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Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation

(DNA binding/c-*rel* gene/transactivation)

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ABSTRACT **The nuclear factor that binds to the κ light-chain enhancer of B cells (NF-κB) is a transcription factor that regulates the expression of a variety of cellular and viral genes. NF-κB is composed of distinct subunits, and at least four independent genes (p105, p100, p65, and c-*rel*) have been isolated that encode related proteins that bind κB sites. Because it is possible that specific interactions of different subunits can allow selective gene activation, we have characterized the specificity of transcriptional activation by various combinations of these subunits. When tested alone, an ≈49-kDa form (p49) of the p100 protein bound weakly to κB, but p49 associated with p65 to bind efficiently to this site. Furthermore, p49 acted in combination with either p65 or a Rel/VP16 fusion protein to activate κB-dependent transcription in Jurkat T leukemia cells. The p49/p65 or p49/Rel combination stimulated transcription mediated by the canonical κB site but did not stimulate reporter genes containing interleukin 2 receptor α or major histocompatibility complex κB elements, despite its ability to bind to these sites. Transactivation mediated by the p49/p100 and p65 NF-κB proteins is therefore sensitive to minor changes in the sequence of the κB site. Specificity determined by the association of NF-κB subunits provides a mechanism to selectively regulate variant κB sites associated with different cellular and viral genes.**

Regulation of κB-dependent transcription displays several levels of complexity. A number of sites have been found in association with various cellular and viral genes. Although they are recognized by the transcription factor NF-κB, these sites exhibit considerable DNA sequence variation (1–8). At the same time, NF-κB consists of several protein species. These include 50-kDa and 65-kDa proteins, which have been highly purified (9–16), and additional proteins defined by UV crosslinking (17). Recently, cDNAs encoding precursors for two distinct ≈50-kDa proteins have been isolated. Each of these products is encoded as a larger precursor molecule, p105 (12–14) or p100 (18); these precursors show sequence similarity but map to distinct chromosomal loci. Proteolytic processing of these proteins is presumably required to generate the ≈50-kDa amino-terminal DNA-binding subunits (12–14, 18). A 49-kDa form of p100 can also be generated by alternative splicing (18). p49, like the processed p105 product, specifically binds the class I major histocompatibility complex (H-2) κB site and associates with the protooncogene c-*rel* (18).

In addition, other cDNAs encoding NF-κB subunits have been defined. These subunits include p65 and Rel, which share amino acid similarity with one another, p100, p105, and the protein encoded by the *Drosophila* maternal-effect gene dorsal (12–16, 18). Both p65 and Rel contain putative transcriptional activation domains in their carboxyl-terminal regions (15, 16, 19–22). In this report, we show that the p49 derivative of p100 associates with p65 to form a complex that binds to the κB site found in the immunoglobulin (Ig) (23) and human immunodeficiency virus (HIV) (24) enhancers and stimulates transcription of such an Ig/HIV κB-containing reporter plasmid in cultured cells. Reporters containing variant κB sites bind to these NF-κB protein complexes but are less responsive when cotransfected with combinations of known NF-κB cDNA expression vectors. Specific combinations of NF-κB/Rel proteins can therefore distinguish between variant κB sites in intact cells.

MATERIALS AND METHODS

NF-κB Eukaryotic Expression Vectors. The eukaryotic expression vector used for the human c-*rel* cDNAs was prepared by insertion of the Rous sarcoma virus (RSV) long terminal repeat and the simian virus 40 polyadenylylation signal in the *Sac* I and *Kpn* I sites of pBluescript KS (Stratagene). Full-length c-*rel* was inserted at the *Sma* I site of the polylinker, whereas a *Not* I–*Apa* I fragment of c-*rel* was inserted at these restriction sites to create the *Apa* I-truncated c-*rel* plasmid (N.R.R., unpublished work). The Rel/VP16 fusion was prepared by digestion of the truncated c-*rel* with *Apa* I, incubation with T4 DNA polymerase, and insertion of a Klenow DNA polymerase-treated 240-base-pair *Acc* I–*Sty* I fragment encoding the acidic transcriptional transactivation domain of herpes simplex viral protein VP16 (25, 26).

