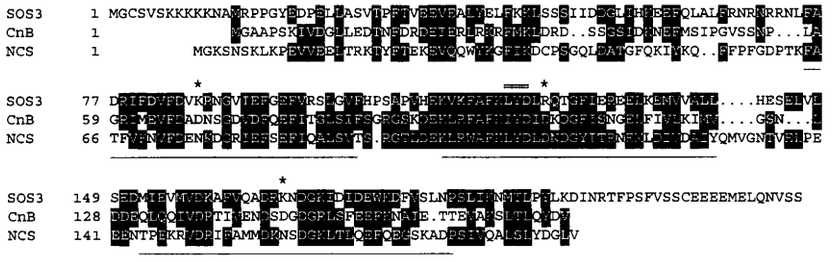

Fig. 3. Sequence comparison of SOS3 (GenBank accession number AF060553) with yeast CnB subunit (*16*) and frog NCS (*19*). Dots indicate gaps introduced to maximize the sequence alignment. Residues identical or similar in at least two of the three sequences are shaded dark or light, respectively. The three predicted EF hands (*15*) of SOS3 are underlined. Asterisks indicate the basic residues in the Ca^{2+}-binding sites of SOS3 where conserved acidic residues are found in other EF hand proteins (*15*). The double line marks residues deleted in the *sos3-1* allele (*21*).

that SOS3 responds to the Ca^{2+} signal by activating a protein phosphatase or inhibiting a protein kinase (or by doing both) that then regulates K^+ and Na^+ transport systems. Although there do not appear to be conspicuous differences between the cytosolic Ca^{2+} signals elicited by drought and salinity (*26*), subtle differences in their kinetics and subcellular spatial arrangement could result in drought- or salinity-specific responses. The specific role of SOS3 in the tolerance of the ionic but not the osmotic component of salt stress (*9*) strongly supports the existence of ionic stress–specific calcium signaling.

For plants, the amount and interactions of three abundant soil cations, Ca^{2+}, K^+, and Na^+, are essential determinants of potassium nutrition and salt tolerance and therefore greatly affect plant productivity. Our evidence suggests that SOS3 mediates the interaction of K^+, Na^+, and Ca^{2+}.

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10. Overlapping YAC clones were isolated from YAC libraries of *Arabidopsis* [E. R. Ward and G. C. Jen, *Plant Mol. Biol.* **14**, 561 (1990); J. R. Ecker, *Methods* **1**, 186 (1990); E. Grill and C. Somerville, *Mol. Gen. Genet.* **226**, 484 (1991)]. Overlapping BAC clones were isolated from BAC libraries of *Arabidopsis* [S. Choi, R. A. Creelman, J. E. Mullet, R. A. Wing, *Weeds World* **2**, 17 (1995)]. End probes were isolated from the YAC and BAC clones by inverse PCR. Restriction fragment length polymorphism (RFLP) analysis was performed with F2 *sos3* plants with recombination break points in either the *nga139-SOS3* region (four recombinants) or the *SOS3-CDPK9* region (14 recombinants).
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14. A labeled Bam HI–Kpn I fragment of *SOS3* genomic DNA (Fig. 1B) was used as probe to screen an *Arabidopsis* cDNA library [J. J. Kieber, M. Rothenburg, G. Roman, K. A. Feldmann, J. R. Ecker, *Cell* **72**, 427 (1993)]. RT-PCR was performed with mRNA from *Arabidopsis* leaves.
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Stabilization of Interleukin-2 mRNA by the c-Jun NH_2-Terminal Kinase Pathway

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Signaling pathways that stabilize interleukin-2 (IL-2) messenger RNA (mRNA) in activated T cells were examined. IL-2 mRNA contains at least two cis elements that mediated its stabilization in response to different signals, including activation of c-Jun amino-terminal kinase (JNK). This response was mediated through a cis element encompassing the 5′ untranslated region (UTR) and the beginning of the coding region. IL-2 transcripts lacking this 5′ element no longer responded to JNK activation but were still responsive to other signals generated during T cell activation, which were probably sensed through the 3′ UTR. Thus, multiple elements within IL-2 mRNA modulate its stability in a combinatorial manner, and the JNK pathway controls turnover as well as synthesis of IL-2 mRNA.

