sion of another activity present in serum, or relieve an inhibition. Our results raise the possibility that a new pituitary factor will join the list of growth-promoting hormones induced by thyroid hormone in vivo. The finding that T3 induces GH_4C_1 pituitary cells to secrete an autocrine growth regulator should provide a basis for future research on the molecular mechanism of growth control by thyroid hormones.

REFERENCES AND NOTES

1. J. H. Oppenheimer, *Science* **203**, 971 (1979).
2. H. H. Samuels, J. S. Tsai, R. Cintron, *ibid.* **181**, 1253 (1973).
3. C. R. DeFesi, E. C. Fels, M. I. Surks, *Endocrinology* **116**, 2062 (1985).
4. H. H. Samuels and J. S. Tsai, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3488 (1973).
5. P. Walker, M. E. Weichsel, Jr., D. A. Fisher, S. M. Guo, D. A. Fisher, *Science* **204**, 427 (1979).
6. S. B. Hoath, J. Lakshmanan, S. M. Scott, D. A. Fisher, *Endocrinology* **112**, 308 (1983).
7. H. H. Samuels, F. Stanley, J. Cassanova, *ibid.* **105**, 80 (1979).
8. P. Walker, J. D. Dubois, J. H. Dussault, *Pediatr. Res.* **14**, 247 (1980).
9. M. J. Miller, E. C. Fels, L. E. Shapiro, M. I. Surks, *Clin. Res.* **34**, 714A (1986).
10. P. M. Hinkle, M. H. Perrone, T. L. Greer, *J. Biol. Chem.* **254**, 3907 (1979).
11. A. S. Schonbrunn, M. Krasnoff, J. M. Westendorf, A. H. Tashjian, Jr., *J. Cell Biol.* **85**, 786 (1980).
12. L. K. Johnson, J. D. Baxter, I. Vlodavsky, D. Gospodarowicz, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 394 (1980).
13. J. Massague, *Trends Biochem. Sci.* **10**, 237 (1985).
14. C. W. Welsch, *Cancer Res.* **45**, 3415 (1985).
15. T. C. Dembinski, C. K. Leung, R. P. Shiu, *ibid.*, p. 3083.
16. D. Danielpour, T. Ikeda, M. W. Kunkel, D. A. Sirbasku, *Endocrinology* **115**, 1221 (1984).
17. T. Ikeda and D. A. Sirbasku, *J. Biol. Chem.* **259**, 4049 (1985).
18. T. Ikeda, D. Danielpour, D. A. Sirbasku, *J. Cell. Biochem.* **25**, 213 (1984).
19. J. Halpern and P. M. Hinkle, *Mol. Cell. Endocrinol.* **33**, 183 (1983).
20. P. Walker, M. E. Weichsel, Jr., S. M. Guo, D. A. Fisher, D. A. Fisher, *Brain Res.* **186**, 331 (1980).

21. Supported in part by NIH grant AM 32847, Cancer Center core research grant CA 11198, and a Research Career Development Award (AM/NS 00827) to P.M.H. We are very grateful to E. Johnson, Washington University, St. Louis, for providing the antiserum to NGF.

9 June 1986; accepted 21 October 1986

Two Different *cis*-Active Elements Transfer the Transcriptional Effects of Both EGF and Phorbol Esters

HARRY P. ELSHOLTZ, HARRY J. MANGALAM, ELLEN POTTER, VIVIAN R. ALBERT, SCOTT SUPOWIT,* RONALD M. EVANS, MICHAEL G. ROSENFELD

Short *cis*-active sequences of the rat prolactin or Moloney murine leukemia virus genes transfer transcriptional regulation by both epidermal growth factor and phorbol esters to fusion genes. These sequences act in a position- and orientation-independent manner. Competitive binding analyses with nuclear extracts from stimulated and unstimulated cells suggest that different *trans*-acting factors associate with the regulatory sequence of each gene. A model is proposed suggesting that both epidermal growth factor and phorbol esters stimulate the transcription of responsive genes via discrete classes of hormone-dependent, enhancer-like elements that bind different *trans*-acting factors, even in the absence of hormone stimulation.

DIFFERENTIATION AND REPLICATION of eukaryotic cells are under complex control and are regulated by diverse families of peptides, including those collectively referred to as growth factors (*1*). One of the best studied of these is epidermal growth factor (EGF) (*2*), which acts by binding to a specific transmembrane receptor that has intrinsic protein kinase activity (*3*). The binding of EGF to its receptor generates a series of very early responses, such as increased sodium flux and stimulation of tyrosine phosphorylation (*1, 3, 4*), and rapidly affects a number of cellular processes involved in the growth response. These include increases in the synthesis of specific proteins (*5*) and rapid stimulation of transcription of specific genes (*6–10*). Because the receptors for all polypeptide hormones are initially localized to the plasma membrane, the regulation of gene transcription by polypeptide hormones requires transduction of the signal to the nucleus.

The prolactin gene is expressed in vivo in a population of pituitary cells and in GH rat pituitary cell lines (*11, 12*), and encodes a polypeptide hormone important for reproduction and osmoregulation. We have reported that EGF, thyrotropin-releasing hormone (TRH), and adenosine 3′,5′-monophosphate (cAMP) rapidly stimulate transcription of the rat prolactin (rPRL) gene within minutes of their addition to cultures of GH cells, resulting in a rate of initiation of new transcripts that is increased seven to ten times (*6, 13, 14*). Because the transcriptional effects of EGF are rapidly attenuated and the rPRL messenger RNA (mRNA) is long-lived, the mature rPRL mRNA increases only 2 to 3.5 times, with a comparable increase in rPRL synthesis (*6, 13*). Phorbol esters, which are reported to activate protein kinase C (*15*) and cause the same pattern of cytoplasmic phosphorylation and morphological changes as EGF in GH cells (*16*), also rapidly activate transcription of the rPRL gene (*17*). A fragment of the rPRL gene containing 3 kb of 5′-flanking sequence transfers regulation by both EGF and the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) to an unresponsive heterologous transcription unit in A431 cells (*18*), suggesting that these *cis*-active sequences transfer transcriptional regulation in a manner analogous to those conferring regulation by steroid hormones (*19, 20*). Evidence for comparable regulatory elements in the bovine PRL gene (*21*) and the c-*fos*^{H} gene (*7*) has been reported. On the basis of these observations and the demonstration that serum growth factors, including EGF, rapidly induce the transcription of multiple gene products (*7, 8*), one might predict that all such genes would have a similar or consensus sequence responsible for their increased transcription.

Cis-active sequences referred to as enhancers markedly stimulate the transcription of eukaryotic genes in a relatively position- and orientation-independent fashion (*22, 23*). It is suggested that enhancer activity is dependent on the binding of *trans*-acting factors present in limiting concentrations (*23, 24*). Although many of these enhancers do not appear to be hormonally regulated and exert only tissue-specific actions on gene transcription, studies of the mammary tumor virus, Moloney murine sarcoma virus, and other genes have shown that some enhancers confer hormonal responsiveness on gene transcription. For example, the glucocorticoid-receptor complex directly regulates gene transcription as a consequence of binding to genomic sequences that act as classic enhancers (*19, 20*).

