JOURNAL OF VIROLOGY, Aug. 1992, p. 5018–5029
0022-538X/92/085018-12$02.00/0

Vol. 66, No. 8

The v-*rel* Oncogene: Insights into the Mechanism of Transcriptional Activation, Repression, and Transformation

WILLIAM H. WALKER,[1] BERND STEIN,[1] PARHAM A. GANCHI,[1]† JAN A. HOFFMAN,[1] PETER A. KAUFMAN,[1] DEAN W. BALLARD,[1]† MARK HANNINK,[2] AND WARNER C. GREENE[1]†*

Howard Hughes Medical Institute, Box 3037, Duke University Medical Center, Durham, North Carolina 27710,[1] and Department of Biochemistry, University of Missouri, Columbia, Missouri 65212[2]

Received 9 December 1991/Accepted 13 May 1992

The v-*rel* oncogene product from the avian reticuloendotheliosis virus strain T corresponds to a member of the Rel-related family of enhancer-binding proteins that includes both the mammalian 50- and 65-kDa subunits of the NF-κB transcription factor complex. However, in contrast to NF-κB, v-Rel has been shown to function as a dominant-negative repressor of κB-dependent transcription in many mature cell types. We now demonstrate that a highly conserved motif within the Rel homology domain of v-Rel containing a consensus protein kinase A phosphorylation site is required for DNA binding, transcriptional repression, and cellular transformation mediated by this oncoprotein. However, replacement of the serine phosphate acceptor within the protein kinase A site with an alanine did not alter any of these functions of v-Rel, suggesting that phosphorylation at this site is not central to the regulation of this oncogene product. Rather, the inactive mutations appear to identify a functional domain within v-Rel required for these various biological activities. It is notable that these same mutations do not impair the ability of v-Rel to heterodimerize with the 50-kDa subunit of NF-κB, suggesting that v-Rel-mediated transcriptional repression likely involves direct nuclear blockade of the κB enhancer rather than indirect alterations in the composition of preformed cytoplasmic NF-κB complexes. Paradoxically, when introduced into undifferentiated F9 cells, v-Rel functions as a κB-specific transcriptional activator rather than as a dominant-negative repressor. These stimulatory effects of v-Rel require both the conserved protein kinase A phosphorylation site and additional unique C-terminal sequences not needed for v-Rel-mediated repression in mature cells. Retinoic acid-induced differentiation of these F9 cells restores the repressor function of v-Rel. These opposing biological actions of v-Rel occurring in cells at distinct stages of differentiation may have important implications for the mechanism of v-Rel-mediated transformation occurring in avian splenocytes.

The avian reticuloendotheliosis virus strain T (REV-T) (59, 68) corresponds to a remarkably virulent retrovirus that induces fatal lymphoid tumors in young birds (54). REV-T encodes a unique 59-kDa phosphorylated oncoprotein, v-Rel, which alone has proven sufficient to transform lymphoid cells (7, 8, 32). It is notable that v-Rel exhibits extensive homology with several mammalian κB enhancer-binding proteins, including the 50- and 65-kDa subunits of the NF-κB transcription factor complex (15, 28, 39, 43). In addition, v-Rel shares structural homology with the *dorsal* gene product of *Drosophila melanogaster*, a maternal morphogen that governs dorsal-ventral axis formation in the developing embryo (60). Specifically, these functionally diverse proteins share an N-terminal region of homology spanning approximately 300 amino acids, termed the Rel homology domain, which is essential for an array of functions including DNA binding, protein oligomerization, and nuclear localization (6, 15, 16, 28). At present, the precise subregions within this large Rel domain responsible for DNA binding and oligomerization with other Rel family members remain undefined.

The 50-kDa (NF-κB p50) and 65-kDa (NF-κB p65) subunits of the NF-κB heterodimeric complex represent well-characterized cellular members of this Rel-related family of polypeptides (18). NF-κB binding sites serve as functional enhancer elements in a large number of immunologically relevant cellular and viral transcription units, including the kappa light chain immunoglobulin (53), the interleukin-2 (IL-2) (22, 30) and IL-2 receptor α (IL-2Rα) genes (9), as well as the long terminal repeat (LTR) of the type 1 human immunodeficiency virus (HIV-1) (36). The same cellular activation signals that stimulate expression of these inducible transcription units (29), including phorbol esters (9, 52), cytokines such as tumor necrosis factor α and IL-1 (34, 40), and the human T-cell leukemia virus type I (HTLV-I) Tax protein (5, 31, 44, 55), all induce nuclear expression of NF-κB. In part, nuclear expression of NF-κB is regulated at a posttranslational level, involving its association with and induced dissociation from a cytoplasmic inhibitor termed IκB (2, 3, 14, 70). On the basis of in vitro analyses, phosphorylation of IκB has been proposed to play a major physiological role in promoting its dissociation from NF-κB (14).

Like NF-κB, c-Rel, and Dorsal (11, 23, 41, 48), v-Rel has been implicated as a transcriptional regulator on the basis of its ability to modulate the activity of certain viral promoters (13, 20). Subsequent transient transfection experiments performed in mature Jurkat T lymphocytes or NIH 3T3 cells have revealed that v-Rel specifically engages the κB enhancer but functions as a dominant-negative repressor of κB-directed transcription from the IL-2Rα promoter and the

* Corresponding author.
† Gladstone Institute of Virology and Immunology, University of California, San Francisco, San Francisco General Hospital, P.O. Box 419100, San Francisco, CA 94141-9100.

HIV-1 LTR (6, 23, 41). In contrast, nontransforming mutants of v-Rel both lacked such DNA binding activity and failed to repress κB-dependent transcription (6). Coupled with the central role that NF-κB plays in normal T-cell growth, these findings raised the intriguing possibility of a mechanistic link between this κB-specific inhibitory activity and cellular transformation mediated by v-Rel.

Although the inhibitory effects of v-Rel on κB-dependent transcription suggest that v-Rel-mediated transformation involves its specific DNA binding activity, this proposal is complicated by at least two factors. First, v-Rel is predominantly expressed as a cytoplasmic protein in REV-T-transformed avian lymphocytes (17) and second, v-Rel is capable of forming heterodimers with NF-κB p50 (23, 28, 33). Thus, v-Rel could interact with and disrupt the normal cytoplasmic NF-κB complex, leading to its inhibition by a mechanism distinct from DNA binding. Thus far, no mutants of v-Rel have been identified that discriminate between these two potential mechanisms for v-Rel-mediated repression of NF-κB function.

Amino acid sequence analysis of v-Rel has revealed the presence of two motifs within its N-terminal Rel homology domain that could potentially serve as targets for phosphorylation by cyclic AMP-dependent protein kinase A (PKA) (27). One of these candidate phosphate acceptor sites is also conserved at a similar position in many (18), but not all (50), recognized members of the Rel-related family. In the present study, we demonstrate that this highly conserved sequence is critically required for κB-specific DNA binding, transcriptional repression, and transformation mediated by v-Rel but is not required for heterodimerization with NF-κB p50. However, our studies have further shown that phosphorylation at this site is not critically involved in the regulation of these various functions. Rather, the consensus PKA site appears to correspond to a part of a critical structural domain needed for these various functions. Using these mutants, we provide evidence that v-Rel-mediated transcriptional regulation involves its direct engagement of the κB enhancer in the nucleus rather than occurring through altered cytoplasmic protein-protein interactions. Finally, we show that v-Rel can function as a κB-specific transcriptional activator rather than as a repressor when expressed in undifferentiated F9 embryonal carcinoma cells or immature U937 promonocytes. These stimulatory effects of v-Rel require both the domain associated with the PKA site and additional carboxy-terminal sequences not needed for repression. In contrast, the induced differentiation of F9 cells mediated by retinoic acid restores the dominant-negative repressor phenotype of v-Rel. These results suggest that v-Rel may exert opposing transcriptional effects via the κB enhancer which are perhaps dependent on the state of cellular maturation and the intracellular milieu of other κB-specific enhancer-binding proteins. These newly recognized properties of v-Rel-mediated transformation thus serve to expand the possible mechanism(s) by which v-Rel exerts its transforming activity.

