MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 5867–5877
0270-7306/91/125867-11$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 11, No. 12

A Protein Kinase-A Recognition Sequence Is Structurally Linked to Transformation by p59$^{v\text{-}rel}$ and Cytoplasmic Retention of p68$^{c\text{-}rel}$

GEORGE MOSIALOS,[1] PETER HAMER,[2] ANTHONY J. CAPOBIANCO,[2]
RICHARD A. LAURSEN,[1] AND THOMAS D. GILMORE[2]*

Departments of Chemistry[1] and Biology,[2] Boston University, Boston, Massachusetts 02215

Received 20 June 1991/Accepted 24 August 1991

The Rel family of proteins includes a number of proteins involved in transcriptional control, such as the retroviral oncoprotein v-Rel, c-Rel, the *Drosophila melanogaster* developmental protein Dorsal, and subunits of the transcription factor NF-κB. These proteins are related through a highly conserved domain of approximately 300 amino acids, called the Rel homology domain, that contains dimerization, DNA binding, and nuclear targeting functions. Also within the Rel homology domain, there is a conserved consensus sequence (Arg-Arg-Pro-Ser) for phosphorylation by cyclic AMP-dependent protein kinase (PKA). We used linker insertion mutagenesis and site-directed mutagenesis to determine the importance of this sequence for the transformation of avian spleen cells by v-Rel and the subcellular localization of c-Rel in chicken embryo fibroblasts (CEF). The insertion of 2 amino acids (Pro-Trp) within this sequence completely abolished transformation and transcriptional repression by v-Rel and resulted in a shift in the localization of c-Rel from cytoplasmic to nuclear in CEF. When the conserved Ser within the PKA recognition sequence was replaced by Ala, there was no significant effect on transformation and transcriptional repression by v-Rel or on cytoplasmic retention of c-Rel. However, when this Ser was changed to Asp or Glu, transformation and transcriptional repression by v-Rel were significantly inhibited and c-Rel showed a diffuse nuclear and cytoplasmic localization in CEF. Although a peptide containing the recognition sequence from v-Rel can be phosphorylated by PKA in vitro, this site is not constitutively phosphorylated to a high degree in vivo in transformed spleen cells incubated with okadaic acid. Our results indicate that the transforming and transcriptional repressing activities of v-Rel and the cytoplasmic retention of c-Rel are dependent on the structure of the conserved PKA recognition motif. In addition, they suggest that phosphorylation at the conserved PKA site could have a negative effect on transformation and transcriptional repression by v-Rel and induce the nuclear localization of c-Rel.

The v-*rel* oncogene of the highly oncogenic avian retrovirus Rev-T specifically transforms cells of myeloid and lymphoid lineages both in vivo and in vitro (4). v-*rel* encodes a phosphoprotein (p59$^{v\text{-}rel}$ or v-Rel) that is located in the nucleus of most cells (reviewed in reference 15).

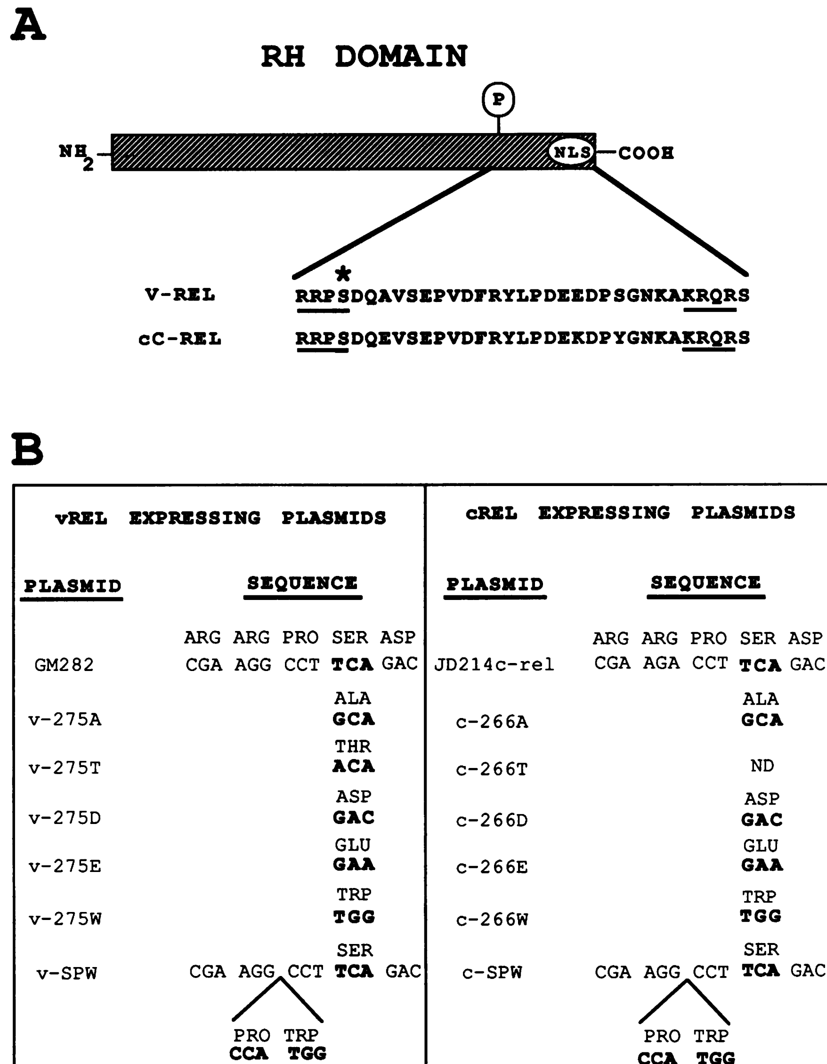
v-Rel is a mutated and truncated form of the avian proto-oncogene-encoded protein p68$^{c\text{-}rel}$ (c-Rel). v-Rel is missing 2 N-terminal and 118 C-terminal amino acids as compared with c-Rel, and there are a number of internal differences between the two proteins (7, 20, 45). In contrast to v-Rel, c-Rel is located in the cytoplasm of most cells (7, 39). The deletion of the 118 C-terminal c-Rel amino acids in v-Rel is known to remove two functional domains. First, it apparently removes a cytoplasmic retention sequence, since a c-Rel protein with a deletion of C-terminal amino acids enters the nucleus of chicken embryo fibroblasts (CEF) as efficiently as does v-Rel (7). Second, most or all of a gene activation domain is removed from c-Rel by the deletion of the C-terminal amino acids in v-Rel (23, 33). v-Rel, therefore, can function as a repressor of transcription from promoters whose activity is dependent on Rel-related transcriptional activating proteins (3, 33; see below).

In addition to v-Rel and c-Rel, the Rel family of proteins includes a number of proteins known to act as regulators of transcription. These include the *Drosophila melanogaster* developmental morphogen Dorsal, both subunits (p50 and p65) of the NF-κB transcription complex, and probably additional proteins that can associate with p50 and p65 of NF-κB (reviewed in reference 14). All members of this family appear to be regulated, at least in part, by subcellular localization. That is, an active form of the protein is located in the nucleus of cells, and the protein is sequestered in an inactive form in the cytoplasm. For example, NF-κB is sequestered in the cytoplasm through an interaction with an inhibitor protein, IκB, and is translocated from the cytoplasm to the nucleus in various cell types upon receipt of an appropriate signal (reviewed in reference 2). Activation of protein kinase C or protein kinase A (PKA) can dissociate IκB from NF-κB and allow the p50-p65 complex to enter the nucleus. Protein kinase C appears to phosphorylate IκB; however, PKA (cyclic AMP-dependent protein kinase) does not (12).

Rel proteins are similar in an approximately 300-amino-acid highly conserved domain which is located towards the N terminus and which is called the Rel homology (RH) domain (Fig. 1A). The C-terminal halves of Rel proteins are generally unrelated but frequently contain sequences important for gene activation (6, 23, 33). The RH domain contains overlapping DNA binding and dimerization functions, at least some of which are contained within the C-terminal 33 amino acids of the RH domain (13, 27, 30, 32). All Rel proteins also contain a conserved nuclear localizing signal located at the extreme C terminus of the RH domain (7, 17).