Gene expression is controlled at the transcriptional and posttranscriptional levels. Posttranscriptional regulation of gene expression in eukaryotic cells includes mRNA processing, turnover, and translation. Although control of gene transcription by extracellular stimuli through DNA-binding proteins has been widely studied (*1*), relatively little is known about regulation of mRNA turnover (*2*). Stability of mRNA is determined by cis-acting elements within the mRNA molecule, believed to be recognized by regulatory proteins (*2*). Such cis elements positively or negatively modulate mRNA stability and are present throughout the mRNA, including the coding region

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and 3′ UTR. The 3′ UTRs of rapidly decaying mRNAs usually contain an adenosine- or uridine-rich element (ARE), characterized by multiple copies of the pentanucleotide AUUUA (*3*). In chimeric constructs, the ARE can destabilize normally stable transcripts (*4*). Many cytokine genes, such as those coding for granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor, interferon-γ, IL-1, IL-2, and IL-3, are regulated transcriptionally, but their mRNAs also contain multiple AUUUA motifs (*4*, *5*), and their stability is regulated in response to extracellular stimuli. When produced in nonstimulated cells, such transcripts are unstable, but their half-lives are prolonged after cell activation (*4*, *6*–*10*). Ca^{2+} ionophores, such as A23187, stabilize IL-3 mRNA (*10*) as well as GM-CSF mRNA, which is also stabilized in cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (*4*, *6*).

The AREs of GM-CSF or IL-3 are sufficient to confer regulation of mRNA turnover in response to Ca^{2+} signals (*9*, *10*). However, ARE-containing transcripts are differentially regulated. In a monocytic tumor cell line, the 3′ UTR of c-*fos* or c-*myc* destabilizes a reporter mRNA, whereas the 3′ UTR of GM-CSF does not (*11*). Stimulation of quiescent T cells with antibodies to the T cell receptor (TCR)–CD3 complex and the CD28 auxiliary receptor increases the stability of several cytokine mRNAs, whereas c-*fos* and c-*myc* mRNAs remain labile (*7*).

The relatively short half-life ($t_{1/2}$ = 30 to 60 min) of IL-2 mRNA in unstimulated T cells (*12*) is prolonged by incubating the cells with antibodies that ligate the TCR-CD3 complex and CD28 (*7*) or by treatment with TPA and A23187 (*12*). Like other short-lived cytokine mRNAs, IL-2 mRNA contains several AUUUA motifs in its 3′ UTR (*4*). To study posttranscriptional regulation of IL-2 gene expression, we prepared a transgene composed of full-length IL-2 cDNA under control of the chicken β-actin promoter (*13*) and transiently transfected it into Jurkat cells derived from a T cell leukemia. Total RNA was isolated at various times after the addition of actinomycin D (ActD), an inhibitor of transcription, and the $t_{1/2}$ of transfected IL-2 mRNA was determined by a semiquantitative competitive reverse transcriptase–polymerase chain reaction (RT-PCR) (*14*). The IL-2 transcripts were unstable, with a $t_{1/2}$ of 40 min, in unstimulated Jurkat cells (Fig. 1, A and C) (*12*). Stimulation of cells with A23187 or

