The NF-κB transcription factors display a high degree of sequence conservation in a domain initially described in the rel oncogene. Two family members, NF-κB1 and NF-κB2, have distinct DNA binding properties and functionally distinct effects on different enhancers. NF-κB1, for example, binds to the κB site from the human immunodeficiency virus (HIV) with ~15-fold higher affinity than NF-κB2. In this study, we have defined regions within the Rel domain which determine DNA binding specificity and interaction with other proteins. We find that the COOH-terminal putative Rel dimerization domain of NF-κB1 is required for preferential binding to the HIV κB site. In contrast, preferential stimulation of the HIV enhancer by NF-κB2 with RelA(p65) is determined by both the NH2- and COOH-terminal Rel domains of NF-κB2. These two regions of NF-κB2 also mediate preferential synergy with Bc13. These data suggest that a specific subdomain of the Rel conserved region has evolved to control the fine specificity of DNA binding, and two distinct subregions within the Rel domain determine the specificity of interaction with other transcription factors. These specific Rel-conserved domains therefore determine the specificity of NF-κB interactions and contribute to selective gene activation.

Transcriptional regulation by the NF-κB/Rel family of proteins is regulated at multiple levels. The diversity of this family has been well established and includes at least five independent gene products in mammalian cells (1-11). Although they are related in their Rel-conserved region, NF-κB/Rel gene products display differences in DNA binding specificity and transcriptional activation (12-14). We have previously described an NF-κB family member which is an ~100-kDa protein and can give rise to an ~50-kDa form which is capable of dimerizing to RelA(p65) and other Rel family members (9, 12, 13). This gene product, NF-κB2, binds less avidly to the canonical κB site from the immunoglobulin (Ig) or HIV-1 enhancers than NF-κB1 (13). NF-κB2 nonetheless preferentially stimulates the HIV enhancer in combination with RelA(p65) compared with NF-κB1 when transfected into Jurkat cells (9). It is unlikely that this preferential activation is due to increased DNA binding affinity, since in the case of NF-κB2/RelA, the DNA binding affinity of the heterodimer does not differ significantly from NF-κB1/RelA (13, 15). In addition, it has been found that NF-κB2 preferentially stimulates transcription in combination with Bc13 (16, 17). Despite their functional differences, NF-κB1 and NF-κB2 are nearly 50% identical at the amino acid level in their Rel-related regions. Within this region are two subdomains which show even higher sequence conservation (Fig. 1). From previous studies, these subdomains, here termed A and B (Fig. 1), were shown to contribute to DNA binding and dimerization, respectively (4, 18-23). Mutations within the NH2-terminal region abrogate DNA binding but not dimerization, whereas those in the COOH-terminal region also affect dimerization (4, 12-14). Although the physical structure of these molecules is not yet known, we sought to define the relative contributions of Rel subdomains to further understand the structure and function of these proteins.

Because they are highly related, a variety of swap mutations between the two NF-κB subunits can be generated between conserved amino acid residues, and the contribution of these domains to the DNA binding specificity and interaction with RelA(p65) or Bc13 can be determined. In this report, we show that the COOH-terminal (putative dimerization) domain of NF-κB1 mediates the preferential binding to canonical κB, whereas both NH2- and COOH-terminal regions of NF-κB2 are required to confer specificity of interactions with other proteins, presumably through protein-protein interactions of both the dimerization and DNA binding domains. These findings suggest that the putative dimerization domain of this DNA-binding protein determines its specificity of DNA binding, and interactions of the NH2- and COOH-terminal Rel domains of NF-κB2 with adjacent proteins contribute to the specificity of transcriptional activation.

