Identification of a distal regulatory element in the 5′ flanking region of the bovine prolactin gene

Julie B.Wolf, Victor A.David and Alan H.Deutch
BioMolecular Research Department, W.R.Grace and Co-Conn., Washington Research Center, Columbia, MD 21044, USA

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ABSTRACT

The 5′-flanking region of the bovine prolactin gene was cloned and sequenced. The expression of chimeric gene constructs containing 5′-flanking DNA fragments from the prolactin gene joined to a reporter gene encoding human growth hormone (hGH) was examined using transiently transfected rat pituitary cells. Prolactin nucleotide sequences located at position −1213 to −925 enhance the basal level of expression of growth hormone by 5-fold and function in a position- and orientation-independent fashion. In addition to increasing the basal level of growth hormone expression, this enhancer element also responds to induction by epidermal growth factor. The nucleotide sequence of the bovine prolactin gene enhancer element is highly similar to an enhancer element located approximately −1.5 kb from the rat prolactin transcription initiation site. Deletion analysis of the enhancer region shows that sequences −1124 to −985 are necessary and sufficient for enhancer activity.

INTRODUCTION

The polypeptide hormone prolactin is synthesized by specialized cells, lactotrophs, in the anterior pituitary and is a major regulator of lactogenesis (1). Studies using a rat pituitary tumor cell line that constitutively expresses prolactin and growth hormone have suggested that the synthesis of prolactin is regulated transcriptionally by a number of factors including cAMP (2, 3), epidermal growth factor (EGF) (4−6), phorbol esters (5), thyrotropic releasing hormone (5−8), Ca^{+2} (9−11), dopamine (2, 5), glucocorticoids (12) and estradiol (13, 14). Deletion analysis of the rat prolactin promoter region has defined two major regulatory regions (15, 16) both of which respond to a number of these inducing/repressing molecules (15, 17−21). Both of these regions have enhancer activity in that they function in either orientation and can activate genes driven by heterologous promoters (15, 20). Several of the regulatory factors have been reported to interact with both of the enhancer regions and, in some cases, in a synergistic fashion (18). The most proximal regulatory region is located within the first 300 bases of the 5′ flanking region; the distal enhancer is approximately 1.5 kb from the transcription start site (16, 22).

The sequence of the first 250 base pairs of the bovine prolactin 5′ flanking region is highly similar to the sequence of the proximal enhancer region of the rat prolactin gene (23). Transcriptional responses to several regulatory molecules including epidermal growth factor, thyrotropic releasing hormone, and dexamethasone have been reported for the first 250 base pairs of the bovine flanking region (17). However, no additional regulatory sequences analogous to the rat distal enhancer region were identified on a bovine prolactin fragment containing 1 kb of 5′ flanking sequence (17).

In order to determine whether the DNA region distal to the bovine promoter could reveal any additional transcriptional regulatory sequences, a region containing more than 2 kb of 5′ flanking sequence was cloned and sequenced. An enhancer element within the bovine prolactin 5′ flanking region was identified that stimulates transcription and that responds in a similar fashion as the proximal region to induction by epidermal growth factor. This distal region is highly homologous to the rat distal enhancer region but highly dissimilar in the region between the enhancer and the proximal regulatory regions. Upstream of the bovine enhancer region is a repetitive DNA element that is homologous to a region of the bovine 1.709 satellite DNA (24).

MATERIALS AND METHODS

Cloning of the bovine prolactin promoter and enhancer regions

A synthetic DNA fragment containing the proximal 248 base pairs of the bovine prolactin 5′ flanking region was cloned using two pairs of overlapping oligonucleotides synthesized on an Applied Biosystems, Inc. 380B DNA Synthesizer. The oligonucleotides were purified by polyacrylamide gel electrophoresis; and the two sets of oligonucleotides A-1/B-1 and C-1/D-1 were separately annealed, extended with T7 DNA polymerase (Sequenase, US Biochemical) to generate blunt ends, and then ligated together. The ligation reaction was digested with *Bam*HI and *Bgl*II to generate ends suitable for cloning, and the desired fragment was gel purified (25). The oligonucleotides were designed such that the 5′ end of the synthetic double-stranded fragment represents the natural *Bgl*II site at position −248; and the 3′ end is an artificial *Bam*HI site created by the insertion of three bases, GGA, between the T at position +18 and the T at position +19 (see

* To whom correspondence should be addressed

Fig. 2). The synthetic DNA fragment was subcloned into the *Bam*HI site of pUC13.