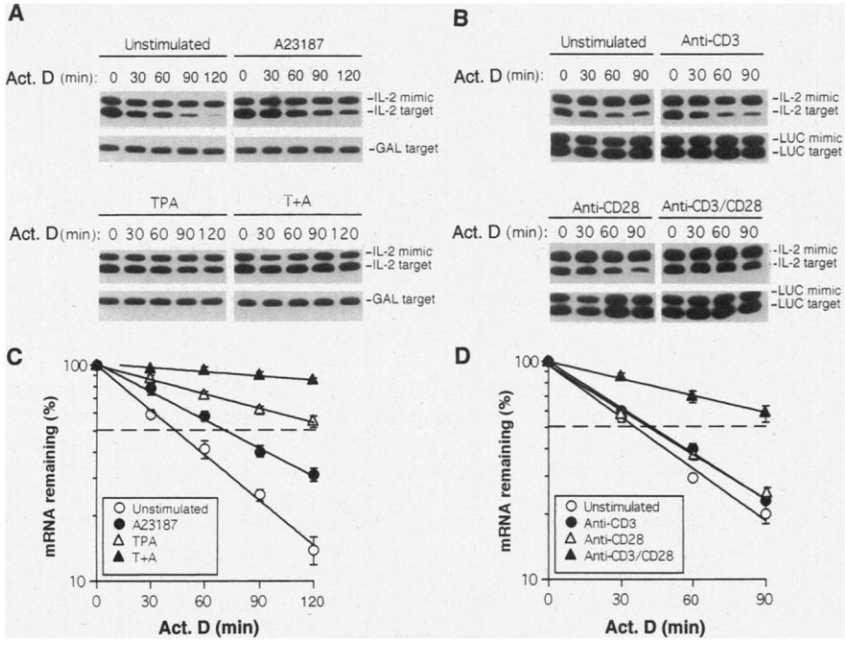

Fig. 1. Stabilization of IL-2 mRNA in activated Jurkat T cells. (**A** and **B**) Stabilization of IL-2 mRNA by T cell activators. Jurkat cells were transiently cotransfected with an IL-2 transgene and with a control plasmid expressing either β-galactosidase (GAL) or luciferase (LUC) mRNAs containing SV40 3′ UTR, which served as an internal control. The cells were left unstimulated or stimulated as indicated with A23187 (1 μg/ml), TPA (15 ng/ml), anti-CD3 (10 ng/ml), or anti-CD28 (2 ng/ml), either alone or in combinations, together with ActD (5 μg/ml). Total RNA was isolated at various times, and the amount of IL-2 mRNA was analyzed by a semiquantitative competitive RT-PCR (*14*). IL-2 mimic is a PCR product derived from a DNA template that was added to each reaction to control for amplification efficiency. IL-2 target is derived from IL-2 mRNA. Both PCR mimic and target were amplified with the same primer set. GAL or LUC mRNAs were also amplified by semiquantitative PCR. (**C** and **D**) IL-2 signals in A and B were quantitated with a PhosphorImager, normalized to the GAL or LUC signals, and plotted on a semilogarithmic scale by a linear regression program against the time of ActD addition. Each point represents the average of two transfection experiments.

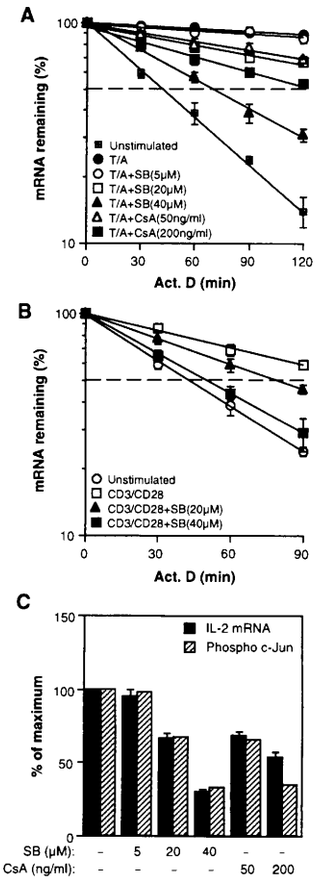

Fig. 2. Effect of anti-inflammatory drugs on stabilization of IL-2 mRNA. (**A**) Decay of IL-2 mRNA in transfected Jurkat cells stimulated with TPA and A23187 and incubated in the presence of the indicated concentrations of SB202190 (SB) or CsA. Each point represents the average of two transfection experiments. (**B**) Decay of IL-2 mRNA in transfected Jurkat cells stimulated with anti-CD3 and anti-CD28 in the presence of the indicated concentrations of SB202190. Each point is the average of two transfection experiments. (**C**) The relative amounts of IL-2 mRNA remaining 3 hours after addition of ActD in the presence of TPA and A23187 and the indicated concentrations of SB202190 or CsA and relative amounts of c-Jun phosphorylation at Ser^{63}. The relative amounts of IL-2 mRNA were determined as described (Fig. 1). Phosphorylation of c-Jun at Ser^{63} was determined by immunoblotting lysates of the same cells used for RNA determination to phospho–c-Jun antibodies (*29*). Immunoreactivity was quantitated with a PhosphorImager after visualization by enhanced chemiluminescence. The amounts of IL-2 mRNA or phospho–c-Jun in cells stimulated with TPA and A23187 in the absence of SB202190 were set at 100%.