Because 5′-flanking sequences of the rPRL gene confer transcriptional regulation

H. P. Elsholtz, H. J. Mangalam, V. R. Albert, S. Supowit, M. G. Rosenfeld, Howard Hughes Medical Institute, Eukaryotic Regulatory Biology Program, University of California at San Diego School of Medicine, La Jolla, CA 92093.

E. Potter, Howard Hughes Medical Institute, Eukaryotic Regulatory Biology Program, University of California at San Diego School of Medicine, La Jolla, CA 92093 and Molecular Biology and Virology Laboratory, Salk Institute, La Jolla, CA 92037.

R. M. Evans, Howard Hughes Medical Institute, Molecular Biology and Virology Laboratory, Salk Institute, La Jolla, CA 92037.

*Present address: Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550.

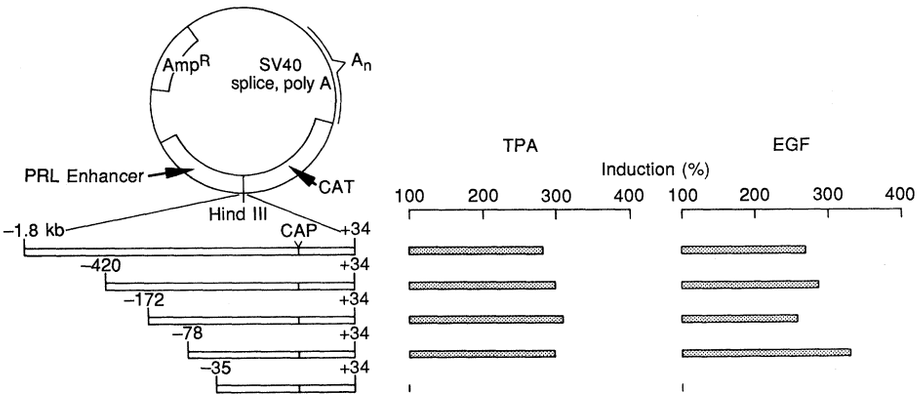

Fig. 1. Mapping the EGF and TPA regulatory region of the rat prolactin gene. Fragments of the 5′-flanking region of the rPRL gene extending from −1.8 kb to −35 bp were placed 5′ of the bacterial CAT gene, and the plasmids were transfected into GH_4 cells. EGF ($10^{-8}M$) or TPA ($10^{-7}M$) were added 24 hours after transfection and cells were harvested 48 hours after transfection. CAT activity was quantitated as described (*26, 36*). Similar results were obtained in ten experiments. The stimulations by both EGF and TPA were statistically significant ($P < 0.001$, $n = 10$); the average stimulation was 280 ± 50 (SEM) percent.

by EGF and TPA on fusion genes in permanent transfectants (*18*), we performed a deletional analysis of this region to identify the putative *cis*-active regulatory element. Deletions of the rPRL 5′-flanking sequence were evaluated for responsiveness to EGF and TPA by fusion to the bacterial chloramphenicol acetyltransferase (CAT) gene (*25*) and measurement of enzyme activity after transient transfection of the constructions into GH cells. The cell-specific rPRL gene enhancer (*26*) was utilized in these constructions to obtain a detectable level of basal expression. The serial deletions exhibited a relatively constant level of promoter efficacy, even in the case of the −78 and −35 constructions, suggesting the absence of upstream sequences that confer suppression of rPRL gene expression in the basal state (Fig. 1). Both EGF- and TPA-induced gene expression was observed with rPRL sequences shortened to −78 bp, but were absent in constructions containing only 35 bp of 5′-flanking information. These data suggested that a *cis*-active element conferring such regulation was either entirely located between −78 and −35 or that a critical region was split at −35. The amount of stimulation in different experiments reflected the variable (2- to 3.5-fold) stimulation of endogenous rPRL transcripts.

To determine whether the regulatory region was acting as a classic enhancer element, a series of fusion genes was constructed with promoters from the rat growth hormone (rGH) (*26*) and herpes simplex virus thymidine kinase (*tk*) genes (*27*). rPRL 5′-flanking genomic sequences extending from −172 to −10, placed in both orientations in front of the rGH promoter (−320 to +8), transferred both EGF and TPA regulation (Fig. 2B), whereas five DNA fragments from pBR322 failed to exert any effect (Fig. 2A). Consistent with this enhancer-like effect, the sequence also conferred regulation when placed 3′ of the CAT gene at more than 1.5 kb from the transcription start site. When placed 5′ of the *tk* promoter (−200 to +70), the identical −172 to −10 fragment conferred a 20- to 40-fold induction by EGF or TPA, consistently higher than that observed with the rGH promoter.

Because the above analysis indicated that the sequence extending from −78 to +34 retained hormonal responsiveness but the sequence −35 to +34 was inactive, a synthetic oligonucleotide corresponding to the sequence −79 to −30 of the rPRL gene was tested for ability to transfer regulation to the *tk* promoter. In the inverted orientation, a fourfold to sixfold induction was observed in the presence of TPA or EGF (Fig. 2C). In the direct orientation, minimal hormonal stimulation was recorded with a single copy of the oligomer, but a threefold to fivefold

