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

et al. 1999). This suggests that *Cyp19* expression in the placenta, unlike that in the ovary and the brain, is not conserved but, instead, may have developed independently several times during evolution.

In recent years, the human placenta-specific promoter has been examined in detail for *cis*-acting transcriptional control elements in reporter-gene studies using human BeWo or JEG-3 choriocarcinoma cell lines or primary cultures of human trophoblast cells as models of placental expression. Toda *et al.* (1990) found that the promoter region from -242 to -183 bp contains activating elements, whereas the region from -500 to -243 bp includes inhibiting elements. These findings were confirmed by other investigators, who additionally identified some of the responsible *cis*-acting elements. Enhancing activity could be assigned to a GC-box and hexameric sequences both of which are located within 246 bp of the sequence flanking the placenta-specific untranslated exon, whereas a cell-type-specific inhibiting element was located in the region between -501 and -246 bp (Kamat *et al.* 1998). An imperfect palindromic sequence including one of the hexameric elements was demonstrated to mediate 9-*cis* retinoic acid (RX) and vitamin D (VD) responsiveness (Sun *et al.* 1998). Toda *et al.* (1995) found that binding of nuclear factor interleukin-6 to a distal element increased promoter activity. Trophoblast-specific elements were identified by Yamada *et al.* (1995). Recently, it was demonstrated that a sequence of only 500 bp of the human placenta-specific promoter is sufficient to direct placenta-specific expression in transgenic mice – a species without any endogenous *Cyp19* expression in the placenta (Kamat *et al.* 1999).

So far, no information on functional *cis*-acting elements of bovine placenta-specific promoter 1.1 is available. Sequence analysis demonstrated that it is completely unrelated to the human promoter, I.1 (Hinshelwood *et al.* 1995, Furbass *et al.* 1997). Elucidation of *Cyp19* regulation in the bovine placenta and comparison with the regulatory mechanisms of the human gene are important prerequisites for obtaining a better understanding of the role of *Cyp19* expression during placental development in both species.

The aim of the present study was to confirm the promoter activity of the region flanking bovine placenta-specific exon 1.1 and to investigate the role of putative regulatory elements that had been identified during earlier investigations (Furbass *et al.* 1997). The human JEG-3 choriocarcinoma cell line was used as an *in vitro* model for trophoblast-specific gene expression.

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 Furbass *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; Furbass *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'-CATCCCTCTGGGCA***ACT***TGTGAATGCAT CAT-3', the E-box motif E2 at -340 bp was mutated with the oligonucleotide 5'-GCTCA AAGAGGTCA***ATT***TGGCCCAATCCCCT-3', the hexameric (H) sequence H1 at -268 bp was mutated with the oligonucleotide 5'-AACCCCACTGTT TGA***ATT***TCAGTCAGTGGCT-3' and the hexameric sequence H2 at -344 bp was mutated with the oligonucleotide 5'-CCACAGCTCAAAGA***ATT***CA TGTGGCCCAAT-3' (putative binding motifs are shown underlined; mutated nucleotides are shown in bold italics).

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).

FIGURE 1. Promoter activity of different exon 1.1 5'-flanking sequences in JEG-3 cells. Top left: fragments of various lengths (arrows) were cloned upstream from the luciferase reporter gene (hatched boxes) and transiently transfected into JEG-3 cells. One fragment was inserted in the complementary orientation (-1071c). Top right: promoter activity of the constructs 48 h after transfection. The data are calculated from corrected luciferase activities (for more details see *Materials and Methods*) of the different constructs relative to the activity of the promoterless vector, pGL3. Constructs -1997, -1071, -907, -553 and -404 showed comparable high-level activity, construct -309 showed intermediate expression, whereas the promoter activity of construct -170 was almost completely abolished. No activity was observed with constructs that included the complementary fragment -1071c. Bottom: sequences of putative regulatory elements as found in promoter 1.1. The position numbers indicating the various 5'-ends of promoter deletion fragments (-1997 to -170 bp) and the 3'-ends of all fragments (+113 bp) refer to the main start site of transcription (+1 bp) of bovine placenta-specific transcripts (Fürbass *et al.* 1997). Ap1, binding site for activating protein 1; Ap2, binding site for activating protein 2; E1 and E2, E-boxes; H1, H2 and H3, core half-site hexameric sequences. The sequence of promoter 1.1 and exon 1.1 has the accession number Z69241 (EMBL/GenBank) and is published in Fürbass *et al.* (1997). The black lines in the bars indicate s.e.m. values. The asterisks indicate constructs with significantly reduced activity relative to construct -404.