TPA increased IL-2 mRNA stability, and stimulation with both A23187 and TPA further increased this stabilization (Fig. 1C). Antibodies to CD3 or to CD28 alone did not stabilize IL-2 mRNA, but stimulation of cells with both antibodies increased the $t_{1/2}$ from 40 min to 90 min (Fig. 1, B and D). Similarly, stimulation of Jurkat cells with combinations of A23187 and TPA or anti-CD3 and anti-CD28 synergistically activated the c-Jun NH_2-terminal kinase (JNK) and p38 or Mpk2 groups of mitogen-activated protein kinases (MAPKs), whereas treatment of Jurkat or normal T cells with anti-CD3 or anti-CD28 alone had little effect on the activation of these kinases (*15*). We therefore examined the effect of SB202190 [a p38 inhibitor (*16*)] and cyclosporin A (CsA) [an immunosuppressive drug that specifically inhibits the Ca^{2+}-sensitive phosphatase calcineurin (*17*) and thereby blocks the Ca^{2+}-dependent increase in JNK or p38 activities (*15*)] on stabilization of IL-2 mRNA. The combination of TPA and A23187 induced IL-2 mRNA stabilization that was inhibited by SB202190 or CsA in a dose-dependent manner (Fig. 2A). The $t_{1/2}$ of IL-2 mRNA in cells treated with both TPA and A23187 decreased to 70 min in the presence of 40 μM SB202190 or to 120 min after treatment with CsA (200 ng/ml). Stabilization of IL-2 mRNA by anti-CD3 and anti-CD28 was also inhibited by SB202190 (Fig. 2B). The concentration of SB202190 required to destabilize IL-2 mRNA was considerably greater than that required to efficiently inhibit p38 activity in other cell types (*16*, *18*). We therefore examined the effect of SB202190 on JNK activity, activation of which leads to phosphorylation of c-Jun at Ser^{63} and Ser^{73} (*19*). Similar concentrations of SB202190 and CsA were required to affect c-Jun phosphorylation and turnover of IL-2 mRNA (Fig. 2C).

We also cotransfected the IL-2 reporter with expression vectors encoding activated MKK6 (MKK6D/D), a MAPK kinase (MAPKK) that activates p38 but not JNK or extracellular signal-regulated kinase (ERK) (*20*); truncated MEKK1, a MAPKK kinase (MAPKKK) that unless overexpressed activates the JNK cascade and has little effect on either p38 or ERK (*21*); activated JNKK2 (JNKK2-act), an activator of JNK but not of p38 or ERK (*22*); or activated Raf-1 (RafBXB), a MAPKKK for the ERK cascade (*23*) that has no effect on JNK or p38. Immune-complex kinase assays confirmed the specificity of these enzymes (Fig. 3A). Although cotransfection of constitutively activated MKK6 or Raf-1 did not lead to stabilization of IL-2 mRNA, cotransfection with MEKK1 or JNKK2-act increased the $t_{1/2}$ of IL-2 mRNA to ~90 min (Fig. 3B). The effect of MEKK1 was smaller than that of A23187 and TPA but still was similar to the effect of anti-CD3 and anti-CD28. These results indicate that the JNK pathway, but not the p38 or ERK MAPK cascades, leads to stabilization of IL-2 mRNA.

We also examined the effects of inactive mutants of JNKK2 or JNK2. Coexpression of either JNKK2(AA), which cannot be activated by upstream stimuli (*22*), or JNK2(GE), which is defective in binding adenosine triphosphate (ATP) (*24*), attenuated the increase in IL-2 mRNA stability that was caused either by expression of MEKK1, stimulation of CD3 and CD28, or incubation with TPA and A23187 (Fig. 3, C to E). Neither mutant adversely affected MEKK1 expression (*25*), and neither mutant

