Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8526–8530, November 1989
Immunology

The same 50-kDa cellular protein binds to the negative regulatory elements of the interleukin 2 receptor α-chain gene and the human immunodeficiency virus type 1 long terminal repeat

(DNA–protein interaction/transcriptional repressor/crosslinking)

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Communicated by Joseph E. Rall, August 11, 1989 (received for review May 25, 1989)

ABSTRACT **We have investigated the biochemical basis for negative regulation of interleukin 2 receptor α-chain (IL-2Rα) gene expression. Transient transfection studies employing internally deleted forms of the IL-2Rα promoter localized a negative regulatory element (NRE) between nucleotides −400 and −368 relative to the major distal transcription start (cap) site. This 31-base-pair (bp) element is involved in the attenuation of both basal and inducible IL-2Rα promoter activity. Comparison of this IL-2Rα NRE with other known regulatory motifs revealed an 11-bp core element (TTCATCCCAGG) that was strikingly similar to a protein-binding domain within the long terminal repeat of the type 1 human immunodeficiency virus (HIV-1). This viral domain has been previously implicated in the negative control of HIV-1 gene expression. *In vitro* protein–DNA binding studies demonstrated that the same constitutively expressed ≈50-kDa protein (SP-50) specifically bound to both the IL-2Rα and HIV-1 NRE core elements. Mutation of the 11-bp IL-2Rα NRE core element, which disrupted protein binding, significantly augmented basal as well as Tax protein- or phorbol ester-induced IL-2Rα promoter activity *in vivo*, suggesting that SP-50 functions as a transcriptional silencer.**

The proliferation of human T lymphocytes is regulated in part by the coordinate expression of the cellular genes encoding the T-cell growth factor interleukin 2 (IL-2) and its high-affinity membrane receptor (IL-2R) (1, 2). The functional high-affinity IL-2R complex is composed of at least two IL-2-binding protein subunits, IL-2Rα (Tac, p55, CD25) (3) and IL-2Rβ (p70–75) (4–8). Since some resting T cells constitutively express IL-2Rβ (7, 8), the transcriptional activation of the IL-2Rα gene contributes importantly to both the regulated display of high-affinity IL-2R and T-cell proliferation. IL-2Rα gene expression is induced by a variety of stimuli, including antigen, cytokines such as tumor necrosis factor α (9), the transactivator protein (Tax) of the type I human T-cell leukemia virus (HTLV-I (10, 11), and various nonspecific mitogens such as phytohemagglutinin and phorbol 12-myristate 13-acetate (PMA) (12).

Prior transfection studies with the human YT-1 leukemic T-cell line revealed that deletion of IL-2Rα promoter sequences between −471 and −317 (numbering relative to +1 at the major distal cap site) resulted in a marked increase in both basal and inducible promoter activity (13). Similar findings have been reported by Cross *et al.* (14) for the HTLV-I-infected human T-cell line MT-2. Together, these results raised the possibility that promoter sequences upstream of −317 contain a negative regulatory element (NRE). In the present study, we have defined this upstream NRE and identified a constitutively expressed nuclear protein of ≈50 kDa that specifically interacts with this IL-2Rα promoter element as well as a related sequence element present in the long terminal repeat (LTR) of type 1 human immunodeficiency virus (HIV-1).

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MATERIALS AND METHODS

Cell Lines and Nuclear Extract Preparation. The human T-cell lines YT-1 and MT-2 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. Nuclear extracts were prepared as described (15) from cells either cultured in medium alone or stimulated with PMA (50 ng/ml) for 4–16 hr.

Plasmid Constructions. The −471 and −317 5′-deletion mutants of the IL-2Rα promoter linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene have been described (13). Additional internal deletions and point mutations were introduced into the −471 IL-2Rα promoter–CAT plasmid (see Fig. 1*A*) by oligonucleotide-directed *in vitro* mutagenesis (16). All mutations were confirmed by DNA sequencing.

