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NF-κB homodimer binding within the HIV-1 initiator region and interactions with TFII-I

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ABSTRACT We show that the binding of Rel p50 and p52 homodimers at sites within the transcriptional initiation region of HIV-1 provides for their ability to interact with other proteins that bind the initiator. The binding of one such protein, the initiator protein TFII-I, to the initiation region of HIV-1 is augmented in the presence of Rel p50 and Rel p52 homodimers. Consistent with this, *in vitro* Rel homodimers potentiate HIV-1 transcription in a manner dependent upon TFII-I. The findings suggest that Rel dimers may regulate HIV-1 transcription in two ways. First, through binding at the κB enhancer sites at (−104 to −80), NF-κB p50:p65 participates in classical transcriptional activation. Second, Rel dimers such as p50 or p52 might bind at initiator sequences to regulate the *de novo* binding of components of certain preinitiation complexes. These findings, and the existence of Rel binding sites at the initiators of other genes, suggest roles for Rel proteins in early events determining transcriptional control.

Transcription of HIV-1 is a controlled process, involving both host and viral factors whose regulatory activities converge at the long terminal repeat (LTR) (1). Within the LTR are multiple cis-acting motifs. The core promoter elements include the TATA and the initiator (Inr) motifs, both of which can position the precise nucleotide at which RNA synthesis initiates (2–6). These and other promoter proximal elements, such as the Sp1 sites (7, 8), the HIV-1 κB enhancer, and the recently recognized C/EBP elements (9), control transcription by regulating the activity of bound TBP and TAFS at the TATA element (10, 11).

Many cellular stimuli that are known to activate HIV-1 gene expression function by increasing the bound levels of Rel-related transcription factors, such as NF-κB p50:p65 and NF-κB p52:p65, to the HIV-1 κB enhancer at −104 to −80 (12–15) or NFAT (S. Kinoshita, M.A.M., and G.P.N., unpublished data). Despite a critical role of the HIV-1 κB enhancer in HIV-1 transcription, some studies indicate that HIV-1 virus remains replication competent (16, 17) and inducible (4, 18) in the absence of the HIV-1 κB enhancer in some cell types. Although it is likely that the κB enhancer motifs are important for activation by mitogens or cytokines, other transcription factors might also control HIV-1 gene expression through separate enhancer or promoter elements, such as the C/EBP binding site (9). As HIV-1 is known to replicate in a variety of cellular contexts, it is probable that the HIV-1 promoter/LTR has evolved a variety of compensatory or redundant mechanisms that ensure viral expression.

During identification of additional control elements within the HIV-1 LTR, we delineated two novel κB sites overlapping the initiator region of the HIV-1 promoter/LTR. The binding of Rel homodimers at these sites suggests an involvement in the *de novo* assembly of preinitiation complexes (PIC) dependent on TFII-I, in contrast to the more familiar role of NF-κB in cis-acting classical transcriptional activation through the upstream κB elements.

MATERIALS AND METHODS

Promoter Constructs and Expression Vectors. The pHIV-lacZ plasmid contains the Arv-2 HIV-1 LTR (*Kpn*I/*Hin*dIII) fragment driving expression of the lacZ gene. Expression vectors for NF-κB p50 (19) and p65 (20) were as described (21, 22); the p52 expression vector was a kind gift of Colin Duckett and G. Nabel (University of Michigan).

Protein Purification and Antibodies. *Escherichia coli* over-expressing Rel p50 or p52 was prepared essentially as described (23). Extracts expressing p50 or p52 were passed over a nonspecific DNA column and then a specific DNA affinity column (GGGGAATTCCC; ref. 23), washed with 50 mM KCl in buffer, and eluted in a KCl gradient. TFII-I was purified as described and used previously (5, 6). Antibodies to NF-κB p50 were prepared as described (21). Rabbit polyclonal antibodies to TFII-I were prepared and used as described (41).

Electrophoretic Mobility-Shift Assays. DNA-binding assays were carried out as previously described (19, 20). The DNA probes used were (*i*) InrκB2: 5′-TTG ACT GGG AGC TCT CTG ACA-3′/5′-TGT CAG AGA GCT CCC AGT CA-3′; (*ii*) InrκB1: 5′-TTG ACT GGG TCT CTC TTG ACA-3′/5′-TGT CAA GAG AGA CCC AGT CAA-3′; and (*iii*) Ig/HIV-1 κB: 5′-TTG ACT GGG ACT TTC CTG ACA-3′/5′-TGT CAG GAA AGT CCC AGT CAA-3′.

