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Interaction of the v-rel protein with an NF-κB DNA binding site

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ABSTRACT **The avian reticuloendotheliosis virus T contains within its genome the oncogene *rel*. The expression of this gene is responsible for the induction of lymphoid tumors in birds. Recently, the *rel* gene was shown to be related to the p50 DNA binding subunit of the transcription factor complex NF-κB. Binding sites for the NF-κB complex are found in the enhancer regions of a number of genes, including the immunoglobulin κ gene and the human immunodeficiency virus long terminal repeat. In this communication we identify an activity from avian reticuloendotheliosis virus T-transformed avian lymphoid cells that binds in an electrophoretic-mobility-shift assay to an NF-κB binding site from the κ enhancer. This activity contains proteins immunologically related to rel, as detected by polyclonal and monoclonal antibodies directed against v-rel. In a DNA affinity precipitation assay using the NF-κB site from the human immunodeficiency virus long terminal repeat, v-rel and several other proteins were identified. These data suggest that oncogenic transformation by v-rel is the result of an altered pattern of gene expression.**

Infection of birds with the avian reticuloendotheliosis virus T (REV-T) results in a fatal lymphoma within days after infection (1). *In vitro* REV-T is also able to transform avian lymphoid cells (2). v-*rel*, the oncogene harbored by REV-T, is a truncated form of its cellular homologue, c-*rel*, that lacks two amino acids at the amino terminus and 118 amino acids at the carboxyl terminus (3, 4). The protein product of the v-*rel* gene $p59^{v\text{-}rel}$ is a phosphoprotein that is found in both the nucleus and cytoplasm in transformed lymphoid cells (5). We (5) and others (6) have demonstrated that v-rel is part of a high molecular mass complex in the infected cell that includes cellular proteins of 124 kDa, 115 kDa, and 36 kDa.

The *rel* gene was shown to be related to the *Drosophila* embryonic polarity gene dorsal. The extent of homology in a 295-amino acid stretch at the amino terminus is 75% when conservative amino acid changes are taken into account (7). The carboxyl termini are unrelated. Reminiscent of v-rel, the dorsal protein is also found either in the cytoplasm or nucleus. However, the localization of dorsal is controlled by positional effects within the developing embryo and carboxyl-terminal sequences (8). The recent cloning of the p50 DNA binding subunits of the transcription factors NF-κB and KBF-1 also revealed homology with *rel* (9, 10). As with dorsal, the homology extends over ≈334 amino acids at the amino terminus. Biochemical evidence suggests further similarities between these proteins. NF-κB is found in the cytoplasm and nucleus and also associates in complex with other cellular proteins (11).

Functional studies support the suggestion that NF-κB, dorsal, and rel are closely related. All three proteins are able to modulate transcription from a variety of promoters (11–13). In addition, the c-rel protein contains a carboxyl-terminal transcriptional activation domain as shown by fusion to LexA or GAL4 DNA binding sequences (14, 15). Thus these data imply that rel is involved in the control of transcription.

The biochemical and functional similarities of rel, dorsal, and NF-κB prompted us to examine the ability of v-rel to bind to DNA, specifically to an NF-κB binding site. In this communication we demonstrate by an electrophoretic-mobility-shift assay (EMSA) that nuclear and cytosolic extracts from a REV T-transformed avian lymphoid cell line (NPB4) contain an activity that is immunologically reactive with both polyclonal and monoclonal antibodies directed against v-rel and that binds to a double-stranded oligonucleotide containing an NF-κB binding site. This activity consists of a set of proteins including v-rel, as determined by a DNA affinity precipitation (DNAP) assay (16) and two-dimensional (2D) gel analysis of proteins immunoprecipitated from NPB4 extracts with a monoclonal antibody directed against v-rel.

MATERIALS AND METHODS

Cells. NPB4 cells are a REV-T-transformed cell line (derived by infection of avian bone marrow) that express low levels of IgM and B lymphoid-specific cell surface markers (L. Morrison, personal communication; ref. 2). The cells were grown as described (5).