In order to obtain additional 5′ flanking sequences, a bovine genomic DNA library in λEMBL3 was prepared from sperm DNA from a Holstein bull (American Breeders Service). The library was screened for prolactin sequences using either a [^{32}P] end-labeled oligonucleotide that represents the first nine amino-terminal amino acids of the mature prolactin protein or a random-primer labeled synthetic promoter fragment. One of the clones, λprlL8, in addition to containing the entire structural gene, contained 2.0 kb of upstream prolactin sequences on a *Bgl*II fragment. This *Bgl*II DNA fragment was subcloned on a larger *Hin*dIII fragment into pBR322, (pL8H2).

DNA Sequencing

The 2.0 kb upstream prolactin DNA fragment was sequenced using Exonuclease III-generated deletions of M13 clones using the procedure described by Henikoff (26). Both the synthetic prolactin promoter fragment and deletions of the 2.0 kb upstream sequences were sequenced by the dideoxynucleotide-chain-termination method (27) using fluorescently-labeled primers (Applied Biosystems, Inc.). Template DNA was prepared as described (28). For the A and C reactions, 0.2 pmole of template DNA was annealed with 0.4 pmole primer in 10 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, and 50 mM NaCl in a total volume of 5 μl. For the G and C reactions, volumes and amounts of these and all subsequent steps were scaled up three fold. The annealing reactions were incubated at 55°C for 5 minutes and allowed to cool to room temperature. Working deoxy- and dideoxynucleotide mixes were prepared as follows: each mix contained 1 mM dATP, 1 mM dCTP, 1.5 mM 7-deaza-dGTP (Boehringer Mannheim), 1 mM dTTP, and 50 μM of one of the four dideoxynucleotides. For the A and C reactions, 1.5 μl of the appropriate nucleotide mix and 1 μl of Sequenase dilution (1.0 μl Sequenase enzyme, 10−12 U/ml, 4.5 μl 0.1 M DTT, 3.5 μl 5× Sequencing Buffer: 50 mM Tris-Cl pH 7.5, 50 mM $MgCl_2$, and 250 mM NaCl) were added and incubated at 37°C for 5 minutes. The reactions were terminated by heating at 65°C for 7−10 minutes. The four extension reactions were then pooled and ethanol precipitated, washed with 70% ethanol and dried briefly. The DNA pellets were resuspended in 5 μl of water, 1 μl of 50 mM EDTA, and 5 μl deionized formamide. Prior to loading, the reactions were heated at 95°C for 8 minutes (with the caps off to reduce the volume). The reactions were then run on an 8.3 M urea, 6% polyacrylamide gel (19:1, acrylamide:bis) and analyzed on an Applied Biosystems, Inc. 370A DNA Sequencer. Overlapping DNA sequences were identified with the Seqman program of DNASTAR (DNASTAR, Inc.).

Construction of PRL:hGH expression vectors

Promoter activity was measured by the ability of the bovine prolactin 5′ flanking sequences to drive the synthesis of hGH in transiently transfected GH_3 rat pituitary cells (29). The expression plasmids used are illustrated in Fig. 1. The control plasmid, p0GH, is pUC12 with the promoter-less hGH structural gene (obtained from Nichols Institute Diagnostics, CA). Plasmid p103 is the synthetic prolactin promoter fragment cloned into p0GH on a *Hin*dIII/*Bam*HI fragment. The plasmid containing an additional 2.0 kb of upstream sequences, pEPH-7, was constructed from a three-way ligation of 1) the *Bgl*II fragment from pL8H2 containing 2.0 kb of 5′ flanking sequence, 2) the synthetic prolactin promoter from p103 (whose *Bgl*II site was destroyed during subcloning) digested with *Bam*HI and *Sau*3A, and 3) *Bam*HI-digested p0GH. The appropriate combination, order, and orientation of ligated fragments were identified by restriction digestion. An analogous plasmid with the 2.0 kb *Bgl*II fragment in the opposite orientation, pOPH-17, was identified in the same ligation mixture. Plasmid p113 containing the upstream enhancer region on a 300 base-pair *Hin*dIII/*Xba*I fragment was constructed from a 5′ deletion of the 2.0 kb *Bgl*II fragment generated in M13mp18. The *Hin*dIII site is part of the M13 multiple-cloning-site with the 5′ end of the deletion at position −1213 in the bovine sequence; the 3′ end of the insert, the *Xba*I site, is at -925. This fragment was then cloned into *Hin*dIII/*Xba*I-digested p103 to place the enhancer directly upstream from the promoter region.

