Transcriptional Regulation of Insulin-like Growth Factor-II Gene Expression by Cortisol in Fetal Sheep during Late Gestation*

(Received for publication, December 5, 1997)

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The objective of this study was to determine the mechanisms by which cortisol down-regulates hepatic insulin-like growth factor-II (IGF-II) gene expression in late gestation. Leader exons 6 and 7 of the ovine IGF-II gene, with their 5′-flanking regions, were first isolated. Characterization of transcription start sites revealed a unique site for exon 6 and three dispersed sites for exon 7. Nuclear run-on assays showed a 5-fold higher transcription rate of the IGF-II gene in liver of adrenalectomized fetuses compared with control animals, suggesting that regulation of IGF-II gene expression by cortisol is at the transcriptional level. RNase protection assays demonstrated hepatic leader exon 7 expression in adrenalectomized fetuses to be more than 2-fold higher than in controls, whereas it was reduced by 50% in cortisol-infused fetuses compared with controls. There was no effect on the expression of other leader exons. Functions of the upstream regulatory region of leader exon 7 (i.e. promoter P4) were investigated by luciferase transient expression. A region of −172 bases downstream relative to the first transcription site of leader exon 7 was shown to retain basal promoter activity and respond to cortisol. These results suggest that cortisol may induce the prenatal decline in ovine hepatic IGF-II expression by suppressing promoter P4 of the IGF-II gene.

The insulin-like growth factors (IGFs) are small single-chain peptides that affect cell proliferation and metabolism. The two major forms, IGF-I and IGF-II, comprise 70 and 67 amino acids for the mature peptides, respectively, and are structurally related to each other and to insulin (1). IGF-I plays an important role throughout development, and postnatally it regulates growth under the control of pituitary growth hormone (2). By contrast, IGF-II is considered to have a major role in fetal growth. Disruption of the IGF-II gene by homologous recombination in transgenic mice produces growth deficiency in fetal growth. Disruption of the IGF-II gene by homologous recombination in transgenic mice produces growth deficiency in fetal growth. Disruption of the IGF-II gene by homologous recombination in transgenic mice produces growth deficiency in fetal growth. Disruption of the IGF-II gene by homologous recombination in transgenic mice produces growth deficiency in fetal growth.

The IGF-II genes in human, rat, and mouse have been characterized (4–7), and a recent report on the structure of the human IGF-II gene (8) showed it to be most similar to that of the ovine IGF-II gene (9). In the human IGF-II gene nine exons including six 5′-untranslated leader exons, and four promoters, P1–P4, have been identified. Promoter P1 is active in adults, whereas the other promoters are active in many fetal tissues, including liver, and are switched off completely in adult (5, 9).

The developmental and tissue-specific regulation of IGF-II mRNA expression is complex. The predominant IGF in the fetal circulation is IGF-II, and levels of IGF-II mRNA and protein are high in most fetal tissues; their concentration in utero is about 10 times greater than that of IGF-I (10). After birth, IGF-II expression is markedly decreased in most tissues (1). However, regulatory factors and mechanisms involved in IGF-II gene expression still remain poorly understood. In the ovine fetus, there is a gradual rise in plasma cortisol levels starting about 2 weeks before birth and culminating in a dramatic rise during the final 3–5 days in utero (11). It has been demonstrated that cortisol fulfills the vital role of initiating many of the important prepartum maturation events that are essential for neonatal survival (11). It is during this latter period that the plasma level of IGF-II and the rate of fetal growth decline. In a previous study, we demonstrated that the surge in fetal plasma cortisol suppresses hepatic IGF-II mRNA expression during late gestation (12). The aims of the present study were, therefore, to gain further insight into the transcriptional control of the ovine IGF-II gene by cortisol and to identify the regulatory region of the gene involved in this response.