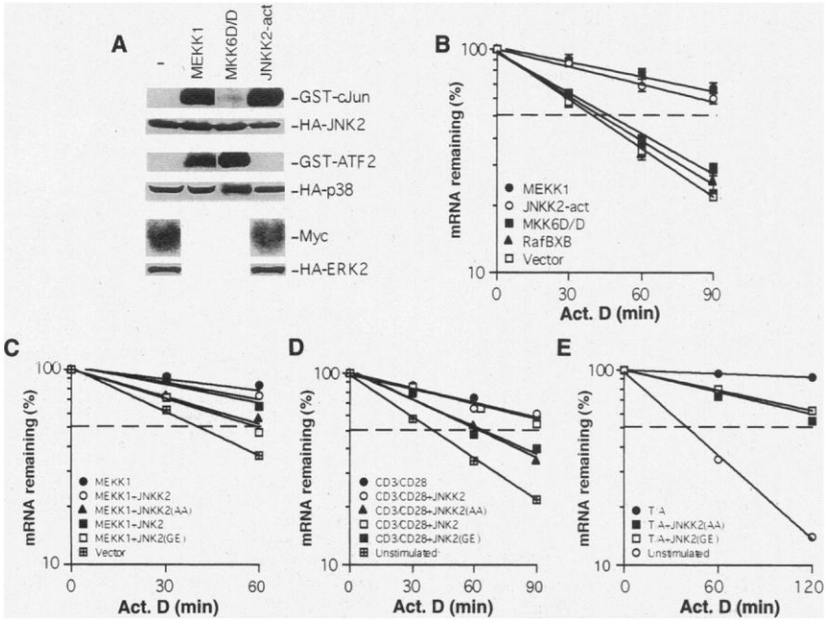

Fig. 3. Stabilization of IL-2 mRNA by activation of the JNK pathway. (**A**) Stimulation of JNK, p38, or ERK activity by MEKK1, JNKK2, or MKK6. Jurkat cells were cotransfected with either hemagglutinin A (HA)–JNK2 (10 μg), HA-p38 (10 μg), or HA-ERK2 (10 μg) in combination with MEKK1 (1 μg), JNKK2-act (5 μg), or MKK6D/D (4 μg) expression vectors. The cells were lysed and the various MAPKs were isolated by immunoprecipitation with anti-HA, and their activity was determined by immune-complex kinase assays (*21*) with GST-cJun(1–79), GST-ATF(1–122), or His-Myc as substrates for JNK, p38, or ERK, respectively. The amounts of JNK, p38, or ERK in each sample were determined by immunoblotting with anti-HA. (**B**) Effect of kinase expression vectors on IL-2 mRNA decay. Results are averages of three transfection experiments. (**C** to **E**) Effect of JNKK2 or JNK2 mutants on IL-2 mRNA stabilization by MEKK1 expression (C), anti-CD3 and anti-CD28 (D), or TPA and A23187 (E). Neither mutant adversely affected MEKK1 expression (*25*). Averages of two transfection experiments are shown.

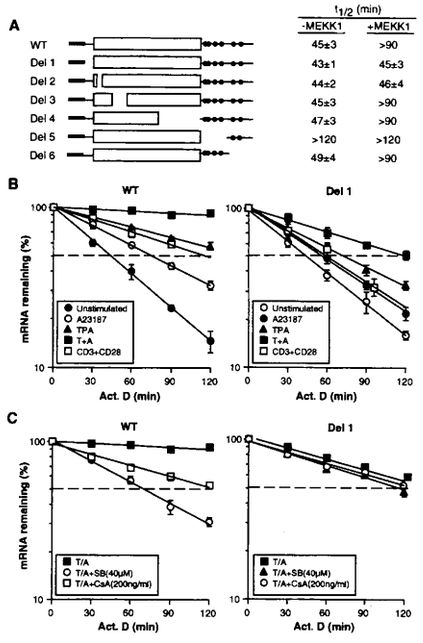

Fig. 4. Identification of a cis element mediating MEKK1-induced IL-2 mRNA stabilization. (**A**) Schematic representation of the WT and mutant IL-2 reporter constructs. Open rectangles represent the IL-2 coding region; the 5′ and 3′ UTRs are indicated by thin lines. Thick lines represent the 5′ UTR of β-actin, which precedes that of IL-2. Filled circles indicate the AUUUA motifs. The average $t_{1/2}$ of each transcript in the absence or presence of MEKK1 is indicated on the right. (**B**) Decay of WT and Del 1 IL-2 mRNAs in mitogen-stimulated Jurkat cells. (**C**) Decay of WT and Del 1 IL-2 mRNAs in mitogen-stimulated Jurkat cells treated with anti-inflammatory drugs.

gave more than 50% inhibition of JNK activation or c-Jun phosphorylation (*22*, *24*, *25*), consistent with their partial destabilizing effect. Coexpression of wild-type (WT) JNKK2 or JNK2 did not result in destabilization (Fig. 3, C and D). The weaker effect of the JNK2 and JNKK2 mutants on the response to TPA and A23187, in comparison with their effects on the responses to MEKK1 or anti-CD3 and anti-CD28, suggests that additional signaling pathways responsive to TPA or Ca^{2+} ionophore (or both) may participate in stabilization of IL-2 mRNA. In addition, this would be consistent with the stronger effect of TPA and A23187 on IL-2 mRNA stability.

