Thyroid Hormone Induction of an Autocrine Growth Factor Secreted by Pituitary Tumor Cells

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Thyroid hormones stimulate the rate of cell division by poorly understood mechanisms. The possibility that thyroid hormones increase cell growth by stimulating secretion of a growth factor was investigated. Thyroid hormones are nearly an absolute requirement for the division of GH_4C_1 rat pituitary tumor cells plated at low density. Conditioned media from cells grown with or without L-triiodothyronine (T3) were treated with an ion exchange resin to remove T3 and were tested for ability to stimulate the division of GH_4C_1 cells. Conditioned medium from T3-treated cells was as active as thyroid hormone at promoting GH_4C_1 cell growth but did not elicit other thyroid hormone responses, induction of growth hormone, and down-regulation of thyrotropin-releasing hormone receptors, as effectively as T3 did. A substance or substances associated with T3-induced growth stimulatory activity migrated at high molecular weight at neutral *p*H and was different from known growth-promoting hormones induced by T3. The results demonstrate that thyroid hormones stimulate the division of GH_4C_1 pituitary cells by stimulating the secretion of an autocrine growth factor.

THYROID HORMONES ARE ESSENTIAL for normal growth and development and exert profound effects on cellular metabolism in almost all organs (*1*). Thyroid hormones may accelerate growth by acting directly on cells to increase their rate of division, by acting permissively for other hormones, or by inducing the synthesis of growth-promoting hormones such as growth hormone, epidermal growth factor (EGF), and nerve growth factor (NGF) (*1–6*). A number of rat pituitary tumor cell lines, including GH_4C_1, respond to physiological concentrations of L-triiodothyronine (T3) and L-thyroxine (T4) with an increase in the rate of cell proliferation and induction of growth hormone (*2–4*). T3 and T4 are reported to act early in the G_1 phase of the cell cycle to increase the rate of GH cell division (*3*). The growth stimulatory effects of thyroid hormones appear to be mediated by the well-characterized specific, high-affinity nuclear thyroid hormone receptors on GH cells since the potency of various iodothyronines to stimulate growth correlates well with the measured affinity for nuclear receptors. However, the mechanism of thyroid hormone stimulation of cell division in the pituitary and other cells remains obscure.

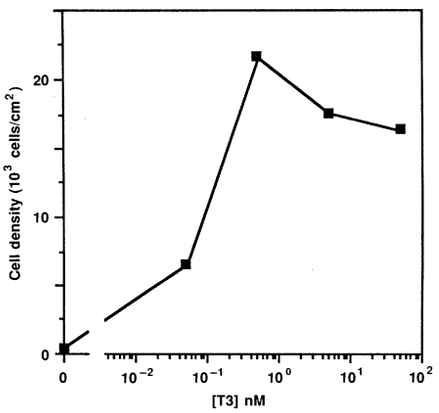

Fig. 1. T3 stimulation of cell division. GH_4C_1 cells were plated in 150-cm^2 flasks in 25 ml of Tx medium supplemented with 0 to 50 n*M* T3. The medium was changed and collected every 3 to 4 days. Cell density obtained during the logarithmic phase of growth is shown. Plating efficiency, approximately 50%, was not affected by T3.

We report here that T3 stimulates division of GH_4C_1 pituitary cells by causing the cells to secrete an autocrine growth factor. We have found that thyroid hormone is almost an absolute requirement for division if GH_4C_1 cells are plated at very low density (<20,000 cells per 35-mm culture dish). GH_4C_1 cells did not divide (less than one doubling per 21 days) when they were plated in Ham's F10 culture medium supplemented with 5% fetal calf serum depleted of thyroid hormones by ion exchange resin (*7*) referred to as Tx medium. T3 caused a dramatic increase in cell growth (doubling time with T3, 60 hours) with a half-maximal effect at 0.1 n*M* (Fig. 1). Since 85% of T3 is bound to serum proteins under the conditions of our experiments, the free T3 concentration at the median effective dose (ED_{50}) was approximately 15 p*M*, within the normal physiological range for a rat (*8*).

We hypothesized that T3 causes GH_4C_1 cells to divide by stimulating the secretion of a growth-promoting factor. To test for the presence of an autocrine growth factor in conditioned media, we collected media from GH_4C_1 cells grown with and without T3 and removed the T3 with a strong anion exchange resin, Dowex AG 1 (*7*). To confirm the adequacy of T3 removal, we added [^{125}I]T3 to the conditioned media, allowed it to equilibrate with serum binding proteins before adding resin, and determined that more than 99% of the radioactivity was extracted.

