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Regulation of interleukin 2 receptor α subunit (Tac or CD25 antigen) gene expression: Binding of inducible nuclear proteins to discrete promoter sequences correlates with transcriptional activation

(DNA–protein interaction/gel retardation assay/tat-I/5′ regulatory region)

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ABSTRACT **Transfection of deleted forms of the human interleukin 2 receptor α subunit (IL-2Rα; also called CD25 or Tac antigen) gene (*IL2RA*) promoter revealed a requirement for sequences 3′ of base −317 for phytohemagglutinin- and phorbol 12-myristate 13-acetate (PMA)-induced promoter activation in CD4$^+$ Jurkat T cells. In contrast, sequences 3′ of base −271 were sufficient for promoter induction in CD4$^-$/CD8$^-$ YT-1 T cells or Jurkat cells expressing the trans-activator protein (tat-I) of human T-cell lymphotropic virus type I (HTLV-I). Gel retardation assays revealed that nuclear extracts from induced, but not uninduced, Jurkat and YT-1 cells mediated the formation of two specific DNA–protein complexes with oligonucleotides spanning the region of the *IL2RA* promoter from position −291 to −245, which contains two imperfect direct repeats (IDRs). Consistent with the different 5′ sequence requirements for promoter activation in Jurkat and YT-1 cells, oligonucleotides corresponding to the region from −267 to −243 (downstream IDR and flanking region) formed only one complex with induced Jurkat extracts but two complexes with induced YT-1 extracts. Oligonucleotides containing the region of the *IL2RA* promoter from −293 to −270 (upstream IDR and flanking region) failed to bind protein in either cell type. In further support of the biological significance of these DNA–protein interactions, the *IL2RA* oligonucleotide from −291 to −245 proved to be sufficient in either orientation to confer PMA inducibility to the mitogen-insensitive thymidine kinase gene promoter in Jurkat cells. Together, these findings suggest that the interaction of inducible DNA binding proteins with the *IL2RA* promoter between bases −291 and −245 plays an important role in mitogen-induced changes in the transcriptional activity of this receptor gene. Furthermore, the requisite 5′ sequences appear to differ in T cells depending upon the nature of the activation signal and perhaps the stage of cellular maturation.**

The proliferation of activated T cells is regulated by the transient secretion of interleukin 2 (IL-2) and the induced expression of high-affinity receptors for IL-2 (IL-2R) (1–4). Resting T cells do not express high-affinity IL-2R but are induced to do so after activation by antigenic or mitogenic stimuli (1–4). Recent studies (5–12) suggest that high-affinity IL-2R corresponds to a complex composed of at least two distinct IL-2 binding subunits including the 55-kDa IL-2R α subunit (IL-2Rα), also called CD25 or Tac antigen (5), and the more recently identified 70-kDa IL-2R β subunit (IL-2Rβ) or p70 chain (6–10). Both of these proteins uniquely contribute to the ability of the high-affinity IL-2R complex to rapidly bind and retain IL-2 (11, 12). In general, resting T cells do not express cell-surface IL-2Rα (CD25, Tac) (1–4); however, some and perhaps all of these cells do display measurable levels of IL-2Rβ (p70) (13). Thus, the regulation of high-affinity IL-2R expression is in part controlled by the dynamic changes in the level of expression of the gene encoding IL-2Rα (CD25, Tac), designated *IL2RA*.

The requirements for induction of *IL2RA* expression in T cells have been well documented (14, 15), and the upstream flanking regions of the *IL2RA* promoter that are involved in transcriptional activation have been recently investigated (refs. 16–18 and this report). We have focused our attention on the potential specific interaction of trans-acting factors (19, 20) with regulatory sequences located within the 5′ flanking region of the gene. In this report, we demonstrate the specific binding of inducible nuclear proteins to defined regulatory sequences of the *IL2RA* promoter and correlate the site of these DNA–protein interactions with regions involved in promoter activation *in vivo*.