To determine which sequences mediated the response to JNK activation, we constructed several IL-2 deletion mutants (*26*). WT and mutant reporters were transfected with MEKK1 or empty expression vectors, and the stability of the corresponding IL-2 transcripts was measured (Fig. 4A). Del 5, in which four closely clustered AUUUA motifs in the 3′ UTR were removed, was constitutively stable, indicating that this region contains the instability determinant (or determinants). Deletion of nucleotides (nt) 130 to 200 (Del 3) or nt 350 to 550 (Del 4) or the last two of the AUUUA motifs (Del 6) did not prevent rapid mRNA decay, and all of these mutants were stabilized in cells transfected with MEKK1. By contrast, expression of MEKK1 did not stabilize transcripts missing the IL-2 5′ UTR (Del 1) or nt 67 to 114 (Del 2). These results indicate that sequences between nt 1 and 130 of IL-2 mRNA contain a cis element that mediates the response to MEKK1. This region is also required for mitogen-induced mRNA stabilization because removal of the 5′ UTR impaired the response to TPA, to A23187, to TPA and A23187, or to anti-CD3 and anti-CD28 (Fig. 4B). The residual response of Del 1 to TPA and A23187 was not inhibited by SB202190 or CsA (Fig. 4C) and is therefore not mediated through the JNK pathway or calcineurin. Thus multiple distinct response elements within the IL-2 mRNA determine stabilization in response to stimulation of various signaling pathways during T cell activation.

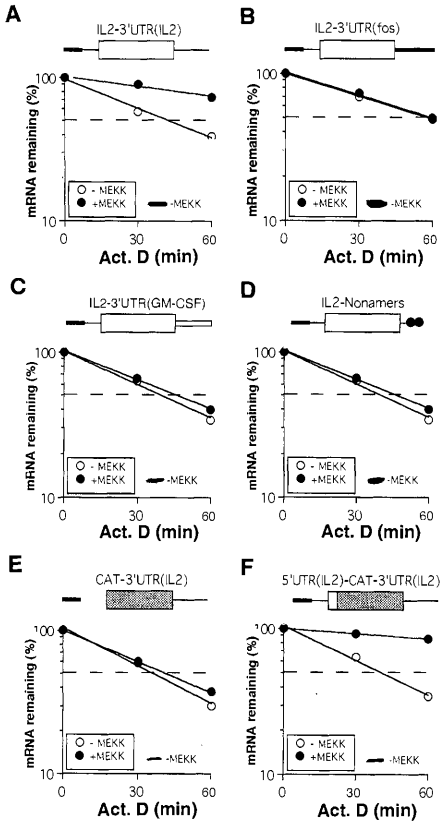

Fig. 5. Role of 5′ and 3′ UTRs of IL-2 in the response to JNK activation. Jurkat cells were transiently cotransfected with (**A**) WT IL-2 construct or (**B**) constructs in which the native IL-2 3′ UTR was replaced with the 3′ UTRs of *c-fos* or GM-CSF (**C**), or with a twofold repeat of UUAUUUAUU nonamers (**D**), or with CAT reporters containing the IL-2 3′ UTR (**E**), or with both the 5′ cis element (nt 1 to 130) and the 3′ UTR of IL-2 (**F**) and either an empty vector or a MEKK1 expression vector. The stability of each transcript in transfected cells was determined in the absence or presence of MEKK1 (Fig. 1). Shown are the averages of two transfection experiments. (**Inset**) The amount of MEKK1 expression in one transfection experiment, measured in the same lysates used for RNA isolation.

To examine whether the 3′ UTR of IL-2 mRNA was also required for MEKK1-induced stabilization, we replaced it with the 3′ UTRs of c-*fos* or GM-CSF (*27*), which also contain two or more AUUUA motifs, or with a synthetic 3′ UTR composed of UUAUUUAUU nonamers, which destabilizes stable transcripts (*28*). With the exception of the chimeric transcripts containing c-*fos* 3′ UTR, which were more stable, the $t_{1/2}$ values of the transcripts were similar to that of IL-2 mRNA but were not stabilized upon coexpression of MEKK1 (Fig. 5, A to D). Thus, sequences within the IL-2 3′ UTR are apparently required to function together with the 5′ cis element to confer JNK-mediated stabilization. To determine whether both 5′ and 3′ elements are the only signals required to confer JNK-mediated stabilization of IL-2 mRNA, we constructed chloramphenicol acetyltransferase (CAT) reporter genes (*27*). Chimeric CAT mRNA containing the 3′ UTR of IL-2 at its 3′ end was unstable and was not stabilized by coexpression of MEKK1. By contrast, chimeric CAT mRNA consisting of nt 1 to 130 and the 3′ UTR of IL-2 at its 5′ and 3′ ends, respectively, was stabilized by MEKK1 coexpression (Fig. 5, E and F).

