trans-Acting Factors Interact with a Cyclic AMP Response Element To Modulate Expression of the Human Gonadotropin α Gene

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The α subunit of the placental hormone chorionic gonadotropin is regulated by cyclic AMP (cAMP) at the transcriptional level. A cAMP-responsive fusion gene (α -CAT) containing 1.5 kilobases of the α gene 5'-flanking sequence linked to the chloramphenicol acetyltransferase (CAT) gene was used as a transcriptional reporter in competition assays in transfected JEG-3 choriocarcinoma cells. Expression of the α -CAT fusion gene increased linearly with increasing amounts of transfected plasmid and was maximal at the same amount of α -CAT DNA (2 μ g) with or without cAMP treatment. Various amounts of different competitor DNA sequences were cotransfected with the α -CAT reporter plasmid to examine the interactions of intracellular *trans*-acting factors with the regulatory elements of the α gene promoter. An 800-base-pair fragment of α gene 5'-flanking sequence inhibited both basal and cAMP-stimulated transcription of the α -CAT reporter plasmid in a dose-dependent manner, indicative of interactions with one or more *trans*-acting factors that activate α gene expression. The α gene sequences that interact with intracellular regulatory factors were defined by using several discrete regions of the 5'-flanking sequence as competitors for α -CAT expression. A proximal promoter sequence (-99 to +44) containing the CCAAT box, TATA box, and transcriptional initiation site was a relatively ineffective competitor of α -CAT transcription. In contrast, an upstream sequence between -236 and -100 was an effective competitor for transcriptional activators of α -CAT expression. Competition for α -CAT expression by this regulatory sequence did not require *cis* interactions with downstream promoter elements and was equally effective with or without cAMP treatment. An 18-base-pair repeated sequence within this region of the α gene (-146 to -111) greatly enhanced both basal gene expression and cAMP responsivity and also competed for limiting cellular transcription factors. These findings suggest that JEG-3 cells contain *trans*-acting factors that interact with a cAMP response element to activate α gene transcription. The chorionic gonadotropin β gene 5'-flanking sequence also competed for α -CAT expression, suggesting that a common *trans*-acting factor is shared by the regulatory sequences of the α and β genes.

The glycoprotein hormones comprise a structurally related family of proteins produced in the pituitary gland (thyroid-stimulating hormone, follicle-stimulating hormone, and luteinizing hormone [LH]) and in the placenta (human chorionic gonadotropin [hCG]). The hormones are heterodimers, each containing a common α subunit and different β subunits, which confer distinct biological activities to the hormones (39). The α and β subunits are encoded by separate genes (7, 14). In humans, there is a single copy of the α subunit gene (13) which is expressed in conjunction with different β subunit genes in several different cell types including thyrotropes and gonadotropes of the pituitary gland, syncytiotrophoblasts of the placenta, and in neoplasms from a number of different tissues (5, 27). In addition, expression of the α subunit gene is regulated by a wide variety of hormones including hypothalamic releasing factors (37, 47), thyroxine (46), gonadal steroids (16), and the second messenger cyclic AMP (cAMP) (6, 25; P. J. Deutsch, J. L. Jameson, and J. F. Habener, *J. Biol. Chem.*, in press).

The regulated expression of eucaryotic genes requires interactions of both general and specific cellular transcription factors with *cis*-acting DNA regulatory elements (11). Deletion-mapping analyses of the promoter regions of a variety of viral and mammalian genes have demonstrated

multiple functional domains, which in some instances interact in *cis* to provide specific gene expression (12, 15, 20, 34). In addition, multiple protein components are involved in the initiation of transcription by RNA polymerase II, some of which bind to specific DNA regulatory sequences to confer regulated gene expression (41). It is likely that the regulated expression of the α gene in different cell types and by a variety of hormones involves specific interactions of *trans*-acting factors with different DNA regulatory elements.

Choriocarcinoma cells produce hCG and provide a useful model system for studying the regulation of hCG α and β gene expression. Production of hCG by the JEG-3 choriocarcinoma cell line is stimulated by cAMP (23). The stimulation of hCG biosynthesis by cAMP occurs at the pretranslational level resulting in a 15- to 30-fold increase in both hCG α and β subunit mRNAs (6, 25). The increase in hCG α and β mRNAs is due, in part, to an effect of cAMP on the rates of α and β gene transcription (25). Expression of a transfected fusion gene (α -CAT), consisting of the 5'-flanking region of the α subunit gene linked to the coding sequence of the enzyme chloramphenicol acetyltransferase (CAT), is stimulated by cAMP (25), a finding that is consistent with transcriptional regulation of the endogenous α gene by cAMP. Deletion mutagenesis studies have delineated a region of the α subunit gene that is necessary for efficient gene expression and for cAMP responsivity (10; Deutsch et al., in press). We are interested in examining the potential

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interactions of these *cis*-acting DNA elements with specific *trans*-acting factors to regulate α gene transcription.

In this report, the interactions of the 5'-flanking sequences of the α subunit gene with intracellular factors were studied by an *in vivo* competition assay. Increasing amounts of competitor α gene 5'-flanking sequence decreased expression of a cotransfected reporter plasmid (α -CAT). These findings are consistent with the binding of limiting amounts of one or more *trans*-acting factors that activate α subunit gene expression. The region of DNA that competed most effectively for intracellular regulatory factors contains the putative cAMP response element of the α subunit gene.