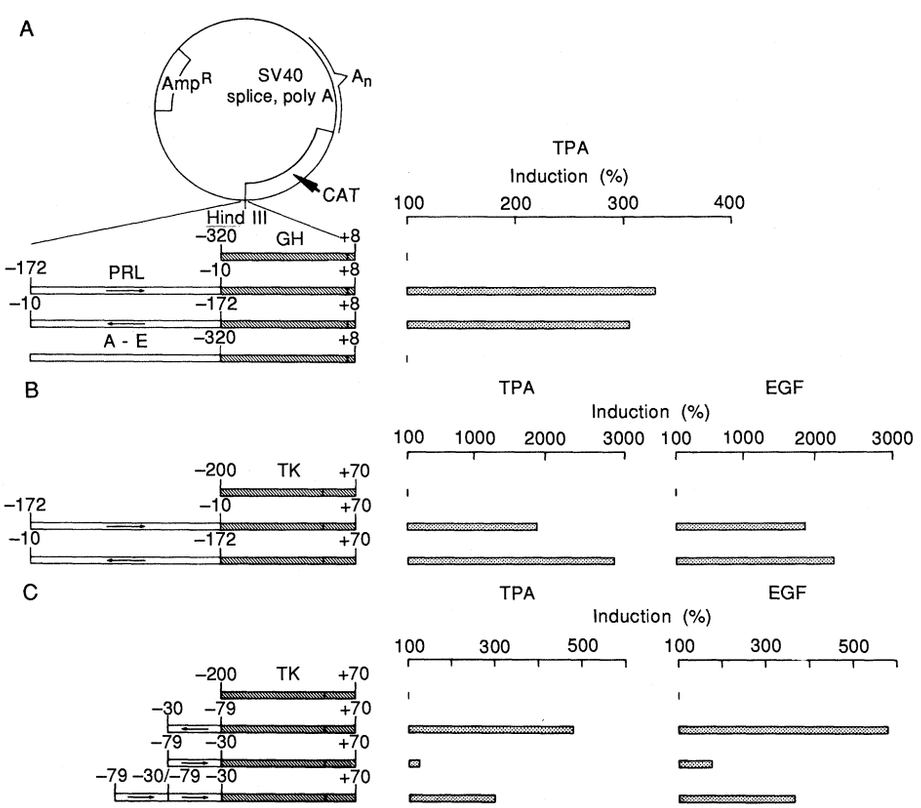

Fig. 2. Position- and orientation-independence of the rPRL *cis*-active element. A rPRL DNA fragment extending from −172 to −10 was placed in both orientations, 5′ to a CAT fusion gene containing (A) the rGH promoter (−320 to +8) or (B) the *tk* promoter (−200 to +70). EGF gave virtually the same results as TPA in the rGH fusion genes. Five Sau 3A fragments of pBR322 (*30*), referred to as A to E (*36*), conferred no regulation. Placement of the rPRL −172 to −10 fragment 3′ of the CAT gene also gave comparable stimulation. (C) A fragment encompassing the region −79 to −30 was inserted in the comparable position in an inverted orientation or as one of two tandem copies in the direct orientation. The effects of EGF or TPA on gene expression were quantitated by CAT assay 20 hours after their addition; results are significant at $P < 0.001$. Similar results were obtained in four additional experiments. The average induction by EGF and TPA was similar for either the GH or the *tk* promoter constructions.

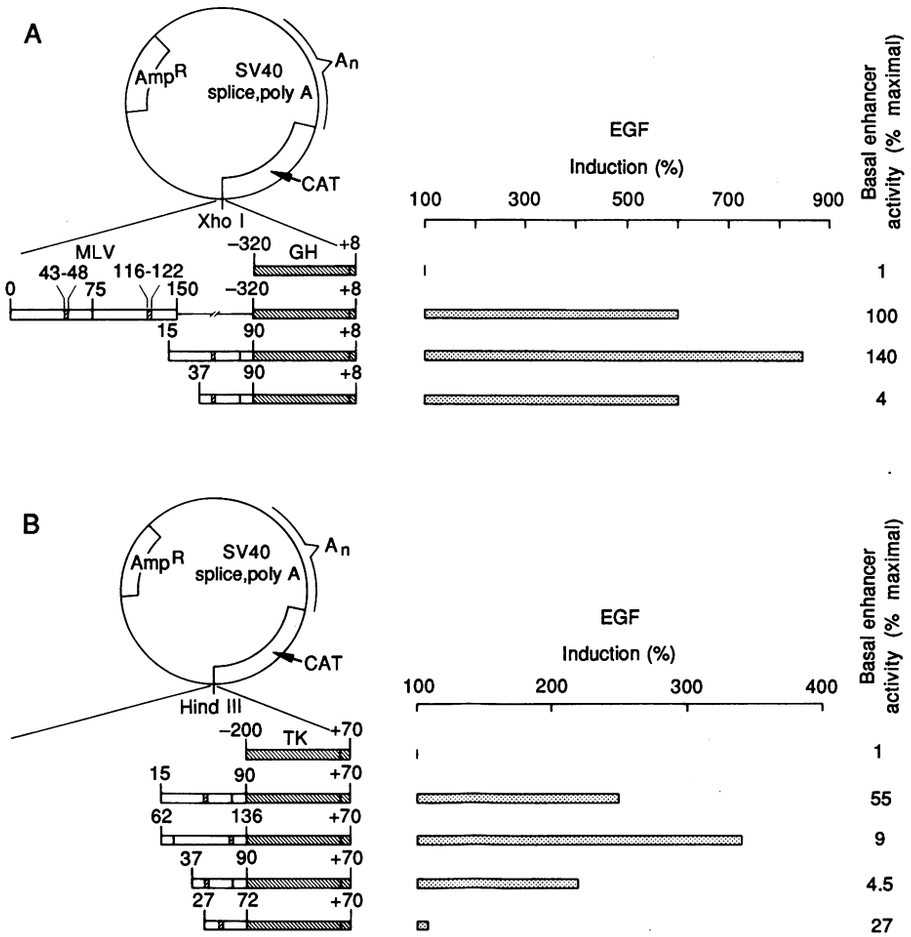

Fig. 3. EGF and TPA regulatory regions in the MoMLV LTR. A fragment of the MoMLV LTR containing the two 75-bp repeats (numbered 0 to 150) or a series of fragments representing 15 to 90, 62 to 136, 37 to 90, and 27 to 72 were placed 5′ of the (A) rGH or (B) *tk* promoter fused to the CAT gene (*37*). EGF or TPA inductions were for 20 hours, 24 hours after transfection of GH_4 cells with 10 μg per plate of each plasmid. The basal activity of the full MoMLV LTR fragment containing both 75-bp repeats was considered 100% basal enhancer activity. Results were repeated in six experiments of similar design and are significant at $P < 0.001$; the average stimulation by the 37 to 90 fragment was not significantly different from the average stimulation by the full MoMLV enhancer or the 62 to 136 fragment.

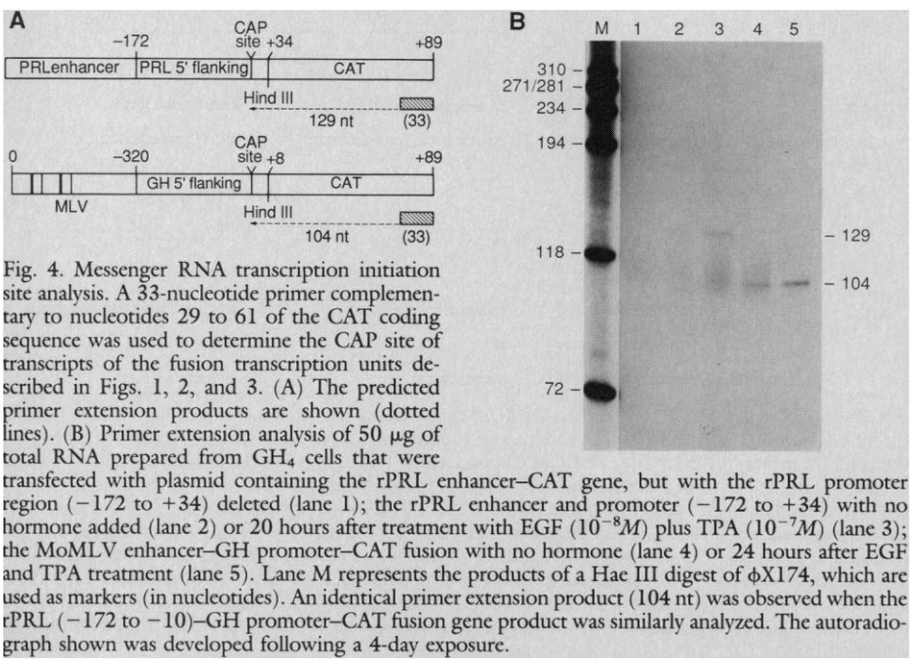

Fig. 4. Messenger RNA transcription initiation site analysis. A 33-nucleotide primer complementary to nucleotides 29 to 61 of the CAT coding sequence was used to determine the CAP site of transcripts of the fusion transcription units described in Figs. 1, 2, and 3. (A) The predicted primer extension products are shown (dotted lines). (B) Primer extension analysis of 50 μg of total RNA prepared from GH_4 cells that were transfected with plasmid containing the rPRL enhancer–CAT gene, but with the rPRL promoter region (−172 to +34) deleted (lane 1); the rPRL enhancer and promoter (−172 to +34) with no hormone added (lane 2) or 20 hours after treatment with EGF ($10^{-8}M$) plus TPA ($10^{-7}M$) (lane 3); the MoMLV enhancer–GH promoter–CAT fusion with no hormone (lane 4) or 24 hours after EGF and TPA treatment (lane 5). Lane M represents the products of a Hae III digest of ϕX174, which are used as markers (in nucleotides). An identical primer extension product (104 nt) was observed when the rPRL (−172 to −10)–GH promoter–CAT fusion gene product was similarly analyzed. The autoradiograph shown was developed following a 4-day exposure.