EMSA. Nuclear or S100 extract (7.5 μg) from NPB4 cells, prepared as described by Dingham *et al.* (17), was incubated on ice for 15 min in the presence of 0.5 μg of salmon sperm DNA and incubation buffer [final concentrations after DNA oligonucleotide addition: 10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (vol/vol) glycerol, and 3 mM dGTP], followed by the addition of 10,000–20,000 cpm of labeled oligonucleotide (with or without unlabeled competitor) and incubation at room temperature for 20 min. The reactions were performed in a final volume of 25 μl and samples were run on 6% native polyacrylamide gels at 200 V in TBE (25 mM Tris base/22 mM boric acid/0.25 mM EDTA, pH 8.0) at 4°C.

Oligonucleotides and Labeling Procedure. Oligonucleotides contained the following sequences: NF-κB, 5′-AGCTTCAACAGAGGGGACTTTCCGAGAGGCTCGAG-3′, and the complementary strand, 5′-AATTCTCGAGCCTCTCGGAAAGTCCCCTCTGTTGAA-3′. NF-κB(M) oligonucleotides are identical to the NF-κB oligonucleotides except that the underlined nucleotide was changed from a guanine to a cytosine (and vice versa for the complementary strand). This sequence has been reported to have a >100 times lower affinity for NF-κB (18). The MYB oligonucleotide, kindly provided by Joe Lipsick (State University of New York at Stony Brook), contained the sequence 5′-GATCCAAATAACGGAAAAGATCT-3′, and the complementary strand was 5′-GATCAGATCTTTTCCGTTATTTG-3′. The HIVEN3c

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Abbreviations: REV-T, reticuloendotheliosis virus T; EMSA, electrophoretic-mobility-shift assay; DNAP, DNA affinity precipitation; 2D, two dimensional.

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oligonucleotide contained the sequence 5′-AGGGACTTTC-CGCTGGGGACTTTCCAG-3′ repeated once and annealed with the complimentary strand. The HIVEN3m mutant oligonucleotide contained the sequence 5′-ACTCACTTTCCGCT-GCTCACTTTCCAG-3′ repeated once and annealed to the complimentary strand. The HIVENc and -m oligonucleotides were used in the DNAP assay described below. The probes used for mobility-shift analyses were labeled using [α-^{32}P]dATP, [α-^{32}P]dGTP, [α-^{32}P]dCTP, and the Klenow fragment of DNA polymerase I.

Antibodies and Western Blots. Rabbit polyclonal antibody SB66 is directed against a trpE–rel fusion protein (pJH12 rel), which has been described (5, 19). P1 represents the pJH12 rel fusion protein. P2 is a nonoverlapping trpE–rel fusion protein.

Production of Monoclonal Antibodies. The polyclonal antibody SB66 was covalently linked to protein A-Sepharose by standard procedures (20). This matrix was incubated with NPB4 cell extract and bound rel protein was eluted at pH 11.5 in 20 mM ethanolamine. BALB/c mice were inoculated with 50 μg of affinity-purified rel protein for the production of monoclonal antibodies. Standard protocols were followed for injection, fusion of spleen cells, and screening of hybridomas (20). Hybridoma supernatants were screened for reactivity to p59$^{v\text{-}rel}$. Western blots were performed essentially as described (21).

DNAP Analysis. NPB4 cells were grown as described (5) and labeled with Tran^{35}S-label (ICN) at 1 mCi/ml (1 Ci = 37 GBq). Immunoprecipitation was carried out as described (5) and samples were prepared for analysis in 2D gels as described (16). Cells were lysed in 600 μl of DNAP buffer as reported (16). The final KCl concentration for extraction was 250 mM and the final NaF concentration was 50 mM. DNAP binding assays were conducted at 75 mM KCl and the same KCl concentration was used for rinsing the streptavidin-agarose–biotinylated oligonucleotide complex. The appropriate biotinylated oligonucleotide (HIVEN3c or HIVEN3m synthesized by the Cold Spring Harbor Laboratory oligonucleotide facility) at 100 pmol and a 40-fold mass excess of poly(dI-dC) (Pharmacia) were used per DNAP assay. Beads were rinsed four times with 400 μl of DNAP buffer containing 75 mM KCl and 5 mM NaF and then rinsed once with 50 μl of DNAP buffer without KCl or NaF. All samples were prepared for 2D gels by addition of 1 μl of a DNase/RNase solution, incubation on ice for 2 min, addition of 40 μl of a SDS solution, and incubation at 37°C for 4 min. Each sample was lyophilized and resuspended in 40 μl of SB 2D gel sample buffer. The solutions above were as described (16). The 2D gels consisted of a pH 3.5–10 isoelectric first-dimension gel and a 10% acrylamide second-dimension gel.