MATERIALS AND METHODS

Cell culture and DNA transfection. JEG-3 cells, a human choriocarcinoma cell line that produces hCG, were obtained from the American Type Culture Collection (ATCC HTB 36; Rockville, Md.). Cells were plated onto 60-mm dishes 24 h before transfection and grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Reporter and competitor plasmid DNAs were cotransfected into JEG-3 cells by the calcium phosphate precipitation technique (19, 51). The total amount of transfected plasmid DNA (10 to 30 μ g) was kept constant in a given experiment by the addition of a carrier plasmid, pGEM-3 (Promega Biotec, Madison, Wis.). In experiments in which 8-bromo-cAMP (8-Br-cAMP) was added, cells were incubated for 28 h after transfection and then exposed to 1 mM 8-Br-cAMP for 20 h before preparation of extracts for CAT assays (18). In the absence of cAMP treatment, cell extracts were prepared 48 h after transfection. Isolation of transfected plasmid DNA (1, 22) and hybridization with a 32 P-labeled oligonucleotide specific for the CAT-coding sequence demonstrated that the amounts of transfected α -CAT reporter plasmid were not altered either by treatment with 8-Br-cAMP or by cotransfection of plasmids containing competitor sequences.

Plasmids. The construction of the α -CAT plasmid has been described previously (25). This plasmid contains ~1.5 kilobases of the human α gene 5'-flanking sequence and 44 base pairs (bp) of 5' untranslated tract (*EcoRI*-*Bam*HI fragment) joined to the coding sequence of the bacterial gene, CAT. Construction of a deletion mutant of α -CAT (α -99/+44CAT) that lacks basal and cAMP-responsive transcriptional activity is described elsewhere (Deutsch et al., *in press*). An additional construct (α -146/+44CAT) contains a synthetic 36-bp sequence (-146 to -111 of the α gene) inserted 5' of α -99/+44CAT (Deutsch et al., *in press*). DNA sequencing (40) and subcloning were performed by standard procedures (30). The following competitor sequences were subcloned into the polylinker sites of pGEM-3: α GEM 1.5, *EcoRI*-*Bam*HI fragment of the human α gene (13) extending from -1500 to +44; pCG β GEM, *EcoRI*-*Sac*I fragment of the hCG β gene 5 (14) extending from -300 to +273; prLH β GEM, *EcoRI*-*Kpn*I fragment of the rat LH β gene (24) extending from -600 to 23 bp of intron 1. In addition, short restriction fragments from the proximal region of the α gene promoter were blunt-end ligated into the unique *Hinc*II site of pGEM-3: α GEM A, *Rsa*I-*Bam*HI (-99 to +44); α GEM B, *Rsa*I fragment (-236 to -100); α GEM C, *Rsa*I fragment (-332 to -252). An 18-bp direct repeat in the human α gene (-146 to -111) was synthesized with *Bam*HI and *Bgl*II cohesive ends and subcloned into the *Bam*HI site of pGEM-3 to form α GEM (-146 to -111).

Additional deletion mutants of the α gene 5'-flanking sequence were prepared by *Bal* 31 nuclease digestion. α GEM 1.5 (containing the *EcoRI*-*Bam*HI fragment of the α gene) was linearized with *Bgl*II and digested with *Bal* 31 nuclease for various times. After the addition of *EcoRI* linkers and digestion with excess *EcoRI*, the plasmids were recircularized and characterized. The plasmid prGluS1 contains 3.5 kilobases of rat glucagon 5'-flanking sequence and extends 400 bp into intron 1 (21).

CAT assays. Extracts of JEG-3 cells were prepared and assayed for CAT enzyme activity by measuring the rate of acetylation of [14 C]chloramphenicol (18, 25). Transfection reactions were performed in duplicate for dose-response experiments and in triplicate for experiments in which different competitor sequences were compared. For a given experiment, transfections were performed on cells derived from a common mass culture, and CAT activities were measured in the same assay. Assays containing dilutions of 1/100 to 1/5 of total cellular extract allowed reliable determinations of CAT activity in 60-min incubations at 37°C. CAT activity is expressed as the percentage of chloramphenicol converted to acetylated derivatives per microgram of total cellular protein (3) per hour $\times$ 100.

RESULTS

Saturation analyses of α -CAT expression in JEG-3 cells. A fusion gene (α -CAT) containing 1.5 kilobases of the α gene 5'-flanking sequence ligated to the coding sequence of the bacterial enzyme CAT was used as a reporter of transcriptional activity of the α gene promoter (18, 25). This plasmid was expressed efficiently in JEG-3 choriocarcinoma cells, and expression was stimulated by 8-Br-cAMP, a finding consistent with transcriptional regulation of the endogenous α gene by cAMP (25). Whereas previous studies of transient expression were performed by DEAE-mediated transfection (25, 28), in these experiments we found that the efficiency of transfection was 50- to 100-fold greater using the technique of calcium phosphate precipitation (19). Furthermore, the results of the cotransfection competition assays were more reproducible with the calcium phosphate technique compared with DEAE-mediated transfection (L. Jameson, unpublished observations). For these reasons, calcium phosphate transfection was used for subsequent studies of α -CAT expression in JEG-3 cells.

Because the efficiency of transfection is affected by the concentration of DNA (29), the amount of carrier DNA added was optimized to reduce variability in the efficiencies of α -CAT transfection and expression in JEG-3 cells (Fig. 1). A constant amount of α -CAT plasmid (2 μ g) was cotransfected with increasing amounts of pGEM-3 DNA. Expression of CAT activity was maximal with 10 to 30 μ g of total DNA and decreased with the addition of greater amounts of DNA. Comparable results were found with either pGEM-3 or pBR322 as the carrier DNA. In subsequent experiments, the amount of total DNA in each transfection reaction was kept constant by the addition of pGEM-3 DNA.

