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Tissue specific *trans*-acting factor interaction with proximal rat prolactin gene promoter sequences

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Using an exonuclease III protection assay, strong, reversible and tissue-specific binding of GH_3 cell nuclear factors to proximal regions of the rat prolactin (r*Prl*) promoter (−31 to −77) has been detected. A second less prominent region of factor binding, that may have a correlate in HeLa cell extracts, was detected in the region (−155 to −180). The binding is eliminated in the presence of excess unlabelled r*Prl* promoter sequences (−423 to +38), excess unlabelled distal r*Prl* 5′-flanking sequences (−1960 to −1260) and SV40-enhancer/promoter sequences; it is largely unaffected by growth hormone (*rGH*) promoter and RSV-LTR sequences. A plasmid containing the proximal r*Prl* promoter sequences (−75 to +38) was also shown to be an avid inhibitor, at low concentrations, of r*Prl* promoter driven chloramphenicol acetyl transferase (CAT) gene expression in transient co-transfection competition studies; under these assay conditions distal r*Prl* 5′-flanking sequences and RSV and r*GH* promoter plasmids do not compete. The results emphasize the critical importance of proximal r*Prl* promoter sequences for prolactin gene expression in GH_3 cells but recognize the related functional potential of more distal sequences.

Key words: rat prolactin gene expression/promoters/tissue-specific transcription factors

Introduction

The regulation of genes that encode mRNA in higher eukaryotes is mediated by two classes of *cis*-acting DNA sequence elements: promoters and enhancers (reviewed in Maniatis *et al.*, 1987). It has recently become clear that the differential tissue specific expression of cellular genes is controlled by these two classes of sequence elements (e.g. Walker *et al.*, 1983; Grosschedl and Baltimore, 1985; Banjeri *et al.*, 1983; Edlund *et al.*, 1985).

Prolactin and growth hormone gene expression would seem to be tissue-specific events confined to lactotrophs and somatotrophs within the anterior pituitary, for instance, rat growth hormone gene expression occurs at a level of $>10^8$ greater in anterior pituitary cells than in rat hepatoma cells (Ivarie *et al.*, 1983). The availability of the GH_3 clonal tumour cell lines (Tashjian, 1967) in which one (or both) of these genes is expressed (Bancroft, 1981) has contributed significantly to our understanding of the control of rat prolactin (r*Prl*) and growth hormone (r*GH*) gene expression. Recent studies by Nelson *et al.* (1986), Elsholtz *et al.* (1986), Lufkin and Bancroft (1987) and Gutierrez-Hartmann *et al.* (1987) have begun to define, using transfection,

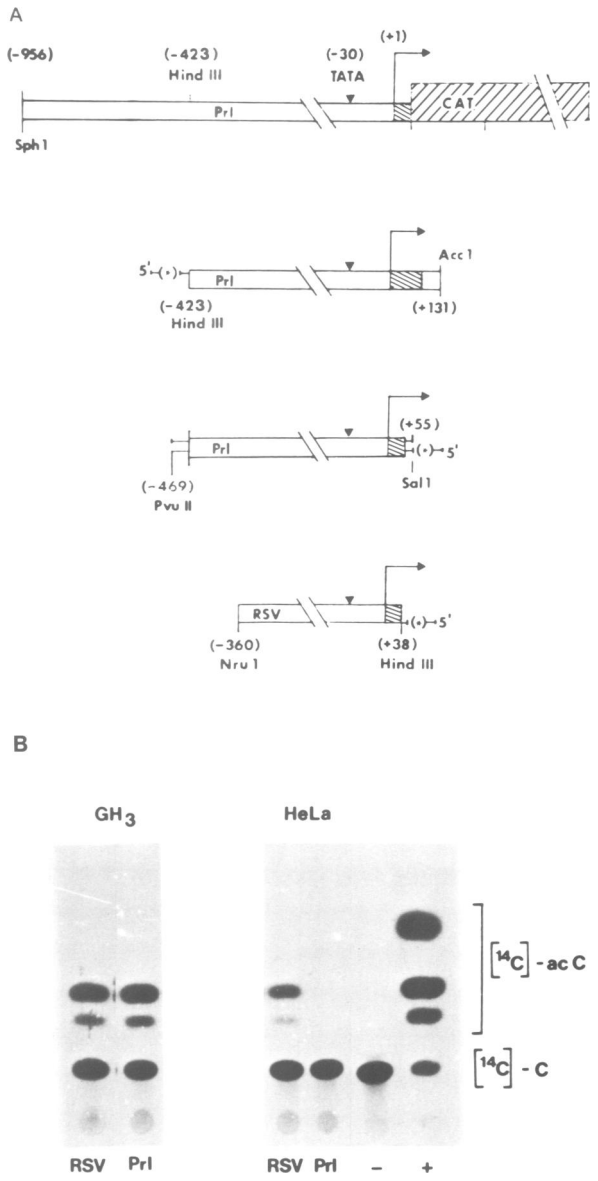


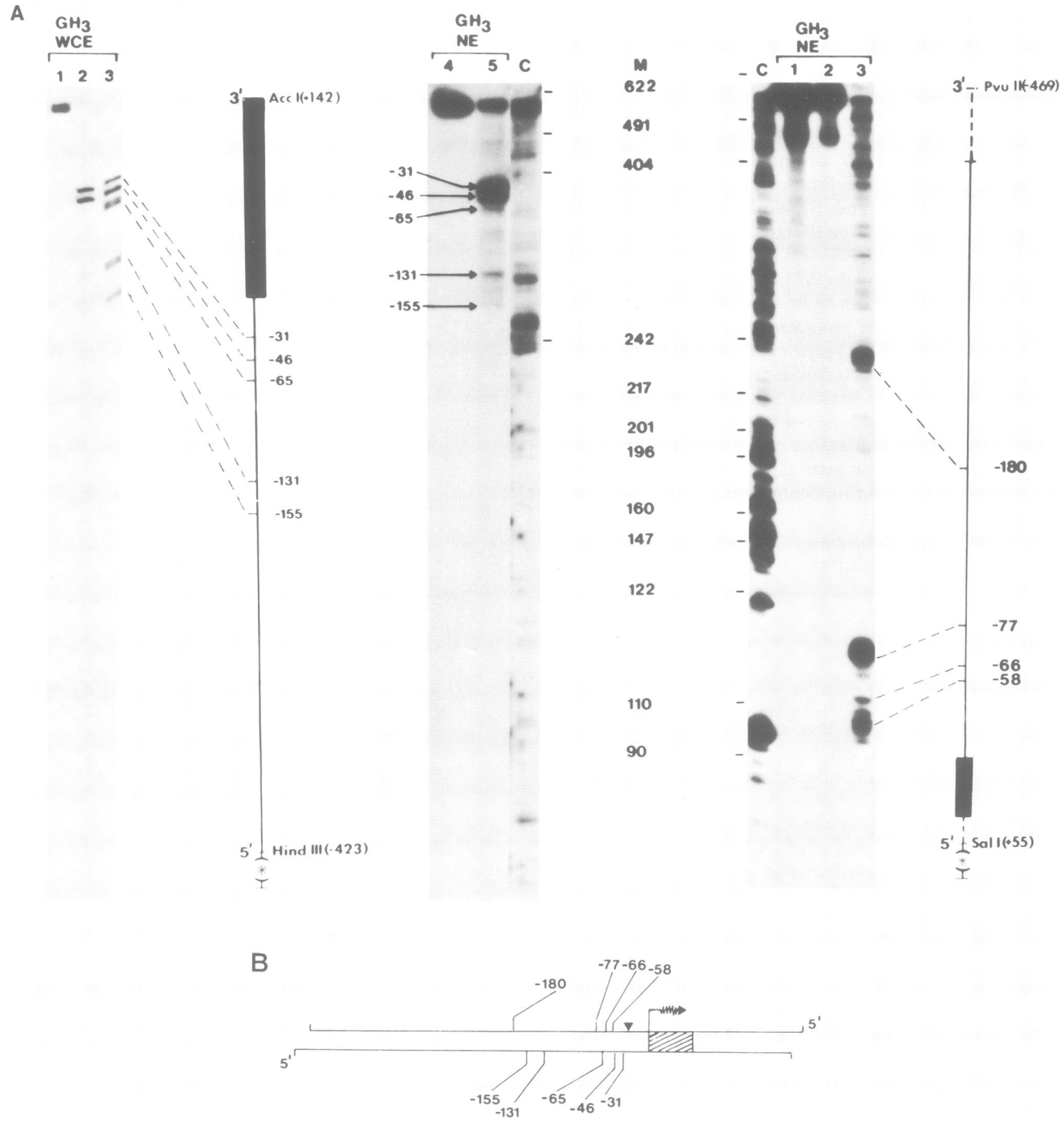
Fig. 1. (**A**) r*Prl* promoter constructs; illustrated is the pr*Prl*(Δ956)−*CAT* construct used in the transient transfection study (**B**) and the selectively end-labelled r*Prl* promoter and RSV-LTR fragments used in the exonuclease III protection analysis. (**B**) Selective expression of pr*Prl*(Δ956)−*CAT* in GH_3 versus HeLa cells. Shown is the autoradiograph of a TLC plate on which [^{14}C]chloramphenicol and its acetylated products from cell extracts of GH_3 and HeLa cells, transiently transfected with pRSV−CAT (RSV) or pr*Prl*(Δ956)−*CAT* (*Prl*), have been separated; also chromatographed was unmetabolized [^{14}C]chloramphenicol (−) and [^{14}C]chloramphenicol after incubation with pure bacterial CAT enzyme (+). The position of the substrate and its acetylated products on the TLC plate are indicated.

transfection–fusion and DNA–protein binding assays, the involvement of a number of *cis*-acting promoter sequences in the first 210 bp of the 5′-flanking DNA in the control of r*Prl* gene expression and their interaction with tissue specific *trans*-acting factors.