MATERIALS AND METHODS

Expression vectors. The *Bgl*II-*Sal*I fragment containing the entire v-*rel* cDNA was subcloned into the polylinker of bacteriophage M13mp19 DNA for use as a template for site-directed mutagenesis (37). This template was annealed with synthetic oligonucleotide primers (27 bp) that introduced clustered point mutations and diagnostic restriction sites. Two independent clones for each mutant were identified by restriction mapping. In v-Rel phosphorylation acceptor mutant 1 (mu-P1), the two conserved arginines at positions 272 and 273 were substituted with glycine and alanine, respectively, and a unique *Nar* I restriction site was concommitantly introduced. To create mu-P2, the serine and aspartic acid at positions 275 and 276 were altered to alanine and glutamic acid, respectively, introducing a diagnostic *Pst*I restriction site. Single-amino-acid substitutions of alanine for serine at position 275 (mu-P3) and glutamic acid for aspartic acid at position 276 (mu-P4) were also prepared. Each of these mutants was excised from M13mp19 by *Xba*I digestion and subcloned into the pCMV4 eukaryotic expression vector (1). The sequence of each v-*rel* mutation was confirmed by DNA sequencing (49).

Wild type v-*rel* cDNA was inserted into the pCMV4 expression vector as a *Bgl*II-*Sal*I fragment. A modified pCMV4 vector (pCMV4 term) was constructed by inserting a 14-bp oligonucleotide duplex containing stop codons in all three reading frames into the *Sma*I site of pCMV4. Carboxy-terminal v-*rel* cDNA truncations Δ230, Δ211, and Δ172 were constructed by digestion with *Stu*I, *Bam*HI, and *Hin*cII, respectively, and inserted directly upstream of the universal terminator present in pCMV4 term. The NF-κB p50 expression vector pCMV4-p50 contains amino acids 1 through 462 of human NF-κB/KBF1 (28) inserted into pCMV4 term upstream of the universal terminator and encodes a protein of approximately 50 kDa.

In vitro transcription and translation of v-Rel. To produce full-length wild-type v-Rel and the carboxy-terminal v-Rel deletion mutants Δ172 and Δ230, a pGEM-v-*rel* cDNA expression vector (6) was digested with *Sal*I (wild type), *Hin*cII (Δ172), or *Stu*I (Δ230). To produce Δ211, a *Bam*HI fragment encoding the amino-terminal 292 amino acids of v-Rel was inserted into pGEM-4 and subsequently linearized with *Sal*I. CV-*rel*, a v-*rel* deletion mutant lacking amino acids 1 through 29 (64), was inserted into pGEM-4 as an *Xba*I fragment (6) and linearized with *Sal*I. Full-length v-*rel* PKA phosphorylation acceptor mutants mu-P1 and mu-P2 were subcloned into pGEM-4 as an *Xba*I fragment and linearized with *Asp*718 or *Sal*I. A truncated NF-κB/KBF1 clone (pGEM-p50Δ) encoding amino acids 1 through 400 (43 kDa) was inserted into pGEM-4 as a *Hin*dIII-*Rsa*I fragment and linearized by digestion with *Sma*I. Linearized wild-type or mutant DNA templates were transcribed with SP6 or T7 polymerase. Runoff RNA transcripts (~2 μg) were translated, using rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]cysteine according to the manufacturer's instructions.

Cell transfections, CAT assays, and cell lysate preparation. Monkey COS-1 and mouse F9 cells were cultured in Iscove's medium. Human Jurkat T cells and the monocytic cell lines U937 and THP-1 were cultured in RPMI medium. All media were supplemented with 7.5% fetal calf serum and antibiotics. F9 cells were differentiated by incubation in retinoic acid (5×10^{-7} M) for 3 days (62). Chloramphenicol acetyltransferase (CAT) reporter plasmids containing full-length LTRs from HIV-1 or HTLV-I have been described previously (57). In addition, CAT reporter constructs driven by the TATA element from the albumin gene in the absence (TATA-CAT) or the presence (κB-TATA-CAT) of the two tandem κB enhancer motifs from the HIV-1 LTR were as described by Stein et al. (58). Jurkat T cells were transfected with reporter and effector plasmid DNA by using DEAE-dextran (21). F9 cells were transfected by calcium phosphate coprecipitation (19). Cell extracts were normalized for protein recovery (10) and assayed for CAT activity at 24 to 48 h posttransfec-

tion as previously described (38). COS cells were transfected with v-*rel* expression vectors by using DEAE-dextran (51) and were lysed after 48 h with 1 ml of ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM dithiothreitol) supplemented with 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation (35,000 × *g*, 30 min) prior to use in immunological analysis and DNA-protein binding studies. The *tax* cDNA expression vector used in some transfection studies has been previously described (42).

Gel retardation and UV cross-linking studies. Photoreactive ^{32}P-labeled probes were prepared as previously described (6). The coding strands of the duplex probes used in this study were 5'-CAACGGCAGGGGAATTCCCCTCTCC TT-3', κB-pd, and 5'-CAACGGCAGATCTATCTCCCTCT CCTT-3', κB-mu. DNA binding reactions were performed by mixing 2 to 5 μl of in vitro-translated products or 2 μl of in vivo-synthesized proteins from COS cell extracts with bromodeoxyuridine-substituted probes (1×10^7 to 5×10^7 cpm/pmol) in the presence of the nonspecific competitors poly(dI-dC) and $p(dN)_6$ (4). DNA-protein complexes were resolved by electrophoresis on native 5% polyacrylamide gels, using a Tris-borate-EDTA buffer system (9). For DNA-protein cross-linking studies, DNA binding reactions were irradiated with UV light (at 300 nm) for 15 min, using a Fotodyne transilluminator, and analyzed directly on sodium dodecyl sulfate (SDS)-polyacrylamide gels (4, 6). In some experiments, nucleoprotein adducts were first immunoprecipitated with specific antisera generated against NF-κB p50 (amino acids 1 to 21) (28) or v-Rel (6) prior to SDS-polyacrylamide gel electrophoresis (PAGE).

Protein analysis. Lysates prepared from COS-1 cells transfected with wild-type or mutant v-*rel* cDNA expression vectors were analyzed as previously described (69). Briefly, 50-μl aliquots of whole-cell lysates were subjected to electrophoresis on SDS–7.5% polyacrylamide gels, and then the proteins were transferred to Immobilon-P (Millipore Corp., Bedford, Mass.) membranes. Membranes were probed with rabbit anti-v-Rel antiserum (17). Colorimetric Western (immunoblot) assays were performed by using anti-rabbit antibodies conjugated to horseradish peroxidase and a diaminobenzidine substrate to form a visible precipitate as previously described (69). For heterodimerization studies, in vitro-translated proteins were immunoprecipitated in ELB buffer with anti-v-Rel antiserum or antipeptide antibodies directed against the amino-terminal 21 amino acids of NF-κB p50 (1:500 dilution). Immunoprecipitation reaction products were mixed with 20 μl (packed volume) of protein A-Sepharose beads, and the resultant immune complexes were washed with ELB buffer and analyzed on SDS-polyacrylamide gels.

Transformation assays. Wild-type and mutant v-*rel* genes were inserted into the REV-T-derived retroviral vector pBS102 (64) as *Xba*I fragments. pSW253 (67) is a plasmid that contains a replication-competent DNA clone of REV-A, the helper virus for REV-T. Virus stocks were generated by cotransfection of 10 μg of the respective wild-type or mutant v-*rel* vector plasmid DNA along with 0.1 μg of pSW253 into chicken embryo fibroblasts by using the dimethyl sulfoxide-Polybrene protocol (26). Virus was harvested from the transfected cell supernatants after 4 to 5 days and used for infection of fresh chicken embryo fibroblast cells and primary spleen cells. All infections were performed in the presence of Polybrene (10 μg/ml) at 37°C for 1 to 2 h. After infection, the primary spleen cells were diluted into 5 ml of RPMI 1640 containing 20% fetal calf serum and incubated at 37°C for 3 to 4 days to allow for maximal virus spread throughout the spleen cell cultures. Either 10 or 90% of the infected cell culture was then plated onto 5 ml of medium containing 0.3% soft agar, 15% fetal bovine serum, 1% chicken serum, 1% beef embryo extract, and 0.028% $NaHCO_3$ and incubated at 39.5°C. The plates were scored for growth after 10 to 14 days.

