vRel Is an Inactive Member of the Rel Family of Transcriptional Activating Proteins

PAUL M. RICHARDSON AND THOMAS D. GILMORE*

Department of Biology, Boston University, Boston, Massachusetts 02215

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The vRel oncoprotein is member of a family of related proteins that also includes cRel, NF- κ B, and Dorsal. We investigated the transcriptional regulatory properties of several Rel proteins in cotransfection assays with chicken embryo fibroblasts (CEF). Retroviral vectors expressing hybrid proteins that contain the DNA-binding domain of LexA fused to portions of the viral oncoprotein vRel or chicken, mouse, human, or *Drosophila melanogaster* (Dorsal) cRel proteins were cotransfected with a reporter plasmid that contains the DNA sequence recognized by LexA, a promoter, and the assayable gene for chloramphenicol acetyltransferase. In transient assays, a LexA-vRel protein did not activate transcription in CEF. Full-length chicken cRel, mouse cRel, and Dorsal fusion proteins all activated transcription weakly; however, deletion of N-terminal Rel sequences from each of these proto-oncogene encoded proteins resulted in strong activation by LexA fusion proteins containing only C-terminal sequences. Inhibition of the C-terminal chicken cRel gene activation domain by N-terminal sequences was seen in CEF and mouse and monkey fibroblasts. These results show that cRel proteins from different species have the same general organization: an N-terminal inhibitory domain and a C-terminal activation domain. Sequence comparison suggests that the inhibitory domain is conserved but the activation domain is species specific. In contrast, vRel lacks a strong C-terminal gene activation function, since a LexA fusion protein containing C-terminal vRel sequences alone only weakly activated transcription. In addition, the wild-type vRel protein (lacking LexA sequences) repressed transcription from reporter plasmids containing NF- κ B target sequences; nontransforming vRel mutants did not repress transcription from these plasmids. Our results suggest that vRel transforms cells by interfering with transcriptional activation by cellular Rel proteins.

v-rel is the oncogene of the avian retrovirus Rev-T (reviewed in reference 32). Avian lymphoid cells are rapidly transformed by v-rel in vivo and in vitro. It is now clear that there is a family of Rel proteins that includes vRel, cRel, the *Drosophila dorsal* gene product, and both major subunits of the NF- κ B transcription complex (reviewed in reference 13). DNA sequence data are available for v-rel; chicken, turkey, mouse, and human c-rel; dorsal; and the mouse and human NF- κ B p50 and p65 subunits (4–6, 12, 18, 19, 23, 27a, 33a, 34, 39–41, 43). Rel proteins share extensive sequence identity over an approximately 300-amino-acid (aa) domain (the Rel homology [RH] domain) located towards the N terminus but are generally unrelated in their long C-terminal halves and frequently in a short domain N terminal to the RH domain (reviewed in reference 13).

v-rel encodes a 59-kDa phosphoprotein, p59^{v-rel} (vRel, herein), that is a truncated form of the normal avian protein p68^{c-rel} (14, 20, 31, 37). Sequence comparison of p59^{v-rel} and p68^{c-rel} shows that p59^{v-rel} is missing 2 N-terminal aa and 118 C-terminal aa that are present in p68^{c-rel}; in place of the c-rel-encoded aa, there are viral envelope-derived aa in p59^{v-rel} (6, 19, 39, 43). p59^{v-rel} is located in the nuclei of chicken embryo fibroblasts (CEF) (14). In contrast, chicken c-rel-encoded protein p68^{c-rel} is located exclusively in the cytoplasm of CEF (6).

The mechanism by which p59^{v-rel} transforms cells has remained obscure, partially because the aa sequence reveals no obvious functional domains in the protein (39, 43). However, certain things are known about Rel proteins. Viral and chicken Rel proteins contain a short basic sequence located near the C-terminal end of the RH domain that can

function as an autonomous nuclear localizing signal (6, 15). This sequence is conserved in all Rel-related proteins. The C terminus of avian p68^{c-rel} (which is deleted in vRel) has two known functions: it can inhibit the cRel nuclear localizing signal in CEF, and it contains a strong gene activation domain that can function in *Saccharomyces cerevisiae* (6, 21). More recently, it has been shown that N-terminal RH sequences of NF- κ B are important for recognition of specific DNA target sequences in vitro and that the vRel protein can also bind to NF- κ B target sequences (3, 12, 23).

That Rel proteins can act as transcription factors is suggested by several lines of evidence. In cotransfection studies, vRel, hybrid vRel-cRel, and Dorsal proteins can activate transcription from certain promoters (11, 19, 34). Genetic studies suggest that Dorsal can activate certain genes and repress others (reviewed in reference 41), and as we have previously shown, DNA-bound vRel, chicken cRel, and Dorsal proteins can activate gene expression in *S. cerevisiae* (21). Recently, Bull et al. (5) have shown that the mouse cRel protein also contains a C-terminal gene activation domain that can function in *S. cerevisiae* and mouse cells. In addition, the sequence similarity to NF- κ B (12, 23, 27a, 33a) and to the bacterial *fmr*-encoded protein (7), which are known transcription factors, indicates that Rel proteins act to control transcription.