In the present report we detail (i) the tissue-specific interaction of nuclear factors from GH_3 cells with, in particular, a proximal region of the r*Prl* gene promoter, (ii) transient co-transfection competition studies which suggest that tissue-specific nuclear factor binding to this proximal promoter region may be a key requirement for r*Prl* gene expression in pituitary cells and (iii) that SV40 early gene enhancer/promoter sequences can compete with r*Prl* promoter sequences for the binding of these presumptive tissue-specific nuclear factors.

Results

The tissue specificity of transcription from transfected plasmids containing the r*Prl* promoter (Nelson *et al.*, 1986; Lufkin and Bancroft, 1987) was confirmed in transient transfection studies by introducing pr*Prl*(Δ956)–*CAT* (Figure 1A) and pRSV–*CAT* into GH_3 and HeLa cells (Figure 1B): the latter conferred *CAT* activity to both cell types, the former to the cells of pituitary origin only; with C127 (fibroblast), C_6 (glioma) and MH_1C_1 (hepatoma) cells results identical to those reported above for HeLa cells were obtained (data not shown). In order to investigate the mechanism of this tissue-specific regulation of prolactin gene expression, the ability of nuclear proteins from GH_3 cells to interact specifically with r*Prl* gene promoter regions was



examined using an exonuclease III digestion protection assay (Wu, 1985): incubation of a GH_3 whole cell extract (WCE) (Manley *et al.*, 1980) or a GH_3 cell nuclear extract (NE) (Dignam *et al.*, 1983) with a r*Prl* promoter containing fragment (−423 to +131) specifically ^{32}P-5′-end labelled on one strand at position −423 [Figure 1A (start site for transcription, +1)] led to a unique pattern of extract factor induced stops on fragment digestion by exonuclease III

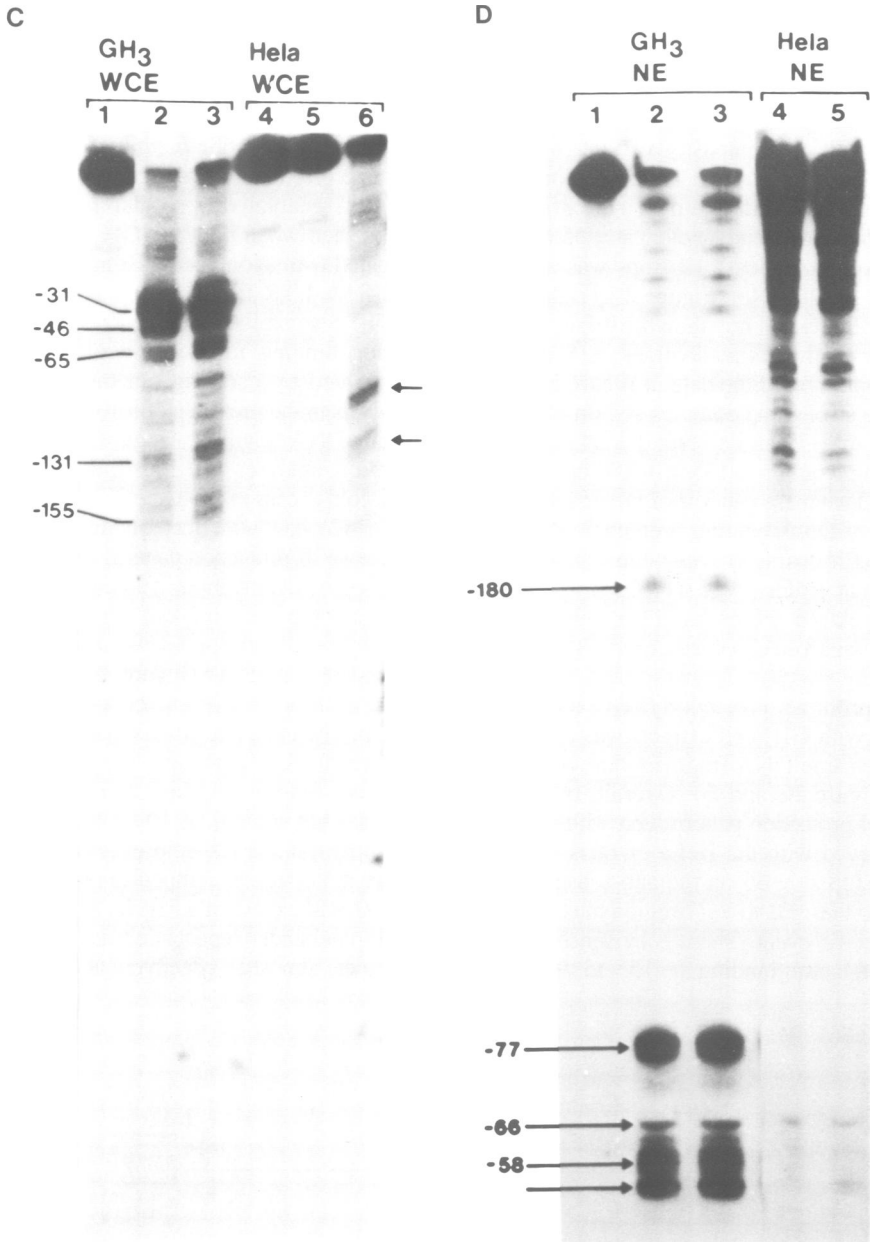
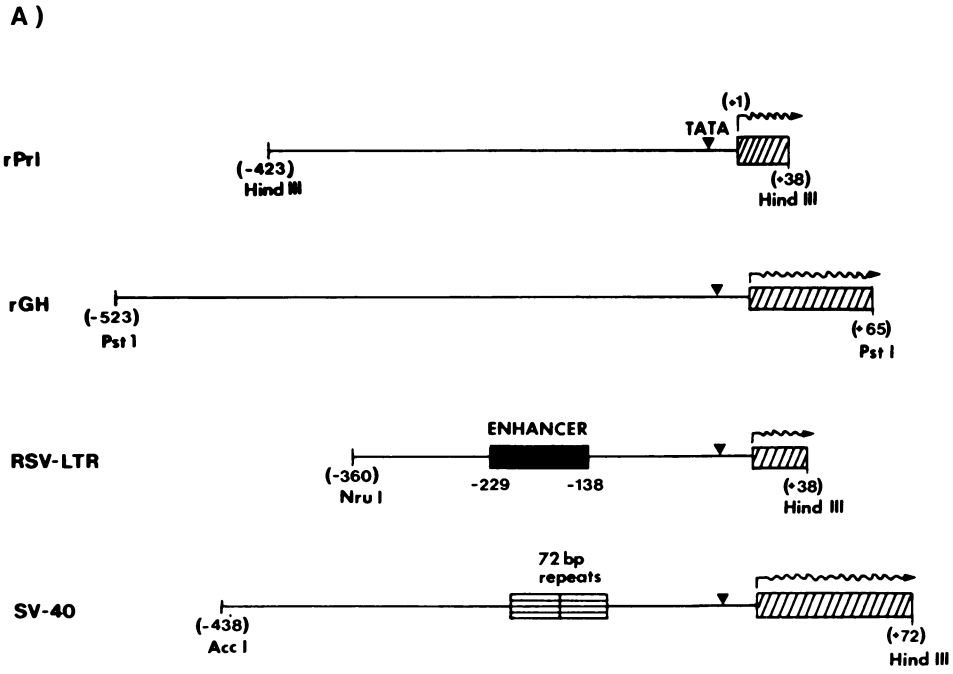


Fig. 2. Exonuclease III analysis of tissue specific binding of GH_3 cell factors to r*Prl* promoter sequences. (**A**) Left-hand panel: analysis of −423 ^{32}P-5′-labelled 565 bp r*Prl* fragment (3 ng) in GH_3 WCE: + carrier (12 μg), + extract (38 μg protein) (**lane 1**); as lane 1, + exonuclease III (160 U) (**lane 2**); as lane 1, + exonuclease III (320 U) (**lane 3**). Centre panel: analysis of −423 ^{32}P-5′-labelled fragment (6 ng) in GH_3 cell NE: + carrier (6 μg), + extract (41 μg protein) (**lane 4**); as lane 4, + exonuclease III (112.5 U) (**lane 5**); + exonuclease III (112.5 U) only (**lane C**). Right-hand panel: analysis of +55 ^{32}P-5′-labelled 524-bp rPrl fragment (6 ng) in GH_3 cell NE: + exonuclease III (80 U) only (**lane C**); labelled fragment alone (**lane 1**); + carrier (6 μg), + extract (41 μg protein) (**lane 2**); as lane 2, + exonuclease III (80 U) (**lane 3**). M is marker, *Hpa*II restricted pAT153; cartoons indicate borders of exonuclease III stops on the respective strand relative to the r*Prl* start-site for transcription (+1). (**B**) Summary of borders of exonuclease III stops on both DNA strands of r*Prl* promoter. (**C**) Comparative analysis of exonuclease III digestion of −423 ^{32}P-5′-labelled 565 bp fragment (3 ng) in GH_3 and HeLa cell WCEs: labelled fragment, + carrier (6 μg), + GH_3 extract (16 μg protein) (**lane 1**); as lane 1, + exonuclease III (80 U) (**lane 2**); as lane 1, + exonuclease III (160 U) (**lane 3**); labelled fragment, + HeLa extract (24 μg protein) (**lane 4**); as lane 4, + carrier (6 μg) (**lane 5**), as lane 5, + exonuclease III (80 U) (**lane 6**). Borders of exonuclease III stops are indicated as in (A), above. Note that the stops at position −131 and −155 are arrowed in lane 6. (**D**) Comparative analysis of exonuclease III digestion of +55 ^{32}P-5′-labelled 524 bp fragment (6 ng) in GH_3 and HeLa cell NE: labelled fragment, + carrier (6 μg), + GH_3 cell extract (46 μg protein) (**lane 1**); as lane 1, + exonuclease III (112.5 U) (**lane 2**); as lane 1, + exonuclease III (225 U) (**lane 3**); + carrier (6 μg), + HeLa cell extract (40 μg protein), + exonuclease III (225 U) (**lane 4**); as lane 4, + exonuclease III (337.5 U) (**lane 5**). Borders of exonuclease III stops are indicated as in (A), above. Note that an additional tissue-specific border, not indicated in (A) is arrowed here, its positions to −56; further, the stop at −66 occurs in both GH_3 and HeLa extract analyses.