***In Vitro* Transcription.** *In vitro* transcription experiments were performed with nuclear extracts from HeLa cells (HeLaScribe, Promega). Preincubations with Rel proteins or antibody were performed on ice for 10 min with the DNA template prior to addition of nuclear extracts. Extracts (8 units per ≈20 μg) were incubated at 30°C for 1 hr in a 20 μl reaction as described by the manufacturer using 0.5 μg DNA template.

Transfections and β-Galactosidase Assays. Target plasmids were transfected at 10–50 ng per well in Costar 24-well plates containing ≈10^5 cells per well using a modified calcium phosphate coprecipitation technique (24). Cells were assayed for β-galactosidase activity using the methyl-umbelliferone-galactoside (MUG) assay (25). Fluorescence was determined in a Fluoroskan plate reader (Flow Laboratories).

RESULTS

NF-κB p50 Binds the Initiator Region of HIV-1. We performed a computer analysis of the Arv-2 HIV-1 LTR for binding sites of known transcription factors and revealed two novel κB sites (Fig. 1*A*). These sites conform, with either one

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Abbreviations: AdML, adenovirus major late; PIC, preinitiation complex; LTR, long terminal repeat.

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A

κB enhancer — Sp1 binding region

-106 agGGGACTTTCCgctgGGGACTTTCCagggaggcgtggcctgggcgggactggggagtggcgagccctcaga -36

+1

-35 tcctgcaTATAAgcagctgctttttgcctgtactGGGTCTCTCTggttagaccagatctgagcctGGGAGCTCTCt +42

InrκB1 InrκB2

B

NF-κB Consensus	GGGRNNYYCC	TdT Inr Consensus	YAYTCYYY
HIVκB	GGGACTTTCC	AdML Inr	CTCACTCTCT
InrκB2 (+32 to +41)	GGGAGCTCTC	InrκB2 (+32 to +42)	GGGAGCTCTCT
InrκB1 (-1 to +9)	GGGTCTCTCT	InrκB1 (-1 to +9)	GGGTCTCTCT

FIG. 1. Comparative sequence analysis of the classically defined NF-κB enhancer site, and two novel Rel binding sites within the regulatory region of HIV-1. (*A*) Schematic of the HIV-1 LTR. The novel κB sites, indicated as InrκB1 and InrκB2, are underlined. The three SP1 binding sites are underlined. (*B*) Sequence alignment of HIV-1 κB enhancer. InrκB1 and InrκB2, and the NF-κB consensus; adenovirus major late (AdML) initiator, and the consensus initiator sequence for the TdT family of initiators.

or two substitutions, to the consensus for κB sites (GGGRNNYYCC). Overlapping these putative κB sites, and relevant to this work, the sites also conform to consensus for initiator motifs of the TdT class, YAYTCYYY (Fig. 1*B*; refs. 2, 5, 6, and 26). For these studies, these sites will be termed HIV-1 initiator-κB1 (InrκB1: −1 to +9, GGGTCTCTCT) and HIV-1 initiator-κB2 (InrκB2: +32 to +41, GGGAGCTCTC).

Both InrκB1 and InrκB2 meet several of the criteria for, and expand the potential role of, bona fide κB sites. In mobility-shift assays, using nuclear extracts prepared from human Jurkat T cells or 293 cells stimulated with or without TNF-α, a single complex was observed bound to both InrκB1 and InrκB2 comigrating with endogenous NF-κB $(p50)_2$ bound to an IgκB oligonucleotide control (Fig. 2*A*, compare lanes 3, 4, 7, and 8 with lanes 1, 2, 5, and 6, and lane 10 with lane 9). Notably, a complex corresponding to NF-κB p50:p65 was not observed using InrκB1 or InrκB2, whereas NF-κB can be observed binding to the classically defined κB element.