The expression of α -CAT depended on the amount of transfected reporter plasmid in both the absence and presence of 8-Br-cAMP (Fig. 2). Increasing amounts of α -CAT plasmid (0 to 15 μ g) were cotransfected with pGEM-3 DNA such that the total amount of plasmid DNA remained constant (15 μ g). At each concentration of transfected α -CAT plasmid, CAT expression was stimulated approximately 10-fold by 8-Br-cAMP (Fig. 2A). Expression of CAT activity increased linearly between 0 and 1 μ g of α -CAT plasmid and

FIG. 1. Effect of carrier plasmid DNA on α -CAT expression. JEG-3 cells were cotransfected with 2 μ g of α -CAT plasmid and different amounts (0 to 50 μ g) of pGEM-3 DNA. CAT activity was determined 48 h after transfection. Average values from two separate transfections were used to determine CAT activity. The units for CAT activity are expressed as the percentage of chloramphenicol converted to acetylated derivatives per hour per microgram of protein $\times$ 100.

was maximal at ~ 2 μ g of α -CAT plasmid in either the absence or presence of 8-Br-cAMP (Fig. 2B). These findings are consistent with the binding of a limiting amount of one or more transcription factors. Because the addition of 8-Br-cAMP induced α -CAT expression at saturating amounts of transfected reporter α -CAT plasmid, expression in the absence of cAMP is not limited by consumption of general transcription factors. Furthermore, the amounts of transfected α -CAT plasmid detected by Southern blot analyses (1, 50) were unaffected by treatment with 8-Br-cAMP or by cotransfection with the pGEM-3 plasmid containing competitor sequences (data not shown).

If 8-Br-cAMP induces α -CAT expression by increasing the affinity of a limiting regulatory factor, one would expect to find maximal expression at lower amounts of transfected

α -CAT plasmid. However, the amount of α -CAT plasmid (0.5 μ g) resulting in half-maximal CAT activity was unchanged by the addition of 8-Br-cAMP (Fig. 2B). Thus, cAMP does not appear to enhance transcription of the α gene by altering the affinity of a limiting transcription factor(s) for the α gene promoter.

Competition of 5'-flanking sequence of α gene for binding of one or more *trans*-acting activators of α gene expression. Cotransfection competition experiments were used to characterize the interactions of *trans*-acting factors with α gene regulatory sequences. Binding of a *trans*-acting activator (43, 44) to competitor sequences would be expected to decrease expression of a reporter plasmid, whereas binding and depletion of repressors (35) would stimulate expression of the reporter plasmid. Because expression of the α -CAT reporter was nearly linear between 0 and 2 μ g of plasmid (Fig. 2), competition experiments were feasible with amounts of reporter plasmid that are readily detected in the CAT assay. Cotransfection of increasing amounts of the α gene 5'-flanking sequence (-800 to $+44$) inhibited expression of the α -CAT reporter plasmid in a dose-dependent manner (Fig. 3). Half-maximal inhibition occurred with 1 μ g of competitor plasmid (approximately twofold molar excess), consistent with the fact that CAT expression was saturable within this range of reporter plasmid (Fig. 2). These results indicate that JEG-3 cells contain one or more limiting *trans*-acting factors that activate α gene expression.

Competition for α -CAT reporter expression by the α gene 5'-flanking sequence was equally effective in the absence or presence of treatment with 8-Br-cAMP (Fig. 3). Although the actual levels of α -CAT expression were 10-fold greater after cAMP stimulation, 50% inhibition of expression occurred with the same amount of competitor α gene sequence. Thus, both basal and cAMP-stimulated α -CAT transcription are inhibited by cotransfection of the competitor 5'-flanking sequence.

Independent competitor elements in the α gene 5'-flanking sequence. Three restriction enzyme fragments derived from the downstream region of the α gene 5'-flanking sequence were subcloned into pGEM-3 and used as competitors for cellular regulatory factors that interact with the α gene promoter. Because the competitor sequences are relatively short (100 to 150 bp), they make up only $\sim 5\%$ of the DNA in

FIG. 2. Saturation analyses of α -CAT expression. JEG-3 cells were transfected with increasing amounts (0 to 15 μ g) of α -CAT plasmid. pGEM-3 DNA was added to maintain a constant amount of total DNA (15 μ g). At 24 h after transfection, cells were either not treated or treated with 8-Br-cAMP for an additional 20 h, and cell lysates were assayed for CAT activity. Average values from two separate transfections were used to determine CAT activity. CAT activity is illustrated as both units (A) and relative to maximal CAT expression (B).

FIG. 3. Inhibition of α -CAT reporter expression with the 5'-flanking sequence of the α gene as a competitor. JEG-3 cells were cotransfected with α -CAT reporter plasmid (2 μ g) containing 1.5 kilobases of α gene 5'-flanking DNA and increasing amounts of a plasmid containing the α gene *Bgl*III-*Bam*HI insert (-800 to +44) subcloned into plasmid pGEM-3. In each transfection reaction, the total amount of DNA was brought to 32 μ g with pGEM-3 DNA. At 24 h after transfection, cells were either not treated or treated with 8-Br-cAMP for an additional 20 h, and cell lysates were assayed for CAT activity. Average values from three separate transfections were used to determine CAT activity. Expression of the α -CAT reporter plasmid is expressed relative to control reactions containing only pGEM-3 DNA. The location of the competitor sequence relative to the reporter 5'-flanking sequence is schematically illustrated below the data plot.

