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Interaction of Basal Positive and Negative Transcription Elements Controls Repression of the Proximal Rat Prolactin Promoter in Nonpituitary Cells

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The proximal rat prolactin (rPRL) promoter contains three cell-specific elements, designated footprints I, III, and IV, which restrict rPRL gene expression to anterior pituitary lactotroph cells. Footprint II (−130 to −120) binds a factor, which we have termed F2F, present in pituitary and nonpituitary cell types. Here we demonstrate that a key role of the footprint II site is to inhibit rPRL promoter activity in nonpituitary cells, specifically, by interfering with the basal activating function of a vicinal element. Gene transfer analysis revealed 20-fold activation of the rPRL promoter in nonpituitary cell types when footprint II was either deleted or specifically mutated. Similar activation of the intact rPRL promoter was obtained by in vivo F2F titration studies. In GH_4 rat pituitary cells, the footprint II inhibitory activity was masked by the redundant, positively acting cell-specific elements and was inhibitory only if the two upstream sites, footprints III and IV, were deleted. Deletion of the −112 to −80 region in the footprint II site-specific mutant background resulted in complete loss of rPRL promoter activity in both pituitary and nonpituitary cell types, mapping a basal activating element that is operative irrespective of cell type to this region. While the basal activating element imparted an activating function in a heterologous promoter assay, the footprint II sequence did not display any inherent repressor function and actually induced several minimal heterologous promoters. However, the inhibitory activity of the footprint II site was detected only if it was in context with the basal activating element. These data underscore the importance of ubiquitous activating and inhibitory factors in establishing cell-specific gene expression and further emphasize the complexity of the molecular mechanisms which restrict gene expression to specific cell types. We provide a novel paradigm to study rPRL promoter function and hormone responsiveness independently of lactotroph cell-specific requirements.

Eukaryotic cells have evolved a striking capacity to completely alter their program of gene expression in response to temporal, developmental, and metabolic signals. Indeed, differential activation and simultaneous repression of specific genes appears to be critical for orderly development of highly specialized tissues (19, 22, 37, 65). While the role of nuclear factors which activate novel patterns of tissue-specific gene transcription during development have been well documented (6, 13, 17, 22, 24, 25, 33, 38, 52, 59, 60), the importance of inhibitory transcription factors in establishing the final, fully differentiated phenotype is not very well understood. Previous studies have indicated that generalized repression, as effected by chromatin structure, DNA methylation, and H1 linker histone, may render gene regulatory regions inaccessible to diffusible activating nuclear factors and RNA polymerase II and thus be the dominant mechanism by which expression of tissue-specific genes is not allowed in inappropriate cell types (65). Additionally, distinct repressors which extinguish pituitary-specific (43, 64), lymphocyte-specific (69), and entire networks of liver-specific (34, 66) genes in fibroblast cells have been described. Current evidence indicates that these extinguishers act by repressing the transcription of certain cell type-specific transcription factors (43). Recently, however, significant progress has been made in the identification of DNA-binding proteins that repress the expression of selective genes (8, 10, 11, 15, 28, 35, 49, 50). Yet our understanding of the precise molecular details by which they control promoter activity remains incomplete. Nevertheless, these data have implicated molecular strategies used by repressor proteins as elaborate as those determined for transcriptional activator proteins (37).

In the simplest scheme, the DNA sequence which binds the repressor overlaps with either the transcription initiation site or an upstream activating element and thus either physically interferes with the formation of a stable initiation complex or blocks the binding of a necessary *trans*-acting factor, respectively (2, 8, 15, 28, 35, 49, 50). The molecular mechanism implicit in this paradigm is the competitive and mutually exclusive binding, due to steric hindrance, of repressor and activator proteins for overlapping DNA elements. Alternative inhibitory mechanisms include quenching, direct inhibition, and squelching (37). Quenching, as manifested by the yeast α2 mating type and the MCM1 proteins, requires that both an activator and a repressor bind the DNA such that the repressor interferes with the ability of the positive factor to interact with the general transcription machinery (32, 37, 54). Direct inhibition is thought to occur when a DNA-bound inhibitory factor directly blocks the action of the basal transcription complex (8, 37, 50). A silencer is a special case of the direct inhibition model in which negative transcription regulation occurs with properties similar to those of an enhancer; that is, repression

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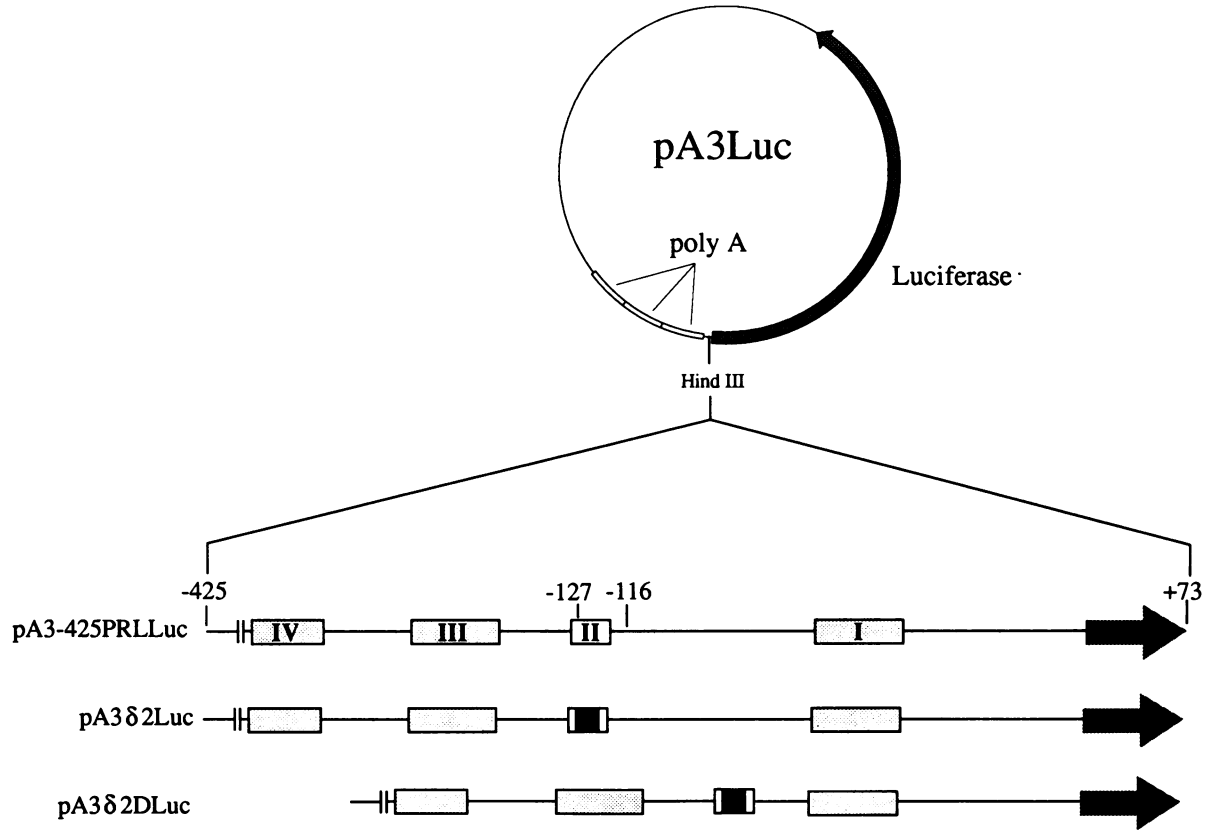