EXPERIMENTAL PROCEDURES

Genomic Cloning and Sequencing—A sheep genomic library in λEMBL3 was screened with a human IGF-II cDNA fragment (13). A positive clone was digested with PstI and EcoRI and subcloned into M13mp18. Further screening produced positive subclones containing fragments of 1.7 and 0.9 kb, which were found to encode exon 8 (the first coding exon) and exon 9, respectively. Another positive genomic clone from the primary screening was digested with EcoRI and SalI, and the fragments were analyzed by Southern blot using an α-32P-radiolabeled 1.3-kb human IGF-II DNA fragment containing full-length leader exon 6 of the human IGF-II gene (kindly given by Dr. Paul Schofield, Department of Anatomy, University of Cambridge, United Kingdom (UK)). Fragments of 3.0 and 2.4 kb that hybridized to the probe were shown to contain leader exons 6 and 7, and their flanking regions, of the ovine IGF-II gene.

DNA Sequencing—The M13mp18 subclones containing exons 8 and 9 were sequenced by the single-stranded method of Sanger (14). The EcoRI/SalI fragments containing leader exons 6 and 7 were cloned into Bluescript KS+ (Stratagene Ltd., Cambridge, UK), and nested deletions generated by exonuclease III (15) were sequenced. Where double-stranded sequencing gave rise to compressions, further subclones were generated in M13 and single-stranded sequencing was carried out. In all cases clones were sequenced from both ends. Splicing sites were determined by comparing the genomic sequence and that of leader-exon-containing cDNAs obtained by reverse transcription-polymerase chain reaction (RT-PCR). The Wisconsin Package system (16) was used for computer sequence analysis.

Animals—Six Welsh Mountain ewes carrying twin fetuses of known gestational age were used. Under halothane anesthesia (1.5% in O2/
TABLE I
Regulation of Fetal Insulin-like Growth Factor-II Gene

| Age at delivery | Number of fetuses | Cortisol levels* |
|-----------------|-------------------|------------------|
|                 |                   | ng/ml            |
| Saline-infused  | 127–130           | 3                |
| Cortisol-infused| 127–130           | 3                |
| Adrenalectomized| 143–145           | 3                |
| Unoperated      | 143–145           | 3                |

* Values of cortisol levels are shown as mean ± S.E.

NADPH, one of two procedures was carried out using the surgical procedures described previously (17): 1) intravascular catheterization of both fetuses in three sets of twins at 115–117 days of gestation, and 2) bilateral adrenalectomy of one fetus in each of three sets of twins at 117–119 days of gestation (term = 145 days). At least 5 days after surgery, the catheterized twin fetuses were infused intravenously with either cortisol (2–3 mg/kg/day in 3 ml of 0.9% (w/v) saline; EF-Cortenal, Glaxo Ltd.) or saline (3 ml/day; 0.9% (w/v) for 5 days beginning at 122–125 days of gestation. Blood samples of 2 ml were taken daily from these fetuses throughout the experiment period, and tissues were collected at the end of the infusion period at 127–130 days. Blood and tissue samples were obtained from the adrenalectomized fetuses and their control twins at 143–145 days. All fetuses, regardless of previous treatment, were delivered by Caesarean section under general anesthesia (sodium pentobarbitone, 20 mg/kg intravenously). Blood samples were taken from the fetus immediately at delivery either through the indwelling arterial catheter or by venipuncture from the umbilical artery. After administration of a lethal dose of anesthetic (sodium pentobarbitone, 200 mg/kg intravenously), blood samples were collected, frozen rapidly in liquid nitrogen, and stored at −80 °C until analysis. Blood samples were centrifuged immediately at 4 °C, and the plasma was stored at −20 °C until determination of cortisol concentrations using a radioimmunoassay validated for use with ovine plasma (20). Table I shows the plasma cortisol levels in the intact and unoperated fetuses at delivery.

**RNA Isolation**—Total RNA was isolated from 1-gram portions of frozen tissue using the guanidinium thiocyanate method of Chomczynski and Sacchi (19). Total RNA was quantified by absorbance at 260 nm (A260, unit = 40 μg). To check equivalence of RNA samples, total poly(A)+ content was also measured, as described previously (20). A constant relation between A260 and polyA+ content was found for RNA from all tissues.