In our previous studies (21), we made plasmids which express fusion proteins containing the DNA-binding portion of the bacterial repressor protein LexA and various portions of the vRel and cRel proteins. Gene activation was measured in *S. cerevisiae* as the ability to stimulate β -galactosidase expression from a reporter plasmid that contains LexA-binding sequences, a yeast promoter, and the *lacZ* gene. The conclusions from those studies with *S. cerevisiae* are that vRel and cRel proteins contain a weak gene activation

* Corresponding author.

sequence in the RH domain and that chicken cRel contains an additional C-terminal strong transcriptional activation domain.

To determine the biological relevance of gene activation by Rel proteins, we performed analogous experiments using LexA hybrid proteins in avian and mammalian cells. In addition, we tested the ability of wild-type vRel to affect transcription from reporter plasmids containing natural and artificial promoters with NF- κ B target sites. Our results have led us to propose a model for transformation by vRel and control of gene expression by normal Rel proteins.

MATERIALS AND METHODS

Plasmids. All plasmids were constructed by conventional procedures (35). Reporter plasmid XXBCO (a gift of K. Lech) contains four LexA operators inserted at the *Xho*I site located approximately 100 bp upstream of the rabbit β -globin promoter in OBCO (16). Reporter plasmid 2NFBCO was made by inserting four copies of a B site NF- κ B target oligonucleotide into OBCO; two copies of a *Sal*I fragment from plasmid 4 (28; a gift of J. Pierce), each containing two copies of the B site, were inserted at the *Xho*I site of OBCO. Reporter plasmids HIV-CAT and κ B mutant HIV-CAT were a kind gift of G. Nabel (27).

Plasmid JDL was made by insertion of a 300-bp Klenow-treated *Eco*RI fragment containing codons for LexA aa 1 to 87 from pJK1521 (21) into the *Sma*I site of JD214 (10). This plasmid has a unique *Sma*I site which cuts after codon 87 of LexA into which all *rel* sequences were subcloned. Sequencing across the LexA fusion point was done when necessary; otherwise, Western blotting (immunoblotting) was performed to confirm that the appropriately sized proteins were synthesized. The numbers above the maps (see Fig. 1A) refer to the Rel aa (from the full-length wild-type protein) contained in the final fusion proteins.

The enzymes used to digest the 5' ends of the indicated *rel* genes were as follows: JDL-V and JDL-5'V, *Hinc*II aa 2; JDL-3'V, *Hinc*II aa 332; JDL-C and JDL-5'C, *Dde*I-Klenow-treated aa 5; JDL-3'C, *Hinc*II aa 323. For JDL-M, full-length mouse *c-rel* was subcloned into the *Sma*I site of JDL as a *Bam*HI-*Eco*RI-Klenow-treated fragment (aa 1 to 588) from PCR5'3'rel (5; a gift of Inder Verma). JDL-3'M contains mouse *c-rel* sequences from a unique *Stu*I site (aa 267). JDL-3'H was created by subcloning into the *Sma*I site of pJDL sequences from a plasmid containing human *c-rel* cDNA 2 (4; a gift of Nancy Rice) from a 5' *Pvu*II site (aa 137). JDL-D was made by inserting an *Sna*BI-*Aha*III (aa 49 to 678) fragment from plasmid D1B6 (a gift of Ruth Steward) into the *Sma*I site of JDL. JDL-3'D starts at Dorsal aa 431 (*Sst*I-T4 treated). JDL-C40 contains chicken cRel aa, starting at aa 40 (an *Sca*I site); JDL-C47 starts at aa 47 (by sequencing); and JDL-C265 starts at cRel aa 265 (an *Stu*I site).

JDL-3'C deletion mutants dX (*Xba*I), dA (*Acc*I), dB (*Bst*XI), and dP (*Pvu*II) were created by subcloning chicken *c-rel* fragments to encode proteins from the *Hinc*II site at aa 323 to the sites indicated in parentheses. Each C-terminal deletion mutant was sequenced across the LexA fusion point and at the 3' end of the gene. Proteins encoded by these plasmids are predicted to contain non-Rel aa encoded by vector sequences at their carboxy termini as follows: dX, 13 additional aa; dA, 24 aa; dB, 1 aa; and dP, 27 aa.

GM282 is a wild-type *v-rel* retroviral vector expression plasmid (6). dStuI/*Hinc*II is a nontransforming mutant with a deletion between unique *Stu*I and *Hinc*II sites in *v-rel* (15).

PH11 is a nontransforming *v-rel* mutant that has a 6-bp *Nco*I linker insertion at the unique *Stu*I site in *v-rel* (26a).

Cell culture, transfection, and CAT assay. CEF were prepared and cultured as described previously (6). NIH 3T3 cells were grown in Temin's modified Eagle's medium supplemented with 10% calf serum, and CV-1 cells were grown in Temin's modified Eagle's medium supplemented with 10% fetal calf serum.