The samples of resin-treated conditioned medium were then tested for ability to replace thyroid hormone and stimulate the division of low density GH_4C_1 cultures in medium lacking any added thyroid hormone. Control wells received Tx medium only or an optimal concentration of T3, and test wells received resin-treated conditioned medium diluted tenfold in Tx medium. Conditioned medium from cultures grown with 0.5 to 50 n*M* T3 fully replaced thyroid hormone in stimulating cell division (Fig. 2). The mean population doubling time decreased from more than 14 days in Tx medium to 48 hours with resin-treated conditioned medium from T3-treated cells. These results suggested that T3 stimulates GH_4C_1 cell division by causing the cells to secrete a substance with growth-promoting activity that is not removed by a strong anion exchanger. Media from cultures treated with 0.5 n*M* T3 would not have had adequate T3 for maximal growth response after tenfold dilution even without resin treatment.

To confirm that the activity we measured in the samples of conditioned medium could

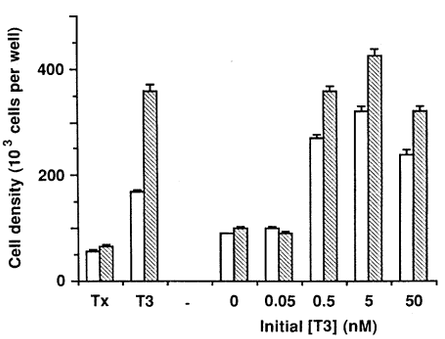

Fig. 2. Evidence that thyroid hormones induce GH_4C_1 cells to secrete an autocrine growth factor. Media samples collected from a 3-day period during the growth experiment shown in Fig. 1 were treated with Dowex Ag1-X8 (2.5 g per 25 ml of medium for 30 minutes at 0°C). GH_4C_1 cells were plated at a density of 20,000 cells per 35-mm well in six-well dishes containing Tx medium supplemented with no further additions (Tx), with 5 n*M* T3 (T3), or with 10% resin-treated conditioned medium (right-hand bars). Open bars represent the mean ± range of duplicate dishes during logarithmic phase (10 days). The same media samples were then subjected to a second, more exhaustive resin extraction (7.5 g of resin per 20 ml of medium for 24 hours at room temperature). A second growth promotion assay was performed in the same way as the one described above (striped bars).

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not have been due to unextracted T3, we subjected the media to a second treatment with ion exchange resin and tested for residual T3 by both radioreceptor assay (Fig. 3, E and F) and radioimmunoassay. For the radioreceptor assay, GH_4C_1 cells were incubated to equilibrium with [^{125}I]T3 in resin-treated conditioned medium before [^{125}I]T3 binding to specific nuclear receptors was measured. For radioimmunoassay, specific antiserum to T3 was incubated with [^{125}I]T3 in resin-treated conditioned media, and antibody-bound [^{125}I]T3 was separated from free hormone by precipitation with a second antibody. Standard curves for these competition displacement assays were run in resin-treated conditioned medium from cultures without T3 to eliminate the effects of serum-binding proteins. Radioreceptor assay and radioimmunoassay confirmed that at least 95% of the T3 had been removed.

The conditioned media that had been extracted twice were then tested for ability to stimulate GH_4C_1 cell division in a second growth assay. The results (striped bars in Fig. 2) were similar to those obtained with media subjected to one resin treatment, and the dose-response relationships were identical. These data confirm that the growth-promoting activity in samples of conditioned medium from T3-treated cells is not due to residual T3 but is due to some secreted factor or factors that stimulate GH_4C_1 cell division. In a recent abstract, Miller *et al.* (*9*) described experiments in which T3 in conditioned medium was complexed with specific antiserum to T3 and the conditioned medium was then shown to stimulate pituitary cell division. They also concluded that the conditioned medium contained an autocrine growth-promoting activity.