The smaller enhancer fragments were generated by PCR amplification (30) of the enhancer region using the following oligonucleotides and plasmid pEPH-7 as template: PRL-1178: 5′TTGAAACTAAAGCTTACAGGCTG3′; PRL-982C: 5′TATCTAGAATTCTCTGACCTC-AAGCC3′; PRL-1041C: 5′CATGGGCTCTAGAGTTCCACTTGC3′; and PRL-1128: 5′AGAAAGCTTTCAGCAACTTGGTC3′. Sequences −1168 to −985 were generated from PRL-1178/PRL-982C; sequences −1168 to −1049 from PRL-1178/PRL-1041C; and sequences −1124 to −985 from PRL-1128/PRL982C. The PCR amplification products were digested with *Hin*dIII and *Xba*I and cloned into plasmid p103 to generate plasmids p117, p119 and p120, respectively, as shown in Figure 5.

Cell culture conditions

GH_3 rat pituitary cells (obtained from American Type Culture Collection) were maintained in the absence of CO_2 in complete WRC 935^{TM} medium (Amicon), 5−10% fetal calf serum (Gibco, #230−6140), and 100 unit/ml Penicillin-Streptomycin (Gibco, #600−5140). Additional supplements as indicated in the text were 10 nM epidermal growth factor (Collaborative Research) and thyrotropic releasing hormone (25−250 ng/ml, Calbiochem).

DNA transfections

GH_3 cells were transfected with cesium chloride-purified plasmid DNA by a modification of the method described by Camper, et. al. (17). Approximately $1-3\times10^6$ cells per well were plated in 6-well tissue culture plates or 60 mm cell culture dishes (Falcon) and grown for 24 hours prior to transfection. The cells were washed three times with WRC 935^{TM} medium without supplements, and the DNA solution was added in a dropwise fashion to the center of the plate. The DNA solution contained the appropriate amount of DNA diluted into a total volume of 200 μl: 100 μl of WRC 935^{TM} medium/50mM Tris pH=7.5 (without supplements) and 100 μl of 200 μg/ml DEAE-Dextran (Pharmacia #17−0595−01) diluted in saline. After 30 minutes at 37°C, the DNA solution was removed, the cells were washed once with WRC 935^{TM} (without supplements) and 5 ml of complete WRC 935^{TM} medium was added with or without inducer. The tissue culture plates were wrapped in plastic wrap and incubated at 37°C for up to 6 days. Aliquots of supernatant (200−300 μl) were removed at various times after transfection and assayed for hGH activity. For cotransfection experiments, the supernatants and cells were harvested at 120 hours. Optimum cell numbers and DNA concentrations were determined using plasmid p103 and pXGH-5, a control plasmid containing the

mouse metallothionein promoter fused to the hGH structural gene (Nichols Institute Diagnostics).

Time course experiments were performed with prolactin:hGH constructs alone in triplicate. Analyses of the prolactin enhancer deletion constructs involved cotransfection with pRSVcat plasmid DNA (ATCC) to correct for transfection efficiencies. Crude extracts were prepared in 0.25 M Tris-HCl pH=8.0 and chloramphenicol acetyltransferase (CAT) enzyme activity was determined using the liquid scintillation counting (LSC) assay with N-butyryl coenzyme A (Promega) as the cofactor (31). Units of hGH activity were calculated as ([hGH]ng/ml)/(CAT units × 100).

Growth hormone assays

The reporter gene in all plasmid constructs is hGH, a eukaryotic gene whose protein product is secreted into the medium (29). Radioimmune hGH assays were performed using a monoclonal antibody-based assay system essentially as described (Nichols Institute Diagnostics, #40−2205). No cross reactivity to bovine growth hormone in the fetal calf serum or to endogenous rat growth hormone produced by the pituitary cells could be detected. Samples were typically diluted at least 1:2 in 100% fetal bovine serum. Accumulation of hGH in the supernatants could be detected up to at least 200 hours after transfection.

RESULTS

Cloning of the bovine prolactin promoter and enhancer

The proximal 250 base pairs of the bovine prolactin 5′ flanking region had been reported to contain all the sequence information necessary for regulation by inducing hormones such as EGF and TRH in transiently transfected rat pituitary cells (17). Based on published sequence information (23), this small DNA segment was prepared by enzymatic extension of chemically synthesized oligonucleotides and cloned into an expression vector, p0GH, containing hGH as the reporter gene. The resulting prl:hGH construct is p103 (Fig. 1).