FIG. 1. Structural organization of rPRL promoter constructs. The rPRL 5′ deletion mutants were constructed by subcloning a DNA fragment that encodes luciferase downstream of rPRL promoter fragments with 5′ endpoints of −425, −127, and −116 in a pGEM4 background, as previously described (30). Additionally, at the top is the pA3*Luc* vector (42), which contains a trimerized poly(A) stop signal upstream of the cloning sites and luciferase reporter gene. This vector was used as the recipient reporter vector for the intact and site-specific mutants of the rPRL promoter indicated below. As with our original prPRL*Luc* (30), the intact rPRL promoter in the pA3*Luc* vector (pA3−425PRL*Luc*) contains the −425- to +73-bp region of the rPRL gene. The reporter construct containing the FPII site-specific mutation (shown as a dark box in FPII) is labelled pA3δ2*Luc*, and the internal deletion mutant devoid of the −112 to −80 region in the δ2 background is labelled pA3δ2D*Luc*. All of these constructs, whether in the pGEM4 or pA3*Luc* background, have a common rPRL 3′ endpoint of +73. The organization of the pituitary-specific footprints are shown as stippled rectangles labelled I (−45/−67), III (−148/−170), and IV (−192/−209); FPII (−120/−130), which binds a ubiquitous factor, is depicted as a white rectangle; and the transcribed portion is indicated by a dark arrow. The position of the 5′ endpoint of each rPRL promoter deletion template is mapped relative to the FPII site.

occurs over a distance and is independent of DNA orientation (9, 10, 37, 51). In its most general terms, squelching is based on protein-protein interactions such that the activation or excessive production of one factor, operationally the repressor, allows it to interact with a separate and required factor, essentially titrating away the activating factor and precluding it from binding to DNA (1, 7, 44). Clearly, the formation of certain heterodimers may amplify the combinatorial possibilities between members of transcription factor families, and some of these heterodimers may function as repressors, particularly if they include nuclear factors that are devoid of the DNA-binding domain (5, 7, 14, 18, 29, 37, 39). It is clear, however, that a single *cis*-acting DNA element or a single *trans*-acting factor can act as either a positive or a negative regulator of transcription and that posttranslational modifications, the context of nearby sequences, and the presence of accessory factors may greatly influence the final activity of any transcription factor.

The anterior pituitary gland serves as a model system for studying tissue-specific gene expression. Prolactin, a peptide hormone that is a member of the growth hormone (GH) gene family, is restricted in its expression to pituitary somatomammotrophs, where it is produced in conjunction with GH, and to pituitary lactotroph cells, where it is uniquely expressed (45). By using DNase I protection analysis, three pituitary-specific factor binding sites, labelled footprint I (FPI), FPIII, and FPIV (Fig. 1), have been localized to the proximal 210 bp of the proximal rat prolactin (rPRL) promoter (21, 23, 40, 47, 57, 58). All three elements bind Pit-1/GHF-1, which is a homeodomain-containing protein that *trans*-activates both GH and PRL promoters and, more importantly, appears to be critical for pituitary stem cell commitment to the somatotroph, lactotroph, and thyrotroph cell lineages (13, 38, 41, 59, 63). We have been able to identify and purify a pituitary-specific factor, which we have designated lactotroph-specific factor 1 (LSF-1), that is distinct from Pit-1/GHF-1 (4, 20, 21). In cell-free transcription-reconstitution experiments, LSF-1 preferentially activates the rPRL promoter and has only negligible effects on the GH promoter (20a). It is of note that affinity-purified LSF-1 is also capable of activating the murine TSHβ promoter in HeLa nonpituitary cell extracts (4). Taken together, these data show that tissue-restricted transcription factors, such as Pit-1/GHF-1 and/or LSF-1, are important not only for lac-

totroph cell ontogeny but also for cell-specific induction of the rPRL gene. However, they do not document whether the simple lack of a cell-specific transactivator is sufficient to explain rPRL promoter inactivity in nonpituitary cells or whether the additional inhibitory action of a repressor is also required. It is of note that the importance of extinguishers (39, 43) and putative silencers (64) of GH gene activity in nonpituitary cells has been shown, yet a similar repression mechanism for the lack of rPRL promoter activity in nonpituitary cells has not been documented.

In this respect, the biological role of the FPII site (−130 to −120; Fig. 1), which binds a ubiquitous factor (F2F) present in pituitary and nonpituitary cells, becomes particularly intriguing. This report focuses on the functional significance of the FPII site in regulating rPRL promoter activity in pituitary and nonpituitary cultured cells by gene transfer analysis and shows that FPII functions as a dominant repressor element in nonpituitary cells. Activation of the rPRL promoter in nonpituitary cells is due solely to a basal activating element which maps adjacent to the FPII site and is released from F2F inhibition by FPII mutagenesis, whereas in pituitary cells the inhibitory activity of the FPII site is masked by the positive effects of the redundant cell-specific elements and its repressive activity is detected only if the two upstream LSF-1 sites are removed. Nevertheless, the basal activating element also displays a critical role in maintaining rPRL promoter activity in pituitary cells. Heterologous-promoter studies show that the FPII element is not inherently inhibitory, but rather its repressive function is manifested only if it is in context with the vicinal basal activating element. On the basis of these data, we present a model for the mechanism of FPII-mediated repression of the rPRL promoter in nonpituitary cells, whereby F2F binding to the FPII site interferes with the stimulatory function of the adjacent basal activating factor. Moreover, establishment of a model system that allows expression of a pituitary-specific gene in nonpituitary cells provides a powerful tool for analysis of the effects of signal transduction and hormone action on rPRL promoter activity independently of lactotroph-specific influences.

MATERIALS AND METHODS

Plasmid constructs. Plasmids referred to as prPRL*Luc*, p-127rPRL*Luc*, and p-116rPRL*Luc* (Fig. 1) were generated by inserting a 2.6-kb *Hin*dIII-*Bam*HI DNA fragment that encodes the firefly luciferase cDNA and simian virus 40 intron and polyadenylation signals from pSV232AL-AΔ5′ (12) downstream of the rPRL promoter in constructs derived from pG6PRL containing the −425, −127, and −116 to +73 regions of the rPRL gene, respectively, as previously described (30). For site-specific mutagenesis, plasmid pG7PRL was first constructed by subcloning a 570-bp *Eco*RI fragment containing the −425 to +73 rPRL promoter region and flanking restriction sites from pG6PRL (23, 30) into phagemid pGEM7zf(+) (Promega). Single-stranded DNA prepared from the pG7PRL phagemid and a 21-mer mutant oligonucleotide, 5′-GATGTT**GTCGAC**TATTGGGGC-3′, was used in an oligonucleotide-directed mutagenesis kit (Amersham) to alter positions −123 to −128 of the rPRL FPII element from TAAAAT to **GTCGAC**, resulting in a diagnostic *Sal*I site in FPII (altered bases are in boldface) (23, 46, 62). Protocols supplied by the manufacturer (Amersham) were utilized, except that the oligonucleotide-to-template ratio was increased 10-fold. Also, to ensure complete repolymerization, 10 ng of an SP6 promoter primer was added to the final synthesis step. Two FPII site-specific mutants identified by *Sal*I digestion were further analyzed by DNA sequencing, resulting in pδ2PRL, which contains only the expected alterations in the FPII site, and pδ2DPRL, which contains not only the same FPII mutation but also a 32-bp deletion from positions −112 to −80 between FPI and FPII (Fig. 1). A 557-bp, *Hin*dIII DNA fragment containing the −425 to +73 rPRL region and a 59-bp polylinker was excised from the wild type and site-specific mutants in the pG7PRL background and inserted into the *Hin*dIII site of pA3*Luc* (42), resulting in plasmids pA3-425PRL*Luc*, pA3δ2*Luc*, and pA3δ2D*Luc* (Fig. 1). The trimerized poly(A) stops upstream of the polylinker and luciferase reporter gene in pA3*Luc* result in a promoterless reporter vector with negligible activity in gene transfer studies, and thus the activity of any promoter fragment cloned into the polylinker can be assayed with increased sensitivity owing to the lack of significant contribution from vector readthrough (3, 4, 42, 68). The orientation of the inserted promoter fragment and documentation of specific rPRL promoter mutations in either the pA3*Luc*- or the pGEM4-based vectors were verified by DNA sequencing using a luciferase oligonucleotide primer as previously reported (3, 4, 68).

