MOLECULAR AND CELLULAR BIOLOGY, Nov. 1990, p. 5736–5746
0270-7306/90/115736-11$02.00/0

Vol. 10, No. 11

On the Mechanism for Efficient Repression of the Interleukin-6 Promoter by Glucocorticoids: Enhancer, TATA Box, and RNA Start Site (Inr Motif) Occlusion

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Received 6 June 1990/Accepted 31 July 1990

The feedback inhibition of interleukin-6 (IL-6) gene expression by glucocorticoids represents a regulatory link between the endocrine and immune systems. The mechanism of the efficient repression of the IL-6 promoter by dexamethasone (Dex) was investigated in HeLa cells transiently transfected with plasmid constructs containing different IL-6 promoter elements linked to the herpesvirus thymidine kinase gene (*tk*) promoter and the bacterial chloramphenicol acetyltransferase gene (*cat*) and cotransfected with cDNA vectors constitutively expressing either the active wild-type or inactive mutant human glucocorticoid receptor (GR). The induction by interleukin-1, tumor necrosis factor, phorbol ester, or forskolin of IL-6-*tk-cat* chimeric constructs containing a single copy of the IL-6 DNA segment from −173 to −151 (MRE I) or from −158 to −145 (MRE II), which derive from within the multiple cytokine- and second-messenger-responsive enhancer (MRE) region, was strongly repressed by Dex in a wild-type GR-dependent fashion irrespective of the inducer used. The induction by pseudorabies virus of an IL-6 construct containing the IL-6 TATA box and the RNA start site ("initiator" or Inr element) but not the MRE region was also repressed by Dex in the presence of wild-type GR. DNase I footprinting showed that the purified DNA-binding fragment of GR bound across the MRE, the TATA box, and the Inr site in the IL-6 promoter; this footprint overlapped that produced by proteins present in nuclear extracts from uninduced or induced HeLa cells. Imperfect palindromic nucleotide sequence motifs moderately related to the consensus GR-responsive element (GRE) motif were present at the Inr, the TATA box, and the MRE II site in the IL-6 promoter; although MRE I and a GR-binding site between −201 and −210 in IL-6 both lacked a discernible inverted repeat motif, their sequences showed considerable similarity with negative GRE sequences in other Dex-repressed genes. Surprisingly, chimeric genes containing MRE II, which lacks a recognizable GACGTCA cyclic AMP- and phorbol ester-responsive motif, were strongly induced by both phorbol ester and forskolin, suggesting that MRE II (ACATTGCACAATCT) may be the prototype of a novel cyclic AMP- and phorbol ester-responsive element. Taken together, these observations suggest that ligand-activated GR represses the IL-6 gene by occlusion not only of the inducible IL-6 MRE enhancer region but also of the basal IL-6 promoter elements.

There has been increasing awareness in recent years of overlap between the endocrine and immune systems. The inhibition of interleukin-6 (IL-6) gene expression by glucocorticoids and by estrogens (25, 37, 53, 57) appears to be part of a feedback regulatory loop that affects the function of the hypothalamus-pituitary-adrenal-gonadal axis (20, 36, 53). IL-6 produced in response to infections, tissue injury, or even psychological stress (16, 28, 29, 45, 46, 48) is a stimulus for the secretion of corticotropin-releasing factor by the hypothalamus, which in turn leads to the enhanced secretion of adrenocorticotrophic hormone (ACTH) by the anterior pituitary and the subsequent increase in levels of circulating corticosteroids (36). Additionally, IL-6, which can itself be produced by the folliculostellate cells of the anterior pituitary (55), has been reported to directly stimulate the release of the anterior pituitary hormones ACTH, prolactin, growth hormone, and luteinizing hormone (52, 55). The administration of IL-6-inducing cytokines (such as tumor necrosis factor [TNF]) or of bacterial endotoxin to human volunteers leads to the appearance of circulating IL-6 and to elevations in plasma ACTH and cortisol levels (20, 33). Elevated levels of circulating glucocorticoids synergize with IL-6 in inducing the increased hepatic synthesis and secretion of "acute-phase" plasma proteins such as fibrinogen, various antiproteinases, complement factors, and scavenger proteins (e.g., haptoglobin, hemopexin, and C-reactive protein) (16, 45, 46). In addition, the role of IL-6 in the activation of B- and T-cell function also contributes to the ability of the host to combat infection and tissue damage (12, 16, 45, 46, 48). However, this acute response is self-regulating in that glucocorticoids strongly inhibit IL-6 gene expression in different tissues (9, 25, 37, 57). Repression of the IL-6 gene is a component in the well-known anti-inflammatory effect of glucocorticoids. The downregulation of IL-6 gene expression by estradiol-17β in estrogen-sensitive tissues such as endometrial stromal cells probably represents an additional feedback regulatory loop affecting circulating IL-6 levels in women (53, 54).

Expression of the IL-6 gene is readily induced in different cell types by a wide range of "noxious" stimuli (40). Bacterial products such as endotoxin strongly enhance IL-6 gene expression in human fibroblasts and monocytes/macrophages (15). IL-6 gene expression in different cell types is also enhanced by infection with different RNA- and DNA-containing viruses, by inflammation-associated cytokines such as IL-1, TNF, platelet-derived growth factor, and interferons and by second-messenger agonists (24, 26, 46,

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47, 56). Endothelial cells, keratinocytes, and a variety of other epithelial and mesenchymal cells also express IL-6 in response to these stimuli.

We have previously investigated the transcription regulatory elements present in the 5'-flanking region of the human IL-6 gene in transfection assays in HeLa cells (38, 39). The IL-6 and c-*fos* promoters are strikingly similar in their overall functions. The c-*fos* serum response element enhancer region exhibits strong nucleotide sequence similarity with the multiple cytokine- (IL-1, TNF, serum) and second-messenger- (cyclic AMP [cAMP], phorbol ester)- responsive enhancer (MRE) region in IL-6 (−173 to −145) (38, 39, 56).

In the present article, we report that the complex IL-6 enhancer region consists of two partially overlapping DNA elements, each of which is responsive to all of the inducers. MRE I, −173 to −151, contains the typical GACGTCA cAMP-phorbol ester-responsive (CRE/TRE) motif. MRE II, −158 to −145, contains an imperfect dyad repeat which bears little resemblance to a CRE/TRE motif. That a chimeric construct containing MRE II is nevertheless strongly induced by both phorbol ester and forskolin suggests that this DNA defines a novel cAMP- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (CRE/TRE II). This element is very similar to an atypical cAMP-responsive element recently identified in the bovine cytochrome P-$450_{17\alpha}$ gene (32) and has also been recently identified to be the binding site of NF-IL6, a nuclear factor hypothesized to function in the IL-1-mediated induction of IL-6 in some cell types (3, 19). The fact that both MRE I and MRE II are independently strongly induced by TPA and forskolin demonstrates extensive cross-talk between components of the two major signal transduction pathways that are involved in the activation of the IL-6 promoter. The NF-κB site in the IL-6 promoter (−73 to −64) also appears to contribute to the activation of this gene in transfected U937 cells and in some but not all batches of L cells in response to lipopolysaccharide and IL-1 (31, 49).