In the rat, an upstream enhancer element located at about -1.5 kb has been described that not only stimulates the basal level of transcription but also contains sequences that respond to cAMP, EGF, TRH, and estradiol (18). Given the high degree of homology between the rat and the bovine DNA sequence in the

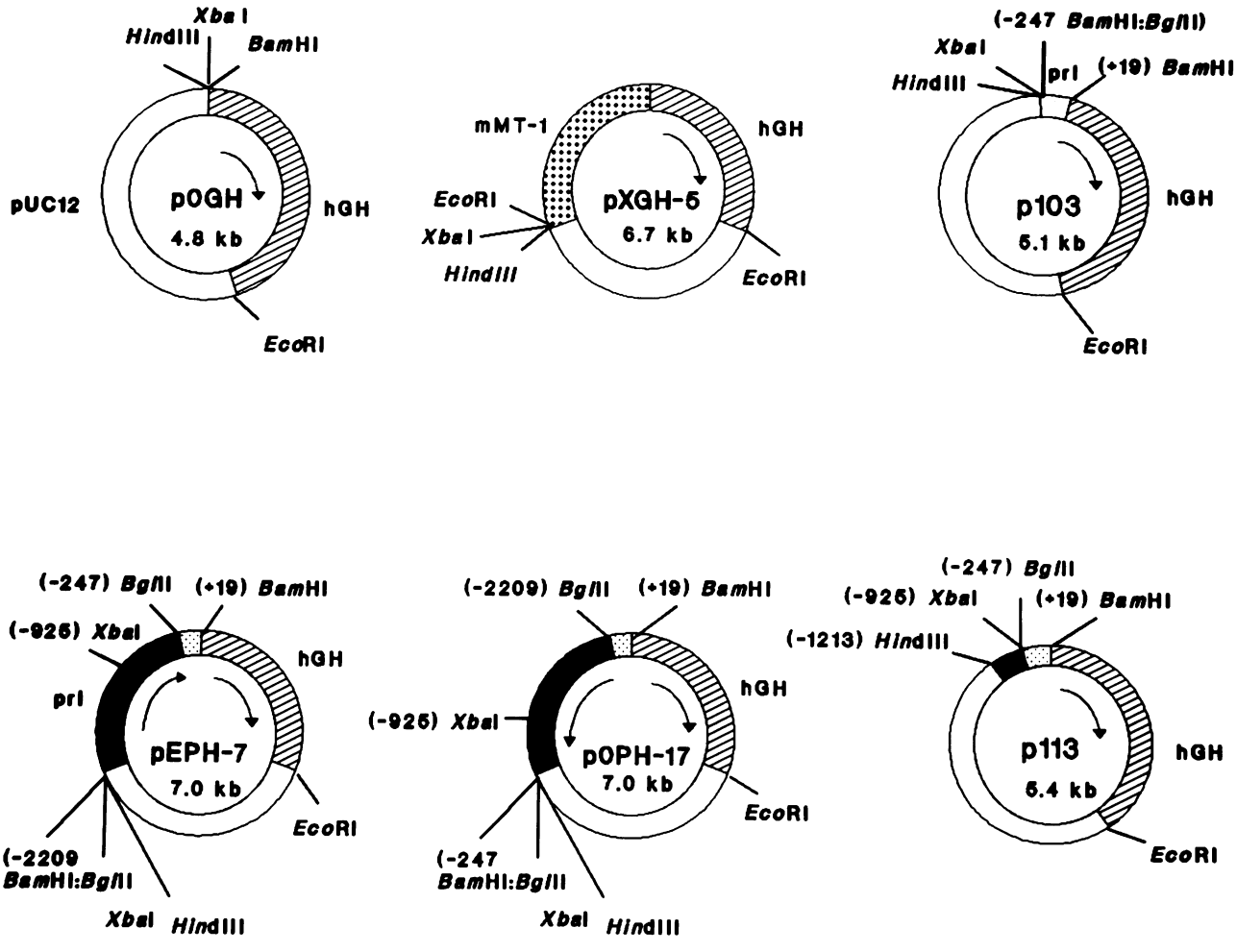

Fig. 1: The hGH expression plasmids used in transient transfection assays. Plasmids p0GH and pXGH-5 contain no promoter and the mouse metallothionein promoter, respectively, and the structural gene encoding hGH. Plasmid p103 is the synthetic prolactin promoter fragment cloned into the *Bam*HI site of p0GH. Plasmids pEPH-7 and pOPH-17 contain 2.0 kb of upstream prolactin sequences on a *Bgl*II fragment cloned in front of the synthetic promoter fragment in the correct and in the opposite orientations, respectively. Plasmid p113 is a deletion of pEPH-7 containing the prolactin enhancer region on a 288-base pair *Hin*dIII/*Xba*I fragment joined to the prolactin promoter.