Focusing on InrκB2, immunoreactivity with polyclonal anti-p50 antibody confirmed that a nuclear protein immunologically related to p50 is complexed to the InrκB2 probe. In mobility-shift assays, polyclonal anti-p50 antibody supershifted a complex bound to the InrκB2 site (Fig. 2*B*, compare lanes 1 and 3), whereas control preimmune sera did not shift the complex (lane 4). Similar results were obtained using the InrκB1 site as a probe, and in control reactions, anti-p50 antiserum supershifted endogenous p50 bound to an IgκB oligonucleotide probe (data not shown). Given the magnitude of the InrκB binding present in nuclear extracts (Fig. 2*A*, lanes 3, 4, 7, and 8) of a complex comigrating with Ig-κB bound p50, and the inability to supershift the entire complex, we hypothesized that the band might be comprised of multiple different protein/DNA complexes. Crosslinking studies with the antibody supershifted complex confirmed the presence of a 50-kDa protein bound to DNA, consistent with the expected size of Rel p50. As hypothesized, additional proteins of $M_r \approx 40$ and ≈ 70 were also observed (data not shown). The identity of these proteins remains unknown, although the 40-kDa protein could be the TDP-43 protein recently cloned by Gaynor and colleagues (29).

The binding specificity of InrκB2 was confirmed using oligonucleotide competition (see Fig. 2*C*), which indicated that motifs for both Oct-1 (lanes 5–7) and AP-1 (lanes 11–13) failed to compete at ratios up to 50:1, whereas InrκB2 (lanes 2–4) competes efficiently. NFAT-1 motifs also compete at low ratios (20:1 and 50:1), consistent with reports that the cytoplasmic component of NFAT has homology with Rel DNA binding domains (27, 28), and would bind in a Rel-dependent manner as first proposed by Nolan (27) and later confirmed by Rao and colleagues (29).

Binding assays with recombinant Rel proteins confirmed that p50 and p52 homodimers bound to either InrκB site (Fig. 2*D*). In contrast, the InrκB sites, unlike the control IgκB oligonucleotide, failed to bind recombinant p50:p65 heterodimers (Fig. 2*D*, compare lanes 4 and 2). This is consistent with what is observed using nuclear extracts from activated cells (Fig. 2*A*). Additionally, DNase I footprint analysis of the initiator region confirmed that p50 and p52 protected regions corresponding to the InrκB sites (data not shown and ref. 30).

Enhancer-Independent Regulation of HIV-1 by Rel p50 and p52. The presence of novel Rel recognition elements in the HIV-1 promoter potentially provides HIV-1 with a form of Rel-regulation that is distinct from the activities of the upstream HIV-1 κB enhancer. We mutagenized the upstream κB element to generate pHIV-1 (enhκB)-*lacZ*, which contains GGG → CTC substitutions in both κB elements to ablate activity of the enhancer (12, 31). Reproducible dose-responsive transactivation of pHIV-1(enhκB$^-$)*lacZ* was observed in cotransfections with expression vectors for both p50 and p52 (Fig. 3 *A* and *B*), but not p65 (Fig. 3 *C* and *D*). Levels of cotransfected expression vectors coding for p50, p52, p65, and wild-type HIV-1 target reporter plasmids were calibrated to reproduce the synergy seen in published cotransfections with p52 and p65. We confirmed the synergy reported by Nabel and colleagues (13, 32) when expression vectors for p52 and p65 were cotransfected with wild-type LTR, as well as ablation of that synergy using the pHIV-1(enhκB$^-$)*lacZ* target (Fig. 3*D*). We observed reproducible activation of the pHIV-1(enhκB$^-$)*lacZ* reporter plasmid by p52 alone (Fig. 3*D*)—similar results were obtained with a luciferase reporter (data not shown). These results are consistent with the Rel binding activities observed with the InrκB oligonucleotides presented in Fig. 2.

Rel p50 and p52 Augment the DNA Binding of TFII-I to the HIV-1 Initiator Site. It would be unusual for Rel proteins binding in this region of HIV-1 act as classical transcriptional activators, given the proximity of these sites to initiator binding elements. Since other factors bound in this region have been suggested to repress transcription by interfering with the binding of initiator factors (33–35), we assessed what influence p50 and p52 Rel homodimers may have on the binding of de facto initiator proteins. TFII-I is a critical initiator protein that