The RSV β-globin expression vector (27) and *Rsa* I-truncated p105, p49, and mouse p65 expression vectors (18) have been described. The p100 and *Xho* I-truncated p100 expression vectors were generated by insertion of Klenow polymerase-treated *Hin*dIII–*Bam*HI fragment (full-length) or *Hin*dIII–*Xho* I fragments into Klenow polymerase-treated, *Hin*dIII/*Bgl* II-cut RSV β-globin plasmid. The p105/VP16 fusion was created by incubation of the RSV p105 expression vector with *Xba* I and Klenow polymerase, followed by insertion of a Klenow polymerase-treated 241-base-pair *Sal* I–*Sty* I fragment encoding the VP16 acidic transcriptional activation domain (25, 26).

κB-Containing Reporter Plasmids. Four copies of each κB site were inserted upstream of the simian virus 40 promoter linked to the chloramphenicol acetyltransferase (CAT) gene (6, 28), with the exception of the variant 1 and H-2 κB sites, which were present in six and three copies, respectively.

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; HIV, human immunodeficiency virus; IL-2Rα, interleukin 2 receptor α chain; PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus.

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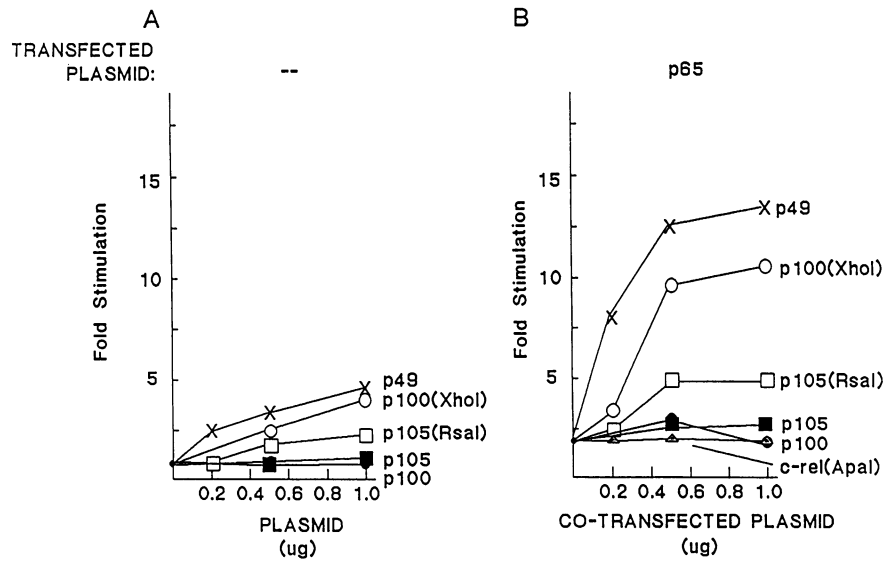

FIG. 1. Transcriptional activation of an Ig/HIV κB CAT reporter plasmid by Rel, p100, or p105 NF-κB derivatives alone (*A*) or in combination with p65 (*B*). (*A*) Jurkat T cells were transfected with reporter plasmid and the indicated amounts of eukaryotic expression vectors encoding p49 (×), *Xho* I-truncated p100 (○), *Rsa* I-truncated p105 (□), p105 (■), or p100 (●). (*B*) Cells were transfected as in *A*, except that all cells received 1 μg of p65 expression plasmid. △, *Apa* I-truncated Rel. In both *A* and *B*, fold stimulation refers to CAT activity relative to that of cells transfected only with the reporter.

Transfections and CAT Assays. Transfections and CAT assays were performed as described (18). Nuclear extracts from transfected cells were prepared as described (6).

