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Interaction of the v-Rel Oncoprotein with Cellular Transcription Factor Sp1

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We previously showed that v-Rel, the oncoprotein of the avian retrovirus Rev-T, can increase expression from promoters containing binding sites for the cellular transcription factor Sp1 in chicken embryo fibroblasts (S. Sif, A. J. Capobianco, and T. D. Gilmore, Oncogene 8:2501–2509, 1993). In those experiments, v-Rel appeared to increase the transactivating function of Sp1; that is, v-Rel stimulated transactivation by a GAL4-Sp1 protein that lacked the Sp1 DNA-binding domain. We have now shown that in vitro-synthesized v-Rel and GAL4-Sp1 form a complex that can be immunoprecipitated with either anti-Sp1 or anti-v-Rel antiserum. We have also shown that a glutathione *S*-transferase (GST)-Sp1 fusion protein can specifically interact with in vitro-translated v-Rel and with in vivo-synthesized v-Rel from transformed chicken spleen cells. In addition, we have found that the abilities of wild-type and two mutant forms of v-Rel to increase transactivation by Sp1 in vivo correlate with their abilities to interact with Sp1 in vitro. The sequences important for the interaction of v-Rel with Sp1 in vitro have been mapped to the first 147 amino acids of v-Rel. Other Rel proteins, such as c-Rel, RelA, p52, and p50, were also able to form a complex with Sp1 in vitro. These results suggest that v-Rel increases expression from Sp1 site-containing promoters by functionally interacting with Sp1 and that cellular Rel proteins and Sp1 are likely to interact to influence transcription from natural promoters.

Regulation of gene expression can be accomplished at different levels, including preinitiation complex formation, start of transcription, elongation, and termination. In the case of class II genes, the formation of the preinitiation complex involves assembly of the general transcription factors TFIID, TFIIA, TFIIB, TFIIE, TFIIF, TFIIJ, TFIIH, and RNA polymerase II (reviewed in reference 56). TFIID, the only activity capable of binding DNA, contains the TATA-binding protein (TBP) and at least seven TBP-associated factors (TAFs) (12, 52). TBP can support basal transcription; however, activated transcription requires the presence of TAFs. Basal transcription can be up- or down-regulated by gene-specific transcription factors that bind upstream *cis*-acting elements, and it is the programmed cross-talk between specific and general transcription factors that determines the levels of gene expression.

The Rel family of gene-specific transcription factors can be subdivided into two classes. One class includes the vertebrate proteins c-Rel, RelA, and RelB, the retroviral oncoprotein v-Rel, and the *Drosophila melanogaster* ventral morphogen dorsal and immunity factor Dif (1, 2, 16, 17, 22). A second class contains the precursor proteins p100 and p105, which are believed to undergo proteolysis to generate the mature DNA-binding proteins p52 and p50, respectively (14, 33, 41). Rel proteins share a highly homologous region located in the N-terminal half of the protein, called the Rel homology (RH) domain (16). The RH domain is approximately 300 amino acids (aa) in length and contains sequences important for DNA binding, dimerization, nuclear localization, and inhibitor binding. The distinction between the two classes of Rel proteins is based on whether the protein contains copies of a 33-aa motif, called the ankyrin repeat, in sequences C terminal to the RH domain.

Structurally, the retroviral oncoprotein v-Rel and the proto-oncoprotein c-Rel differ in that v-Rel is missing 2 aa at its N terminus and 118 aa at its C terminus (reviewed in reference 17). Furthermore, v-Rel has several internal changes compared with c-Rel, including amino acid substitutions and small deletions. The deletion of C-terminal c-Rel sequences in v-Rel has resulted in the loss of sequences important for transcriptional activation and cytoplasmic retention (5, 20, 42).

Sp1 is an approximately 100-kDa ubiquitous transcription factor that binds GC-rich sequences (10, 11, 24, 25). The DNA-binding domain of Sp1 consists of three C-terminal zinc fingers, and the Sp1 transactivation domain contains multiple glutamine-rich sequences located toward the N terminus (7, 8, 24, 25). Sp1 can affect basal transcription by physically interacting with at least one TAF, TAF110 (21, 40, 54), and possibly TBP (13). Sp1 has also been shown to interact with viral proteins such as the human immunodeficiency virus type 1 Tat protein and the bovine papillomavirus enhancer E2 protein (23, 27, 32) and with cellular proteins such as the NF-κB RelA subunit (39) and the initiator-binding protein YY1 (44).

We have recently shown that v-Rel can increase expression from Sp1 site-containing promoters by increasing the transactivation potential of Sp1 (45). We now report that v-Rel, and certain other cellular Rel proteins, can specifically form a complex with Sp1 sequences (aa 83 to 621) that include the Sp1 transactivation domain. These results suggest that v-Rel increases expression from Sp1 site-containing promoters by functionally interacting with Sp1 and that interaction with Sp1 is common to most Rel proteins.