RESULTS AND DISCUSSION

NF-κB Binding Activity in NPB4 Cells. To determine if the REV-T-transformed cell line NPB4 contained an activity that could bind to the NF-κB binding site, nuclear extracts were prepared from NPB4 cells and used in the EMSA (22). A radioactively labeled oligonucleotide containing a single NF-κB site was used as a probe in this analysis. Two major protein–DNA complexes (B_1 and B_2) were observed after incubation of the oligonucleotide with nuclear extract (Fig. 1*A*, lane 1), suggesting that the NPB4 cell line did indeed contain an NF-κB-like binding activity. The relationship between B_1 and B_2 is not clear at present since the EMSA precludes an analysis of the protein composition of B_1 and B_2. To demonstrate the specificity of the B_1 and B_2 complexes for the NF-κB oligonucleotide, competition experiments were performed. Unlabeled oligonucleotides containing the wild-type NF-κB site, a mutant NF-κB site containing a single point mutation, or a myb binding site were used to compete with labeled NF-κB oligonucleotides. The wild-type oligonucleotide competed effectively at a 100-fold molar excess (Fig. 1*A*, lanes 2–4). The mutant NF-κB oligonucleotide, which has been reported to have an $\approx$100 times lower affinity for NF-κB (18), competed moderately at a 1000-fold molar excess (Fig. 1*A*, lanes 5–7). In contrast, the oligonucleotide containing the myb binding site did not compete when present at a 1000-fold molar excess (Fig. 1*A*, lanes 8–10).

To investigate whether the specific complexes (B_1 and B_2) contained the rel protein, an EMSA was performed in the presence of polyclonal antibody SB66 directed toward the rel protein. A new slower migrating complex (B*) and a corresponding decrease in the B_1 and B_2 protein–DNA complexes was observed in the presence of SB66 but not preimmune serum (Fig. 1*B*, lanes 3 and 4). To demonstrate the specificity of this interaction, protein–DNA complexes were incubated with antiserum SB66 in the presence of the trpE–rel fusion protein (P1) (5, 19) used to generate the antibody. The slower migrating species B* was no longer detectable (Fig. 1*B*, lane 5). Incubation of the protein–DNA complexes with antibody SB66 and an unrelated trpE–rel fusion protein had no effect on the formation of the B* complex (Fig. 1*B*, lane 6). These results suggested that the protein(s) interacting with the NF-κB oligonucleotide probe were antigenically related to rel. It was possible that the polyclonal antibody recognized cross-reactive proteins related to the NF-κB binding proteins. To demonstrate the specificity of the SB66 polyclonal antibody, Western blot analysis of NPB4 cell extract was performed. The nitrocellulose filter containing NPB4 cell lysate was probed with affinity-purified SB66. As can be seen in Fig. 1*D*, lane 3, the antibody recognizes p68$^{c\text{-}rel}$, p59$^{v\text{-}rel}$, and several other proteins in the molecular mass range of 40–50 kDa in NPB4 cells. These proteins may represent proteolytic cleavage products of rel or cross-reactive related proteins. Therefore, to demonstrate that v-rel was at least one component in the protein–DNA complexes, two v-rel-specific monoclonal antibodies were also tested for their ability to reduce the mobility of the protein–DNA complex(es) (Fig. 1*C*, lanes 3 and 4). The specificity of the anti-rel monoclonal antibodies 6 and 33 is shown in Fig. 1*D*, lanes 1 and 2. When NPB4 cell extracts were subjected to Western blot analysis and probed with monoclonal antibodies 6 and 33, these antibodies recognized only p59$^{v\text{-}rel}$. Each monoclonal antibody was able to reduce the mobility of the protein–DNA complexes to a position equivalent to B* (Fig. 1*C*, lanes 3 and 4), supporting the conclusion that v-rel is at least one of the constituents binding to the NF-κB site. As can be seen, incubation with the monoclonal antibodies does not dramatically reduce bands B_1 or B_2. This may be due to one of several possibilities. The titers or affinity of monoclonal antibodies 6 and 33 are significantly lower than SB66, perhaps accounting for the weak intensity of the B* band observed. Alternatively, the monoclonal antibodies may recognize only a subset of the protein–DNA complexes. It is also possible that the epitopes recognized by the monoclonal antibodies are partially masked in the protein–DNA complexes. Because SB66 also recognizes c-rel, it is conceivable that some of the binding activity reactive with the SB66, but not the monoclonal antibodies, is c-rel. However, preliminary examination of the binding activity from the NPB4 cell line with a c-rel-specific peptide antibody did not reveal any detectable c-rel-specific binding activity (data not shown). Thus these data suggest that nuclear extracts from transformed lymphoid cells possess an NF-κB binding activity that contains v-rel. Incubation with an unrelated monoclonal antibody, M4, specific to avian immunoglobulin (IgM, ref. 23), did not result in the appearance of a B* band (Fig. 1*C*, lane 5).