FIG. 4. Competition for α -CAT expression with different regions of the 5'-flanking sequence of the α gene as competitors. (A) JEG-3 cells were cotransfected with α -CAT reporter plasmid (2 μ g) and increasing amounts of competitor plasmid and pGEM-3 to bring the total amount of DNA to 32 μ g. The α gene competitor sequences include -99 to +44, -236 to -100, and -332 to -252, each cloned into plasmid pGEM-3. At 24 h after transfection, cells were treated with 8-Br-cAMP for an additional 20 h. At each concentration of competitor, CAT activity was determined from two separate transfections. The relative positions of the competitor sequences are schematically illustrated by the shaded boxes to the right and below the data plot.

FIG. 5. Inhibition of α -CAT reporter expression with different regions of the α gene 5'-flanking sequence alone and in combination as competitors. JEG-3 cells were cotransfected with α -CAT plasmid (0.2 μ g) and a 25-fold molar excess of the indicated competitor plasmid(s), each cloned into plasmid pGEM-3. The amount of total DNA in each transfection was brought to 15 μ g with pGEM-3 DNA. The fragments indicated by open boxes are 5' deletion mutants generated by using restriction enzyme sites or *Bal* 31 nuclease digestion. The solid and hatched boxes represent internal fragments that were subcloned and cotransfected as separate plasmids. For each competitor, CAT activity was determined from three separate transfections and is expressed as mean $\pm$ standard error (SEM). Experiment 1 was performed without cAMP treatment. Experiment 2 was performed with 8-Br-cAMP treatment. ND, Not done.

CAT Activity

Expt. #1 (- cAMP)			Expt. #2 (+ cAMP)		
Mean	$\pm$ SEM	(% Control)	Mean	$\pm$ SEM	(% Control)
196	$\pm$ 18	(100%)	1186	$\pm$ 390	(100%)
43	$\pm$ 2	(22%)	71	$\pm$ 7	(6%)
44	$\pm$ 6	(22%)	ND		
115	$\pm$ 7	(59%)	ND		
155	$\pm$ 11	(78%)	781	$\pm$ 214	(66%)
62	$\pm$ 1	(32%)	400	$\pm$ 122	(33%)
119	$\pm$ 16	(61%)	791	$\pm$ 121	(67%)
43	$\pm$ 1	(22%)	106	$\pm$ 4	(9%)
40	$\pm$ 4	(20%)	ND		

TABLE 1. Comparison of transcriptional activity and competition efficacy of plasmids containing different α gene 5'-flanking sequences^a

CAT fusion gene	Transient expression CAT activity (U)	Competitor sequence	Competition for α -CAT expression (% control)
(-99/+44) α -CAT	3	pGEM-3 control	100
(-146/+44) α -CAT	4,170	-99 to +44	66
		-236 to -100	27
		-146 to -111	33
(-1500/+44) α -CAT	38,000	-800 to +44	6

^a The plasmids expressed in transient assays are described in the text. A 10- μ g sample of each plasmid was transfected into JEG-3 cells and treated with 1 mM 8-Br-cAMP for 24 h before assay for CAT activity. The results are the mean from three separate transfections (units = percent conversion of [¹⁴C]chloramphenicol per 60 min per milligram of protein). In the competition assays, a 25-fold molar excess of each competitor sequence was cotransfected with 0.2 μ g of reporter α -CAT and the total amount of DNA was brought to 15 μ g with pGEM-3. The transfected cells were treated with 1 mM 8-Br-cAMP for 20 h before assay for CAT activity. The results are expressed relative to control transfections containing pGEM-3 DNA without α gene competitor sequences. The data for the competitor sequences -99 to +44 and -800 to +44 were derived from the experiments shown in Fig. 5. The data for the competitor sequences -146 to -111 and -236 to -100 were determined in an independent experiment.

the subcloned plasmids. Therefore, effects of the various recombinant plasmids on expression of the reporter α -CAT can be attributed to the α gene inserts rather than to differences in the type of plasmid vector or to the size of the plasmid. The subcloned sequences include the following. (i) A fragment (-99 to +44) that contains a consensus CCAAT box sequence, the TATA box, and the transcriptional initiation site. Deletion mutants of the 5'-flanking sequence of the α -CAT indicate that this region of the promoter (p α -99/+44CAT) is not sufficient for expression in JEG-3 cells (see Table 1) (Deutsch et al., in press). (ii) An adjacent region of the α gene (-236 to -100) contains a 36-bp element (-146 to -111) consisting of two 18-bp direct repeats which confer both basal expression and responsiveness to cAMP (Deutsch et al., in press). (iii) A more upstream fragment (-332 to -252), for which no regulatory function has yet been determined.

The effects of increasing amounts of each of these three regions of the α gene on α -CAT expression are shown in Fig. 4. The downstream promoter sequence (-99 to +44) was a weak competitor, producing ~25% inhibition of α -CAT expression. Both of the more upstream fragments of the α gene promoter were more effective competitors for α -CAT expression. The sequence -236 to -100 that contains enhancer elements for both basal and cAMP-regulated expression was an effective competitor: a 25-fold molar excess of this DNA fragment produced 70 to 80% inhibition in different experiments. The sequence -332 to -252 also inhibited α -CAT expression, but the inhibition did not exceed 40 to 50% in several different experiments (Fig. 4 and 5). Thus, different regions of the proximal α gene promoter compete for binding of limiting transcription factors, but with differing degrees of effectiveness, suggesting that multiple cellular factors bind to the α gene promoter.