MATERIALS AND METHODS

Plasmids. Plasmids for the in vitro expression of v-Rel, v-SPW, dStu/Hinc, c-Rel, RelA, p52, p50, and the chicken retinoblastoma protein (RB) have been described previously (4, 15, 34, 46). Plasmid SP6/GAL4-Sp1, for the expression of the DNA-binding domain of GAL4 [aa 1 to 147; designated GAL4(1–147)] fused to aa 83 to 621 of human Sp1, was made by digesting pSG4+Sp1N (kindly provided by G. Gill) with

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*Hin*dIII and *Xba*I; the *Xba*I end was treated with Klenow enzyme before subcloning of the fragment into the *Hin*dIII and *Sma*I sites of pGEM-3 (Promega). The template for the expression of GAL4(1–147) was generated by linearizing the SP6/GAL4-Sp1 plasmid with *Eco*RI. SP6/pGAL4-VP16, a plasmid for the expression of GAL4-VP16, was kindly provided by P. Morin. Plasmid JD5′V-GAD for the in vivo expression of 5′v-Rel (aa 1 to 331) fused to the activation domain of GAL4 (aa 753 to 881) was kindly provided by S. Sarkar. Plasmid SP6/pGAL4-3′v-Rel, encoding GAL4(1–147) fused to the C-terminal sequences of v-Rel (aa 273 to 503), was constructed by digesting pSG-3′vRel (43) with *Hin*dIII and *Xba*I; the *Hin*dIII-*Xba*I insert was then subcloned into the corresponding sites of pGEM-3. Templates for the in vitro expression of truncated v-Rel proteins v-Rel/*Hin*cII, v-Rel/*Stu*I, v-Rel/*Hin*dIII, and v-Rel/*Cla*I were generated by linearizing the v-Rel expression plasmid CG129 (4) with *Hin*cII, *Stu*I, *Hin*dIII, and *Cla*I, respectively. The glutathione *S*-transferase (GST)-Sp1 expression plasmid, pGEX-Sp1, was constructed by digesting pJDG+Sp1N (45) with *Sma*I and *Xba*I; the *Xba*I end was treated with Klenow enzyme before subcloning of the insert encoding aa 83 to 621 of Sp1 in frame with the coding region of GST at the *Sma*I site of pGEX-2TK (Pharmacia).

In vitro transcription and translation. All in vitro transcriptions and translations were carried out in the TnT coupled wheat germ system (Promega) as described previously (46).

Immunoprecipitations. Immunoprecipitations were performed essentially as described previously (46). In vitro-translated or cotranslated proteins were mixed with either anti-Sp1 or anti-v-Rel antiserum in buffer A (see below) containing 0.2 or 0.3% sodium dodecyl sulfate (SDS), respectively, and were incubated on ice for 1 h. Protein A-Sepharose beads were added, and samples were incubated at 4°C for 4 to 6 h. Beads were washed five times with buffer A containing SDS, boiled in SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

GST pull-down experiments. Bacterial cells transformed with either pGEX-2TK or pGEX-Sp1 were grown overnight and were then diluted 1:10 in fresh Luria broth and grown at 37°C. One hour later, cells were induced with 1 mM isopropylthiogalactopyranoside (IPTG) and allowed to grow for an additional 3 to 4 h. Cells were then pelleted and resuspended in 1/50 culture volume of buffer containing 150 mM KCl, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% (vol/vol) Nonidet P-40 (NP-40), and 0.5% (vol/vol) aprotinin (Sigma). Cells were lysed by sonication, and samples were centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were collected and stored in 20% glycerol at −70°C. Bacterial extracts containing 1 to 3 μg of GST or GST-Sp1 were mixed with 5 μl of a 50% slurry of glutathione-agarose beads at room temperature for 20 min. The beads were then washed three times with buffer A (20 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 0.5% [vol/vol] NP-40, 1% [vol/vol] aprotinin) supplemented with 1% bovine serum albumin (BSA) and 0.5% Carnation nonfat dry milk, resuspended in 250 μl of buffer A containing 1% BSA, 0.5% milk powder, 1 mg of bacterial protein extract per ml, and 400 μg of ethidium bromide per ml, and incubated at 4°C. One to two hours later, ^{35}S-labeled proteins were added and samples were incubated at 4°C for 2 to 4 h. Beads were then washed five times with buffer B (20 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 250 mM NaCl, 1 mM EDTA, 0.5% [vol/vol] NP-40, 1% [vol/vol] aprotinin) supplemented with 1% BSA and 0.5% milk powder and two times with buffer B. SDS sample buffer was added, and samples were heated at 100°C for 5 min and analyzed on an SDS–12% polyacrylamide gel. Gels were treated with 1 M sodium salicylate for 20 min, and proteins were visualized by fluorography.

To assay for the interaction of in vivo-synthesized v-Rel with GST-Sp1, v-Rel-transformed chicken spleen cell lysates were prepared in buffer A and were centrifuged at 14,000 rpm for 15 min at 4°C. Twenty-five microliters of cell lysate (containing ~60 μg of cellular protein) was then clarified by incubation with 20 μl of glutathione-agarose beads and 400 μg of ethidium bromide per ml at 4°C for 2 h. The clarified cell lysate was then incubated with 1 to 3 μg of either GST-Sp1 or GST protein coupled to 5 μl of glutathione-agarose beads as described for the in vitro-translated proteins. Cellular proteins retained on the beads were separated by SDS-polyacrylamide gel electrophoresis and detected by Western immunoblotting using anti-v-Rel antiserum as a primary antibody as described previously (45).