The relative titers of the wild-type and mutant viruses were determined by immunoprecipitation of v-Rel protein from infected chicken embryo fibroblasts, using antiserum directed against the v-Rel protein (17). Alternatively, the level of v-Rel protein present in the transfected cells was determined by immunoprecipitation or by indirect immunofluorescence. The level of each v-Rel protein present in the transfected cells was found to be proportional to the level of v-Rel protein in infected cells (data not shown). Immunoprecipitation and indirect immunofluorescence analyses were performed as previously described (20).

In vivo phosphorylation. COS-1 cells (2×10^6) were transfected with the pCMV4 expression vector alone or pCMV4 containing wild-type v-Rel, mu-P1, or mu-P2. Two days after transfection, cells were washed three times with phosphate-free RPMI medium and incubated for 1 h at 37°C in 2 ml of phosphate-free RPMI containing 10% dialyzed fetal calf serum. $[^{32}P]_i$ (1 mCi) (ICN Biomedicals) was added for a further 2 h of incubation. Cells were washed three times in ice-cold phosphate-buffered saline containing phosphatase inhibitors (0.4 mM sodium vanadate, 0.4 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate) and lysed in 1 ml of radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% SDS, 1% Nonidet P-40, 1% deoxycholate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing phosphatase inhibitors. Cell lysates were first precleared by using 2 μl of irrelevant antiserum and protein A-Sepharose and then subjected to immunoprecipitation with v-Rel-specific antiserum (1:500 dilution). Immune complexes were electrophoresed on SDS–7.5% polyacrylamide gels, and the v-Rel proteins were visualized either by fluorography or by the colorimetric assay described above.

RESULTS

The v-Rel oncoprotein (Fig. 1) contains an amino-terminal 300-amino-acid segment that is homologous with other Rel-related proteins, including the *dorsal* gene product of *Drosophila melanogaster* (60), the p65 (39, 43) and p50 (15, 28) subunits of NF-κB, and the recently described p49 protein (50). This v-Rel domain contains a functional nuclear localization signal (KAKRQR) (16) as well as poorly defined subregions mediating DNA binding and oligomerization with other Rel-related proteins. In addition, this domain contains two potential phosphorylation sites for cyclic AMP-dependent PKA, an enzyme that preferentially phosphorylates serine residues within the sequence RRXSX or KRXXSX (71). One of these consensus PKA phosphate acceptor sites is located at amino acid 304, immediately adjacent to the nuclear localization signal, whereas the second, which is conserved in all but one of the recognized Rel-related proteins (50), is located at position 275 (Fig. 1).

Carboxy-terminal v-Rel deletion mutants lacking the highly conserved PKA phosphorylation sequence are defective in κB-specific DNA binding and κB-specific transcriptional repression. Previous studies (6) have shown that the v-Rel oncoprotein binds independently to the κB enhancer. Anal-

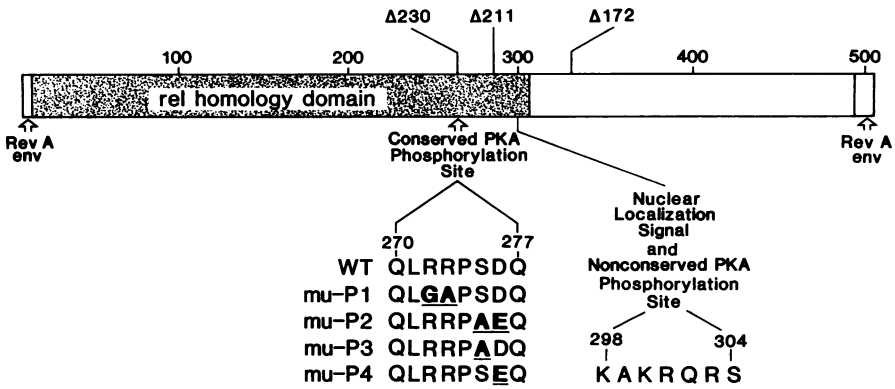

FIG. 1. Structure of the v-*rel* oncogene product. The 503-amino-acid v-Rel protein is depicted, including 12 N-terminal and 19 C-terminal residues derived from the REV-A *env* gene. End points for the Δ172, Δ211, and Δ230 carboxy-terminal deletion mutants, as well as the relative location of the nuclear translocation signal and cyclic AMP-dependent PKA phosphorylation sites, are indicated. Amino acid substitutions introduced to form the mu-P1, mu-P2, mu-P3, and mu-P4 mutants are also indicated.

ysis of deletion mutants of the 503-amino-acid v-Rel protein has allowed localization of the v-Rel DNA binding domain to the amino-terminal 331 amino acids, corresponding roughly to the Rel homology domain (6, 28). However, this Rel domain does not contain any previously characterized DNA binding motifs. To further localize residues within v-Rel required for DNA binding, additional carboxy-terminal deletion mutants of v-Rel were prepared and tested, including Δ172 (amino acids 1 to 331), Δ211 (amino acids 1 to 292), and Δ230 (amino acids 1 to 273). It should be noted that the Δ172 v-Rel mutant retains both PKA phosphate acceptor sites and the nuclear localization motif, whereas the Δ211 mutant retains only the proximal, highly conserved PKA phosphorylation site at position 275. The Δ230 mutant lacks both PKA phosphorylation acceptor sites (Fig. 1). To characterize their respective DNA binding properties, each of these deletion mutants was transcribed and translated in vitro. The resultant [^{35}S]cysteine-radiolabeled products were subsequently tested for κB-specific DNA binding activity in DNA-protein cross-linking assays by using photoreactive radiolabeled oligonucleotides corresponding to either a functional palindromic variant of the wild-type IL-2Rα enhancer (κB-pd) or a nonfunctional mutant (κB-mu) κB enhancer sequence (6). As shown in Fig. 2A, unprogrammed rabbit reticulocyte lysates (lane 1) contained endogenous κB-specific enhancer-binding proteins migrating with apparent molecular sizes of 50 to 55 kDa (likely p50) and 65 to 72 kDa (likely p65). However, the Δ172 (lanes 2 to 4) and Δ211 (lanes 5 to 7) truncated v-Rel proteins formed distinctly smaller, sequence-specific DNA-protein complexes. These findings indicate that neither the nuclear localization signal nor the nonconserved PKA phosphorylation acceptor site at position 304 of v-Rel is obligately required for DNA binding activity in vitro. In contrast, removal of 19 additional amino acids in the Δ230 v-Rel protein virtually abolished the DNA binding activity (lanes 8 to 10). These results highlight the importance of residues located between amino acids 274 and 292 for DNA binding. It is notable that this region encompasses the highly conserved proximal PKA phosphorylation acceptor site.

Using combinations of these v-Rel deletion mutants in gel retardation assays, we next investigated sequences required for v-Rel multimerization. In addition to DNA-protein complexes formed by intrinsic κB enhancer-binding proteins present in the rabbit reticulocyte lysates, incubation of the Δ172 or Δ211 mutants with the κB enhancer resulted in single unique complexes designated C1 and C3, respectively (Fig. 2B, lanes 1 to 3). As expected, the Δ230 mutant failed to bind to the κB enhancer (lane 4). Cotranslation of the Δ172 and Δ211 v-Rel mutants produced a novel DNA-protein complex exhibiting intermediate mobility (C2; lane 5), indicative of the effective multimerization of these two polypeptides. In contrast, no new complexes were produced with the combination of Δ172 and Δ230, suggesting that the Δ230 truncation removed not only DNA binding activity but also perhaps residues required for multimerization. In situ cross-linking confirmed that the C1 and C3 complexes contained the Δ172 and Δ211 v-Rel proteins, whereas the novel C2 complex contained both Δ172 and Δ211 v-Rel truncated proteins (data not shown). Together, these results suggest that v-Rel residues 274 to 292 are critical for v-Rel multimerization and are consistent with dimerization studies of the related NF-κB p50 protein described by Logeat and colleagues (33).