Cell Transfection. Plasmid DNA (10 μg per 10^7 cells) was transfected into cells with DEAE-dextran (13). After 24 hr, the cultures were either incubated in medium alone or stimulated with PMA (50 ng/ml). After an additional 24 hr, cell extracts were prepared and assayed for CAT activity and total protein recovery (17).

Oligonucleotide Preparation. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and cleaved with ammonia. These synthetic DNAs were recovered by using oligonucleotide-purification cartridges (Applied Biosystems) and further purified by preparative polyacrylamide gel electrophoresis (18).

***In Vitro* DNA–Protein Binding Studies.** Gel shift assays were performed as described (19), using ≈5 μg of extracted nuclear proteins. DNA–protein crosslinking studies were performed using a 27-base-pair (bp) IL-2Rα (−399 to −373) or HIV-1 LTR (−179 to −153) probe substituted with 5-bromo-2′-deoxyuridine and α-^{32}P-labeled deoxynucleotides on the noncoding strand (20). These photoreactive probes were incubated with nuclear extracts under ultraviolet light (300 nm) at a distance of 5 cm for 30 min in the presence or absence of a 500-fold molar excess of unlabeled oligonucleotide competitors. DNA–protein complexes were resolved by electrophoresis through low-ionic-strength 5% polyacrylamide gels. The relevant complexes were subsequently excised and further analyzed in SDS/12.5% polyacrylamide gels under reducing conditions.

Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; IL-2Rα, IL-2 receptor α subunit; NRE, negative regulatory element; HTLV-I, type I human T-cell leukemia virus; HIV-1, type 1 human immunodeficiency virus; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase.

RESULTS

To define more precisely the NRE upstream of nucleotide −317 in the IL-2Rα gene, transfection studies were performed using a series of internally deleted promoter mutants (Fig. 1*A*). Comparison of the activity of these plasmid constructs in MT-2 cells (Fig. 1*B*) revealed that the removal of the region between nucleotides −471 and −317 produced a marked increase in reporter gene activity. The internal deletion of sequences between −440 and −317 (Δ440/317) or −400 and −317 (Δ400/317) conferred a similar 4- to 5-fold increase in promoter activity. In contrast, deletion of sequences between −369 and −317 (Δ369/317) resulted in little or no change in promoter activity relative to the −471 construct. These results suggested that a functional NRE is located between nucleotides −400 and −369. Consistent with this interpretation, deletion of the 31-bp segment between −400 and −368 (Δ400/368) resulted in a marked increase in reporter gene activity. A similar pattern of promoter activity was observed in YT-1 cells (Fig. 1*C*). In both cell lines, the relative inducibility by PMA was similar for each deletion mutant. The increases in promoter activity produced by deletion of the 31-bp element in YT-1 cells indicate that both the basal and PMA-inducible activities of the IL-2Rα promoter are negatively regulated. Since MT-2 cells are infected with HTLV-I and constitutively produce Tax protein (14), the results with MT-2 suggest that this 31-bp element is also capable of downregulating Tax-induced promoter activity. The inducibility of the IL-2Rα promoter by PMA in MT-2 cells may reflect, in part, increased production of Tax protein, since the HTLV-I LTR is activated by PMA (21). Of note, promoter activity induced by combinations of Tax and PMA was also negatively regulated by IL-2Rα sequences located between −400 and −368.

Comparison of this 31-bp IL-2Rα negative control region with other known regulatory motifs revealed an 11-bp core element (TTCATCCCAGG, −390 to −380) that shared significant sequence similarity (9 out of 11 nucleotides) with a domain in the HIV-1 LTR (TTCATCACATG, −170 to −160; (Fig. 2*A*). Interestingly, this HIV-1 domain overlaps with the 3′ boundary of a NRE of the HIV-1 LTR previously identified by functional analysis of a series of 5′ deletion mutants of the HIV-1 LTR (22). Additionally, *in vitro* (23), and *in vivo* (24) DNase I protection studies have demonstrated that this HIV-1 domain represents a binding site for one or more constitutively expressed host cellular factors.