CAT assays. Chicken embryo fibroblasts (CEF) were grown and maintained as described previously (45). CEF were transfected with 5 μg of reporter plasmid tkCAT9, which contains the CAT gene, the herpes simplex thymidine kinase minimal promoter and four Sp1-binding sites, and 10 μg of the retroviral plasmid JD214BS+ (45), dH172 (v-Rel aa 1 to 331 [43]), or GM282BS+ (v-Rel [45]). For GAL4 assays, CEF were cotransfected with 5 μg of reporter plasmid G5BCAT, which contains five upstream GAL4-binding sites, 1 μg of producer plasmid pJDG+Sp1N (45) for the expression of GAL4-Sp1, and 10 μg of the retroviral plasmid JD214BS+, dH172, GM282BS+, or JD5′V-GAD. Approximately 48 h later, chloramphenicol acetyltransferase (CAT) activity was determined from lysates that had been normalized to β-galactosidase activity from a control plasmid (MSV-βgal) included in all transfections as described previously (45). CAT activity was determined as the percent acetylated chloramphenicol from the total of the acetylated and nonacetylated chloramphenicol.

RESULTS

Interaction of v-Rel and GAL4-Sp1 in vitro. We have previously shown that v-Rel specifically increases expression from reporter plasmids containing DNA-binding sites for the cellular protein Sp1 in CEF (45). We have also shown that v-Rel increases the transactivation potential of a GAL4-Sp1 fusion protein, which contains GAL4(1–147) fused to sequences of Sp1 (aa 83 to 621 of human Sp1) that contain the Sp1 transactivation domain but lack the C-terminal DNA-binding domain (45).

To elucidate the molecular mechanism underlying activation of Sp1 by v-Rel, we first attempted to determine whether v-Rel and GAL4-Sp1 could interact. v-Rel and GAL4-Sp1 were cotranslated in vitro and subjected to immunoprecipitation with an anti-v-Rel antiserum. GAL4-Sp1 was immunoprecipitated only in the presence of v-Rel (Fig. 1A, lane 5). Anti-v-Rel antiserum did not recognize GAL4-Sp1 (Fig. 1A, lane 7; see also Fig. 3A, lane 9). When cotranslated v-Rel and GAL4-Sp1 were immunoprecipitated with the anti-Sp1 antiserum, v-Rel was also immunoprecipitated (Fig. 1A, lane 13), presumably through its interaction with GAL4-Sp1 because the anti-Sp1 antiserum did not recognize v-Rel (Fig. 1A, lane 15).

To determine whether the interaction of v-Rel with Sp1 is dependent on cotranslation of both proteins, v-Rel and GAL4-Sp1 were translated separately and then mixed prior to immunoprecipitation with an anti-v-Rel antiserum. v-Rel and GAL4-Sp1 were still able to form a complex, but generally less efficiently than when these proteins were cotranslated (Fig. 1A, lane 8).