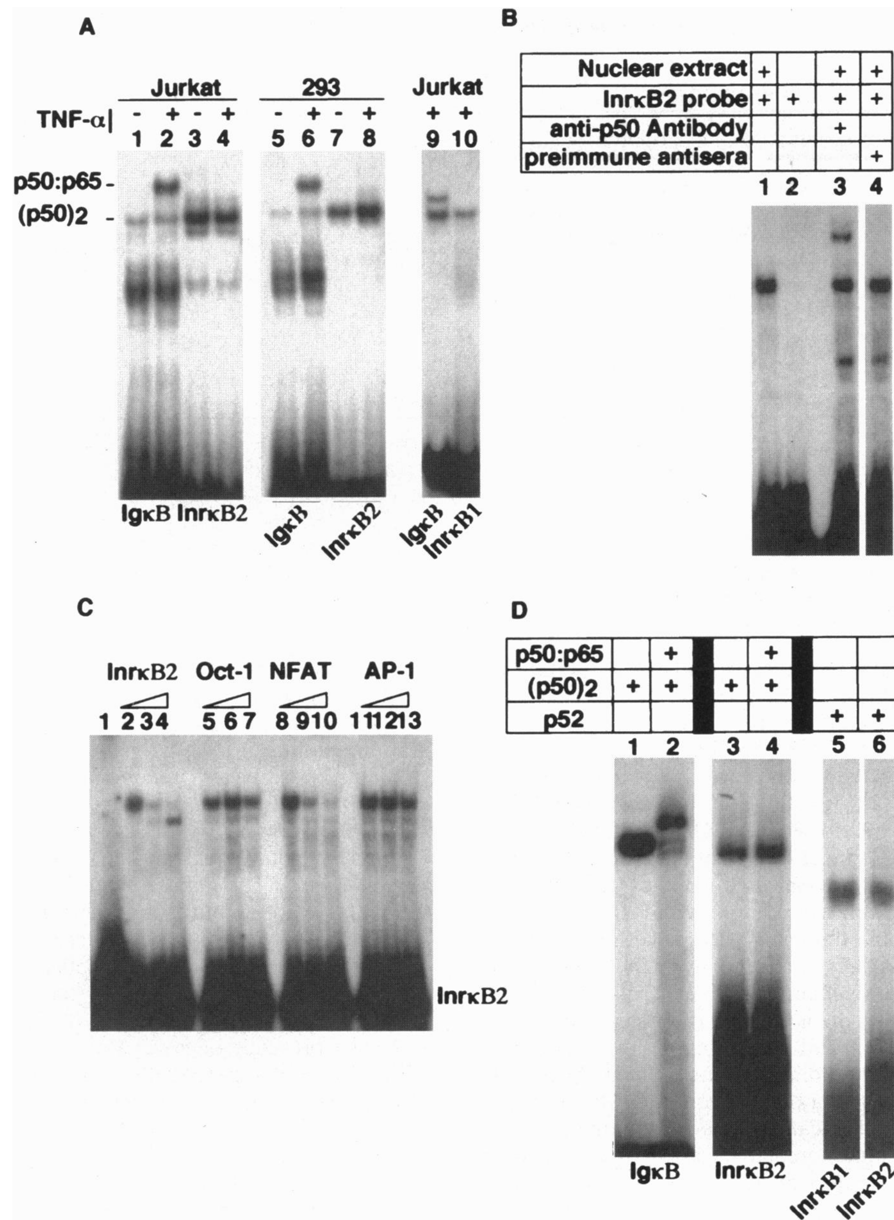

FIG. 2. Comparative protein binding properties of nuclear extract to HIV-1 InrκB 1 and InrκB 2, and HIV-1κB enhancer site. (*A*) Jurkat and 293 cell nuclear extracts were added as shown, with or without TNFα stimulation. Probes are as indicated. (*B*) A p50-related complex binds to HIV-1 InrκB2. Supershift analysis was performed by the addition of nuclear extract to radiolabeled InrκB2 probe, incubation for 30 min at room temperature, and subsequent addition of anti-50 antibody for 15 min. Lanes are as indicated. (*C*) Competition for binding to InrκB2. Lanes contain probe, nuclear extract and a dilution series of competitor indicated. Competitor oligonucleotides were added prior to the addition of radiolabeled probe at final molar ratios of 0:1, 20:1, and 50:1. Oct-1, 5′-TGT CAG AGA GCT CCC AGT CA-3′/5′-AAA TGT TTT ACA TAT TA-3′; NFAT-1, 5′-TAA GGA GGA AAA ACT GTT TCA TG-3′/5′-CAT GAA ACA GTT TTT CCT CC-3′. AP-1: 5′-GTG ACT CAG CGC G-3′/5′-CGC GCT GAG TCA C-3′. (*D*) Purified p50 homodimer, p52 homodimer, and p50:p65 heterodimer proteins bind to InrκB1 and InrκB2. Proteins and probes were added as indicated.