Additionally, a synthetic double-stranded oligonucleotide encompassing the rPRL FPII site, from positions −137 to −114, was inserted via *Sal*I ends into pGEM7zf(+) (Promega), resulting in p3xFP2, which contains a trimerized FPII oligonucleotide. Additionally, the same FPII oligonucleotide was cloned upstream of several minimal heterologous viral promoter constructs driving the luciferase reporter gene (48, 68). Cloning of the FPII oligonucleotide at position −130 of an enhancerless Rous sarcoma virus (RSV) promoter (pA3RSV130*Luc*) (68) resulted in pFP2RSV130*Luc*, and FP2 insertion at positions −109 and −81 of the Herpes simplex virus thymidine kinase (TK) promoter (pTK109*Luc* and pTK81*Luc*) (48) resulted in pFP2TK109*Luc* and pFP2TK81*Luc*, respectively (see Fig. 7). In the RSV construct, the FPII oligonucleotide is in the same orientation as the RSV promoter, whereas in both of the TK constructs, the FPII oligonucleotide is in the opposite orientation. In a similar fashion, a synthetic oligonucleotide encompassing the −117- to −71-bp region of the rPRL promoter was cloned in the correct orientation just upstream of the RSV promoter in both the pRSV130*Luc* and pFP2RSV130*Luc* constructs, resulting in plasmids p(−117/−71)RSV130*Luc* and pFP2(−117/−71)RSV130*Luc*. Position −85 was changed from a T to a C in the −117 to −71 oligonucleotide to generate a *Sau*3A site that cuts the 52-bp oligonucleotide into two fragments of 33 and 19 bp that were used in gel shift analysis.

Tissue culture and electroporation. Monolayer cultures of GH_4 rat pituitary tumor cells and nonpituitary cell lines of HeLa human cervical carcinoma cells and Rat2 fibroblast cells were maintained in culture in Dulbecco's modified Eagle's medium–10% fetal calf serum–and 50 μg each of penicillin and streptomycin per ml. Cells were harvested at approximately 70% confluence and electroporated by using conditions previously determined as optimal (30) and the plasmid DNA amounts indicated in the figure legends. Following electroporation, cells were maintained in culture for 8 to 24 h, depending on the optimal time of luciferase expression for each cell line (30). Cells were then harvested and lysates were measured for luciferase activity as previously reported (3, 12, 30, 31, 68). When making comparisons across cell lines, the various cells lines were electroporated in parallel by using the same number of cells and the same

plasmid DNA preparation. Within each experiment, each plasmid construct was electroporated in triplicate and the mean luciferase activity of the three individual electroporations was calculated and expressed relative to that obtained with the respective wild-type vector within that experiment. Identical experiments were then repeated in triplicate, as described above, on at least three separate occasions by using different plasmid DNA preparations. Data for each plasmid construct were then combined across experiments to give the mean ± the standard error of the mean (SEM). Having compared the various methods to minimize variation between replicate transfections, such as expressing the data relative to an internal control expressing β-galactosidase, relative to a parallel control, or relative to the total protein content, we found that simply repeating the experiment many times on separate days by using different plasmid DNA preparations and analyzing the data by statistical methods provided the best consistency of agreement, i.e., SEMs of 20% or less (unpublished data). Furthermore, our earlier studies have shown that using the electroporation method of introducing DNA into cells results in transfection efficiencies into GH or GC rat pituitary cells and HeLa cells that are fairly uniform (3, 30, 31, 68).

Gel shift analysis. Whole-cell and nuclear extracts were prepared, in accordance with previously published procedures (3, 4, 21, 23, 68), from GH_4 and GC rat pituitary cell lines and HeLa and Rat2 nonpituitary cell lines. Various amounts of cellular extracts were incubated with 250 ng of sheared herring sperm nonspecific competitor DNA and about 0.1 ng ([1 to 2] × 10^4 cpm) of radiolabeled oligonucleotides encompassing either the FPII (−137 to −114) site or different regions (−117 to −71 or −117 to −85) of the rPRL basal transcription element (BTE), as previously described (3). The −117/−85 oligonucleotide was generated by cutting the −117/−71 DNA with *Sau*3A and purifying the 33-bp fragment. Specific competition was performed with increasing amounts of the unlabeled FPII oligonucleotide or with an oligonucleotide encompassing either the most proximal Pit-1/GHF-1-binding site of the rat GH promoter, GC1 (67), or the MyoD-binding site of the muscle creatine kinase promoter. After 20 min of incubation at room temperature, loading buffer was added to each reaction and the samples were immediately loaded onto a 4% polyacrylamide gel in 45 mM Tris-borate (pH 8.3)–1.25 mM EDTA and electrophoresed at 150 V in 22.5 mM Tris-borate (pH 8.3)–0.625 mM EDTA running buffer. The dried gel was exposed to film, and labeled DNA was visualized by autoradiography.

RESULTS

rPRL FPII is a dominant inhibitory element in nonpituitary cells. Our previous analysis of 5′ deletion mutants of the rPRL promoter revealed significant promoter activity of a −178 deletion, yet further deletion to −127 resulted in essentially complete lack of promoter activity (30). This loss of basal rPRL promoter activity with 5′ truncation to the −130 region was also reported by other investigators who used gene transfer analysis (39, 40, 53, 57). While significant reduction in promoter activity could be due to removal of a critical positive transcription element, such as FPIII, the almost complete loss of promoter activity with the −127 deletion suggested to us that this 5′ deletion endpoint unmasked a strong repressor element. To test this possibility directly, the promoter activities of 5′ deletion mutants that either retained (p−127PRL*Luc*) or were devoid of (p−116PRL*Luc*) the FPII site and an FPII site-specific mutant (pA3δ2*Luc*) were compared with that of the intact promoter (p−425PRL*Luc*) in both pituitary and nonpituitary cell lines (Fig. 2). The luciferase activities obtained for the various rPRL promoter constructs are shown relative to the activity of the −425 construct in GH_4 rat pituitary tumor cells. The results shown in Fig. 2 corroborate and extend our previous findings (30), indicating that the promoter activity of the −127 deletion is only 5% of that of the intact promoter in pituitary cells and that the intact rPRL promoter is 20-fold more active in pituitary than nonpituitary cells. In striking contrast, further deletion of the FPII site, from positions −127 to −116, resulted in about 13-fold (from 5 to 65%) reactivation of rPRL promoter activity in GH_4 rat pituitary tumor cells. Particularly surprising was the effect of either deleting or site-specifically mutagenizing the FPII site on rPRL promoter function in HeLa cells, which was about 20-fold (from 1 to 20%) or 50-fold activation in these nonpituitary cells, respectively. Similar data were obtained with Rat2 fibroblasts cells (data not shown). By contrast, the same FPII site-specific mutation did not activate the rPRL promoter in GH_4 pituitary cells but rather resulted in about a 25% reduction in activity (Fig. 2). These data imply that rPRL FPII DNA acts as a dominant inhibitory element in nonpituitary cells, whereas in pituitary cells it becomes a dominant negative element only if upstream cell-specific elements are deleted. Furthermore, these data provide further support of our original observation that a ubiquitous, FPII-binding factor is present in both pituitary and nonpituitary cells (21).

F2F competition derepresses wild-type rPRL promoter activity in nonpituitary cells. By using DNase I protection analysis, we previously reported the identification, in pituitary and nonpituitary cell lines, of a ubiquitous factor (called F2F here) which binds to the FPII site (21). To determine directly whether the differential effect of FPII mutagenesis on rPRL promoter activity in the various cell types could be due to differences in the relative amounts of F2F, a gel shift assay was performed. As shown in Fig. 3A, lanes B to D, a radiolabeled double-stranded oligonucleotide encompassing the FPII site (from positions −137 to −114) is shifted to the same position by an equivalent amount (2.5 μg) of GH_4 and HeLa cell nuclear extract, or Rat2 whole-cell extract. The relative intensity of the shifted band indicates that HeLa cells contain slightly increased levels of F2F compared with that present in GH_4 cells. However, since whole-cell extracts were used to assess F2F levels in Rat2 cells, it is likely that these nonpituitary cells also have increased amounts of F2F compared with GH_4 pituitary cells. Furthermore, increasing the amount of HeLa nuclear extract from 2 to 16 μg (Fig. 3B, lanes B to E) revealed an increased intensity of complex 3 and the appearance of three other shifted bands, indicated as complexes 1, 2, and 4. Despite the use of the highest protein input (16 μg), specific competition with increasing amounts of the unlabelled FPII oligonucleotide (Fig. 3B, lanes F to H) revealed that the protein-FPII DNA interaction resulting in complex 3 was diminished with as little as 10 ng and completely eliminated with 250 ng of competitor DNA, whereas complexes 1, 2, and 4 were much less affected. By contrast, 50 ng of an irrelevant oligonucleotide (GC1) encompassing the most proximal rGH Pit-1/GHF-1 site did not show any significant competition of any of the four complexes (Fig. 3B, lane I), compared with the specific competition obtained with a similar amount of FPII DNA (Fig. 3B, lane G). These data show that a stable and specific interaction between FPII DNA and HeLa nonpituitary nuclear protein(s) resulted initially in complex 3 and