Fig. 2: DNA sequence of 2.2 kb of prolactin 5′ flanking sequence. Single base pair differences between our sequence and that reported by Sakai, et al. (40) are shown with the base pair differences in lower case under the affected base. A caret (^) denotes the insertion of a base and a dash (−) a missing base. The region of the bovine sequence that displays similarity to the rat enhancer region is shown in bold and underlined. The bold and starred bases between +18 and +19 are the bases inserted to generate a *Bam*HI site and are not part of the native prolactin sequence. The repetitive DNA element homologous to a portion of the bovine 1.709 satellite DNA is in lower case.

first 250 bases of the prolactin 5′ flanking sequence (23), the existence of additional regulatory sequences more than 1 kb from the start of the bovine transcription initiation start site, analogous to those seen in rat, was investigated.

To isolate additional sequences upstream of the bovine prolactin promoter, a bovine DNA library was constructed in λEMBL3. An hGH expression plasmid, pEPH-7, containing a 2.0 kb *Bgl*II fragment containing prolactin gene 5′ flanking sequences joined to the previously synthesized prolactin fragment was constructed as described in Materials and Methods. A similar construct (POPH-17) containing the 2.0 kb *Bgl*II fragment in the opposite orientation was also isolated.

Sequencing of the 2.0 kb prolactin promoter region and localization of the enhancer by DNA homology

The sequence of the entire 2.0 kb *Bgl*II fragment as well as the synthetic promoter region is shown in Figure 2. The rat enhancer sequence has been localized to a fragment extending from −1769 to −1531 relative to the start of transcription (16). The 2.0 kb *Bgl*II fragment was searched for sequence homologies to the rat enhancer sequence using the DNA alignment program of Wilbur and Lipman (32). The results of the homology search revealed a region of similarity (Fig. 3) between the rat enhancer and positions −1175 to −996 in the bovine sequence.

Effect of the 2.0 kb enhancer fragment on expression of hGH

To assess the effect of the additional 5′ prolactin sequences, plasmids p103, pEPH-7 and pOPH-17 were transfected into GH_3 rat pituitary cells and expression of hGH was measured by assaying the amount of hGH protein that accumulated in the medium. Parallel transfections were performed with plasmid pXGH-5, a construct containing the mouse metallothionein promoter fused to hGH, and p0GH as controls. Immediately following transfection, the cells were cultured with or without EGF and aliquots of cell supernatant were taken at various times after transfection and assayed for hGH activity. Values for p0GH were at background levels, <0.1 ng/ml (data not shown). The addition of EGF to cultures transiently transfected with the synthetic promoter fragment construct, p103, boosts expression levels by as much as 10-fold (Fig.4). Similar experiments using TRH also resulted in a two- to four-fold induction of hGH (data not shown). Expression of the control plasmid pXGH-5 containing the mouse metallothionein promoter was unaffected by the addition of EGF. The effect of the additional 5′ flanking

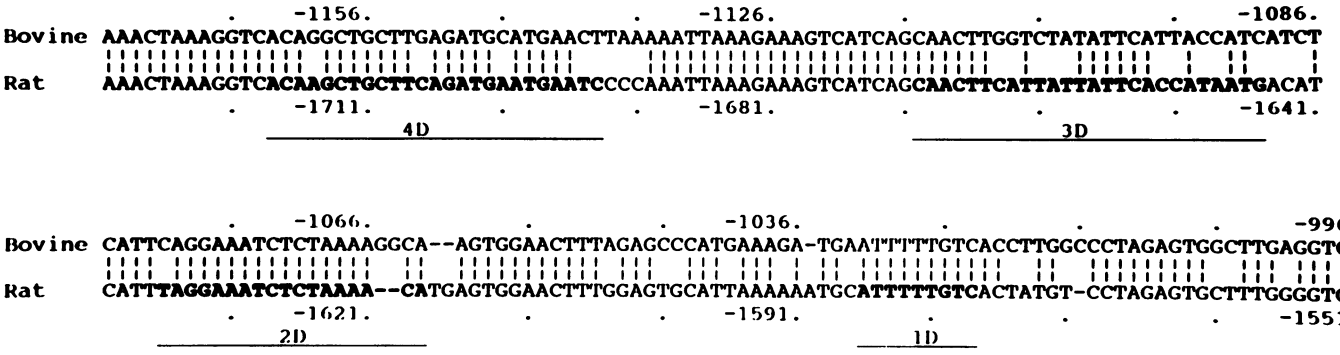

Fig. 3: DNA sequence homology between the bovine 5′ flanking sequence and the rat enhancer region sequence. The four areas of protein binding identified in the rat: 4D, 3D, 2D, and 1D, are highlighted (33).