MATERIALS AND METHODS

Plasmids—The RSV eukaryotic expression plasmids containing the human NF-κB1 (RSV p105 truncated at XbaI, truncated at SalI after introduction of a stop codon at amino acid 434), NF-κB2 (RSV p49 and RSV p100 truncated at XhoI, RelA/RSV p65) and RelA/RSV Bc3 DNA have been previously described (9, 12, 13). A stop codon was introduced at amino acid 440 of NF-κB2 to generate a protein containing only the NH2-terminal part of NF-κB2(p100) using site-directed mutagenesis. To create swap mutations, silent restriction sites were introduced in the RSVp49 and RSVp60 plasmids by site-directed mutagenesis. The following oligonucleotides were used to introduce an Apr1 restriction site in RSVp49: AGC CCC AGA AAC GGC CGA CCA GCC CTA CCT CCT GTG G and in RSVp50: GCC ACT GCC AAC GGC CGA CGG GCC CTA CCT TGA ATT AG; Sp41 in RSVp49: CAG TGA CCC ACC GAG GGC GCC GCA TGC CCA CAG TC; Apr1 in RSVp49: CTG CCC AAC TTA ACC TAG CGG TAC TGG ATG TCA C and in RSVp50: GTG CGG CTT CCC GAA CCT CAG CAT CTC TCA TCT GAC; MHC, major histocompatibility complex; CAT, chloramphenicol acetyltransferase.
NF-κB Protein Structural and Functional Domains

**Fig. 1. Schematic representation of NF-κB1 and NF-κB2 domains and expression vectors.** Comparison of the major structural features of the NH2 terminus of NF-κB1 and NF-κB2 (upper panel) and depiction of the swap mutations (lower panel). The two conserved blocks of the Rel domain are shaded. Sites of swaps are indicated by arrows. Amino acids of NF-κB1 and NF-κB2 which generate each chimeric protein are indicated. NF-κB2 has more amino acid sequence upstream of the Rel-conserved domains, and in some cases, numbering is not consecutive, although all fusions were made precisely at conserved residues.

**Table 1.**

| **Sequence 1** | **Sequence 2** |
|----------------|----------------|
| 5' AGCTTGGGAGATCCCAACTGAGTCG -3' | 5' AGCCCTAACAGGGATCATGCTTAA -3' |

**Sequence 1** and a double-stranded oligonucleotide probe encoding a single MHC class I site

**Sequence 2**

Oligonucleotides were radiolabeled using Tj kinase and [γ-32P]ATP. ~1 ng was used in an EMSA reaction.

**Immunoprecipitations**—Immunoprecipitations were performed on in vitro synthesized proteins in a buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and protease inhibitors. An antibody was used directed against RelA(p65) (Santa Cruz). The antigen-antibody complexes were precipitated with protein G bound to Sepharose beads. Laemmli buffer was added, complexes were dissociated by boiling, and separated on a 10% SDS-polyacrylamide gel. Dried gels were exposed to Kodak XAR-5 film.

**Transfections and CAT Assays**—Transfections using DEAE-dextran with Jurkat T leukemia cell and CaPO4 transfection of 293 cells were performed and standardized for transfection efficiency as described previously (24, 26, 27). CAT assays were also performed as described previously (24).

**RESULTS**

The Rel-conserved regions of NF-κB1 and NF-κB2 were examined. A series of swap mutations at conserved amino acids spanning the Rel-conserved region (Fig. 1) were expressed using a eukaryotic expression plasmid with the RSV enhancer/promoter. Swap mutants which showed comparable levels of DNA binding activity following transfection of 293 cells (Fig. 2A) and Western blotting (data not shown) were analyzed. Nuclear protein extracts were prepared, and their DNA binding activity determined by EMSA. Similar to the processed wild type NF-κB1 gene product, swap mutants which contained the COOH-terminal Rel-conserved region of NF-κB1 bound to the canonical κB site of the HIV/Ig enhancers. These proteins also bound well to the κB-related site in the class I MHC gene (Fig. 2A, lanes 2–7 versus 10–15). In contrast, the NF-κB2 gene product (p52 or p49) bound poorly to the HIV/Ig κB site (Fig. 2A, lane 8). Swap mutants with the COOH-terminal regions of NF-κB2 substituted for this region of NF-κB1
showed reduced binding to the HIV κB site, whereas binding to the MHC κB site remained comparable with either wild type protein (Fig. 2B, lanes 2 to 5 versus 9–12). These findings demonstrate that the COOH-terminal region of NF-κB1 is necessary and sufficient to confer DNA binding specificity for the HIV κB site to NF-κB2. Interestingly, the mobilities of the different swap mutant proteins upon binding to the MHC κB site were different among these mutants. Possibly,
these differences could be due to variation in conformation, among these mutant proteins. Regardless of these differences, however, the complexes formed with the HIV xB sites suggest that the COOH-terminal putative dimerization domains of NF-xB1 or NF-xB2 determine their fine specificity of DNA binding, presumably by indirectly altering the conformation of the region which contacts DNA.