For chloramphenicol acetyltransferase (CAT) assays, cells were transfected with 5 μ g of a reporter plasmid (XXBCO, OBCO, or 2NFBCO) and 5 μ g of a producer plasmid by the dimethyl sulfoxide-Polybrene method (22). Cotransfections with reporter plasmids containing the human immunodeficiency virus 1 (HIV-1) long terminal repeat (LTR) were performed as described in the figure legends. Approximately 48 h posttransfection, cells were scraped and lysates were prepared by freeze-thawing the cells three to five times in 25 mM Tris, pH 7.4. CAT activity in extracts containing equal amounts of protein was assayed essentially as previously described (17). After thin-layer chromatography and autoradiography, cellulose was excised. CAT activity is expressed as a percentage of the acetylated form of chloramphenicol compared with the total number of counts in the acetylated and nonacetylated forms as determined by liquid scintillation counting.

Western blotting and immunofluorescence. Western blotting and immunofluorescence were performed essentially as described previously (6). CEF were transfected with 5 μ g of producer virus DNA and 0.1 μ g of reticuloendotheliosis virus strain A helper virus DNA (pSW253; reference 42). Four days posttransfection, cells were analyzed by Western blotting or immunofluorescence with a rabbit anti-LexA antibody as the primary antibody.

RESULTS

Gene activation by avian and viral Rel proteins in CEF. To test the abilities of Rel proteins to activate gene expression in chicken cells, we cotransfected CEF with producer and reporter plasmids. Producer plasmids were spleen necrosis virus-derived retroviral vectors expressing aa 1 to 87 of LexA (the DNA-binding portion) fused to the Rel sequences indicated in Fig. 1A (and elsewhere). The reporter plasmid, XXBCO, contains four copies of the DNA sequence recognized by LexA positioned approximately 100 bp upstream of the rabbit β -globin promoter and the CAT-encoding gene. At 48 h posttransfection, extracts were prepared from cells and CAT activity was measured. CAT activity, as measured by percent acetylation of chloramphenicol, is interpreted as an indication of transcriptional activity from the promoter in the reporter plasmid.

A typical result is shown in Fig. 1B. CEF transfected with a vector expressing only LexA aa 1 to 87 gave a low basal level of expression from the reporter plasmid (0.2% conversion, JDL). JDL-V (vRel aa 2 to 503 fused to the LexA sequences) also gave a low level of CAT activity, similar to that seen with JDL. Transfection with JDL-C (chicken cRel aa 5 to 598) resulted in a small increase in CAT activity (2.4%), whereas a LexA fusion protein expressing the C-terminal half of the cRel protein (JDL-3'C) increased CAT activity very dramatically (94.5% conversion). The activation seen with JDL-3'C was as strong as that seen with a LexA-vFos producer plasmid (data not shown). Conversely, the corresponding C-terminal aa of vRel (in JDL-3'V) increased expression from the reporter plasmid only weakly (2.5%) compared with the increase seen with JDL-3'C.

FIG. 1. Gene activation by LexA-Rel fusion proteins. (A) The striped box at the left of each construct represents LexA aa 1 to 87. The numbers above the open boxes are the aa numbers of the Rel proteins indicated. The reporter plasmid (XXBCO) contains four copies of the target sequence for LexA (Lex-ops) positioned approximately 100 bp upstream of the start site of transcription (arrow) from the rabbit β -globin promoter (B-globin), the CAT-encoding gene (CAT), and the simian virus 40 polyadenylation sequence (SV40 term). (B) Gene activation by LexA-vRel and LexA-cRel fusion proteins. Five micrograms of the reporter plasmid and 5 μ g of each producer plasmid indicated (as described for panel A) were transfected into CEF. Approximately 48 h posttransfection, cells were lysed, equal amounts of protein were incubated with [14 C] chloramphenicol for 60 min at 37°C, and samples were separated on thin-layer chromatography plates. Values correspond to the percent chloramphenicol acetylated as explained in Materials and Methods. This is a representative assay from many independent assays.

Sequences from the N-terminal half of vRel or cRel (JDL-5'V and JDL-5'C) did not activate gene expression above basal levels. The sequences expressed in JDL-5'C and JDL-5'V correspond very nearly to the sequences of the RH domain (see Introduction). LexA-Rel fusion proteins did not

affect transcription from a reporter plasmid (OBCO) that contained no LexA target sequences (data not shown).

In the representative assay whose results are shown in Fig. 1B, CAT activity for JDL-3'C (94.5%) was out of the linear range; this was done to detect the low activity present in JDL-3'V and JDL-C lysates (2.5 and 2.4%, respectively). Therefore, the difference in gene activation by JDL-3'V and JDL-C versus JDL-3'C is probably an underestimate of the real difference in gene activation by these proteins. When comparing strongly activating proteins (such as JDL-3'C), incubation times or the amounts of cell lysate were reduced so that the CAT activity was in the linear range (see Fig. 3 and 4).