binds to the initiator sites of HIV-1, AdML and TdT (5, 6, 36) where it promotes assembly of a PIC. In mixing experiments with purified native TFII-I (Fig. 4), we observed reproducible Rel-responsive recruitment of TFII-I binding to an oligonucleotide spanning the HIV-1 initiator (Fig. 4*A*, compare lanes 2, 4, and 6); overexposure demonstrates a TFII-I specific band in Fig. 4*A*, lane 2. Binding of TFII-I to the HIV-1 Inr element was restricted so as to observe maximum stimulatory effects of p50 and p52 Rel homodimers on its bindng. Similar recruitment of TFII-I binding was observed using Rel p50 (see Fig. 4*B*). Binding enhancement for TFII-I with either 50 or p52 was greater than 50-fold. In contrast to the HIV-1 initiator (GGGTCTCTCT), the closely related AdML initiator (CTCACTCTCT, lacking sequences necessary for Rel binding, see underlined) failed to demonstrate significant Rel homodimer binding and exhibited only 2- to 3-fold augmented binding of TFII-I (explainable by low-affinity nonspecific interactions of p50 with the AdML probe DNA) in the presence of Rel homodimers (see Fig. 4*B*). Hence, the data suggests that Rel dimer binding sites can specifically augment the binding of TFII-I.

Rel Proteins Stimulated Enhancer-Independent, TFII-I-Dependent HIV-1 Transcription *in Vitro*. To explore the biochemical outcome of an interaction between Rel and TFII-I, *in vitro* transcription assays were performed with recombinant Rel proteins, HeLa nuclear extracts (Promega), specific inhibitors, and an HIV-1 template. We used an *Acc*III/*Kpn*I fragment (−147 to +281) of pHIV-1

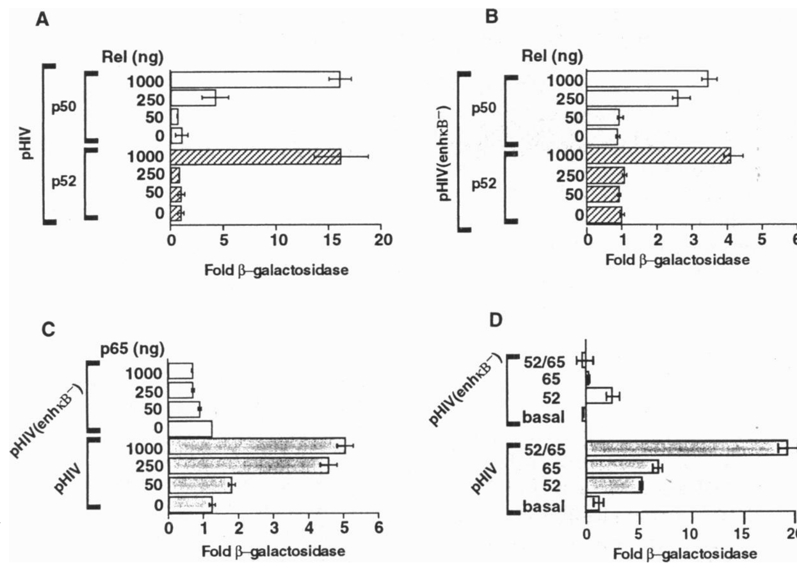

FIG. 3. Rel activation of HIV-1 in the absence of the the upstream κB elements. (*A*) Dose-dependent activation of pHIV-lacZ with p50 and p52. (*B*) Dose-dependent activation of pHIV(enhκB$^-$)-lacZ with p50 and p52. (*C*) Dose-dependent activation of pHIV-lacZ and pHIV(enhκB$^-$)-lacZ with p65. (*D*) Activation of pHIV-1-lacZ with synergistic combinations of p52 and p65. The graphs presented are representative of at least two, or as many as six, independent experiments. All transfections were assayed at 24 hr.

