleotides carrying the same mutation which significantly reduced reporter-gene expression are still efficient competitors for complex-forming protein(s) (Fig. 3b, lane 6). Only those mutations affecting the nucleotides
that are located immediately upstream of the H1 element dramatically reduced the protein-binding capacity. This is clearly demonstrated by the inability of oligonucleotides carrying this mutation to remove the complex (Fig. 3b, lanes 5, 7 and 8).

**DISCUSSION**

**The exon 1.1 5'-flanking region directs luciferase expression in JEG-3 cells**

The study demonstrates that a sequence of 404 bp upstream from untranslated exon 1.1 which is mainly expressed in placenta can direct strong constitutive reporter-gene expression in JEG-3 cells. The sequence between −405 and −1997 bp does not include additional enhancing or silencing elements that are active in these cells. Thus it can be concluded that the sequence upstream from exon 1.1 is a functional promoter region. This conflicts with the observation of Hinshelwood et al. (1997), who found (by using an almost identical experimental design) that this promoter is not active in JEG-3 cells (in contrast to the human placental promoter, I.1). This is even more surprising because both
An E-box and a hexameric sequence motif are important for promoter activity

From deletion experiments, it is evident that the sequence from −170 to −404 bp is essential for high-level constitutive expression in JEG-3 cells. In addition, it could be demonstrated that at least two different enhancing elements are present. The promoter region from −309 to −404 bp presumably harbours one of these elements. Sequence analysis demonstrated the presence of two overlapping motifs within this region (Fürbass et al. 1997), i.e. a half-site hexameric sequence (AGGTCA) at −344 bp (H2) and an E-box motif (CATGTG) at −340 bp (E2), a potential binding site for bHLH (basic helix–loop–helix) transcription factors. From the promoter deletion series, it cannot be determined if one, none, or even both, elements mediate enhancing activity. Mutagenesis experiments, however, clearly revealed that only the alteration of the E2 motif significantly reduced promoter activity, whereas that of the H2 element had no effect on reporter-gene expression. Another line of evidence showing the functional importance of the E2 element was provided by competition EMSA. It could be demonstrated that oligonucleotides harbouring this sequence motif form a specific protein/DNA complex with factors from JEG-3 nuclear extracts. Competition was abolished when the E-box motif was completely mutated. On the other hand, with an oligonucleotide including the identical E-box motif E1 the complex could also be efficiently competed, even though the flanking sequences were completely different. These results demonstrate that the six nucleotides constituting the E-box motif are essential for the formation of the protein/DNA complex observed, and they suggest that this complex mediates enhancing activity within bovine promoter 1.1.

The E-box consensus sequence has been defined as CANNTG (Braun et al. 1992, Robinson & Lopes 2000). For the virtual abolition of the protein-binding capacity of the E2 element in competition EMSA experiments it was necessary to alter this motif extensively. In contrast, the enhancing activity of this element in JEG-3 cells was already significantly reduced by the exchange of only the central two nucleotides which are not specified by the general consensus sequence. This observation suggests that the function of the E2 element is not sufficiently defined by the consensus sequence but is critically dependent on the central nucleotides, TG. Furthermore, by comparing the results of reporter-gene experiments with those of EMSA experiments it can be suggested that the transcription machinery of living cells seems to be more sensitive to sequence alterations than are in vitro protein binding assays. This could be due to additional co-factors which act in the cell but are either inactive or lost under EMSA conditions.

In other species, it has been demonstrated that the E-box-binding bHLH transcription factor, MASH2, the antagonistically acting bHLH cofactor, Hand1, and also the inhibitory HLH protein, Id-2, play important roles in the regulation of trophoblast differentiation (Janatpour et al. 2000, Scott et al. 2000). The presence of the trophoblast-specific bHLH factor, Hand1, in the JEG-3 cell line has been reported by Knoller et al. (1998). Further experiments are necessary to identify the factor(s) which actually bind(s) to the E2 element of bovine Cyp19 promoter 1.1. So far, it can only be speculated that bHLH factors which are present in JEG-3 cells interact with the E2 element and thus mediate enhancing activity in this model system. Further studies, possibly including bovine trophoblast cell lines (Munson et al. 1988), are also necessary to determine if the bovine Cyp19 is also regulated in the placenta, by trophoblast-specific bHLH transcription factors via the E-box-sequence motif. This would be an interesting difference with respect to the human gene. Reporter-gene studies in this species gave no indication that E-box motifs actively regulate the non-homologous placenta-specific promoter, I.1, of the human Cyp19 gene.

A second enhancer region was located between −309 and −221 bp, as clearly revealed by promoter-deletion experiments. Site-directed mutagenesis of putative cis-acting elements showed that nucleotides within half-site hexameric motif H1 are important for enhancer activity. With EMSA, it could be demonstrated that this region in fact is a target for DNA-binding nuclear factors. However, these experiments also revealed that the hexameric sequence motif AGGTCA (element H1) itself is obviously not necessary in order to remove the protein/DNA complex by competition. Instead, it
became evident that nucleotides TG immediately flanking H1 have a crucial role in protein binding. This suggests that the protein/DNA complex observed during EMSA is not identical to that which mediates the enhancing activity in reporter-gene assays. Additional factors may be recruited in the living cell.

Reporter-gene studies, however, clearly demonstrate that nucleotides located within the H1 motif are important for the enhancing activity, thus suggesting that half-site hexameric sequence H1 is part of this enhancer element. This is consistent with the observation that an identical hexameric motif located within the human Cyp19 placenta-specific promoter is an important enhancing element which, in the context of an imperfect palindromic sequence, mediates RX and vitamin D3 responsiveness by binding RXRα (RX receptor) and VDR (VD receptor) heterodimers (Kamat et al. 1998, Sun et al. 1998). Recently it was found that RXRα and RXRβ are, in fact, essential regulators of placentogenesis in mice (Wendling et al. 1999), though it is not yet clear if these factors are involved in regulating placental Cyp19 expression. It is also an interesting, but still open, question as to whether the H1 element of the bovine promoter is a target for nuclear receptors such as RXR or VDR.

The exact extension of this second enhancer element is not yet clear. However, results from reporter-gene assays and EMSA experiments suggest that hexameric sequence H1 and nucleotides flanking this element upstream are also involved. Interestingly, during a recent study the nonameric sequence TGAGGTCAG was published as a novel transcriptional regulatory element of the keratinocyte growth-factor gene (Zhou & Finch 1999). This sequence is identical to the region from −270 to −262 bp of bovine Cyp19 promoter 1.1 which includes the H1 element and flanking nucleotides. Also, other consensus sequences of regulatory elements have been defined which exactly match this sequence. Interestingly, most of them include the upstream flanking nucleotides TG (Zhou et al. 1995, Barton et al. 1996), which have been demonstrated, during this study, to be important in protein binding.

During the present study, results were presented demonstrating, first, that the placenta-specific promoter of the bovine Cyp19 gene includes two active regulatory elements. Additional evidence was obtained to show that one of these elements is likely to be identical to the E-box motif at −340 bp and that the second enhancer region includes the half-site hexameric motif at −268 bp and additional upstream flanking nucleotides. These results now enable further investigations towards the characterisation of transcription factors that are actually involved in binding these elements and thus mediate the enhancing activity in JEG-3 cells. Additional experiments will determine if these elements and corresponding transcription factors are, in fact, responsible for the regulation of Cyp19 in the bovine placenta.

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