Figure 2A shows a Western blot, using anti-LexA antibody, of CEF transfected with the producer plasmids in the presence of reticuloendotheliosis virus strain A helper virus. All of the plasmids shown in Fig. 1 expressed proteins of the appropriate sizes.

Figure 2B shows immunofluorescence of representative cells expressing the LexA-Rel fusion proteins using the anti-LexA antibody. The proteins were located in the nuclei to various degrees. JDL-V-, JDL-5'V-, and JDL-5'C-encoded proteins were all predominantly nuclear. The JDL-3'V-encoded protein was located exclusively in the nuclei of some cells and in both the nuclei and cytoplasm of others; although this protein lacks the primary nuclear localizing sequence of vRel, it does contain the second weak nuclear localizing sequence located in the C-terminal half of vRel (15), and LexA sequences have a limited ability to direct proteins to the nucleus (36). JDL-C- and JDL-3'C-encoded proteins showed heterogeneity of staining: in some cells, the staining was exclusively cytoplasmic, and in other cells the staining was nuclear or distributed between the nucleus and the cytoplasm. Presumably this is a reflection of the facts that overexpressed wild-type cRel protein, because of the C-terminal cRel aa, is normally a cytoplasmic protein in CEF (6) and LexA contains nuclear localizing sequences (36). Therefore, the fusion proteins containing the LexA limited nuclear signal and the cytoplasmic retention signal of cRel are distributed in both the nuclear and cytoplasmic compartments. Nevertheless, in all cases some LexA-Rel fusion protein was located in the nuclei of CEF. Furthermore, localization is not sufficient to explain activation, since JDL-5'V and JDL-5'C proteins are exclusively nuclear but do not activate.

Suppression of the C-terminal gene activation sequences present in cRel by N-terminal Rel sequences. The results presented in Fig. 1 indicated that N-terminal chicken cRel sequences could suppress the C-terminal cRel activation domain and that vRel lacked the gene activation function present in the C terminus of cRel.

The JDL-C-encoded protein contains aa 5 to 598 of chicken cRel and was a weak activator of transcription; JDL-3'C contains aa 323 to 598 of chicken cRel and was a strong activator of transcription. To confirm that suppression of the C-terminal gene activation function was due to sequences in the RH domain, we assayed gene activation by four plasmids that contain deletions in sequences encoding N-terminal cRel sequences: JDL-C (aa 5 to 598), JDL-C40 (aa 40 to 598), JDL-C47 (aa 47 to 598), and JDL-C265 (aa 265 to 598). Only JDL-C265 activated transcription as strongly as JDL-3'C (Fig. 3A). Therefore, the sequences necessary for suppression of the C-terminal gene activation domain of chicken cRel are located completely within the RH domain between aa 47 and 265 of chicken cRel.

To map the C-terminal gene activation domain present in

FIG. 2. Western blotting and immunofluorescence of fusion proteins expressed in CEF. Western blotting and immunofluorescence were performed with anti-LexA antibody as the primary antibody as described in Materials and Methods. The single-digit numbers in panels A and B correspond to the following: 1, JDL-V; 2, JDL-5'V; 3, JDL-3'V; 4, JDL-C; 5, JDL-5'C; and 6, JDL-3'C. In panel A, 68 and 59 correspond to the positions of p59^{v-rel} and p68^{c-rel} from a v-rel-transformed spleen cell line lysate run on the same gel and stained with anti-Rel primary antibody (data not shown; see reference 6 for details). In panel A, the approximate sizes of the fusion proteins are as follows: JDL-V, 68 kDa; JDL-5'V, 50 kDa; JDL-3'V, 30 kDa; JDL-C, 75 kDa; JDL-5'C, 50 kDa; and JDL-3'C, 45 kDa.

chicken cRel, we made a series of C-terminal truncations in JDL-3'C. The result of a typical CAT assay is shown in Fig. 3B. JDL-3'CdX- and JDL-3'CdA-encoded proteins (missing 56 and 103 C-terminal cRel aa, respectively) were as strongly activating as the protein encoded by JDL-3'C that contains an intact C terminus. However, when 173 or 216 aa from the cRel C terminus were deleted, gene activation was greatly reduced (JDL-3'CdB and JDL-3'CdP). Western blotting showed that all deletion mutants synthesized appropriately sized proteins in CEF (data not shown). These results

FIG. 3. Characterization of the N-terminal inhibitory and C-terminal gene activation domains in chicken cRel. (A) Deletion mapping shows that the inhibitory domain is located between aa 47 and 265 of cRel. As in Fig. 1A, the numbers above the drawings refer to chicken cRel aa. The values for percent acetylation (% ACET.) in this representative assay were determined as described in the footnote to Fig. 1 and Materials and Methods. (B) Deletion mapping shows that the cRel C-terminal gene activation domain is located between aa 425 and 495. The values are for a representative assay, as in panel A. CAT assays were performed as described for Fig. 1B, except that the conditions (total protein and incubation times) were reduced to obtain activities in the linear range.

indicate that most of the C-terminal gene activation domain of chicken cRel is located between aa 425 and 495 (defined by unique *Bst*XI and *Acc*I sites in chicken c-rel).