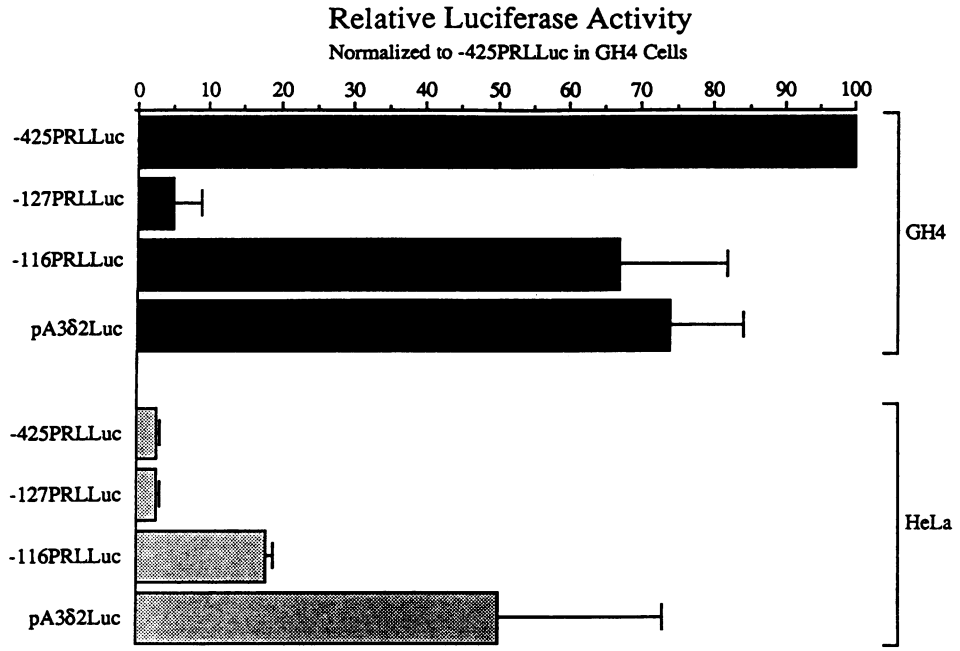

FIG. 2. Effect of the FPII motif on relative expression of the rPRL promoter in the GH_4 pituitary and HeLa nonpituitary cell lines. Cells were transiently transfected, each time in triplicate, with 5 to 20 μg of the indicated rPRL-*Luc* reporter constructs (Fig. 1), which contained either an intact FPII element (−425 and −127 constructs) or an altered FPII element (deleted in the −116 construct and site specifically mutated in the δ2 construct). Cells were harvested at 8 h postelectroporation for GH_4 cells (solid black bars) and at 12 h postelectroporation for HeLa cells (stippled bars), since these times of harvest have previously been determined to be optimal for luciferase expression in the given cell type (30). The rPRL promoter activity of each reporter construct is expressed relative to that of the intact −425 rPRL promoter construct (in either prPRL*Luc* or pA3−425PRL*Luc*, since these have equivalent rPRL promoter activity) in GH_4 rat pituitary tumor cells (value set at 100%). The data are shown as the mean ± SEM from 3 to 10 separate transfections performed with different plasmid preparations on different days.

that GH_4 pituitary and Rat2 nonpituitary cells also contain this factor. Complexes 1, 2, and 4 appear to result from less specific protein-FPII DNA interactions, since these complexes could not be fully eliminated by the FPII oligonucleotide.

As a separate test for the biological role of F2F in rPRL promoter activity in HeLa nonpituitary and GH_4 pituitary cells, an in vivo factor competition assay was performed by cotransfecting increasing amounts of a pGEM7 vector containing a trimerized FPII oligonucleotide (p3xFP2) together with a constant amount of the wild-type rPRL promoter ligated to the luciferase reporter gene (pA3−425PRL*Luc*). The amount of total DNA in the transfection was kept constant by including pGEM7 DNA. Although the level of luciferase activity expressed by pA3−425PRL*Luc* in nonpituitary cells is minimal (as in Fig. 2), cotransfecting increasing amounts of p3xFP2 DNA unmasked wild-type rPRL promoter activity in a dose-dependent manner, resulting in 22-fold promoter activation in HeLa nonpituitary cells at the maximal input of competitor DNA (Fig. 4). A similar titration experiment resulted in only 1.8-fold activation of the rPRL promoter in GH_4 cells (Fig. 4). These studies document the presence of a diffusible factor (F2F) in HeLa nonpituitary cells that binds to the FPII region and serves to repress wild-type rPRL promoter activity. Despite rPRL promoter reactivation in nonpituitary cells by either FPII mutation or F2F titration, the resultant pPRL promoter activity was only about 20 to 50% of that obtained in GH_4 rat pituitary tumor cells, indicating that cell-specific influences remain critical for optimal promoter activity. Moreover, the minimal effect of F2F titration in GH_4 cells further indicates that pituitary cell-specific factors play a dominant role compared with F2F in these cells. The question remains, however, of whether removal of the F2F repressor allows rPRL promoter activation to occur via the basal transcription machinery (TATA box and TFIID) or whether other activating sequences are present.

Identification of a BTE and a cognate factor (basal transcription factor [BTF]) which mediates rPRL promoter activity in both pituitary and nonpituitary cell types. To determine whether the relatively large interfootprint region between the cell-specific FPI site and the ubiquitous FPII element contains any DNA sequences necessary for optimal rPRL gene transcription, an FPII site-specific mutant from which the −112 to −80 region was also deleted was obtained (pδ2DPRL). This mutant promoter was cloned into the pA3*Luc* vector, and its activity was compared with that of the rPRL intact (pA3−425PRL*Luc*) and parental FPII site-specific mutant (pA3δ2PRL*Luc*) promoters in both GH_4 pituitary and HeLa nonpituitary cells (Fig. 5). We chose to make the −112/−80 deletion in the FPII site-specific mutant background so as to be able to assess rPRL promoter activity in nonpituitary cells. As in Fig. 2, the data are plotted relative to the activity of the intact −425 promoter in GH_4 rat pituitary cells, which is set at 100% (Fig. 5). While the effect of the FPII mutation was minimal in GH_4 cells, further deletion of the −112 to −80 region resulted in significant loss of rPRL promoter activity, from 75% of the wild-type level for the FPII mutant to 12% of the wild-type level for the combined FPII mutation plus the −112/−80 deletion. It is of note that all three pituitary-specific sites are retained in this δ2D construct, and furthermore, they remain