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)

FIGURE 2. Promoter activity of construct -404 variants with different targeted point mutations of elements E1, E2, H1 and H2. The sequences of the wild-type and mutated elements and their positions relative to the main start site of transcription are indicated. The data are calculated from corrected luciferase activities (for more details see *Materials and Methods*) of the different constructs relative to the activity of the promoterless vector, pGL3. Mutations of elements E2 and H1 significantly reduced the activity, whereas those of elements E1 and H2 did not change the level of expression. The black lines in the bars indicate S.E.M. values. The asterisks indicate constructs with significantly reduced activity relative to construct -404 (wild type).

and washed twice in MEM with serum. Usually 4×10^6 trypsinised cells were mixed with 10 μ g reporter plasmid (pGL3) in 400 μ l buffered sucrose solution (272 mM sucrose, 7 mM sodium phosphate, 1 mM $MgCl_2$, 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 μ g 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 μ g DNA. Ten minutes after electroporation, the cells were washed twice in MEM with serum and subsequently incubated at 37 °C with 5% CO_2 .

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 [32 P]ATP and T4 polynucleotide kinase (Sambrook *et al.* 1989). Binding reactions contained 35 fmol labelled DNA, 10 μ g nuclear extract in a total volume of 20 μ l 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 $\times$ 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 -1997, -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

FIGURE 3. Competition EMSA demonstrating binding of JEG-3 nuclear proteins to sequences of promoter 1.1. Kinased double-stranded oligonucleotides were incubated with JEG-3 nuclear extract with or without unlabelled competitor oligonucleotides and were analysed on polyacrylamide gels. (a) Promoter sequence -323 to -352 forms a specific protein/DNA complex (lane 2) which could be removed efficiently by competition with identical, but unlabelled, oligonucleotides (lane 3). No competition was seen when the same oligonucleotides including mutated E-box motif E2 were used (lane 4). With oligonucleotides representing promoter region -39 to -68 bp, which includes the E-box motif E1 at -56 bp, the complex could also be removed (lane 5). (b) With oligonucleotides representing promoter region -253 to -282 bp including half-site hexameric motif H1, a strong protein/DNA complex was observed (lane 2). Competition was possible with identical, but unlabelled, oligonucleotides (lane 3). The partial or complete mutation of hexameric motif H1 did not eliminate competition capacity (lanes 4 and 6), although a mutation identical to that of competitor oligonucleotide m3 significantly reduced reporter-gene expression (see Fig. 2; -404E1 mut). Only the additional or exclusive alteration of two nucleotides flanking the H1 element upstream strongly reduced competition efficiency (lanes 5, 7 and 8). The molar excess of competitor DNA in all experiments was 50-fold. Specific complexes and free probes are indicated (arrows). Sequences of wild-type and mutated competitor oligonucleotides (only plus strands are shown) are aligned. The asterisks indicate identical nucleotides. Putative binding motifs are underlined. Position numbers referring to the start site of transcription of promoter 1.1 are indicated.

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

studies were based on the same reporter system (luciferase). However, different 3'-ends of the promoter were fused to the luciferase gene in both studies. Whereas the constructs of Hinshelwood *et al.* (1997) end at position -17 bp, which includes only transcription start site 1, all of the constructs described here end at position +113 bp, thus including all possible start sites for transcription – among them, the main placental site, site 2 (Fürbass *et al.* 1997). These observations suggest that one, or several, unidentified regulatory element(s) is/are located within the sequence between positions -17 to +113 bp.

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 co-factor, 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 Knoefler *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 RXR α (RX receptor) and VDR (VD receptor) heterodimers (Kamat *et al.* 1998, Sun *et al.* 1998). Recently it was found that RXR α and RXR β 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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