The results presented in this section show that the chicken cRel protein contains a strong gene activation domain either largely absent or nonfunctional in the vRel protein and that cRel N-terminal RH sequences can inhibit this activation domain.

Similarity in protein organization between mouse and *Drosophila* Rel proteins and chicken cRel. As mentioned above, chicken, mouse, human, and *Drosophila* (Dorsal) cRel proteins are highly similar in their N-terminal halves

FIG. 4. Gene activation by Rel family proteins. The numbers above the drawings correspond to the aa from the wild-type Rel family protein. In each protein, the RH domain extends up to approximately aa 300. C, chicken cRel; M, mouse cRel; D, *Drosophila* Dorsal; H, human cRel. The assays were performed as described in the legend to Fig. 3, and the values correspond to the CAT activity in transfected cell lysates.

but very dissimilar in their C-terminal halves. Therefore, we wished to determine whether mammalian and fly Rel proteins also contained C-terminal gene activation domains that could be suppressed by N-terminal Rel sequences.

Similar to the results obtained with chicken cRel, essentially full-length mouse and *Drosophila* Rel proteins were only weak activators of transcription in CEF when fused to LexA sequences (Fig. 4, JDL-M and JDL-D). However, deletion of much of the N-terminal RH sequences in mouse, human, and fly Rel proteins resulted in proteins that strongly activated transcription. The mouse JDL-3'M-encoded protein and the fly JDL-3'D protein were as strongly activating as the chicken JDL-3'C protein. The human cRel protein (in JDL-3'H) was not as strongly activating as the other C-terminal Rel fusion proteins; however, the protein encoded by JDL-3'H contains a large portion of the human cRel RH domain which may have partially suppressed its activation function.

These results show that Rel proteins from different species all contain strong C-terminal activation domains that are inhibited by N-terminal sequences located in the RH domain.

Functionality of chicken cRel gene activation and inhibitory domains in mouse and monkey cells. To test for gene activation and inhibitory Rel domains in cell types other than CEF, we cotransfected mouse NIH 3T3 and monkey CV-1 cells with producer and reporter plasmids as described above for CEF. The results of CAT assays performed with these cells are shown in Table 1. Essentially, we obtained the same results as with CEF. Full-length chicken cRel protein fused to LexA aa 1 to 87 was only a weak activator of transcription

TABLE 1. Functioning of the chicken cRel C-terminal activation domain in mouse and monkey cells and its inhibition by N-terminal sequences^a

Producer plasmid	Fold activation with:	
	3T3 cells	CV-1 cells
JDL-V	1	1
JDL-3'V	1	1
JDL-C	2	2
JDL-3'C	40	10

^a Mouse 3T3 and monkey CV-1 cells were cotransfected with 5 µg of the reporter plasmid (XXBCO) and 5 µg of each of the indicated producer plasmids, and cell lysates were assayed for CAT activity 48 h posttransfection. Values correspond to fold activation compared with the CAT activity seen with JDL (0.5% acetylation = 1 for 3T3 cells; 0.3% acetylation = 1 for CV-1 cells).

(JDL-C in Table 1), whereas the chicken cRel fusion protein containing only C-terminal aa (JDL-3'C) was a much stronger activator of transcription in these mammalian cells. Therefore, the C-terminal gene activation domain of chicken cRel can function in mammalian and avian cells and this gene activation domain can be also inhibited by N-terminal Rel sequences in both cell types.

Repression by vRel of transcription from promoters requiring NF-κB-binding sites for activity. Kieran et al. (23) and Ballard et al. (3) have shown that vRel can bind to NF-κB target sites in vitro. Therefore, we wished to determine whether vRel could affect transcription from promoters containing NF-κB target sites. We performed cotransfection studies with CEF similar to those described above.

In one set of reporter plasmids, the wild-type HIV-1 LTR (which contains two functional NF-κB sites) or a mutant HIV-1 LTR (with mutated NF-κB target sites) are positioned upstream of the CAT-encoding gene (27). Figure 5A shows that transcription from the HIV-1 LTR in CEF is dependent on functional NF-κB sites: transcription from the wild-type HIV-1 LTR was rather efficient in CEF, whereas the mutant HIV-1 LTR was virtually inactive. Expression from the wild-type HIV-1 LTR was not affected by cotransfection of a retroviral vector expressing the vMyb nuclear oncoprotein. However, Fig. 5B shows that cotransfection of the HIV-CAT plasmid with an increasing amount of a retroviral vector expressing vRel (GM282) greatly decreased expression from the HIV-1 LTR. vRel expression did not significantly affect expression of a β-galactosidase reporter plasmid used as an internal control in these experiments, nor did vRel affect expression from plasmid XXBCO (data not shown).

A second set of reporter plasmids contained four copies of the NF-κB target sequence (2NFBCO) in place of the LexA target sequences (in XXBCO) or no target sequences (OBBCO) at all. The NF-κB target sequences in 2NFBCO are like those found in the NF-κB B site in the kappa light-chain enhancer (28).