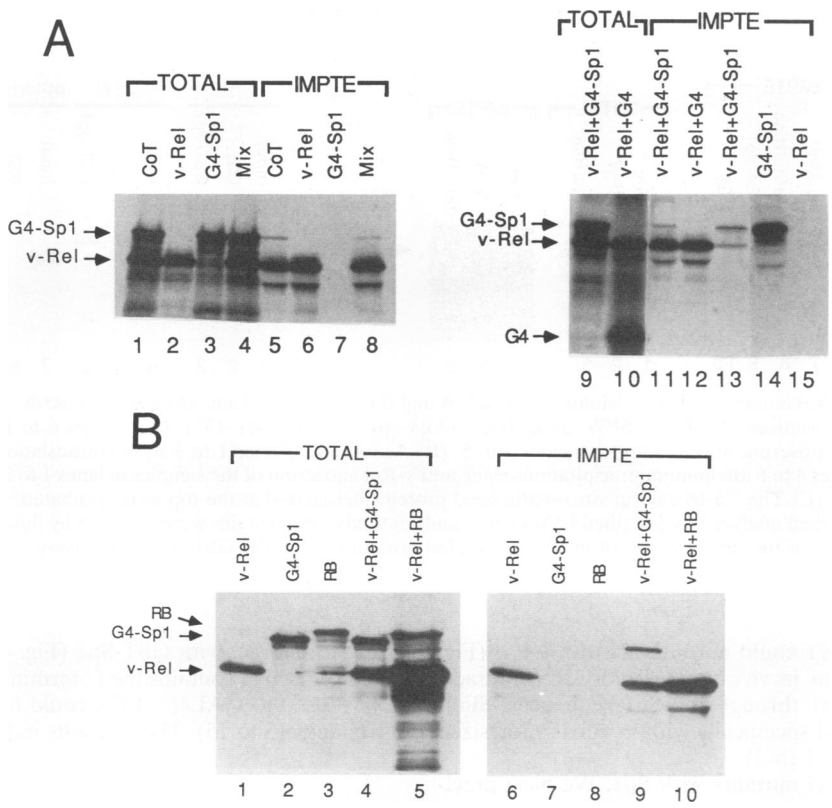

FIG. 1. v-Rel and GAL4-Sp1 can interact in vitro. Proteins were translated in vitro by using the Promega TNT coupled wheat germ system (TOTAL) as described in Materials and Methods. Where indicated (IMPTE), proteins were immunoprecipitated with anti-v-Rel or anti-Sp1 antiserum. Relevant bands are indicated. (A) Lanes: 1 to 4, 1, cotranslated (CoT) v-Rel plus GAL4-Sp1 (G4-Sp1); 2, v-Rel alone; 3, GAL4-Sp1 alone; 4, posttranslationally mixed (Mix) v-Rel and GAL4-Sp1; 5 to 8, immunoprecipitations using anti-v-Rel antiserum of the samples shown in lanes 1 to 4; 9 and 10, cotranslated v-Rel plus GAL4-Sp1 and v-Rel plus GAL4(1–147) (G4), respectively; 11 and 12, immunoprecipitations of lanes 9 and 10 using anti-v-Rel antiserum; 13 to 15, immunoprecipitations using anti-Sp1 antiserum of cotranslated v-Rel plus GAL4-Sp1 (lane 13), GAL4-Sp1 alone (lane 14), and v-Rel alone (lane 15). (B) Lanes: 1, v-Rel; 2, GAL4-Sp1; 3, chicken RB; 4, cotranslated v-Rel plus GAL4-Sp1; 5, cotranslated v-Rel plus RB; 6 to 10, immunoprecipitations using anti-v-Rel antiserum of the samples shown in lanes 1 to 5.

To exclude the possibility that v-Rel was interacting with the GAL4(1–147) portion of the GAL4-Sp1 fusion protein, we immunoprecipitated cotranslated v-Rel and GAL4(1–147) with the anti-v-Rel antiserum. GAL4(1–147) did not coimmunoprecipitate with v-Rel (Fig. 1A, lane 12). Similarly, when v-Rel was cotranslated with chicken RB and the reaction was subjected to immunoprecipitation with the anti-v-Rel antiserum, RB was not detected in the immunoprecipitate (Fig. 1B, lane 10). These results indicate that v-Rel interacts with GAL4-Sp1 through Sp1 sequences that include the transactivation domain.

Interaction of in vivo- and in vitro-synthesized v-Rel with bacterially expressed Sp1. To confirm the results obtained with the in vitro-translated proteins, we tested the interaction of v-Rel with bacterially expressed Sp1. Sequences encoding aa 83 to 621 of Sp1 were fused to the GST coding sequence, expressed in bacteria, and purified by using glutathione-agarose cross-linked beads (Fig. 2, lane 1). First, we determined whether bacterially expressed GST-Sp1 was capable of interacting with in vivo-synthesized v-Rel. GST-Sp1 was incubated with whole cell lysates from v-Rel-transformed chicken spleen cells and processed as described in Materials and Methods. After washing, proteins retained on the beads were analyzed by Western blotting with the anti-v-Rel antiserum. Under these conditions, in vivo-synthesized v-Rel was able to interact specifically with GST-Sp1 (Fig. 2, lane 4). To show that this interaction was occurring through Sp1 sequences, an approximately fivefold molar excess of bacterially expressed GST was incubated with the same amount of whole cell lysate from v-Rel-transformed chicken spleen cells and analyzed as de-

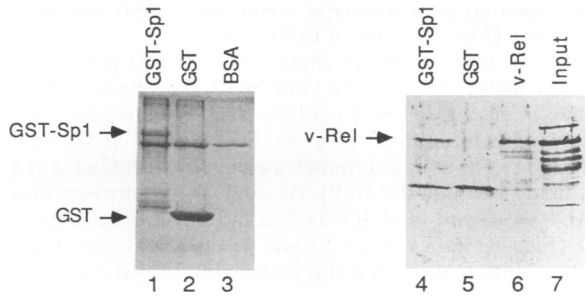

FIG. 2. Bacterially expressed Sp1 can interact specifically with in vivo-synthesized v-Rel. Lanes 1 to 3 show a Coomassie blue-stained gel of GST-Sp1 (lane 1), GST (lane 2), and 1 μg of BSA (lane 3). Lanes 4 to 8 show a Western blot using anti-v-Rel antiserum of the following: GST pull-down reactions of whole cell lysates from v-Rel-transformed chicken spleen cells using GST-Sp1 (lane 4) or GST (lane 5); 2 μl of in vitro-translated v-Rel (lane 6); and 25% of the total amount of whole cell lysate from v-Rel-transformed chicken spleen cells used in the GST pull-down (lane 7). Relevant bands are indicated.