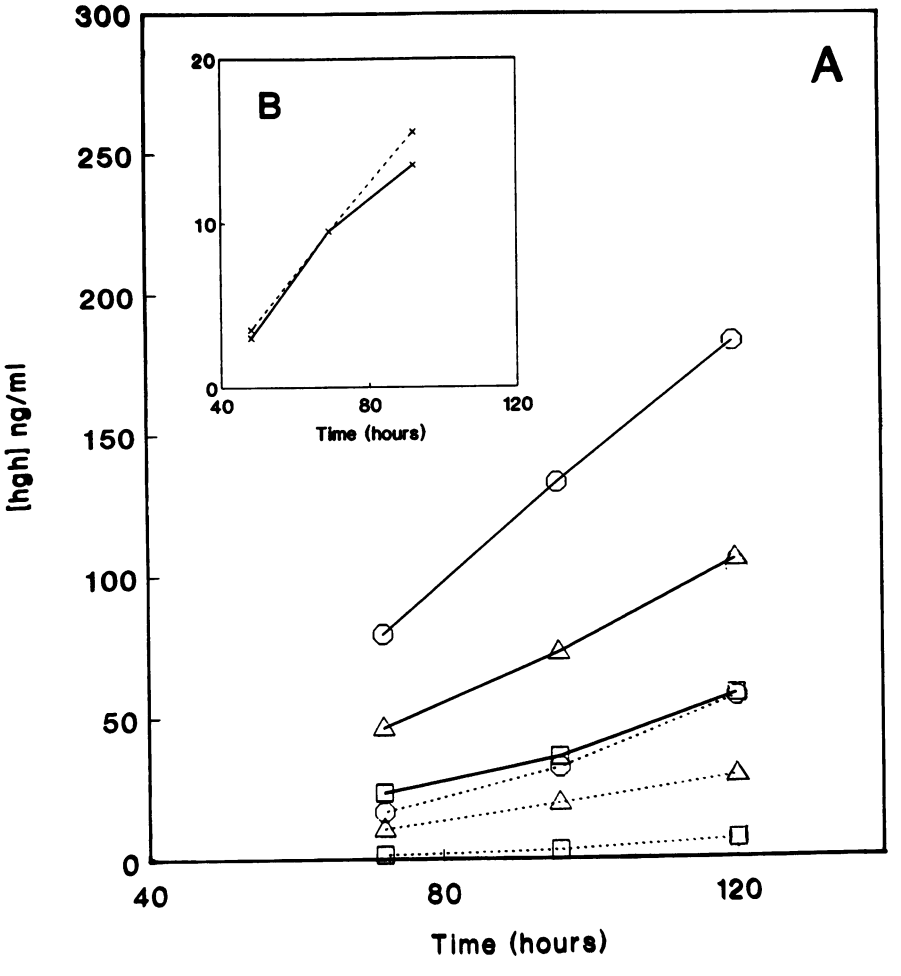

Fig. 4: Accumulation of hGH in cell culture medium following transfection of GH_3 cells with PRL:hGH (A) or mMT1:hGH (B) constructs. A. 10^6 GH_3 cells were transfected with 0.5 μg plasmid pXGH-5 (x) in 100 mm culture dishes and cultured in the presence (——) or absence (– –) of EGF. B. 2×10^6 GH_3 cells were transfected with 0.25 μg of plasmid p103 (□), pEPH-7 (△), and POPH-17 (○) in 60 mm culture dishes and cultured in presence (——) or absence (– –) of EGF.

sequences is to boost basal expression of hGH by about five-fold. An increase in expression is also seen in the construct containing the upstream sequences in the opposite orientation suggesting that this regulatory element is functioning like an enhancer element. The effect of the enhancer in the presence of the inducer is a two-to-four-fold stimulation in hGH expression.

Deletion analyses of the bovine enhancer region

If the structure of the bovine prolactin 5′ flanking region is analogous to the rat, then the bovine enhancer is likely to be localized to the region defined by homology to the rat enhancer. Indeed, plasmid p113, a 300-base pair subclone of the 2.0 kb *Bgl*II fragment containing this region alone fused to the prolactin