Plasmid OBBCO gave a very low level of CAT activity when transfected into CEF either alone (data not shown) or in the presence of control retroviral vector DNA with no inserted gene (JD214) (Fig. 6). However, cotransfection of plasmid 2NFBCO and control DNA (JD214) gave a much higher basal level of CAT activity, indicating that there are endogenous proteins in CEF which bind to and activate transcription from promoters containing NF-κB target sites. Cotransfection of 2NFBCO and the wild-type v-rel-express-

FIG. 5. Repression of expression from the HIV-1 LTR by vRel. (A) Representative CAT assay of lysates from CEF cotransfected with 1 µg of HIV-CAT (+) or κB mutant HIV-CAT (M) and 20 µg of retroviral plasmid DNA expressing no gene (JD214) or *v-myb* (V-MYB). (B) CEF were cotransfected with 1 µg of HIV-CAT and the indicated amounts of plasmid GM282, a retroviral vector which expresses *v-rel* (6). CAT activity (% ACET.) was determined as described in Materials and Methods.

ing plasmid (GM282) resulted in approximately 95% reduction of CAT activity (Fig. 6).

We also tested the abilities of two nontransforming vRel mutants (dStuI/HincII and PH11) to affect transcription from reporter plasmids 2NFBCO and HIV-CAT. The wild-type vRel protein and the protein made by PH11 are both exclusively nuclear in CEF (14, 26a). The protein made by dStuI/HincII is distributed in the nuclei and cytoplasm of CEF (15). Figure 6 also shows that the nontransforming vRel proteins made by PH11 and dStuI/HincII had little effect on transcription from reporter plasmids 2NFBCO and HIV-CAT.

These results indicate that vRel can repress transcription from promoters containing NF-κB target sites. It remains unclear whether vRel represses transcription by binding to the NF-κB target sequences (see Discussion). Since two nontransforming vRel mutants did not repress transcription, it suggests that this activity is important for transformation by vRel.

	PRODUCER	SPLEEN TRANSF.	REPORTER	RELATIVE CAT ACTIVITY	REPORTER	RELATIVE CAT ACTIVITY
JD214	LTR — LTR	—	OBCO	0.6	M-κBF	0.5
JD214	LTR — LTR	—	2NFBCO	100	HIV-CAT	100
GM282	LTR — v-rel — LTR	+	2NFBCO	4.7	HIV-CAT	16
dStuI/HincII	LTR — — LTR	—	2NFBCO	98	HIV-CAT	92
PH11	LTR — PRO TRP — LTR	—	2NFBCO	67	HIV-CAT	120

FIG. 6. Repression of promoters containing NF-κB-binding sites by vRel. Reporter plasmids (OBCO, no κB sites; 2NFBCO, four κB sites; HIV-CAT, wild-type HIV-1 LTR; and M-κBF, HIV-1 LTR with mutant κB sites) were cotransfected into CEF with producer plasmids expressing no gene (JD214; reference 10), wild-type vRel (GM282), or a nontransforming vRel protein (dStuI/HincII or PH11). CAT assays were performed as described in Materials and Methods and the legend to Fig. 5. CAT activity is expressed relative to the value obtained with either 2NFBCO and JD214 or HIV-CAT and JD214. The values are the averages of at least three independent assays in each case. The spleen cell transformation (SPLEEN TRANSF.) data (+, transforming; —, nontransforming) were determined previously (GM282, reference 6; dStuI/HincII, reference 15; and PH11, reference 26a). The boxes at the ends of the drawings correspond to the retroviral LTRs.

DISCUSSION

We have shown that cRel sequences from chickens, mice, humans, and flies activated gene expression in avian and mammalian cells when bound to target DNA through the heterologous DNA-binding domain of LexA. Although activation by the full-length Rel proteins was rather weak, removal of N-terminal sequences revealed a strong C-terminal activation domain. The C-terminal gene activation function of chicken cRel is missing in the vRel protein, and neither full-length nor C-terminal sequences from vRel significantly activated gene expression in avian cells. Moreover, vRel repressed transcription from promoters containing NF-κB sites.

Comparison of our results with previous results. Our experiments with avian cells failed to detect the weak N-terminal gene activation domain in vRel and chicken cRel proteins that we previously demonstrated in *S. cerevisiae* (21). There are several possible explanations for the discrepancy between the results obtained with *S. cerevisiae* and those obtained with higher eukaryotic cells. It may be that the CAT assay is not sensitive enough to detect this weak activation domain, it is possible that the weak N-terminal activation domain that we described in *S. cerevisiae* is not truly a functional gene activation domain in higher cells, that is, that these sequences can weakly activate transcription in *S. cerevisiae* but do not do so in avian cells in which they are normally expressed. Alternatively, it is possible that the domain we described as an activation domain in *S. cerevisiae* is inactivated in higher cells (by modification or binding to a protein) but is not inactivated in *S. cerevisiae* or that it affects transcription in some other way in higher cells (for example, as a repressor). Recently, it has been shown that the vErbA protein activates transcription in *S. cerevisiae* but represses transcription in mammalian cells (29). Along these lines, we have shown that vRel mutants that do not activate transcription in *S. cerevisiae* (21) failed to repress transcription from promoters that contain NF-κB sites (Fig. 6). Therefore, we believe that the N-terminal activation domain

seen in *S. cerevisiae* represents a functionally important part of the vRel protein since there was a strict correlation between activation by this domain in *S. cerevisiae* and the ability of v-rel mutants to transform avian spleen cells. We are currently trying to determine whether the N-terminal vRel activation domain in *S. cerevisiae* is identical to the vRel-repressing domain that functions in higher cells on promoters containing NF- κ B-binding sites.