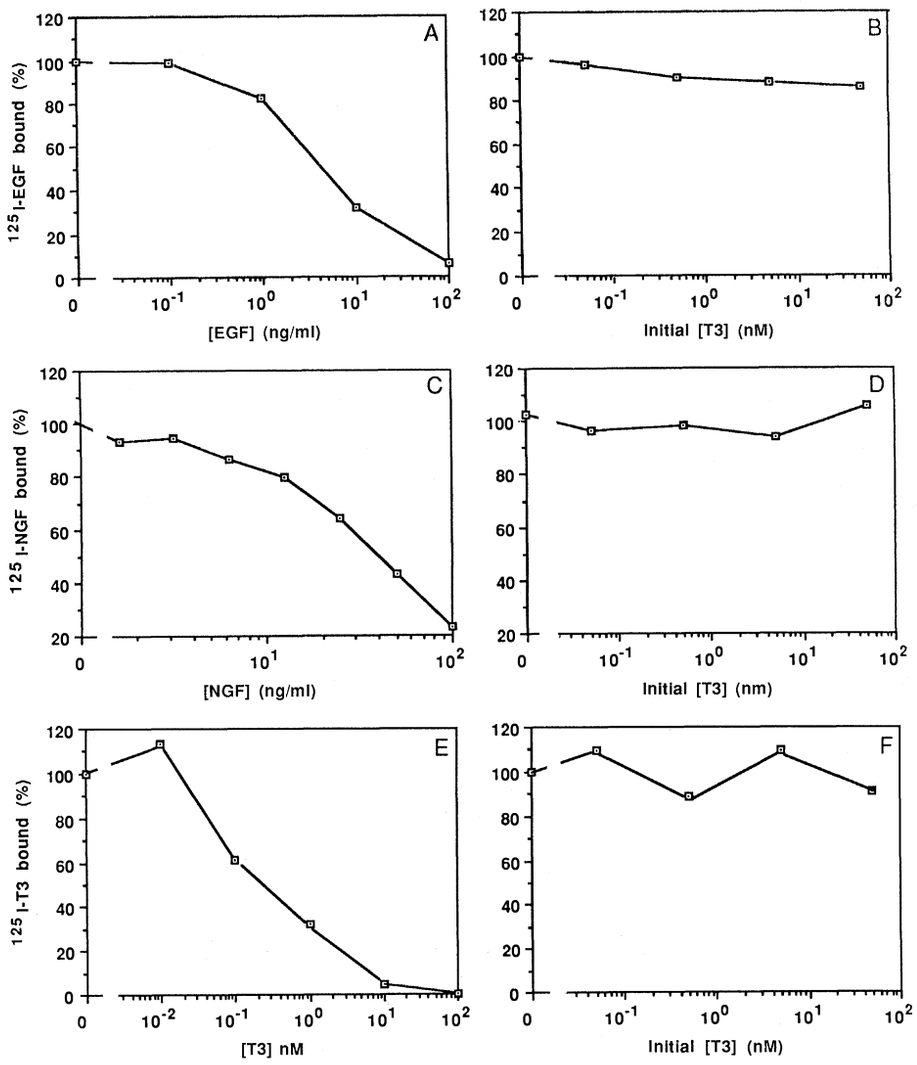

Fig. 3. Evidence that conditioned media do not contain EGF, NGF, or T3. (A and B) Radioreceptor assay of EGF. GH_4C_1 cells were incubated for 1 hour at 37°C in serum-free F10 medium containing ^{125}I-labeled EGF (200,000 cpm/ml) and (A) unlabeled EGF or (B) 10% resin-treated conditioned medium. Binding of ^{125}I-labeled EGF to cells was measured as described earlier (*19*). (C and D) Radioimmunoassay of NGF. ^{125}I-labeled NGF-β (25,000 cpm; 150 mCi/μg) was incubated for 24 hours at 4°C with a specific guinea pig antibody to mouse NGF-β (1/5000 final dilution) and (C) unlabeled NGF-β or (D) 10% resin-treated conditioned medium. Antibody-bound ^{125}I-labeled NGF-β was separated from free NGF-β by second antibody precipitation with the use of minor modifications of published procedures (*20*). (E and F) Radioreceptor assay of T3. GH_4C_1 cells were incubated in serum-free F10 medium containing [^{125}I]T3 (50 p*M*) and either (E) unlabeled T3 diluted in 10% resin-treated conditioned medium from Tx cultures or (F) 10% resin-treated conditioned medium. Specific nuclear-bound [^{125}I]T3 was measured after 1.5 hours as described earlier (*10*). All panels show the mean of duplicates which differed by less than 10%.

The samples of resin-treated medium that stimulated cell division were also tested for their ability to exert other thyroid hormone activities. One of the best characterized thyroid hormone responses in GH_4C_1 cells is the induction of growth hormone (*4*). We incubated GH_4C_1 cells with Tx medium, an optimal concentration of T3, or samples of resin-treated conditioned media. Conditioned media samples from T3-treated cells did stimulate a rise in growth hormone production (1.6- to 2.4-fold), but the response was much less than the sevenfold increase caused by T3 in the same experiment (Table 1). A second well-characterized thyroid hormone response in GH_4C_1 cells is the down-regulation of surface receptors for thyrotropin-releasing hormone (TRH) (*10*). Conditioned medium from T3-treated cells decreased TRH binding (7% to 54%) but less extensively than thyroid hormone did (77%). Thus the growth-promoting activity in medium from T3-treated cells can be dissociated from other thyroid hormone activities.