Our results demonstrate that in addition to its role in activation of IL-2 transcription (*15*), JNK promotes stabilization of IL-2 mRNA in activated T cells. The 5′ UTR of IL-2 mRNA, which does not contain destabilizing elements, may contain a cis element that interacts with a cytoplasmic protein targeted by the JNK pathway. As with combinatorial control of transcription initiation (*1*), turnover of mRNA is apparently regulated through distinct cis-acting elements that respond to distinct signaling pathways.

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13. To construct the IL-2 reporter, we isolated a Bgl II–Hind III fragment, containing the promoter sequences and exon 1, intron 1, and part of exon 2 of the chicken β-actin gene that encodes its 5′ UTR, from pRc/βact [A. Helmberg, N. Auphan, C. Caelles, M. Karin, *EMBO J.* **14**, 452 (1995)] and subcloned it between the Bam HI and Hind III sites of pBluescript II KS (Stratagene) to create pβACT. The full-length IL-2 cDNA, including the 5′ UTR, coding region, 3′ UTR, and polyadenylation signal, was amplified by RT-PCR with oligonucleotides (sense, 5′-CAGAAGCTTATCACTCTCTTTA-ATCACTACTC-3′; antisense, 5′-CCAGGTACCTAT-ATTTATCAAATTTATTAAATAGTTTTAC-3′) with RNA isolated from TPA/A23187-stimulated Jurkat cells, digested with Hind III and Kpn I, and subcloned between the Hind III and Kpn I sites of pβACT to create pβACT-IL2.
14. Normal Jurkat cells were electroporated with pβACT-IL2 (2 μg) and either pβACT-LUC (2 μg) or pβACT-GAL (2 μg) as described (*15*). Total RNA was isolated by RNAzol (Tel-Test) at various times after addition of ActD, and samples (5 μg) were reverse-transcribed with oligo(dT) and Moloney murine leukemia virus reverse transcriptase (Promega). The amounts of IL-2 or LUC mRNA present were determined by competitive PCR [I. Kramnik, E. Skamene, D. Radzioch, *J. Immunol. Methods* **162**, 143 (1993)], in which one-tenth of the RT reaction was co-amplified with a determined amount of IL-2 or LUC DNA templates (mimic), which were constructed from pβACT-IL2 or pβACT-LUC and can be amplified by the same set of primers used to amplify IL-2 or LUC cDNA (target) to control for amplification efficiency. PCR products of mimic and target differ in size, as distinguished by polyacrylamide gel electrophoresis (PAGE). PCR amplification was done in 50 μl of 1

× PCR buffer [10 mM Tris (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each dinucleoside triphosphate) containing 5 μCi of [α-^{32}P]dCTP, 0.5 μM of each primer set, and 2.5 units of AmpliTaq Gold (Perkin-Elmer). The amount of each mimic DNA added to the reaction to obtain similar amplification between mimic and target [usually 0.1 to 0.5 amol (10^{-18} mol) of mimic DNA was added depending on transfection efficiency] and the number of amplification cycles required to avoid overamplification were determined by initial amplification reactions. Samples were amplified by repeated cycles (usually 30 cycles) at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. A portion (10 μl) of each reaction mixture was subjected to electrophoresis on 5% polyacrylamide gels, dried, and visualized by autoradiography. The following primers were used: 5′-TCTGACTGACCGCGTTACTC-3′ and 5′-TTCT-TCTAGACATAGAAGATGTTTCAGTTC-3′ for IL-2; 5′-TCTGACTGACCGCGTTACTC-3′ and 5′-CACT-GCATA CGACGATTCTGT-3′ for LUC; and 5′-TCT-GACTGACCGCGTTACTC-3′ and 5′-TGCAAGGC-GATTAAGTTGGG-3′ for GAL.