In our previous experiments with *S. cerevisiae* (21), we did not detect inhibition of the chicken cRel C-terminal gene activation domain by N-terminal sequences with a protein identical to that encoded by JDL-C40 whose C-terminal gene activation domain is inhibited in CEF (Fig. 3A). Similarly, a LexA fusion protein containing chicken cRel aa 1 to 598 (like JDL-C [Fig. 1]) is fully active in yeast cells, like fusion proteins containing only the C-terminal chicken cRel sequences (20a). Thus, LexA-full-length chicken cRel fusion proteins behave differently in CEF versus *S. cerevisiae*. By using Gal4 fusion proteins, Bull et al. (5) have demonstrated that inhibition of the mouse C-terminal cRel activation domain occurs in both *S. cerevisiae* and mouse cells. It is not clear why this discrepancy occurs, but it may be due to the yeast strains used or there may be a difference in *S. cerevisiae* between the inhibitory domains of chicken and mouse cRel proteins.

There is an additional discrepancy between the C-terminal chicken cRel gene activation domains in *S. cerevisiae* and CEF. In *S. cerevisiae*, a deletion that removed the final 55 aa of chicken cRel (LexA-delXbaI in reference 21) inactivated the C-terminal gene activation domain. However, our results obtained with CEF (Fig. 3B) indicate that the C-terminal gene activation domain in chicken cRel maps upstream of the XbaI site used to make both of these deletions. One possible explanation for this discrepancy is that the additional vector-derived 52 aa in the LexA-delXbaI protein inhibited the C-terminal gene activation domain in *S. cerevisiae*. Consistent with this, a JDL-3'C deletion mutant at the XbaI site which encodes additional non-Rel aa compared with JDL-3'CdX (because of insertion of a polylinker) did not activate transcription in CEF (32a). Therefore, we believe that the results presented here more accurately map the cRel gene activation domain. Furthermore, our results place the chicken cRel C-terminal gene activation domain in a position that is very similar to where it is found in mouse cRel (5).

Inhibition of the cRel C-terminal gene activation domain by N-terminal RH sequences. There are several possible mechanisms by which the N termini of cRel proteins could inhibit gene activation by the C termini. The N termini may interact in some direct way with the C termini to mask the gene activation function. However, it is difficult to imagine how the C termini of cRel proteins from mice, chickens, and flies, which share no obvious sequence similarity, could all be suppressed by very similar N-terminal Rel sequences.

It is also possible that the N terminus blocks activation by interaction with a cellular inhibitory factor. Since the N termini of cRel proteins are highly conserved, the ability of these proteins to interact with this factor in many cell types would also be conserved. Chicken cRel and vRel proteins are known to bind to a number of cellular proteins (9, 26, 38), and one of these may be responsible for inhibition of the C-terminal gene activation domain. Baichwal and Tjian (2) have shown that a cell type-specific factor acting through the delta domain is involved in repression of the cJun gene activation domain. However, overexpression of the viral RH sequences did not relieve the suppression seen with JDL-C

(data not shown); Bull et al. (5) have reported similar results obtained with mouse cRel.

It is known that Rel family proteins can form heterodimers. For example, Kieran et al. (23) have shown that mouse NF- κ B p50 and vRel can associate in vitro. Thus, it is possible that LexA-Rel proteins containing the RH domain form heterodimers with endogenous Rel proteins, which would titrate out the active LexA-Rel homodimers. JDL-3'C proteins, lacking the RH sequences, would not form these inactive complexes. It will be interesting to determine whether RH sequences can suppress activation domains from heterologous Rel proteins or activation domains not ordinarily found in Rel family proteins.

Gene activation by cRel proteins. Transcriptional activating domains generally contain regions rich in acidic aa, proline residues, or glutamines (25, 30). Although the C-terminal halves of mouse and chicken cRel proteins show no obvious sequence similarity, the C-terminal half of each protein does have a net negative charge. However, the region between aa 425 and 495 of chicken cRel, where we have mapped the C-terminal gene activation domain (Fig. 3B), has only scattered negatively charged residues (with an overall net negative charge of -5) and thus may contain a novel gene activation sequence. The C-terminal half of the *Drosophila* Dorsal protein is very rich in glutamine residues (40), as are other *Drosophila* proteins that function as activators of transcription. Our results indicate that although the C-terminal gene activation domains of Rel proteins may be unrelated, they can all function in chicken cells.