MATERIALS AND METHODS

Expression Vectors and *IL2RA* (Tac Gene) Promoter Constructs with the Gene Encoding Chloramphenicol Acetyltransferase (CAT Gene). The pTac-CAT plasmid was constructed in the CAT gene expression vector JymCAT0 by insertion of a 580-base-pair (bp) fragment of the *IL2RA* promoter that extends from a *Bam*HI site 471 bp upstream to a *Hin*dIII site 109 bp downstream of the major cap site (21). The spFMTLTR/82-2Ctat-I cDNA expression plasmid (22) directs the synthesis of biologically active human T-cell lymphotropic virus type I (HTLV-I) trans-activator protein. The spFMTLTR/82-4NC antisense tat-I expression plasmid contains the same tat-I cDNA cloned in the opposite and nonfunctional orientation (22).

Generation of 5′ Deletion Mutants of the *IL2RA* Promoter. The pTac-CAT plasmid was linearized by *Bam*HI digestion, and a nested series of 5′ deletions was generated by BAL-31 (New England Biolabs) treatment. *Kpn* I linkers (Pharmacia) were added and *Kpn* I–*Hin*dIII fragments were ligated into JymCAT0 (previously modified to contain a *Kpn* I site at the original *Cla* I site). The 5′ border of each deletion mutant was determined by DNA sequencing with the dideoxynucleotide chain-termination method. The deletion mutants are desig-

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Abbreviations: IL-1 and IL-2, interleukins 1 and 2; IL-2R, IL-2 receptor; IL-2Rα and IL-2Rβ, α and β subunits of IL-2R; HTLV-I, human T-cell lymphotropic virus type I; IDR, imperfect direct repeat; tat-I, trans-activator protein of HTLV-I; CAT gene, chloramphenicol acetyltransferase gene; TK gene, thymidine kinase gene; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; NRE, negative regulatory element; oligo I, II, or III, oligonucleotide I, II, or III.

nated based on the most 5′ nucleotide of the *IL2RA* promoter sequence present with the numbering system beginning at the major cap site (position +1.)

Cell Transfection and CAT Assay. The human T-cell lines Jurkat (IL-2R α^-, $-\beta^-$), YT-1 (IL-2R$\alpha^{+/-}$, $-\beta^+$) (23), HUT 102B2 (24), and MT-1 (25) were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum and 2 mM L-glutamine. Plasmid DNA (5 μg per 5 $\times$ 10^6 cells) was transfected into cells by using DEAE-dextran as described (26). After 24 hr, phorbol 12-myristate 13-acetate (PMA; 50 ng/ml, Sigma), phytohemagglutinin (PHA; 1 μg/ml, Burroughs Wellcome), interleukin 1 β (IL-1β; Immunex, 10 units/ml) or forskolin (10 μM, Sigma) were added to different cultures. After a further 24-hr period, cell extracts were assayed for CAT activity as described (27). [^{14}C]Chloramphenicol conversion from nonacetylated to acetylated forms was measured after a 6- to 14-hr incubation period, during which time the rate of acetylation was linear (22). All transfection results were confirmed with at least two independent CsCl-banded plasmid preparations.

Nuclear Extracts and Gel Retardation Assay. Nuclear extracts were prepared from induced and noninduced cells (28, 29). Three different double-stranded oligonucleotides (oligos) spanning various regions of the *IL2RA* promoter were synthesized and gel-purified. Oligos I, II, and III contain the *IL2RA* promoter sequences from position -293 to -270, from -267 to -243, and from -291 to -245, respectively. Binding reactions were performed in a volume of 20 μl containing 30% (wt/vol) glycerol, 50 mM KCl, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 1 mg of bovine serum albumin per ml, 2 μg of nuclear extract, and 1 μg of poly[d(I-C)] as nonspecific competitor DNA. After a 10-min incubation at room temperature, ^{32}P-labeled oligonucleotide probe (specific activity, 2–10 $\times$ 10^7 cpm/μg; 10,000 cpm per reaction) was added. After a further 10-min incubation period, the mixture was electrophoresed on a 5% nondenaturing polyacrylamide gel in Tris borate buffer at 10 V/cm. Protein–DNA complexes were visualized after 6–18 hr of exposure to Kodak XAR-2 film at -70°C.