Competition with several different fragments of the α gene alone and in combination are shown in Fig. 5. In this experiment, 0.2 μ g of reporter α -CAT plasmid was used to allow cotransfection of a greater molar excess (25-fold) of competitor plasmid. Deletion of the α gene 5'-flanking sequence from -1500 to -325 bp did not alter the effectiveness of the competitor sequence. However, further deletion of the

α gene sequence to -125 or -100 bp caused a substantial reduction in the effectiveness of the flanking sequence as a competitor. As indicated in Fig. 4, several regions of the α gene 5'-flanking sequence compete independently for α -CAT expression. However, the sequence extending from -236 to -100 accounts for most, but not all, of the competition observed with the entire α gene 5'-flanking sequence in both the absence and presence of cAMP stimulation (Fig. 5). Thus, *cis* interactions of this sequence with downstream promoter elements are not required for interactions with intracellular regulatory factors.

Competition with combinations of different regions of the α gene promoter together, but on separate plasmids, caused inhibition of α -CAT expression similar to that observed when the same sequences were arranged in *cis* in a single plasmid (Fig. 5). These results are consistent with the idea that these sequences independently interact with cellular factors and thereby limit α -CAT transcription in an additive manner. However, because the single fragment from -236 to -100 was a highly effective competitor sequence when used alone, these results do not exclude the possibility that *cis* interactions between these sequences alter the binding of factors that do not limit transcription.

The dose response for competition by the -236 to -100 fragment in either the absence or the presence of 8-Br-cAMP is shown in Fig. 6. As observed with larger fragments of the α gene 5'-flanking sequence (Fig. 3), the addition of 8-Br-

FIG. 6. Effect of cAMP on inhibition of α -CAT expression by the -236 to -100 competitor sequence. JEG-3 cells were cotransfected with α -CAT reporter plasmid (0.2 μ g) and increasing amounts of a competitor plasmid containing a fragment (-236 to -100; hatched box) of the α gene. The amount of total DNA was brought to 15 μ g with pGEM-3 DNA. At 24 h after transfection, cells were either not treated or treated with 8-Br-cAMP for an additional 20 h, and cell lysates were assayed for CAT activity. At each concentration of competitor, CAT activity was determined from two separate transfections. The sequence of an 18-bp direct repeat (-146 to -111) within this region of the α gene is shown at the bottom of the figure. An 8-bp palindromic element is boxed.

cAMP did not alter the amount of specific competitor sequence required for half-maximal inhibition of α -CAT expression. As illustrated schematically in Fig. 6, this region of the α gene contains a repeated 18-bp sequence that contains an 8-bp palindromic element found in several cAMP-responsive genes (9, 32). To determine whether this 18-bp repeat was sufficient for interacting with limiting cellular transcription factors, we chemically synthesized it and subcloned it into the pGEM-3 plasmid for use as a competitor. A 25-fold molar excess of competitor plasmid containing this sequence (−146 to −111) also inhibited α -CAT expression and was similar in activity to the longer restriction fragment that extends from −236 to −100 (Table 1).

The correlation between the effects of different α gene 5'-flanking sequences on transcriptional activity and as competitors for cellular regulatory factors is summarized in Table 1. The proximal promoter sequence extending from −99 to +44 retained minimal transcriptional activity. Thus, although this region of the promoter contains the TATA box and CCAAT box sequences, it seems that additional upstream regulatory elements are required for expression and cAMP responsiveness (Deutsch et al., in press). Moreover, the cellular factors that interact with these sequences do not appear to be limiting for transcription, as the proximal promoter was a relatively poor competitor in the cotransfection competition assay (Fig. 4 and 5). Addition of the 18-bp repeated sequence (−146 to −111) to the proximal promoter markedly stimulated transcription and effectively limited transcription of the reporter plasmid when cotransfected as a competitor. Addition of upstream α gene sequences (−1500 to +44) further stimulated transcription as well as serving as

a more effective competitor of α -CAT transcription (Fig. 5). These findings suggest that additional sequences upstream of the 18-bp repeat are important for regulating α gene transcription.

Effect of gonadotropin β subunit 5'-flanking sequences on α -CAT expression. One potential explanation for the coordinant expression of glycoprotein hormone α and β subunit genes would be for a common cellular regulatory factor to interact with both genes. To explore this possibility, a 25-fold molar excess of the 5'-flanking sequence of the hCG β gene 5 (−300 to +273) was cotransfected with the reporter, α -CAT. In three independent experiments, cotransfection of hCG β 5'-flanking sequence inhibited α -CAT expression by 50 to 60% relative to control plasmids (pGEM-3 or pBR322) ($P < 0.001$) (Fig. 7). The rat LH β gene 5'-flanking sequence, which is the evolutionary precursor for the hCG β gene (14, 24, 26), also inhibited α -CAT expression by 50 to 60%. However, the α gene 5'-flanking sequence was a much more effective competitor than either of the β subunit 5'-flanking sequences ($P < 0.05$), causing 80 to 90% inhibition of α -CAT expression relative to control plasmids. The glucagon 5'-flanking region, a sequence that is not known to be related to the glycoprotein hormones, was a less effective competitor sequence and did not significantly inhibit α -CAT expression. Similar results were obtained when competition assays were performed in the presence of 8-Br-cAMP (data not shown). These results suggest that the 5'-flanking region of the hCG β subunit genes interacts with one or more cellular factors that also bind to the α subunit gene promoter.

DISCUSSION

Competition assays for limiting cellular transcription factors allow insight into the complex interactions between the *cis*-acting regulatory elements of a gene and the *trans*-acting factors that modulate formation of an active transcription complex (4, 8, 31, 35, 41–43, 50). Importantly, these assays are performed in intact cells, and competition may therefore occur for a single or several different transcription factors. Thus, while some regulatory factors may bind directly to specific DNA sequences, others may recognize specific DNA-protein complexes and would still be assessed in competition assays performed in transfected cells.