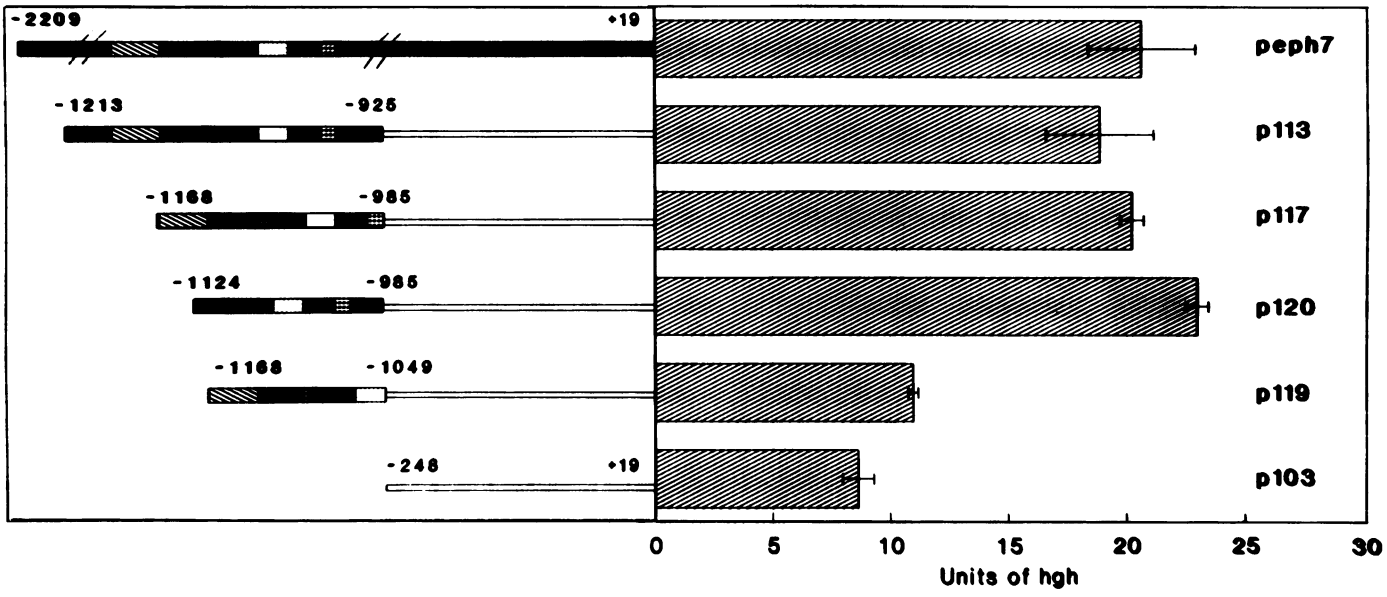

Fig. 5: Accumulation of hGH at 120 hours following transfection of GH_3 cells with prolactin enhancer constructs and culturing with EGF. The endpoints of the enhancer region are shown with the regions homologous to the DNA binding regions in rat indicated—4D (stripes); 3D (hatched); 2D (pinpoint dots); 1D (large dots). The open box represents the synthetic prolactin promoter fragment extending from −248 to +19. All constructs are fused to the hGH structural gene. Units of hGH were determined as described in Materials and Methods.

proximal promoter, gave transient expression levels similar to that seen with pEPH-7 (Fig. 5). To further localize the sequences necessary for enhanced gene expression, a deletion analysis of the enhancer region was performed. Three enhancer deletion constructs were assayed for their ability to enhance hGH expression in transiently transfected GH_3 cells induced with EGF. Deletion construct p120 containing sequences −985 to −1124 fused to the proximal 250 base pairs of the 5′ flanking region shows full enhancer activity suggesting that this 139 base pair region is necessary and sufficient for enhancement of prolactin expression. However, deletion of sequences −1049 to −985, as in construct p119, results in an almost complete loss of enhancer activity as the expression levels are similar to construct p103 containing only the proximal 250 base pairs. The region deleted is homologous to the rat DNA binding region 1D (33) in rat, also shown to be important in expression of rat prolactin.

DISCUSSION

A genomic DNA clone containing 2.0 kb of bovine prolactin 5′ sequence was isolated and the entire DNA sequence of this DNA fragment was determined. In transiently transfected rat pituitary cells, the addition of this fragment to the synthetic promoter region driving the synthesis of hGH resulted in a stimulation in basal hGH expression of five-fold. A construct containing this fragment in the opposite orientation also stimulated transcription suggesting that the element identified was functioning like an enhancer. Interestingly, the opposite orientation construct stimulated hGH accumulation as much as two-fold higher than the correct orientation. In the opposite orientation construct, the actual enhancer sequences are positioned farther from the transcription start site by about 300 bases. It is possible that the spacing difference alters the helical position of the enhancer relative to the promoter causing this difference in expression. The addition of EGF to the cells transfected with the enhancer constructs stimulated expression of hGH over that seen with the proximal 250 base pairs alone suggesting that EGF interacts with DNA sequences in both the promoter region and the enhancer. Similar results have been reported in the rat prolactin enhancer regions (18).