Bacterial Expression of NF-κB cDNAs. p49 and *Rsa* I-truncated p105 were expressed in *Escherichia coli* using the pET system essentially as described (18). Bacterial pellets were resuspended in buffer Q [20 mM Hepes, pH 7.9/10 mM $MgCl_2$/20% (vol/vol) glycerol/0.01% Nonidet P-40/1 mM dithiothreitol containing DNase I (10 μg/ml), aprotinin (0.2 μg/ml), pepstatin A (0.7 μg/ml), leupeptin (0.5 μg/ml), phenylmethylsulfonyl fluoride (0.1 mM), benzamidine (1 mM), and antipain (1 μg/ml)], and the cells were lysed by sonication. EDTA was added to a final concentration of 1 mM, and lysates were clarified by microcentrifugation for 15 min.

Purification of Recombinant p49 and Truncated p105 Proteins. These recombinant proteins were partially purified from bacterial cell lysates by gel filtration on an S-200 FPLC column (Pharmacia).

Heteromerization of p49 or Truncated p105 with p65. Partially purified fractions of recombinant p49 or truncated p105 were diluted to various concentrations in buffer Q and incubated with partially purified recombinant mouse p65 protein (gift of T. Fujita, G. Nolan, and D. Baltimore, Rockefeller University) for 30 min at 37°C. After incubation, radiolabeled probe was added to a final volume of 10 μl, and complex formation was analyzed by electrophoretic mobility-shift assay (EMSA) in a nondenaturing 4% acrylamide gel in 22.5 mM Tris/22.5 mM boric acid/0.5 mM EDTA (0.25× TBE). EMSAs were performed essentially as described (18).

RESULTS

p49/p100 NF-κB Preferentially Transactivates Through the Ig/HIV κB Site in Combination with p65. To determine which

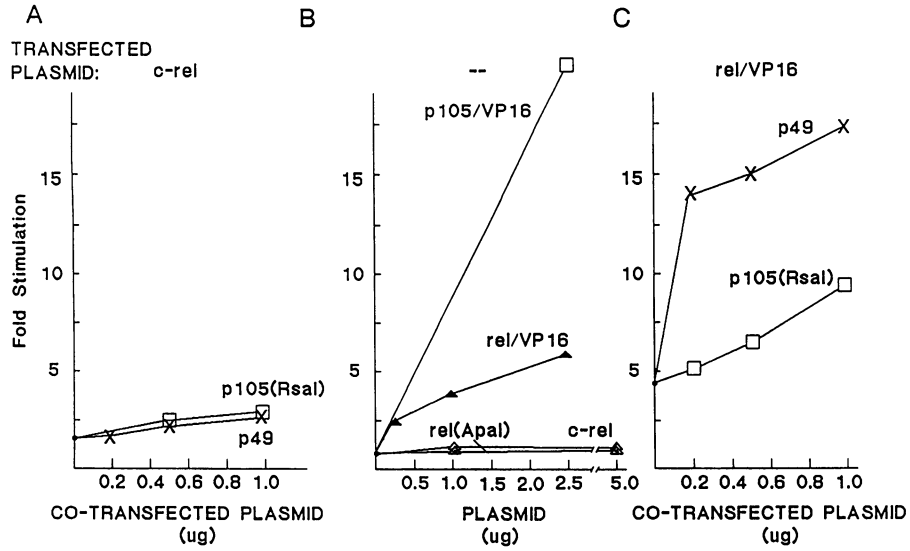

FIG. 2. Stimulation of an Ig/HIV κB reporter plasmid by Rel derivatives alone or in combination with p100 and p105 derivatives. Jurkat T cells were transfected with reporter plasmid and eukaryotic expression vectors as indicated. (*A*) Rel plasmid (1 μg) and the indicated amounts of p49 (×) or *Rsa* I-truncated p105 (□) plasmid. (*B*) Rel (△), *Apa* I-truncated Rel (▲), Rel/VP16 fusion (▲), or p105 (*Xba* I)/VP16 fusion (□) plasmid. (*C*) Rel/VP16 plasmid (1 μg) and the indicated amounts of p49 (×) or *Rsa* I-truncated p105 (□) plasmid.