The glucocorticoid receptor (GR), like other steroid receptors, undergoes transformation upon hormone binding (21), which enables it to bind to regulatory elements in appropriate target genes (5, 8, 41). Positive glucocorticoid-responsive elements (GREs) identified in glucocorticoid-inducible genes (e.g., mouse mammary tumor virus, human metallothionein II_A, and human growth hormone gene I) share a related 15-bp palindromic nucleotide sequence motif (23, 44, 50) (see Fig. 7). Although the mechanism of transcriptional activation mediated by GR remains unclear, positive GR-binding elements have been shown to mediate induction of heterologous promoters by glucocorticoids (5, 8, 41). Genes whose expression is inhibited by glucocorticoids (e.g., prolactin, α subunit gene of glycoprotein hormone, pro-opiomelanocortin, proliferin) (1, 2, 7, 34, 42) do not appear to contain a clear consensus motif at the sites that interact with GR (designated negative GREs) (4, 22, 30).

We have investigated the molecular basis for the repression of the human IL-6 promoter by the glucocorticoid dexamethasone (Dex). The results obtained indicate that the binding of the activated GR to the inducible enhancer as well as to the basal transcription regulatory regions (TATA box and RNA start sites) in the IL-6 promoter interferes with the binding of positive-acting inducible and basal transcription factors and accounts for the highly efficient repression of this gene by Dex. The major RNA start site in the IL-6 promoter corresponds to the "initiator" or Inr motif that, in common with the TATA box, is responsive to the transcription factor TFIID (51). Strikingly, GR binds to both the IL-6 TATA box and the Inr element. Imperfect palindromic sequence motifs moderately related to the consensus GRE motif are present at the Inr site and the TATA box and across the cytokine- and second messenger-inducible enhancer region (MRE) in the IL-6 promoter.

MATERIALS AND METHODS

Cell culture and DNA transfection assays. Procedures for the growth of HeLa cells, transient transfection assays, and quantitation of chloramphenicol acetyltransferase (CAT) and neomycin phosphotransferase (NPT II) enzyme activities and the concentrations and origins of the various reagents used as inducers were described earlier (38). HeLa cells (2×10^6 to 3×10^6 cells) in 100-mm plates were transfected with IL-6-*cat* reporter plasmids (10 μg each) together with constitutive human GR (hGR) expression vectors (17, 18) containing cDNAs for the intact wild-type GR receptor (RSVhGRα), a mutant receptor (I465*; the glucocorticoid binding domain and a part of the DNA-binding domain are deleted) or a constitutive receptor (I550*; the glucocorticoid binding domain is deleted) (5 μg each), and pSV2neo (3 μg) (marker for transfection efficiency) by the calcium phosphate coprecipitation procedure (14). At 16 to 18 h after transfection, the precipitate was washed off and the plates were replenished with serum-free medium in the presence or absence of appropriate inducers or Dex (10^{-6} M) in different combinations. The cells were harvested 24 h later and CAT and NPT II activities were measured in cell extracts containing equal amounts of protein (38, 39). The acetylated derivatives of chloramphenicol formed in the CAT assays were resolved from unreacted chloramphenicol by thin-layer chromatography, and the NPT II activity in each extract was measured by a dot assay as described earlier (39). The fold increase in CAT activity in induced cultures relative to uninduced control cultures was normalized to the NPT II activity in each extract (38, 39).

Plasmid DNAs and oligonucleotides. The construction of the IL-6-*cat* (IL-6/CAT) plasmids containing IL-6 promoter fragments from −1180, −596, −225, and −110 to +13 fused with the bacterial CAT coding region (designated pIC1180, pIC596, pIC225, and pIC110, respectively) and of the IL-6–thymidine kinase (*tk*)–*cat* (IL-6/TK/CAT) plasmids pTKCi225/111 and pTKCi111/225 has been described earlier (38). pAR10TKC and pAR11TKC were constructed by using plasmid $pTK_{-105}CAT$ as the parent vector instead of $pTK_{-80}CAT$, which was used earlier to construct the IL-6/TK/CAT chimeric constructs (38). The region between −173 and −151 in the IL-6 promoter (AR10) was inserted between the *Sph*I and *Sal*I sites upstream of $pTK_{-105}CAT$ to give pAR10TKC. AR11 (−158 to −145) was inserted between the *Hin*dIII and *Bam*HI sites upstream of $pTK_{-105}CAT$ to give pAR11TKC. Nuclease (S1 or RNase) protection assays were used to verify that major transcription from these reporter constructs initiated from the correct RNA start site.

The expression vectors for the wild-type hGR (RSVhGRα) and its mutant derivatives (I465* and I550*) were obtained from Hollenberg and Evans (17, 18). Plasmids RSVhGRα, I550*, and I465* all use the same Rous sarcoma virus long terminal repeat constitutive promoter to express wild-type or mutant hGR cDNA inserts (17, 18). The constitutive mutant I550* was reported to have 40% of the transcription-enhancing activity of ligand-activated RSVhGRα in assays for *trans* activation of the mouse mammary tumor virus promoter (17).