stimulation was observed when we used two tandem copies, implying that this region contained sufficient information to transfer hormonal regulation, although suboptimally. Inefficient regulation by short DNA sequences that encompass the critical element for transcriptional regulation has been observed in the transfer of regulation by both steroid hormones and metals (*28, 29*). This decrease in efficiency may be due to the truncation of flanking sequences required for optimal protein-DNA interaction.

Before characterization of the rPRL enhancer (*26*), the enhancer from the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) was utilized to elevate basal CAT gene expression to a detectable level, but it became apparent that the MoMLV enhancer element itself (*30*) conferred both TPA and EGF regulation in GH cells. Deletional analysis of the MoMLV enhancer element revealed that the *cis*-active sequence responsible for such regulation resided in a 53-bp region within one of the 75-bp repeats (*30*) (Fig. 3A). This element had virtually no enhancer activity as determined in the absence of hormonal stimulation, even though the fragment retained a core enhancer consensus sequence (Fig. 3A). Similar data were obtained in fusion genes in which the *tk* promoter was substituted for the rGH promoter; however, the amount of stimulation in response to TPA and EGF was lower than in the case of fusion genes containing the rGH promoter (Fig. 3B).

Correct usage of the transcriptional CAP site in the above constructions was examined by the primer extension method (*31*) and was confirmed for transcription units containing either the rPRL or rGH promoters (Fig. 4). The effect of EGF and TPA was to increase the number of mature transcripts that utilized the identical CAP site.

The identification of EGF- and TPA-responsive elements in the rPRL 5′-flanking region and in the MoMLV enhancer permitted us to investigate whether these two *cis*-active elements were regulated by the identical *trans*-acting factor. The binding of proteins to the rPRL regulatory sequence was assessed by the exonuclease III (Exo III) protection assay (*32*) with a rPRL DNA probe extending from −172 to +34, including the −78 to −35 regulatory element. As shown in Fig. 5, and as seen in additional experiments labeling the fragment at each 5′ terminus, nuclear extracts from GH cells contained a protein or proteins that bound to the region between −73 and −28. Specificity of binding was suggested by competition with various DNA fragments; both genomic sequences and synthetic homopolymers failed to compete for binding even

when they were in 100-fold molar excess. The rPRL genomic fragment from −172 to −79 also failed to compete for binding, at the same molar excess (Fig. 5B, lane 4); however, a fragment from −78 to +34 competed at 10- and 100-fold molar excess (Fig. 5B, lanes 5 and 6).

The MoMLV enhancer, which also transfers EGF and TPA regulation, did not compete with the binding of proteins to the rPRL region, even when present at a 100-fold molar excess (Fig. 5B, lane 7). The MoMLV 75-bp region, defined by Pvu II restriction sites within the two 75-bp repeats (MoMLV-P75), itself exhibited two regions of protein binding, as assessed by the Exo III protection assay, both of which were unaffected by the addition of 100-fold molar excess of the rPRL regulatory sequences (−172 to +34). Because cAMP also regulates rPRL gene transcription (*14*), a 53-bp sequence that transfers regulation by forskolin to the herpes simplex virus *tk* promoter was also tested as a competitor and failed to compete binding to the rPRL probe at 100-fold molar excess (Fig. 5B, lane 9). These data suggest that at least two different nuclear proteins bind the rPRL and MoMLV regulatory regions. Because the major protein binding sites in the rPRL sequence coincide with those sequences that transfer EGF and TPA regulation, a potential role for the DNA-binding protein or proteins in hormone-dependent transcriptional activation is suggested.

These conclusions were tested further with the gel retardation assay described by Fried and Crothers (*33, 34*). Incubation of extracts from GH_4 cells with the −79 to −30 rPRL DNA fragment resulted in two major retarded species, with binding proportional to the amount of extract used (Fig. 6A). Binding was competed by the rPRL fragment −172 to +34 at tenfold molar excess, but was not effectively competed by the MoMLV-P75 fragment, a 205-bp fragment of the SV40-VP1 gene, the herpes *tk* promoter (Fig. 6B), or by a poly d(A-T) heteropolymer. These data further support the conclusion that the sequences −72 to −36 are both required and sufficient for binding of specific *trans*-acting factors and that the factors that bind the rPRL regulatory region are not competed by the comparable MoMLV regulatory regions or by an unrelated *cis*-active DNA sequence that confers cAMP responsiveness. Conversely, with the MoMLV-P75 fragment regulatory region as the labeled probe, binding detected in the gel retardation assay was effectively competed by the MoMLV region, but not by a 50-fold molar excess of the rPRL regulatory region (−172 to +34).

Because the elements that conferred EGF and TPA regulation were localized to short sequences that bound specific factors, we could investigate whether hormonal treatment altered the pattern of protection seen during Exo III digestion of the regulatory element. There was no discernible difference in binding of factors to the rPRL regulatory region with extracts prepared from unstimulated GH_4 cells or GH_4 cells treated with TPA and EGF for either 60 minutes (Fig. 5B, lanes 2 and 3) or 10 to 30 minutes. Similar results were obtained with the gel retardation assay (Fig. 6A) with no differences observed in patterns of retarded species between control and hormone-treated cells. The absence of a shift in the migration of DNA-protein complexes would suggest that no change in stable protein-protein interactions involving the DNA-binding factor or factors occurred in nuclear extracts from hormone-treated cells. Allosteric alterations of these factors by covalent modification or noncovalent interactions may be required for their stimulatory effects on initiation of transcription. However, the caveat remains that activation or inactivation of proteins during preparation of nuclear extracts may prevent detection of hormone regulated *trans*-acting factors in our assay system.