**Construction of Probes for Mapping Transcription Start Sites**—The transcription start sites for leader exons 6 and 7 were mapped by RNase protection assays using a radioimmunoassay validated for use with ovine plasma (20). The purity of the RNA was determined spectrophotometrically following solubilization in 0.1 N NaOH. In vitro transcription reactions were carried out following the method essentially as described by Linial et al. (23). Nuclear suspensions containing 90 μg of DNA were mixed with the reaction buffer and incubated at 30 °C for 30 min. RNase-free Dnase I (Amersham Pharmacia Biotech) was added to the reaction which was then incubated at 30 °C for 15 min. Radiolabeled RNAs were extracted using the method of Chomczynski and Sacchi (19). Ovine IGF-II exon 8 DNA (20 μg) in alkaline solution (1 M NaCl, 0.1 N NaOH, and 10 mM EDTA) was heat-denatured, dot-blotted onto Hybond-N, and hybridized with radioactively labeled transcripts at 65 °C for 24 h. After hybridization, the filters were washed at 37 °C for 30 min in 2x SSC + 0.5% SDS and further washed at 65 °C for 30 min in 0.1x SSC. The filters were autoradiographed by exposing to x-ray film at −70 °C and also counted directly in a scintillation counter.

**Cell Culture and Transfections**—NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum. The purity of the DNA was determined spectrophotometrically following solubilization in 0.1 N NaOH. In vitro transcription reactions were carried out following the method essentially as described by Linial et al. (23). Nuclear suspensions containing 90 μg of DNA were mixed with the reaction buffer and incubated at 30 °C for 30 min. RNase-free Dnase I (Amersham Pharmacia Biotech) was added to the reaction which was then incubated at 30 °C for 15 min. Radiolabeled RNAs were extracted using the method of Chomczynski and Sacchi (19). Ovine IGF-II exon 8 DNA (20 μg) in alkaline solution (1 M NaCl, 0.1 N NaOH, and 10 mM EDTA) was heat-denatured, dot-blotted onto Hybond-N, and hybridized with radioactively labeled transcripts at 65 °C for 24 h. After hybridization, the filters were washed at 37 °C for 30 min in 2x SSC + 0.5% SDS and further washed at 65 °C for 30 min in 0.1x SSC. The filters were autoradiographed by exposing to x-ray film at −70 °C and also counted directly in a scintillation counter.

**RESULTS**

**Genomic Cloning and Sequencing**—Plasmid DNA was isolated from a sheep genomic library following screening with a probe for human IGF-II cDNA. Restriction enzyme digestion and Southern blot analysis with both the human IGF-II cDNA and ovine IGF-II cDNA was performed to identify the subcloning of four different fragments of the ovine IGF-II gene, with sizes of 3, 2.4, 1.7, and 0.9 kb, respectively. DNA sequence analysis revealed that the 3-kb fragment contains part of exon 6 and its 5′-flanking region and is contiguous with the 2.4-kb fragment, which contains sequences spanning the rest of exon 6, exon 7, and 5′- and 3′-flanking region of exon 7. The 1.7- and 0.9-kb fragments contain coding exons 8 and 9 of the ovine IGF-II gene, respectively. Fig. 1 (a and c) summarizes the positions of the DNA fragments sequenced on the genome and the DNA se-
sequence of exons 6 and 7 and their flanking regions. These data enabled antisense riboprobes to be constructed and then used to map the transcription start sites for the leader exons and investigate expression and regulation of the ovine IGF-II gene.

Identification of Transcription Start Sites—To determine the transcription start sites for exons 6 and 7, RNase protection analyses were carried out with antisense probes to their respective 5’ upstream sequences, the schematics of which are shown in Fig. 2. The probes contain both 5’ intron and leader exon sequence, and since the genomic clones had been completely sequenced beforehand and the exon/intron boundaries were determined from the sequence of the RT-PCR products for exons 6–8 and exons 7–8-specific mRNAs, the position of the 3’ end of the protected fragment could be predicted unequivocally. The sizes of the protected fragments were determined accurately by co-electrophoresis of a known sequencing reaction and the position of the start site worked back from the 3’ end of the protected fragment for the leader exon. For exon 6, a template that spanned the first 122 bp of exon 6 and 473 bp of 5’ intronic sequence was obtained by linearizing Bluescript plasmid DNA containing the 3-kb fragment DNA by digestion with PstI (position 460 in Fig. 1c). The riboprobe was internally labeled by in vitro transcription with T3 RNA polymerase, and gave a full-length antisense transcript of 509 bases. For exon 7, a template spanning exon 7 and 163 bp of 5’ intronic sequence was obtained by linearizing Bluescript plasmid DNA containing the 3-kb fragment DNA by digestion with PstI (position 460 in Fig. 1c).