Another difference between NF-xB1 and NF-xB2 is their ability to functionally transactivate reporter genes in combination with RelA(p65). The efficiency may vary among cell types and is dependent upon the relative concentration of RelA(p65) (9, 12); however, in the presence of a constant and suboptimal amount of RelA(p65), NF-xB2 stimulates gene expression of the HIV enhancer more effectively than NF-xB1 (Fig. 3A; see also Refs. 9 and 12). This ability of NF-xB2 to more efficiently synergize with RelA was similar whether a processed form of p100 or the alternatively spliced transcript, p49, was used (Fig. 3A, NF-xB2 (p100; 1-446) versus NF-xB2 (p49; 1-444). The difference in cooperativity with RelA(p65) was not due solely to sequences in the COOH-terminal Rel domain of NF-xB2 (Fig. 3B, C-D). In fact, all swap mutants showed reduced ability to act in concert with RelA(p65) (Fig. 3B). These results indicated that the NH2- or COOH-terminal Rel region of NF-xB2 alone did not specify its ability to preferentially activate transcription in concert with RelA(p65) and suggested that more than one part of the Rel-conserved domain of NF-xB2 may be required.

To define the regions of the NF-xB1 and NF-xB2 responsible for these effects, swap mutants of both the COOH- and NH2-terminal Rel regions were used (Fig. 4A). Several of these proteins demonstrated a higher level of transactivation in combination with RelA(p65) (Fig. 4A, NF-xB 2-1-2, I-V). Interestingly, two of these chimeric proteins transactivated the HIV enhancer better than wild type NF-xB2 in combination with RelA(p65) (Fig. 4A, NF-xB 2-1-2, III and IV). Possibly, this difference is due to changes in the binding of these double swap chimeric proteins to the HIV xB site (Fig. 4B, lanes 4 and 5). Alternatively, the conformation of these swap mutants which improves DNA binding might facilitate other protein-protein interactions that promote transcriptional initiation. NF-xB and swap mutants showed comparable levels of protein expression following transfection both in Jurkat cells (Fig. 4C, see also Ref. 12) and in 293 cells by Western blot analysis (data not shown), confirming that the functional differences were not the result of differential protein expression.

A similar effect was seen with Bcl3, which had been shown previously to function more effectively with NF-xB2 (16, 17). In Jurkat cells, co-transfection of NF-xB2 and Bcl3 expression vectors showed greater stimulation of CAT activity than NF-xB1 and Bcl3, and the NH2- or COOH-terminal Rel region of NF-xB2 was not sufficient to restore function with Bcl3 (Fig. 5). However, double swap mutants III and V were able to stimulate HIV enhancer activity to levels comparable with wild type NF-xB2 with Bcl3 (Fig. 5). This synergistic effect was seen in a dose-dependent fashion, peaking when 1 µg of RSV Bcl3 was used (data not shown). Of interest, similar subregions of the NF-xB2 Rel domains conferred preferential activation for both RelA(p65) and Bcl3.

To determine whether NF-xB2 dimerizes more efficiently with RelA compared with NF-xB1, immunoprecipitations were performed with in vitro synthesized proteins. This analysis showed comparable abilities of both proteins to interact with RelA in solution (Fig. 6). These results suggest that dimerization properties alone are unlikely to account for the differential effects of NF-xB subunits. It is thus likely that NH2- and COOH-terminal domains contribute to the specificity of interactions with RelA(p65) and Bcl3 by affecting protein conformation.