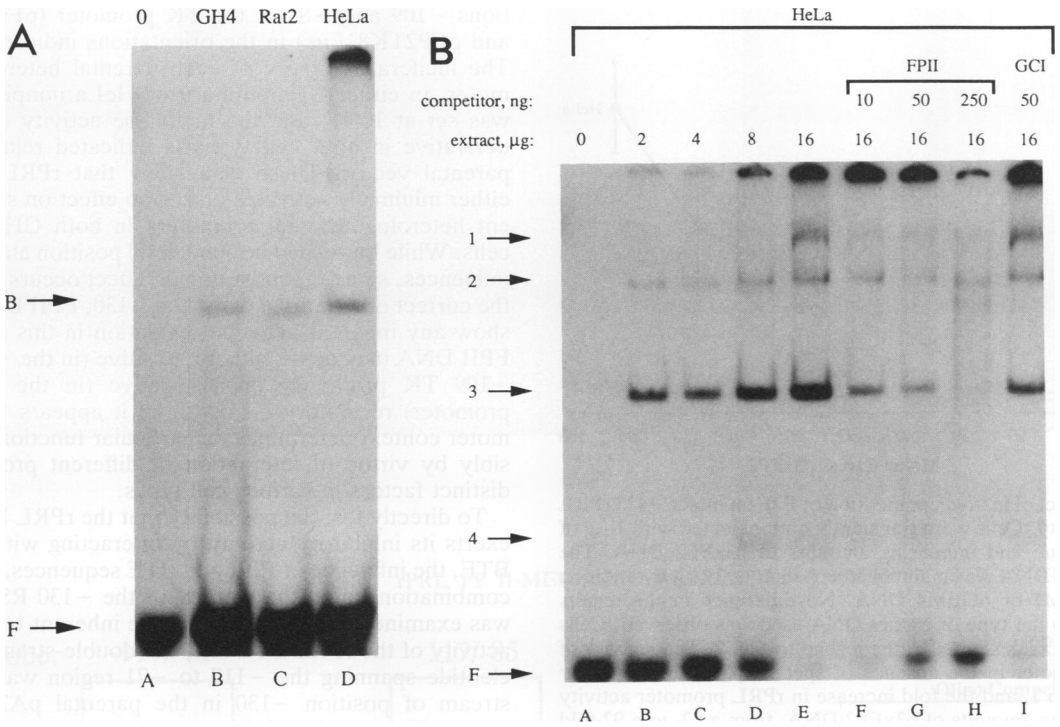

FIG. 3. Gel shift analysis of the FPII oligonucleotide. (A) Identification of FPII-binding factor F2F in 2.5 μg of protein of GH_4 pituitary nuclear extract (lane B), Rat2 whole-cell extract (lane C), and HeLa nuclear extract (lane D). The protein-DNA complex is indicated by the arrow marked B, and the free oligonucleotide is indicated by the arrow marked F. Lane A shows the migration of FPII DNA in the absence of added protein. (B) Characterization of the F2F protein-FPII DNA binding interaction. Increasing amounts of HeLa nuclear extract, from 0 to 16 μg of protein (lanes A to E), were incubated in the standard gel shift assay as described in Materials and Methods. In lanes F to H, increasing amounts (10 to 250 ng) of unlabelled, specific competitor FPII oligonucleotide DNA were included during the protein-DNA binding reaction. In lane I, 50 ng of an irrelevant oligonucleotide (GCI) spanning the proximal Pit-1/GHF-1-binding site of the rGH promoter was included. The amount of total competitor DNA was kept constant at 500 ng in all gel shift reactions by addition of sheared herring sperm DNA. The various protein-DNA complexes are indicated by arrows labelled 1 to 4, and the position of the free probe is shown by the arrow labelled F.

in correct DNA helical phase (23). The consequences of progressive mutations in rPRL promoter function in HeLa nonpituitary cells are also shown in Fig. 5. The derepressing effect of the FPII site-specific mutation compared with the wild-type promoter, as in Fig. 2, is indicated. However, further deletion of the 32-bp region from −112 to −80 resulted in essentially complete loss of the previously derepressed rPRL promoter activity. These data indicate that the −112 to −80 region is critical for basal activity of both the disinhibited rPRL promoter in HeLa nonpituitary cells and optimal rPRL promoter activity in GH_4 rat pituitary cells. However, a nuclear factor(s) that binds to this region, which we have designated rPRL BTE, has not been previously identified (13, 20, 21, 38–40, 47, 58, 59).

To determine whether the rPRL BTE is able to recognize a nuclear factor(s) contained in rat pituitary tumor cells, a gel shift assay using double-stranded oligonucleotides encompassing the −117/−71 and −117/−85 regions of the BTE was performed. As shown in Fig. 6A, lanes A and B, GC pituitary cell extracts partially purified through heparin-agarose resulted in two shifted bands. Also, by taking advantage of the *Sau*3A site that was engineered into the −117/−71 oligonucleotide, we were able to further define the region of factor binding to the −117 to −85 region of the rPRL promoter (Fig. 6A, lanes C and D). It is of note that GH_4 rat pituitary and HeLa nonpituitary cell extracts also contain a nuclear factor which shifts the −117/−71 and −117/−85 rPRL oligonucleotide DNA probes to the same positions depicted in Fig. 6A (data not shown). Furthermore, passing HeLa nuclear extracts through an FPII (−137/−114) DNA affinity column did not subtract the BTE-binding activity (data not shown), implying that this factor recognizes a distinct element and further refining the binding site location to the −114 to −85 region. However, the protein-BTE DNA (−117/−71) interaction does not appear to be of very high affinity, since an excess nonspecific (muscle creatine kinase) competitor oligonucleotide competed almost equally in the gel shift assay (Fig. 6B). Thus, at a 100-fold excess of a specific competitor, the B1 complex was essentially eliminated by competition and the B2 complex was greatly reduced (Fig. 6B, lanes 2 and 4), whereas a 100-fold excess of the nonspecific competitor did not eliminate the B1 complex but did diminish the B2 complex. The low affinity (or specificity) of this interaction may explain the inability to identify this site by DNase I protection assays.

We have termed this ubiquitous factor BTF, since it binds to the rPRL BTE, which was genetically defined as being required for basal activation of the rPRL promoter in both pituitary and nonpituitary cell types (Fig. 5). Mapping of BTF binding to a region of rPRL DNA that is vicinal and slightly overlaps the FPII repressor-binding site may have functional implications regarding the molecular mechanism of action of the F2F repressor and the BTF *trans* activator.

The inhibitory action of rPRL FPII is expressed only in the context of the BTE. Having documented the role of FPII as a repressor element in nonpituitary cells and in the context of

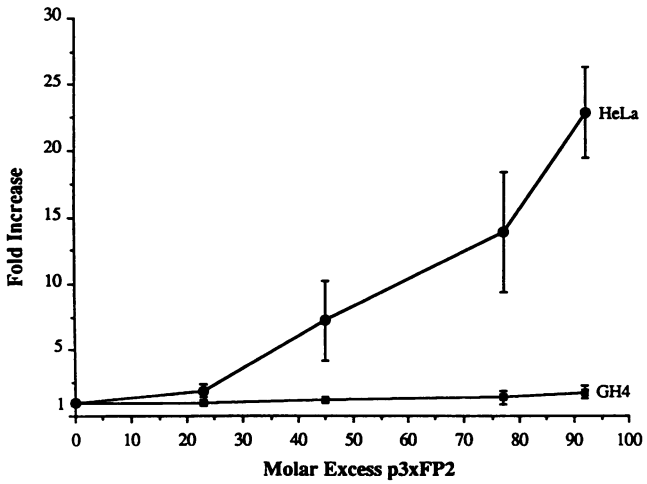

FIG. 4. Effect of in vivo competition of F2F on intact −425 rPRL promoter activity. Cells were transiently cotransfected with 1 μg of pA3−425PRL*Luc* and increasing amounts of p3xFP2 DNA. The amount of total DNA was maintained constant at 39 μg by addition of either pGEM7 or pGEM4 DNA. No difference in the results obtained due to the type of carrier DNA used was observed. Cells were harvested as described in the legend to Fig. 2. The activity of the rPRL promoter in the absence of specific p3xFP2 competitor DNA was set to 1, and the fold increase in rPRL promoter activity due to increasing amounts of p3xFP2 DNA, from a 22- to a 92-fold molar excess, is shown. The datum points are depicted as the mean ± SEM from three separate transfections for HeLa cells and two separate transfections for GH_4 cells, each performed in triplicate.

the rPRL promoter, we next wished to determine the effects of FPII DNA in different minimal heterologous promoters, at various distances from the transcription initiation site, and in different cell types (Fig. 7 and 8). To this end, the FPII oligonucleotide was inserted at position −130 of an enhancerless RSV promoter (pFP2RSV130*Luc*) and at positions −109 and −81 of the TK promoter (pFP2TK109*Luc* and pFP2TK81*Luc*) in the orientations indicated in Fig. 7. The luciferase activity of each parental heterologous promoter, in either GH_4 pituitary or HeLa nonpituitary cells, was set at 100%, and the luciferase activity of each FPII derivative in both cell types is indicated relative to each parental vector. These data show that rPRL FPII DNA either minimally activates or has no effect on several different heterologous viral promoters in both GH_4 and HeLa cells. While there may be some mild position and orientation influences, since the most drastic effect occurs when it is in the correct orientation at position −130, FPII DNA does not show any inherent negative regulation in this assay. Thus, FPII DNA may act as either a positive (in the −130 RSV or −109 TK promoter) or a negative (in the −130 rPRL promoter) regulatory element and it appears that the promoter context determines its particular functional role, possibly by virtue of interaction of different promoters with distinct factors in various cell types.