DNase I footprint analyses. DNA fragments were labeled

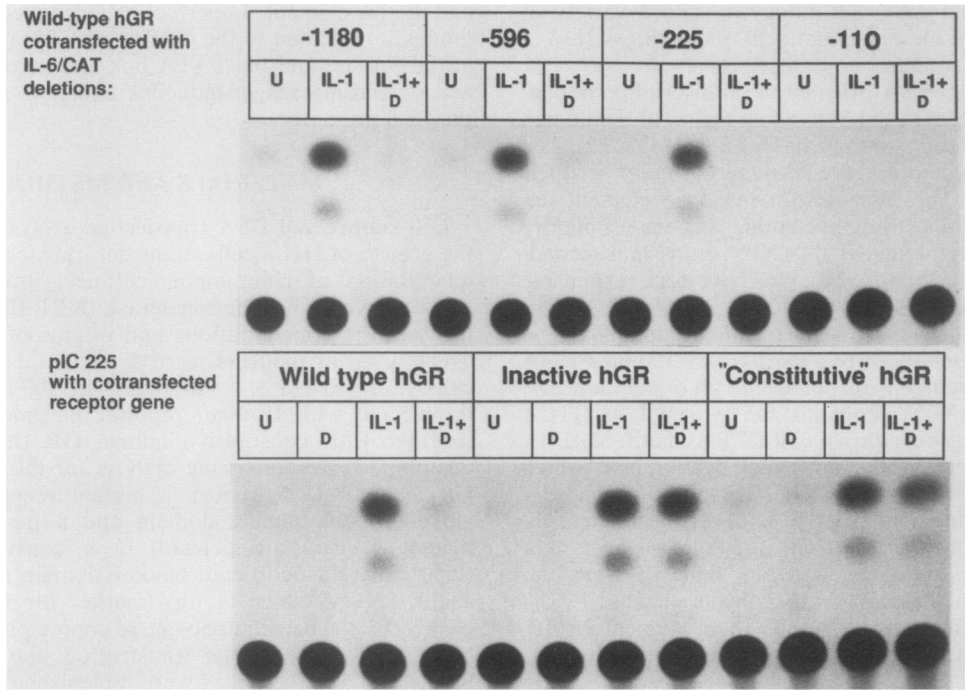

FIG. 1. Repression of IL-1-induced IL-6/CAT gene expression by Dex. HeLa cells (2×10^6 to 3×10^6 cells) in 100-mm plates were transfected with IL-6/CAT reporter plasmids (pIC1180, pIC596, pIC225, or pIC110; 10 μg) together with constitutive hGR expression vectors (17, 18) containing cDNAs for the intact wild-type GR receptor (RSVhGRα), a mutant receptor (I465*; the glucocorticoid-binding domain and a part of the DNA-binding domain are deleted), or a constitutive receptor (I550*; the glucocorticoid-binding domain is deleted) (5 μg), and pSV2neo (3 μg) (marker for transfection efficiency) by the calcium phosphate coprecipitation procedure (14, 38, 39). At 16 to 18 h after transfection, the precipitate was washed off and the plates were replenished with serum-free medium in the presence or absence of IL-1α (5 ng/ml) or Dex (D; 10^{-6} M) in different combinations. The cells were harvested 24 h later, and CAT and NPT II activities were measured in the cell extracts (38, 39). The fold increase in CAT activity in induced cultures relative to uninduced control cultures was normalized to the NPT II activity in each extract (38, 39). (Top) IL-6/CAT plasmids except pIC110 were induced approximately 10- to 12-fold by IL-1α (the percent conversion of [^{14}C]chloramphenicol to its acetylated forms was, on average, 0.5% in uninduced [U] lanes and 5 to 6% in induced lanes). Cells treated with Dex and IL-1 contained 0.5- to 0.8-fold as much CAT activity as was present in uninduced cells. (Bottom) Dex repression requires wild-type hGR. CAT activity observed normalized to the NPT II activity is shown in Table 1, experiment 1.

by filling in restriction-enzyme cleaved ends with the Klenow fragment of DNA polymerase I and [α^{32}P]dATP. Fragments (5 to 10 fmol per footprint reaction) were incubated at room temperature in 20 μl of reaction mixtures containing 10 mM Tris hydrochloride (pH 7.5), 60 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 100 μg of bovine serum albumin per ml, and 2 μg of salmon sperm DNA per ml in the presence (0.15 to 1.5 μg) or absence of the purified DNA-binding domain of the GR (11). Binding was allowed to proceed for 20 min, and DNase I was added at a concentration of 5 ng/ml to tubes which did not receive GR and 50 to 100 ng/ml to those which contained GR. After digestion for 2 min at room temperature, the samples were extracted with a mixture of phenol and chloroform, ethanol precipitated with 5 to 10 μg of carrier glycogen (Boehringer Mannheim), and electrophoresed on 6% polyacrylamide–8 M urea gels. HeLa cell nuclear extracts used in footprinting experiments were prepared as described previously (38).

RESULTS

We took advantage of the fact that HeLa cells possess very low levels of transcriptionally active endogenous GR (27; our unpublished data). Thus, we could transfect these cells with expression vectors containing cDNAs for either the wild-type hGR (RSVhGRα), an inactive mutant (I465*), or a constitutive mutant (I550*) (17, 18) in order to study the influence of Dex on the induction of cotransfected IL-6 promoter constructs in a receptor-specific manner.

Two different series of chimeric IL-6 promoter constructs were used in these functional experiments. In one series, DNA fragments from the 5′-flanking region of the IL-6 promoter deleted to different extents on the 5′ side but all extending up to position +13 on the 3′ side were linked to the bacterial *cat* reporter gene (IL-6/CAT constructs) (39). In the other series, single copies of different segments of the IL-6 5′-flanking region were linked to the basal herpesvirus thymidine kinase gene (*tk*) promoter, which was in turn linked to the *cat* gene (IL-6/TK/CAT constructs) (38). All of these constructs had been characterized previously for their inducibility in transiently transfected HeLa cells by a variety of different stimuli, including IL-1, TNF, serum, TPA, forskolin, and pseudorabies virus (38–40; unpublished data). These experiments in transiently transfected HeLa cells served to define a short multiple cytokine-responsive enhancer element (MRE, −173 to −145) in the IL-6 promoter. A single copy of MRE could render the IL-6/TK/CAT constructs inducible by serum, IL-1, TNF, TPA, and forskolin.

Figure 1 (top) shows that IL-6/CAT constructs containing 1180, 596, and 225 but not 110 bp of the IL-6 promoter were strongly induced by IL-1 and that this inducible expression

TABLE 1. Repression of IL-1-induced IL-6/CAT expression by Dex[a]

Expt	Plasmids	CAT activity (% conversion of chloramphenicol) in uninduced cells	CAT activity in induced cells (fold activity in uninduced cells)		
			Dex	IL-1	Dex + IL-1
1	IL-6/CAT constructs				
	pIC225				
	RSVhGRα	0.24	0.5	10.0	1.0
	I465*	0.24	0.8	15	13
	I550*	0.2	1.0	8.0	7.0
2	IL-6/CAT/TK constructs				
	pTKCi225/111				
	RSVhGRα	0.9	0.6	4.0	0.6
	I465*	1.0	1.0	3.0	2.8
	I550*	0.7	0.8	2.0	2.0
	pTKCi111/225				
	RSVhGRα	0.7	0.6	4.5	0.6
	I465*	1.2	0.9	3.5	3.2
	I550*	0.5	1.0	2.0	2.0

[a] HeLa cells were transfected with IL-6/CAT reporter plasmids or IL-6/CAT/TK plasmids and hGR expression vectors as described in the legends to Fig. 1. CAT activity data are shown for Fig. 1 (bottom) (experiment 1) and Fig. 2 (experiment 2).