In addition, Rel proteins have a sequence that corresponds to a consensus site for serine phosphorylation by PKA (26). This sequence, Arg-Arg-Pro-Ser, is conserved in all Rel proteins (with the exception of a recently described NF-κB–p50-related protein [38]). The Ser of this consensus sequence is located precisely 24 amino acids N terminal to the core of

* Corresponding author.



vREL EXPRESSING PLASMIDS		cREL EXPRESSING PLASMIDS	
PLASMID	SEQUENCE	PLASMID	SEQUENCE
	ARG ARG PRO SER ASP		ARG ARG PRO SER ASP
GM282	CGA AGG CCT **TCA** GAC	JD214c-rel	CGA AGA CCT **TCA** GAC
v-275A	ALA **GCA**	c-266A	ALA **GCA**
v-275T	THR **ACA**	c-266T	ND
v-275D	ASP **GAC**	c-266D	ASP **GAC**
v-275E	GLU **GAA**	c-266E	GLU **GAA**
v-275W	TRP **TGG**	c-266W	TRP **TGG**
v-SPW	CGA AGG CCT SER **TCA** GAC; insertion PRO TRP **CCA TGG**	c-SPW	CGA AGG CCT SER **TCA** GAC; insertion PRO TRP **CCA TGG**

FIG. 1. Structures and sequences within the RH domain. (A) The RH domain contains sequences important for oligomerization, DNA binding, inhibitor binding, and nuclear localization (see references 14 and 15 for reviews). The relative positions of the consensus site for phosphorylation by PKA (P) and of the nuclear localizing signal (NLS) are shown. The C-terminal 33 amino acids of the RH domain from v-Rel (V-REL) (41, 45) and chicken c-Rel (cC-REL) (7) have been aligned for comparison. The PKA recognition sequence and the core sequence of the NLS are underlined. The asterisk shows the position of the Ser that falls within the consensus recognition sequence for PKA. NH_2 and COOH denote the variable N- and C-terminal sequences in Rel proteins. (B) Mutations created within the PKA recognition motif in v-Rel and chicken c-Rel. The v-Rel- and c-Rel-producing plasmids are shown, along with the corresponding nucleotide and amino acid sequences at the PKA recognition sequence. The Ser codon corresponds to amino acids 275 and 266 in v-Rel and chicken c-Rel, respectively. The relevant codon changes for each plasmid are shown in boldface type and were created by site-directed mutagenesis as described in Materials and Methods. ND, not done.

basic residues of the nuclear localizing sequence in all Rel proteins. The conservation of the sequence and spacing of these two sequences suggested that modification of the Ser residue could affect the subcellular localization of Rel proteins.

The Ser residue in this consensus sequence corresponds to amino acid 275 in v-Rel and amino acid 266 in chicken c-Rel. It was previously shown, by deletion analysis, that the region of the v-Rel oncoprotein between amino acids 274 and 323 (which includes the PKA recognition sequence) is important for transformation, DNA binding, and transcriptional repression of promoters containing NF-κB sites (3, 17, 33). Moreover, the insertion of 4 or 15 amino acids between amino acids 273 and 274 (between the Arg and Pro of the consensus PKA recognition sequence) of v-Rel completely abolishes the transforming potential of v-Rel (11, 17). However, as we previously reported (23), a mutation that changed Ser-275 of v-Rel to Ala did not affect transformation by v-Rel or gene activation in *Saccharomyces cerevisiae*.

We have now systematically determined the effect of linker insertion mutagenesis and site-directed mutagenesis within the PKA recognition sequence on transformation and transcriptional repression by v-Rel and on the subcellular localization of avian c-Rel. Our results demonstrate that disruption of the consensus PKA recognition sequence by a 2-amino-acid insertion completely abolishes transformation of avian spleen cells and transcriptional repression by v-Rel and results in exclusively nuclear localization of c-Rel in

CEF. In addition, when the potential site of serine phosphorylation within this sequence is replaced by acidic amino acids, transformation and transcriptional repression by v-Rel are inhibited and c-Rel shows an altered subcellular localization. These results suggest that phosphorylation at the conserved PKA recognition site may affect both transformation by v-Rel and the cytoplasmic retention of c-Rel.

MATERIALS AND METHODS

Plasmids. All recombinant DNA techniques were performed conventionally (37). DNA sequencing was done by dideoxy sequencing of single-stranded or double-stranded DNA (37).

JD214 is a spleen necrosis virus vector designed for the expression of exogenous genes in avian cells (10). The construction of spleen necrosis virus vectors expressing wild-type v-Rel (GM282) and c-Rel (JD214c-rel) has been described previously (7).

Site-directed mutagenesis at the conserved Ser codon in c-*rel* and v-*rel* was performed by the Kunkel method (29) with oligonucleotides that contained the mutated bases and approximately 10 bases on each side. The oligonucleotides used for site-directed mutagenesis were synthesized on a MilliGen/Biosearch 7500 DNA synthesizer. For mutagenesis of v-*rel*, an *Xba*I-*Hinc*II fragment containing the 5′ half of v-*rel* (from plasmid TG7-SH, a plasmid containing full-length v-*rel* subcloned into the *Xba*I site of pUC12 [17]) was subcloned into M13mp18. After mutagenesis, a *Cla*I-*Hinc*II fragment of v-*rel* was used to replace the corresponding fragment of wild-type v-*rel* in TG7-SH. In a subsequent subcloning step, a fragment of TG7-SH containing mutated codon 275 in v-*rel* was used to replace the corresponding fragment in GM282. For point mutations in c-*rel*, a *Hpa*II fragment containing the entire coding domain of a c-*rel* cDNA was blunt ended by treatment with the Klenow fragment and subcloned into the *Sma*I site of M13mp18. Following mutagenesis, a unique *Cla*I-*Bst*XI fragment of c-*rel* derived from M13mp18 was substituted for the corresponding fragment in JD214c-rel. To create the Pro-Trp insertion between amino acids 264 and 265 in c-Rel, we created a *Stu*I site by changing the 3′ adenine to guanine within the Arg-264 codon. v-SPW and c-SPW were created by inserting a 6-bp *Nco*I linker (CCATGG; Pharmacia) at the *Stu*I site in v-*rel* and modified c-*rel*, respectively (v-SPW is called PH11 in reference 33). All mutations were confirmed by DNA sequence analysis of the final retroviral vector plasmid.

v-HPW was created by inserting the *Nco*I linker at the *Hinc*II site that cleaves v-*rel* after codon 331. CVC, CVC-TG18, and CVC-HPW were constructed by substituting the *Eco*RI-*Bst*XI middle v-*rel* fragment, derived from GM282, TG18 (17), and v-HPW, respectively, for the corresponding fragment in JD214c-rel.

2NFBCO, which contains four NF-κB binding sites positioned about 100 bp upstream of the rabbit β-globin promoter and chloramphenicol acetyltransferase (CAT) gene, has been described previously (33). MSV-βgal was a gift from N. Rosenthal.

Cell culturing and CAT assays. CEF were cultured in Temin's modified Eagle's medium (TMEM) containing 10% fetal bovine serum (7). Transformed spleen cells were maintained in TMEM containing 20% fetal bovine serum. CEF were transfected by the dimethyl sulfoxide-Polybrene method (25).

For CAT assays, approximately 2×10^6 CEF were cotransfected with 1 μg of a reporter plasmid (2NFBCO), 0.5 μg of MSV-βgal (used as an internal control to normalize transfection efficiencies), and 5 μg of a retroviral expression plasmid. Approximately 48 h posttransfection, cells were scraped and lysed by being frozen-thawed five times in 25 mM Tris (pH 7.4). CAT activity in extracts containing equal units of β-galactosidase activity was determined as described previously (18, 33). After chromatography and autoradiography, spots were excised and quantitated by liquid scintillation counting. CAT activity was determined as the percentage of radioactivity in the acetylated form of chloramphenicol as compared with the total radioactivity in the acetylated and nonacetylated forms of chloramphenicol. Values are presented as relative CAT activity, which was determined by comparing the CAT activity for a given sample to the CAT activity seen in a cotransfection of 2NFBCO and control retroviral expression plasmid JD214 (see Table 1, footnote *b*).