DISCUSSION

The role of NF-xB in transcriptional regulation is complex. A number of different xB sites have been found in the regulatory regions of multiple viral and cellular genes. It has been shown that specific interactions of different NF-xB/Rel subunits can allow selective activation of xB-regulated genes (12). In addition, it has recently been realized that a variety of protein-protein interactions occur between NF-xB and other transcription factors. In this report, we have determined the contributions of different regions of the Rel domains of NF-xB1 and NF-xB2 to their DNA binding specificity and interaction with other transcription factors. In a separate study, we have determined that an interaction of the RelA(p65) transcription factor with Sp1, also mediated through the Rel-conserved domain, is
FIG. 4. NF-κB swap mutants and functional interaction with RelA(p65). A, Stimulation of the HIV-enhancer by transfection of double swap mutants in Jurkat cells. Conditions are the same as in Fig. 3B, and results represent mean with S.D. based on four independent experiments. Schematic diagram of double swap mutants is shown. The site of the amino acid swap is indicated.

B, DNA binding activity of double swap mutants and wild type proteins was determined by EMSA analysis in 293 cells expressed protein on the HIVIg κB or MHC κB probes. Cells transfected with wild type NF-κB (lanes 1 and 8), swap mutants I (lanes 2 and 9), II (lanes 3 and 10), III (lanes 4 and 11), IV (lanes 5 and 12), V (lanes 6 and 13), or NF-κB1 (lanes 7 and 14) were analyzed. C, DNA binding activity of selected NF-κB1 and -2 mutants determined by EMSA analysis of recombinant proteins expressed in Jurkat T leukemia cells with an MHC κB radiolabeled probe. These proteins include NF-κB2-1(105/111)-B, NF-κB2-1(272/298)-F, NF-κB2-1(105/111)-2/299/III, NF-κB2-1(272/298)-2/43/297/273/IV, β-galactosidase-transduced negative control (lane 9), and tumor necrosis factor-stimulated cells (lane 10). 100-Fold molar excess of wild type MHC κB competitor DNA was used in lanes 1, 3, 5, and 7. The arrow indicates the position of the specific NF-κB complexes.

required for transcriptional activation of HIV (26, 28).

These data show that specific subdomains of the Rel-conserved region of NF-κB determine the specificity of interactions with DNA and with other transcription factors. Remarkably, the putative dimerization domain of NF-κB1 influences the specificity of DNA binding. This protein represents an example wherein binding specificity can be mapped to a region thought not to be directly involved in contacting DNA. Despite the fact that the COOH-terminal domain confers DNA binding specificity, both subunits, NF-κB1 and NF-κB2, seem to interact with RelA with comparable strength in solution (Fig. 6). These data suggest that Rel domains which show differences in DNA binding of the homodimer do not differ significantly in their ability to form heterodimers with another family member, RelA(p65). Differences in homodimer DNA binding specificity are therefore likely determined by the conformation of the subunits of the homodimer. In contrast to the NF-κB homodimer binding, we find that additional regions promote optimal functional interactions with RelA(p65) or Bcl3, suggesting that heterologous protein interactions can occur through the NH2-terminal region of the Rel-conserved domain. It is likely that the NH2-terminal subdomain (A) is required for interaction with other transcription factors as well (29).

Whether DNA binding is required for this interaction in vivo is unknown, although it is not required for these interactions at high protein concentration in vitro. Physiologically, within a cell, it is likely that DNA is required to stabilize these interactions and promote the appropriate conformation of the RelA(p65) protein. These findings and several recent observations have begun to suggest that the DNA binding domains of
transcription factors are not limited simply to an interaction with nucleic acid. Our data suggest that these regions of the protein, in addition to their associations with nucleic acids, allow them to interact with other transcription factors. It is probable that the spacing of adjacent transcriptional regulatory sites helps to determine whether such interactions will facilitate productive assembly of the transcription complex. In the case of the HIV enhancer, we have shown previously that spacing of the κB and Sp1 sites is essential for transcriptional activation and is not compensated by mutations which restore the dyad symmetry of the DNA (26). In this sense, the DNA serves as a template to facilitate specific protein-protein interactions which will subsequently allow cooperative binding of TBP-associated factors. It is likely that different combinations of NF-κB gene products and cis-acting regulatory sequences therefore can be used to activate specific genes during cellular activation and in development. The ability to interfere with such protein interactions selectively may also provide for mechanisms to selectively regulate gene expression in vivo and lead to further understanding of their roles in differentiation and development.

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