[Figure 2A, lanes 2, 3 (left-hand panel) and 5]; these fragment patterns were significantly different from that produced by exonuclease III digestion of the naked DNA fragment (no extract added) (Figure 2A, lane C). The fragment patterns observed with both the GH_3 WCE and NE suggest proximal borders (relative to start site for transcription) of extract factor binding at nucleotides −31, −46, −65, −131 and −155 on the coding strand of the r*Prl* promoter; however, in a large number of experiments (cf. Figure 2C, lanes 2 and 3) this pattern was dominated by the more proximal group of stops with their intensity varying with extract and carrier DNA/exonuclease III concentrations, but not in a predictable fashion. With a similar r*Prl* promoter fragment (−423 to +55) but 5′-end labelled at +55 (Figure 1A) a unique pattern of exonuclease III stops was also induced in the presence of GH_3 NE (Figure 2A, right-hand panel, lane 3) and WCE (results not shown): these suggest distal borders of factor binding at nucleotides −180, −77, −66 and −58. A compilation of the data in Figure 2A suggests the existence of a strong and complex region of factor binding from bp −31 to −77 and a second weaker region of extract factor binding from bp −155 to −180 on the r*Prl* promoter (Figure 2B). That these patterns of protection truely reflect the interaction of DNA binding proteins in the GH_3 cell extracts with the r*Prl* promoter fragments is suggested by the fact that a similar pattern was seen in the presence of a GH_3 cell nuclear extract which had been subjected to purification by heparin-agarose chromatography (results not shown).

The pattern of r*Prl* promoter protection from exonuclease III digestion observed with GH_3 cell extracts was compared to that obtained on the same DNA fragment but with HeLa cell WCE and NE (Figure 2C and D). Overall, the strong characteristic proximal protection pattern seen with the GH_3 extracts was not observed with the HeLa extracts with the exception of a weak stop at −66 on the anti-sense strand. Thus, factor binding to the −31 to −77 region must be considered a GH_3 cell specific event. There is evidence that the more distal region of factor binding (−155 to −180) is weakly protected in HeLa extracts (Figure 2C and D) but relative to GH_3 extracts the intensity of the signal at −180 (anti-sense strand) is always low. Several factors suggest that the absence of these r*Prl* promoter binding proteins from the HeLa extract is a cell specific phenomenon and is not due to the quality of the HeLa extracts used. Firstly, the HeLa extract did display a number of the 'protection' fragments seen in studies with the GH_3 cell extracts and some unique 'protection' fragments (Figure 2C and D). Secondly, the HeLa cell extracts used were competent to accurately transcribe *in vitro* from both the adenovirus major late promoter and the r*Prl* promoter (results not shown); thirdly, qualitatively similar protection fragment patterns were seen when both the GH_3 and HeLa cell NE were incubated with exonuclease III in the presence of an end labelled RSV-LTR fragment (results not shown).

The reversibility and sequence specificity of GH_3 nuclear factor binding to the r*Prl* promoter sequences was investigated by carrying out the exonuclease III protection analysis in the presence of a 10- and 100-fold excess of competing DNA fragments (Figure 3A). The results of such competition analyses are illustrated in Figure 3B. A 10- or 100-fold excess of 'non-specific' competitor DNA (linearized pGEM2) had no effect on the GH_3 extract specific exonuclease III protection pattern. A 100-fold excess of a 461 bp r*Prl* proximal promoter fragment completely eliminated the protection pattern on both strands while a partial effect was clearly seen on the anti-sense strand protection with a 10-fold excess of competitor (Figure 3B). This ability of the 100-fold excess of the r*Prl* promoter sequence fragment to eliminate the protection pattern was also observed under conditions where labelled fragment−factor binding had been allowed to proceed before the addition of unlabelled competitor DNA (results not shown). A 100-fold excess of an r*GH* promoter fragment and a 100-fold excess of an RSV-LTR fragment were not effective competitors for factor binding. However, a 100-fold excess of a 510 bp SV40 fragment containing the 72 bp enhancer repeats and the 21 bp repeats was found to compete almost as effectively as the r*Prl* promoter sequences



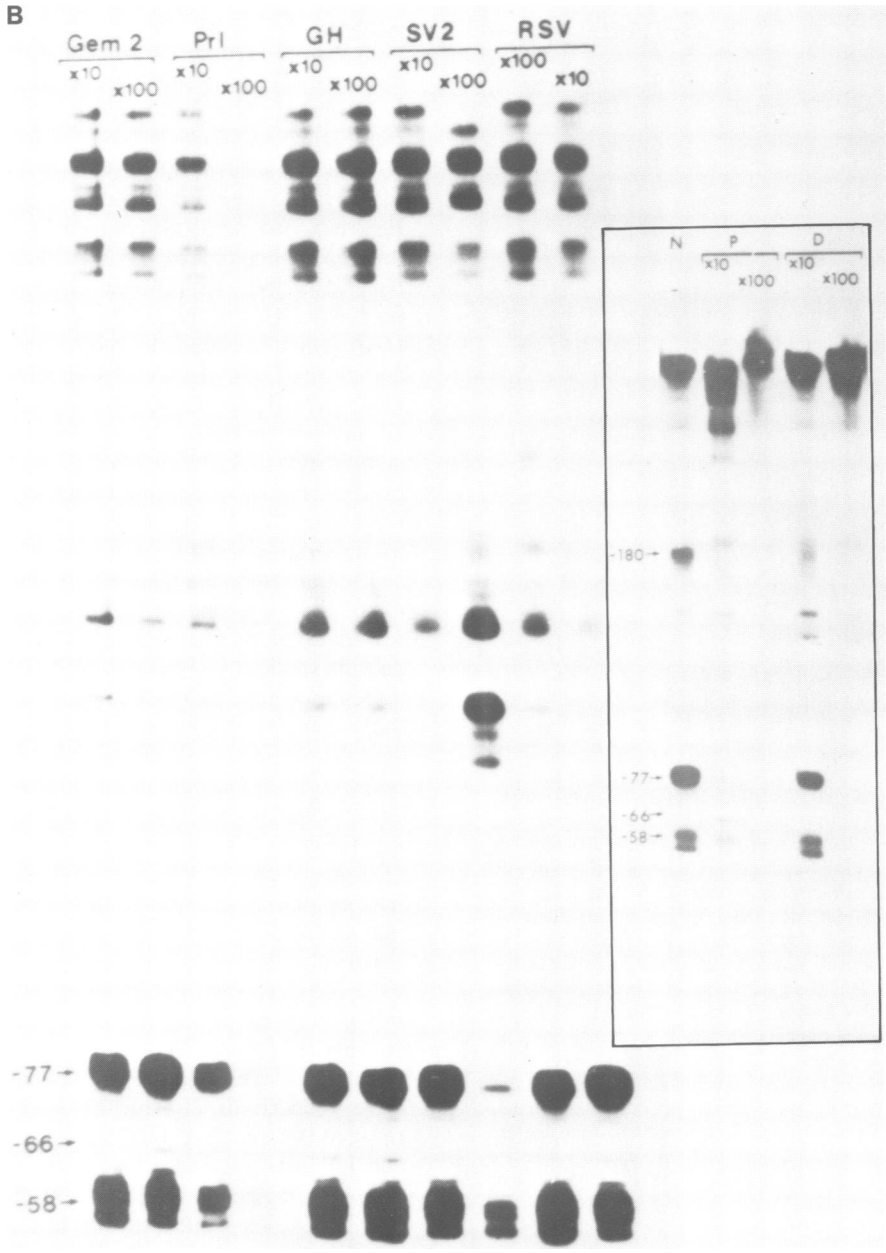


Fig. 3. Exonuclease III digestion protection. Competition analysis. (**A**) Competitor fragments utilized (in addition to those shown, linearised pGEM2 was also used). (**B**) Competition analysis using + 55 ^{32}P-5′-labelled 524 bp r*Prl* fragment (3 ng) in GH_3 cell NE (40 μg protein), + carrier (12 μg), + exonuclease III (225 U); and 30 or 300 ng of the indicated competitor. Proximal promoter borders of exonuclease III stops are indicated as in Figure 2A. Note: the bands (~ −150, arrowed) whose intensity is strongly increased in the SV2 (100-fold) lane are characteristic naked DNA stops (see, for example, Figure 2A, lane c). We have sometimes observed with successful competitors this phenomenon of reduced intensity extract specific stops being associated with the appearance of bands characteristic of naked DNA exonuclease III stops on the same fragment. Inset: competition analysis using +55 ^{32}P-5′-labelled 524 bp r*Prl* fragment (3 ng) in GH_3 cell NE (40 μg protein) + carrier (12 μg), + exonuclease III (225 U), with no competitor (**lane N**) or 30 and 300 ng of the 510 bp r*Prl* proximal promoter fragment (Figure 3A) (**lanes P**) or 30 or 300 ng of the 700 bp (−1960 to −1260) r*Prl* 5′-flanking sequence 'distal' fragment (Figure 5A) (**lanes D**). Borders indicated as in Figure 2A.

for factor binding. It was also shown [Figure 3B (inset)] that an excess of fragment containing distal r*Prl* 5′-flanking sequences (−1960 to −1260) was a very effective competitor in this exonuclease III protection assay; these sequences span the region that has been shown to contain a tissue specific enhancer of r*Prl* transcription in a related cell line (Nelson *et al.*, 1986) (see Discussion and Figure 6).