(enhκB$^-$)*lacZ* predicted to yield an expected RNA transcript size of 281 ribonucleotides. In nuclear extracts, this template gives rise to a basal transcript (Fig. 5 *A*, lanes 1 and 5, and *B*, lane 1) of the expected length (≈280 ribonucleotides). Addition of p52 specifically increased transcription (Fig. 5 *A*, lanes 3 and 6, and *B*, lane 2). Consistent with previously published results, a control AdML template, pMLIcat (36), failed to show significant Rel-responsiveness (ref. 37 and Fig. 5*A*, lanes 7 and 8). Rel-induced transcription of the HIV-1 (enhκB$^-$) template was ≈5-fold above basal transcription levels. Since p50 is constitutively present in the nuclei of most cell types, we pretreated nuclear extracts with an IgκB-derived oligonucleotide (Fig. 5*A*, lanes 2 and 4) to determine the role of p50 in the levels of basal transcription observed. Addition of IgκB oligonuceotide appeared to compete both basal and Rel-induced RNA transcription, whereas AdML control oligonucleotides failed to compete (data not shown). These *in vitro* transcription results recapitulate the observation of Rel-responsiveness in the cotransfection assays, and are consistent with a role for Rel proteins at the initiator.

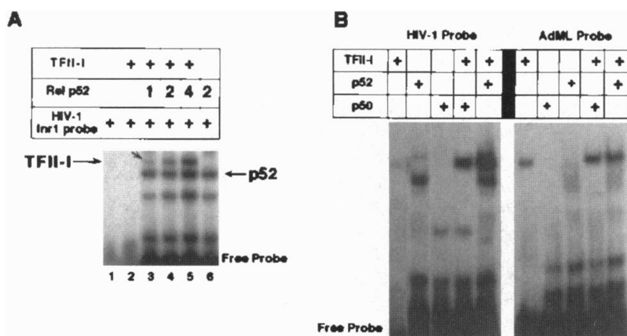

FIG. 4. Effect of Rel p50 and p52 on TFII-I binding. (*A*) Binding reactions were performed with recombinant p50 or p52, highly purified TFII-I, and an HIV-1 initiator oligonucleotide. Additions were made as follows: TFII-I: lanes 2–5, 1 μl/100 ng. p52: lane 3, 1 μl/100 ng; lanes 4 and 6, 2 μl; lane 5, 4 μl. (*B*) p50 and p52 each stimulate the binding of TFII-I to the HIV-1 initiator but not the AdML initiator. Overexposure of the gel in *B* demonstrates a p50-specific band (not shown).

Rel-induced transcription using the HIV-1(enhκB$^-$) template exhibited TFII-I dependency. Pretreatment of nuclear extracts with a polyclonal antibody to TFII-I ablated both Rel-induced transcription (Fig. 5*B*, compare lanes 2 and 3) and basal transcription (lane 5). In control transcriptions, mutations in both initiators ablated basal and TFII-I induced transcription (A.L.R., unpublished results). Taken together with the binding results observed in the previous experiments, Rel proteins seem capable of modulating the binding of TFII-I to the HIV-1 initiator and might be involved in early events affecting transcriptional initiation.

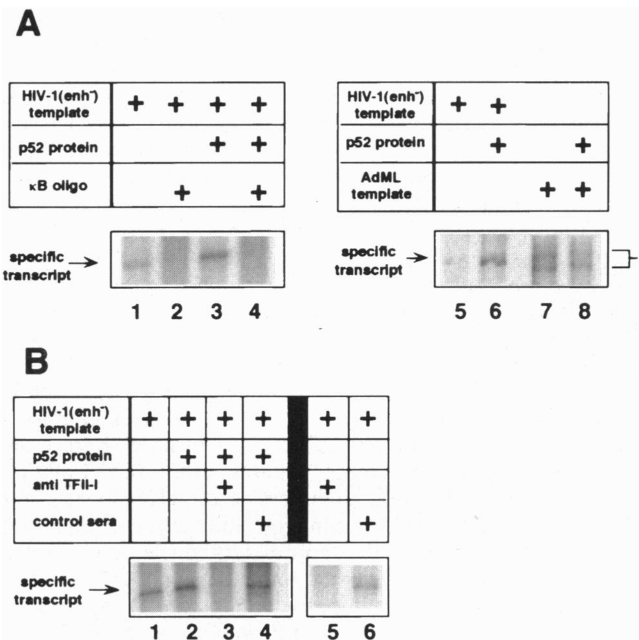

FIG. 5. *In vitro* transcriptions using Rel homodimers specifically induce HIV-1(enhκB$^-$) RNA transcription in a TFII-I-dependent manner. (*A*) HIV-1(enhκB$^-$) template: lanes 1–4, 0.5 μg; p52, lanes 3 and 4, 1 μl/100 ng; unlabeled canonical κB competitor oligonucleotide (IgκB site) (lanes 2 and 4). (*B*) Template: lanes 1–6, 0.5 μg; p52: lanes 2–4, 1 μl/100 ng; anti-TFII-I antibody, lanes 3 and 5; preimmune control sera, lanes 4 and 6.