Fig. 1. Isolation and structural analysis of leader exons 6 and 7 of the ovine IGF-II gene and their flanking regions. Panel a, restriction map of ovine genomic DNA (upper), which contains exons 6–9 of the ovine IGF-II gene and their flanking regions. The solid bars indicate the regions of DNA sequenced. Positions of exons are indicated below, as open and filled boxes, representing non-coding and coding exons, respectively. Panel b, transcription start sites for leader exons 6 and 7 analyzed by RNase protection assays are shown in panels exon 6 and exon 7, respectively. DNA sequencing reactions were co-electrophoresed as size markers (lanes M). Panel c, the sequence of exons 6 and 7, and their flanking region presented with exon sequences written as uppercase and intron sequences as lowercase, comprises promoter P3 region (1–931), exons 6 (932–2057), promoter P4 region (2058–2943), and exon 7 (2944–3053). Sites of restriction enzymes used for subcloning are shown. Positions of transcription start sites are indicated in the sequence by asterisks. Recognizable putative promoter and regulatory elements are underlined.
ing the 2.4-kb fragment by digestion with NheI (position 2781; Fig. 1c), and this gave a full-length antisense T7 transcript of about 723 bases. Fig. 1b shows the analysis of transcription start sites for exons 6 and 7, using RNase protection assay with the riboprobes. A single protected fragment of 122 nt in length for exon 6 and three protected fragments of 109, 104, and 98 nt for exon 7 were seen. The deduced transcription start sites for exons 6 and 7 are indicated in the DNA sequence (Fig. 1c). These indicate that transcription starts at a single point of 1125 bp upstream from the 3' end of exon 6 and at three points, 109, 104, and 98 bp upstream from the 3' end of exon 7.

Identification of a Cortisol-responsive Leader Exon of the Ovine IGF-II Gene—Previous data showed that cortisol down-regulates the ovine IGF-II gene in the fetus during late gestation (12). In the present study, a preliminary assessment of the effect of cortisol on the abundance of mRNA transcripts of the ovine IGF-II gene was carried out by Northern analysis. Total RNAs isolated from the liver of 143-day-old adrenalectomized fetuses and their control twins were hybridized with a labeled exon 8 probe. The IGF-II gene comprises alternate leader exons and multiple polyadenylation sites, which results in multiple mRNA species. The results showed that there was no qualitative change in mRNA species but there was a quantitative decrease in the levels of certain mRNA species, most notably transcripts of 1.3, 1.9, and 2.9 kb, in the livers of control compared with those of adrenalectomized fetuses at 143 days of gestation (Fig. 3). To determine how cortisol affects differential leader exon expression of the ovine IGF-II gene, RNase protection analysis was carried out with a riboprobe which extends across the sequence of exons 7–8 as shown in Fig. 2. In principle, all leader exon 7-derived mRNAs will give full protection to this riboprobe, whereas the mRNAs derived from others will protect only the exon 8 region. Hence, this probe produces two protected bands, one of 262 nt representing full-length protection by leader exon 7 containing transcripts and a second, smaller product of 162 nt, which arises from partial protection from the exon 8 sequence. This allowed simultaneous monitoring of expression of alternative leader exon derived transcripts within a single analysis. Liver RNA was prepared from sets of twin fetuses. In one fetus from each pair, the cortisol levels were manipulated by either exogenous cortisol infusion before 130 days (i.e. prior to the natural surge in cortisol) to increase fetal plasma cortisol levels or by fetal adrenalectomy, which prevents the prepartum cortisol surge. The other fetus from each pair was used as a control and was either saline-infused or unoperated. The RNase protection analysis results are shown in Fig. 4. When fetal cortisol levels were elevated either by exogenous infusion at 127–130 days or endogenously at 143–145 days, there was a significant decrease (p < 0.01) in the abundance of mRNA arising from the leader exon 7-specific transcripts, which gave the full-length protection (upper band), but no significant change in the abundance of the smaller fragments protected by mRNAs originating from other leader exons (p > 0.05). This result suggests that the regulation of IGF-II gene expression by cortisol in the late gestation fetus operates preferentially through regulating the expression of leader exon 7.