To assess the functional effects of the three v-Rel deletion mutants on κB enhancer-directed transcription, human Jurkat T cells were cotransfected with wild-type or mutant v-*rel* cDNA expression vectors and the HIV-1 LTR-CAT reporter plasmid. As shown in Fig. 2C, basal levels of expression from the HIV-1 LTR were not significantly enhanced by expression of wild-type v-Rel or any of the v-Rel deletion mutants. However, consistent with prior studies (6), the HIV-1 LTR was markedly induced by cotransfection of the HTLV-I *tax* expression vector. These effects of Tax have been shown to involve the induced nuclear expression of NF-κB and subsequent binding of this factor to the tandem κB enhancers present in the HIV LTR (5, 31, 44). Also consistent with previous results (6), coexpression of wild-type v-Rel in these recipient cells markedly inhibited the Tax-induced response. Similarly, the Δ172 truncation, which retains DNA binding activity, inhibited the Tax response at levels comparable to that of wild-type v-Rel. The Δ230 truncation mutant, which lacks DNA binding activity, had no inhibitory effect on the Tax response, whereas the Δ211 v-Rel mutant, which binds DNA in vitro, exhibited an intermediate functional phenotype (partial inhibition). It is possible that these findings with the Δ211 mutant reflect a relative dilution effect in vivo caused by removal of the nuclear localization signal. Previous studies have shown that v-Rel proteins lacking functional nuclear localization sequences exhibit a whole-cell pattern of distribution in

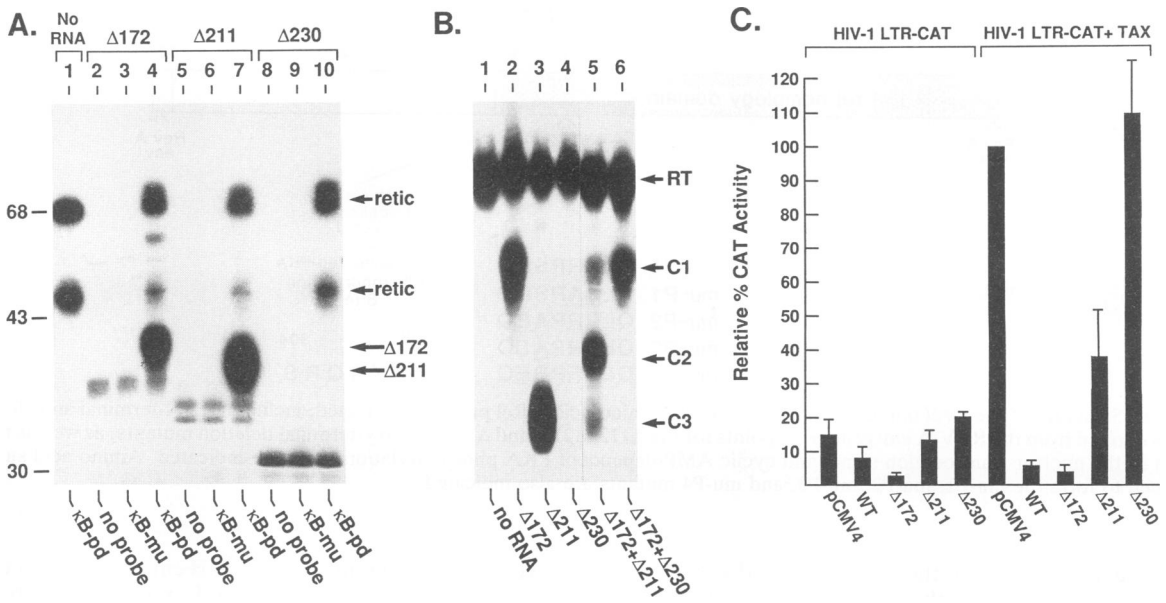

FIG. 2. (A) κB-specific binding activity of v-Rel carboxy-terminal deletion mutants. A nested series of runoff v-*rel* transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]cysteine. Unprogrammed lysate (lane 1) or programmed lysates (2 μl) containing the corresponding C-terminally truncated v-Rel polypeptides Δ172 (lanes 2 to 4), Δ211 (lanes 5 to 7), or Δ230 (lanes 8 to 10) were incubated with buffer, a mutant κB probe (κB-mu), or a functional κB probe (κB-pd) (10^6 cpm) as indicated. DNA binding reactions were irradiated with UV light, and cross-linked adducts were resolved on a denaturing SDS–8.75% polyacrylamide gel and detected by autoradiography. Adducts derived from κB-specific DNA binding activities endogenous to unprogrammed rabbit reticulocyte lysates (retic) are indicated. (B) Dimerization of v-Rel truncation mutants. Unprogrammed rabbit reticulocyte lysates (lane 1) or lysates programmed with C-terminal v-*rel* transcripts Δ172, Δ211, or Δ230 (lanes 2 to 4), Δ172 and Δ211 (lane 5), or Δ172 and Δ230 (lane 6) were incubated with κB probe (10^5 cpm). Resultant DNA-protein complexes were resolved on native 5% acrylamide gels. C1, C2, and C3 refer to nucleoprotein complexes containing Δ172, Δ172/Δ211, and Δ211 truncated v-Rel proteins, respectively. (C) Transcriptional repressor activity of v-Rel deletion mutants. Jurkat T cells (5×10^6) were cotransfected with the HIV-1 LTR-CAT reporter plasmid (5 μg) and control vector (pCMV4) or the indicated v-Rel deletion mutants in the absence or presence of a vector encoding the HTLV-I Tax protein. Transfected cells were assayed for CAT activity 48 h later. Values were normalized for protein recovery and expressed as percent CAT activity relative to the value of HIV-1 LTR-CAT induced by the HTLV-I Tax protein alone. Results reflect the mean of at least four independent experiments employing two plasmid preparations of each plasmid construct. Error bars depict standard errors of the mean. WT, wild type.

chicken embryo fibroblasts (16). Immunofluorescence studies in COS cells have confirmed such a whole-cell pattern of expression for the Δ211 and Δ230 v-Rel proteins in contrast to the exclusively nuclear expression of wild-type v-Rel and the Δ172 truncation mutant (data not shown). The complete lack of repressor activity discerned for the Δ230 truncation, which eliminates the conserved PKA phosphorylation site, suggests that the region between amino acids 274 and 292 is important for transcriptional repression mediated by v-Rel.

Site-directed mutations affecting the PKA consensus sequence but not the serine acceptor site alone disrupt DNA binding and transcriptional repression. To more directly assess the functional importance of the PKA phosphorylation acceptor site, we selectively introduced amino acid substitutions in this region by site-directed mutagenesis. As shown in Fig. 1, mutation mu-P1 altered the two conserved arginines within the PKA phosphorylation site at positions 272 and 273, changing these residues to glycine and alanine, respectively. Mutation mu-P2 contained a second two-amino-acid substitution of alanine and glutamic acid for serine and aspartic acid at positions 275 and 276, thus eliminating the putative phosphate acceptor site while preserving the overall charge within this region. In addition, single amino acid mutants designated mu-P3 (serine 275 to alanine) and mu-P4 (aspartic acid 276 to glutamic acid) were created to assess the potential functional effects of each individual amino acid altered in the dipeptide mu-P2 mutation. cDNA expression vectors directing the synthesis of these various v-Rel proteins were transiently expressed in monkey COS-1 cells. Western blotting analysis of extracts prepared from these recipient cells confirmed that the wild-type and mutant v-Rel proteins were expressed at comparable levels (Fig. 3A, lanes 1 to 5). In UV cross-linking assays employing a photoreactive radioactive κB enhancer probe and transfected COS cell extracts (Fig. 3B), the mu-P1 (lane 2) and mu-P2 (lane 3) proteins exhibited markedly diminished DNA binding compared with that of the wild-type (lane 1) v-Rel protein. In contrast, the mu-P3 (lane 4) and mu-P4 (lane 5) mutant proteins, which contained point mutations at the serine 275 phosphorylation acceptor residue or the adjacent aspartic acid residue, retained essentially wild-type κB enhancer-binding activity. Thus, although two different dipeptide alterations within the conserved PKA site disrupt the DNA binding activity of v-Rel, alteration of the putative serine phosphorylation acceptor alone exerts no apparent effect on this function.