RESULTS

Different Upstream Regions Are Required for *IL2RA* Promoter Activation in Jurkat and YT-1 Cells. To identify cis-acting sequences involved in the regulation of *IL2RA* expression, Jurkat and YT-1 cells were transfected with a series of 5′ deletion mutants of the *IL2RA* promoter. Activation with a number of stimuli including PMA and PHA induced a 4- to 12-fold increase in the level of CAT activity in Jurkat cells transfected with either the -471 or -317 deletion mutant (Fig. 1*A*). In contrast, only a low level of mitogen induction ($\approx$ 2-fold) was observed with the -271 mutant. These results suggested that the 46-bp segment between -317 and -271 was importantly involved in PHA and PMA activation of this promoter. More physiological stimuli, including monoclonal antibodies directed against the Jurkat α/β heterodimeric antigen receptor (MX-6), the CD3 (T3) antigen, and the CD2 (T11) receptor produced similar profiles of response; however, the magnitude of these responses was consistently less than that obtained with PMA (data not shown).

In sharp contrast to the findings in Jurkat cells, the -271 pTac-CAT plasmid proved readily inducible in YT-1 cells stimulated with PMA, IL-1, forskolin (an adenylate cyclase agonist), or combinations of these agents (Fig. 1*B*). Although the -271 construct was about half as active as the -317 deletion mutant in YT-1 cells, it was consistently as active as or more active than the -471 pTac-CAT plasmid. It is also noteworthy that the -471 construct was one-third to one-fourth as active in YT-1 cells as the -317 pTac-CAT plasmid, thus raising the possibility that a negative regulatory element (NRE) exists within this 154-bp region. Interestingly, this NRE did not appear to function in Jurkat cells.

Cotransfection of Jurkat cells with cDNA expression plasmids encoding tat-I induced marked expression of the -271 pTac-CAT plasmid (Fig. 1*C*). In contrast, control plasmids containing the tat-I cDNA cloned in an antisense (noncoding) orientation did not mediate these stimulatory effects. Although PMA was unable to stimulate directly the -271 pTac-CAT plasmid in Jurkat cells, this agent synergized with tat-I, leading to a 5- to 6-fold increase in CAT activity above the level obtained with tat-I alone. Similar results were obtained with PHA (data not shown). In contrast, cotransfection of YT-1 cells with tat-I cDNA did not result in pTac-CAT activation, which is consistent with the notion that the action of the tat-I gene product may be restricted to certain subpopulations of human T cells (22, 30).

Binding of Nuclear Proteins to Specific Regions of the *IL2RA* Promoter. The results of the functional studies focused our attention on the promoter sequences surrounding base -271.