Resin-tested conditioned media were tested at different dilutions from 5- to 50-fold for ability to stimulate GH_4C_1 cell division. Using the samples tested in Fig. 2 we obtained a half-maximal growth-stimulating effect with 2% resin-treated conditioned medium from cells originally incubated with 5 or 50 n*M* T3. The total protein contributed by the conditioned medium at this dilution was 35 μg/ml. Such quantitation is affected by the batch of serum used for both the initial growth with T3 and for the test of growth-promoting activity in resin-treated medium, and the relative growth-stimulating activities of conditioned media and an optimal concentration of T3 may vary.

To obtain an estimate of the size of the growth-promoting activity, we filtered samples of conditioned medium through Sephadex G-75 at *p*H 7.6 and 0.15*M* NaCl and tested the column fractions for growth stimulatory activity. Resin-treated conditioned medium from control cultures did not con-

tain stimulatory or inhibitory activity, whereas resin-treated conditioned medium from T3-treated cells contained material with growth-promoting activity that migrated at high apparent molecular weight (>50,000) (Fig. 4A). This suggests that the active substance is either of high molecular weight or that it is aggregated or bound to a high molecular weight protein at neutral *p*H and physiological salt. The substance (or substances) with T3-induced growth-promoting activity migrated slightly faster than [^{125}I]T3 bound to serum proteins and was well separated from either ^{125}I-labeled rat growth hormone or free [^{125}I]T3 (Fig. 4B). We also showed that the fractions containing growth-promoting activity did not displace [^{125}I]T3 from nuclear receptors on GH_4C_1 cells (Fig. 4C), confirming that the activity is not due to unextracted T3. The T3-induced factor is quite stable since we have noted no loss of activity in samples stored for more than 6 months.

Since thyroid hormones are known to induce the synthesis of three growth-stimulating hormones, EGF, NGF, and growth hormone (*4–6*), we examined the possibility that one of these could be associated with the activity secreted from GH_4C_1 cells in response to T3. T3 does cause GH_4C_1 cells to secrete growth hormone. However, the division of low density GH_4C_1 cell cultures in Tx medium was not stimulated over a 3-week period by rat growth hormone at 0.1 to 10 μg/ml and the T3-induced activity and growth hormone were separable by gel filtration (Fig. 4A).

Epidermal growth factor binds to specific receptors on GH_4C_1 cells and is a potent stimulator of prolactin synthesis. In agreement with published reports (*11, 12*) we found that EGF at 10 to 600 ng/ml did not increase cell division in a 3-week period but rather decreased cell number by 50% to 80%. Using radioreceptor assays, we also demonstrated that neither EGF nor α-transforming growth factor, which binds to the EGF receptor with high affinity (*13*), is secreted by these cells. Conditioned media did not contain any substance capable of displacing ^{125}I-labeled EGF from receptors (Fig. 3B), whereas unlabeled EGF displaced the label potently [median inhibitory concentration (IC_{50}), 5 ng/ml] and completely (Fig. 3A).

Similarly, we found that NGF (100 ng/ml) did not increase the growth of GH_4C_1 cells in Tx medium over 3 weeks and demonstrated by radioimmunoassay that GH_4C_1 cells do not secrete NGF. Conditioned media did not contain any activity that reacted with antibody to NGF-β (Fig. 3D) whereas unlabeled NGF displaced antibody-bound ^{125}I-labeled NGF with an IC_{50} of 30 ng/ml (Fig. 3C). Therefore the growth-promoting factor (or factors) secreted by GH_4C_1 cells in response to T3 is not one of the known growth-promoting hormones increased by thyroid hormones, namely growth hormone, EGF, or NGF.