Transformation assays. Fresh spleen cells were prepared from 19-day-old SPAFAS (Norwich, Conn.) virus-free chickens as described previously (17, 21). Cells were resuspended at approximately 3×10^7 cells per 0.4 ml of TMEM containing 20% fetal bovine serum in sterile 0.4-cm electroporation cuvettes (Bio-Rad). Plasmid DNA (15 to 20 μg of *rel*-expressing plasmid DNA and 10 μg of pSW253 helper virus DNA) was added directly to the cells, and the cells were incubated on ice for 10 min. Cells were electroporated in a Bio-Rad Gene Pulser apparatus at 250 V and 960 μF. These settings were determined empirically after a number of experiments with different settings. Cells were incubated for an additional 10 min on ice, and the cell suspension was placed in 2 ml of warm TMEM containing 20% fetal calf serum.

Cells were incubated for approximately 72 h (to allow for limited viral replication) at 40.5°C in a standard tissue culture incubator in an atmosphere containing 5 to 6% CO_2. After this time, the cells were pelleted gently, resuspended in 5 ml of TMEM containing 0.3% Bacto-Agar (Difco), 20% fetal bovine serum, and 1% chicken serum, and plated in 60-mm petri dishes. Cells in agar were maintained in a very moist tissue culture CO_2 incubator at 40.5°C, and colonies were counted approximately 10 days later. Generally, samples were assayed in duplicate.

In four independent experiments in which fresh spleen cells were electroporated with various amounts of GM282 DNA and a constant amount (10 μg) of helper virus DNA, the number of v-*rel*-transformed colonies was roughly proportional to the amount of input GM282 DNA up to 20 μg of GM282 DNA. However, higher amounts of DNA (40 μg) inhibited the formation of transformed colonies. In these four experiments, the following average numbers of colonies were obtained: 1 μg of GM282 DNA, 13 colonies; 5 μg, 50 colonies; 10 μg, 114 colonies; 20 μg, 145 colonies; and 40 μg, 70 colonies. Electroporation with either helper virus DNA or GM282 DNA alone never produced any colonies (data not shown).

In our experience, approximately 200 colonies was the greatest number of colonies that could be obtained in this type of assay, presumably because the number of target cells was limiting (13a). With the optimal ratio of 15 to 20 μg of GM282 DNA to 10 μg of pSW253 DNA, we frequently obtained roughly 200 colonies. We never obtained more than 80 colonies with virus stocks generated from cotransfection of CEF.

JD214c-rel transformed only about 2% as efficiently as did GM282 in this assay (Table 1). In previous assays with virus

TABLE 1. Transformation and transcriptional repression by mutant v-Rel proteins and c-Rel

Plasmid	Relative transforming efficiency[a]	Relative CAT activity[b]
JD214	ND[c]	100
GM282	100	18
v-SPW	<0.06[d]	158
v-HPW	98	ND
v-275A	112	25
v-275T	103	ND
v-275W	78	31
v-275D	4.5	55
v-275E	5.9	61
JD214c-rel	1.9[e]	ND

[a] The relative transforming efficiency for each virus is relative to the number of colonies seen with GM282 in a given spleen cell transformation assay performed as described in Materials and Methods. Values are the averages of several independent assays for each plasmid: GM282, 32 assays; v-SPW, 19 assays; v-HPW, 7 assays; v-275A, 12 assays; v-275T, 3 assays; v-275W, 7 assays; v-275D, 7 assays; v-275E, 7 assays; and JD214c-rel, 13 assays.

[b] The relative CAT activity for each plasmid is relative to transcription from 2NFBCO seen with cotransfected JD214, a spleen necrosis virus vector with no inserted gene (10). Values are the averages of independent assays performed as described in Materials and Methods: GM282, 5 assays; v-SPW, 4 assays; v-275A, 4 assays; v-275W, 5 assays; v-275D, 5 assays; and v-275E, 5 assays.

[c] ND, not determined.

[d] No colonies were ever seen with v-SPW.

[e] JD214c-rel-transformed colonies were difficult to propagate in liquid cultures and frequently senesced after several passages. Western blot analysis with anti-Rel antiserum of 11 independently derived JD214c-rel-transformed spleen cell colonies detected two types of colonies: ones that expressed truncated c-Rel proteins of approximately 65 kDa and ones that expressed apparently full-length c-Rel proteins.

stocks from CEF, we had not seen colonies with JD214c-rel (23). A transformed spleen cell clone expressing a full-length c-Rel protein was used in the biochemical assays described in this paper.

Western blotting (immunoblotting) and immunofluorescence. Western blotting and immunofluorescence were performed as previously described (7). CEF were transfected with 5 μg of the replication-defective *rel*-expressing plasmid and 0.1 μg of the replication-competent reticuloendotheliosis virus strain A plasmid pSW253 (44). Six days posttransfection, cells were analyzed by Western blotting or immunofluorescence with a polyclonal anti-Rel antibody as the primary antibody (16).

Immunoprecipitation, tryptic peptide mapping, and phosphoamino acid analysis. Before being labelled, approximately 5×10^6 transformed spleen cells were starved for 1 h in 1 ml of phosphate-free minimum essential medium (GIBCO) containing 5% dialyzed fetal bovine serum. For preparative purposes, cells were radiolabelled for 5 h in the same medium containing 2 mCi of $^{32}P_i$ (28,500 Ci/mmol; ICN Biomedicals, Inc.) per ml. After being radiolabelled, the cells were lysed and immunoprecipitated with anti-Rel antiserum as previously described (16), except that the sodium dodecyl sulfate (SDS) concentration in the lysis buffer was raised to 0.5%. For analytical purposes, cells were labelled with 0.5 mCi of $^{32}P_i$ per ml and samples were normalized according to the number of trichloroacetic acid-precipitable counts in the lysate. For quantitation, bands were excised from nitrocellulose and counted by liquid scintillation counting. When indicated, okadaic acid (Calbiochem) was used at 500 nM in the culture medium and lysis buffer.

Following immunoprecipitation, the samples were analyzed on 7.5% SDS-polyacrylamide gels. The Rel bands were identified by autoradiography and excised, and protein was eluted from the gel and analyzed essentially as described elsewhere (5). After oxidation with performic acid, the protein was digested with 20 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone-trypsin (Sigma) overnight at room temperature, 10 μg of trypsin was then added, and the mixture was incubated for 4 h at 37°C. Phosphotryptic peptides were analyzed in the first dimension by thin-layer electrophoresis at pH 8.9 and in the second dimension by ascending chromatography in *n*-butanol–pyridine–acetic acid–water (75:50:15:60) (5).

Phosphoamino acid analyses of ^{32}P-labelled v-Rel and c-Rel were performed as described elsewhere (9).

In vitro phosphorylation of peptides. Peptides corresponding to v-Rel from positions 271 to 287 were synthesized on a MilliGen/Biosearch Excell peptide synthesizer with fluorenylmethyloxycarbonyl-protected amino acids and activation with benzotriazolyloxy-trisdimethylaminophosphonium hexafluorophosphate. The sequences of the peptides are described in Results.

The purified catalytic subunit of cyclic AMP-dependent protein kinase (Promega) was used for the in vitro phosphorylation of the peptides. In brief, 15 μg of peptide was incubated at 30°C for 30 min in assay buffer (40 mM Tris hydrochloride [pH 7.4], 20 mM magnesium acetate, 0.2 mM ATP) containing 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and 100 casein units of PKA. The labelled peptide was subsequently analyzed or purified by thin-layer electrophoresis at pH 1.9 as described previously (5).

RESULTS

Mutagenesis of the conserved PKA consensus phosphorylation sequence (Arg-Arg-Pro-Ser) in v-Rel and c-Rel. To identify more precisely the structural elements that are functionally important in the PKA recognition sequence of Rel proteins, we performed linker insertion mutagenesis and site-directed mutagenesis in v-*rel* and chicken c-*rel*. Figure 1B shows the mutations that we created. Insertion of a 6-bp *Nco*I linker at a unique *Stu*I site resulted in an in-frame insertion of Pro and Trp codons between the Arg and Pro codons of the PKA recognition sequence, thus creating the sequence Arg-Arg-Pro-Trp-Pro-Ser in v-Rel (v-SPW) or c-Rel (c-SPW). The insertion of 2 amino acids at this position therefore disrupted the consensus phosphorylation sequence, but the overall Arg-Arg-Pro sequence was retained. As a control, an *Nco*I linker was also inserted in-frame between amino acids 331 and 332 of v-Rel (v-HPW), a nearby region that we have previously shown to be insensitive to small alterations (17).