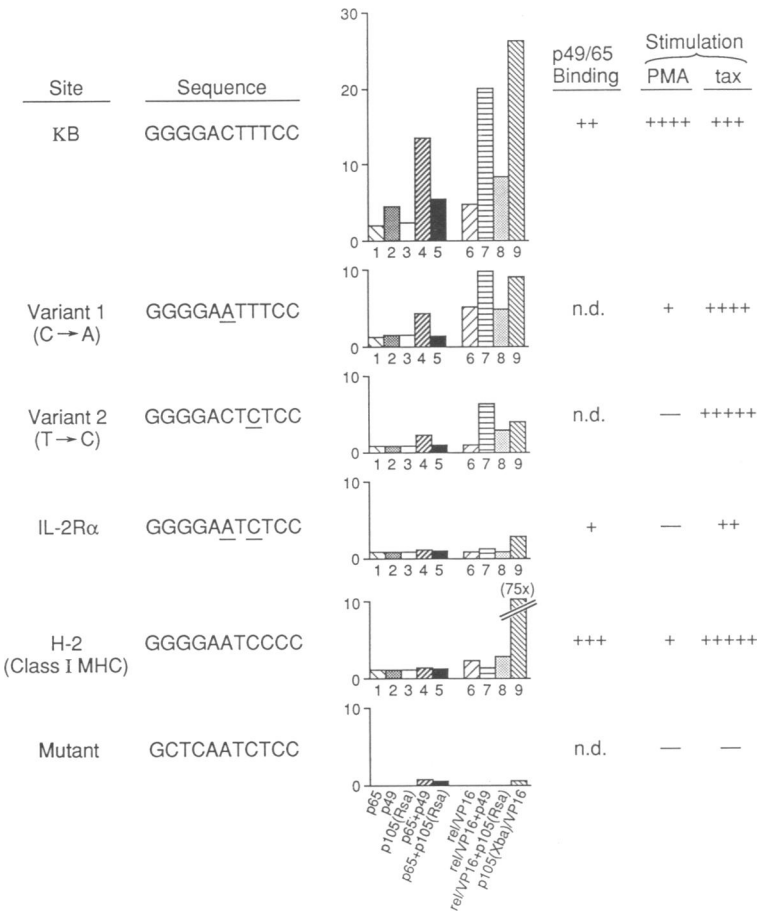

FIG. 3. Stimulation of variant κB sites by transfection of different combinations of NF-κB cDNA expression vectors. Jurkat T cells were transfected with CAT reporter plasmids containing different multimerized κB sites as indicated. MHC, major histocompatibility complex. The cotransfected NF-κB cDNAs [1 μg, except p105(*Xba* I)/VP16, which was 2 μg] are indicated below the histograms showing fold stimulation of CAT activity. Relative p49/65 binding activities and transcriptional stimulation by PMA or Tax are indicated at right (n.d., not determined).

combinations of NF-κB subunits were required to stimulate transcription from the Ig/HIV κB site, various NF-κB or Rel expression vectors were transfected into Jurkat T cells, together with a CAT reporter plasmid containing four copies of the Ig/HIV κB element. When only p49 or a *Xho* I-truncated p100 (a 48.5-kDa protein) was transfected along with the reporter, transactivation did not exceed 5-fold (Fig. 1*A*); however, when p49 or truncated p100 was cotransfected with p65, an 11- to 13-fold stimulation of CAT activity above that found with the reporter plasmid alone was observed, an increase that was appreciably more than the sum of the increases with each vector alone (Fig. 1). In contrast, a truncated form of p105 in combination with p65 was less active and showed an additive effect (Fig. 1). A mutant κB reporter whose site fails to bind NF-κB *in vitro* (6) was not transactivated by these NF-κB cDNAs (ref. 18; see also Fig. 3). Cotransfection of p65 with c-*rel* truncated at the *Apa* I site also did not stimulate CAT activity (Fig. 1*B*). Neither full-length p100 nor full-length p105 transactivated the Ig/HIV κB CAT plasmid when transfected alone or in combination with p65 (Fig. 1), demonstrating that processing of these precursor molecules is required to generate a transcriptionally active form.