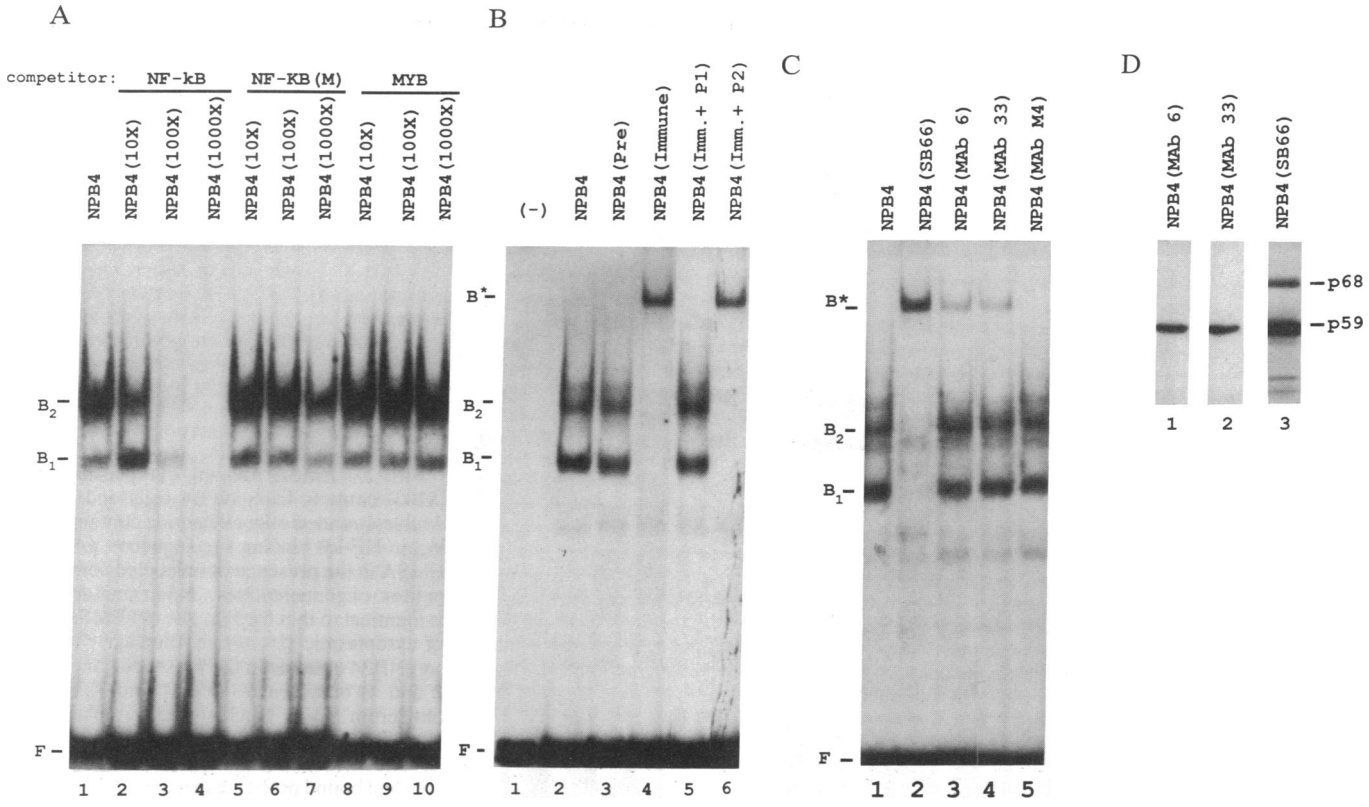

FIG. 1. EMSA utilizing nuclear extracts from NPB4 cells and a double-stranded oligonucleotide containing an NF-κB binding site sequence. (*A*) EMSA in the presence of unlabeled competitor oligonucleotides. The molar excess of competitor is indicated in parentheses above the respective lanes. Lane 1 does not contain added competitor. Bands F and B are free and bound probe DNA, respectively. NF-κB, MYB, and NF-κB(M) are unlabeled double-stranded competitor oligonucleotides containing a wild-type NF-κB binding site, a myb binding site, or an NF-κB site with a single-point mutation, respectively. (*B*) EMSA done in the absence of nuclear extract (lane 1) or in the presence of 7.5 μg of nuclear extract (lanes 2–6). DNA binding reactions were followed by incubation with preimmune sera (lane 3), polyclonal immune serum SB66 directed toward the rel protein (lane 4), immune serum in the presence of P1, a trp E–rel fusion protein used to immunize the rabbits in the generation of antiserum SB66 (lane 5), or immune serum in the presence of P2, an unrelated trpE–rel fusion protein (lane 6). Bands F and B are free and bound probe, respectively. B* indicates a band with retarded migration after the addition of polyclonal antibody directed to the rel protein. (*C*) EMSA of extracts incubated with polyclonal antisera SB66 (lane 2) or monoclonal antibodies (MAb) 6 and 33 (lanes 3 and 4, respectively). Lane 5 contains monoclonal antibody M4, specific to avian immunoglobulin, as a negative control (23). Lane 1 contains no added immunoglobulin. (*D*) Western blot of lysates from NPB4 cells probed with monoclonal antibody 6 (lane 1), monoclonal antibody 33 (lane 2), or polyclonal antibody SB66 (lane 3). Bands p59 and p68 identify avian v-rel and c-rel proteins, respectively.