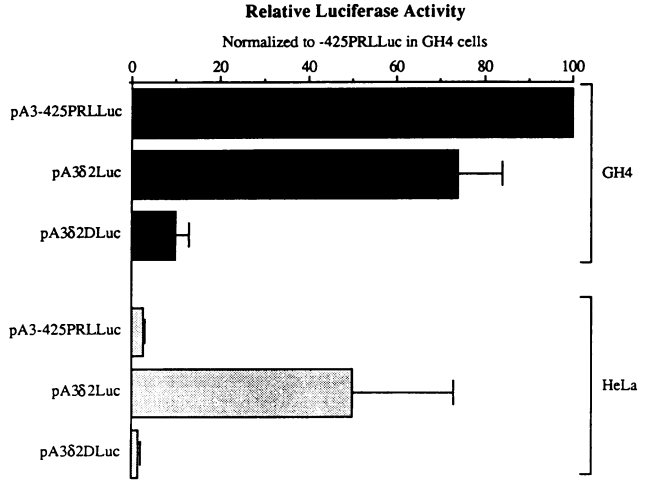

FIG. 5. Effect of deleting the BTE (−112 to −80) region on the activity of the δ2 rPRL promoter. GH_4 rat pituitary tumor cells (solid black bars) and HeLa cervical carcinoma cells (stippled bars) were transiently transfected with 5 to 20 μg of the indicated rPRL-*Luc* reporter construct, and the data are depicted as described in the legend to Fig. 2. The results are from 3 to 10 separate transfections.

To directly test the possibility that the rPRL FPII element exerts its inhibitory activity by interacting with the vicinal BTE, the influence of FPII and BTE sequences, alone and in combination, upon the activity of the −130 RSV promoter was examined. (i) To determine the inherent transcriptional activity of the rPRL BTE site, the double-stranded oligonucleotide spanning the −117 to −71 region was cloned upstream of position −130 in the parental pA3RSV130*Luc* vector. The activity of the parental vector was set at 100%, and insertion of the BTE site alone resulted in about 1.6-fold stimulation (Fig. 8). (ii) Insertion of the FPII (−137/−114) site into the same position of the −130 RSV vector resulted in 1.3-fold stimulation of heterologous promoter activity (Fig. 8), as shown in the previous section. (iii) Cloning of the FPII oligonucleotide just upstream of the BTE, so that the normal context of these rPRL regulatory elements was obtained, did not result in additive nor synergistic activation of the heterologous promoter, but rather the combined presence of the two elements reduced the 1.6-fold stimulation back to baseline activity. Although these effects were not dramatic, they nevertheless show that the manifestation of negative regulation by FPII DNA requires the rPRL promoter context, specifically, the presence of BTE DNA.

DISCUSSION

Repression of rPRL promoter activity in nonpituitary cells. In this report, we have identified the rPRL FPII site as an inhibitory element which binds a ubiquitous factor, F2F, and serves to repress rPRL promoter activity in nonpituitary cells. Several lines of evidence show that the FPII site, in the context of the rPRL promoter, functions as a negative control element. Specifically, 5′ deletion or site-specific mutagenesis of the FPII site derepresses rPRL promoter activity in HeLa nonpituitary cells. We obtained similar results when FPII mutants were transfected into Rat2 fibroblasts and 293 human embryonal kidney cells (data not shown). Moreover, sequestration of F2F by plasmid DNA containing an FPII trimer resulted in activation of the otherwise inactive wild-type rPRL promoter in HeLa cells. These data indicate not only that the lack of rPRL gene expression in nonpituitary cells is due to the absence of cell-specific transcription factors but also that a negative control mechanism mediated by FPII and F2F plays a critical role. The implication is that the proximal rPRL promoter contains a basal positive control element that is functional in different cell types but that its activity is

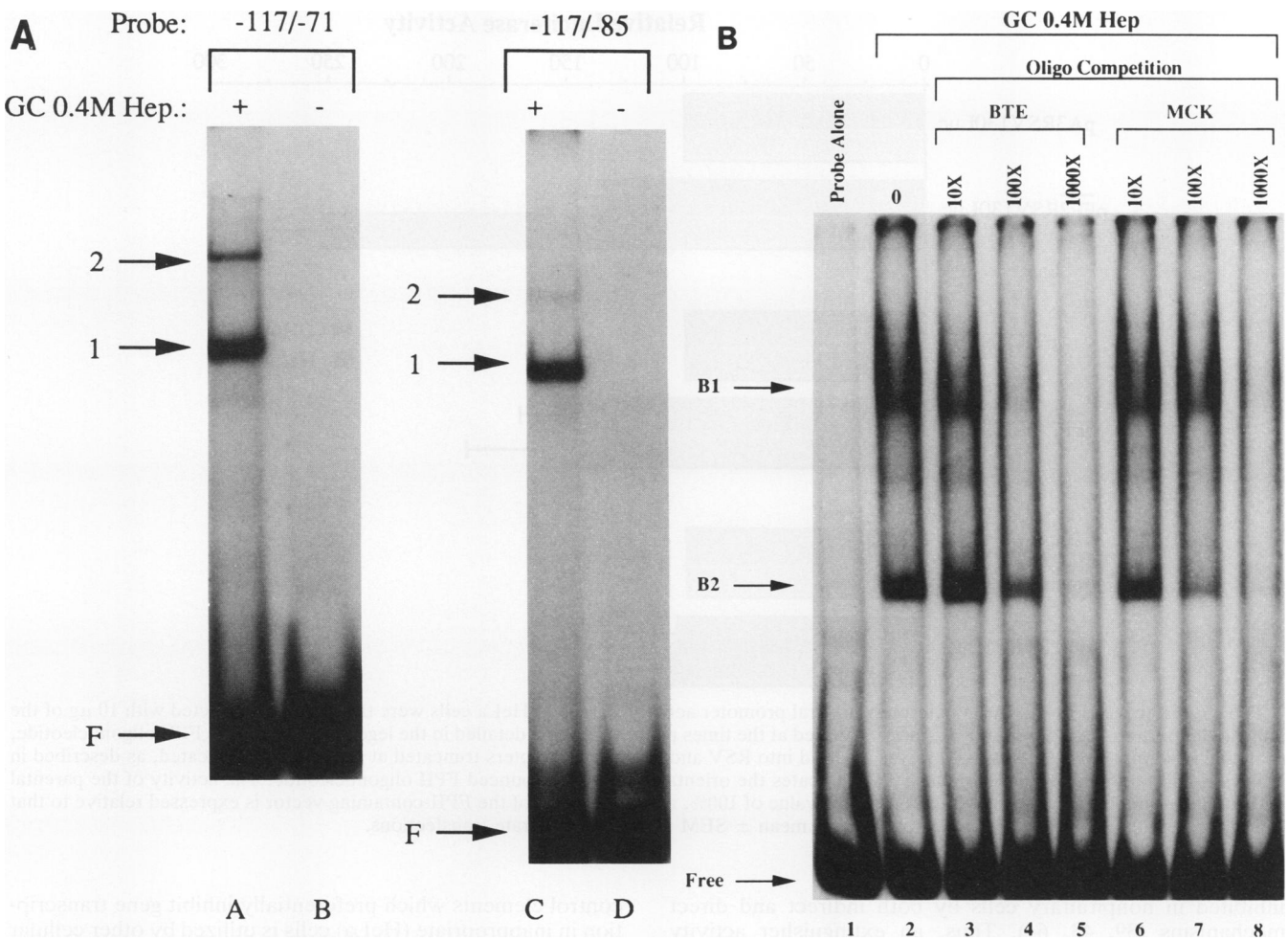

FIG. 6. (A) Gel shift analysis of BTE oligonucleotides. Gel shift assays were performed with either a labelled BTE oligonucleotide spanning the −117 to −71 rPRL region (lanes A and B) or a subfragment spanning the −117 to −85 rPRL region (lanes C and D) in the presence (+) or absence (−) of GC protein which had been partially purified by heparin (Hep.)-agarose chromatography. The positions of the protein-DNA complexes are denoted by arrows labelled 1 and 2, and the free probe is indicated by an arrow marked F. These gel shifts were performed on the same gel, and the relative migrations of the free and protein-bound probes are shown as on the original autoradiogram. (B) Oligonucleotide competition of BTE-BTF complexes. The −117 to −71 oligonucleotide was used as a probe in gel shift studies using the same GC pituitary extract as in panel A. Specific competition with increasing amounts (as fold molar excess) of the unlabelled homologous BTE oligonucleotide (lanes 3 to 5) and the heterologous muscle creatine kinase (MCK) oligonucleotide (lanes 6 to 8) is shown. All lanes contained 500 ng of sheared herring sperm DNA as a nonspecific competitor, and lane 2 contained no additional oligonucleotide competitor.