was strongly repressed by Dex to levels even lower than those observed in uninduced cells (Table 1). To test whether the observed Dex repression was dependent on the transfected GR, the repression of pIC225 was further analyzed in HeLa cells cotransfected with either wild-type (RSVhGRα) or mutant (I465* or I550*) GR cDNA vectors (17, 18). Figure 1 (bottom) shows that efficient Dex repression of the IL-1-induced IL-6/CAT chimeric gene required the wild-type GR. Similar results were obtained with IL-6/TK/CAT constructs that contained the 115-bp IL-6 fragment from −225 to −111 inserted in either orientation (pTKCi225/111 and pTKCi111/225, Fig. 2, Table 1). IL-1-induced transcription from the *tk* promoter in both of these constructs was repressed by Dex to levels lower than those observed in uninduced cells in a manner completely dependent on the presence of the wild-type GR (Fig. 2). In gel shift assays, the DNA-binding fragment of GR (11) bound to the 115-bp IL-6 enhancer fragment from −225 to −111 (data not shown).

Figure 3A illustrates the DNase I footprint of the DNA-binding fragment of GR on both strands of this 115-bp IL-6 fragment. Binding of GR to the IL-6 enhancer generated a major footprint encompassing nucleotides −170 through −144 and a second footprint between −210 and −201. The GR footprint between −170 and −144 was found to overlap the one obtained by using nuclear extracts from uninduced or induced (IL-1, TPA, or serum) HeLa cells (Fig. 3B). The region of the IL-6 promoter between −173 and −151 was shown by us earlier to behave as a serum-, multiple cytokine-, and second messenger-responsive enhancer element (MRE) when linked to $pTK_{-80}CAT$ (pAR1TKC [38]). The

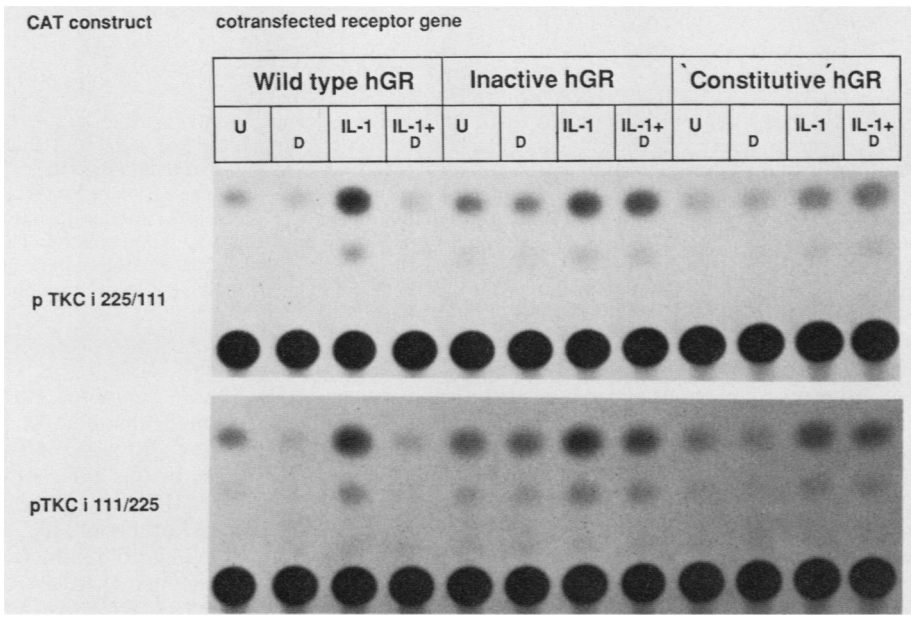

FIG. 2. Repression of IL-1-induced IL-6/CAT gene expression by Dex in an orientation-independent manner from plasmids carrying IL-6 sequences linked to the herpesvirus *tk* promoter (pTKCi225/111 and pTKCi111/225; 10 μg). All other conditions were the same as in the legend to Fig. 1. CAT activity observed normalized to the NPT II activity is shown in Table 1, experiment 2.