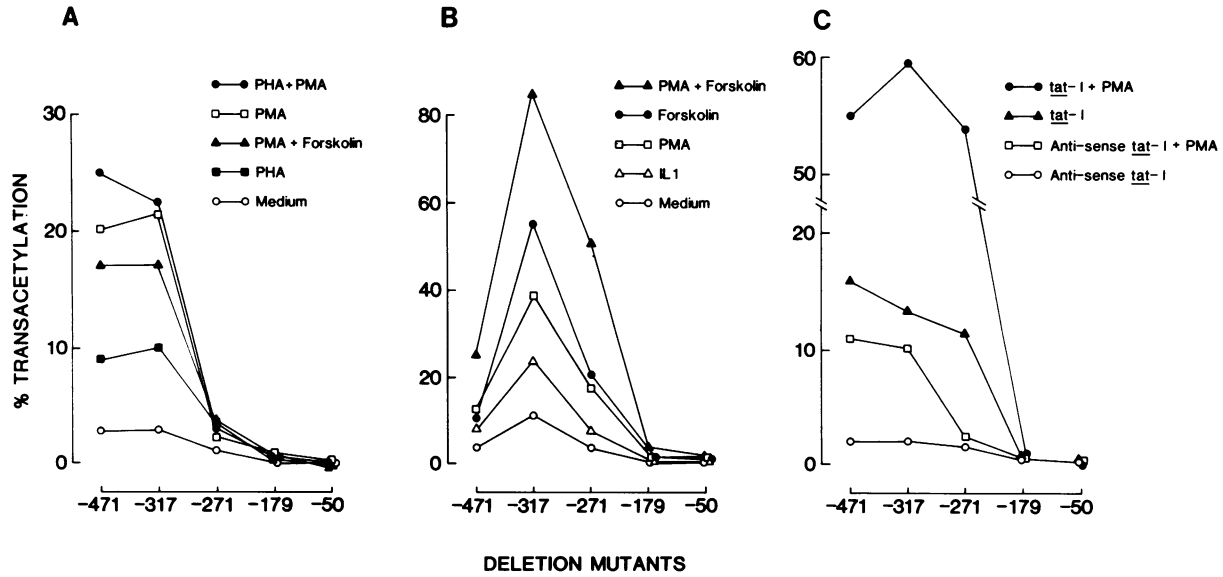

FIG. 1. CAT activity after transfection of cells with various pTac-CAT deletion mutants and induction with different stimuli. (*A*) Jurkat cells. (*B*) YT-1 cells. (*C*) Jurkat cells cotransfected with tat-I or antisense tat-I cDNA expression plasmids. Cells were transfected as described and stimulated as indicated. Similar results were obtained in three to six independent experiments.

The region between bases −293 and −241 is remarkable for the presence of two imperfect direct repeats (IDR). As direct repeat motifs have been implicated in the binding of transcriptional factors in other systems (31), the IDRs within the *IL2RA* promoter represented potential sites of interaction with DNA binding proteins (17). Three different oligonucleotides corresponding to sequences within this region were used as radiolabeled probes in gel retardation assays. Oligo I and oligo II contained sequences spanning the upstream and downstream IDR, respectively, while oligo III encompassed both IDRs (see Fig. 2*C*). Incubation of radiolabeled oligo III with nuclear extracts isolated from induced (Fig. 2*A*, lane 3) but not uninduced (Fig. 2*A*, lane 2) Jurkat cells produced two discrete electrophoretically retarded DNA–protein complexes. The formation of these inducible complexes was completely inhibited by preincubation with a 200-fold molar excess of unlabeled oligo III (Fig. 2*A*, lane 5) but not by equivalent amounts of a size-matched oligonucleotide from the ampicillin-resistance gene (Fig. 2*A*, lane 4). The participation of proteins in the formation of these complexes was indicated by the finding that treatment of the nuclear extracts with proteases, heating for 10 min at 80°C, or adding 1% $NaDodSO_4$ abolished complex formation (data not shown).

Two retarded complexes were also identified after incubation of radiolabeled oligo III with extracts from YT-1 cells stimulated with PMA (Fig. 2*B*, lane 6) or PMA/forskolin (Fig. 2*B*, lane 7). The pattern obtained was similar to that seen with induced Jurkat extracts (Fig. 2*B*, lanes 3 and 4). Extracts from uninduced Jurkat and YT-1 cells failed to produce detectable complexes (Fig. 2*B*, lanes 2 and 5). Similarly, two complexes were also identified using nuclear extracts from the tat-I-producing HTLV-I-infected T-cell lines HUT 102B2 and MT-1 (Fig. 2*B*, lanes 8 and 9). Both of these cell lines constitutively express large quantities of IL-2Rα (CD25 or Tac antigen) (12, 24, 25). In some experiments a much fainter band, which migrated more rapidly than the prominent bands, was also detectable.