The exonuclease III digestion protection analysis presented above highlights the tissue-specific binding of pituitary cell specific factors to a proximal region of the r*Prl* gene promoter (−77 to −31). It was of interest therefore to verify this tissue-specific and promoter-specific binding by an independent analysis. In Figure 4A the binding of factors in a GH_3 cell WCE to an end-labelled DNA fragment containing the proximal r*Prl* promoter sequence as analysed by gel retardation (Strauss and Varshavskey, 1984) is presented; in this experiment increasing the concentration of non-specific DNA [poly(dI−dC)·poly(dI−dC)], added to decrease non-specific protein binding to the labelled probe, emphasized the formation of two specific DNA-factor complexes (I and II, Figure 4A, lanes 3 and 4). A third complex (III) was more prominent at lower non-specific DNA

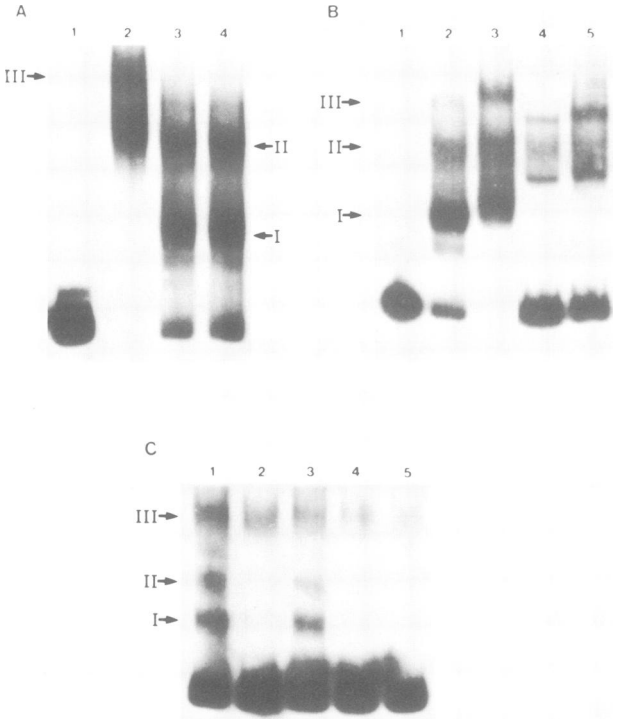


Fig. 4. Gel retardation analysis. (**A**) Effect of p(dI−dC)·p(dI−dC) concentration on complex formation: ^{32}P-end-labelled 134 bp r*Prl* fragment (−75 to +38) (12 000 c.p.m.) + 3 μg p(dI−dC)·p(dI−dC) (**lane 1**); ^{32}P-end-labelled p*Prl* fragment + 1 μg p(dI−dC)·p(dI−dC) + 14 μg whole cell GH_3 extract protein (**lane 2**); as lane 2 but containing 2 μg p(dI−dC)·p(dI−dC) (**lane 3**); and as lane 2 but containing 3 μg p(dI−dC)·p(dI−dC) (**lane 4**); all reactions were buffered and brought to a final volume of 25 μl (as described in Materials and methods). (**B**) Tissue specificity of complex formation: ^{32}P-end-labelled 134 bp r*Prl* fragment (−75 to +38) (12 000 c.p.m.) + 3 μg p(dI−dC)·p(dI−dC) (**lane 1**); as lane 1 but containing 8 μg GH_3 nuclear extract protein (**lane 2**); as lane 1 but containing 16 μg GH_3 nuclear extract (**lane 3**): as lane 1 but containing 8 μg HeLa cell nuclear extract protein (**lane 4**): and as lane 1 but containing 16 μg HeLa cell nuclear extract protein (**lane 5**), all reactions were buffered and brought to a final volume of 25 μl. (**C**) DNA sequence specificity of complex formation. Competition analysis. ^{32}P-End-labelled 134 bp r*Prl* fragment (−75 to +38) (12 000 c.p.m.) + 3 μg p(dI−dC)·p(dI−dC) + 16 μg GH_3 nuclear extract protein (all lanes) + competitor DNA (10-fold excess, by weight over labelled fragment): pUC19 (*Pvu*II fragment, 322 bp) (**lane 1**); r*Prl* promoter fragment (−423 to +38) (Figure 3A) (**lane 2**); r*GH* promoter fragment (−523 to +65) (Figure 3A) (**lane 3**); SV40 enhancer/promoter fragment (−438 to +72) (Figure 3A) (**lane 4**); and, r*Prl* 5′-flanking sequence 'distal' fragment (−1960 to −1260) (Figure 5A) (**lane 5**), all reactions were buffered and brought to a final volume of 38 μl.

concentrations. A qualitatively similar retardation pattern was also seen using a number of independently prepared GH_3 NE (e.g. Figure 4B, lanes 2 and 3) and with NE of the related GC cell line (not shown). HeLa cell NE (Figure 4B, lanes 4 and 5) and WCE (not shown) display a weaker and unrelated gel retardation pattern under identical conditions emphasizing the tissue specific nature of the observed DNA−factor interactions. Finally, the sequence specificity requirements for the formation of complexes I and II was established in a competition analysis (Figure 4C): in the presence of a 10-fold excess of a non-specific DNA fragment (322 bp *Pvu*II fragment from pUC19) (lane 1) formation of the three complexes is unimpaired; r*GH* promoter sequences (−523 to +65) (10-fold) also have little reproducible effect on complex formation (lane 3), while a 10-fold excess of r*Prl* promoter sequences (−423 to +38) (lane 2), a 10-fold excess of SV40 early promoter/enhancer sequences (see Figure 3A) (lane 4) and a 10-fold excess of r*Prl* distal 5′-flanking sequence [−1960 to −1260, containing the 'distal tissue specific enhancer' (Nelson *et al.*, 1986)] (see Figure 5A) (lane 5) all competed successfully against the formation of complexes I and II. (Identical results were obtained in four experiments using three independently prepared GH_3 cell NE.) These findings suggest a similar DNA sequence specificity requirement for the formation of complexes I and II as was seen for the GH_3 cell specific factor-proximal r*Prl* interactions detected by the exonuclease III analysis (Figure 3) and further suggests the biological significance of the latter analysis. As the formation of complex III (Figure 4) was not competed against by the excess of unlabelled r*Prl* fragment sequences (Figure 4C, lane 2) its importance must be questioned.

The transcriptional relevance of this GH_3 cell nuclear factor binding to proximal regions of the r*Prl* gene promoter was investigated in a series of transient co-transfection competition studies (Seguin *et al.*, 1984): pr*Prl*−*CAT* was used as reporter gene and CAT enzyme activity was measured in the transfected GH_3 cells to reflect transcription from the r*Prl* promoter. All competitor sequences (Figure 5A) were co-transfected with the reporter gene and in all cases total transfected plasmid was made up to 50 μg with pGEM2. From Figure 5B it can be seen that distal fragments of the r*Prl* promoter (−1960 to −1260 and −1960 to −423) do not impair transcription from transfected pr*Prl*−*CAT* and may in fact mildly enhance it under certain circumstances. Proximal r*Prl* promoter sequences (−423 to +38) and (−75 to +265) (Figure 5A) potently and almost equivalently inhibited transcription from the r*Prl* promoter as judged by suppression of CAT activity (Figure 5B): at the 5 μg competitor level mean chloramphenicol acetylation levels were reduced to 30 and 22% of control, respectively ($P < 0.005$ and $P < 0.001$). As both these fragments contain TATA box sequences the ability of pRSVneo and a r*GH* promoter fragment (−523 to +160) (both of which also contain TATA box sequences) was also investigated: at higher concentrations, >15 μg, both partially impair transcription from the r*Prl* promoter but neither had a significant effect at 5 or 10 μg. We would conclude that GH_3 cell nuclei contain a factor or factors in limiting amounts which bind to sequences in the proximal 75 bp of the r*Prl* promoter which are required to permit transcription from the promoter. This co-transfection competition was also demonstrated to be evident at the mRNA level when examined by RNase protection analysis (Figure 5C and D): this analysis reveals that transcription from the transfected r*Prl* promoter is being correctly initiated [as predicted by the protection of 302 nt of the anti-sense CAT mRNA probe (lanes 1 and 5)]; co-transfection with pGEM2 or the distal r*Prl* (−1960 to −423) fragment had no effect on transcriptional activity (lanes 2−4) while a proximal r*Prl* promoter fragment (−423 to +38) effectively competed away (pr*PRL*−*CAT*) transcription as judged by the decreasing intensity of the 302 nt protected band (lanes 6−9).

The ability of the chimeric gene construct pr*Prl*(Δ75)−*CAT* (Lufkin and Bancroft, 1987) which contains only the first 75 bp of the r*Prl* promoter to drive *CAT* expression

(but at a low level) in GH cells was also demonstrated: 48 h after transient transfection with pr*Prl*(Δ*75*)−*CAT* GC cells displayed significant CAT activity (5.1% acetylation of [^{14}C]chloramphenicol added to cell extracts); GH_3 cells displayed lower activity (mean value 1.64% acetylation) (mock transfected GH_3 cell extracts showed a mean acetylation of added [^{14}C]chloramphenicol of 0.5%).

Discussion

Using an exonuclease III protection assay we have detected significant binding of GH_3 cell nuclear factors to DNA fragments encompassing the r*Prl* promoter. This binding is most significant over a small proximal area of the promoter (−30 to −77) over which at least three boundaries of factor binding can be detected on each strand (Figure 2A and B) which suggests the binding of multiple factors or multiple elements of a single factor. This GH_3 cell factor specific pattern of exonuclease III stops differs from the pattern of exonuclease III stops seen on the naked DNA fragments (Figure 2A) and has been detected in GH_3 cell nuclear extracts, whole cell extracts, a heparin-agarose purified GH_3 cell nuclear extract and also in GC cell extracts. The binding is tissue-specific (Figure 2C and D) and reversible, suggesting that the factors involved are either absent or present in very low abundance in, for example, transcriptionally competent HeLa cell nuclear extracts. It is worth noting that the same pattern of factor binding in both GH_3 and HeLa cell nuclear extracts was seen over an RSV-LTR fragment (results not shown).