Cotransfection of the α gene 5'-flanking region inhibited expression of the α -CAT reporter plasmid in transfected JEG-3 cells in a sequence-specific and dose-dependent manner (Fig. 3). These findings indicate that JEG-3 cells contain one or more *trans*-acting factors that activate α gene expression by interacting with DNA or DNA-protein complexes involving the α gene 5'-flanking sequence. To minimize potential nonspecific effects of competitor DNA on α -CAT expression, relatively short DNA sequences were subcloned into the same plasmid vector and the total amount of DNA in the transfection reactions was kept constant. Thus, in a typical competition experiment, a 25-fold molar excess of the competitor sequence (−236 to −100) accounted for <1% of the total DNA. Furthermore, the observation that cAMP can still stimulate reporter α -CAT expression for any given amount of competitor sequence indicates that competition does not reflect depletion of general transcription factors.

Several different regions of the α gene 5'-flanking sequence were found to inhibit expression of the α -CAT reporter plasmid, suggesting that there are multiple functional domains in the α gene promoter. The proximal promoter region (−99 to +44), which contains the CCAAT box

FIG. 7. Competition for α -CAT expression by the 5'-flanking sequences of gonadotropin β subunit genes. JEG-3 cells were cotransfected with a 25-fold molar excess of competitor 5'-flanking sequences relative to α -CAT reporter plasmid (0.2 μ g). The amount of total DNA in each transfection was brought to 15 μ g with pGEM-3 DNA. Control transfections contained 0.2 μ g of α -CAT reporter plasmid and 15 μ g of either pGEM-3 or pBR322. The 5'-flanking sequences for the competitors were subcloned into the plasmid pGEM-3 and include: human α , −800 to +44; hCG β 5, −300 to +273; rat LH β (rLH β), −600 to 23 bp of intron 1; rat glucagon, −3500 to 400 bp of intron 1. At 48 h after transfection, cell lysates were assayed for CAT activity. The values for CAT activity are the mean $\pm$ standard error (bars) from three separate transfections. A one-way analysis of variance was used to analyze differences between the different competitor sequences relative to pGEM-3 as a control. NS, Not significant ($P > 0.05$); ●, $P < 0.001$.

and the TATA box, was relatively ineffective for titrating binding factors, while the adjacent upstream 5'-flanking sequences were more effective as competitors. Although it cannot be determined from the competition experiments whether the different regions of the α gene promoter bind common or different regulatory factors, inspection of the different competitor sequences does not reveal any apparently conserved motifs. Because the sequences compete with different efficacies and inhibit α -CAT expression to different extents, it is likely that multiple factors are bound, more than one of which may limit transcription when depleted. Consistent with the view that multiple factors bind to the α gene promoter, we have found that several different sequences in the α gene 5'-flanking region specifically bind nuclear proteins in band-shift experiments (48) and in DNase I footprinting assays (48; L. Jameson, R. Jaffe, and J. Habener, unpublished observations).

A relatively limited sequence (-236 to -100) of the α gene 5'-flanking sequence effectively inhibited expression of the α -CAT reporter. This indicates that a limiting *trans*-acting factor can be bound without additional *cis*-linked sequences of the proximal promoter. Specific competition by a limited region of a promoter has also been noted for the simian virus 40 early gene in which a 72-bp repeat element is sufficient for effective binding of a limiting *trans*-acting factor (42). In contrast, competition for regulatory factors of the simian virus 40 late promoter or the metallothionein IIa promoter require interactions of *cis*-linked DNA regulatory elements with the proximal promoter region for maximal binding (4, 43). Cotransfection of different α gene regulatory sequences on separate plasmids was as effective as equimolar amounts of these sequences arranged in *cis* (Fig. 5). These findings suggest that *cis* interactions between these regions of the α gene are not required for binding limiting *trans*-acting factors, but do not exclude the possibility that interactions between different DNA-protein complexes are important for activating transcription. Thus, for some genes, including the α gene, effective binding and competition can occur with a limited region of the promoter, while other genes may require cooperative interactions for maximal binding (4, 44, 50).

As was found previously for the simian virus 40 early promoter (42) and metallothionein II promoter (43), there is a good correlation between the sequences required for promoter function and the sequences that interact with limiting *trans*-acting regulatory factors in competition assays (Table 1). An 18-bp repeated element in the α gene 5'-flanking sequence (-146 to -111) that competed effectively for α -CAT expression corresponds to a region that stimulates basal gene expression and confers cAMP responsiveness (10; Deutsch et al., in press). Thus, this region of the α gene interacts either directly or indirectly with factors that mediate cAMP stimulation of α gene transcription. Within this 18-bp direct repeat there is an 8-bp palindromic element (5'-TGACGTCA-3') (Fig. 6) that is found in several other eucaryotic cAMP-responsive genes (9, 32, 33, 45) and may represent a core recognition sequence for cellular factors that mediate transcriptional responses to cAMP. Because the palindromic element is within a direct repeat, it has dyad symmetry, raising the possibility of forming alternative DNA secondary structures. Dyad symmetry has been noted in the recognition sequences for DNA-binding proteins in several viral and eucaryotic genes (17, 36, 48) and may be an important feature of the α gene recognition sequence.