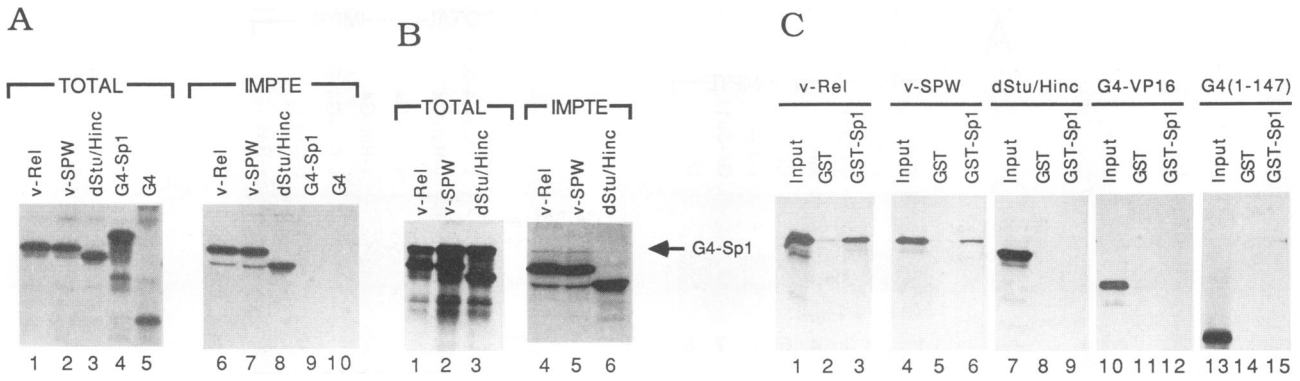

FIG. 3. Interaction of v-Rel mutants with Sp1. Samples in panels A and B were prepared and analyzed as described for Fig. 1. (A) As indicated, lanes 1 to 5 are in vitro translations of v-Rel, v-SPW, dStu/Hinc, GAL4-Sp1, and GAL4(1–147) (G4). Lanes 6 to 10 are immunoprecipitations (IMPTE) using anti-v-Rel antiserum of the samples in lanes 1 to 5. (B) As indicated, lanes 1 to 3 show cotranslations of GAL4-Sp1 with v-Rel, v-SPW, and dStu/Hinc. Lanes 4 to 6 are immunoprecipitations using anti-v-Rel antiserum of the samples in lanes 1 to 3. Samples in this experiment were washed with buffer B. (C) The ^{35}S-labeled in vitro-synthesized proteins designated at the top were incubated with either GST or GST-Sp1 as indicated. Samples were then analyzed as described in Materials and Methods, and proteins were detected by fluorography. The input lane for each protein represents 33% of the total amount of in vitro-translated protein used in the GST pull-down assay.

scribed for GST-Sp1. GST could not interact with v-Rel (Fig. 2, lane 5), indicating that in vivo-synthesized v-Rel interacts specifically with GST-Sp1 through the Sp1 sequences. Similarly, GST-Sp1 interacted specifically with in vitro-synthesized v-Rel (see Fig. 3C, lanes 1 to 3).

Interaction of two v-Rel mutants with Sp1. We have previously analyzed the effect of two nontransforming v-Rel mutants on transcriptional activation by cellular Sp1 and GAL4-Sp1 (45). In those experiments, v-SPW, which has a 2-aa insertion in the RH domain, still increased transactivation by Sp1, whereas dStu/Hinc, which has a 58-aa deletion at the C terminus of the RH domain, did not stimulate the transactivation function of Sp1 (45). Figure 3A shows that wild-type v-Rel and the two v-Rel mutants can be immunoprecipitated equally well with the anti-v-Rel antiserum and that GAL4-Sp1 and GAL4(1–147) are not recognized by this antiserum. To determine whether the interaction of v-Rel with GAL4-Sp1 correlates with its ability to increase transactivation by Sp1, we tested v-Rel mutants v-SPW and dStu/Hinc for the ability to form a complex with GAL4-Sp1. v-SPW and dStu/Hinc were each cotranslated with GAL4-Sp1 and immunoprecipitated with the anti-v-Rel antiserum. In this assay, GAL4-Sp1 was coprecipitated with wild-type v-Rel and v-SPW but not with dStu/Hinc (Fig. 3B, lanes 4 to 6).

We also tested whether these v-Rel mutant proteins could interact with Sp1 using the GST-Sp1 fusion protein. In these assays, in vitro-translated v-SPW and dStu/Hinc proteins were incubated with either GST or GST-Sp1. v-SPW, but not dStu/Hinc, was able to interact specifically with GST-Sp1 (Fig. 3C, lanes 4 to 6 and 7 to 9). As controls, in vitro-synthesized GAL4-VP16 and GAL4(1–147) could not interact with GST-Sp1 (Fig. 3C, lanes 10 to 12 and 13 to 15). Taken together, these results suggest that the interaction of v-Rel and v-SPW with Sp1 is specific and that the interactions of v-Rel mutants with Sp1 correlate with their abilities to increase transactivation by Sp1.

Definition of sequences in v-Rel important for interaction with Sp1. We next sought to define the v-Rel sequences that are important for interaction with Sp1. N- and C-terminally truncated forms of v-Rel were expressed in vitro and tested for the ability to interact with GST-Sp1. v-Rel/*Hinc*II, which contains the entire RH domain (aa 1 to 331) (Fig. 4A), was able to interact with GST-Sp1 (Fig. 4B, lanes 1 to 3). GAL4–3′v-Rel, which contains the C-terminal half of v-Rel (aa 273 to 503) fused to GAL4(1–147), could not interact with Sp1 (Fig. 4B, lanes 13 to 15). These results indicated that the sequences