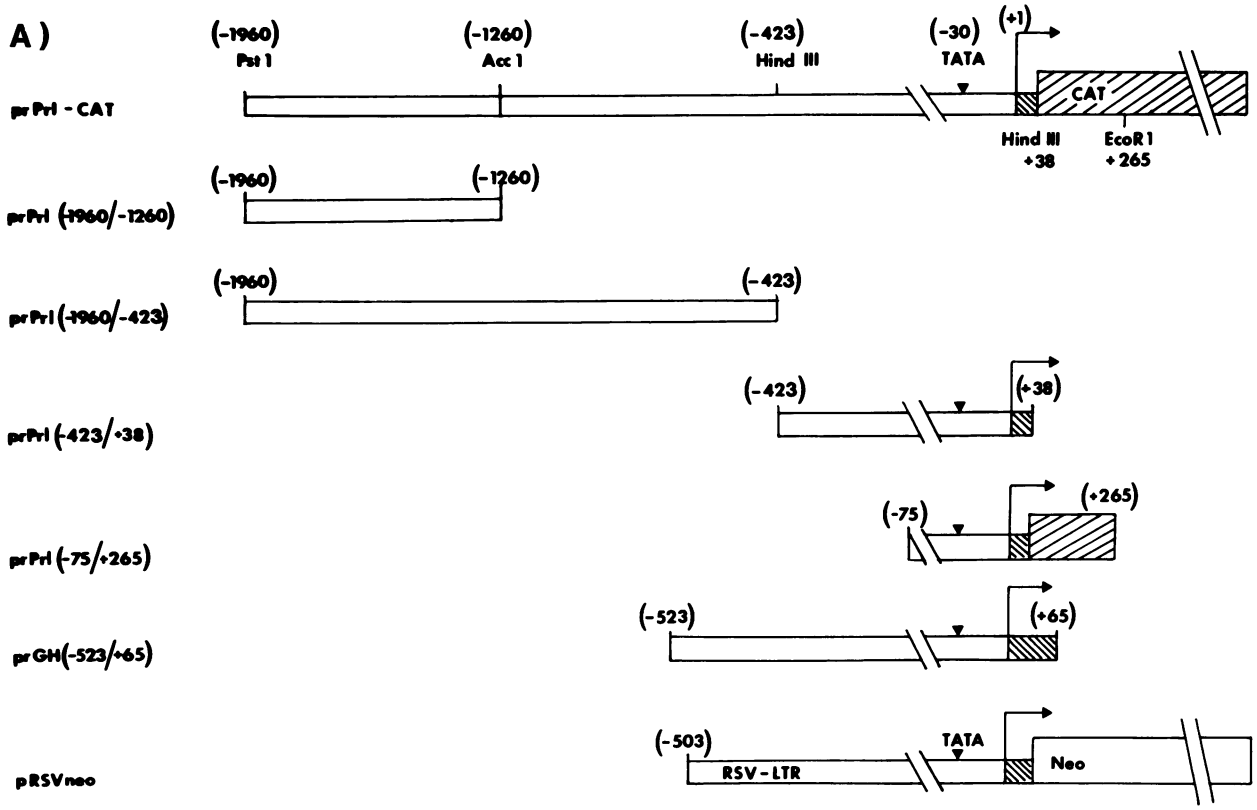
A second but less prominent boundary of GH_3 cell nuclear factor binding was detected in the region from −155 to −180 (Figure 2A). Protection from exonuclease III digestion of this region is significantly more prominent in GH_3 cell extracts than in HeLa cell extracts.

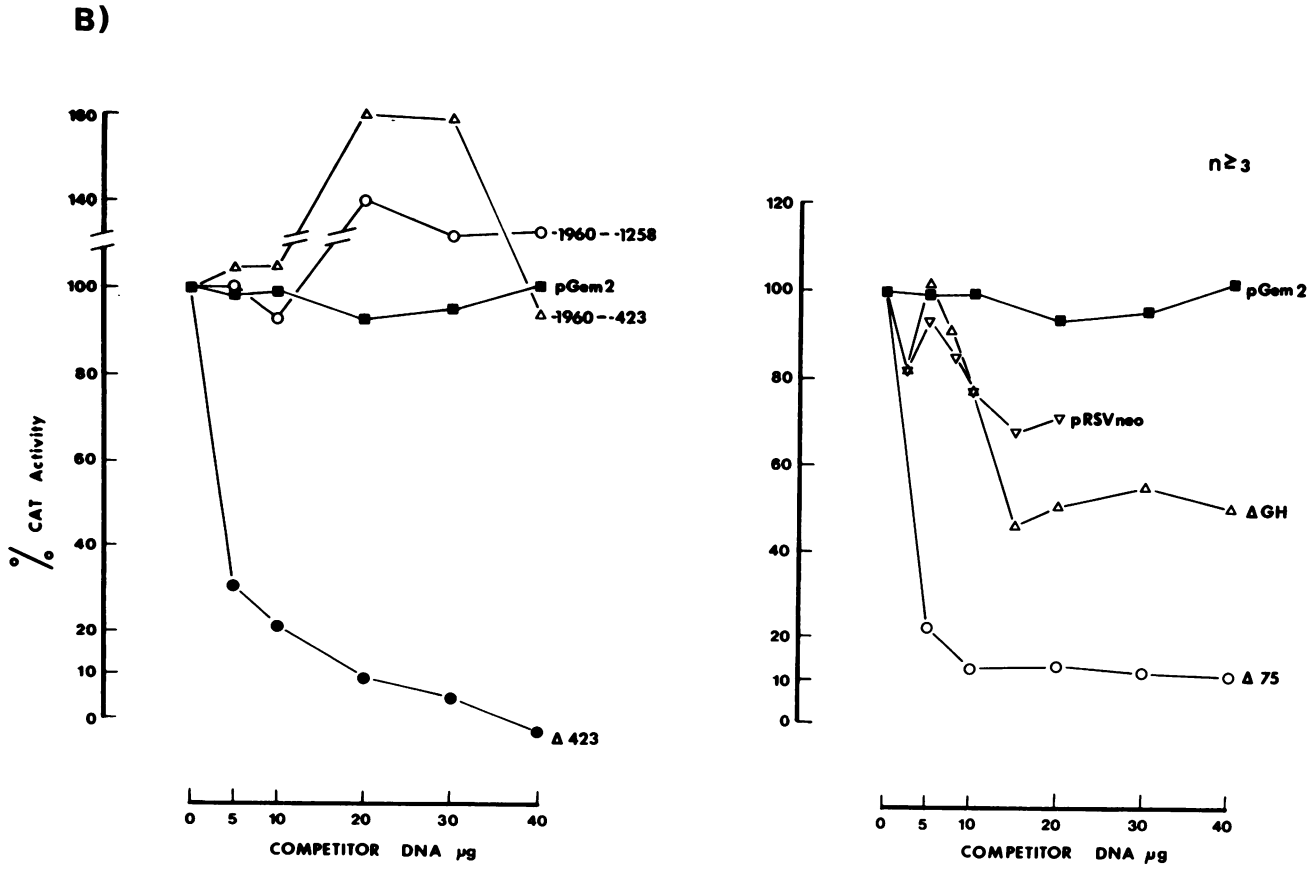
The binding of nuclear factors from another pituitary tumour cell line (GH_4 cells) to this proximal r*Prl* promoter region has also been detected by the exonuclease III protection technique, by Elshotz *et al.* (1986), but its tissue specificity was not established. Gutierrez-Hartmann *et al.* (1987), using DNase I footprinting, have identified GH_3 cell specific factor binding to part of this proximal promoter region (−45 to −65). Their footprinting studies defined a second region of cell specific factor binding (−145 to −175) which equates approximately with the second region of binding (−155 to −180) defined in these studies (illustrated in Figure 6). It is interesting that the exonuclease III protection analysis is quantitatively dominated by the binding over the proximal promoter region while the DNAse I footprinting studies of Gutierrez-Hartmann *et al.* (1987) would seem to detect factor binding over the two regions equally; it is not as yet clear whether the exonuclease III protection technique is distinguishing differing degrees of tightness of factor binding between the two regions or is reflecting concentration differences in factors which bind to the two regions in the GH_3 cell extracts.

The pattern of six borders of factor binding seen over the proximal promoter region (−31 to −77) is highly complex (Figure 2A and B). These sequences and, indeed, those of the more distal binding region (−155 to −180) are strongly conserved in both the bovine (b) (Camper *et al.*, 1984) and human (h) (Truong *et al.*, 1984) *Prl* genes (Figure 6B). At the centre of the proximal binding region is the sequence ···CCTGATTATATATATTCAT···: this contains a 13 nt region of dyad symmetry which although not faithfully conserved in the human and bovine genes exists in one copy in each (Figure 6B). This sequence, in one copy (with one mismatch) also occurs in the r*GH* (−116 to −122) and h*GH* (−117 to −123) gene promoters. In the h*GH* promoter this region is covered by the more distal of two DNase I footprints induced by the partially purified pituitary somatotroph specific *trans*-acting factor, GHF-1, which can stimulate transcription from the h*GH* promoter, *in vitro*, in extracts of non-expressing cells (Bodner and Karin, 1987). In the r*GH* promoter this sequence (−116 to −122) also forms part of a tissue-specific DNase I footprint formed with GH_3 cell nuclear extracts (West *et al.*, 1987). A related sequence occurs in the proximal tissue-specific DNase I footprint reported with GH_3 cell nuclear extracts on the r*GH* promoter (−75 to −100) (Ye *et al.*, 1987; Catanzaro *et al.*, 1987) and within the proximal GHF-1 *trans*-acting factor binding region on the h*GH* promoter (−66 to −92) (Bodner and Karin, 1987). The 5′-end of this central protected sequence (see above) contains the 8 bp motif ···CCTGATTA···; this is also completely conserved in the bovine gene (Figure 6B) and the rat, bovine and human genes contain a completely conserved and closely related motif ···CCTGAATAT··· which in the rat gene occurs in the distal exonuclease III defined binding region (Figure 2B) and in the distal DNase I footprint observed with GH_3 cell NE by Gutierrez-Hartmann *et al.* (1987). It is of further interest (see below and Figure 6C) that the ···CTGATTA··· motif also occurs (in reverse orientation) on the late gene coding strand in binding region P of the SV40 enhancer 72 bp repeat (reviewed by Maniatis *et al.*, 1987).