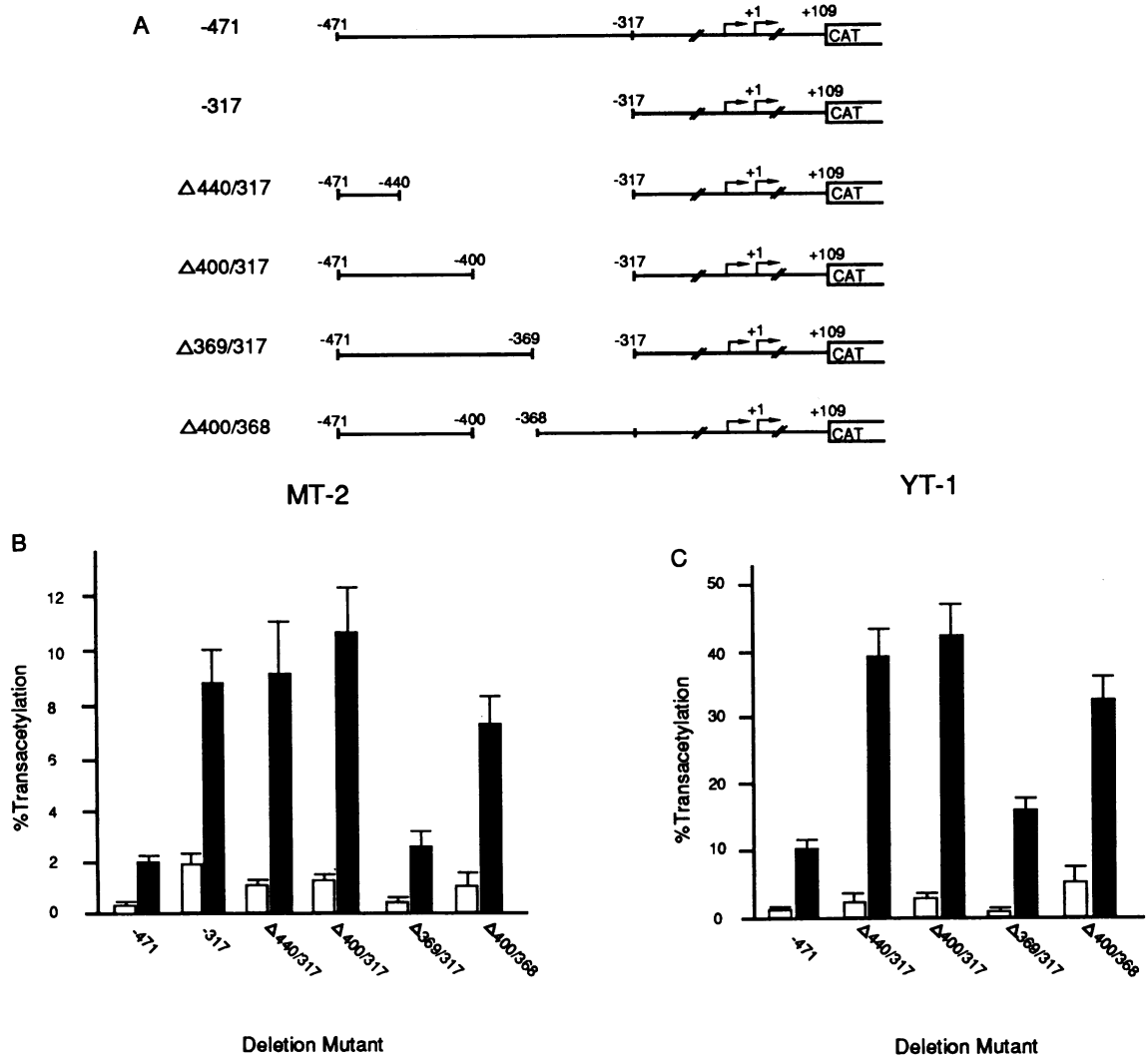

FIG. 1. CAT activity in YT-1 and MT-2 cells transfected with various IL-2Rα NRE deletion mutants. (*A*) Schematic representation of the various IL-2Rα promoter–CAT expression plasmids. Position of the major 3′ cap site is identified as +1. (*B* and *C*) Each plasmid was transfected into MT-2 cells (*B*) or YT-1 cells (*C*) cultured in medium alone (open bars) or stimulated with PMA (solid bars). Percent transacetylation of chloramphenicol was determined and normalized for protein recovery. Data shown represent the mean CAT activity from three different transfections. Error bars indicate the standard error of the mean. Similar results were obtained with at least two independent preparations of each plasmid DNA.