To localize the enhancer element on the 2.0 kb fragment, a DNA homology search comparing the 2.0 kb bovine fragment with the region identified as the rat enhancer element was performed. Comparison of the two DNA sequences showed that the maximum sequence homology can be seen when base pair −1730 to −1559 in the rat is aligned with base pair −1175 to −996 in the bovine sequence (Fig. 3). The four distal regions (1D-4D) identified by DNase footprinting (33) as areas of protein binding in the rat are 87, 88, 88, and 87% homologous to analogous regions in the bovine sequence for regions 4D, 3D, 2D and 1D, respectively. These elements have been shown to bind tissue-specific trans-acting factors and to be required for maximal expression of the prolactin gene in rat pituitary cells cultured *in vitro*. These factors, although tissue-specific, do not appear to be species specific since rat trans-acting factors can interact with the bovine 5′ flanking region to activate transcription.

Confirmation of the localization of the bovine enhancer was obtained by a deletion analysis of the region displaying DNA homology to rat. The boundaries of the deletion p120 subclone localized the boundaries of the enhancer to a region from −1124 to −985. Interestingly, this region does not include the putative DNA binding region 4D which does appear to be necessary for maximum expression in rat (33). Deletion of sequences −985 to −1049 results in loss of most of the enhancer activity suggesting that this region (containing the sequences homologous to region 1D) are essential for expression.

Although the rat and bovine enhancer elements are highly homologous, their relative distance from their respective

transcription start sites differs by 500 bases, the bovine enhancer region being closer to the bovine prolactin start site. A more extensive DNA alignment was performed on the entire rat and bovine prolactin 5′ flanking regions to determine where the sequences diverged (34). The region from −1175 to −996 (the enhancer region) is highly homologous, 79%; an even greater similarity than the two prolactin structural genes which show 71% homology. There is less DNA homology (42%) in the region between the enhancer and the next 450 bases of flanking sequence. This region includes two large gaps in the bovine sequence that accounts for the difference in enhancer-promoter distance. One gap is about 120 base pairs in length and in the rat includes a large stretch of poly GT repetitive sequences. This region has been shown to be involved in the down-regulation of prolactin transcription in the rat as well as to form left-handed (Z) DNA (35). An additional large gap of more than 400 bases in the bovine sequence is also present but these sequences have not been identified as containing any regulatory function in the rat.

At the most 5′ end of the cloned distal prolactin region (positions −2090 to −1828) is a repetitive DNA element. This sequence is 87% homologous to the dimeric dispersed repeated sequences in bovine 1.709 satellite DNA (24). Homologous repetitive elements have also been found in the 5′ flanking region of other bovine genes (36−39).

The bovine DNA sequence was also compared with the 1 kb of previously published bovine genomic sequence (23, 40). Seventeen individual base pair differences were found between the two sequences, all of which occurred between positions −975 and −570. Confirmation of our sequence data was obtained by cloning and sequencing this region from a Holstein unrelated to our original clone. The identical sequence was obtained. Based on restriction fragment length polymorphisms (23, 41) and DNA sequence analysis (42, 43), heterogeneity at the prolactin locus has been described and breed-related differences have been found. If the differences are due to nucleotide sequence polymorphisms, they may not affect expression as none of the changes were located in regions known to be involved in regulation of transcription. The published sequence did not extend into the region we have defined as the upstream enhancer element so no sequence comparison of this essential region could be performed.

The distal prolactin enhancer in rats has been reported to account for 99% of promoter activity (33). In this study, only about 50% of the total activity is due to the presence of the upstream bovine enhancer. In addition, some of the sequences essential for maximal activity in the rat are dispensable in bovines. An explanation may be a reflection of species differences in the pattern of prolactin expression. In bovines and other ruminants, prolactin is the major pituitary hormone in the adult (44−46), in contrast to rodents and other mammalian species where growth hormone is the predominant hormone (47). In light of this, it is interesting that there is so much homology between the rat and bovine promoter sequences and that the rat cells can regulate expression from the bovine promoter. This may suggest that the trans-acting factors that bind the DNA and regulate expression are very similar in both species but that the relative amount of the proteins in the cell as well as the individual affinities of the DNA binding sites for these factors are different. Since the experiments reported here reflect the expression of bovine sequences in rat cells, we would like to determine whether the response of the bovine cis-acting elements would be different if they were expressed in bovine pituitary cells.

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