Tissue-specific factor binding to both of these regions in GH_3 cells must be suggested, therefore, to be of functional importance. The apparent contribution of nuclear factor binding to the more proximal of these promoter regions (−31 to −77) to facilitating tissue-specific r*Prl* gene transcription was further emphasised in transient co-tranfection competition studies (Figure 5A−D): a plasmid containing only 75 bp of the r*Prl* promoter sequence was sufficient, in low concentrations, to effectively inhibit transcription from co-transfected pr*Prl*−*CAT*; a plasmid containing 423 bp of the r*Prl* promoter showed similar inhibitory activity but more distal r*Prl* 5′-flanking sequences (e.g. −1960 to −423) had no such activity. The competing 75 bp promoter sequence contained sequences from −30 to the start site for transcription but it is unlikely that competition for general transcription factors (e.g. TATA box binding factor) which bind these sequences is responsible for its inhibitory effectiveness, as co-transfected r*GH* promoter and RSV-LTR sequences which also contain the latter sequences competed poorly in low concentration and were only partial inhibitors of pr*Prl*−*CAT* expression at the highest co-transfected concentrations (Figure 5B). RNase protection studies (Figure 5C and D) showed that transcription of the reporter gene, pr*Prl*−*CAT*, is being correctly initiated from the CAP site and that the reduced levels of CAT activity seen with the successful competitor plasmids is arising due to reduced transient production of CAT mRNA. Furthermore, it was shown that the presence of only the first 75 bp of the r*Prl* promoter was sufficient to drive a significant but low level of CAT gene expression in GC cells, in particular, but also in GH_3 cells (see Results).

The cell fusion/transfection studies of Lufkin and Bancroft (1987) suggest that at least 187 bp of r*Prl* immediate 5′-flanking sequence (Figure 6) are required to permit r*Prl*−*CAT* expression in fused fibroblast GH_3 cell hybrids; constructs containing < 187 bp of immediate 5′-flanking sequence (e.g. 175 bp) did not permit r*Prl*−*CAT* expression under these conditions. This might suggest that even partial loss of the distal binding region (−181 to −155) identified





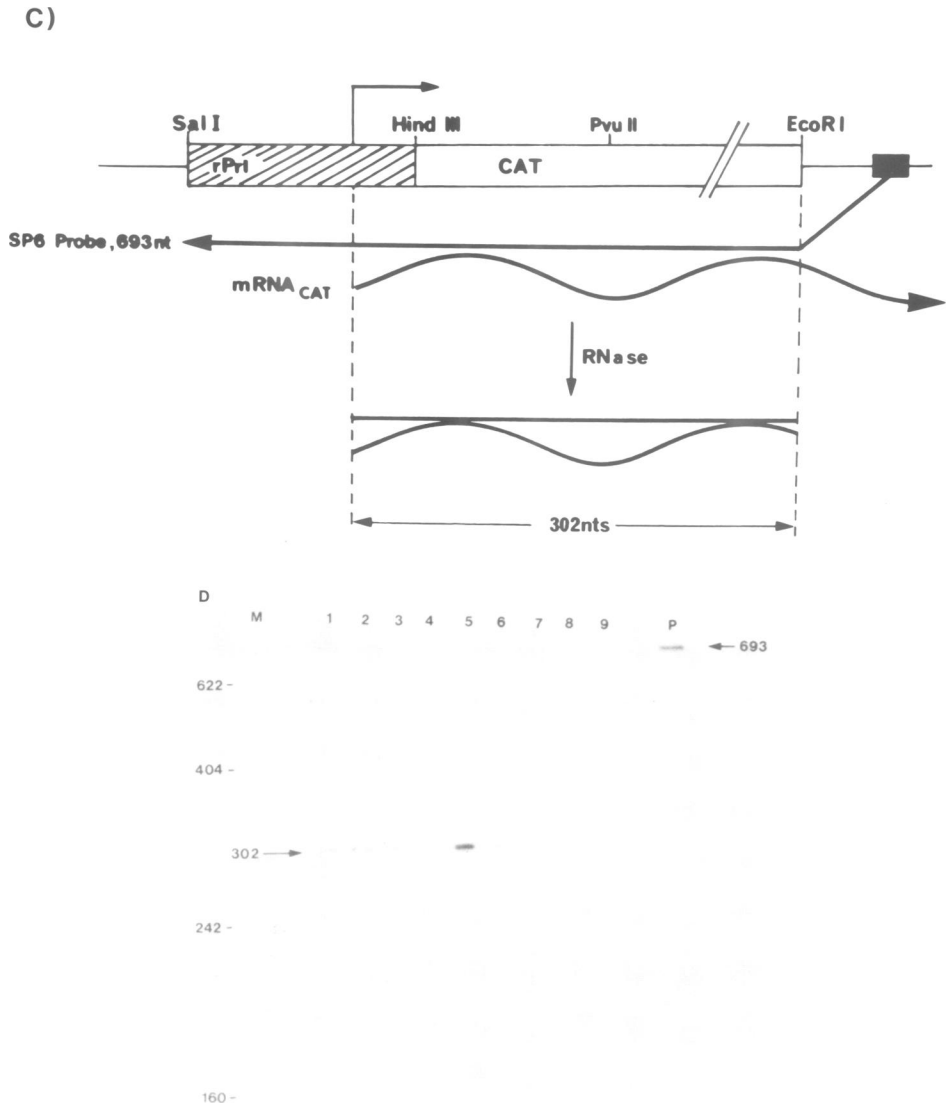
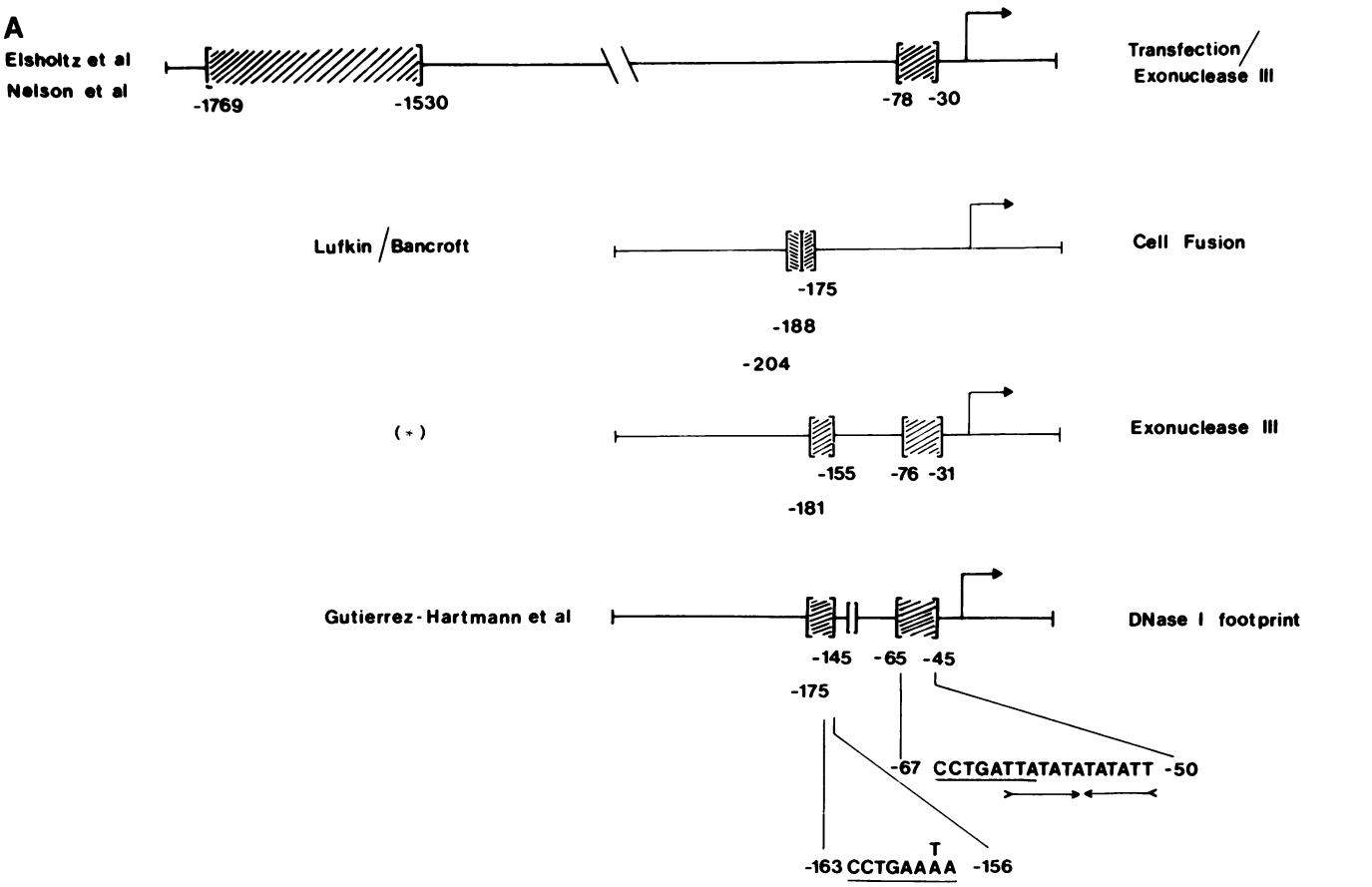


Fig. 5. Transient co-transfection competition analysis. (**A**) The reporter gene pr*Prl*−*CAT* and the competing sequences within the co-transfected competition plasmids. (**B**) Quantitative analysis of transient co-transfection competition analysis. Values represent the mean of three to five experiments. A value of 100% is assigned to the mean CAT activity assessed for 10 μg transfected pr*Prl*−*CAT* with no added competitor. (**C**) Strategy for RNase protection analysis of CAT specific mRNA generated in the transient co-transfection competition experiment. (**D**) RNase protection analysis of transient co-transfection competition experiment. Marker, as in Figure 2A (**lane M**); [^{32}P]UTP labelled anti-sense RNA probe (1/100 that used per hybridization) (**lane P**); transient co-transfection with no competitor (**lanes 1** and **5**), with 40 μg pGEM2 (**lane 2**), with 10 and 20 μg pr*Prl* (−1960/−423) (**lanes 3** and **4**), with 5, 10, 20 and 30 μg pr*Prl* (−423/+38) (**lanes 6**–**9**).