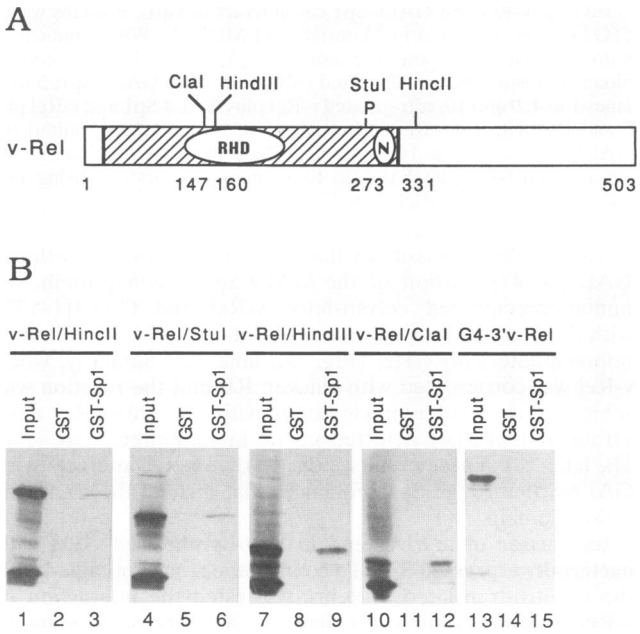

FIG. 4. Definition of sequences in v-Rel important for interaction with Sp1. (A) Schematic representation of v-Rel. Diagonal hatching represents the RH domain (RHD), which contains the nuclear localization sequence (N), and a protein kinase A consensus phosphorylation site (P). Numbers denote amino acid positions defined by unique restriction enzyme cleavage sites used to generate N- and C-terminally truncated forms of v-Rel. (B) GST pull-down experiments of in vitro-translated v-Rel/*Hinc*II, v-Rel/*Stu*I, v-Rel/*Hin*dIII, v-Rel/*Cla*I, and GAL4 (G4)–3′v-Rel, using either GST or GST-Sp1 as indicated. As described in the legend to Fig. 3, approximately 33% of the total amount of in vitro-translated protein used in the GST pull-down experiments is shown in the input lane.

important for interaction with Sp1 are located within the N-terminal half of v-Rel. Therefore, additional C-terminal truncations of v-Rel were generated and tested for interaction with GST-Sp1. v-Rel mutant proteins containing as little as the first 147 aa were able to interact with GST-Sp1 (Fig. 4B, lanes 4 to 12), suggesting that the v-Rel sequences mediating the interaction with Sp1 are located within the first 147 aa.

Sequences in the RH domain and C-terminal sequences of v-Rel are involved in enhancing transactivation by Sp1. v-Rel contains sequences C terminal to the RH domain that can activate transcription (42, 43) and interact with the general transcription factors TBP and TFIIB (55). Therefore, we wanted to determine whether the sequences in the C-terminal half of v-Rel were involved in enhancing transcriptional activation by Sp1. A retroviral plasmid (dH172) expressing v-Rel sequences up to the *Hin*cII site (aa 1 to 331) (Fig. 4A) was cotransfected into CEF with reporter plasmid tkCAT9 containing four Sp1-binding sites. In this experiment, the dH172 v-Rel protein, which contains the entire RH domain but lacks the C-terminal sequences of v-Rel needed for interaction with TBP and TFIIB, still increased expression from the reporter plasmid tkCAT9, although less efficiently than full-length v-Rel (Fig. 5A). This finding demonstrates that sequences in the C-terminal half of v-Rel are partly involved in the induction of expression from reporter plasmids containing Sp1-binding sites.

To determine whether a heterologous transcriptional activation domain could substitute for the C-terminal activation domain of v-Rel in enhancing transcriptional activation by Sp1, we tested the ability of the chimeric protein 5′v-Rel-GAD, which contains the RH domain of v-Rel (aa 1 to 331) fused to the GAL4 activation domain (aa 753 to 881), to enhance transcriptional activation by GAL4-Sp1. CEF were cotransfected with a GAL4 site-containing reporter plasmid (G5BCAT), the GAL4-Sp1 expression plasmid, and retroviral plasmids for the expression of full-length v-Rel (GM282), 5′v-Rel (aa 1 to 331) (dH172) and 5′v-Rel-GAD (JD5′V-GAD). Similar to our previous results (45), full-length v-Rel increased transactivation by GAL4-Sp1 approximately 16-fold (Fig. 5B). When the C-terminal sequences of v-Rel were removed (mutant dH172), transactivation by GAL4-Sp1 was increased only eightfold, suggesting that the C-terminal sequences of v-Rel are partially involved in increasing transactivation by GAL4-Sp1. When sequences in the C-terminal half of v-Rel were replaced with the GAL4 activation domain (JD5′V-GAD), transactivation by GAL4-Sp1 was increased to a greater extent than with wild-type v-Rel (22-fold). Therefore, it appears that the extent of activation varies with the type of activation domain present in the C-terminal half of v-Rel. That is, transactivation by GAL4-Sp1 is enhanced less strongly by dH172 lacking the C-terminal transactivation domain than by full-length v-Rel; however, transactivation by GAL4-Sp1 is enhanced more strongly when the C-terminal activation domain of v-Rel is replaced by the activation domain of GAL4. These in vivo results suggest that v-Rel increases expression from the Sp1 site-containing promoters by functionally interacting with Sp1 and that sequences in the C-terminal half of v-Rel are partly involved in enhancing transactivation by Sp1.