To determine the biological activity of these missense v-Rel mutants on κB-directed transcription, Jurkat T cells were cotransfected with graded amounts of each mutant or wild-type v-*rel* expression vector together with HTLV-I *tax* and the HIV-1 LTR-CAT reporter plasmid. As shown in Fig. 3C, the wild-type, mu-P3, and mu-P4 v-Rel proteins markedly inhibited Tax-induced activation of the HIV-1 LTR over a broad range of input vector concentrations (1 to 12

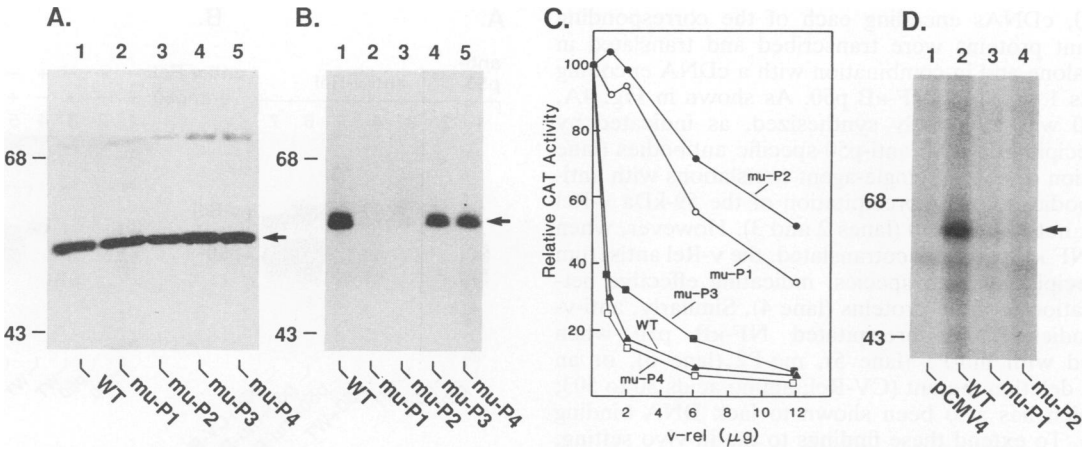

FIG. 3. (A) Expression of wild-type and mutant v-Rel proteins. Whole-cell lysates (50 μl) from COS-1 cells transfected with pCMV4 expression vectors encoding wild-type v-Rel (WT; lane 1), mu-P1 (lane 2), mu-P2 (lane 3), mu-P3 (lane 4), or mu-P4 (lane 5) were subjected to electrophoresis through an SDS–7.5% polyacrylamide gel and transferred to Immobilon-P membranes. Rel proteins were detected by using a colorimetric Western blot assay employing anti-v-Rel antibodies and secondary goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. (B) DNA binding activity of wild-type and mutant v-Rel proteins. Whole-cell extracts (2 μl) from COS-1 cells transfected with expression vectors encoding wild-type v-Rel (WT; lane 1), mu-P1 (lane 2), mu-P2 (lane 3), mu-P3 (lane 4), or mu-P4 (lane 5) were incubated with radiolabeled κB-pd probe and then irradiated with UV light. Adducts were fractionated by SDS-PAGE (7.5% gel) and detected by autoradiography. The arrow indicates the v-Rel-κB enhancer complex. (C) Functional effects of site-directed v-Rel phosphate acceptor mutants. Jurkat T cells (5×10^6) were cotransfected with the HIV-1 LTR-CAT reporter plasmid (5 μg), cytomegalovirus-driven expression vectors encoding the HTLV-I Tax protein (5 μg), and graded doses of the indicated v-*rel* expression plasmids. The total amount of transfected DNA was held constant by the addition of various amounts of parental pCMV4 expression vector. Results of four independent experiments are expressed as percent CAT activity relative to the value of the HIV-1 LTR-CAT reporter induced by the HTLV-I Tax protein only. In all cases, standard errors of the mean were less than 25%. (D) v-Rel phosphorylation in vivo. COS-1 cells transfected with either unmodified pCMV4 (lane 1) or pCMV4 expression vectors encoding the wild-type (WT; lane 2), mu-P1 (lane 3), and mu-P2 (lane 4) v-Rel proteins were labeled with $[^{32}P]_i$. Cell lysates were immunoprecipitated with anti-v-Rel antibodies and subjected to electrophoresis through an SDS–7.5% polyacrylamide gel. Comparable levels of these v-Rel proteins were detected with a colorimetric Western assay employing rabbit anti-v-Rel antibodies and secondary goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (data not shown).

μg). In contrast, the mu-P1 and mu-P2 v-Rel proteins produced essentially no inhibition at low expression vector inputs (1 to 2 μg) and only modest inhibition at high vector concentrations (6 to 12 μg). These findings suggest that the mu-P2 and mu-P1 mutants of v-Rel, which display attenuated DNA binding activity, also exhibit at least 8- to 10-fold less functional repressor activity compared with that of the wild-type v-Rel protein. Similar results were obtained in cotransfection assays performed in chicken embryo fibroblasts (data not shown). The attenuated repressor function of the mu-P1 and mu-P2 mutants is not due to grossly altered subcellular localization, because immunofluorescence studies have confirmed that these proteins are predominantly expressed in the nuclei of transfected COS cells (data not shown). The functional effects of these various mutants thus parallel their DNA binding activities and support a critical role for this subregion domain in v-Rel function. However, these data further argue against a major regulatory role for phosphorylation at serine 275 in the control of these v-Rel functions.

Consistent with our findings, Mosialos and colleagues (35) have shown that replacement of serine 275 with alanine does not affect transcriptional repression by v-Rel. However, in this study, transcriptional repression by v-Rel was significantly inhibited by changing serine 275 to aspartic or glutamic acid, which could mimic the negative charge introduced by phosphorylation. To determine whether the altered phenotypes of the inactive mu-P1 and mu-P2 mutations reflect changes in phosphorylation patterns, levels of in vivo phosphorylation were measured for the wild-type and mutant v-Rel proteins. cDNA expression vectors directing the synthesis of mutant and wild-type v-Rel were transiently expressed in COS-1 cells and analyzed for their overall level of expression and degree of phosphorylation. Transfected cells were radiolabeled with $[^{32}P]_i$ for 2 h, and whole-cell extracts were immunoprecipitated by using v-Rel antiserum. Colorimetric Western blot analysis of these immunoprecipitates using anti v-Rel and anti-rabbit antibodies conjugated to horseradish peroxidase confirmed that the wild-type and mutant v-Rel proteins were expressed at comparable levels (data not shown). In contrast, fluorographic analysis of these immunoprecipitates (Fig. 3D) revealed significantly diminished phosphorylation of mu-P1 and mu-P2 (lanes 3 and 4) compared with the wild-type protein (lane 2). These findings suggest that the region surrounding the PKA site at position 275 is important for in vivo phosphorylation of v-Rel. However, rather than being controlled by phosphorylation at serine 275, it seems likely that these inhibitory effects involve altered protein conformation affecting phosphorylation at other sites within v-Rel.