Examination of Transcriptional Regulation of Cortisol on Ovine IGF-II Gene Expression—Nuclear run-on experiments were carried out to determine whether the observed changes occurred at the level of transcription, mRNA processing or by

![Fig. 2. Schematic representation of positions of riboprobes. Riboprobes for exon 6 and exon 7 were used for determination of transcription start sites for leader exons 6 and 7. Riboprobes for exons 7 and 8 were used for examining leader exon expression in response to cortisol. Solid bars represent the region the riboprobes spanned.](image)

![Fig. 3. Northern analysis of mRNA transcripts of the ovine IGF-II gene affected by cortisol. 20 µg of total RNAs of liver from adrenalectomized (AX) and control (Con) twin fetuses at 143 days of gestation were electrophoresed, blotted, and hybridized with radiolabeled exon 8 fragment of the ovine IGF-II gene. Seven transcripts were detected of 6, 5, 3.8, 2.9, 2.3, 1.9, and 1.3 kb. Positions of 28 and 18 S ribosome RNAs are indicated.](image)

![Fig. 4. Determination of cortisol-responsive leader exon expression by RNase protection assays. Analysis was carried out using liver RNAs isolated from adrenalectomized (AX) and control (CON) twin fetuses at 143–145 days of gestation, after infusion with either cortisol (CORT) or saline (SAL) at 127–130 days. Panel a, ovine IGF-II gene expression examined by RNase protection using riboprobe exons 7–8, which spanned exons 7 to exon 8. Upper bands represent fragments protected by mRNAs derived from leader exon 7, and lower bands represent fragments protected by mRNAs derived from the other leader exons. The protected signals were quantified by image analysis and represent mRNA levels. The histogram (panel b) shows the levels (presented by mean ± S.E.; n = 3) of differential leader exon expression.](image)
Selective alteration of mRNA stability. Nuclei were prepared from fresh liver obtained from two pairs of 143-day-old adrenalectomized and control twin fetuses and incubated in the presence of $\alpha-\text{P}UTP$ to complete the transcription of nascent RNA chains. The radiolabeled RNAs were isolated and hybridized to an excess of ovine IGF-II exon 8 DNA dot-blotted onto nitrocellulose. Results from the two pairs of animals were similar, and Fig. 5 therefore gives the results from one set of animals. Hybridization signals, which reflect the in vivo transcription rates, are shown in Fig. 5. Quantitative analysis showed that the transcription rate of the IGF-II gene was approximately 5 times higher in the liver of adrenalectomized compared with control animals. These data suggest that, in vivo, expression of the ovine IGF-II gene in late gestation is controlled at least in part by cortisol at the transcriptional level.