NF-κB was originally identified as a lymphoid-specific nuclear activity capable of binding to the immunoglobulin κ enhancer (24). It was subsequently demonstrated to exist ubiquitously in an inactive cytoplasmic form (for review, see ref. 11). Treatment of cells with phorbol esters causes the dissociation of cytoplasmic NF-κB from an inhibitor protein (IκB) resulting in translocation to the nucleus and an increase in NF-κB DNA binding activity (25, 26). Our previous biochemical analyses of the NPB4 cell line have shown that 80–90% of the v-rel protein is present in the cytoplasm (5). Therefore, we were interested in comparing the binding activity of the cytoplasmic and nuclear extracts from the NPB4 cell line to an oligonucleotide containing the NF-κB site.

Two protein–DNA complexes (B_{c1} and B_{c2}) were observed after incubation of the oligonucleotide with the cytoplasmic (S100) extract (Fig. 2*A*, lane 1). Competition analysis was then performed to determine the specificity of the B_{c1} and B_{c2} complexes for the NF-κB oligonucleotide. The wild-type oligonucleotide competed for both B_{c1} and B_{c2} effectively at 100-fold molar excess (Fig. 2*A*, lanes 2–4). The oligonucleotide containing the mutant NF-κB site competed for the B_{c2} complex at a 100-fold molar excess (Fig. 2*A*, lanes 5–7), although less effectively than the wild-type oligonucleotide. Oligonucleotides containing the myb binding site competed for B_{c2} at a 1000-fold molar excess. The B_{c1} complex was not competed significantly by the myb or mutant NF-κB oligonucleotides (Fig. 2*A*, lanes 5–10).