Interaction of other Rel family proteins with Sp1. Rel proteins bind DNA as homo- or heterodimers through sequences in their RH domains, which are common among all Rel transcription factors. Because our deletion analysis revealed that the v-Rel sequences important for the interaction with Sp1 are located within the N-terminal 147 aa of v-Rel, we wanted to determine whether other Rel proteins could also interact with Sp1. In vitro-translated chicken c-Rel, human

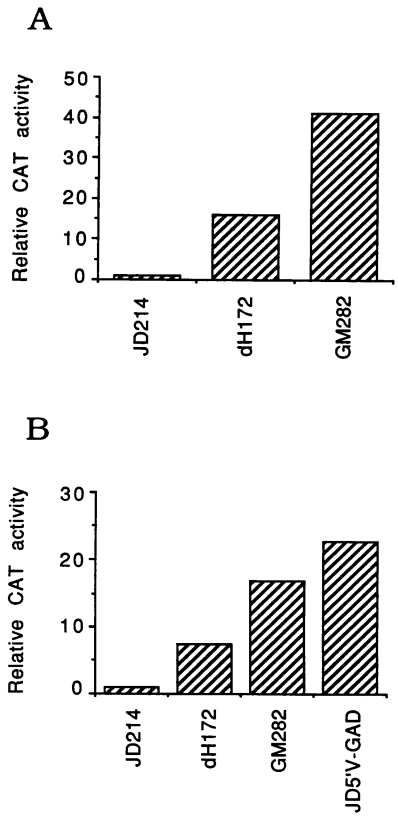

FIG. 5. The C-terminal sequences of v-Rel are involved in enhancing transactivation by Sp1. (A) CEF were cotransfected with 5 μg of Sp1 site-containing reporter plasmid tkCAT9 and 10 μg of the indicated retroviral plasmids for the expression of no protein (JD214), v-Rel aa 1 to 331 (dH172), or wild-type v-Rel (GM282). CAT assays were performed as described in Materials and Methods, and CAT activity is expressed relative to the activity (1.0) seen with the reporter plasmid plus control plasmid JD214. (B) CEF were cotransfected with 5 μg of a reporter plasmid G5BCAT (which contains five GAL4 DNA-binding sites), 1 μg of GAL4-Sp1 expression plasmid JDG+ Sp1N, and 10 μg of retroviral plasmid JD214, dH172, GM282, or JD5′V-GAD. CAT activity was determined as described for panel A and is relative to the activity (1.0) seen with control plasmid JD214.

RelA (p65), chicken NF-κB p52, and chicken NF-κB p50 were tested for the ability to complex with Sp1 using bacterially expressed GST-Sp1. All four Rel proteins were able to interact specifically with GST-Sp1 (Fig. 6). Although different percentages of the various Rel proteins interacted with GST-Sp1 in this assay, it is not clear whether these differences reflect true differences in their affinities for Sp1; for example, it is not known what fraction of each in vitro-translated Rel protein is competent to bind to GST-Sp1. Nevertheless, these results demonstrate that interaction with Sp1 is a common feature of many Rel proteins.

DISCUSSION

In this report, we show that v-Rel can interact with sequences in the cellular transcription factor Sp1 that are known to be important for transcriptional activation. We showed previously that v-Rel can increase expression from reporter plasmids containing upstream *cis*-acting elements known to bind Sp1 by enhancing the transcriptional activation potential of Sp1 (45). Taken together, our results suggest that v-Rel

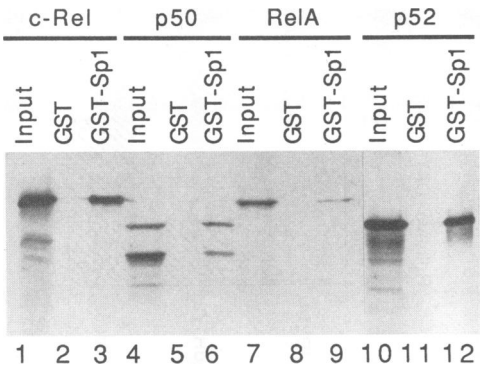

FIG. 6. Many Rel/NF-κB proteins can interact with Sp1. c-Rel, NF-κB p50, RelA, and NF-κB p52 were translated in vitro and incubated with either GST or GST-Sp1 as indicated. Samples were then analyzed as described for Fig. 3C. The input lane for all samples represents 33% of the total amount of in vitro-translated protein used.

increases expression from Sp1 site-containing promoters by functionally interacting with Sp1.

The analysis of v-Rel mutants suggests that interaction with Sp1 is necessary for v-Rel proteins to increase transcriptional activation by Sp1 and that the sequences necessary for interaction with Sp1 are located within the first 147 aa of v-Rel. Two mutants, v-SPW and dH172 (v-Rel aa 1 to 331), that can activate transcription from Sp1 site-containing promoters in vivo also interacted with Sp1 in vitro. However, the v-Rel mutant dStu/Hinc, which does not increase expression from Sp1 site-containing promoters, did not interact with Sp1. As mentioned above, dStu/Hinc has a 58-aa deletion near the C terminus of the RH domain (Fig. 4A). The first 147 aa of v-Rel are still present in dStu/Hinc, and yet it is unable to interact with Sp1; this finding suggests that the C-terminal sequences in dStu/Hinc, which do not interact with GST-Sp1 (Fig. 4B), are interfering with its interaction with Sp1. It is possible that the internal deletion in dStu/Hinc causes a conformational change that affects its ability to interact with Sp1.

The mutations in v-SPW and dStu/Hinc render both proteins unable to form homodimers, bind DNA, or transform chicken spleen cells (Table 1). However, v-SPW can still form heterodimers with NF-κB p105, but dStu/Hinc cannot (4). Although interactions of wild-type and mutant forms of v-Rel with Sp1 correlate with their abilities to form heterodimers and to increase transcriptional activation by Sp1, there is no correlation between the transforming potentials of v-Rel, v-SPW, dH172 (v-Rel aa 1 to 331), and dStu/Hinc and their abilities to interact with Sp1 (Table 1). In light of these results, we conclude that interaction of v-Rel with Sp1 is not sufficient for transformation by v-Rel. However, modulation of the activity of cellular transcription factors such as Sp1 might be part of the mechanism by which v-Rel alters normal cellular growth.