Gel retardation assays were performed with induced Jurkat cell extracts and radiolabeled oligo III in the presence of excess amounts of unlabeled oligonucleotides or DNA fragments. As expected, unlabeled oligo III blocked the formation of both complexes (Fig. 3*A*, lane 2). Interestingly, competition with oligo II also prevented the formation of both complexes (Fig. 3*A*, lane 3); however, competition with oligo I was without effect (Fig. 3*A*, lane 4). DNA fragments from the ampicillin-resistance gene were noninhibitory (Fig. 3*A*,

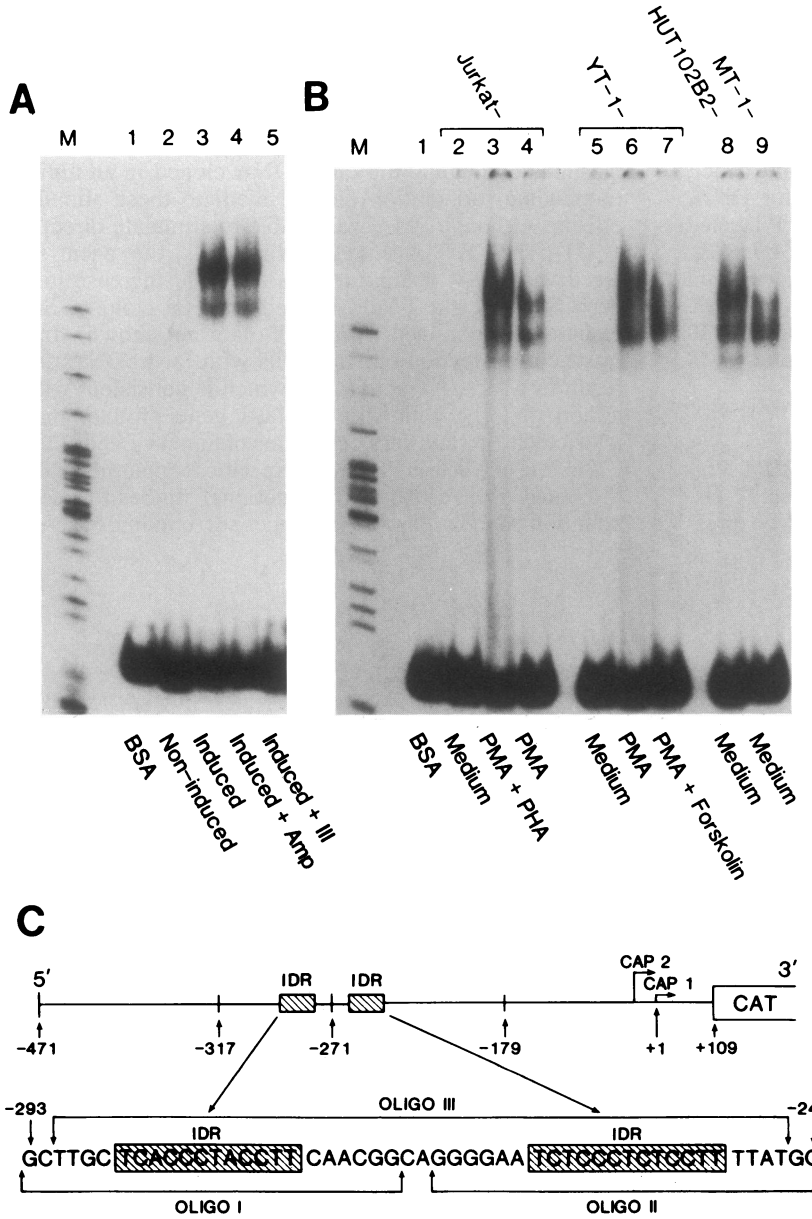

FIG. 2. Gel retardation assays using nuclear extracts from various T-cell lines and ^{32}P-end-labeled double-stranded oligonucleotides corresponding to the sequence of the *IL2RA* promoter from −291 to −245 (oligo III; see *C*). (*A*) Markers are ^{32}P-end-labeled *Hpa* II fragments of pBR322 (lane M). Radiolabeled oligo III was incubated with bovine serum albumin (BSA) (lane 1) or with nuclear extracts from uninduced Jurkat cells (lane 2) or from Jurkat cells induced with PHA (1 μg/ml) and PMA (50 ng/ml) for 5 hr (lane 3). Specificity of protein binding to radiolabeled oligo III was studied by preincubating nuclear extracts with a 200-fold molar excess of unlabeled, size-matched oligonucleotide from the ampicillin (Amp)-resistance gene of pBR322 (lane 4) or with a similar molar excess of unlabeled oligo III. Free radiolabeled oligo III migrated near the bottom of the gel, while two retarded complexes migrated near the top of the gel (lanes 3 and 4). (*B*) Markers are as in *A*. Radiolabeled oligo III was incubated with BSA (lane 1); with nuclear extracts isolated from uninduced Jurkat cells (lane 2) or from Jurkat cells induced with PHA and PMA for 5 hr (lane 3) or with PMA alone for 20 hr (lane 4); or with nuclear extracts isolated from uninduced YT-1 cells (lane 5) or YT-1 cells activated with PMA for 20 hr (lane 6) or with combinations of PMA and forskolin for 20 hr (lane 7). Radiolabeled oligo III was also incubated with nuclear extracts isolated from the HTLV-I-infected, tat-I-producing HUT 102B2 (lane 8) and MT-1 (lane 9) cell lines. (*C*) Schematic of the 0.6-kb *IL2RA* promoter fragment present in the −471 pTac-CAT plasmid. The sequence of the two IDRs and flanking regions located between −293 and −243 as well as the sequences corresponding to oligos I, II, and III utilized in the gel retardation assays are shown.