Functional Analysis of IGF-II Fetal Promoter Regions in Response to Cortisol—Having established that the ovine IGF-II gene is down regulated by cortisol at the transcriptional level as well as via affecting leader exon 7 expression preferentially, the promoter activity of the putative 5′ regulatory region (i.e. promoter P4) of the leader exon 7 was investigated in response to cortisol. As a control, the regulatory region (i.e. promoter P3) of the leader exon 6 was also included in this study. Both promoter sequences were constructed into the plasmid vector pGL-2-basic in both orientations to drive the luciferase reporter gene in transient expression. Functional analysis of the promoter activities was performed by transfection into NIH 3T3 cells (Fig. 6). Full-length constructs for P3 (pGL-P3) and P4 (pGL-P4a) supported approximately the same degree of luciferase expression when cloned into the vector in the 5′ to 3′ orientation, but not in the opposite orientation (data not shown). The presence of 50 ng/ml dexamethasone in the medium caused a 50% reduction in the promoter activity of P4 but had no effect on the activity of promoter P3. Since leader exon 7 was shown to respond to cortisol the function of promoter P4 was investigated in further detail by ligation of a series of nested deletions to the vector pGL-2 basic. In this study, analysis of the P4 deletions showed that the promoter could be deleted to within about 172 bp of the transcriptional start site without affecting the relative inhibition by dexamethasone, although an overall reduction of promoter activity of about 30% was observed when the sequence upstream of −388 bp was removed. When all but 32 bp of the P4 leader sequence was deleted, a complete abolition of luciferase expression was seen. The basal P4 promoter and glucocorticoid repression functions therefore appear to reside in a minimal construct, which includes only a 172-bp sequence immediately upstream of the transcriptional start site of P4.

**DISCUSSION**

In a previous study, the prepartum rise in fetal plasma cortisol was shown to be responsible for down-regulating IGF-II gene expression in the fetal ovine liver close to term (12). Generally, glucocorticoid hormones are thought to exert their effects at the transcriptional level by inducing or repressing expression of target genes (25). However, glucocorticoids have also been reported to regulate mRNA expression at the post-transcriptional level by affecting mRNA stability and by facilitating extranuclear transport of specific mRNA to the cytoplasm (26). In the current study, in vivo run-on assays in isolated nuclei indicated that the transcription rate of the IGF-II gene was suppressed about 5-fold in liver nuclei from control fetuses with high plasma cortisol levels compared with those from fetuses in which the natural prepartum rise in plasma cortisol was prevented by adrenalectomy (Fig. 5). To investigate IGF-II gene regulation further, the regions of genomic DNA containing the fetal promoters were cloned and their expression characterized. Two potential regions of control are inferred from the existence of two untranslated leader exons (exons 6 and 7), which are alternately spliced to the first IGF-II coding exon (exon 8). The presence of alternatively spliced mRNA species was confirmed by sequencing the RT-PCR products from fetal liver RNA using exon-specific primers. The contiguous sequence for exons 6 and 7 and their 5′ regions shown in Fig. 1 extends the partial sequences of Ohlsen et al. (8) for the ovine IGF-II gene and provides information for mapping transcriptional start sites. The initiation of transcription of exon 6 appears to be from a unique site 1125 bp upstream of the 3′ splice site for this exon. The corresponding exon for the human IGF-II gene has a single start site in a comparable position and, like its ovine counterpart, is situated within 30 bp downstream of a TATA box (27). Three discrete start sites were found for exon 7 of the ovine IGF-II gene (Fig. 1b), with approximately 50% of them initiating from a point 109 bp upstream of the 3′ splice site of exon 7, and the remainder mapping to points 5 and 15 bp downstream. These map closely to the corresponding regions for the rat IGF-II gene for which two single start sites differing by 10 bp of each other have been reported by two separate groups (6, 28).

The complex structure of the human IGF-II gene with the presence of 5′ alternate leader exon splicing and multiple 3′
The absence of cortisol from NIH 3T3 cells provides independent triplicate transfections of leader exon 7. Plasmid constructs were transfected into NIH 3T3 cells, and transcriptional activities of the promoters were assayed by measuring luciferase activity. Similar transfection experiments with human mammary tumor virus promoter-driven luciferase expression assay (Fig. 4) showed that leader exon 7, but not other leader exons, is sensitive to the presence of cortisol. Decreased levels of IGF-II mRNA containing exon 7 were found to be related to increased cortisol levels resulting either from the natural prepartum cortisol surge or from premature elevation of cortisol by infusion into fetuses earlier in gestation. These results demonstrate that cortisol exerts preferential control over the alternate leader exon expression and suggests the existence of leader exon-specific promoters, which can be controlled independently of each other.