We have also shown that c-Rel, RelA, NF-κB p50, and NF-κB p52 are able to interact with Sp1 in vitro. This finding indicates that interaction with Sp1 is conserved among many Rel proteins. Our results are consistent with those of Perkins et al. (38a, 39), who showed that interaction between the NF-κB subunit RelA and Sp1 is required for the inducible activation of the human immunodeficiency virus type 1 promoter. However, in their studies, the interaction between RelA and Sp1 occurs while both proteins are bound to their cognate DNA sites. In addition, Rel/NF-κB proteins have also been shown to be involved in the induction of Sp1 activity by phorbol esters (50) and oligonucleotides (38). Therefore, we believe that while interaction with Sp1 might be conserved among the different members of the Rel/NF-κB family of transcription factors, it may not always require DNA binding by both proteins. For example, v-Rel appears to increase the transactivation potential of Sp1 without binding DNA (45).

Transcriptional activation by Sp1 appears to require at least one TAF, TAF110, which functions as a coactivator of Sp1 (21, 40, 54). In the presence of TAF110, Sp1 can increase the levels of transcription from an Sp1 site-containing promoter approximately 15-fold in *Drosophila* Schneider cells (21). Furthermore, there appears to be a correlation between the strength of the interaction of Sp1 with TAF110 and transcriptional activation (21). We have shown previously that v-Rel can increase expression from Sp1 site-containing promoters in a dose-dependent fashion (45). In addition, when v-Rel was cotransfected with GAL4-Sp1 in CEF, we detected a 10- to 15-fold increase in expression from a reporter gene that is dependent on GAL4 DNA-binding sites (45) (Fig. 5B). Like TAF110, the presence of v-Rel appears to overcome a limiting step during transcriptional activation by Sp1.

v-Rel might be acting as a positive regulator of Sp1 in one of several ways. An interaction between v-Rel and Sp1 could displace inhibitors of v-Rel or Sp1. We consider this unlikely for the following reasons. First, the sequences in v-Rel required for interaction with Sp1 and with IκB-α appear to be different; i.e., two v-Rel mutants (mutants 2721 and 3069 [31]) that do not bind IκB-α (47) still bind Sp1. Second, displacement of an inhibitor of Sp1 by RB increases DNA binding by Sp1 (6); however, v-Rel-expressing CEF do not show increased DNA binding by Sp1 (45). Alternatively, v-Rel could be increasing the concentration of Sp1 near the promoter region by nucleating the assembly of Sp1 multimers. Although Sp1 can bind DNA as a monomer, it is known to form multimers that can activate transcription more strongly than Sp1 monomers (37). Finally, v-Rel could be increasing activation by Sp1 by bridging an interaction between Sp1 and some component

TABLE 1. Properties of wild-type and mutant v-Rel proteins

Protein	Transforming ability[a]	Dimerization and DNA binding[b]	Heterodimers with p50/p105[c]	Interaction with Sp1[d]	Activation of Sp1[e]
v-Rel	+	+	+	+	+
v-SPW	−	−	+	+	+
dStu/Hinc	−	−	−	−	−
dH172 (v-Rel aa 1–331)	−	+	+	+	+

[a] Ability to transform chicken spleen cells in vitro (18, 36, 43).
[b] Ability to form homodimers and bind DNA in vitro (35, 43).
[c] Ability to form heterodimers with NF-κB p50/p105 (4, 30).
[d] Ability to interact with GST-Sp1 in vitro (Fig. 3 and 4).
[e] Ability to activate transcription from Sp1 site-containing promoters in cotransfection assays (reference 45 and Fig. 4C).

of the basal transcription complex. Consistent with the latter possibility, Kamens et al. (26) have shown that sequences in the v-Rel RH domain can activate transcription in yeast cells, and Xu et al. (55) have shown that v-Rel can interact with TBP and TFIIB. In addition, we have been able to show that the C-terminal sequences of v-Rel are partly involved in transactivation by Sp1. Therefore, it seems reasonable to think that v-Rel could be mediating an interaction between the transactivation domain of Sp1 and some component(s) of the general transcription complex. However, we and others (38a, 39, 47) have been unable to isolate a Rel-Sp1 complex from cells, suggesting that this complex may be unstable under most commonly used lysis procedures.

In summary, Sp1 appears to be one of a number of cellular and viral transcription factors that can functionally interact with Rel proteins. This list also includes the high-mobility group I (Y) protein (9), cyclic AMP-independent ATF family members (9, 28), Fos/Jun (48), C/EBP family members (49), the general transcription factors TBP and TFIIB (29, 55), the human T-cell leukemia virus type I-encoded Tax protein (3, 51, 53), and the bZIP transactivator of Epstein-Barr virus, BZLF1 (19). Future work will be directed toward mapping the Sp1 sequences important for interaction with Rel proteins. In addition, we are in the process of looking for cellular genes whose expression might be increased by v-Rel through interaction with cellular Sp1 in v-Rel-transformed chicken spleen cells.

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