lane 5), whereas *Kpn* I/*Hin*dIII restriction fragments from the −317 and −271 pTac-CAT plasmids inhibited the binding of both proteins (Fig. 3*A*, lanes 6 and 7). These data suggest that the segment from −267 to −243 spanned by oligo II contains an important site(s) of protein–DNA interaction. Only one DNA–protein complex was identified with radiolabeled oligo II when extracts from induced Jurkat cells were used (Fig. 3*B*, lane 1). In contrast, this probe yielded two complexes when extracts from induced YT-1 cells were tested (Fig. 3*B*, lane 5, and Fig. 3*C*, lane 1). With both extracts, excess unlabeled oligo II or III blocked the formation of both of these complexes while oligo I did not. Incubation of radiolabeled oligo I with PMA-induced YT-1 extract (Fig. 3*C*, lane 2) or PMA-induced Jurkat extract (data not shown) did not result in complex formation.

To test further the biological significance of the DNA–protein interactions observed with oligo III, this 47-bp DNA segment was cloned immediately upstream of the herpes simplex virus thymidine kinase (TK) gene promoter (32) in both orientations as well as a tail-to-tail dimer. Activities of these modified TK-CAT plasmids and the −471 and −317 pTac-CAT constructs were then compared in Jurkat cells in the presence and absence of PMA stimulation. Oligo III, in both orientations, conferred a level of PMA inducibility (3.7- and 3.8-fold induction) to the PMA-insensitive TK gene promoter that was only slightly less than that observed with the −471 and −317 pTac-CAT plasmids (6.8- and 5.7-fold induction) (Table 1). Furthermore, the construct containing the double reiteration of oligo III was induced 11.5 fold by PMA. Similar levels of induction were observed when tat-I was used as the stimulatory agent (data not shown).

Table 1. The region spanning −291 to −245 of the *IL2RA* promoter (oligo III) imparts PMA inducibility to the TK gene promoter in Jurkat cells

	Chloramphenicol conversion in plasmid construct transfected,* %					
	pTac-CAT		Oligo III-TK-CAT			
PMA stimulation	−471	−317	5′–3′ →	3′–5′ ←	←→†	TK-CAT
Without	2.1	1.8	7.5	6.1	3.4	4.7
With	14.2	10.3	27.8	23.2	39.1	7.5
	Fold induction‡					
	6.8	5.7	3.7	3.8	11.5	1.6

Similar results were obtained in three independent experiments with six independently prepared oligo III-TK-CAT plasmids.
*Percent conversion of nonacetylated to acetylated forms of chloramphenicol in transfected Jurkat cells.
†Double reiteration of oligo III.
‡Fold increase in induced CAT activity relative to noninduced cells.