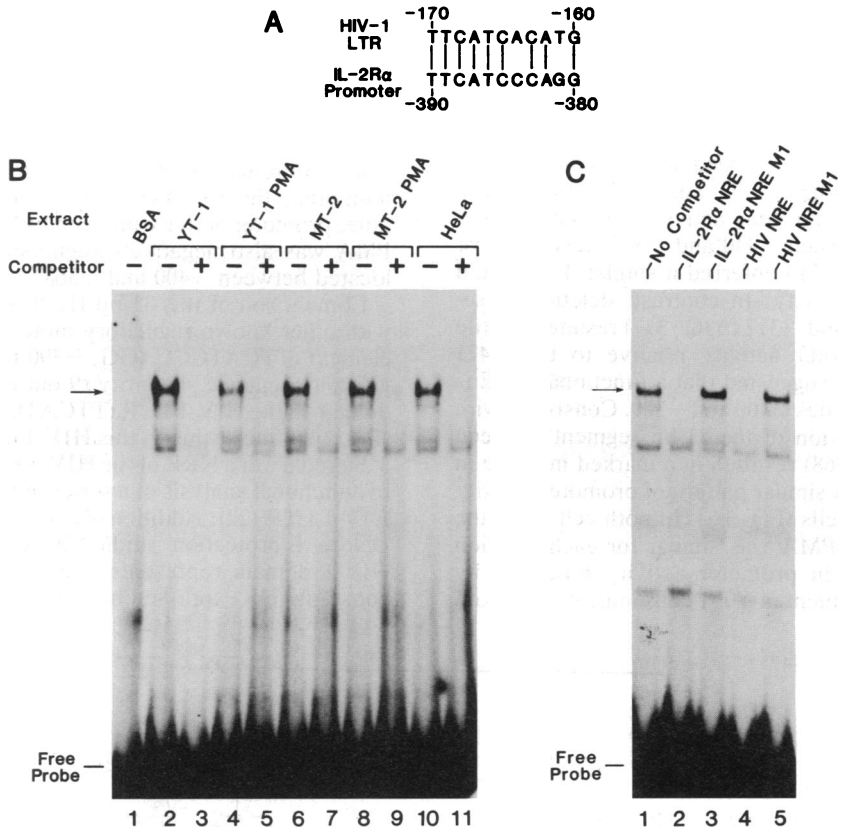

FIG. 2. Identification of sequence-specific trans-acting nuclear factors. (*A*) Sequence similarity between a subregion of the IL-2Rα NRE and sequences within a cellular protein-binding domain in the HIV-1 LTR. Positions of nucleotide identity are indicated by vertical lines. This 11-bp region of sequence similarity is designated the NRE core element. (*B*) Gel shift assays performed with ^{32}P-end-labeled IL-2Rα NRE probe (−406 to −367) and nuclear extracts prepared from various cell lines. Radiolabeled IL-2Rα NRE probe was incubated with bovine serum albumin (BSA) (lane 1) or extracts prepared from YT-1 cells (lanes 2 and 3), YT-1 cells stimulated with PMA (lanes 4 and 5), MT-2 cells (lanes 6 and 7), MT-2 cells stimulated with PMA (lanes 8 and 9), or HeLa cells (lanes 10 and 11). Reaction mixtures analyzed in lanes 3, 5, 7, 9, and 11 were preincubated with a 250-fold molar excess (relative to radiolabeled probe) of unlabeled probe. This preincubation step inhibited formation of the single retarded complex identified by the arrow. (*C*) Competition for DNA–protein complex formation by IL-2Rα and HIV-1 oligonucleotide competitors. Nuclear extracts prepared from YT-1 cells were incubated with a 250-fold molar excess of various competitors prior to the addition of ^{32}P-end-labeled IL-2Rα NRE probe. Competitors included IL-2Rα NRE (lane 2), IL-2Rα NRE M1 (an IL-2Rα NRE mutant competitor containing a 5-bp substitution, CATCCC → AGATCT, at positions −388 to −383 within the NRE core element; lane 3), HIV NRE (nucleotides −185 to −147; lane 4), and HIV NRE M1 (an HIV NRE mutant containing a 6-bp substitution, CATCAC → AGATCT, at positions −168 to −163 within the HIV-1 NRE core element; lane 5). No specific competitor DNA was added to the binding reaction analyzed in lane 1.