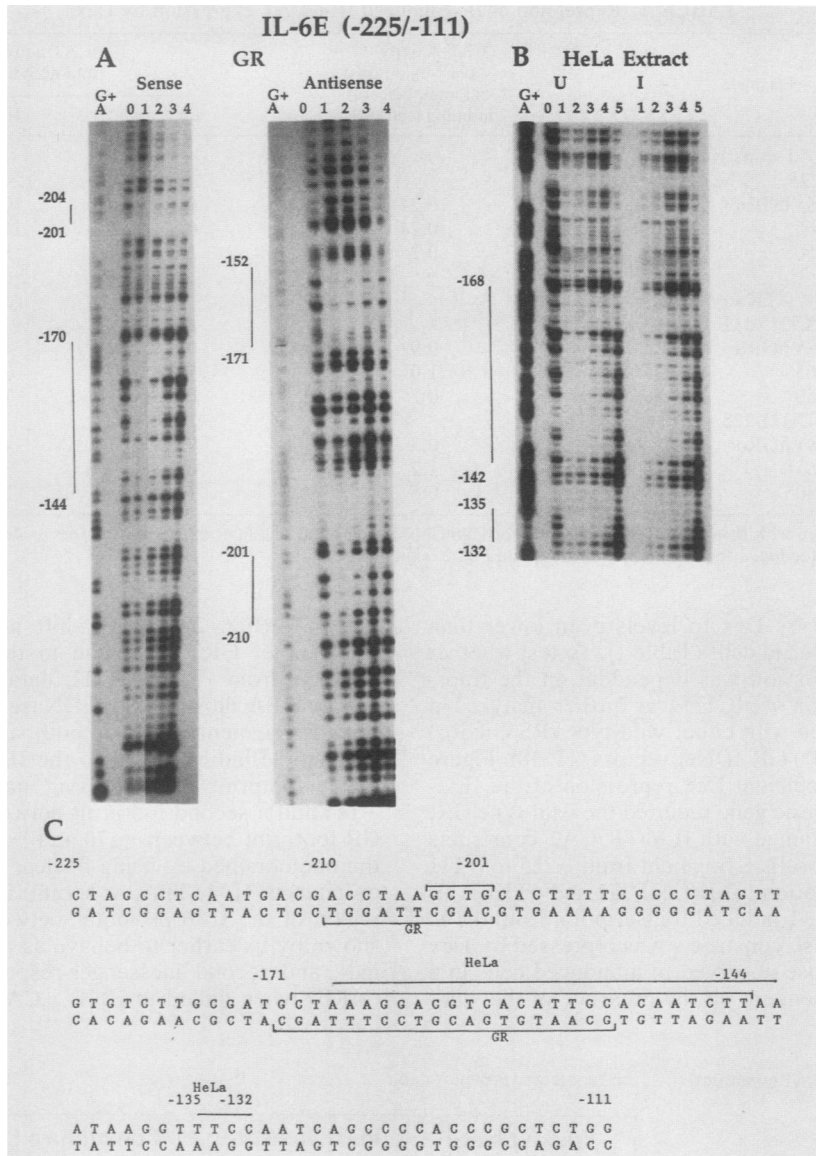

FIG. 3. Binding of GR and HeLa cell nuclear proteins to IL-6 promoter sequences between −225 and −111 in vitro. (A) DNase I protection experiments were carried out separately with either one of the IL-6 DNA strands labeled with ^{32}P. Labeled DNA fragments were incubated without (lane 0) or with the DNA-binding fragment of GR (11) at 1,500, 625, 312, or 156 ng (lanes 1, 2, 3, and 4, respectively) in 20 μl of reaction mixture for 20 min at room temperature and then treated with DNase I at a concentration of 5 ng/ml (lane 0) or 50 ng/ml (lanes 1 to 4) and analyzed on a 6% polyacrylamide gel. (B) DNase I protection analysis of the sense strand with nuclear extracts from either uninduced (U) or serum-induced (I) HeLa cells (80, 40, 20, 10, and 5 ng of protein in lanes 1 to 5, respectively). The footprint obtained was the same whether IL-1 (5 ng/ml) or TPA (100 ng/ml) was used as the inducer (not shown). Lane 0 is the pattern of DNase I-cleaved DNA without the addition of nuclear extract. (C) DNA sequence of the 115-bp IL-6 fragment, indicating the GR-binding regions (brackets) and HeLa nuclear extract footprints (overlines).

generation of a large footprint by proteins present in HeLa cell nuclear extracts extending all the way up to −142 and our earlier observation that the GACGTCA motif present in the region between −173 and −151 was not responsible for conferring serum, IL-1, and TNF responsiveness led us to make the additional constructs pAR10TKC and pAR11TKC to explore this region further.

The region between −173 and −151 in the IL-6 promoter (AR10) was inserted between the *Sph*I and *Sal*I sites upstream of $pTK_{-105}CAT$ to give pAR10TKC. AR11 (−158 to −145) was inserted between the *Hin*dIII and *Bam*HI sites upstream of $pTK_{-105}CAT$ to give pAR11TKC. A different construct, pAR1TKC, contained IL-6 sequences between −173 and −151 cloned upstream of $pTK_{-80}CAT$ between *Sph*I and *Eco*RI sites (38). A comparison of the IL-1 and TNF responsiveness of the different constructs containing sequences from the MRE region showed that the IL-1 and TNF responsiveness of pAR1TKC and pAR11TKC was comparable (four- to eightfold induction), while pAR10TKC showed a lower response than pAR11TKC (Table 2 and reference 38). In pAR1TKC, the AATT sequence at the *Eco*RI site recreates sequences downstream of −151 (−149 to −146) very similar to the sequence AATC at the same location in the IL-6 promoter. Also, mutation of the CGTCA

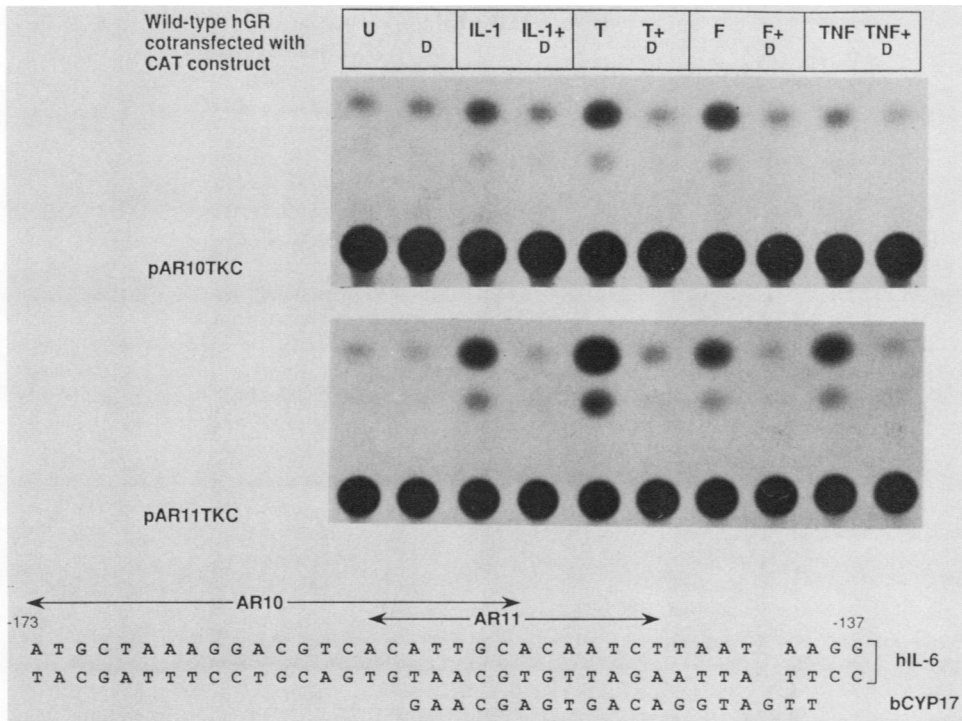

FIG. 4. Repression of IL-1-, TPA-, forskolin-, and TNF-induced MRE I/TK/CAT and MRE II/TK/CAT gene expression by Dex. IL-6 promoter oligonucleotides AR10 (−173 to −151) and AR11 (−158 to −145) were linked to $pTK_{-105}CAT$. HeLa cells were transfected with a mixture of control pSV2neo (3 μg), RSVhGRα (5 μg), and either pAR10TKC (2.5 μg) or pAR11TKC (2.5 μg). The cells were then left untreated (U) or treated with IL-1α (5 ng/ml), TPA (T, 100 ng/ml), a combination of forskolin (50 μM) and isobutylmethylxanthine (IBMX; 0.5 mM) (F), or TNF (100 ng/ml) in the presence or absence of Dex (D, 1 μM). Induction of CAT activity after adjustment for NPT II activity is shown in Table 2. The sequences of AR10 and AR11 are presented in the lower part of the figure. The similarity of the CRE/TRE II motif in AR11 to an atypical CRE identified recently in the bovine CYP17 gene (32) is also shown.

motif to GTTCA only affects responsiveness to TPA and forskolin in pAR1TKC (38). Taken together, these observations indicate that sequences between −158 and −145 and different from the CGTCA motif are major contributors to the IL-1 and TNF induction of the IL-6 promoter in HeLa cells, although the imperfect dyad symmetry is not required for this responsiveness.