To investigate the role of the conserved, potentially phosphorylated Ser, we made the following substitutions. A Ser-to-Trp change was made in v-Rel and c-Rel to determine the effect of a bulky side chain at this position and compare it with the effect of the Pro-Trp insertion (described above). The importance of phosphorylation at this position was addressed by substituting Ala (a nonphosphorylatable amino acid) for Ser in v-Rel and c-Rel (clones v-275A and c-266A, respectively) or Thr (a phosphorylatable amino acid) for Ser in v-Rel (clone v-275T). In addition, we substituted Asp or Glu for Ser in v-Rel (clones v-275D and v-275E, respectively) and c-Rel (clones c-266D and c-266E, respectively) in an attempt to mimic the presence at this position of a negative charge that could not be removed by a phosphatase.

Effects of mutations within the PKA recognition sequence on

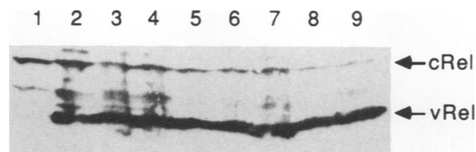


FIG. 2. Western blot of mutant v-Rel proteins. Shown is Western blot analysis of whole-cell lysates from CEF cotransfected with the indicated v-Rel expression plasmids and Rev-A helper virus DNA. Cells were lysed 6 days posttransfection, and equal amounts of total protein were loaded on a 7.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with anti-Rel antiserum as the primary antibody. The positions of endogenous c-Rel and recombinant v-Rel proteins are designated by arrows. Lanes: 1, no plasmid; 2, GM282 (wild-type v-Rel); 3, v-275A; 4, v-275T; 5, v-275D; 6, v-275E; 7, v-275W; 8, v-SPW; 9, v-HPW.

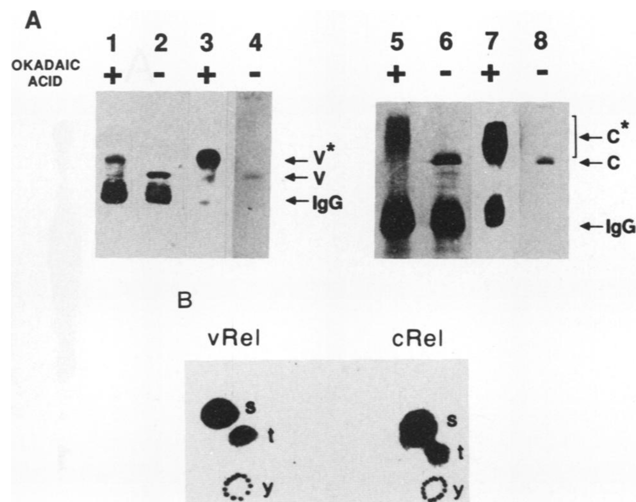


FIG. 3. Effects of okadaic acid on the phosphorylation of v-Rel and c-Rel. (A) Western blot of immunoprecipitated ^{32}P-labelled v-Rel (lanes 1 to 4) and c-Rel (lanes 5 to 8) from okadaic acid-treated (+) and nontreated (−) spleen cells transformed by GM282 (lanes 1 to 4) and JD214c-rel (lanes 5 to 8). Equal numbers of cells were labelled with 0.5 mCi of $^{32}P_i$ per ml in the presence or absence of okadaic acid and lysed, and normalized samples were immunoprecipitated with a limiting amount (5 μl) of anti-Rel antiserum as described in Materials and Methods. Samples were electrophoresed on a 7.5% SDS-polyacrylamide gel. Western blotting with anti-Rel antiserum as the primary antibody and a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was performed to detect the immunoprecipitated Rel protein (lanes 1, 2, 5, and 6). This Western blot was subjected to autoradiography to determine the extent of phosphorylation of Rel protein (lanes 3, 4, 7, and 8). The lower large band in lanes 1, 2, 5, 6, and 7 is the heavy chain of immunoglobulin (immunoglobulin G [IgG]). The positions of v-Rel (V), c-Rel (C), and the hyperphosphorylated forms of these proteins (V* and C*, respectively) are indicated by arrows. Lanes 4 and 8, showing the weakly labelled Rel bands in the absence of okadaic acid, were exposed longer than lanes 3 and 7, showing the strongly labelled Rel bands in the presence of okadaic acid. (B) Phosphoamino acid analysis of immunoprecipitated v-Rel and c-Rel from transformed spleen cells labelled with $^{32}P_i$ in the presence of okadaic acid. Autoradiography was performed with an intensifying screen. The positions of cold markers are indicated as follows: s, phosphoserine; t, phosphothreonine; y, phosphotyrosine. Phosphoserine and phosphothreonine were quantitated by liquid scintillation counting: v-Rel, s = 138 cpm, t = 29 cpm; c-Rel, s = 168 cpm, t = 37 cpm.

transformation and transcriptional repression by v-Rel. To determine the effect of mutations in the PKA recognition sequence on the transforming ability of v-Rel, we electroporated primary chicken spleen cells with plasmids encoding mutant v-Rel proteins in the presence of helper virus DNA. We have found that this method is more convenient, quicker, and more sensitive in detecting weakly transforming *rel* genes than are previous methods that rely on infection of spleen cells with virus stocks generated by transfection of CEF (17). This novel method for assaying transformation by *rel* genes is described in detail in Materials and Methods.

Table 1 shows the results of this in vitro Rel transformation assay. Clone v-SPW, which contains the Pro-Trp insertion within the PKA recognition sequence, was completely nontransforming, whereas clone v-HPW, which contains the Pro-Trp insertion after amino acid 331, was as transforming as was wild-type v-Rel. These results indicate that the Pro-Trp insertion within the PKA recognition sequence completely abolishes the transforming potential of v-Rel and are consistent with previous results obtained when potentially more disruptive deletions and insertions were made at this site (11, 17).

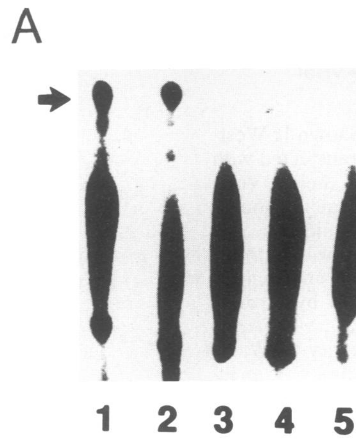
Clones v-275A, v-275T, and v-275W transformed avian spleen cells approximately equally as well as did wild-type v-Rel. The results with v-275A and v-275W demonstrate that phosphorylation at this site is not necessary for transformation by v-Rel; the result with v-275W indicates that the presence of an amino acid with a large hydrophobic side chain in place of Ser-275 has little or no effect on transformation. When Ser-275 was substituted with Asp or Glu (in v-275D or v-275E, respectively), transformation was inhibited by approximately 95%. These results show that the presence of a negative charge at this position inhibits the transforming potential of v-Rel.