This sequence similarity raised the possibility that the IL-2Rα NRE core element might constitute a functional binding site for one or more nuclear proteins. To investigate this possibility, gel shift assays were performed with a 40-bp radiolabeled IL-2Rα NRE probe (−406 to −367) containing this core element. Incubation of this probe with nuclear extracts prepared from unstimulated YT-1 cells resulted in the formation of a single specific DNA–protein complex (Fig. 2*B*, lane 2; indicated by arrow) that was inhibited by preincubation with a 250-fold molar excess of homologous unlabeled probe (Fig. 2*B*, lane 3). Nuclear extracts prepared from unstimulated and PMA-stimulated YT-1 and MT-2 cells and from HeLa cells produced DNA–protein complexes with indistinguishable electrophoretic mobilities (Fig. 2*B*, lanes 2–11), suggesting that this nuclear protein(s) is expressed in a constitutive and non-lymphoid-specific manner.

To confirm the involvement of the 11-bp NRE core element in these DNA–protein interactions, competitive binding studies were performed with a mutant competitor that contained a 5-bp substitution within this element (IL-2Rα NRE M1, CATCCC → AGATCT). While complex formation was inhibited by preincubation with an excess of the wild-type IL-2Rα NRE competitor, the IL-2Rα NRE M1 mutant competitor failed to inhibit complex formation (Fig. 2*C*, compare lanes 2 and 3). Additional gel shift assays using IL-2Rα NRE M1 as the radiolabeled probe confirmed that this 5-bp substitution eliminated protein binding (data not shown). Of note, complex formation was also inhibited by a 40-bp oligonucleotide competitor containing the HIV-1 NRE core element (HIV NRE, −185 to −147), whereas a mutant HIV NRE competitor containing a 6-bp substitution in the NRE core element (HIV NRE M1, CATCAC → AGATCT) failed to compete (Fig. 2*C*, compare lanes 4 and 5). These findings suggest that IL-2Rα and HIV-1 NRE core elements may interact with the same constitutively expressed nuclear protein(s).

To analyze the functional significance of this sequence-specific DNA–protein interaction, transfections were performed using an IL-2Rα promoter construct (−471 M1) that contained the same 5-bp substitution (CATCCC → AGATCT, −388 to −383) that eliminated DNA–protein complex formation *in vitro*. Introduction of this mutation into the −471 construct resulted in a marked increase in promoter activity in MT-2 cells (Fig. 3*A*), comparable to the increase produced by deletion of sequences between −400 and −368. Similar results were obtained in transfected YT-1 cells incubated in medium alone or stimulated with PMA (Fig. 3*B*). These findings suggest that the interaction of nuclear protein(s) with the IL-2Rα NRE core element plays an important role in the negative regulation of this growth factor receptor gene.