Analyses of the different IL-6 constructs with different inducers indicated that the region between −173 and −145 consisted of two partially overlapping functional segments with different DNA motifs, each of which was able to confer inducibility to IL-1, TNF, TPA, and forskolin (Fig. 4). MRE I, present in the construct pAR10TKC, contained the cAMP- and TPA-responsive (CRE/TRE) consensus motif GACGTCA, whose mutation to GAGTTCA blocks the response of MRE I-containing constructs to TPA and forskolin (38). The GACGTCA motif in MRE I was very similar to the negative GRE present in the α-subunit gene of the glycoprotein hormone (2) (see Fig. 7). MRE II (−158 to −145), present in the construct pAR11TKC, lacked the GACGTCA motif but contained a 14-bp imperfect dyad symmetry (Fig. 4 and 7). MRE II, which had no discernible similarity with the GACGTCA motif, identified a novel functional DNA element (designated CRE/TRE II), utilized by activators of both the protein kinase A and protein kinase C pathways as well as by cytokines (Fig. 4). Strikingly, induction of either pAR10TKC or pAR11TKC in response to any of the inducers tested was strongly repressed by Dex in a wild-type GR-specific manner (Fig. 4, Table 2; additional data not shown). Preliminary data suggest that the GR-binding region from −201 to −210 contributes to repression of the IL-6 promoter in uninduced cells. The expression of the control plasmid pTKC was unaffected by any of the inducers in the presence or absence of Dex plus GR (38) (data not shown). Taken together, our observations suggest that Dex repression of these inducible IL-6/TK/CAT constructs involves interference by GR with the binding of positive-acting transcription factors to the inducible IL-6 enhancer segment between −173 and −145.

In addition to the MRE region, the NF-κB site, located

TABLE 2. Repression of CAT induction by Dex[a]

Plasmid	CAT activity in induced cells (fold activity in uninduced cells)								
	Dex	IL-1	IL-1 + Dex	TPA	TPA + Dex	F	F + Dex	TNF	TNF + Dex
pAR10TKC	1.0	2.0	1.0	5.0	1.0	5.0	1.1	1.5	0.9
pAR11TKC	0.7	8.0	0.7	20.0	1.4	5.0	0.7	6.0	0.7

[a] HeLa cells were transfected and treated as described in the legend to Fig. 4.

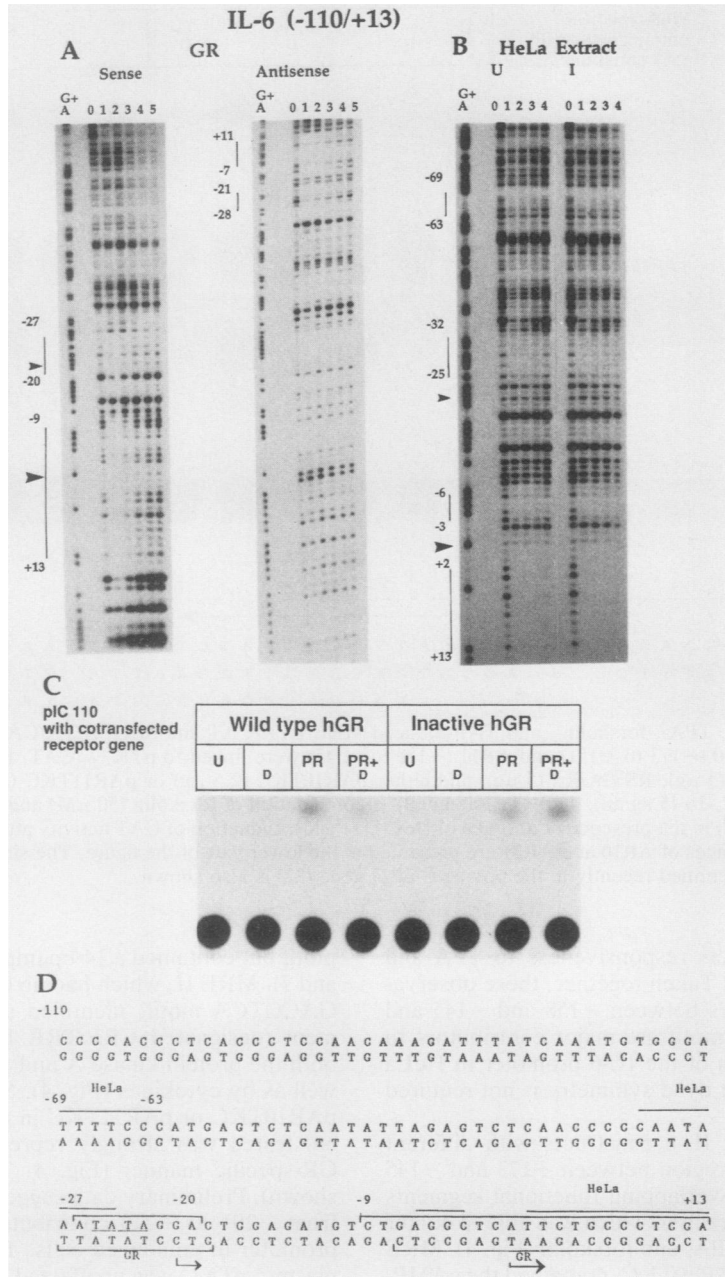

FIG. 5. Binding of GR (A) and of HeLa cell nuclear proteins (B) to IL-6 promoter sequences between −110 and +13. All experimental conditions were as indicated in the legend to Fig. 3. Lane 5 in panel A contained approximately 80 ng of the GR fragment. Arrowheads indicate the major and minor start sites at +1 and −21, respectively. (C) Representative illustration indicating that the repression of pseudorabies virus-induced pIC110 gene expression by Dex requires the wild-type GR. Relative CAT activities (fold activity in untreated extracts) in extracts of cells cotransfected with pIC110, pSV2neo, and either RSVhGRα or I465* and then left untreated (U) or treated with pseudorabies virus (PR) at a multiplicity of infection of 1.0 in the presence or absence of Dex (D) were as follows. RSVhGRα: Dex, 1.0-fold; virus, 14.0-fold; virus plus Dex, 7.0-fold. I465*: Dex, 1.0-fold; virus, 14.0-fold; virus plus Dex, 16.0-fold. (D) DNA sequence of the −110/+13 IL-6 fragment indicating the GR and HeLa nuclear extract footprints.