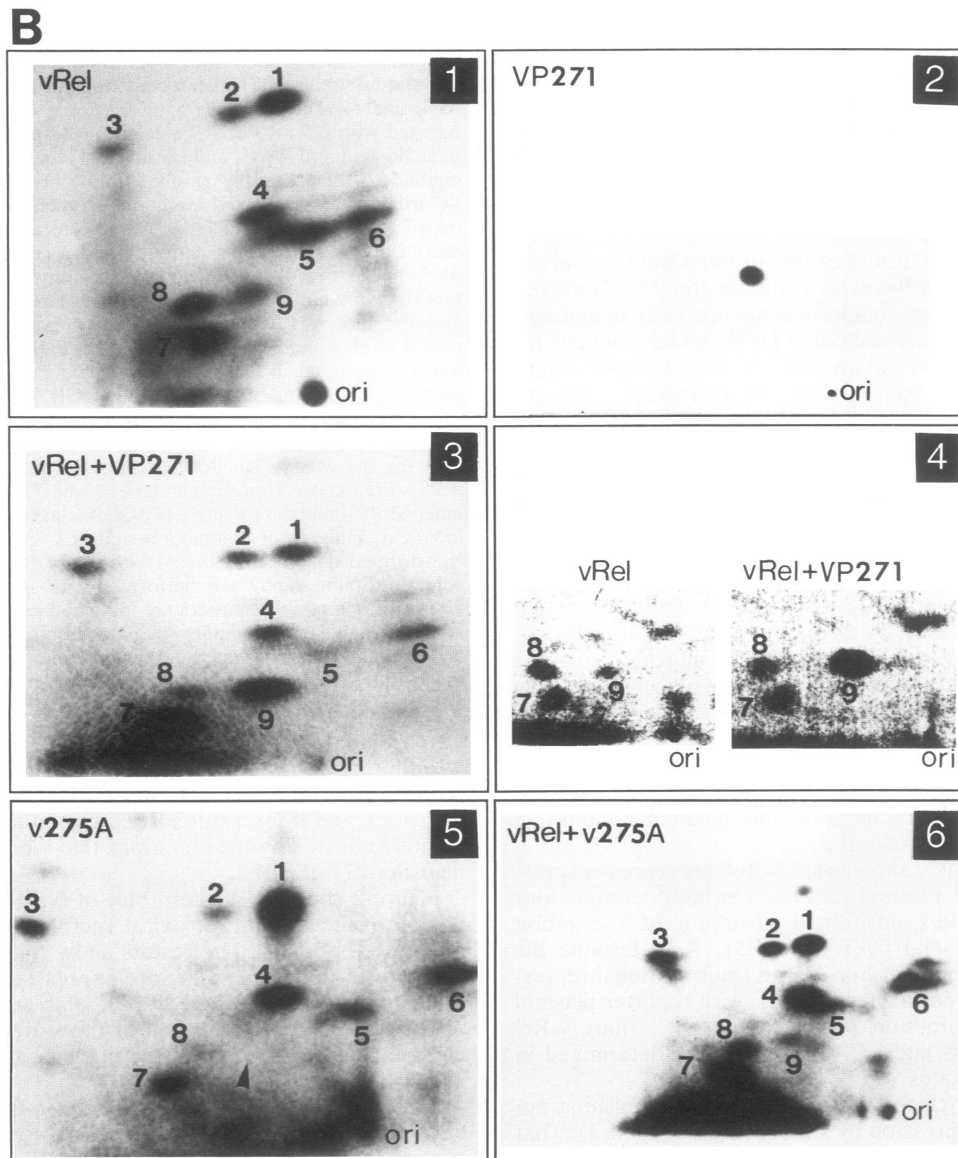
We have previously shown that v-Rel can repress expression from reporter plasmid 2NFBCO, which contains four NF-κB binding sites positioned upstream of the rabbit β-globin promoter and CAT gene (33). To determine the effect of the mutations at Ser-275 on transcriptional repression by v-Rel, we cotransfected CEF with reporter plasmid 2NFBCO and expression plasmids for the various v-Rel mutants. Two days later, CAT activity was determined in normalized cell lysates.

There was a strict correlation between transforming potential and gene repression by v-Rel proteins (Table 1). That is, fully transforming v-Rel proteins (v-Rel, v-275A, and v-275W) strongly repressed expression from 2NFBCO, weakly transforming proteins (v-275D and v-275E) were weakly repressing, and the nontransforming v-SPW protein did not affect expression from 2NFBCO. Therefore, transformation and transcriptional repression appear to be coordinately affected by the mutations that we have created at or near Ser-275 of v-Rel.

Figure 2 shows a Western blot of lysates from CEF that were transfected with retroviral vectors expressing the various v-Rel mutants. The results show that the mutant proteins are of the proper size, are expressed at levels comparable to that of wild-type v-Rel, and are as stable as is wild-type v-Rel. Similarly, all of the v-Rel mutant proteins were exclusively located in the nucleus of CEF (see below and Table 2).

Phosphorylation of v-Rel and chicken c-Rel proteins. It has been previously shown that v-Rel and c-Rel are phosphoproteins (16, 31, 40). However, under standard labelling conditions, very limited amounts of ^{32}P are incorporated into these proteins in vivo.


A
1
2
3
4
5


B
vRel
1
VP271
2
ori
vRel+VP271
3
4
vRel
vRel+VP271
v275A
5
vRel+v275A
6

In an effort to increase the efficiency of labelling of Rel proteins, transformed spleen cells were labelled and lysed in the presence of okadaic acid, a potent inhibitor of phosphatases 1 and 2A (8), prior to immunoprecipitation with anti-Rel antiserum. The amount of phosphate incorporated into v-Rel and c-Rel in spleen cells transformed by these proteins was increased by approximately 15-fold in the presence of okadaic acid (Fig. 3A). In addition, hyperphosphorylation caused a significant retardation in the electrophoretic mobilities of v-Rel and c-Rel on SDS-polyacrylamide gels. v-Rel and c-Rel proteins showed identical mobility shifts in CEF incubated in the presence of okadaic acid (data not shown).

In contrast to v-Rel, c-Rel appears to be converted to a large number of differentially phosphorylated isoforms. That is, c-Rel from transformed spleen cells incubated with okadaic acid runs as a smear of bands ranging in molecular mass from 72 to 85 kDa, while hyperphosphorylated v-Rel runs as a single band at approximately 65 kDa. Since the number of conformational isoforms of c-Rel created upon treatment with okadaic acid is larger than that seen with v-Rel, we believe that it is likely that the additional C-terminal sequences of c-Rel are directly involved in the c-Rel structural alterations that result from hyperphosphorylation.

Figure 3B shows phosphoamino acid analyses of v-Rel and c-Rel labeled in vivo in transformed avian spleen cells in the presence of okadaic acid. Both v-Rel and c-Rel are primarily labelled at serine and threonine residues, with approximately five times as much phosphoserine as phosphothreonine in each protein. A similar ratio of phosphoserine to phosphothreonine was seen in v-Rel labelled in the absence of okadaic acid, even though the overall extent of phosphorylation was greatly reduced (13a, 43).

Characterization of the tryptic peptide containing Ser-275 of v-Rel. To determine whether Ser-275 in v-Rel could be phosphorylated by PKA, we attempted to phosphorylate synthetic peptides corresponding to amino acids 271 to 287 of v-Rel in vitro. The wild-type peptide (VP271) had the following sequence: LRRPSDQAVSEPVDFRY. We also used two peptides that had altered sequences as compared with peptide VP271: one had an Ala at the position corresponding to Ser-275 (VP271/275A), and one had an Ala at the position corresponding to Ser-280 (VP271/280A). These peptides were incubated with radiolabelled ATP and purified PKA, trypsinized, and analyzed by thin-layer electrophoresis and autoradiography. Figure 4A shows that peptides VP271 and VP271/280A were phosphorylated under these conditions but that peptide VP271/275A was not. Phosphoamino acid analysis showed that both VP271 and VP271/280A contained solely phosphoserine (data not shown). Edman degradation of the in vitro-labelled tryptic phosphopeptide indicated that trypsin cleaved between Arg-272 and Arg-273 (data not shown), consistent with findings indicating that Arg-Pro is a poor substrate for trypsin (5). These results demonstrate that Ser-275 lies within a sequence that can be phosphorylated by PKA.

Figure 4B shows two-dimensional tryptic phosphopeptide maps of in vivo-labelled v-Rel from transformed spleen cells labelled with ^{32}P in the presence of okadaic acid, in vitro-labelled VP271, and a mixture of in vitro-labelled VP271 and in vivo-labelled v-Rel from transformed spleen cells. We consistently detected nine tryptic phosphopeptides from in vivo-labelled v-Rel. The tryptic phosphopeptide derived from VP271 comigrated with a relatively minor phosphopeptide, number 9, of v-Rel (Fig. 4B, panels 3 and 4). Furthermore, this phosphopeptide was not found in v-275A (Fig. 4B, panels 5 and 6). These results demonstrate that Ser-275 can be phosphorylated by PKA in vitro. However, it does not appear to be a major site of phosphorylation in v-Rel in vivo.

Effects of mutations within the PKA recognition motif on the cytoplasmic retention of c-Rel. The close proximity and identical positioning of the conserved PKA recognition sequence relative to the nuclear localizing signal in Rel proteins prompted us to investigate the potential influence of this sequence on the subcellular localization of c-Rel. Figure 5 shows the results of indirect immunofluorescence experiments with anti-Rel antiserum and CEF transfected with plasmids encoding wild-type and mutant c-Rel proteins.

As determined previously (7), c-Rel and c-Rel protein containing sequences from the middle of v-Rel (clone CVC) showed exclusively cytoplasmic staining in CEF, despite the presence of a functional nuclear localizing signal in each of these proteins (Fig. 5A and D). The insertion of Pro-Trp within the PKA recognition site had a very dramatic effect on the subcellular localization of c-Rel; the protein encoded by c-SPW showed exclusively nuclear staining in CEF (Fig. 5B).