A Truncated Rel/VP16 Fusion Protein Can Functionally Substitute for p65. p65 and Rel are more similar to one another than to other members of the NF-κB family, showing extensive amino acid similarity in their amino-terminal regions (15, 16). Although Rel has a carboxyl-terminal transactivation domain (19–22), it differs from p65 because it apparently contains a cytoplasmic retention sequence (19, 20, 29, 30), which may interfere with its ability to transactivate in the nucleus. To investigate the specificity and potential function of Rel, a fusion gene was made linking the amino-terminal region of Rel, which is localized to the nucleus (N.R.R., unpublished observation), to the herpes simplex virus VP16 transactivation domain (25, 26). Transfection of this Rel/VP16 expression plasmid stimulated transcription through the Ig/HIV κB site 4-fold (Fig. 2*B*); however, cotransfection with p49 stimulated CAT activity about 17-fold above controls (Fig. 2*C*). Similar to the result observed with p65, truncated p105 proved to be less capable than p49/100 of stimulating transcription when cotransfected with Rel/VP16 (Fig. 2*C*). Full-length Rel (Fig. 2*B*) or truncated Rel lacking the transactivation domain (Fig. 2*A*) failed to activate transcription, suggesting that Rel may normally require processing or release from a cytoplasmic inhibitory protein to yield a transcriptionally active form.

Sequence Specificity of Transactivation by NF-κB cDNAs. The κB site of the gene encoding the interleukin 2 receptor α chain (IL-2Rα) differs from the Ig/HIV κB site by only two base pairs. Although it binds to NF-κB and is stimulated by the Tax I protein encoded by human T-cell leukemia virus type I (6, 8), this site fails to respond to phorbol 12-myristate 13-acetate (PMA) stimulation in some Jurkat cell lines (6, 7). To determine its response to various combinations of NF-κB subunits, p49 or truncated p105 was cotransfected with p65 or Rel/VP16. At levels that strongly transactivate Ig/HIV κB–CAT (Figs. 1*B* and 2*C*), minimal transactivation was seen with IL-2Rα κB–CAT (Fig. 3). To explore this specificity further, additional reporters with single base-pair changes in the κB elements (28) were analyzed. Mutants with C → A at position 6 (variant 1) or T → C at position 8 (variant 2) also markedly decreased transcription when co-