Full-length and truncated vRel proteins can recognize the same sequence bound by the p50 subunit of NF- κ B (3, 23). Furthermore, NF- κ B p50 can form heterodimers with the vRel protein (23). Our results show that the C-terminal sequences of cRel and Dorsal proteins can activate gene expression when bound to DNA through LexA. It is not clear whether cRel and Dorsal proteins activate transcription by being brought to DNA by a heterologous Rel-related protein or whether they bind DNA directly and activate gene expression. It is interesting that an active NF- κ B complex appears to consist of a 50-kDa DNA-binding protein and a 65-kDa Rel family protein that may not bind to DNA (1).

As we have previously shown, the overexpressed chicken cRel protein is located in the cytoplasm of CEF (6). It is not known whether or under what circumstances avian or mammalian cRel proteins enter nuclei. However, it is clear that the Dorsal protein does enter the nuclei of cells on the ventral side of the fly blastoderm (but remains in the cytoplasm of dorsally located cells) (33, 34, 41).

Gene activation by cRel proteins may be inhibited in two ways. (i) The protein (like NF- κ B or cRel) can be sequestered in the cytoplasm, or (ii) the C-terminal activation domain can be inhibited by N-terminal sequences. The exact mechanisms by which these processes occur for cRel or Dorsal are not known; however, in the case of NF- κ B, the complex is sequestered in the cytoplasm because of the association with an inhibitor, I κ B (reviewed in reference 24). Upon some signal, the Rel protein could enter the nucleus and bind to DNA either by itself or through a heterologous DNA-binding protein. However, the inhibition of the C-terminal activation domain by the N-terminal sequences must also be relieved. The same signal that causes the cRel protein to enter the nucleus could also inactivate the N-terminal gene activation-suppressing effect. Alternatively, it may be that cRel proteins are simply weak activators of transcription and that the N-terminal sequences ensure a

relatively low level of transcriptional activation by cRel proteins.

Transformation by mutant Rel proteins. The results obtained with JDL-3'V (Fig. 1B) show that vRel does not have a strong gene activation function at its C terminus. We mapped the cRel gene activation domain between aa 425 and 495. vRel contains all but 15 of these aa. Why, then, does JDL-3'V activate transcription so weakly compared with JDL-3'C? We are currently testing the following three possibilities: (i) that the 15 additional aa in cRel are important for the gene activation function, (ii) that internal changes (there are three) between vRel and cRel in this region (cRel aa 425 to 480) inactivate the gene activation domain in vRel, and (iii) that the 18 out-of-frame *env* aa at the C terminus of vRel inactivate the gene activation domain.

Since p59^{v-rel} lacks the strong C-terminal gene activation function of p68^{c-rel}, it is possible that p59^{v-rel} transforms cells by interfering with transcription directed by Rel family proteins. That is, the inactive vRel protein might occupy regulatory DNA sequences normally regulated by active cRel or NF- κ B complexes. In this sense, p59^{v-rel} would transform cells by acting as a dominant negative factor, not unlike what has been described for the vErbA protein (8). This model would require that at least some of the vRel protein be in the nuclei of avian spleen cells. However, various lines of evidence suggest that fully cytoplasmic Rel proteins are transforming (14, 15, 19). Furthermore, transforming Rel proteins need not be defective for the gene activation function: Hannink and Temin (19) have shown that some Rel proteins with intact cRel C termini are transforming.

cRel proteins may interact with a variety of proteins in a cell. For example, a Rel protein might interact with DNA-binding proteins and, perhaps, cytoplasmic retention proteins. In this sense, transforming Rel proteins might titrate out proteins that normally regulate Rel family proteins (e.g., the 124-, 115-, or 40-kDa Rel-associated protein or the p65 and I κ B NF- κ B-associated proteins). According to this model, either cytoplasmic or nuclear mutant Rel proteins could be transforming.

Either model would explain the repression of transcription from promoters containing NF- κ B sites by vRel described in this report (Fig. 5 and 6). That is, vRel could repress transcription from 2NFBCO and the HIV-1 LTR by either binding to the NF- κ B sites occupied by endogenous factors or forming heterodimers with and inactivating these same factors. In addition, we found that vRel can repress transcription from the chicken *c-rel* promoter, which also contains a consensus NF- κ B-binding site (5a). The finding that nontransforming vRel proteins did not repress transcription from 2NFBCO suggests that this type of competition is important for transformation. While this work was in progress, Ballard et al. (3) obtained essentially identical results with repression of the HIV-1 LTR by vRel and a lack of repression in an NF- κ B-responsive promoter by the mutant protein encoded by dStuI/HincII.

Rel proteins are in a family of oncoproteins that can act in the nucleus to affect transcription directly. In addition to the product of *rel*, this family now includes the products of *myb*, *fos*, *jun*, *ets*, *p53*, *Ela*, and (possibly) *myc*. Insight into transformation by these proteins should come from identification of the genes controlled by them.

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