As a control, the Pro-Trp insertion was placed 48 amino acids C terminal to Ser-266 of c-Rel (i.e., after amino acid 314) in clone CVC-HPW; this insertion did not affect the cytoplasmic localization of c-Rel (Fig. 5C). However, when we inserted 14 amino acids corresponding to the simian virus 40 large T antigen nuclear targeting sequence after amino acid 314 in CVC (in plasmid CVC-TG18), the c-Rel protein entered the nucleus very efficiently in CEF (Fig. 5E). This result indicates that the c-Rel cytoplasmic retention sequence can be overcome by the addition of the strong simian virus 40 large T antigen nuclear targeting signal. A similar result was reported by Hannink and Temin (20) with a v-Rel–c-Rel recombinant protein.

FIG. 4. Analysis of a tryptic phosphopeptide containing Ser-275 of v-Rel. (A) In vitro phosphorylation of v-Rel peptides by PKA. Shown is one-dimensional thin-layer electrophoresis of peptides phosphorylated in vitro by PKA. Lanes: 1, VP271; 2, VP271/280A; 3, VP271/275A; 4, VP271 in the absence of PKA. [γ-^{32}P]ATP was electrophoresed in parallel for comparison (lane 5). Equal amounts of peptides (15 μg) were phosphorylated in vitro as described in Materials and Methods, and 1/50 of the reaction mixture was analyzed by one-dimensional thin-layer electrophoresis at pH 1.9 (cathode on top) after digestion with trypsin. Autoradiography was done for 1 day with an intensifying screen. The arrow indicates the position of the phosphorylated peptides. (B) Two-dimensional phosphotryptic peptide mapping. Immunoprecipitated, ^{32}P-labelled v-Rel from okadaic acid-treated transformed spleen cells or in vitro-labelled peptide VP271 was digested with trypsin, and the resultant peptides were separated by electrophoresis (horizontal dimension) and chromatography (vertical dimension) as described in Materials and Methods. Autoradiography was performed with intensifying screens at −70°C. The nine phosphopeptides consistently seen in v-Rel are numbered (peptide 2 may represent an incompletely digested or differentially phosphorylated form of peptide 1). Panels: 1, in vivo-labelled v-Rel (4,500 cpm, 5-day exposure); 2, in vitro-labelled peptide VP271 (100 cpm, 4 days); 3, v-Rel + VP271 (6,400 cpm of v-Rel and 320 cpm of VP271, 5 days); 4, enlargement of the region containing peptide 9 from v-Rel and a mixture of v-Rel and VP271; 5, in vivo-labelled v-275A (3,600 cpm, 6 days); 6, v-Rel + v-275A (approximately 2,400 cpm of each, 5 days). The arrowhead indicates the expected position of peptide 9, which was absent in panel 5. Ori, origin.

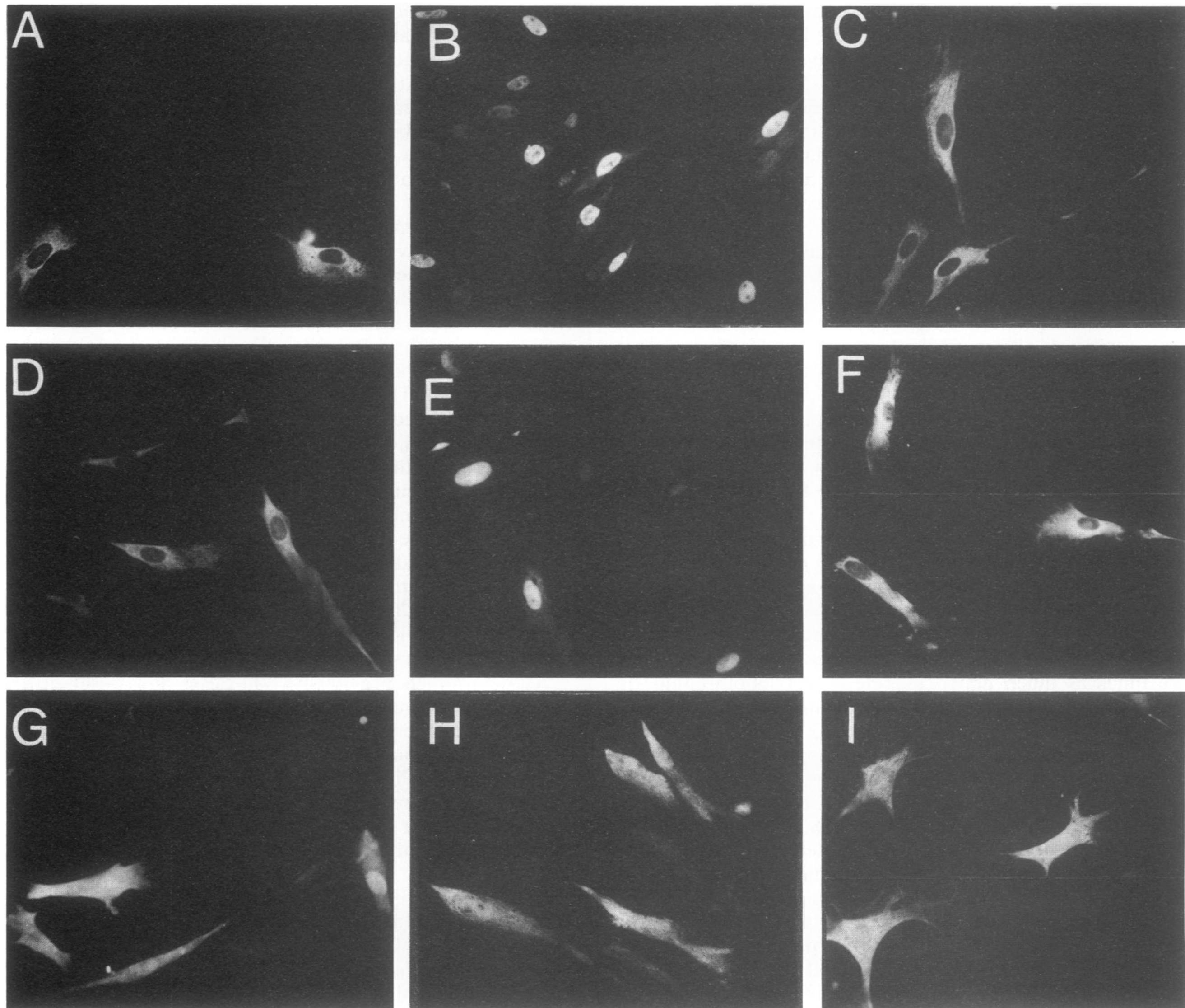


FIG. 5. Indirect immunofluorescence of CEF transfected with c-Rel-expressing plasmids. CEF were transfected with c-Rel-expressing plasmids and helper virus DNA, and immunofluorescence with anti-Rel primary antiserum was performed on cells 6 days after transfection as described in Materials and Methods. Panels correspond to CEF transfected with the following expression plasmids: A, JD214c-rel; B, c-SPW; C, CVC-HPW; D, CVC; E, CVC-TG18; F, c-266A; G, c-266D; H, c-266E; and I, c-266W.

To determine the effect of single amino acid changes at Ser-266 of c-Rel (analogous to Ser-275 of v-Rel) on subcellular localization, we performed indirect immunofluorescence on CEF transfected with plasmids encoding c-Rel proteins with substitutions at Ser-266. The protein expressed by c-266A, containing an Ala at position 266, showed exclusively cytoplasmic staining, identical to that seen with wild-type c-Rel (Fig. 5F). However, when Ser-266 was substituted with Asp, Glu, or Trp (in clones c-266D, c-266E, or c-266W, respectively), a significant amount of c-Rel entered the nucleus. In general, these c-Rel mutant proteins were approximately equally distributed between the nucleus and the cytoplasm (Fig. 5G, H, and I). All c-Rel-producing plasmids expressed stable proteins of the expected sizes, as determined by Western blotting with anti-Rel antiserum (data not shown). These results (summarized in Table 2) demonstrate that changes at Ser-266 of c-Rel can affect its subcellular localization in CEF, even though the analogous changes do not affect the subcellular localization of v-Rel in these cells.

DISCUSSION

The data presented here indicate that the structure of a conserved PKA recognition sequence is important for transformation and transcriptional repression by the v-Rel oncoprotein and cytoplasmic retention of the c-Rel proto-oncoprotein in CEF. When this sequence was disrupted by certain mutations, the transforming and transcriptional repressing abilities of v-Rel were impaired and c-Rel showed reduced cytoplasmic retention in CEF.