DISCUSSION

HIV-1 transcription, both basal and activated, requires the establishment of promoter proximal and distal DNA-bound protein complexes that coordinate developmental progression of the viral life cycle. We have identified novel κB binding motifs at the initiator of HIV-1, demonstrated that they can bind a subset of Rel dimers, and characterized the ability of these sites to mediate apparent interaction between Rel proteins and a basal transcription component, TFII-I. The context of these κB elements, near the transcription start site of HIV-1, is in contrast to the activities previously postulated for NF-κB motifs at the enhancer.

Numerous proteins have been demonstrated to bind in the vicinity of the critical HIV-1 initiator (2, 5, 6). These include *de facto* initiator proteins YY1 (35), USF (36), and TFII-I (5, 6), as well as other proteins such as the LBP family of proteins (34), PRDII-BF1 (38), CTF/NF-1 (7), and the recently characterized TDP-43 protein (33). PRDII-BF-1, a protein with zinc-finger domains that was isolated through its ability to bind NF-κB-like motifs, binds the upstream κB enhancer elements and activates transcription; PRDII-BF-1 also binds a region of the initiator that overlaps InrκB2 (38). Although no activity is conferred by binding of PRDII-BF-1 at the downstream element the binding of a protein that recognizes κB elements to a region containing the the InrκB motif is consistent with the findings presented in this report. YY1 represses HIV-1 expression, possibly by inhibiting the assembly of initiation complexes (35). The role of CTF1/NF-1 and the LBP-1 class of factors at the initiator elements remains to be determined (7, 34), although some evidence suggests LBP-1 also acts as a repressor. TDP-43 also repressed transcription at the HIV-1 promoter when bound (33); the size of TDP-43 is consistent with the size of a protein observed in crosslinking experiments in our hands using the InrκB1 and InrκB2 sites as crosslinking probes (data not shown).

In contrast to the repressors, TFII-I is responsible for recruiting basal transcription machinery (5, 6) in what has been proposed to be a TFII-A independent mechanism. Thus, it was important to determine biochemically whether the activity of TFII-I was affected by the binding of Rel proteins. Fig. 4 suggested that p50 and p52 Rel homodimers enhance the binding of TFII-I to its motif in the HIV-1 Inr. Rel p52 increased transcription from an enhancerκB$^-$ construct, and this activation was dependent upon the presence of TFII-I (Fig. 5*B*). This is consistent with a model in which certain Rel proteins recruit TFII-I to the initiator region and then are rapidly displaced by TFII-I (no stable p52-TFII-I or p50-TFII-I complex has been observed in any of our studies). At that point TFII-I would then complete PIC assembly as previously proposed by Roy *et al.* (5, 6). Sequence dependent protein–protein interactions between members of the Rel families and other transcription factors have been previously observed (for review, see ref. 27). Multiple proteins have been proposed to be involved in HIV-1 regulation (5–7, 33, 36, 38, 39), and in some cell types, low levels of active TFII-I might necessitate interaction with factors, such as p50, that enhance TFII-I binding to the initiator. The complex nature of the HIV-1 promoter, and the fact that initiator function still defies explanation, indicates that further studies are warranted if a better understanding of HIV-1 gene regulation is to be gained.

Do Rel proteins act at initiator regions of other genes? In certain other strains of HIV-1 and simian immunodeficiency virus, in other retroviruses such as the human spumaretrovirus (HSRV) (40), and in promoters for certain cellular genes, namely *bcl3* and *NF-κB 105* there is clear evidence of κB elements overlapping or within a few nucleotides of the transcriptional start site. Binding studies confirm that Rel proteins do in fact associate with the "InrκB" sites in *bcl-3* and *NF-κB p105* (unpublished observations). Therefore, the presence of composite Inr/κB sites in genes may functionally classify them as a novel group of promoter subtypes, regulated in part by Rel protein binding. A more fully representative analysis of promoter regions (characterized for defined transcriptional start sites) might determine whether κB motifs at initiators are restricted to genes with classical κB enhancers or whether these sites can independently act in a modular manner to regulate preinitiation function.

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