The ability of the 5′ regions of exon 6 (P3) and exon 7 (P4) to drive the expression of a luciferase reporter gene in an orientation specific manner (Fig. 6) confirms the presence of promoter activity in these sequences. Moreover, glucocorticoid repression of the minimal P4 promoter could be demonstrated in NIH 3T3 cells. Similar transfection experiments with human P3 and P4 reporter constructs in Hep3B and HeLa cell lines indicate that the proximal regions of the promoters direct a basal level of transcription and that regions further upstream contain cell-specific enhancer elements (9). In our experiments (Fig. 6), there is evidence from transfection experiments that the distal part of P4 has an enhancing effect on the transcriptional activity of the proximal region, and it is possible that the EGR-1 consensus sequence (Fig. 1c) may be involved in this. However, the mechanisms that control the repressive effects of cortisol appear to reside almost exclusively within the latter region. This contrasts with the corresponding experiment with P3, which shows no significant influence of cortisol on transcriptional activity. It is interesting to note that, in comparable experiments with the rat (28) and human (9), no difference was seen in the transcriptional potency of constructs transfected into cultured cells in which the endogenous IGF-II gene was either active or inactive. This suggests that the factors necessary for the activation of the IGF-II P4 promoter are ubiquitous and are readily accessible to exogenously introduced DNA and that there is another level of control, possibly involving higher order chromatin structure, which determines the accessibility of these factors to the endogenous IGF-II promoter. In this regard, it is worth noting that, although we could demonstrate the same trend in transcriptional activity of the deletion constructs in NIH 3T3, HepG2, and HuH 7 cells (data not shown for the latter two cell types), only in NIH 3T3 cells was there clear transcriptional repression in the presence of cortisol. The fact that NIH 3T3 cells are of fetal origin whereas the others are adult in nature may be significant in this differential response, and future studies should be undertaken in fetal hepatocytes. In addition, the NIH 3T3 cells contain an active glucocorticoid regulatory system, as demonstrated using mouse mammary tumor virus promoter-driven luciferase expression in transfection analysis (31).

A comparison of ~200 bp of sequence immediately upstream of the ovine P3 and P4 promoters with their homologs in other species is shown in Fig. 7, a and b, respectively. In P3 the single start sites in the ovine and human sequence are the same, and each has a distinct TATA box about 20 bp upstream. The overall homology of this region is 76.7%, and the sequences of DNA involved in protein interaction described by van Dijk et al. (9) for human P3 (PE3–1 to PE3–4) are poorly conserved in the ovine P3. It remains to be seen whether they share common or distinctly different transcriptional control mechanisms. Of particular interest to the present study is the structure of promoter P4 in relation to its sensitivity to glucocorticoids. There is strong homology (65.5%) between human and ovine P4 over 170 bp upstream of the transcriptional start sites which map almost coincidentally (Fig. 7b). This region of the rat IGF-II gene has been shown to support basal transcription and glucocorticoid regulation in transfection experiments and, like the ovine gene, has four Sp1-like and two non-consensus AP1 DNA binding sites. The Sp1 sites give two broad DNA footprints, and...
studies of van Dijk consensus AP1 DNA binding sites in the response. However, sion assay and demonstrated the participation of two non-
to human P4) was transactivated by AP1 in a transient expres-
casole and Ward (33) showed that mouse IGF-II P3 (equivalent transcrip-
tional start that contains a putative TATA box. Car-
PE4–1, a stretch of AT-rich sequence about 30 bp from the this promoter in humans. The only region to footprint was
response studies indicate that the two distal sites are particu-
larly important for promoter activity (32). However, the corre-
sponding Sp1 footprints on human P4 were unable to be de-
tected, and it is surmised that this is due to the weak nature of this promoter in humans. The only region to footprint was
PE4–1, a stretch of AT-rich sequence about 30 bp from the transcriptional start that contains a putative TATA box. Car-
ica and Ward (33) showed that mouse IGF-II P3 (equivalent transcriptional start that contains a putative TATA box. Car-
region and whether they have a role in the cortisol-dependent repression of IGF-II expression.

Acknowledgments—We thank P. Hughes for help during surgery; A. Graham, I. Cooper, S. Nicolls, and D. Lindsay for care of the animals; and M. Bloomfield and V. Whittaker for assistance with the biochemical analyses.

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