The mechanism by which cAMP enhances gene transcription is not known. In analogy with its effects on metabolic

pathways such as glycogen breakdown, cAMP-dependent phosphorylation of specific enzymes or binding proteins may also modulate gene expression (49). For example, the catalytic subunit of protein kinase stimulates synthesis of the enzyme tyrosine aminotransferase when microinjected into hepatoma cells (2). The observation that cAMP-responsive somatostatin fusion genes are not stimulated by cAMP in cell lines deficient in type II protein kinase activity supports a role for type II protein kinase in the transcriptional regulation of certain cAMP-responsive genes (32).

The competition assays provide further evidence for interactions of *trans*-acting factors with DNA regulatory elements to modulate cAMP-stimulated gene transcription. For the α gene, both basal and cAMP-stimulated transcription were inhibited by competitor sequences containing the cAMP response element, suggesting that interactions of a *trans*-acting activator with the cAMP response element are an important requirement for basal and cAMP-stimulated transcription. Thus, it is possible that both basal and cAMP-regulated transcription are mediated by this sequence via a common mechanism. Because cAMP treatment did not affect the amount of transfected α -CAT plasmid required for half-maximal expression, cAMP does not appear to increase transcription by altering the affinity of limiting transcription factors for the α gene 5'-flanking sequence. Nevertheless, cAMP increases the absolute amount of α -CAT expression over a broad range of specific competitor DNA sequences. These results suggest that cAMP increases the efficiency of α gene transcription either by increasing the amount of active *trans*-acting factor or by amplifying the effect of the factor on α gene transcription after interaction with the transcription complex.

Because both the hCG α and β genes are coordinately expressed JEG-3 cells, and because transcription of both genes is stimulated by cAMP (25), we assessed whether the β subunit 5'-flanking sequences could compete for factors required for α -CAT expression. Competition for α -CAT expression with the hCG β 5'-flanking sequence caused ~50% of the inhibition observed with the α gene flanking sequence. In contrast, the 5'-flanking sequence of the glucagon gene, which is not responsive to cAMP (38), caused only minimal reduction in α -CAT expression. The small effects of the glucagon sequence on α -CAT expression may reflect partial depletion of ubiquitous transcription factors or a weak interaction with factors specific for α gene expression. The finding that the 5'-flanking sequences of the hCG α and β subunit genes both reduce α -CAT expression raises the possibility that the coordinate expression of certain cellular genes involves binding of common regulatory factors. The localization of the regulatory elements in the hCG β gene will be required to define fully the possible interactions of the α and β subunit genes with common regulatory factors.

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LITERATURE CITED

1. Alwine, J. C. 1985. Transient gene expression control: effects of transfected DNA stability and *trans*-activation by viral early proteins. *Mol. Cell. Biol.* 5:1034-1042.
2. Boney, C., D. Fink, D. Schlichter, K. Carr, and W. D. Wicks. 1983. Direct evidence that the protein kinase catalytic subunit mediates the effects of cAMP on tyrosine aminotransferase