To determine whether either B_{c1} or B_{c2} complexes contained rel or rel-related proteins, an EMSA was performed. Protein–DNA complexes were incubated with the affinity-purified SB66 antisera after the DNA binding reaction. A band with reduced mobility (B*) was observed after incubation with the antibody, with a concommitant decrease in the B_{c1} band, suggesting that this complex, but not the B_{c2} complex, contained proteins recognized by the anti-rel antisera (Fig. 2*B*, lanes 1 and 2). B_{c1} appears to comigrate with the nuclear band B_2 (Fig. 2*B*, lanes 3 and 4) and both are reactive with antibody SB66. However, it is not clear from this analysis that these two comigrating bands represent the same protein–DNA complex. Analysis with the monoclonal antibodies 6 and 33, which are only reactive to the v-rel protein, also resulted in a band with reduced mobility (data not shown). When cell equivalent amounts of NPB4 nuclear and cytoplasmic extract were compared by EMSA for binding to the NF-κB oligonucleotide, approximately equivalent binding activity was observed (data not shown). However, the majority of SB66-reactive proteins (predominantly v-rel; ref. 5) are in the cytoplasm, suggesting that the specific binding activity of the cytoplasmic proteins is lower than that from the nuclear fraction.

DNAP Assay. The EMSA data presented are suggestive that v-rel is at least part of the protein complex that binds to the

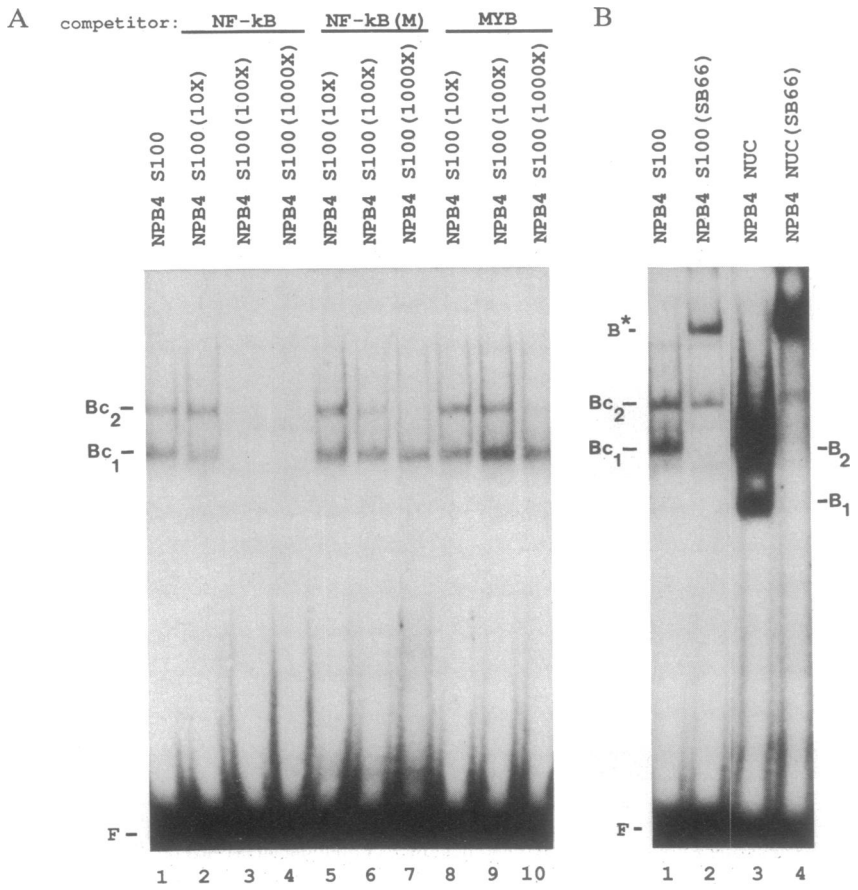

FIG. 2. EMSA utilizing cytoplasmic (S100) extracts from NPB4 cells and a double-stranded oligonucleotide containing an NF-κB binding site sequence. (*A*) EMSA in the presence of unlabeled competitor oligonucleotides. Nomenclature is identical to that for Fig. 1*A*. (*B*) EMSA of cytoplasmic (15 μg) and nuclear (7.5 μg) NPB4 extracts in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of antiserum SB66. Nuclear and cytoplasmic (S100) extracts were prepared as described (17). Bands F, B, and B_c are free and bound probe, respectively. Band B* is a probe with reduced mobility after the addition of antibody specific to the rel protein.