Mutations within the PKA phosphorylation site do not impair heterodimerization of v-Rel with the 50-kDa subunit of NF-κB. Prior studies have demonstrated that v-Rel and the 50-kDa subunit of NF-κB can interact in vitro and form a stable heterodimeric complex (23, 28, 33). This finding provides an alternative explanation for the repressive functional effects of v-Rel, whereby its dimerization with NF-κB p50 might lead to the formation of a heterodimeric complex that lacks normal biological function and precludes assembly of NF-κB p50 and NF-κB p65. To determine whether mutations mu-P1 and mu-P2 attenuate v-Rel-mediated repression by altering its capacity to form heterodimers with

NF-κB p50, cDNAs encoding each of the corresponding v-Rel mutant proteins were transcribed and translated in vitro both alone and in combination with a cDNA encoding amino acids 1 to 400 of NF-κB p50. As shown in Fig. 4A, NF-κB p50 was effectively synthesized, as indicated by immunoprecipitation with anti-p50-specific antibodies (lane 1). Incubation of control single-agent translations with anti-v-Rel antibodies allowed precipitation of the 59-kDa v-Rel protein but not NF-κB p50 (lanes 2 and 3). However, when v-Rel and NF-κB p50 were cotranslated, the v-Rel antiserum immunoprecipitated both species, indicating effective heterodimerization of these proteins (lane 4). Similarly, anti-v-Rel antibodies immunoprecipitated NF-κB p50 when cotranslated with mu-P1 (lane 5), mu-P2 (lane 6), or an N-terminal deletion mutant (CV-Rel; amino acids 30 to 503; lane 7) which has also been shown to lack DNA binding activity (6). To extend these findings to the in vivo setting, COS-7 cells were transfected with the corresponding eukaryotic cDNA expression vectors, radiolabeled with [^{35}S]methionine, and analyzed in immunoprecipitation studies using anti-peptide antiserum specific for the N terminus of NF-κB p50 (amino acids 1 to 21). As shown in Fig. 4B, anti-p50 reacted strongly with radiolabeled NF-κB p50 (lane 1) but not with v-Rel (compare lanes 3 and 4) in single-agent transfections. However, this antiserum immunoprecipitated comparable amounts of radiolabeled v-Rel, mu-P1, and mu-P2 (lanes 5 to 7) present in extracts derived from cotransfected COS-7 cells. On the basis of several independent experiments, the mu-P1 and mu-P2 mutations did not appear to alter significantly the capacity of v-Rel to dimerize with NF-κB p50. Taken together, these results clearly define three independent mutations (mu-P1, mu-P2, and CV-Rel) that segregate the DNA binding and heterodimerization functions of v-Rel and provide strong evidence that the wild-type v-Rel protein can heterodimerize with NF-κB p50 in vivo.

We next investigated whether these v-Rel-NF-κB heterodimeric complexes could bind the κB enhancer by using two approaches. First, extracts from COS-7 cells cotransfected with NF-κB p50 and v-Rel expression vectors were incubated with a radiolabeled κB enhancer and analyzed in band shift assays (Fig. 4C). Coexpression of the wild-type v-Rel and NF-κB p50 proteins in these recipient cells resulted in an additional complex with intermediate mobility (lane 1) relative to that of wild-type v-Rel or NF-κB p50 alone. In situ UV cross-linking analysis confirmed that this intermediate band corresponded to an NF-κB p50-v-Rel heterodimer (data not shown). In contrast, heterodimers composed of NF-κB p50 and either mu-P1 or mu-P2 exhibited virtually no κB enhancer-binding activity (lanes 2 and 3). However, κB binding by the NF-κB p50 homodimers present in these extracts was not significantly diminished. To independently confirm the subunit composition of the novel complex revealed in lane 1, DNA-protein cross-linking reactions were performed and subjected to immunoprecipitation analysis by using NF-κB p50-specific antiserum. As shown in Fig. 4D, radiolabeled nucleoprotein adducts containing both NF-κB p50 and v-Rel (lane 10) were specifically immunoprecipitated with this antiserum, using extracts from cells cotransfected with the NF-κB p50 and v-Rel expression vectors. In contrast, the v-Rel adduct was absent in immunoprecipitates derived from cells transfected with either the unmodified pCMV4 expression vector (lane 2) or the NF-κB p50 expression vector alone (lane 8). These results strongly suggest that wild-type v-Rel and NF-κB p50, when synthesized in vivo, can directly engage the κB enhancer.

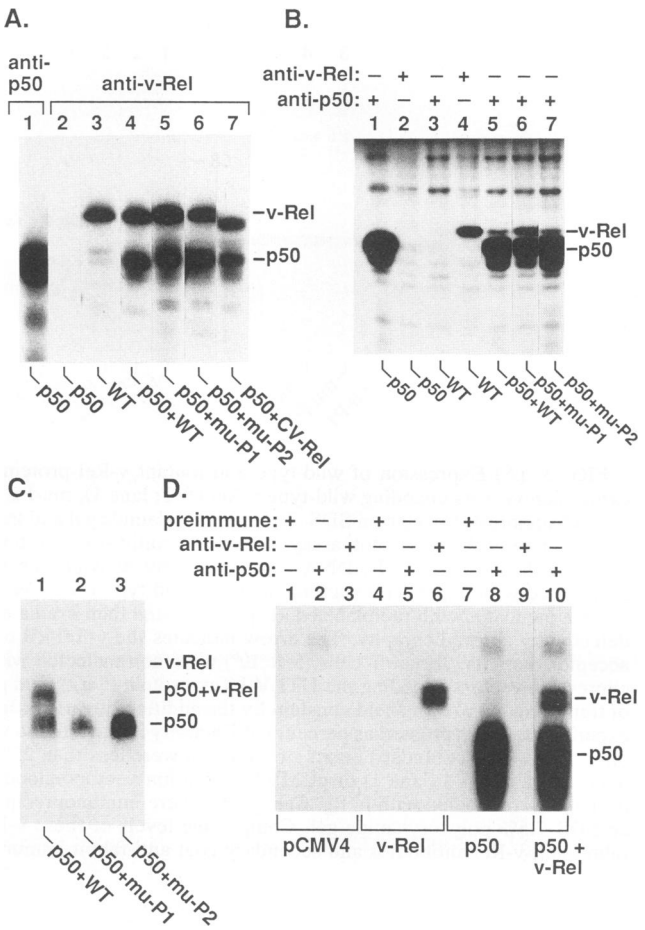

FIG. 4. (A) In vitro-synthesized NF-κB p50 coimmunoprecipitates with mu-P1, mu-P2, and wild-type v-Rel. Runoff transcripts derived from cDNAs encoding NF-κB p50 (amino acids 1 to 400) (lanes 1 and 2), wild-type v-Rel (WT; lanes 3 and 4), or v-Rel mutants including mu-P1 (lane 5), mu-P2 (lane 6), and CV-Rel, a deletion mutant lacking amino acids 1 to 29 (lane 7), were translated in vitro either alone or in combination. The resultant ^{35}S-labeled proteins were immunoprecipitated in ELB buffer with anti-NF-κB p50 (lane 1) or anti-v-Rel (lanes 2 to 7) antibodies and washed three times in ELB buffer, and the resultant immune complexes were resolved by SDS-PAGE (12.5% gel) and analyzed by autoradiography. (B) Formation of v-Rel-NF-κB p50 heterodimers in vivo. Whole-cell extracts (100 μl) from ^{35}S-labeled COS cells cotransfected with the indicated cDNA expression vectors (bottom) were subjected to immunoprecipitation and analyzed on an SDS–7.5% polyacrylamide gel. The specific antibodies used in each reaction are indicated above each lane of the autoradiogram. (C) Binding of NF-κB p50-v-Rel and NF-κB p50-v-Rel mutant heterodimers to the κB enhancer. Whole-cell extracts (2 μl) from COS cells cotransfected with expression vectors encoding NF-κB p50 and wild-type v-Rel (WT; lane 1), mu-P1 (lane 2), or mu-P2 (lane 3) were incubated with a radiolabeled κB-pd probe (6). DNA-protein complexes were resolved by electrophoresis on native 5% polyacrylamide gels. The relative positions of nucleoprotein complexes containing either v-Rel or NF-κB p50 homodimers are indicated. (D) DNA binding activity of wild-type v-Rel-NF-κB p50 heterodimers. COS cells were transfected with either pCMV4 (lanes 1 to 3) or recombinant pCMV4 expression vectors encoding v-Rel (lanes 4 to 6), NF-κB p50 (lanes 7 to 9), or v-Rel and NF-κB p50 in combination (lane 10). Whole-cell extracts from recipient cells were mixed with a ^{32}P-labeled κB enhancer probe, cross-linked with UV light, and subjected to immunoprecipitation with the indicated specific antiserum (top). Immunoreactive adducts were fractionated on an 8.5% polyacrylamide gel and detected by autoradiography.