between −73 and −64 in the IL-6 promoter (39), has recently been implicated in its induction in transfected murine L-TK$^-$ cells (49) and U937 cells (31). In HeLa cells, the IL-6/CAT construct pIC110, which contains the NF-κB site, the TATA box, and the two inducible RNA start sites (see Fig. 6) (40), was partially induced by pseudorabies virus but not by any other inducer, as reported earlier (39). We footprinted GR on the IL-6 fragment between −110 and +13 in order to search for additional sites of interaction (Fig. 5A). Surprisingly, GR gave footprints covering both the TATA box and the major and minor RNA start sites (Fig. 5A). It is noteworthy that in the IL-6 promoter, the major RNA start site corresponds to a perfect initiator or Inr motif, which, in the terminal deoxynucleotidyltransferase gene, has been shown to be functionally interchangeable with the TATA box (51) (Fig. 6), while the minor IL-6 RNA start site actually overlaps the

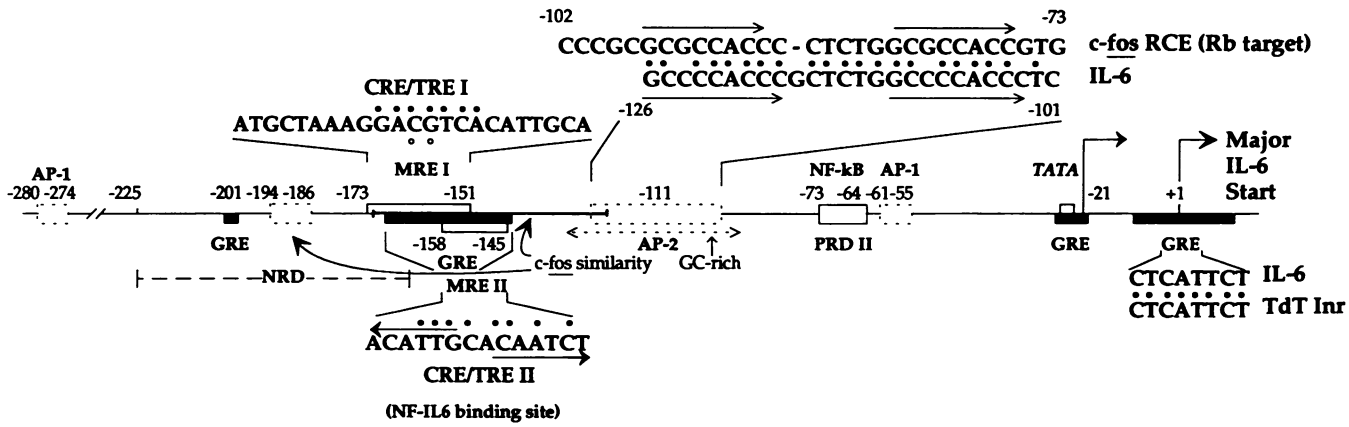

FIG. 6. Schematic representation of positive and negative transcription regulatory elements in the 5'-flanking region of the IL-6 gene. Solid lines (either boxes or arrows) indicate DNA regulatory elements that have already been functionally implicated in IL-6 gene expression, while those marked by broken lines or boxes are based on DNA sequence analyses. The inducible transcription start sites were derived by S1 nuclease mapping (ratio of major +1 to minor −21 was 99:1.0) (40). The presence of a negative regulatory domain (NRD) between −225 and −165 was inferred from results published earlier (38). The typical GACGTCA CRE/TRE motif in MRE I and the nucleotides in the novel CRE/TRE in MRE II which match with nucleotides in the CRE identified in bovine CYP17 (32) (also see Fig. 4) are highlighted by solid circles. The mutation of the CG residues (open circles) to GT reduces the responsiveness of MRE I to TPA and forskolin (38). PRDII refers to the NF-κB-like domain in the beta interferon promoter (31); Inr refers to the initiator RNA start site motif as functionally characterized in the terminal deoxynucleotidyltransferase (TdT) gene (51). RCE is the Rb-repressible DNA target in the c-*fos* promoter (41a).

major IL-6 TATA box. That GR bound across both the IL-6 TATA box and Inr element is consistent with the observation that both the TATA and Inr elements are responsive to the same transcription factors (e.g., TFIID [51]). No footprint of the GR fragment was detected across the NF-κB site (Fig. 5A). Nuclear extracts from uninduced or induced HeLa cells generated a footprint across the NF-κB site, the TATA box, and transcription initiation sites (Fig. 5B).

These observations led us to test whether induction of the IL-6/CAT construct pIC110 by pseudorabies virus would be repressed by Dex in the presence of the wild-type GR. Figure 5C is a representative illustration of repression by Dex of the responsiveness of pIC110 to virus only in the presence of the wild-type receptor, suggesting that the binding of GR across the Inr site and the TATA element in the IL-6 promoter can also contribute to the downregulation of gene expression. This twofold repression of pseudorabies virus-induced expression of the chimeric gene in pIC110 was highly reproducible in additional experiments. The large quantities of transcription-activating immediate-early proteins present in pseudorabies virus-infected HeLa cells (6) may account for the fact that repression was not greater than 50% in this experiment. The immediate-early proteins produced by pseudorabies virus have been shown to bind tightly to the core promoter elements (TATA element and RNA start sites) in different mammalian genes and thus to activate their transcription (6). GR could interfere with the function of these immediate-early viral proteins by binding directly to the TATA box and the Inr element in the IL-6 promoter.

DISCUSSION

Figure 6 summarizes the transcription regulatory elements present in the 5'-flanking sequence of the IL-6 gene. The GR-binding regions overlap sequences that are important for the positive regulation of this gene in HeLa cells. Imperfect palindromic sequence motifs related to the consensus GRE motif are present at the major RNA start site (Inr element), the TATA box, and the MRE II site in the IL-6 promoter (Fig. 7). Although MRE I and the GR-binding site between −210 and −201 in the IL-6 promoter lack discernible inverted repeats, both sequences show similarity with a negative GRE sequence identified in the enhancer of the α-subunit gene of the glycoprotein hormone (2) and with a "preliminary" negative GRE consensus motif (4) (Fig. 7). That a chimeric construct containing MRE II which lacks a recognizable CRE/TRE motif (the GACGTCA motif) was strongly induced by both TPA and forskolin suggests that MRE II may be the prototype of a novel cAMP- and TPA-responsive element (CRE/TRE II). This element is highly similar to an atypical cAMP-responsive element re-