NF-κB site. To show definitively that v-rel was part of the protein complex binding to the NF-κB site, a microscale DNAP assay was employed (16). Whole-cell extracts, prepared as described (16), were incubated with biotinylated oligonucleotides containing either wild-type (HIVEN3c) or mutant (HIVEN3m) NF-κB binding sites from the human immunodeficiency virus long terminal repeat. Proteins bound to the probes were analyzed by 2D gel electrophoresis. Fig. 3 is an example of such an analysis. Only the relevant portions of the gels (i.e., proteins of 40–65 kDa) are shown from this experiment. As can be seen in Fig. 3*B*, several proteins are identified that bind to the wild-type (HIVEN3c) but not to the mutant oligonucleotide (Fig. 3*A*, HIVEN3m oligonucleotide). These include the circled and bracketed spots. This result is consistent with previous reports that have identified a number of cellular proteins that bind to the NF-κB site in the DNAP assay (16, 27, 28), including the HIVEN86A protein, which has recently been shown to correspond to human c-rel (29).

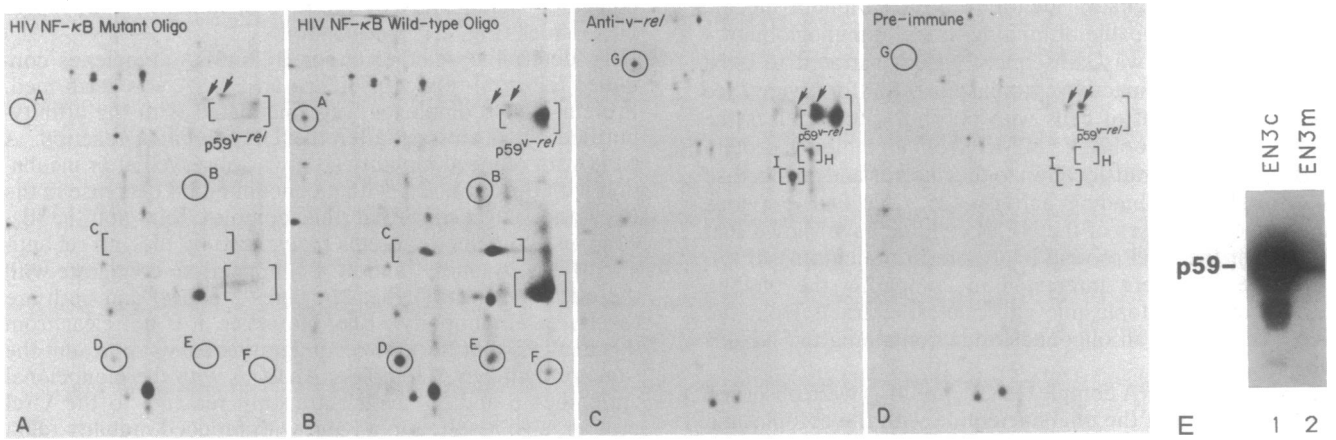

FIG. 3. 2D gel comparison of human immunodeficiency virus enhancer binding proteins to proteins in an anti-rel immune complex. (*A*) DNAP analysis using the HIVEN3m (GGG to CTC mutant oligonucleotide). (*B*) DNAP analysis using the HIVEN3c oligonucleotide. (*C*) Anti-rel immunoprecipitation using monoclonal antibody 33, which recognizes v-rel but not proteins of 40–50 kDa. (*D*) Immunoprecipitation with HAT medium (no added monoclonal antibody). $p59^{v\text{-}rel}$ is identified in *B* and *C*. Proteins in *B* that specifically interact with the human immunodeficiency virus type 1 enhancer core oligonucleotide are designated by letter. Proteins in *C* that are members of the anti-rel immune complex are designated by letter. Exposure of fluorographed gels was 20 days at −70°C. Only relevant regions of the gels (i.e., proteins of 40–65 kDa) are shown in this figure. (*E*) NPB4 protein–DNA complexes were formed with oligonucleotides HIVEN3c or HIVEN3m, as described above, and subjected to analysis on 10% denaturing acrylamide gels. The gel was analyzed by Western blot (21) and probed with the SB66 antibody. Lanes 1 and 2 are proteins complexes with HIVEN3c and HIVEN3m oligonucleotides, respectively. The location of $p59^{v\text{-}rel}$ is indicated (p59).