TABLE 1. Transformation properties of mu-P1 and mu-P2 v-*rel* mutants[a]

Virus	Expt	Soft agar colonies at the following plating dilution[b]:	
		10^0	10^{-1}
Wild-type v-rel	A	>200	50
	B	>200	56
mu-P1	A	0	0
	B	0	0
mu-P2	A	0	0
	B	0	0

[a] Results of additional independent transformation studies have revealed transformation activities for mu-P3 and mu-P4 equivalent to that of wild-type v-Rel. Virus stocks expressing either wild-type or mutant v-*rel* genes were used to infect primary spleen cells and the infected spleen cells. Four days after infection spleen cells were plated into soft agar. The results shown are from two independent experiments (A and B) and are representative of more than 10 independent experiments. No transformed colonies were obtained in the soft agar assays from spleen cells infected with either mutant v-*rel* virus.

[b] Either 10% (10^{-1}) or 90% (10^0) of the infected cells was plated onto 5 ml of soft agar per 60-mm-diameter plate.

v-Rel-mediated transformation is inhibited by the mu-P1 and mu-P2 mutations. Since the mu-P1 and mu-P2 mutations significantly diminished κB-specific transcriptional repression by v-Rel, studies were next performed to analyze the transforming potential of these mutants relative to that of the wild-type v-Rel oncoprotein. Wild-type v-*rel*, mu-P1, and the mu-P2 mutants were each inserted into a retroviral expression vector. Viral stocks prepared by cotransfection of chicken embryo fibroblasts with these constructs and a second plasmid encoding a replication-competent clone of REV-A were used to infect fresh chicken embryo fibroblasts and primary avian spleen cells. Immunoprecipitation analysis of radiolabeled proteins from infected cells confirmed equivalent levels of expression of the wild-type and mutant v-Rel proteins (data not shown). The ability of the wild-type v-Rel and mutant proteins to induce colony formation in soft agar was assayed as a measure of transformation potential. Splenocytes infected with wild-type v-*rel* virus readily formed colonies in the soft agar (Table 1). These colonies could also be established as cell lines by placement in suspension culture. In contrast, splenocytes infected with the mu-P1 or mu-P2 mutants failed to form colonies in soft agar indicative of their lack of transforming activity.

Opposing functional effects of v-Rel in undifferentiated and differentiated F9 embryonal carcinoma cells. As noted, prior studies have clearly demonstrated that v-Rel functions as a dominant-negative repressor of κB-directed transcription in mature Jurkat T lymphocytes and NIH 3T3 cells (6, 23, 41). However, REV-T transformation has been shown to involve both immature and mature avian lymphoid cells (7, 8, 32). These observations regarding the transformation of immature cells prompted our evaluation of the functional effects of v-Rel in undifferentiated cells, specifically F9 embryonal carcinoma cells. Surprisingly, transfection of these undifferentiated F9 cells with the wild-type v-*rel* expression vector produced κB-specific transcriptional activation rather than repression, as measured with CAT reporter plasmids containing either two HIV-1 κB enhancer elements (κB-TATA-CAT) or the full-length HIV-1 LTR (HIV-1 LTR-CAT) (Fig. 5A). In contrast, neither the mu-P1 nor the mu-P2 mutants of v-Rel functioned as transcriptional activators in the undifferentiated F9 cells. These activating effects of v-Rel also

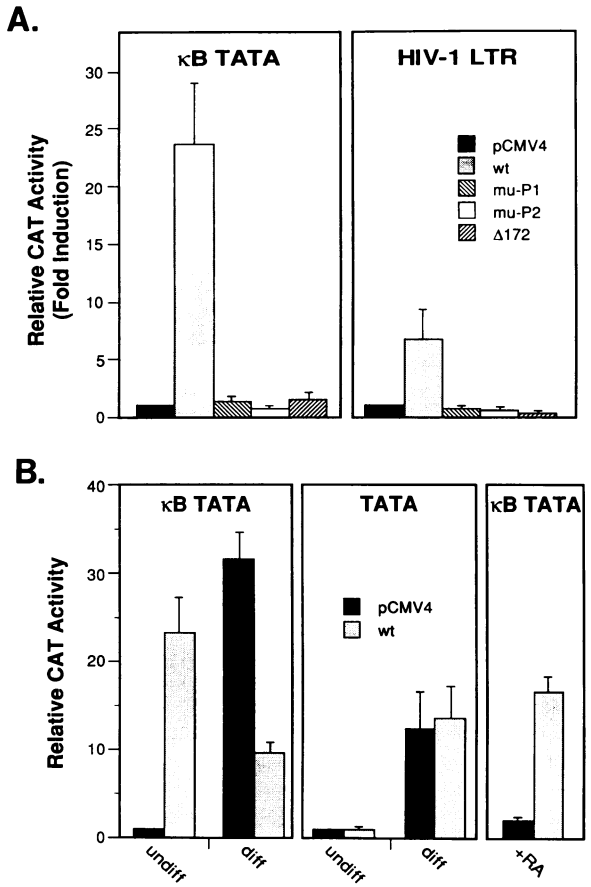

FIG. 5. (A) Wild-type v-Rel activates κB-directed transcription in undifferentiated F9 embryonal carcinoma cells. F9 cells were cotransfected with either the κB-TATA-CAT or HIV-1 LTR-CAT reporter plasmids (8 μg) and cytomegalovirus-driven expression vectors (12 μg) encoding wild-type (wt) v-Rel, mu-P1, mu-P2, or a truncated form of v-Rel missing the carboxy-terminal 172 amino acids (Δ172). Basal activity of each promoter was assigned a value of 1. CAT activity values represent the mean ± the standard error of the mean of three experiments employing two independently derived v-Rel mutants. (B) v-Rel represses κB-directed transcription in differentiated F9 cells. F9 cells were differentiated by the addition of retinoic acid (RA) (5×10^{-7} M) 3 days prior to transfection. Differentiated F9 cells were cotransfected with the κB-TATA-CAT or TATA-CAT reporter plasmid (8 μg) and 12 μg of the pCMV4 expression vector lacking (solid bars) or containing (stippled bars) a wild-type v-*rel* cDNA (wt). In control experiments (right panel), undifferentiated F9 cells were cotransfected with κB-TATA-CAT and effector plasmids followed by treatment with retinoic acid (5×10^{-7} M) 24 h after transfection. For comparison, the basal activity of each reporter in the absence of retinoic acid was normalized to 1. CAT activity values represent the mean ± the standard error of the mean of three experiments with two plasmid preparations.

appeared to require additional carboxy-terminal sequences not needed for v-Rel repression, since the Δ172 deletion mutant of v-Rel failed to support activation. These stimulatory effects of v-Rel were dependent on the presence of the wild-type functional κB enhancer, because neither a κB-deleted promoter nor a HIV-1 LTR-CAT construct containing point mutations in both κB motifs was activated by v-Rel in undifferentiated F9 cells (data not shown).

The regulatory phenotype of v-Rel was next investigated

in F9 cells induced to differentiate by the addition of retinoic acid (62). In such differentiated F9 cells, the basal activities of several promoter constructs, including κB-TATA-CAT and TATA-CAT, increased approximately 10-fold (Fig. 5B). However, contrary to these generalized stimulatory effects accompanying F9 differentiation, coexpression of v-Rel now produced marked inhibition of κB-TATA-CAT activity while exerting little or no effect on expression of the control TATA-CAT plasmid (Fig. 5B). These κB enhancer-specific inhibitory effects were dependent on the differentiated F9 phenotype and not simply an artifact of retinoic acid treatment, as the addition of this inducing agent to undifferentiated F9 cells 1 day after transfection did not inhibit κB-TATA-CAT expression (Fig. 5B). To determine whether these opposing biological functions of v-Rel extended to other cell types of various degrees of maturity, v-Rel action was studied in immature human promonocytic U937 cells (63) and the relatively more differentiated monocytic cell line THP-1 (66). Of note, recent studies have suggested that many v-Rel-induced tumor cell lines are, in fact, monocytic in origin (7). In the immature U937 promonocytic cells, v-Rel activated basal activity and phorbol myristate acetate stimulated κB-TATA-CAT activity, whereas in the more mature THP-1 monocytic cells v-Rel repressed κB-TATA-directed transcription (data not shown). Thus, the opposing functional effects of v-Rel observed in undifferentiated and differentiated F9 cells were recapitulated in human monocytic cell lines phenotypically exhibiting different states of maturation.