- synthesis. *J. Biol. Chem.* **258**:4911-4918.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **42**:248-254.
 4. Brady, J., M. R. Loeken, and G. Khoury. 1985. Interaction between two transcriptional control sequences required for tumor-antigen-mediated simian virus 40 late gene expression. *Proc. Natl. Acad. Sci. USA* **82**:7299-7303.
 5. Braunstein, G. D., J. L. Vaitukaitis, P. P. Carbone, and G. T. Ross. 1973. Ectopic production of human chorionic gonadotropin by neoplasms. *Ann. Intern. Med.* **78**:39-45.
 6. Burnside, J., S. B. Nagelberg, S. S. Lippman, and B. D. Weintraub. 1985. Differential regulation of hCG α and β subunit mRNAs in JEG-3 choriocarcinoma cells by 8-bromo-cAMP. *J. Biol. Chem.* **260**:12705-12709.
 7. Chin, W. W. 1985. Organization and expression of the glycoprotein hormone genes, p. 103-126. In H. Imura (ed.), *The pituitary gland*. Raven Press, New York.
 8. Chung, J., E. Sinn, R. R. Reed, and P. Leder. 1986. Trans-acting elements modulate expression of the human c-myc gene in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA* **83**:7918-7922.
 9. Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman. 1986. A cyclic AMP- and phorbol ester-inducible DNA element. *Nature (London)* **323**:353-356.
 10. Darnell, R. B., and I. Boime. 1985. Differential expression of the human gonadotropin α gene in ectopic and eutopic cells. *Mol. Cell. Biol.* **11**:3157-3167.
 11. Dynan, W. S., and R. Tjian. 1986. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature (London)* **316**:774-778.
 12. Edlund, T., M. D. Walker, P. J. Barr, and W. J. Rutter. 1985. Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5'-flanking elements. *Science* **230**:912-916.
 13. Fiddes, J. C., and H. M. Goodman. 1981. The gene encoding the common alpha subunit of the four human glycoprotein hormones. *J. Mol. Appl. Genet.* **1**:3-18.
 14. Fiddes, J. C., and K. Talmadge. 1984. Structure, expression, and evolution of the genes for the human glycoprotein hormones. *Recent Prog. Horm. Res.* **40**:43-78.
 15. Garcia, J. V., L. Bich-Thuy, J. Stafford, and C. Queen. 1986. Synergism between immunoglobulin enhancers and promoters. *Nature (London)* **322**:383-385.
 16. Gharib, S. D., S. M. Bowers, L. R. Need, and W. W. Chin. 1986. Regulation of rat luteinizing hormone subunit messenger ribonucleic acids by gonadal steroid hormones. *J. Clin. Invest.* **77**:582-589.
 17. Giniger, E., S. M. Varnum, and M. Ptashne. 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell* **40**:767-774.
 18. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.
 19. Graham, F., and A. van der Erb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-457.
 20. Grosschedl, R., and D. Baltimore. 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. *Cell* **41**:885-897.
 21. Heinrich, G., P. Gross, and J. F. Habener. 1984. Glucagon gene sequence. Four to six exons encode separate functional domains of rat pre-proglucagon. *J. Biol. Chem.* **259**:14082-14087.
 22. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
 23. Hussa, R. O. 1980. Biosynthesis of human chorionic gonadotropin. *Endocr. Rev.* **1**:268-294.
 24. Jameson, J. L., W. W. Chin, A. N. Hollenberg, A. S. Chang, and J. F. Habener. 1984. The gene encoding the β -subunit of rat luteinizing hormone. Analysis of gene structure and evolution of nucleotide sequence. *J. Biol. Chem.* **259**:15474-15480.
 25. Jameson, J. L., R. C. Jaffe, S. L. Gleason, and J. F. Habener. 1986. Transcriptional regulation of chorionic gonadotropin α and β -subunit gene expression by 8-bromo-adenosine 3',5'-monophosphate. *Endocrinology* **119**:2560-2567.
 26. Jameson, J. L., C. M. Lindell, and J. F. Habener. 1986. Evolution of different transcriptional start sites in the human luteinizing hormone and chorionic gonadotropin beta subunit genes. *DNA* **5**:227-234.
 27. Jameson, J. L., C. M. Lindell, and J. F. Habener. 1987. Gonadotropin and thyrotropin α and β subunit gene expression in normal and neoplastic tissues characterized using specific mRNA hybridization probes. *J. Clin. Endocrinol. Metab.* **64**:319-327.
 28. Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**:5707-5717.
 29. Loyter, A., G. A. Scangos, and F. H. Ruddle. 1982. Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc. Natl. Acad. Sci. USA* **79**:442-426.
 30. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 31. Mercola, M., J. Goverman, C. Mirell, and K. Calame. 1985. Immunoglobulin heavy-chain enhancer requires one or more tissue-specific factors. *Science* **227**:266-270.
 32. Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682-6686.
 33. Nagamine, Y., and E. Reich. 1985. Gene expression and cAMP. *Proc. Natl. Acad. Sci. USA* **82**:4606-4610.
 34. Nelson, C., E. B. Crenshaw, R. Franco, S. A. Lira, V. R. Albert, R. M. Evans, and M. G. Rosenfeld. 1986. Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone genes. *Nature (London)* **322**:557-562.
 35. Nir, U., M. D. Walker, and W. J. Rutter. 1986. Regulation of rat insulin 1 gene expression: evidence for negative regulation in nonpancreatic cells. *Proc. Natl. Acad. Sci. USA* **83**:3180-3184.
 36. Okamoto, T., and F. Wong-Staal. 1986. Demonstration of virus-specific transcriptional activator(s) in cells infected with HTLV-III by an *in vitro* cell-free system. *Cell* **47**:29-35.
 37. Papavasiliou, S. S., S. Zmeili, S. Khoury, T. D. Landefeld, W. W. Chin, and J. C. Marshall. 1986. Gonadotropin-releasing hormone differentially regulates expression of the genes for luteinizing hormone α and β subunits in male rats. *Proc. Natl. Acad. Sci. USA* **83**:4026-4029.
 38. Philippe, J., D. J. Drucker, and J. F. Habener. 1987. Glucagon gene transcription in an islet cell line is regulated via a protein kinase C-activated pathway. *J. Biol. Chem.* **262**:1823-1828.
 39. Pierce, J. G., and T. F. Parsons. 1981. Glycoprotein hormones: structure and function. *Annu. Rev. Biochem.* **50**:465-495.
 40. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 41. Sawadogo, M., and R. G. Roeder. 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43**:165-175.
 42. Scholer, H. R., and P. Gruss. 1984. Specific interaction between enhancer-containing molecules and cellular components. *Cell* **36**:403-411.
 43. Scholer, H., A. Haslinger, A. Heguy, H. Holtgreve, and M. Karin. 1986. *In vivo* competition between a metallothionein regulatory element and the SV40 enhancer. *Science* **232**:76-80.
 44. Sequin, C., B. K. Felber, A. D. Carter, and D. H. Hamer. 1984. Competition for cellular factors that activate metallothionein gene transcription. *Nature (London)* **312**:781-785.
 45. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson. 1986. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. II. Identification of cAMP and glucocorticoid regulatory domains. *J. Biol. Chem.* **261**:9721-9726.

46. **Shupnik, M. A., W. W. Chin, D. S. Ross, M. F. Downing, J. F. Habener, and E. C. Ridgway.** 1983. Regulation by thyroxine of the mRNA encoding the α subunit of mouse thyrotropin. *J. Biol. Chem.* **258**:15120–15124.
47. **Shupnik, M. A., S. L. Greenspan, and E. C. Ridgway.** 1986. Transcriptional regulation of thyrotropin subunit genes by thyrotropin-releasing hormone and dopamine in pituitary cell culture. *J. Biol. Chem.* **261**:12675–12769.
48. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature (London)* **319**:154–158.
49. **Waterman, M., G. H. Murdoch, R. M. Evans, and M. G. Rosenfeld.** 1985. Cyclic AMP regulation of eukaryotic gene transcription by two discrete molecular mechanisms. *Science* **229**:267–269.
50. **Weintraub, H., P. F. Cheng, and K. Conrad.** 1986. Expression of transfected DNA depends on DNA topology. *Cell* **46**:115–122.
51. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenosine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* **76**:1373–1376.