Transformation and transcriptional repression by v-Rel. The molecular basis for the transformation of avian spleen cells by the v-Rel oncoprotein is not known. It has been

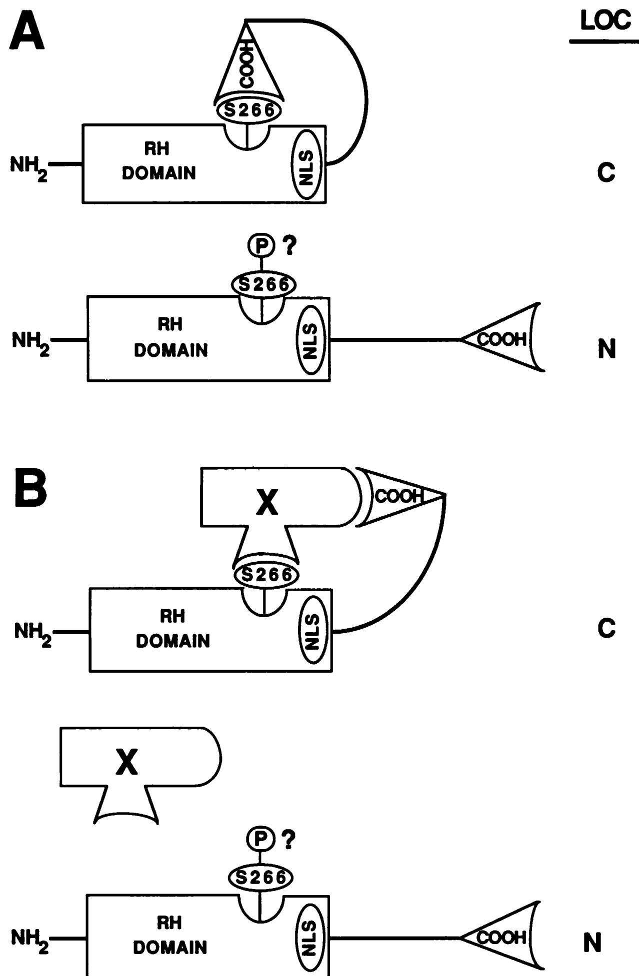


FIG. 6. Models for the regulation of the subcellular localization (LOC) of c-Rel. Serine 266 (S266) of chicken c-Rel is depicted as lying within a structural pocket. (A) A direct interaction between the C terminus (COOH) of c-Rel and the region surrounding Ser-266 obscures the nuclear localization signal (NLS) of c-Rel and keeps c-Rel located in the cytoplasm (C). Upon modification of Ser-266 (e.g., by phosphorylation [P]) or deletion of the C terminus (e.g., as in v-Rel or c-Rel proteins with deletions [7]), the c-Rel protein can enter the nucleus (N) by means of the exposed NLS. (B) A cytoplasmic retention protein (X; for example, IκB) is depicted as interacting with the region around Ser-266 and the C terminus of c-Rel. As in panel A, an alteration of Ser-266 or the C terminus of c-Rel can result in the nuclear localization of c-Rel through the release of protein X. In addition, in model A or B, regulatory modifications can occur either at the C terminus of c-Rel or on protein X, and such modifications can have the same effect as modification at Ser-266.

suggested that the oncogenicity of v-Rel is based on its ability to repress promoters containing NF-κB binding sites, either through DNA binding or protein-protein interactions (3, 14, 15, 22, 33). For example, one could imagine that v-Rel is able to down-regulate genes involved in programmed cell death or tumor suppression, thus allowing uncontrolled growth of the cell. Our results present very strong support for the model that correlates transformation by v-Rel with the ability of v-Rel to repress NF-κB enhancers, since we have analyzed transformation and transcriptional repression by v-Rel mutants with only very small changes.

TABLE 2. Summary of Rel protein localization in CEF

Protein	CEF localization[a]
v-Rel	Nuclear
v-SPW	Nuclear
v-275A	Nuclear
v-275D	Nuclear
v-275E	Nuclear
v-275W	ND[b]
c-Rel	Cytoplasmic
c-SPW	Nuclear
CVC	Cytoplasmic
CVC-TG18	Nuclear
c-266A	Cytoplasmic
c-266D	Nuclear and cytoplasmic
c-266E	Nuclear and cytoplasmic
c-266W	Nuclear and cytoplasmic

[a] The subcellular localization of each protein was determined in CEF by indirect immunofluorescence as described in Materials and Methods.
[b] ND, not determined.

If v-Rel transforms cells by repressing NF-κB-regulated genes, then it is likely that the ability of v-Rel to bind to NF-κB sites and/or dimerize with Rel family proteins (such as p50 or p65 of NF-κB) is crucial for its transforming ability. Since Ser-275 is located within a region of v-Rel that, by analogy with NF-κB p50, is likely to be involved in DNA binding and/or dimerization (13, 30), it is possible that mutations in this region interfere with these processes. More specifically, our results show that the insertion of Pro-Trp within the PKA recognition motif abolishes a function important for transformation and transcriptional repression. The Pro-Trp insertion in v-SPW could either change the local structure or change the spacing between sequences. Since the Ser-to-Trp change was not as inhibitory as the Ser-to-Glu or Ser-to-Asp change, we conclude that a negative charge at this position is more disruptive than a bulky side chain. Experiments are in progress to determine whether the above-described mutations affect the binding of v-Rel to NF-κB target sequences.

Our results suggest that the phosphorylation of Ser-275 could have an inhibitory effect on transformation by v-Rel. Consistent with this suggestion, Ser-275 does not appear to be a major phosphorylation site in vivo in v-Rel in transformed spleen cells, even in the presence of okadaic acid (Fig. 4B). Along these lines, it is interesting that actively proliferating B and T lymphocytes contain low levels of cyclic AMP-dependent protein kinase activity (24). The low level of phosphorylation of this site in v-Rel in spleen cells could indicate that only a special population of v-Rel molecules is phosphorylated at Ser-275 at any one time. For example, phosphorylation of this site could be linked to a specific stage of the cell cycle or a specific location of v-Rel within the cell. Furthermore, it is known that v-Rel can show distinct subcellular localizations within a population of v-Rel-transformed spleen cells (16).

Subcellular localization of c-Rel. There are at least two possible mechanisms for the cytoplasmic retention of chicken c-Rel in CEF (Fig. 6). c-Rel may be retained in the cytoplasm of CEF because its C-terminal sequences, which are deleted in v-Rel, mask the nuclear localizing signal and render it nonfunctional. Consistent with this hypothesis, C-terminal deletions in chicken c-Rel cause it to become localized in the nucleus of CEF (7). Alternatively, c-Rel may be retained in the cytoplasm by means of association with a cytoplasmic anchoring protein and translocated to the nu-

cleus upon dissociation from this protein. This cytoplasmic anchor is likely to be an IκB-like molecule, since c-Rel shows extensive homology with the p65 subunit of NF-κB, which is known to associate with IκB through the RH domain (13, 32), and c-Rel complexes in vivo in transformed spleen cells with a 40-kDa protein that can show IκB activity in vitro (9a, 31). However, very little of the v-Rel protein appears to be associated with this 40-kDa protein in CEF, and v-Rel is an exclusively nuclear protein in CEF (16, 28).

Our results demonstrate that certain structural alterations within the PKA recognition motif, such as an insertion or substitutions of acidic or large hydrophobic amino acids for the conserved Ser-266 residue, allow c-Rel to enter the nucleus. Furthermore, our results suggest that nuclear translocation of c-Rel could be mediated by phosphorylation of Ser-266, since a negative charge at this position allows partial nuclear localization of c-Rel. It is possible that phosphorylation at Ser-266 leads to more efficient nuclear transport of c-Rel, since the phosphate group provides two negative charges, as opposed to one contributed by acidic amino acids. Phosphorylation of a serine residue located adjacent to the nuclear localizing signal of simian virus 40 large T antigen increases its rate of nuclear localization (34). Similarly, a site of phosphorylation by the cdc2 kinase immediately precedes the nuclear localizing sequence of p53 (1). In both cases, the potentially regulatory sites of phosphorylation are located immediately next to the nuclear targeting signal. In c-Rel, Ser-266 seems to have a rather long-range effect on the nuclear localizing sequence. Alternatively, it is possible that the critical modification for the nuclear localization of c-Rel occurs at a domain that interacts with Ser-266 rather than at Ser-266.