in this study results in loss of r*Prl* gene promoter function in GH_3 cells. These observations taken together with the fact that the fragment containing only −75 pb of the promoter is a potent inhibitor of expression of r*Prl*−*CAT* in the co-transfection competition assay suggest that these distal (−187 to −155) and proximal (−75 to +1) sequences are sufficient to permit tissue specific basal r*Prl* promoter gene expression in GH_3 cells; that the presence of only the proximal promoter sequences (to −75) can drive low level expression is also recognized. This would infer that the factor binding to these promoter regions detected in this study and in the DNase footprint study of Gutierrez-Hartmann *et al.* (1987) has a significant functional correlate. However, this is not the only solution to basal tissue-specific expression from the r*Prl* gene 5′-flanking sequences in pituitary cells: Elsholtz *et al.* (1986) have shown that in GH_4 cells, another pituitary tumour cell line, the distal (−1790 to −1550) r*Prl* tissue specific enhancer linked to a fragment containing only 35 bp of proximal r*Prl* promoter sequence was sufficient to sustain basal expression of *CAT* on transient transfection. It is of interest that an excess of fragment (−1960 to −1260) containing this enhancer region competed effectively for nuclear factor binding to the −180 to −155 and −77 to −31 regions in the exonuclease III protection assay (Figure 3B). Furthermore, an excess of the same fragment competed against formation of the tissue-specific complexes I and II in the gel-retardation study (Figure 4C). This suggests that both are competing to bind the same factors. However, there is no strikingly apparent homology between these distal sequences (−1960 to −1260) and the proximal promoter region −75 to −30. The fact that plasmids containing these distal enhancer sequences failed to reduce r*Prl*−*CAT* expression in the co-transfection competition studies complicates matters: it is clear that r*Prl* promoter function in GH_3 cells has no requirement for the distal enhancer sequences (e.g. Figure 1B); thus, while the distal and prox-



B

```
rPrl        -180 .... TTTCTGGCCACTATGTCTTCCTGAATATGAATA .... -148
bPrl        -179 .... TTTCTGGTCAGTATGTCTTCCTGAATATGAATA .... -147
hPrl        -180 .... TTTCTGGCCAGTATGTCTTCCTGAATATGAATA .... -148

rPrl        -77  .... GGGAAGAGGATGCCTGATTATATATATATTCATGAAGGTGTCGAAGGTT .... -29
bPrl        -74  .... GGGAAGGGAATGCCTGATTA-A-ATATATTCATGAAGATGTCAAAGCCT .... -28
hPrl        -75  .... GGGAAACGAATGCCTGAAT-CA-TTATATTCATGAAGATATCAAAGGTT .... -29
```

C

```
                  5'      3'                                  5'              3'
rPrl        -66 ... CTGATTA .... -61    rPrl    -45 ..............TGAAGGTGTCGAAGGT---- -30
                  3'          5'                    3'                              5'
SV40                ACTGATTAACT         SV40          TGAAAGGTGTGGGATTGACTGTGTGTAAGGTGTCG
(72-bp repeat)                          (72-bp repeat)
                    region P                          GT-I                GT-II
```

Domain B

Fig. 6. (A) Review of known transcriptionally important sequence elements in the r*Prl* 5'-flanking sequence. Data is summarized from: Elsholtz *et al.* (1986), Nelson *et al.* (1986), Lufkin and Bancroft (1987) and Gutierrez-Hartmann *et al.* (1987). (B) and (C) Sequence homologies with h*Prl* and b*Prl* sequences and SV40 72 bp repeat sequences. The r*Prl* sequences are taken from Cooke and Baxter (1982) and Mauer (1985), the b*Prl* sequences are taken from Camper *et al.* (1984) and the h*Prl* sequences from Truong *et al.* (1984). The SV40 72 bp repeat sequences quoted in (B) are from Maniatis *et al.* (1987) and are the late gene coding strand running 3'→5'.

imal sequences may bind the same or similar *trans*-acting transcriptional components *in vitro*, in GH_3 cells *in vivo* a functional tissue-specific transcriptional complex is formed which incorporates the proximal promoter sequences [to ~ −204 bp (Figure 6 and Lufkin and Bancroft, 1987)] and which can transcribe from the r*Prl* promoter in the presence of an excess of competing distal enhancer sequences, without interference. However, an alternative interpretation of the *in vivo* co-transfection competition data (Figure 5) could also be proposed: the successful competitors (r*Prl* −75 to +38 and −423 to +38) might be binding and limiting the availability of a TATA box binding factor to the r*Prl*−*CAT* reporter gene; the r*GH* promoter and RSV-LTR competitor constructs, although both contain a TATA box, might bind this TATA box factor less well, hence their poor competition.

A most interesting observation was the fact that, in addition to the proximal and distal fragments of the r*Prl* 5′-flanking sequences, a fragment containing SV40 early promoter sequences competed quite avidly for factor binding to the proximal r*Prl* promoter binding region as judged by exonuclease III protection analysis (Figure 3). This was an unexpected finding as it is well known that the SV40 early gene enhancer/promoter is inactive in GH_3 cells, as judged by transient transfection studies (Camper *et al.*, 1985; Schirm *et al.*, 1987). The existence of sequence homologies between the proximal binding region of the r*Prl* promoter and the SV40 enhancer 72 bp repeat may provide an explanation for this successful competition: from Figure 6C it can be seen that the 7 bp motif ···CTGATTA··· (−66 to −61) of the r*Prl* promoter which is conserved in the b*Prl* gene (Figure 6B) is present in the P region of the 72 bp repeat (Zenke *et al.*, 1986) (on the late coding strand but running in reverse orientation). Also, the 16 bp sequence from −45 to −30 of the r*Prl* promoter shows a 75% homology with a sequence within the GT rich segment of domain B of the 72 bp repeat; furthermore, a 5 bp motif ···AAGGT··· occurs twice in the r*Prl* −45 to −30 sequences and in the centre of the two functionally defined regions, GT-I and GT-II, in the SV40 GT rich sequences (Zenke *et al.*, 1986) (Figure 6C); in fact, in its more distal form in the r*Prl* sequences (i.e. ···AAGGTGT···) there is a 7 bp homology with the GT-I and GT-II motif. The AAGGT sequence, thus similarly, forms part of the so-called 'core' sequence which Weiher *et al.* (1983) have suggested to play a key role in enhancer activity.

The P, GT-I and GT-II regions have been defined as significant for SV40 enhancer function in mutational analysis studies (Zenke *et al.*, 1986) and many studies strongly implicate the role of the various SV40 motifs in binding specific *trans*-acting factors involved in enhancer function (Scholer and Gruss, 1984; Mercola *et al.*, 1985; Wildeman *et al.*, 1984; Sergeant *et al.*, 1984; Sassone-Corsi *et al.*, 1985; Wildeman *et al.*, 1986). However, it must again be stressed that these homologous SV40 sequences, on the late gene coding strand, run in the reverse orientation to those seen in the r*Prl* promoter. These sequence homologies may provide an explanation for the observed competition for the GH_3 cell specific factors and there is little doubt that the −31 to −77 r*Prl* region is of significant importance for r*Prl* promoter function. However, our findings do not establish that the *trans*-acting factors which bind to the proximal region of the r*Prl* promoter are related to those which mediate SV40 enhancer function. But, nevertheless, in the future the highly defined nature of the SV40 enhancer factor interactions may be of significant technical help in further defining the interaction of tissue specific *trans*-acting transcriptional factors with the r*Prl* promoter.

Finally, gel retardation analysis (Figure 4A−C) showed the formation of two tissue specific and sequence specific complexes (complexes I and II) between a DNA fragment containing the proximal r*Prl* promoter sequences and GH_3 cell nuclear factors. The formation of these complexes was successfully competed against by an excess of r*Prl* proximal and distal sequences and by SV40 early promoter/enhancer sequences but not by r*GH* promoter sequences (Figure 4C). The larger r*Prl* proximal sequence fragment (−423 to +38) rather than the gel retardation probe fragment (−75 to +38) was used as competitor to retain uniformity with the exonuclease III competition studies (Figure 3). However, a smaller promoter fragment probe [−69 to −44: inclusive of the ···CCTGATTATATATATATT··· motif (see above)] has been shown to generate a tissue specific complex, equivalent to complex I, in additional gel retardation analyses (results not shown), and the formation of this complex is competed against by an excess of the −69 to −44 fragment in addition to other competitors previously used. Overall, the specificity of binding observed in our gel retardation studies reflects that seen in the strong tissue specific r*Prl* proximal promoter−GH_3 cell nuclear factor interactions detected in the exonuclease III based analyses (Figures 2 and 3) and provides independent evidence supporting the biological significance of those latter findings.

Materials and methods

Cell culture; extract preparation, transient transfection and CAT assay

GH_3 cells (Bancroft, 1981) were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 12.5% horse serum and 2.5% fetal calf serum (FCS) or in spinner culture in Joklik's modification of MEM containing the same serum complement. HeLa cells were maintained as monolayers in DMEM containing 10% FCS or in spinner culture in Joklik's MEM containing 10% FCS.

Nuclear extracts from GH_3 and HeLa cells were prepared as described by Dignam *et al.* (1983); nuclei extracted at a final NaCl concentration of 0.3, 0.36 and 0.42 M showed no difference in their pattern of protecting r*Prl* promoter fragments from exonuclease III digestion. Whole cell extracts were prepared as described by Manley *et al.* (1980). Heparin agarose purification of a GH_3 NE was carried out as described by Davison *et al.* (1983) (the pooled 0.24 and 0.6 M NaCl eluate fractions used were provided by Tom Lufkin).