The demonstration that specific sequences act in a position- and orientation-independent fashion to confer responsivity to EGF and TPA suggests that they might be functionally analogous to those sequences required for transcriptional activation by steroid hormones (*28*), double-stranded RNA (*35*), and heavy metals (*29*). In the case of glucocorticoids, a single mediator, the hormone receptor, appears to regulate transcription of responsive genes via a single class of *cis*-active DNA sequences (*28*). In contrast, the EGF and TPA regulatory elements of MoMLV and rPRL do not share any striking homologies (Fig. 7A), nor do they compete for identical binding proteins, suggesting that different regulatory sequences can exhibit functional similarities. The rPRL sequence $5'^{-73}$AGAGGATGCCTGAT$^{-60}3'$ is related to the sequence $5'^{-323}$ACAGGATGTCCATAT$^{-309}3'$ of the c-*fos*^{H} gene, which resides in a 56-bp fragment that transfers serum inducibility to heterologous promoters in NIH-3T3 cells. In GH cells also, a 66-bp region of c-*fos*^{H} (−345 to −280) including this sequence confers regulation by EGF and TPA to otherwise unresponsive promoters. As yet, the relationship between the rPRL and the c-*fos*^{H} regulatory elements is

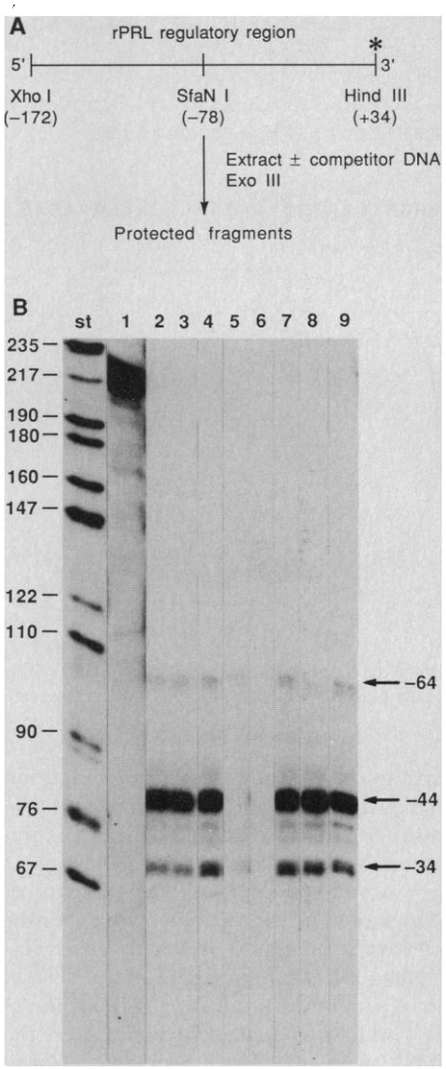

Fig. 5. Exonuclease footprint analysis of the rPRL regulatory region. (A) A rPRL genomic fragment extending from −172 to +34 was labeled at the Hind III site (+34), incubated with nuclear extract from GH_4 cells, digested with Exo III, and (B) electrophoresed on 5% denaturing polyacrylamide gels (*32*). The autoradiograph represents a 3-day exposure. st, DNA standards from an Hpa II digestion of pBR322 (in nucleotides); lane 1, DNA probe with extract, but without Exo III; lane 2, fragment incubated with nuclear extract from unstimulated GH_4 cells; lane 3, fragment incubated with nuclear extract from GH_4 cells treated with EGF ($10^{-8}M$) plus TPA ($10^{-7}M$) for 30 minutes; lanes 4 to 9, competitor DNA plus nuclear extract from TPA plus EGF-stimulated GH_4 cells (*32*); lane 4, 100-fold molar excess of a −172 to −79 rPRL genomic fragment; lanes 5 and 6, 10-fold and 100-fold molar excess of a −78 to +34 rPRL genomic fragment, respectively; lane 7, 100-fold molar excess of the 75-bp MoMLV repeat element; lane 8, 100-fold molar excess of a 424-bp fragment from the rPRL gene, (−1956 to −1530); lane 9, 100-fold molar excess of a 53-bp sequence that transfers regulation by forskolin to *tk* (*32*). Similar results were obtained in three additional experiments in which the probe was labeled at the Xho I site. The same experiments were performed with the MoMLV-P75 fragment. Similar results were obtained when nuclear extracts were prepared in the presence of the phosphatase inhibitor sodium bisulfite (50 m*M*).

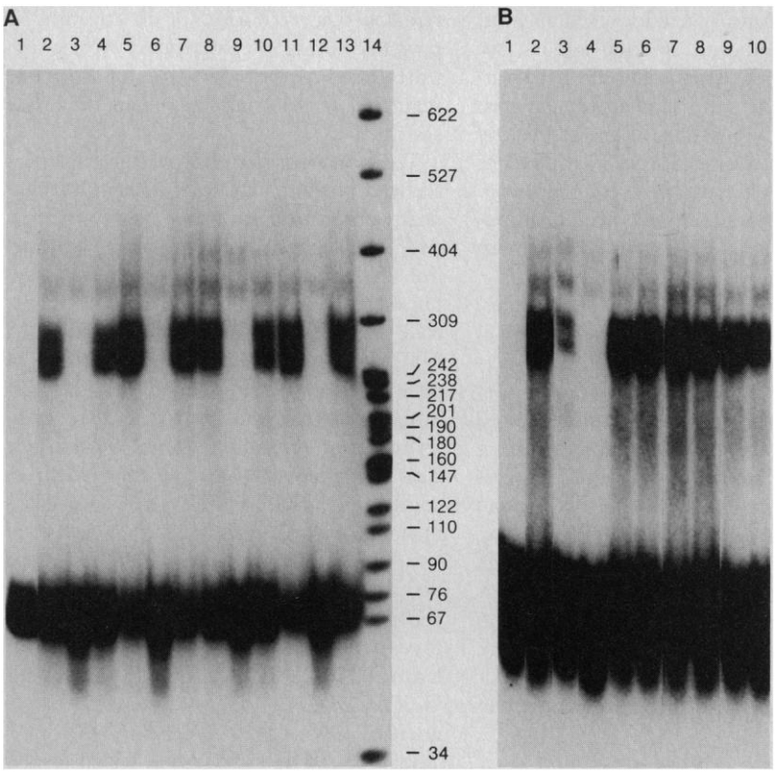

Fig. 6. Analysis of the rPRL regulatory region by a gel retardation assay. An oligomer containing 50 bp of rPRL sequence −79 to −30 was used for the gel retardation analysis (*34*). (A) Concentration dependence of protein binding. Lane 1, probe only; lanes 2 to 7, extract from untreated cells; lane 2, 5 μg extract; lane 3, 5 μg extract plus 20-fold molar excess rPRL fragment −172 to −10; lane 4, 5 μg extract plus 20-fold molar excess SV40-VP1 gene fragment (205 bp); lane 5, 10 μg extract; lane 6, 10 μg extract plus 40-fold molar excess rPRL fragment −172 to −10; lane 7, 10 μg extract plus 40-fold SV40-VP1 gene fragment; lanes 8 to 13, extract from EGF-treated cells; lane 8, 5 μg extract; lane 9, 5 μg extract plus 20-fold molar excess rPRL fragment −172 to −10; lane 10, 5 μg extract plus 20-fold molar excess of the SV40-VP1 gene fragment; lane 11, 10 μg extract; lane 12, 10 μg extract plus 40-fold molar excess of rPRL fragment −172 to −10; lane 13, 10 μg extract plus 40-fold molar excess of the SV40-VP1 genomic fragment; lane 14, size markers, which are fragments of a Hae III digest of ϕX174 (in nucleotides). (B) DNA specificity of protein binding. Lane 1, probe only; lane 2, 5 μg extract; lanes 2 to 10, 5 μg extract plus; lanes 3 and 4, 10-fold and 50-fold molar excess rPRL fragment (−172 to −10), respectively; lanes 5 and 6, 10-fold and 100-fold molar excess MoMLV fragment (75-bp repeat), respectively; lanes 7 and 8, 10-fold and 50-fold molar excess of SV40-VP1 fragment (205 bp), respectively; lanes 9 and 10, a 10-fold and 50-fold molar excess of the *tk* promoter region (−200 to +70), respectively. The autoradiographs represent 12-hour exposures. Similar results were obtained in three additional experiments.