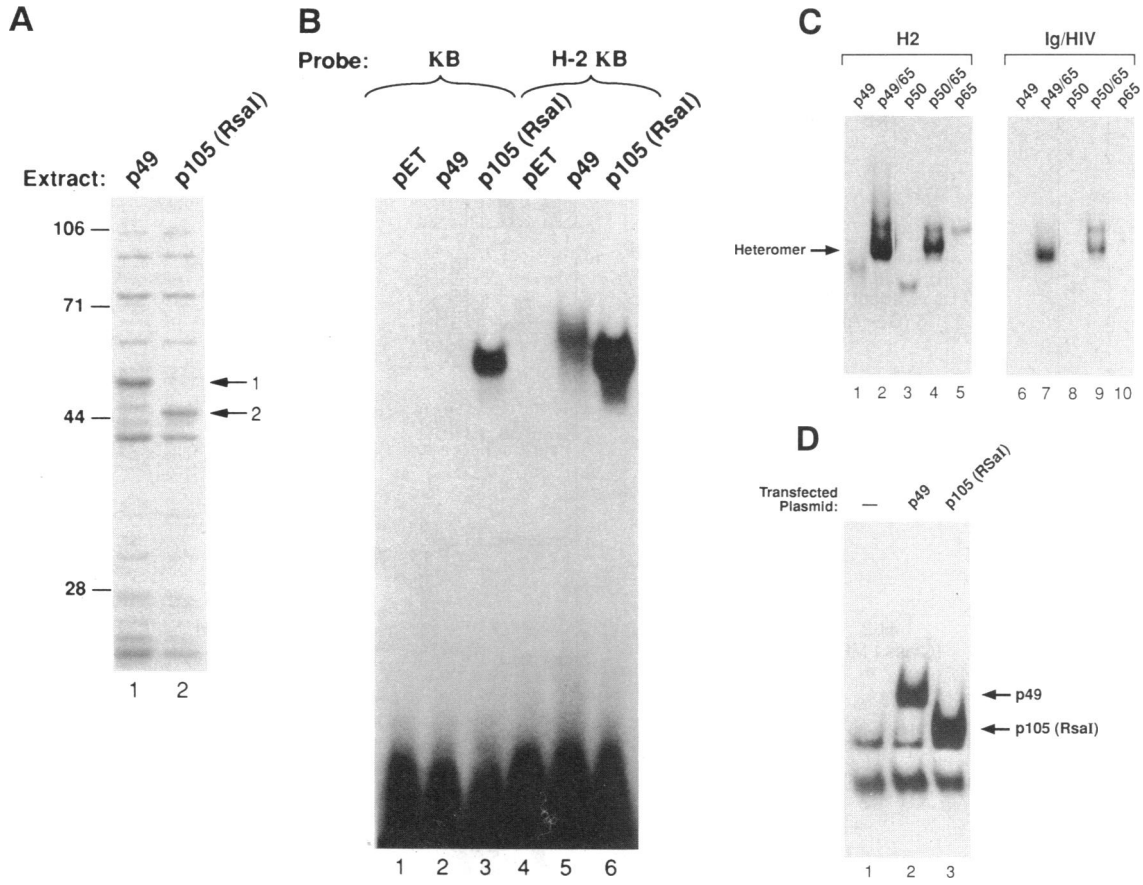

FIG. 4. Preferential binding of p49 protein to the H-2 κB site and association with p65. (*A*) SDS/PAGE analysis of extracts from bacteria with induced p49 or *Rsa* I-truncated p105 pET expression vector. Molecular size markers (kDa) are at left; arrows at right indicate p49 (arrow 1) and truncated p105 (arrow 2). (*B*) DNA-binding activity of p49 or p105(*Rsa* I) added in equal amounts (≈5 ng) as determined by EMSA with the Ig/HIV or H-2 κB element as radiolabeled probe with 1 μg of poly[(dI-dC)·(dI-dC)] in the reaction mixture. (*C*) EMSA analysis of the indicated combinations of partially purified recombinant p49, truncated p105 (p50), or p65 with either H-2 or Ig/HIV κB radiolabeled probe. The same quantity of each protein was used in lanes 1–5 as in lanes 6–10. (*D*) EMSA analysis of nuclear protein extracts (5 μg) prepared from Jurkat cells transfected with 5 μg of the indicated p49 or p105(*Rsa* I) expression plasmids with the H-2 κB radiolabeled probe.

transfected with the NF-κB cDNAs (Fig. 3). Interestingly, a reporter plasmid containing the symmetric H-2 class I κB site (2–5), which binds strongly to p49/p65 heteromers (see Fig. 4*C*), failed to respond significantly to cotransfected NF-κB cDNAs (Fig. 3). This reporter remained strongly responsive to a p105(*Xba* I)/VP16 fusion protein and to PMA or Tax (Fig. 3), suggesting that repression by another factor was unlikely. This finding demonstrates that small changes in DNA binding sequence can dramatically alter the ability of NF-κB to activate transcription, providing a mechanism to selectively regulate expression of genes with variant κB sites.