GH_3 cells in monolayer culture (60 mm dishes, 2×10^6 cells/dish) were transiently transfected (with 10 μg plasmid) using the DEAE-dextran technique (Sompayrac and Danna, 1981) and HeLa cells by the Ca_2PO_4 technique (Parker and Stark, 1979). For the transient co-transfection competition studies (Seguin *et al.*, 1984) GH_3 cells were transfected with a total of 50 μg plasmid, made up to 10 μg reporter gene (pr*Prl*−*CAT*, Lufkin and Bancroft, 1987) and 5.0−40.0 μg competitor plasmid (see Figure 5A) with the balance being made up to 50 μg with pGEM2 (Promega Biotec). None of the competitor plasmids contained a functional CAT gene. At 48 h after all transfections cells were harvested and assayed for CAT activity essentially as described by Gorman *et al.* (1982a,b) with the exception that the harvested cells were disrupted by two 10 s bursts of sonication and that the extracts were incubated for 2 h at 37°C with 0.2 μCi [^{14}C]-choramphenicol (sp. act. 53 mCi/mmol) in a final volume of 60 μl which contained 50 μl (half the total) cell extract. After TLC separation and autoradiography [^{14}C]chloramphenicol and acetylated product radioactivity was quantitated by liquid scintillation counting.

RNase protection assay (Melton et al., 1984)

Total RNA was isolated from transiently transfected GH_3 cells (60 mm dishes; after 48 h) as described by White *et al.* (1981). The anti-sense ^{32}P-labelled RNA probe was prepared using *Sal*I linearized pr*Prl* (SP6) (see below) and SP-6 RNA polymerase as described by Melton *et al.* (1984),

except that the transcription buffer used was pH 8.2 [technical note (Dupont), 1987]. Hybridization (45°C, overnight), RNase treatment (32°C, 2 h) and product isolation followed that suggested by Melton *et al.* (1984). However, the isolated total RNA was dissolved in hybridization buffer at ~5 μg/μl and subsequently diluted for analysis; and carrier yeast tRNA (20 μg/tube) was added before the final product precipitation in 200 μl H_2O. Products were analysed on urea saturated 5% acrylamide sequencing gels.

Exonuclease III protection assays

These were carried out essentially as described by Wu (1985). Between 3 and 6 ng of agarose gel purified selectively 5'-end-labelled DNA fragment was incubated at 24°C for 15 min in 12.5–50 μl binding buffer [15 mM Tris–HCl, pH 7.4, 55 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 2.0 mM Na_2PO_4 (pH 7.0) and 5% glycerol] containing 6 or 12 μg carrier DNA [*Sau*3AI + *Mbo*I cut pUC19 (1 part, by weight)], yeast tRNA (10 parts), and mixed deoxyribonucleotides [pdNdN (Pharmacia), 1 part] and nuclear proteins (25–60 μg). The components were mixed by gentle vortexing. At the end of the binding step exonuclease III (80–360 U) was added and the mixture incubated for a further 10 min at 30°C. The reaction was then terminated by the addition of 150 μl of Tris–HCl, pH 8.0 (10 mM), EDTA (1.0 mM), sodium dodecyl sulphate (1%) and sodium chloride (125 mM) (TENS) and 40 μg yeast tRNA. Nucleic acids were purified by phenol–chloroform and chloroform extraction and precipitated overnight after the addition of two volumes of ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol and taken up in 5 μl formamide/EDTA/dye, denatured in a water bath at 85°C for 15 min and analysed by electrophoresis on a urea saturated 5% polyacrylamide sequencing gel. The gel was exposed to X-ray film at −70°C in the presence of an intensifying screen for 1–5 days.

In vitro transcription

Analysis (Manley *et al.*, 1980) was carried out in HeLa cell NE and GH_3 cell WCE using an adenovirus major late promoter containing DNA sequence (pBalE, *Sma*I linearized template, predicted run-off: 535 nt (Manley *et al.*, 1980)] and a r*Prl* promoter containing template [pr*Prl* (−423/+436), *Hin*cII linearized, predicted run-off: 436 nt]. The reaction mixture contained 20 or 40 μg/ml linearized template, HeLa cell nuclear extract (30 μg protein) or GH_3 whole cell extract (60 μg protein), creatinine phosphate 4.0 mM, ATP 400 μM, CTP and GTP 40 μM, UTP 5 μM (+5 μCi [^{32}P]-UTP, sp. act. 800 Ci/mmol) and was 12 mM Hepes (pH 7.6), 75 mM KCl, 0.3 mM DTT, 0.12 mM EDTA and 12% glycerol (final volume 25 μl). It was incubated at 30°C for 30 min. The reaction was stopped by the addition of TENS (4 volumes), proteinase K (80 μg) and yeast tRNA (40 μg) and then incubated at 58°C for 15 min. Thereafter, the nucleic acids were purified and analysed on urea-saturated 5% acrylamide gels as described above.

Gel retardation analysis (Strauss and Varshavsky, 1984)

This was carried out essentially as described by Singh *et al.* (1986). The ^{32}P-end-labelled (see above) *Hin*dIII fragment from pr*Prl*(Δ75)–*CAT* (134 bp, containing r*Prl* sequences −75 to +38) (12 000 c.p.m.) was incubated with NE or WCE of GH_3 or HeLa cells. Binding reactions [25 μl normally, but 38 μl in the competition analysis (Figure 4C)] contained 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1.0 mM dithiothreitol, 1.0 mM EDTA, 5% glycerol and 7.0–35 μg of NE or WCE protein. The reaction constituents were mixed by gently vortexing and incubated at 22°C for 30 min. The resulting complexes were resolved in a low ionic-strength 4% polyacrylamide gel (acrylamide:bisacrylamide, 30:1) containing 6.7 mM Tris–HCl (pH 7.5), 3.3 mM sodium acetate and 1 mM EDTA. The gel was pre-electrophoresed for 90 min (20 mA, constant current). Electrophoresis was carried out for 90 min (30 mA, constant current) at room temperature with buffer recirculation. Thereafter, gels were dried and autoradiographed at −70°C with intensifying screens.

Plasmid constructions

Four plasmids containing 5'-flanking sequences of r*Prl* were obtained as gifts from Tom Lufkin and Carter Bancroft: pr*Prl*–*CAT*, pr*Prl*(Δ395)–*CAT* and pr*Prl*(Δ75)–*CAT* (Lufkin and Bancroft, 1987) and pr*Prl* (−423/+436) which contains r*Prl* sequences from −423 (*Hin*dIII site) to +436 (*Hin*cII site, in intron A) cloned into *Hin*dIII/*Hin*cII restricted pSP64.

For the exonuclease III protection analyses pr*Prl* (−423/+436) was selectively 5'-^{32}P-end-labelled at −423 by *Hin*dIII cutting, alkaline phosphatase treating and incubation with [γ-^{32}P]ATP (sp. act. 800 Ci/mmol) and T_4-polynucleotide kinase (Wu, 1985); the labelled plasmid was then cut with *Acc*I and the selectively 5'-end-labelled 554 bp fragment (Figure 1A) purified by agarose gel electrophoresis. To end label the other strand a *Hin*dIII fragment (−423 to +38) was isolated from pr*Prl*–*CAT* and subcloned into pGEM2 [pr*Prl* (−423/+38)]; this was *Sal*I cut and selectively 5'-end labelled by the same procedure at position +55 (relative to r*Prl* transcription start site); a 524 bp labelled fragment (Figure 1A) was purified following subsequent restriction with *Pvu*II. A selectively 5'-end-labelled RSV-LTR fragment was prepared by cutting pRSV–*CAT* (Gorman *et al.*, 1982a) with *Hin*dIII, labelling and subsequently isolating a 398 bp fragment after restriction with *Nru*I (Figure 1A).

The following agarose gel purified DNA fragments were used as binding factor competitors in exonuclease III protection assays: the 470 bp (−423 to +38) *Hin*dIII r*Prl* promoter fragment from pr*Prl* (−423/+38); the 700 bp (−1960 to −1260) *Acc*I distal r*Prl* 5'-flanking sequence fragment from pr*Prl*–*CAT*; the 588 bp (−523 to +65) *Pst*I r*GH* promoter fragment from pr*GH* (−523/+65); the 398 bp (−360 to +38) *Nru*I–*Hin*dIII fragment from pRSV–*CAT*; and the 510 bp (−438 to +72) *Acc*I–*Hin*dIII fragment from pSV2–*CAT* (Gorman *et al.*, 1982b) (Figure 3A). Linearized pGEM2 was also used as a non-specific competitor fragment.

For the transient transfection studies pr*Prl*(Δ958)–*CAT* (Figure 1A) was prepared by *Sph*I restricting pr*Prl*–*CAT*, blunting the ends generated, *Sma*I cutting and religating. As competitor plasmids for the transient co-transfection competition assays; pr*Prl* (−1960/−1260) was prepared by subcloning a 700 bp *Acc*I fragment from pr*Prl*–*CAT* into the *Acc*I site in pGEM2; pr*Prl* (−1960/−423) was prepared by subcloning a 1537 bp *Pst*I–*Hin*dIII fragment from pr*Prl*–*CAT* into *Pst*I–*Hin*dIII cut pGEM2; pr*Prl* (−423/+38) was prepared as described above; pr*Prl* (−75/+265) was prepared by cutting pr*Prl*(Δ75)–*CAT* with *Eco*RI and religating; pr*GH* (−523/+65) contains the 588 bp *Pst*I fragment isolated from a λ r*GH* clone [Clone 50, Chien and Thompson (1980)] and subcloned into pSP64; and pRSV–*neo* was a gift from Tom Lufkin.

A 660 bp *Sal*I–*Eco*RI fragment from pr*Prl*(Δ395)–*CAT* (Lufkin and Bancroft, 1987) was subcloned into pGEM2 to generate pr*Prl* (SP6). This allowed a 693 nt anti-sense RNA probe to be generated for RNase protection assay.

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Note added in proof

Nelson *et al.* [Nelson,C., Albert,V.R., Elsholtz,H.P., Lu,L.I.-W. and Rosenfeld,M.G. (1988) *Science*, **239**, 1400–1405] have established that a single tissue specific factor, Pit1, binds to multiple sites in both the r*Prl* promoter and distal 5′-flanking sequences thus providing an explanation for the competition for factor binding by distal sequences reported in this study.