DISCUSSION

Transfection of Jurkat cells with plasmids containing a nested series of deletion mutants of the *IL2RA* promoter revealed that removal of the 46-bp region located between −317 and −271 was associated with a precipitous decrease in inducibility with PMA, PHA, and other T-cell mitogens. Although nearly inactive in PHA or PMA-induced Jurkat cells, the −271 pTac-CAT construct was readily induced in YT-1 cells after stimulation with PMA, IL-1, or forskolin. The level of activity obtained with this construct was consistently as high as or higher than that achieved with the −471 construct but was about half as active as the −371 construct. As the phenotype of Jurkat cells corresponds to that of a mature T-cell population, while YT-1 cells resemble very immature thymocytes (33), it is tempting to speculate that the activation requirements for *IL2RA* expression may differ depending upon the stage of lymphocyte differentiation.

The transfection studies in Jurkat and YT-1 cells revealed another difference involving the function of an NRE within the *IL2RA* promoter. Removal of sequences between −471 and −317 consistently led to the induction of greater CAT activity in YT-1 cells, whereas promoter activity in Jurkat cells was not significantly altered by this deletion. Similar evidence for function of an NRE in the *IL2RA* promoter in HTLV-I-infected MT-2 cells has been described by Cross *et al.* (17). It remains unknown what precise sequences are required and what cellular factors determine whether this NRE is functional.

The tat-I protein of HTLV-I is also capable of activating endogenous *IL2RA* expression (30) and transfected plasmids containing the *IL2RA* promoter in Jurkat cells (refs. 16–18, 22 and this report). However, this viral protein appears unable to exert these effects in YT-1 cells (ref. 22 and our unpublished results). Production of tat-I protein is required for viral replication (34) and may well play a role in leukemic transformation mediated by this retrovirus, perhaps by subverting the normal tightly controlled expression of certain cellular genes, such as those encoding IL-2 and IL-2R. Cotransfection of tat-I cDNA expression plasmid into Jurkat cells led to