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26. Various regions of IL-2 cDNA were deleted. Del 1 was constructed by creating an Eco RV site at codon 4 and codon 5 of IL-2 with PCR-based method to obtain pβACT-IL2RV. The Hind III to Eco RV fragment was replaced with an oligonucleotide that restored the start codon of IL-2. Del 2 was constructed by removing a Eco RV to Afl II fragment from pβACT-IL2RV and replacing it with a PCR fragment digested with Apa LI/Afl II, in which nt 67 to 114 were removed. Del 3 was constructed by subcloning two PCR fragments—nt 1 to 130, which had been digested with Hind III and Bgl II, and nt 200 to 350, which had been digested with Bgl II and Afl II—between the Hind III and Afl II sites of pβACT-IL2. Del 4 and Del 5 were constructed by digesting pβACT-IL2 with Afl II and Stu I, and with Stu I and Sty I, respectively, and then religating with T4 DNA ligase. Del 6 was constructed by removing a Sty I-Kpn I fragment pβACT-IL2 and replacing it with an oligonucleotide containing the polyadenylation signal of IL-2. None of the IL-2 mutants altered the reading frame.
27. The entire 3′ UTR, including the polyadenylation signal of human *c-fos* or GM-CSF amplified by RT-PCR, and a chemically synthesized double-stranded oligonucleotide containing two nonamers, UUAUUUAUU-gauccUUAUUUAUU, and a polyadenylation signal were subcloned between the Stu I and Kpn I sites of pβACT-IL2. CAT reporters were constructed by subcloning the entire coding region of the *CAT* gene, amplified by PCR, between the Hind III and Stu I sites of pβACT-IL2 to obtain CAT-3′ UTR (IL2) or between the Bgl II and Stu I sites of Del 3 to obtain 5′ UTR (IL2)–CAT–3′ UTR (IL2), in which the reading frame of CAT is in-frame with that of IL-2.
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29. Jurkat whole-cell extract (50 μg of protein) was resolved by SDS-PAGE and transferred to an Immobilon P membrane (Millipore). After blocking, the filter was incubated with an antibody specific to phospho-Ser^{63}-c-Jun (KM-1, Santa Cruz Biotechnology), and the antibody-antigen complexes were visualized by enhanced chemiluminescence (Amersham). The membrane was stripped and reprobed with an antibody to c-Jun (N-G, Santa Cruz Biotechnology).
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A Conserved HIV gp120 Glycoprotein Structure Involved in Chemokine Receptor Binding

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The entry of primate immunodeficiency viruses into target cells depends on a sequential interaction of the gp120 envelope glycoprotein with the cellular receptors, CD4 and members of the chemokine receptor family. The gp120 third variable (V3) loop has been implicated in chemokine receptor binding, but the use of the CCR5 chemokine receptor by diverse primate immunodeficiency viruses suggests the involvement of an additional, conserved gp120 element. Through the use of gp120 mutants, a highly conserved gp120 structure was shown to be critical for CCR5 binding. This structure is located adjacent to the V3 loop and contains neutralization epitopes induced by CD4 binding. This conserved element may be a useful target for pharmacologic or prophylactic intervention in human immunodeficiency virus (HIV) infections.

Most naturally occurring primate immunodeficiency viruses [HIV and simian immunodeficiency virus (SIV)] bind the β-chemokine receptor CCR5 as an obligate step in virus entry into target cells (*1*, *2*). The gp120 glycoproteins of primary, macrophage-tropic HIV-1 strains have been shown to bind specifically to cells expressing CCR5 (*3*, *4*). Inhibiting this binding constitutes an attractive means of intervening in HIV-1 infection, but the gp120 glycoprotein is a challenging target because of its conformational flexibility and variability. Incubation with soluble CD4 (sCD4) results in a 100- to 1000-fold increase in the affinity of HIV-1 gp120 for CCR5 (*3*), indicating that the CCR5-binding site on HIV-1 gp120 is fully formed or exposed only after CD4 binding. Efficient CCR5 binding is dependent on the presence of the V3 variable loop of gp120 (*3*), and the sequence of the V3 loop influences the specific chemokine receptors used by different primate immunodeficiency viruses (*2*). In contrast, the gp120 V1 and V2 variable loops and NH_2- and COOH-termini were shown to be dispensable for high-affinity binding to CCR5 (*3*). No significant CCR5 binding has been observed for gp120 glycoproteins derived from laboratory-adapted HIV-1 isolates, which do not use CCR5 as a coreceptor (*3*, *4*).

Specific groups of HIV-1–neutralizing antibodies directed against the gp120 V3 loop or CD4-induced (CD4i) epitopes are able to block the binding of gp120-sCD4 complexes to CCR5-expressing cells (*3*, *4*). The CD4i epitopes are conserved, discontinuous gp120 structures that are better exposed after CD4 binding (*5*). Mutagenic analysis suggested that elements of the conserved stem of the V1/V2 stem-loop and of the fourth conserved region of gp120 make up the CD4i epitopes (*5*). Here we tested the hypothesis that conserved gp120 residues near or within the CD4i epitopes are critical for CCR5 binding.

We developed an assay to assess the CCR5-binding ability of a panel of HIV-1 gp120 glycoprotein mutants. The mutants were created by the introduction of single amino acid changes in gp120 residues near or within regions previously shown to be

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