normally repressed in nonpituitary cells by F2F protein binding to FPII DNA. Indeed, our results show that once the FPII site is altered, derepression occurs in nonpituitary cells via a functional BTE which is juxtaposed to the FPII site. While the reactivation of rPRL promoter activity in nonpituitary cells by mutation of FPII DNA sequences is remarkable, the role of F2F and FPII DNA in rat pituitary cells appears to be minimal and subservient to cell-specific influences (Fig. 2, 4, and 5). Clearly, rPRL promoter function does not approach the wild-type levels reached in pituitary cells, corroborating the importance of cell-specific factors such as Pit-1/GHF-1 and/or LSF-1 (13, 20, 30, 40, 59). Also, the FPII-F2F-mediated repression of the rPRL BTE occurs in GH_4 pituitary cells only if the upstream cell-specific elements (FPIII and FPIV) are absent (e.g., in p−127PRL*Luc* in Fig. 1 or in the heterologous-promoter study shown in Fig. 8), suggesting that possibly the redundant nature of these cell-specific sites dominates over F2F effects. Moreover, Iverson et al. (27) have found that linker-scanner mutations in the FPII region result in significant loss of rPRL promoter activity in GH_3 rat pituitary tumor cells, although the activity of these FPII mutants was not analyzed in nonpituitary cell lines. The discrepancy in our findings could be due to the use of different pituitary cell lines, the use of different rPRL 5′ endpoints to serve as the wild type for comparison, and the difference in the resulting DNA sequence (and restriction site) upon site-specific versus linker-scanner mutagenesis of the FPII site (27).

Ivarie and O'Farrell (26) have suggested that the presence or absence of cell-specific transcription-activating factors is not sufficient to account for the 7 to 8 orders of magnitude of differential GH gene expression in pituitary versus nonpituitary liver cells and proposed that GH gene repression in nonpituitary cells is required to achieve such extremes in transcription levels. Recent progress has borne out this prediction, and GH gene expression has been shown to be

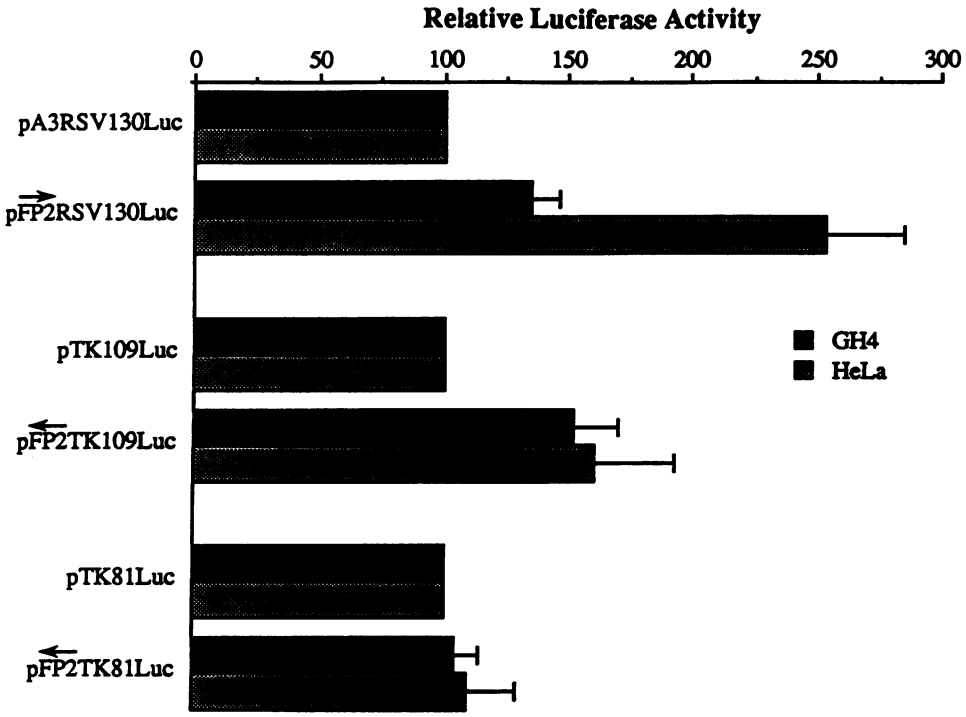

FIG. 7. Effects of FPII DNA on heterologous viral promoter activity. GH_4 and HeLa cells were transiently transfected with 10 μg of the indicated reporter plasmid, in triplicate, and harvested at the times posttransfection detailed in the legend to Fig. 2. The FPII oligonucleotide, spanning rPRL positions −134 to −114, was inserted into RSV and TK viral promoters truncated at the positions indicated, as described in Materials and Methods. The arrow above FP2 indicates the orientation of the introduced FPII oligonucleotide. The activity of the parental RSV or TK promoter in each cell type was set to a value of 100%, and the activity of the FPII-containing vector is expressed relative to that of each parental vector. The data are shown as the mean ± SEM from three separate transfections.

inhibited in nonpituitary cells by both indirect and direct mechanisms (39, 43, 64). Thus, an extinguisher activity which acts indirectly to inhibit endogenous GH gene transcription by repressing Pit-1/GHF-1 gene expression (43) and a silencer region which acts directly to inhibit GH promoter activity have been identified (64). However, the identification of similar inhibitory factors or negative rPRL control elements which serve to repress rPRL gene expression in nonpituitary cells has heretofore been lacking. It is likely that the presence of any inhibitors in nonpituitary cells was overlooked, owing to the ability of the rPRL promoter to be activated in reconstituted nonpituitary in vitro transcription and gene transfer cell systems, by complementing with either pituitary cell extracts, cell-specific factors, or vectors that encode Pit-1/GHF-1 (21, 39, 41, 47, 58, 63). Indeed, these complementation-mixing studies were interpreted to suggest that if any transcription inhibitors were present in nonpituitary cells, they were not dominant to the effects of cell-specific factors (21, 39). These earlier conclusions are consistent with the findings presented in this report, where we show that the manifestation of F2F repression of rPRL promoter function in GH_4 rat pituitary cells requires deletion of the two upstream pituitary-specific DNA elements (Fig. 2). Thus, F2F binding to the rPRL FPII element appears to be important for targeted inhibition of the BTE, which would otherwise be functional, even in nonpituitary cells (Fig. 2, 4, and 5). Furthermore, because F2F repressor action is recessive to cell-specific factor function, fairly tight inhibition of rPRL promoter activity is achieved in nonpituitary cells, with only a minimal effect on rPRL promoter activity in pituitary cells. Apparently, this elegant strategy of negative control elements which preferentially inhibit gene transcription in inappropriate (HeLa) cells is utilized by other cellular systems to achieve highly restricted gene expression in the appropriate cell type, including erythroid cell-specific expression of the human ε-globin and chick β-globin genes, liver-specific expression of the retinol-binding protein gene, and lymphocyte-specific activation of the kappa gene enhancer (10, 11, 15, 51). It is of note, however, that there does not appear to be any homology in these various negative control elements.