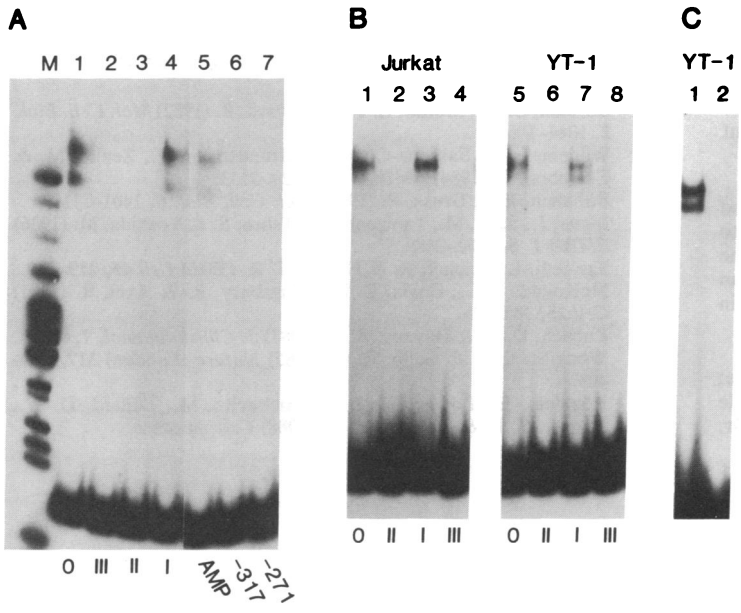

FIG. 3. Competition studies with unlabeled oligonucleotides and DNA fragments in the gel retardation assay. (*A*) Markers are ^{32}P-end-labeled *Hpa* II fragments of pBR322 (lane M). Radiolabeled oligo III (bases −291 to −245) was incubated with nuclear extracts isolated from Jurkat cells induced with PHA and PMA and analyzed either in the absence of competitor oligonucleotides (lane 1) or in the presence of a 200-fold molar excess of unlabeled oligo III (lane 2), unlabeled oligo II (lane 3), unlabeled oligo I (lane 4), ampicillin-resistance gene oligonucleotide (lane 5), or with *Kpn* I/*Hin*dIII DNA fragments isolated from the pTac-CAT deletion plasmids containing bases −317 to +109 (lane 6) or −271 to +109 (lane 7) of the Tac promoter. (*B*) Radiolabeled oligo II (bases −257 to −243) was incubated with nuclear extracts from PHA- and PMA-induced Jurkat cells in the absence of competitor (lane 1) or in the presence of a 200-fold molar excess of unlabeled oligo II (lane 2), unlabeled oligo I (lane 3), or unlabeled oligo III (lane 4). Radiolabeled oligo II was incubated with nuclear extracts isolated from PMA-induced YT-1 cells either in the absence of competitor (lane 5) or a 200-fold molar excess of unlabeled oligo II (lane 6), unlabeled oligo I (lane 7), or unlabeled oligo III (lane 8). (*C*) Radiolabeled oligo II (lane 1) and oligo I (lane 2) were incubated with extracts isolated from PMA-induced YT-1 cells in the absence of competitor.

functional activation of the −271 pTac-CAT plasmid, which was not activated in these cells by various T-cell mitogens. Furthermore, this tat-I-induced response was markedly enhanced by the addition of PMA. Findings in agreement with these regarding the differences in sequence requirements for mitogen and tat-I induction of the *IL2RA* promoter have been described by Cross *et al.* (17) but differ from the report of Maruyama *et al.* (16). The unexpected synergistic effects with PMA and tat-I raises the possibility that additional but conditional PMA-responsive sequences remain in the −271 deletion mutant. Alternatively, this effect may reflect PMA-induced increases in the level of tat-I protein production by the expression plasmid.

These functional studies with deleted forms of the *IL2RA* promoter prompted our examination of the region surrounding base −271 as a possible site for the binding of inducible transcriptional factors. Using a radiolabeled oligonucleotide that contained sequences spanning the two IDRs found within this region (−291 to −245; oligo III), we detected the formation of two sequence-specific protein–DNA complexes with nuclear extracts from induced, but not from uninduced, Jurkat or YT-1 cells. An unlabeled oligonucleotide containing only the downstream IDR and six bases of 5′ and 3′ flanking region (−267 to −243; oligo II) effectively competed for the binding of both proteins to radiolabeled oligo III, whereas an oligonucleotide containing only the upstream IDR and six flanking bases (−293 to −270; oligo I) failed to compete and, when directly tested, did not mediate detectable complex formation. Oligo II supported the formation of two complexes with extracts from induced YT-1 cells, but only a single complex was formed when this probe was incubated with extracts from induced Jurkat cells. These differences in the apparent pattern of protein binding to oligo II paralleled the different 5′ regions required for promoter activation in PMA-induced YT-1 and Jurkat cells.

The potential biological significance of the DNA–protein interactions detected with oligo III was further strengthened by the results of studies linking this small *IL2RA* promoter sequence to the TK gene promoter. The addition of this oligonucleotide in either orientation was sufficient to confer substantial PMA and tat-I inducibility to this mitogen-unresponsive promoter. Furthermore, a double reiteration of oligo III imparted even higher levels of inducibility. Together these results suggest that this region of the *IL2RA* promoter resembles a conditional enhancer element.

In summary, the results of these binding and functional assays suggest that the DNA–protein interactions we have detected play an important role in the mitogen-induced activation of *IL2RA*. However, the involvement of other DNA–protein interactions in activation of this gene is not excluded and in fact seems likely. It is unresolved at present whether the two complexes involve the same or different proteins.

Note Added in Proof. Subsequent to these studies, we (35) mapped the binding site of an inducible protein(s) to a 12-bp region (−267 to −256) of the *IL2RA* promoter. This sequence is homologous to the NF-KB binding sites present in the enhancer elements of human immunodeficiency virus type 1 and the κ light chain immunoglobulin gene.

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