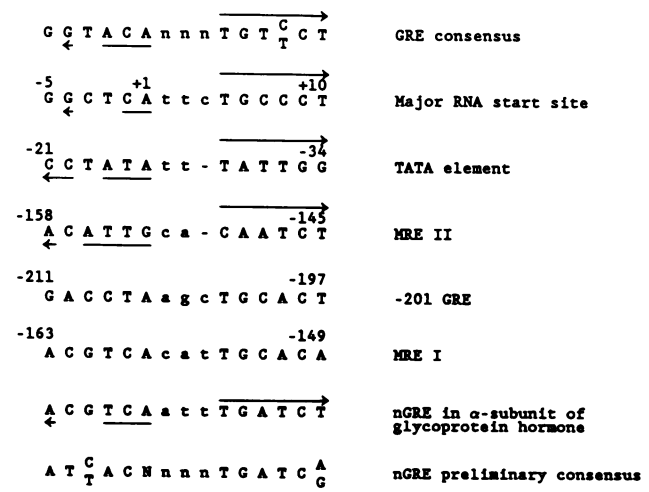

FIG. 7. Nucleotide sequence comparison of the GR-binding sites in the IL-6 promoter with the positive GRE consensus motif (4, 5, 8) and a preliminary negative GRE (nGRE) consensus sequence (4) and the negative GRE sequence described for the promoter of the α-subunit gene of the glycoprotein hormone (2). The major RNA start site contains the Inr motif (CTCANTCT, where N is any nucleotide).

cently identified in the bovine cytochrome P-450$_{17\alpha}$ gene (Fig. 4, bCYP17) (32). The fact that both MRE I and MRE II are induced by phorbol ester and forskolin demonstrates extensive cross-talk (43) between components of the two major signal transduction pathways in activation of the IL-6 promoter.

In the case of MRE I, the CG residues in GACGTCA have been demonstrated to be required for both TPA and forskolin responsiveness (38). IL-6 MRE II identifies a novel DNA motif containing a 14-bp imperfect inverted repeat that is also the target for activation by both protein kinase A and protein kinase C pathways. Furthermore, the fact that induction by IL-1 of pAR11TKC is blocked by Dex in the presence of wild-type GR suggests that the inhibition by GR includes interference with the binding of the newly described nuclear factor NF-IL6 (3, 19) across MRE II.

The theoretical possibility that the TATA box and transcription start site regions of mammalian promoters may be candidate targets for glucocorticoid repression, first suggested from computer analyses of the sequence of the osteocalcin gene promoter (35), has been substantiated by our experiments. Additionally, GR binding appears to interfere with the function of the MRE enhancer region, which is a target for activation by multiple distinct signal transduction pathways. The presence of several different GR-binding target sequences in key functional regions of the IL-6 promoter probably accounts for the efficiency of downregulation of IL-6 gene transcription by glucocorticoids.

The ability of GR to bind to both the TATA box and the major RNA start site in the IL-6 promoter highlights the presence of a perfect Inr motif (51) at the IL-6 RNA start site (Fig. 6). The TATA box and the deoxynucleotidyltransferase Inr motif have been shown to function interchangeably in enhancing in vitro transcription in a TFIID (HeLa-cell derived)-responsive fashion (51), suggesting that some transcription factors (e.g., TFIID) bind to both the TATA box and Inr motifs. Our data show that the transcriptional regulator GR also binds to both the TATA and Inr elements. It is also noteworthy that the minor RNA start site in the IL-6 promoter at −21 overlaps the TATA box, which in turn is overlapped by the GR footprint.

The Inr motif (Fig. 6) is completely conserved in the human, murine, and rat IL-6 genes. Furthermore, sequence motifs resembling the Inr element depicted in Fig. 6 are also found at the RNA start sites in the genes for the glucocorticoid-repressible human cytokines IL-1α (CTTAAGCT) and TNF (CCCAGCCA), raising the possibility that the Inr motif may represent a general target for modulation by GR.

There are different alternative molecular mechanisms by which ligand-activated GR could inhibit IL-6 gene transcription. GR could complex directly with positive-acting transcription factors, interfering with their activity, as was suggested by Adler et al. (1) for glucocorticoid repression of rat prolactin gene expression. An alternative mechanism involves the binding of GR to DNA sequences that either overlap with or serve as targets for positive-acting transcription factors, leading to interference with their function. This appears to be the mechanism for glucocorticoid repression of the bovine prolactin gene (42), the α-subunit gene of the glycoprotein hormone (2), and the pro-opiomelanocortin gene (7).

Our experimental data suggest that glucocorticoid repression of IL-6 gene expression involves direct binding of GR to functional DNA elements in the IL-6 promoter. Dex not only inhibits enhancer activity but also represses IL-6 constructs irrespective of the particular cytokine or second-messenger agonist used. Unlike glucocorticoid repression of other genes, GR inhibits basal IL-6 promoter activity; Dex plus wild-type GR reduces the inducible activity of IL-6/CAT constructs to levels below the basal IL-6 promoter activity in uninduced cells. Dex repression of pseudorabies virus-induced expression of the chimeric gene in pIC110, which lacks the MRE region but contains the basal IL-6 promoter elements, draws attention to GR binding across the TATA box and the Inr elements in repressing the IL-6 promoter. Thus, the highly efficient repression of IL-6 gene expression by glucocorticoids appears to be largely due to the binding of GR to DNA across both the inducible and basal transcription regulatory regions of the IL-6 promoter, which results in interference with gene transcription.

ACKNOWLEDGMENTS

We thank I. Tamm for enthusiastic support and helpful discussions, R. Evans and S. Hollenberg for GR expression plasmids, K. Yamamoto for the purified GR fragment, P. Sassone-Corsi for pTK$_{-105}$CAT, and S. Ceballos for excellent technical assistance.

This work was supported by Public Health Service research grant AI-16262 from the National Institutes of Health and a contract from the National Foundation for Cancer Research.

ADDENDUM IN PROOF

The 30-bp DNA fragment (−102)CCCGCGCGCCAC CCCTCTGGCGCCACCGTG(−73) in the c-*fos* promoter (now designated Rb-control element or RCE) has been functionally identified as a target for repression by the wild-type but not the mutant retinoblastoma susceptibility gene product Rb (41a). This region, which includes an 8-bp direct repeat, was previously shown to be important for basal expression of the c-*fos* gene (41a). Strikingly, the IL-6 DNA sequence (−126)GCCCCACCCGCTCTGGCCCCACCCTC (−101), previously noted to contain a direct repeat with strong similarity to the c-*fos* basal transcription element (38–40), contains a 21 of 26 nucleotide match with the Rb-repressible RCE target motif. This observation raises the possibility that Rb may be involved in the regulation of IL-6 gene expression during development and oncogenesis.

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