Identification of ubiquitous F2F repressor and BTF activator proteins. After mapping of FPII as the negative control element and the −117 to −71 region as the BTE of the rPRL promoter by gene transfer analysis, the nuclear factors that bind to these two DNA regions were identified by gel shift studies (Fig. 3 and 6). These data show that F2F is present in a variety of cell lines and that the relative abundances of F2F are similar among cell types. However, since we originally described it (21), there has been some confusion regarding the cell specificity of the factor that binds to the FPII element (27, 41, 47, 63). We emphasize that the gene transfer (Fig. 2, 5, and 7), gel shift (Fig. 3), and F2F in vivo titration (Fig. 4) results depicted in this study, document that F2F is not restricted to pituitary cells. Moreover, the observation by Iverson et al. (27) that a linker-scanner mutant in the rPRL FPII site is activated as well as the wild-type rPRL promoter by a cotransfected Pit-1 expression vector reveals that Pit-1/GHF-1 *trans* activation of the rPRL promoter is not mediated via the FPII site. More importantly, however, an implication of the current data is that the ubiquitous distribution of the F2F and BTF nuclear proteins in a variety

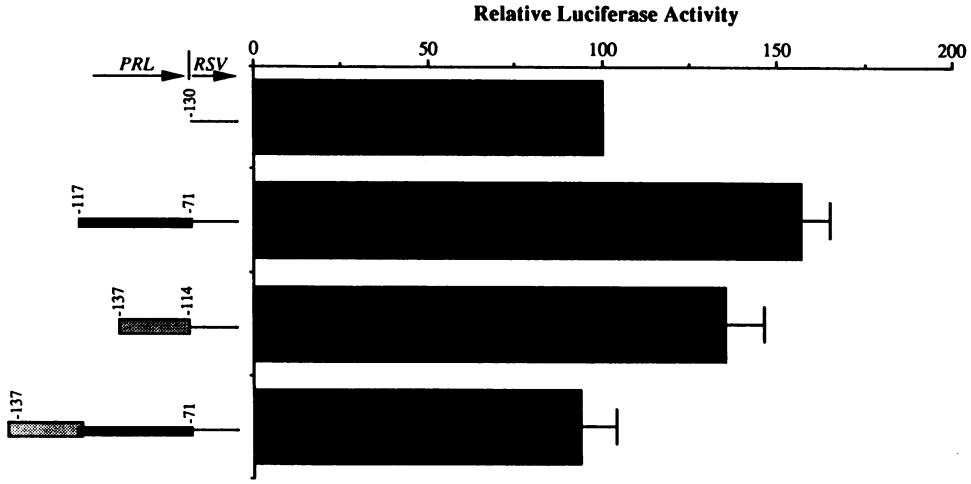

FIG. 8. Effect of FPII and BTE oligonucleotides on heterologous RSV promoter activity. GH_4 rat pituitary tumor cells were transiently transfected with 10 μg of the indicated reporter construct, and cells were harvested as described in Materials and Methods. The arrows labelled *RSV* and *PRL* at the left top margin indicate the source and position of the DNA fragments cloned upstream of the luciferase reporter gene in the pA3*Luc* vector. The black line with a −130 endpoint indicates the structure of the truncated RSV promoter in the pA3RSV130*Luc* vector. An oligonucleotide encompassing the BTE, from positions −117 to −71 of the rPRL promoter (separate small, solid black rectangle), was inserted upstream of position −130 of the truncated RSV promoter. The vector containing the FPII oligonucleotide is the same as that shown in Fig. 7 as pFP2RSV130*Luc*, but the relative position of the FPII element is shown here schematically as a separate stippled rectangle. The vector containing both the FPII and BTE rPRL motifs (joined stippled and black rectangles, respectively) in the correct juxtaposition is also depicted. The activity of the parental −130 RSV promoter was set at 100%, and the activities of the derivative BTE- and/or FPII-containing reporter constructs are expressed relative to that of the parental vector. The data are presented as the mean ± SEM from three separate transfections, each performed in triplicate.

of distinct cell types indicates that these factors may serve a more general function and, possibly, influence the transcription activity of several other cellular genes. As we have pointed out previously, DNA sequence homology to the rPRL FPII site is found in the same relative position in other members of the GH gene family, including the GH and placental lactogen (PL) promoters (21). The role of this sequence in these other genes, however, remains unclear. For example, in the GH promoter, this sequence is just downstream of an Sp-1-binding site and just upstream of and slightly overlapping with the distal Pit-1/GHF-1-binding site (36, 55, 56, 61). Furthermore, the binding of two other factors, GHF-5 and GHF-7, has been mapped to this same DNA region (55, 56). Thus, the −140 to −110 stretch of GH promoter DNA may bind up to four distinct factors, resulting in a very complex *cis*-acting region. Although we have only a minimal understanding of PL promoter function, owing to the lack of an appropriate placental syncytiotrophoblast cell line, recent data indicate that deletion of the homologous FPII site results in slight (twofold) activation of the human PL_3 promoter in the JEG-3 choriocarcinoma placental and Hep-G2 nonplacental cell lines (16). By contrast, the GH and PL genes contain no obvious homology with the rPRL BTE sequences (45). Although these data are suggestive, it must be emphasized that (i) the FPII-equivalent DNA site may serve a very different function in the GH and PL genes, possibly dictated by the slight differences in the FPII DNA sequence or by neighboring sequences which result in the binding of other factors, and (ii) a combinatorial interaction of F2F with yet other transcription factors can potentially result in diverse effects.

The BTE was delimited to the −112 to −80 region by site-specific deletion studies (Fig. 5), to the −117 to −71 region by a heterologous-promoter assay (Fig. 8), and to the −117 to −85 region by gel shift analysis (Fig. 6). Therefore, the BTE is likely to reside within positions −112 to −85 of the rPRL promoter. This coincides almost precisely with the −97 to −84 rPRL region identified by linker-scanner mutations as important for basal and agonist (cyclic AMP and phorbol ester)-induced rPRL promoter activity in GH_3 rat pituitary cells (27). It is of note that the −97 to −84 rPRL DNA sequence (5′-ACGGAAATAGATGA-3′) does not contain a canonical Pit-1/GHF-1, CREB, AP-1, or AP-2 site. Moreover, neither Pit-1/GHF-1 (41, 63), LSF-1 (4), nor CREB (31b) binds to this rPRL DNA region. Although the current studies have not eliminated the possibility that the BTF we have identified may be either AP-1 or AP-2, the −112 to −80 rPRL deletion construct is still regulated by both cyclic AMP and phorbol esters (31a). Nevertheless, the precise identity of the ubiquitous BTF and its potential function in activating other genes remain to be elucidated directly.

Model of F2F-mediated repression. The work presented here indicates that the rPRL BTE functions constitutively, irrespectively of the cell type, and its activity appears to be modulated by F2F. In nonpituitary cells, this constitutive BTE action is repressed by the upstream and adjacent FPII sites, whereas in pituitary cells, the FPII element does not appear to influence BTE activity unless two of the redundant upstream cell-specific elements are deleted (Fig. 2, 5, and 8). From these data, we have formulated a model based on interactions of vicinally bound factors that result in either positive or negative effects, depending on the particular final combination of bound factors. Thus, we propose that in nonpituitary cells which are devoid of pituitary-specific factors, F2F binding to the FPII site directly interferes with the ability of the BTF to *trans* activate. It is clear from the heterologous-promoter studies (Fig. 7 and 8) that the FPII site-F2F does not directly interact with the basal transcription machinery, since FPII DNA alone does not repress the

minimal RSV or TK viral promoters. By contrast, the requirement of BTE DNA for FPII-mediated repression to become manifest implies that F2F acts by interfering with BTF action. Therefore, these data are most consistent with a quenching model of F2F-mediated repression rather than a direct inhibition or squelching effect. However, the possibility remains that F2F acts by sterically interfering with the ability of the BTF to bind DNA instead of quenching its activity. In pituitary cells, we propose that the negative effect of F2F on BTF action is neutralized by the positive transcription effects of cell-specific factors, such as Pit-1/GHF-1 and/or LSF-1. Although the precise molecular mechanisms by which pituitary-specific transcription factors overcome the negative effects of F2F are yet to be elucidated, we have to incorporate into any model a process by which pituitary-specific factors bound to FPIII and FPIV nullify F2F-mediated repression. While the proposed model is simply a working hypothesis, it is evident that the mechanisms used by distinct and terminally differentiated cell types to achieve highly restricted gene expression are very complex.

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