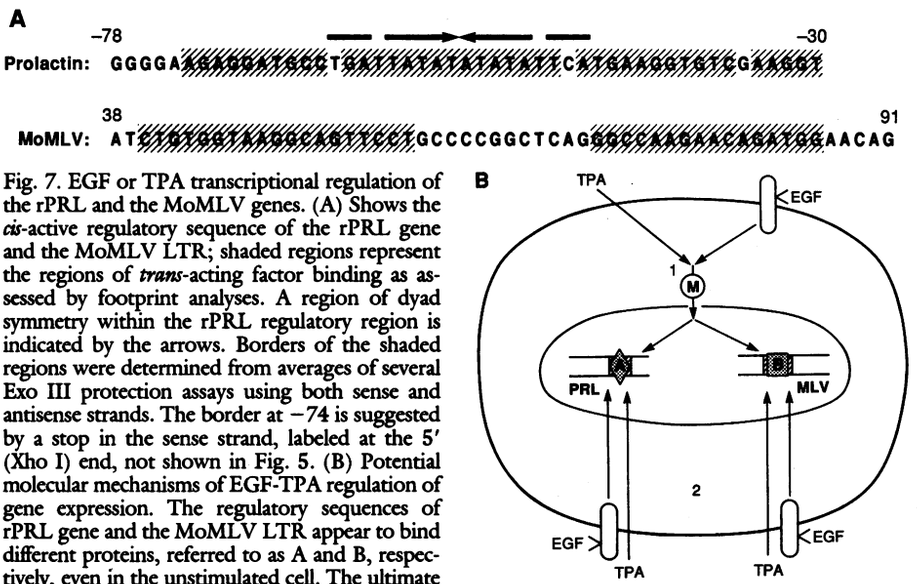

Fig. 7. EGF or TPA transcriptional regulation of the rPRL and the MoMLV genes. (A) Shows the *cis*-active regulatory sequence of the rPRL gene and the MoMLV LTR; shaded regions represent the regions of *trans*-acting factor binding as assessed by footprint analyses. A region of dyad symmetry within the rPRL regulatory region is indicated by the arrows. Borders of the shaded regions were determined from averages of several Exo III protection assays using both sense and antisense strands. The border at −74 is suggested by a stop in the sense strand, labeled at the 5′ (Xho I) end, not shown in Fig. 5. (B) Potential molecular mechanisms of EGF-TPA regulation of gene expression. The regulatory sequences of rPRL gene and the MoMLV LTR appear to bind different proteins, referred to as A and B, respectively, even in the unstimulated cell. The ultimate allosteric activation of these discrete proteins (1) by a single mediator (M) or (2) as a consequence of the generation of distinct second messengers at the level of the plasma membrane are discussed in the text.

unclear, as the rPRL sequence is homologous to only the 5′ half of the dyad necessary for full activity of the c-*fos*^{H} regulatory region (*10*).

Because polypeptide regulators such as EGF interact with plasma membrane receptors that are proximal with respect to their nuclear actions, more than one strategy for control of gene transcription might be utilized. Thus, rapid activation of specific protein kinases by EGF or phorbol esters may lead to changes in intracellular *p*H and ion fluxes, internalization of receptors, and production of metabolites, all of which represent potential mechanisms for regulation of gene transcription (*1–4*). The existence of classes of regulated genes would be consistent with the ability of certain agents, including calcium ionophores and potassium ions, to mimic the effect of EGF on the c-*fos*^{H} and c-*myc* genes (*7*), but not on the rPRL gene (*13*). In view of the similarity between the rPRL and c-*fos*^{H} regulatory regions, such differences may indicate a complex level of control of gene expression that may depend on cell type, for example.

Our data suggest the possibility that at least some of the genes that are induced by a single agent such as EGF, TPA, or another growth factor (*8*) require different DNA-binding proteins for transcriptional activation. This is schematically represented in Fig. 7B, which postulates at least two classes of TPA and EGF regulatory regions, represented by the rPRL and MoMLV sequences, each binding a discrete protein or set of proteins. The range of transcriptional responses could reflect activation of a single pathway (Fig. 7B, lane 1), in which one mediator (M) activates transcription by interacting with different classes of DNA-binding proteins. EGF and TPA could activate this mediator by mechanisms dependent on or independent of protein kinase C. Regulation of the *trans*-acting factors could occur by the modification of these factors or by the activation of additional factors that interact with them. Alternatively, because several second messenger systems are rapidly activated by the specific binding of EGF at the plasma membrane, each could independently regulate different types of *cis*-active regulatory sequences. In this case, EGF and TPA would both utilize one biochemical pathway for transcriptional regulation of the rPRL, and a second, distinct pathway in the case of the MoMLV gene (Fig. 7B, lane 2).

The location of polypeptide hormone receptors in the plasma membrane appears, therefore, to permit a diversity of mechanisms for regulating the ultimate nuclear effects on transcription.