To determine which of these proteins corresponded to v-rel, immunoprecipitates with monoclonal antibody 33 were made from the NPB4 cell line. Immunoprecipitated protein was then analyzed by 2D gel electrophoresis. As can be seen in Fig. 3*C*, $p59^{v\text{-}rel}$ and three other minor proteins (labeled G, H, and I) were specifically precipitated. However, the three minor species did not bind to the wild-type NF-κB oligonucleotide (Fig. 3*B*). Fig. 3*D* is a control immunoprecipitation carried out with hypoxanthine/aminopterin/thymidine (HAT) medium alone to show nonspecific background proteins. Comparison of Fig. 3 *B* with *C* shows definitively that $p59^{v\text{-}rel}$ binds to the wild-type NF-κB oligonucleotide. In addition, it appears that only one isoform of the protein participates in the binding.

Further confirmation of v-rel binding was obtained by immunoblot analysis of the DNAP complex. NPB4 lysate was used to form protein–DNA complexes on wild-type (HIVEN3c) or mutant (HIVEN3m) oligonucleotides. Eluted protein was subjected to analysis on denaturing polyacrylamide gels and Western blots. The probe, in this case, was SB66, which recognizes $p68^{c\text{-}rel}$, $p59^{v\text{-}rel}$, and several other cross-reactive species of 40–50 kDa (Fig. 1*D*, lane 3). Fig. 3*E* is an example of such a blot. $p59^{v\text{-}rel}$ and several other cross-reactive species of 40–50 kDa can be seen (Fig. 3*E*, lane 1). Comparatively low amounts of binding to the mutant oligonucleotide (HIVEN3m) are detected in this assay as expected (Fig. 3*E*, lane 2).

These data definitively demonstrate that $p59^{v\text{-}rel}$ is a member of a set of proteins that are able to bind to the NF-κB site. As demonstrated, several other proteins are also involved in this interaction (encircled and bracketed proteins in Fig. 3*B*). These proteins were not found in immunoprecipitates from NPB4 cells using monoclonal antibody 33, which is specific for v-rel and not proteins in the lower molecular mass range (Fig. 1*D*, lane 2). Clearly, it will be of interest to determine if polyclonal antibody SB66 recognizes proteins such as D, E, or F (Fig. 3*B*) since it recognizes proteins in that molecular mass range in immunoblot analysis (Fig. 1*D*, lane 3). Given the apparent molecular mass of these proteins (40–50 kDa), it is possible that one of them is the rel-related p50/KBF-1/NF-κB.

The data presented demonstrate conclusively that v-rel from a REV-T-transformed lymphoid cell line is involved with other proteins in binding to the NF-κB site. We have not yet shown that v-rel binds directly to DNA. However, Kieran *et al.* (10) have shown that a truncated form of *in vitro*-translated v-rel interacts with the KBF-1 site. Purified v-rel should be tested for its ability to bind directly to DNA. It will be of interest to determine if c-rel is involved in an interaction at the NF-κB site. Because there is no normal cell counterpart to the NPB4 cell line, direct comparison of v-rel and c-rel was precluded. However, preliminary analysis of extracts from normal bursal lymphoid cells revealed binding activity that resulted in protein–DNA complexes that did not appear to comigrate with those formed with NPB4 extracts. At least a portion of these bursal cell complexes were reactive with a c-rel-specific peptide antibody, suggesting that c-rel is also able to interact with the NF-κB binding site. Determination of the specificity of v-rel and c-rel binding and the functional consequences of that binding on transcription will undoubtedly lead to a better understanding of transformation by the *rel* gene.

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