The striking conservation of the PKA recognition motif among all members of the Rel family of proteins suggests that this sequence is functionally important in other members of the family. For example, one critical step for the function of NF-κB and the *Drosophila* morphogen Dorsal is nuclear translocation (reviewed in reference 19). It has been proposed that this process is dependent on the posttranslational modification of these proteins (35, 36, 42). Our results suggest that the conserved Ser within the PKA recognition sequence may regulate the subcellular distribution of NF-κB and Dorsal through phosphorylation and dephosphorylation. It is interesting that PKA is unable to phosphorylate IκB in vitro, yet it can induce the dissociation of IκB from NF-κB (12). Therefore, PKA-mediated activation and nuclear translocation of NF-κB could occur through phosphorylation of the p65 subunit (which mediates the interaction with IκB), most likely at the conserved PKA recognition sequence. It would be interesting to determine whether mutations analogous to the ones that we have described above affect transcriptional activation by NF-κB and developmental processes controlled by the *Drosophila* morphogen Dorsal.

ACKNOWLEDGMENTS

We are grateful to J. D. Dixon for the synthesis of the peptides. We thank Chris Li for help with immunofluorescence and photography and members of our laboratory for critical reading of the manuscript.

This work was supported by Public Health Service grant CA47763 from the National Cancer Institute (to T.D.G.) and by National Science Foundation grant DIR-8900216 (to R.A.L.). T.D.G. was supported in part by an American Cancer Society Junior Faculty Research Award.

ADDENDUM IN PROOF

Preliminary data show that the v-*rel* mutations described here also affect DNA binding in a way that is similar to the effects we have described for transformation and transcriptional repression, i.e., in an electrophoretic mobility shift assay using an NF-κB target oligonucleotide, wild-type v-Rel and v-275A proteins bind strongly and v-SPW does not bind at all (31a).

REFERENCES

1. **Addison, C., J. R. Jenkins, and H.-W. Sturzbecher.** 1990. The p53 nuclear localisation signal is structurally linked to a p34^{cdc2} motif. Oncogene **5**:423–426.
2. **Baeuerle, P. A.** 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072**:63–80.
3. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**:803–814.
4. **Barth, C. F., D. L. Ewert, W. C. Olson, and E. H. Humphries.** 1990. Reticuloendotheliosis virus REV-T (REV-A)-induced neoplasia: development of tumors within the T-lymphoid and myeloid lineages. J. Virol. **64**:6054–6062.
5. **Boyle, W. J., P. Van Der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin layer cellulose plates. Methods Enzymol. **201**:110–149.
6. **Bull, P., K. L. Morley, M. F. Hoekstra, T. Hunter, and I. Verma.** 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional activation domain. Mol. Cell. Biol. **10**:5473–5485.
7. **Capobianco, A. J., D. L. Simmons, and T. D. Gilmore.** 1990. Cloning and expression of a chicken c-*rel* cDNA: unlike p59$^{v\text{-}rel}$, p68$^{c\text{-}rel}$ is a cytoplasmic protein in chicken embryo fibroblasts. Oncogene **5**:257–265.
8. **Cohen, P., C. F. B. Holmes, and Y. Tsukitani.** 1990. Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem. Sci. **15**:98–102.
9. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.

9a. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the Rel family of transcription factors. Science **253**:1268–1271.

10. **Dougherty, J. P., and H. M. Temin.** 1986. High mutation rate of a spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. **6**:4387–4395.
11. **Garson, K., and C.-Y. Kang.** 1990. Mapping of the functional domains of the v-*rel* oncogene. Oncogene **5**:1431–1434.
12. **Ghosh, S., and D. Baltimore.** 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344**: 678–682.
13. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62**:1019–1029.

13a. **Gilmore, T.** Unpublished results.

14. **Gilmore, T. D.** 1990. NF-κB, KBF1, *dorsal*, and *rel*ated matters. Cell **62**:841–843.
15. **Gilmore, T. D.** 1991. Malignant transformation by mutant Rel proteins. Trends Genet. **7**:318–322.
16. **Gilmore, T. D., and H. M. Temin.** 1986. Different localization of the product of the v-*rel* oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell **44**:791–800.
17. **Gilmore, T. D., and H. M. Temin.** 1988. v-*rel* oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. J. Virol. **62**:703–714.
18. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyl-

transferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
19. **Govind, S., and R. Steward.** 1991. Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. Trends Genet. 7:119–125.
20. **Hannink, M., and H. M. Temin.** 1989. Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. Mol. Cell. Biol. 9:4323–4336.
21. **Hoelzer, J. D., R. B. Lewin, C. R. Wasmuth, and H. R. Bose, Jr.** 1980. Hematopoietic cell transformation by reticuloendotheliosis virus: characterization of the genetic defect. Virology **100**: 462–474.
22. **Inoue, J.-I., L. D. Kerr, L. J. Ransone, E. Bengal, T. Hunter, and I. M. Verma.** 1991. c-rel activates but v-rel suppresses transcription from κB sites. Proc. Natl. Acad. Sci. USA **88**: 3715–3719.
23. **Kamens, J., P. Richardson, G. Mosialos, R. Brent, and T. Gilmore.** 1990. Oncogenic transformation by vRel requires an amino-terminal activation domain. Mol. Cell. Biol. **10**:2840–2847.
24. **Kammer, G. M.** 1988. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. Immunol. Today 9:222–229.
25. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. 4:1172–1174.
26. **Kemp, B. E., and R. B. Pearson.** 1990. Protein kinase recognition sequence motifs. Trends Biochem. Sci. **15**:342–346.
27. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62**:1007–1018.
28. **Kochel, T., J. F. Mushinski, and N. R. Rice.** 1991. The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. Oncogene 6:615–626.
29. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
30. **Logeat, F., N. Israel, R. Ten, V. Blank, O. Le Bail, P. Kourilsky, and A. Israel.** 1991. Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. EMBO J. **10**:1827–1832.
31. **Morrison, L. E., N. Kabrun, S. Mudri, M. J. Hayman, and P. J. Enrietto.** 1989. Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. Oncogene 4:677–683.
31a. **Mosialos, G., and T. D. Gilmore.** Unpublished results.
32. **Nolan, G. P., S. Ghosh, H.-C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64**:961–969.
33. **Richardson, P. M., and T. D. Gilmore.** 1991. vRel is an inactive member of the Rel family of transcriptional activating proteins. J. Virol. **65**:3122–3130.
34. **Rihs, H.-P., and R. Peters.** 1989. Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the simian virus 40 T-antigen. EMBO J. 8:1479–1484.
35. **Roth, S., D. Stein, and C. Nusslein-Volhard.** 1989. A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the Drosophila embryo. Cell **59**:1189–1202.
36. **Rushlow, C. A., K. Han, J. L. Manley, and M. Levine.** 1989. The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in Drosophila. Cell **59**:1165–1177.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352**:733–736.
39. **Simek, S. L., and N. R. Rice.** 1988. Detection and characterization of the protein encoded by the chicken c-*rel* protooncogene. Oncogene Res. 2:103–119.
40. **Simek, S. L., and N. R. Rice.** 1988. $p59^{v\text{-}rel}$, the transforming gene of reticuloendotheliosis virus, is complexed with at least four other proteins in transformed chicken lymphoid cells. J. Virol. **62**:4730–4736.
41. **Stephens, R. M., N. R. Rice, R. R. Hiebsh, H. R. Bose, Jr., and R. V. Gilden.** 1983. Nucleotide sequence of v-*rel*: the oncogene of reticuloendotheliosis virus. Proc. Natl. Acad. Sci. USA **80**:6229–6233.
42. **Steward, R.** 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell **59**:1179–1188.
43. **Tung, H. Y. L., W. J. Bargmann, and H. R. Bose, Jr.** 1988. Serine phosphorylation of the v-*rel* oncogene product/pp40 complex. Biochem. Biophys. Res. Commun. **152**:441–448.
44. **Watanabe, S., and H. M. Temin.** 1982. Encapsidation sequences for spleen necrosis virus, an avian retrovirus, are between the 5′ long terminal repeat and the start of the gag gene. Proc. Natl. Acad. Sci. USA **79**:5986–5990.
45. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the protooncogene c-*rel*. J. Virol. **52**:172–182.