REFERENCES AND NOTES

1. E. Rozengurt, *Curr. Top. Cell. Regul.* **17**, 59 (1980); C.-H. Heldin and B. Westermark, *Cell* **37**, 9 (1984); R. James and R. Bradshaw, *Annu. Rev. Biochem.* **53**, 259 (1984); H. Herschman, in *Control of Animal Cell Proliferation*, A. L. Boynton and H. L. Leffert, Eds. (Academic Press, New York, 1985), vol. 1, pp. 169–199.
2. S. Cohen, *J. Biol. Chem.* **237**, 1555 (1962); C. R. Savage, Jr., T. Inigami, S. Cohen, *ibid.* **247**, 7612 (1972); G. Carpenter and S. Cohen, *Annu. Rev. Biochem.* **48**, 193 (1979).
3. G. Carpenter, L. King, Jr., S. Cohen, *Nature (London)* **276**, 409 (1979); H. Ushiro and S. Cohen, *J. Biol. Chem.* **255**, 8363 (1980); T. Hunter and J. A. Cooper, *Cell* **24**, 741 (1981); J. Downward *et al.*, *Nature (London)* **307**, 521 (1984); Y. Xu *et al.*, *ibid.* **309**, 806 (1984); A. Ullrich *et al.*, *ibid.*, p. 418; C. R. Lin *et al.*, *Science* **224**, 843 (1984).
4. E. Rozengurt and L. A. Heppel, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4492 (1975).
5. A. Schonbrunn, M. Krasnoff, J. M. Wetendorf, A. H. Tashjian, Jr., *J. Cell Biol.* **85**, 786 (1977); L. J. Johnson, J. D. Baxter, I. Vlodavsky, D. Gospodarowicz, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 394 (1980); R. Benveniste *et al.*, *J. Clin. Endocrinol. Metab.* **46**, 169 (1978); L. B. Chen, R. C. Gudor, T.-T. Sun, A. B. Chen, M. W. Mosesson, *Science* **197**, 776 (1977).
6. G. H. Murdoch, E. Potter, A. K. Nicolaisen, R. M. Evans, M. G. Rosenfeld, *Nature (London)* **300**, 192 (1982).
7. R. Muller, R. Bravo, J. Burckhardt, T. Curran, *ibid.* **312**, 716 (1984); M. C. Greenberg and E. B. Ziff, *ibid.* **311**, 433 (1984); W. Kruijer, J. A. Cooper, T. Hunter, I. M. Verma, *ibid.* **312**, 711 (1984); J. I. Morgan and T. Curran, *ibid.* **322**, 552 (1986).
8. K. Kelly, B. H. Cochran, C. D. Stiles, P. Leder, *Cell* **35**, 603 (1983); B. H. Cochran, J. Zullo, I. M. Verma, C. D. Stiles, *Science* **226**, 1080 (1984); B. H. Cochran, A. C. Reffel, C. D. Stiles, *Cell* **33**, 939 (1983).
9. W. A. Guyette, R. J. Matusik, J. M. Rosen, *Cell* **17**, 1013 (1979); D. Granner, T. Andreone, K. Sasaki, E. Beale, *Nature (London)* **305**, 549 (1983); W. H. Lamers, R. W. Hanson, H. M. Meisner, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5137 (1982).
10. R. Treisman, *Cell* **42**, 889 (1985); *ibid.* **46**, 567 (1986).
11. E. J. Gubbins, R. A. Maurer, M. Lagrimini, C. R. Erwin, J. E. Donelson, *J. Biol. Chem.* **255**, 8655 (1980); N. E. Cooke and J. D. Baxter, *Nature (London)* **297**, 603 (1982).
12. A. H. Tashjian, Jr., *Methods Enzymol.* **58**, 527 (1979); F. C. Bancroft, in *Functionally Differentiated Cell Lines*, G. Sato, Ed. (Liss, New York, 1982), pp. 47–59; G. A. Evans, D. N. David, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1294 (1978); P. S. Dannies, A. H. Tashjian, Jr., *Biochem. Biophys. Res. Commun.* **70**, 1180 (1976); E. Potter, A. K. Nicolaisen, E. S. Ong, R. M. Evans, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6662 (1981); B. A. White and F. C. Bancroft, *J. Biol. Chem.* **258**, 4618 (1983); R. A. Maurer, *Nature (London)* **294**, 94 (1981); *J. Biol. Chem.* **257**, 2133 (1982).
13. G. H. Murdoch, R. Franco, R. M. Evans, M. G. Rosenfeld, *J. Biol. Chem.* **258**, 15329 (1983); G. H. Murdoch, R. M. Evans, M. G. Rosenfeld, in *Biochemical Actions of Hormones*, G. Litwack, Ed. (Academic Press, New York, 1985), vol. 12, pp. 37–68.
14. G. H. Murdoch, M. G. Rosenfeld, R. M. Evans, *Science* **218**, 1315 (1982); M. L. Waterman, G. H. Murdoch, R. M. Evans, M. G. Rosenfeld, *ibid.* **229**, 267 (1985).
15. Y. Nishizuka, *Trends Biochem. Sci.* **8**, 13 (1983).
16. R. Osborne, and A. H. Tashjian, Jr., *Endocrinology* **108**, 1164 (1981).
17. G. H. Murdoch, M. L. Waterman, R. M. Evans, M. G. Rosenfeld, *J. Biol. Chem.* **260**, 11852 (1985).
18. S. Supowit, E. Potter, R. M. Evans, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2975 (1984).
19. V. L. Chandler, B. Maler, K. R. Yamamoto, *Cell* **33**, 489 (1983); M. C. Ostrowski, A. L. Huang, M. Kessel, R. G. Woolford, G. L. Hager, *EMBO J.* **3**, 1891 (1984).
20. M. V. Govindin, G. Spiess, J. Major, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5157 (1982); C. Sheidereit, S. Geisse, H. M. Westphal, M. Beato, *Nature (London)* **304**, 749 (1983); M. Pfahl, *Cell* **31**, 475 (1982); F. Payvar *et al.*, *ibid.* **35**, 381 (1983).
21. S. A. Camper, Y. A. S. Yao, F. M. Rottman, *J. Biol. Chem.* **260**, 12246 (1985).
22. P. Chambon and C. Benoist, *Nature (London)* **290**, 304 (1981); P. Gruss, R. Dhar, G. Khoury, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 943 (1981); J. Banerji, S. Rusconi, W. Schaffner, *Cell* **27**, 299 (1981); M. Fromm and P. Berg, *J. Mol. Appl. Genet.* **1**, 457 (1982); M. Yaniv, *Nature (London)* **297**, 17 (1982); Y. Gluzman and T. Shenk, Eds., *Enhancers and Eukaryotic Gene Expression* (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1983); G. Khoury and P. Gruss, *Cell* **33**, 313 (1983).
23. R. Hen, E. Borrelli, P. Sassone-Corsi, P. Chambon, *Nucleic Acids Res.* **11**, 8735 (1983); H. Weiher, M. König, P. Gruss, *Science* **219**, 626 (1983); A. Nordheim and A. Rich, *Nature (London)* **303**, 674 (1983); H. R. Scholer and P. Gruss, *Cell* **36**, 403 (1984).
24. M. Mercola, J. Goverman, C. Mirell, K. Calame, *Science* **227**, 266 (1985); P. Sasonne-Corsi, A. Wildeman, P. Chambon, *Nature (London)* **313**, 458 (1985); A. Ephrussi, G. M. Church, S. Tonegawa, W. Gilbert, *Science* **227**, 134 (1985).
25. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
26. C. Nelson *et al.*, *Nature (London)* **322**, 557 (1986).
27. B. L. Graves, R. N. Eisenman, S. L. McKnight, *Mol. Cell. Biol.* **6**, 993 (1985).
28. K. R. Yamamoto, *Annu. Rev. Genet.* **19**, 209 (1985).
29. M. Karin *et al.*, *Nature (London)* **308**, 513 (1984); K. E. Mayo, R. Warren, R. D. Palmiter, *Cell* **29**, 99 (1982); G. W. Stuart, P. F. Searle, H. Y. Chen, R. L. Brinster, R. D. Palmiter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7318 (1984).
30. T. M. Shinnick, R. A. Lerner, J. G. Sutcliffe, *Nature (London)* **293**, 543 (1981).