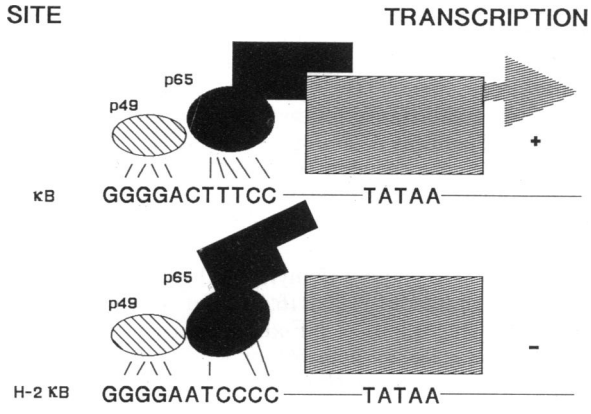

FIG. 5. Model of differential transcriptional activation by NF-κB subunits. Sequences in the 3′ half-site of the κB element could change the structural conformation of p65 and disrupt its interaction with the transcription complex.

p49 and p50 Display Different DNA-Binding Characteristics. DNA–protein complex formation by p49 and p50 was investigated *in vitro* by EMSA with Ig/HIV or H-2 κB probes. With equivalent amounts of protein (Fig. 4*A*), p105(Rsa I) formed complexes of similar intensity with probes from each site. In contrast, p49 bound preferentially to the H-2 κB site (Fig. 4*B*). When recombinant p49 protein was incubated with recombinant p65 protein, a more intense complex of intermediate mobility was formed that bound to the Ig/HIV κB element (Fig. 4*C*, lane 7), suggesting that association of p49 with p65 results in a complex with greater affinity for the κB element, thus allowing p49 to contribute to transcriptional activation. The identical p49/p65 complex shows greater affinity for the H-2 κB element than for the Ig/HIV κB element (Fig. 4*C*; lanes 2 and 7). Similar results were observed with truncated p105 (Fig. 4*C*, lanes 3, 4, 8, and 9). EMSA using nuclear extracts from Jurkat cells transfected with the p49 or p105(*Rsa* I) expression plasmids demonstrates that equivalent quantities of these proteins are synthesized in cells that exhibit differential activation of variant κB reporter plasmids (Fig. 4*D*).

DISCUSSION

Our results suggest that the specificity of NF-κB-mediated transcriptional activation is determined by the combinatorial

associations of NF-κB subunits and the specific sequence of the κB site. The data also suggest that the function of variant κB sites could change, depending on the promoter and the other elements with which it interacts. The level of transcriptional activation of the variant κB reporters by p49/65 strongly correlated with the degree of PMA activation obtained in transfections of the reporter plasmids alone (Fig. 3). In contrast, no correlation was seen with the degree of Tax inducibility displayed by the variant sites, suggesting that Tax activates these NF-κB subunits differentially or stimulates other, as yet undefined, members of the NF-κB family.

Previous studies (31) have demonstrated that the p65 protein binds κB elements with strong preference for right-hand half-site sequence. Taken together with p49 binding preferentially to the palindromic H-2 κB site (Fig. 4), this suggests that p49 binding in the heteromeric p49/p65 complex is directed to the GGGGA sequence, whereas p65 (or Rel) is directed to the CTTTCC sequence. This complex may facilitate other protein–protein interactions that stabilize the transcriptional initiation complex and are sensitive to minor changes in DNA sequence of the 3′ κB half-site (CTTTCC) (Fig. 5) since, despite its ability to bind to other κB sites (Fig. 4*C* and data not shown), the p49/65 heteromer shows a high degree of specificity for transactivation of the Ig/HIV κB element.

The truncated p105 protein displays stronger κB binding activity but lower activity in cotransfections with p65 or Rel/VP16 than does p49/100. It is possible that this lack of transactivation could be attributed to differences in DNA-binding affinity of the heteromeric complexes. Another possibility is that homodimers formed by the p105 product could repress transcription from Ig/HIV κB elements by competing with heteromers for binding to the κB site. In contrast, p49 protein displays very weak affinity for the Ig/HIV κB element (Fig. 4*B*) and would thus be expected not to inhibit transcriptional activation mediated by the p49/p65 heteromers. The observations presented here suggest that sophisticated strategies have evolved to regulate the expression of diverse genes that contain related κB sites. The combinatorial association of NF-κB subunits provides a specific mechanism to regulate expression of the Ig and HIV genes.

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