DISCUSSION

Select mutations within a conserved PKA phosphorylation site inhibit v-Rel-mediated DNA binding, transcriptional regulation, and cellular transformation. Carboxy-terminal deletion mapping of v-Rel has clearly indicated that the region between amino acids 274 and 292 is essential for DNA binding, heterodimerization, and transcriptional repression. Inspection of this 19-amino-acid region revealed the presence of a putative PKA phosphorylation site (RRPS) that is conserved among virtually all Rel family members (18). The introduction of two different dipeptide mutations that disrupt critical residues within this consensus PKA phosphorylation signal markedly diminished DNA binding, transcriptional repression, and transformation. However, mutation of the serine phosphate acceptor at position 275 (mu-P3) had no effect on v-Rel function, suggesting that phosphorylation directly at this site does not subserve a primary regulatory role in controlling κB enhancer binding and transcriptional repression. In this regard, Kamens and colleagues (25) have also shown that mutation of this serine residue fails to alter the transforming properties of v-Rel. Thus, although it appears that serine 275 phosphorylation does not directly regulate v-Rel function, the lack of function observed with the two independent dipeptide mutations within this consensus phosphorylation site indicates that this conserved region forms a structural domain that is critically required for the biological activities of v-Rel. It remains possible that the down-regulating effects that these dipeptide mutations (mu-P1 and mu-P2) exert on v-Rel-mediated repression may result from structural perturbations that inhibit v-Rel phosphorylation events at distant acceptor sites. Indeed, we have found that these dipeptide mutations significantly inhibit the overall phosphorylation of v-Rel occurring in vivo. Consistent with the severe phenotypic changes induced by the dipeptide mutations, Richardson and Gilmore (41) have shown that v-Rel-mediated transcriptional repression and transformation are abolished by insertion of two different amino acids within the consensus PKA sequence. These results and the strict conservation of this PKA sequence within nearly all Rel family members raise the possibility that this structural domain may be critically involved in regulating the function of other Rel-related proteins.

v-Rel inhibits κB-directed transcription by directly engaging the κB enhancer. In an earlier study, we proposed two potential mechanisms for transcriptional repression mediated by v-Rel (6). One mechanism involved the direct binding of the oncoprotein to the κB enhancer, resulting in its occlusion. Supporting this argument was the experimental identification of three independent mutations disrupting DNA binding that also attenuated the repressor activity of v-Rel (6). Alternatively, a second mechanism for v-Rel inhibition was suggested by the recognized ability of Rel-related proteins to oligomerize with each other. Thus, v-Rel association with NF-κB p50 (23, 28, 33) in the cytoplasm might serve to prevent its normal interaction with NF-κB p65, thereby blocking the assembly of the fully functional heteromeric NF-κB complex.

Arguing strongly against this latter model of v-Rel inhibition are our current findings that (i) v-Rel mutants mu-P1 and mu-P2 exhibit greatly diminished DNA binding and inhibitory properties yet retain the ability to dimerize with NF-κB p50, (ii) heterodimerization between NF-κB p50 and either mu-P1 or mu-P2 does not efficiently rescue their DNA binding activity, and (iii) deletion of the amino-terminal 29 amino acids of v-Rel similarly yields a mutant v-Rel protein (CV-Rel) that dimerizes normally with NF-κB p50 yet fails to bind DNA or inhibit κB-directed transcription (this report and reference 6). Together, these results provide support for v-Rel-mediated repression involving direct competitive effects in the nucleus at the level of the κB enhancer rather than through alterations in the composition of cytoplasmic forms of NF-κB. These results do not, however, formally preclude the possibility that v-Rel-NF-κB p50 heterodimers mediate these inhibitory effects on κB-specific transcription. This distinction may be functionally important as our findings indicate that distinct subregions of the Rel homology domain mediate DNA binding and oligomerization.

Our finding that DNA binding is central to v-Rel-mediated transcriptional repression in Jurkat T cells suggests that cellular transformation by v-Rel may be dependent on a similar mechanism. While this paradigm conflicts with the predominantly cytoplasmic distribution of v-Rel in transformed avian cells as assessed by immunofluorescence (16), it is certainly possible that a small fraction of the biologically active oncoprotein gains access to the nuclear compartment. In fact, nuclear extracts from REV-T-transformed cell lines and v-Rel-induced avian tumors have been shown to possess a κB enhancer-binding activity involving v-Rel (24, 66a). In addition, v-Rel mutants that lack DNA binding activity also fail to transform primary avian spleen cells (6; this report). Finally, v-Rel mutants that are predominantly retargeted to the nuclei of avian splenocytes fully retain their transformation potential (16). Together, these findings are most consistent with a nuclear site of action for v-Rel-mediated transformation whereby the v-Rel oncoprotein might modulate transcription of growth-regulating genes through its sequence-specific DNA binding capacity.

v-Rel activates κB-directed transcription in immature cells but acts as a dominant-negative repressor in more mature cell types. The discovery that v-Rel corresponds to a repressor of NF-κB-controlled gene expression has prompted consider-

able interest in the potential link between this biological function and its potent transforming potential (6, 23, 41). We now demonstrate that the v-Rel oncoprotein not only functions as a κB-specific repressor but in the appropriate cellular environment can also function as a κB-specific transcriptional activator. These contrasting biological functions of v-Rel apparently depend on the state of intrinsic host cell differentiation. Specifically, we have shown that v-Rel functions as a transcriptional activator in two relatively immature cell lines (undifferentiated F9 cells and U937 promonocytic cells); however, this oncoprotein acts as a dominant-negative repressor in retinoic acid-differentiated F9 cells and mature THP-1 monocytic cells. Both these stimulatory and inhibitory effects of v-Rel are mediated through the κB enhancer and are lost following mutation of a well-conserved segment of the Rel homology domain containing a consensus PKA phosphorylation site. In addition, the inductive but not repressive effects of v-Rel require additional carboxy-terminal amino acids located between positions 331 and 503. Of note, efficient v-Rel-mediated transformation has been shown to depend on these carboxy-terminal sequences (12, 16). This dual functionality of v-Rel is also consistent with earlier findings showing that, dependent on cell type, v-Rel could activate or repress transcription from certain viral promoters (13).

These opposing functional effects of v-Rel are rather reminiscent of the *Drosophila dorsal* gene product, which acts as both a positive and a negative regulator of different genes involved in early embryonic development. Specifically, *dorsal* represses the expression of *zerknüllt* and *decapentaplegic*, while activating *twist* and *snail* (46, 47, 56, 61, 65). Furthermore, the regulatory activities of the *dorsal* gene product itself are restricted in development and are influenced by other gene products, such as *cactus* and additional *dorsal* group genes (45). It seems likely that v-Rel action may be similarly modulated by the selective presence or absence of other cellular factors during specific stages of development. For example, v-Rel-mediated transcriptional activation may be restricted to immature cells because only in this cellular milieu are the requisite ancillary factors present. Upon differentiation, the expression of other cellular factors may alter v-Rel action, leading to its opposing function as a dominant-negative repressor. These opposing functional effects of v-Rel certainly expand the potential mechanisms by which this oncoprotein produces its transforming effects.

ACKNOWLEDGMENTS

We thank E. Dixon and N. Peffer for technical support, T. Gilmore for discussion of unpublished results, S. Dörre, P. Sista, and J. Molitor for critical comments, and B. Kissell for manuscript preparation.

M. Hannink is supported by the Molecular Biology Program of the University of Missouri and by a Basil O'Conner Starter Scholar Research Award.

ADDENDUM

During the completion of this study, Mosialos et al. (35) reported that although serine 275 of v-Rel is phosphorylated in transformed spleen cells, phosphorylation at this site is not essential for v-Rel-mediated transcriptional repression or transformation. These results are fully consistent with the wild-type phenotype of the mu-P3 mutant that we have characterized (Fig. 3B and C).

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