31. Messenger RNA transcription initiation site analysis was performed with total RNA isolated from GH_4 cells 48 hours after transfection, 24 hours after addition of EGF ($10^{-8}M$) or TPA ($10^{-7}M$). The cells were homogenized in guanidinium thiocyanate and RNA was pelleted through cesium chloride gradients. A single-stranded DNA primer (5′-GTTCTTTACG ATGCCATTGG GATATATCAA CGG-3′), complementary to nucleotides 29 to 61 of the CAT coding sequence, was labeled by treating with kinase until the primer had a specific activity of 10^9 cpm/μg. For each reaction 50 μg of total RNA and 3 ng of a labeled primer were heated to 70°C in 12 μl of 50 m*M* tris (*p*H 8.0), 8 m*M* $MgCl_2$, 50 m*M* KCl, 4 m*M* dithiothreitol, and slowly cooled to 55°C. The annealed primer and RNA were precipitated with one-tenth volume of 3*M* sodium acetate and one volume of isopropanol, then resuspended in the same buffer containing deoxynucleotides at a final concentration of 1 m*M* each in a final volume of 20 μl. Two units of reverse transcriptase were added and the reaction was allowed to proceed for 30 minutes at 42°C. After phenol-chloroform extraction, the extension products were analyzed on 8% denaturing polyacrylamide gels (*26*).
32. The exonuclease III protection assay was performed essentially as described [C. Wu, *Nature (London)* **317**, 84 (1985)]. GH_4 cells were treated with EGF ($10^{-8}M$) and/or TPA ($10^{-7}M$) for 10 to 60 minutes prior to the preparation of nuclear extracts. Double-stranded probes, labeled with T4 kinase at one 5′ end to approximately 10^5 cpm/ng, were prepared by standard procedures. Binding reactions were performed as described by Wu with the exception that 400 ng of supercoiled pBR322 and 3 μg of bovine serum albumin were added to each 50 μl reaction. Preincubation with various amounts of competitor DNA for 15 minutes at 25°C was followed by addition of 0.1 ng (about 10^5 cpm) of probe and further incubation for 15 minutes at 25°C. 150 units of exonuclease III (Boehringer) were added and, after a 1-hour digestion at 30°C, the reaction was stopped by adding 50 μl of 1% SDS and 20 m*M* EDTA. After organic extraction and ethanol precipitation, the products were separated on a 0.5 mm 8*M* urea polyacrylamide (6%) gel at 1000 V in 45 m*M* tris, 45 m*M* borate, and 2 m*M* EDTA (0.5× TBE). A double-stranded synthetic oligonucleotide, 5′-ATCGGATCCG ATCTGGGGGC GCCTCCTTGG CTGACGTCAG AGAGAGATCT GAT-3′, that was based upon a 5′-flanking sequence in the rat somatostatin gene [M. A. Tavianini, T. E. Hayes, M. D. Magazin, C. D. Minth, J. E. Dixon, *J. Biol. Chem.* **259**, 11798 (1984); M. R. Montminy, K. A. Sevarino, R. H. Goodman, *Endocr. Soc. Abstr.* **68**, 50 (1986)] and which confers a 20- to 30-fold induction by forskolin in GH cells when placed 5′ of the *tk*-CAT fusion gene, was used as competitor DNA.
33. M. G. Fried and D. M. Crothers, *Nucleic Acids Res.* **11**, 141 (1982).
34. The gel retardation assay was performed with a double-stranded oligonucleotide containing 50 bp of rPRL sequence (−79 to −30) Klenow-labeled with ^{32}P [$\alpha^{32}P$]dCTP. Binding reactions were carried out according to Carthew *et al.* [R. W. Carthew, L. A. Chodosh, P. A. Sharp, *Cell* **43**, 439 (1986)]; each 20 μl reaction contained 0.5 ng of probe (10^5 cpm), 5 to 10 μg of GH_4 nuclear extract prepared as described in Fig. 5, and 5 μg of poly(dI-C) (Pharmacia) in 12 m*M* Hepes-NaOH, *p*H 7.9, 12% glycerol, 0.6 m*M* EDTA, 60 m*M* KCl, 5 m*M* $MgCl_2$, and 0.6 m*M* dithiothreitol. After a 30-minute incubation at 30°C, samples were loaded immediately on a 5% polyacrylamide gel (acrylamide:bis-acrylamide, 80:1) that had been previously electrophoresed for 1 hour at 400 V in 0.5× TBE buffer. Gels were run for 3 hours at 400 V, placed onto blotting paper (Schleicher and Schuell), and analyzed by autoradiography.
35. S. Goodbourn, K. Zinn, T. Maniatis, *Cell* **41**, 509 (1985); T. Fujita, S. Ohno, H. Yasumitsu, T. Taniguchi, *ibid.*, p. 489; J. Ryals, P. Dierks, H. Ragg, C. Weissman, *ibid.*, p. 497.
36. The rPRL-CAT fusion genes were constructed by converting an Ava II site 33 nucleotides 3′ of the CAP site to a Hind III site, and the indicated 5′ sites (Dra I at −1.8 kb; Hind III at −420; Hae III at −172; Sfa NI at −78; Taq I at −35) were ligated to Bam HI linkers following T4 polymerase–catalyzed fill reactions, and placed into the Bam HI and Hind III sites of pSV2-CAT. The fusion gene containing the *tk* (−200 to +70) promoter was generated by placing this region as a Bam HI to Hind III fragment into the corresponding sites of pSV2-CAT with conventional techniques; in constructions containing the rGH promoter (−320 to +8), the Hind III site was converted to an Xho I site. As controls, five Sau 3A fragments of pBR322 (50 to 320 bp) were inserted 5′ of the rGH promoter. The −172 to −10 rPRL fragment, excised as a Hae III to Pst I fragment, was ligated to linkers and inserted as a Bam HI fragment. An oligonucleotide corresponding to sequences −79 to −30 of the rPRL 5′-flanking region was inserted as a Bam HI to Bgl II fragment into the *tk*-CAT fusion. Where indicated, fragments were inserted 3′ of the CAT gene at an Apa I site converted into a Bgl II site. Plasmids were purified by two cesium chloride gradient purifications and transfections were performed with minor modifications of the DEAE-dextran sulfate procedure (*26*), utilizing 10 μg of plasmid DNA per plate. GH_4 cell cultures were placed into fresh medium 4 hours prior to transfection. The protein concentration and time of CAT assays were varied dependent upon transfectional efficiency, but were always in the linear range. Optimal transfections permitted analysis of 50 μg of protein per 1- to 2-hour reaction with 0.1 μCi of [^{14}C]chloramphenicol. Conversion of chloramphenicol to acetylated forms ranged from 5 to 30% of the substrate.
37. MoMLV fragments prepared from the LTR corresponded to a Pvu II fragment (15 to 90, numbered from the start of the first repeat), an Eco RV to Pvu II fragment (37 to 90), a Hpa II fragment (62 to 136), and a Hae III fragment (27 to 72) were ligated to Bam HI linkers, and were inserted into the Bam HI site of the rGH-CAT and *tk*-CAT vectors described in Fig. 2.

38. We thank C. A. Nelson for contributing to these experiments and M. Richards for the preparation of the manuscript. H.E. is supported by a postdoctoral fellowship from the Medical Research Council of Canada. Supported by grants from the National Institutes of Health. Some of the computer resources used in this study were provided by the NIH-sponsored BIONET™ National Computer Resource for Molecular Biology (1 U41 RR-61685-03).

21 July 1986; accepted 29 October 1986