There is evidence that GH cells can produce growth factors different from growth hormone and prolactin. Some mammary tumors grow faster in nude mice if GH cells are coimplanted in the same animals and estrogen levels are adequate; prolactin does not appear to be responsible (*14, 15*). Conditioned medium from GH cell cultures also increases the rate of DNA synthesis of mammary and GH cells in culture (that is, the medium has autocrine growth-promoting activity), and this activity requires estrogen (*15*). This activity has been attributed to a peptide of 2000 to 6000 daltons (*16*) which, like the T3-induced factor, migrates at high molecular weight on Sephadex at neutral *p*H, and may be identical to peptides purified from normal pituitary and uterus (*17, 18*). However, the previous isolated peptide is inactive in serum-containing medium (*16–18*) whereas we assay the T3-induced activity in medium containing 5% serum, and it appears to be present at much lower levels (*16*). Despite these apparent differences, further work is required to determine whether the factor secreted by GH_4C_1 cells in response to thyroid hormones is identical to previously described growth factors.

Our results demonstrate that a T3-induced secretory product is entirely responsible for the stimulation of GH_4C_1 cell division caused by thyroid hormones. The T3-induced factor may act directly as a growth stimulator, act permissively to allow expres-

Table 1. Activities of T3-induced growth factor. GH_4C_1 cells were incubated for 4 days with no further additions (Tx), 5 n*M* T3, or 10% resin-treated conditioned medium (twice-extracted samples tested in the experiment depicted in Fig. 2). TRH receptor concentration, measured by published procedures (*10*) with 5 n*M* [^{3}H]methyl-TRH, and cell protein were determined at the end of the experiment. Growth hormone concentrations in the media from the last 2 days of the experiment were measured by radioimmunoassay. Results are mean ± SEM of three to five determinations.

Media tested	Protein (μg)	Growth hormone (ng/μg)	[^{3}H]methyl-TRH bound (fmol/μg)
Control	43	2.9 ± 0.6	197 ± 5
T3	67	20.0 ± 4.9	46 ± 2
Resin-treated medium:			
Initial [T3] 0.05 n*M*	74	2.9 ± 0.7	183 ± 12
0.5 n*M*	102	4.6 ± 1.6	98 ± 7
5 n*M*	102	7.1 ± 1.0	91 ± 6

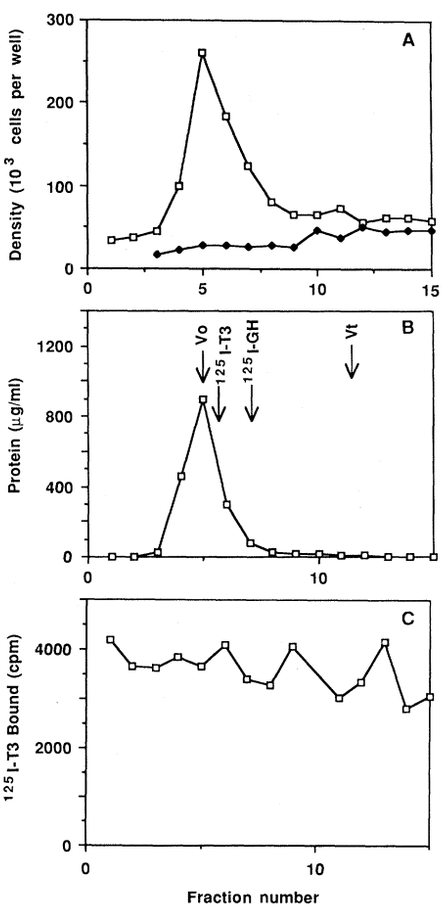

Fig. 4. Gel filtration of T3-induced growth factor. Samples of resin-treated conditioned medium from cells maintained in Tx medium (◆) and cells maintained with 5 n*M* T3 (□) were filtered through Sephadex G-75 column at *p*H 7.6 in a mixture of 0.01*M* sodium phosphate and 0.15*M* NaCl. (A) Fractions were diluted tenfold in Tx medium and tested for their ability to stimulate division of GH_4C_1 cells as described in Fig. 2. The cell number after 7 days is shown. (B) Protein concentrations of the column fractions before dilution in the growth promotion assay (□). Arrows show the elution of blue dextran (V_0, void volume), ^{125}I-labeled rat growth hormone (^{125}I-GH), and Na ^{125}I (V_t, included volume). ^{125}I-labeled T3 was equilibrated with resin-treated conditioned medium and run on the same column. The elution of bound [^{125}I]T3 is shown by the arrow (^{125}I-T3); free [^{125}I]T3 eluted beyond the included volume. (C) The fractions containing growth factor activity [□ in (A)] were diluted tenfold in Tx medium containing 0.25 n*M* [^{125}I]T3. Fresh cultures of GH_4C_1 cells in Tx medium were incubated for 2.5 hours at 37°C before determination of specific nuclear-bound [^{125}I]T3.

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.

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

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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

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