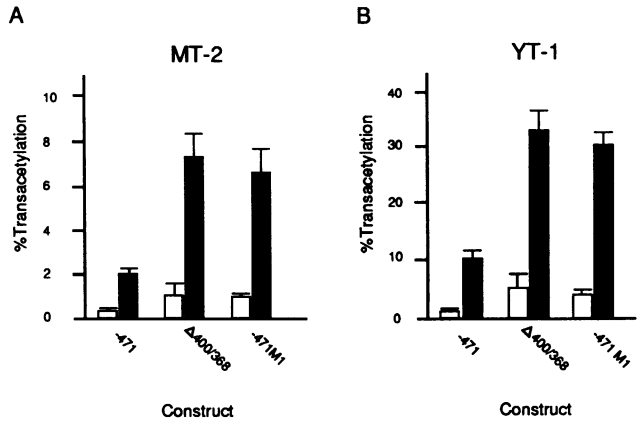

FIG. 3. Upregulation of IL-2Rα promoter activity by mutation within the IL-2Rα NRE core element. A 5-bp substitution (CATCCC → AGATCT, positions −388 to −383) was introduced into the −471 plasmid by oligonucleotide-directed *in vitro* mutagenesis to generate the −471 M1 construct. Transfection studies using this vector were performed with MT-2 cells (*A*) and YT-1 cells (*B*) cultured in medium alone (open bars) or stimulated with PMA (closed bars). Results represent the mean CAT activity detected in three transfections.

Placement of this IL-2Rα NRE upstream of the herpes virus thymidine kinase promoter failed to downregulate promoter activity relative to plasmids containing the mutated NRE core element (data not shown).

To further characterize the nuclear protein(s) involved in this transcriptional attenuation, DNA–protein crosslinking studies were performed using a 27-bp photoreactive probe containing the NRE core element of the IL-2Rα promoter (−399 to −373). The resultant covalent DNA–protein complexes were resolved by electrophoresis through low-ionic-strength gels and further analyzed by SDS/PAGE under reducing conditions. In the absence of oligonucleotide competitor (Fig. 4, lane 1), three major crosslinked products were observed. Formation of the crosslinked species migrating at ≈50 kDa was inhibited by preincubation with an excess of the wild-type IL-2Rα NRE competitor but not the mutant IL-2Rα NRE M1 competitor (Fig. 4, compare lanes 2 and 3), indicating this 50-kDa crosslinked species represents an NRE-specific interaction. In contrast, formation of the more slowly migrating crosslinked products was inhibited by both the wild-type and mutant IL-2Rα NRE competitors. Since the mutant IL-2Rα oligonucleotide competitor contains the same mutation that was sufficient to activate the IL-2Rα promoter *in vivo*, it would appear that the binding of the 50-kDa protein is required for negative regulation. Analogous crosslinking studies using a 27-bp photoreactive probe containing the NRE core element of the HIV-1 LTR (−179 to −153) demonstrated that a constitutively expressed protein of 50 kDa also specifically interacts with this related HIV-1 element (Fig. 4, lanes 4–6). These findings suggest that the same constitutively expressed 50-kDa nuclear protein, designated SP-50 (silencer protein of 50 kDa), binds to the IL-2Rα and HIV-1 NRE core elements and functions as a transcriptional silencer.

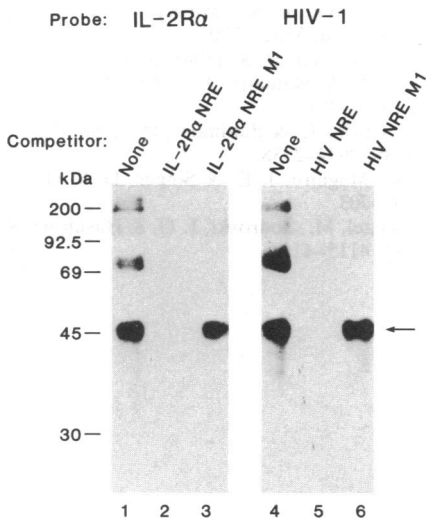

FIG. 4. Ultraviolet crosslinking of DNA-binding proteins to the NRE core elements of the IL-2Rα promoter and HIV-1 LTR. A 27-bp photoreactive IL-2Rα probe (−399 to −373) was incubated with nuclear extract prepared from unstimulated HeLa cells in the absence of oligonucleotide competitor (lane 1) or in the presence of a 500-fold molar excess of the wild-type IL-2Rα NRE (lane 2) or mutant IL-2Rα NRE M1 competitor (lane 3). Analogous experiments were performed with a photoreactive 27-bp HIV-1 LTR probe (−179 to −153) (lanes 4–6). DNA–protein complexes were irradiated with ultraviolet light, resolved by electrophoresis in low-ionic-strength polyacrylamide gels, and analyzed by SDS/12.5% PAGE under reducing conditions. Formation of the 50-kDa crosslinked product was inhibited by wild-type but not mutant NRE competitors. In contrast, formation of the more slowly migrating crosslinked species was inhibited by both wild-type and mutant oligonucleotide competitors.

DISCUSSION

In this study, we have identified and characterized a functional NRE present in the IL-2Rα promoter. An 11-bp core element (TTCATCCCAGG) of this IL-2Rα NRE shares significant sequence similarity with a protein-binding domain in the HIV-1 LTR that has been implicated in the negative regulation of HIV-1 gene expression (22). The introduction of a point mutation within this core element enhanced basal and Tax- or PMA-inducible activity of the IL-2Rα promoter in the human T-cell lines studied. Further, this mutation also disrupted the binding of a constitutively expressed 50-kDa nuclear protein, SP-50, to this site. These data suggest that this element mediates the negative regulation of IL-2Rα gene transcription by a mechanism involving at least one sequence-specific DNA–protein interaction.

It remains to be determined whether the 50-kDa protein identified in our DNA–protein crosslinking studies is functionally involved in downregulating HIV-1 gene expression via an interaction with the retroviral LTR. Binding of the 50-kDa protein, and perhaps additional factors, may be responsible for the previously reported DNase I protection of these related sequences in the HIV-1 LTR (23, 24). Further, it is conceivable that the binding of these nuclear factor(s) to the HIV-1 element may play an important role in transcriptional attenuation of HIV-1 gene expression and maintenance of retroviral latency.

Several recent studies of other inducible cellular genes have illustrated potential mechanisms and biological roles for transcriptional attenuation by trans-acting factors. For example, transcriptional induction of the β-interferon gene appears to involve the displacement of a silencer protein from the promoter, thereby permitting the binding of other transcription factors to adjacent or overlapping positive regulatory domains (25). Similarly, the IL-2 gene is negatively regulated by the sequence-specific binding of a silencer protein to an NRE, and transcriptional activation includes the inactivation or displacement of this silencer protein (26). Since this silencer protein is displaced only in activated T lymphocytes, negative regulation of this cellular gene may play an important role in tissue-specific expression of IL-2.

The mechanism of negative regulation of the IL-2Rα gene appears to be distinct from that of the β-interferon and IL-2 genes, since the NRE core element does not overlap with any

known positive promoter elements. However, transcriptional attenuation by the IL-2Rα NRE appears to require additional specific cis-acting elements, since promoter fragments spanning the NRE core element are insufficient to downregulate the herpes virus thymidine kinase promoter. In this regard, we note that in addition to related NREs, the IL-2Rα promoter and HIV-1 LTR share several positive promoter elements, including binding sites for the transcription factor NF-κB (19). Negative regulation by SP-50 could involve effects on the κB enhancer, or another cis element common to both promoters. As a precedent for this model, studies of a porcine class I major histocompatibility gene have identified a NRE whose function is dependent upon a distant enhancer element (27).

In summary, our results demonstrate that negative regulation of the IL-2Rα gene involves the sequence-specific binding of a constitutively expressed 50-kDa transcriptional silencer protein to an upstream promoter element. Sequence similarity of this cis-acting element with a segment of the NRE present in the HIV-1 LTR suggests that SP-50 may mediate related transcriptional phenomena in HIV-1.

Note Added in Proof. Lu *et al.* (28) have recently demonstrated that deletion of the HIV-1 NRE (−419 to −157) markedly augments viral replication in both Jurkat (T-cell lymphoma) and U-937 (monocytic) cell lines.

We thank Hal Bogerd and Janice Hoffman for expert technical assistance, Drs. Dean W. Ballard and Ernst Böhnlein for valuable discussions, Dr. Richard Randall and Kathleen Theisen for oligonucleotide synthesis, and Dr. Junji Yodoi for the YT cell line. M.R.S. is supported by the Medical Scientist Training Program.

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