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Transactivation of Gene Expression by Nuclear and Cytoplasmic *rel* Proteins

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Transcriptional activation of gene expression by oncogenic proteins can lead to cellular transformation. It has recently been demonstrated that the protein encoded by the v-*rel* oncogene from reticuloendotheliosis virus strain T can transactivate gene expression from certain promoters in a cell-specific manner. We have examined the cytological location, transforming properties, and transactivation properties of proteins encoded by chimeric turkey v-*rel*/chicken c-*rel* genes. We found that whereas the v-*rel* protein was nuclear in both chicken embryo and rat fibroblasts, the presence of the C terminus of the c-*rel* protein inhibited nuclear localization of the *rel* protein in these fibroblasts. Cytoplasmic *rel* proteins containing C-terminal c-*rel* sequences transactivated gene expression from the polyomavirus late promoter as efficiently as did similar *rel* proteins located in the nucleus. These results indicate that the cellular location of *rel* proteins is not important for transactivation of gene expression and suggest that transactivation by *rel* proteins is indirect, perhaps by affecting an intracellular signal transduction pathway that eventually results in the alteration of gene expression. The transforming properties of the *rel* protein were unaltered by the presence of the c-*rel* C terminus, but, as previously reported for turkey c-*rel* sequences, substitution of chicken c-*rel* sequences for internal v-*rel* sequences reduced the transforming activity of the *rel* protein and eliminated the immortalization ability. However, all of the chimeric v/c-*rel* proteins were able to transactivate gene expression, indicating that transactivation does not correlate with transformation. These results suggest that transactivation may be necessary but is not sufficient for transformation by *rel* proteins.

Recent evidence demonstrates that alteration of gene expression by oncogenic proteins can lead to cellular transformation. There are several mechanisms by which oncogenic proteins can alter gene expression. Certain oncogenes are thought to encode proteins that function as direct transactivators of transcription (23). A prototype of this group of oncogenic proteins is the v-*jun* oncogene. The v- and c-*jun* proteins are located in the nucleus, bind to DNA in a sequence-specific manner, and interact with other cellular proteins involved in transcription (3, 4). Both the oncogenic and the cellular proto-oncogenic *jun* proteins are able to activate transcription (3, 4), and cellular transformation by these oncogenic proteins probably results from subtle differences between the activation of genes by the oncogenic proteins and by the proto-oncogenic proteins. Alterations in gene expression have also been shown to result from expression of oncogenic proteins that are not directly involved in transcription. Autocrine expression of the v-*sis* oncogene results in the activation of transcription of many genes, including c-*fos* and c-*myc* (1, 15, 16, 25, 27). The v-*mos* oncogene has been shown to alter expression from the alpha-2 collagen promoter (32), and both the oncogenic and proto-oncogenic c-*ras* proteins have been shown to activate expression from the polyomavirus late promoter (45). These cytoplasmic (v-*mos*) and plasma membrane (*ras*, v-*sis*) proteins are presumably able to activate gene expression via intracellular signaling pathways that ultimately modulate the activity of cellular transcription factors.

The v-*rel* protein, encoded by reticuloendotheliosis virus strain T (Rev-T), has recently been shown to transactivate gene expression in a cell- and promoter-specific manner (10). Rev-T is an acutely transforming avian retrovirus isolated from a turkey lymphoma (42). Transformation by Rev-T is specific for avian lymphoid cells (8, 11, 21). Infection of chicken embryo fibroblasts (CEF) with Rev-T results in a high level of expression of the v-*rel* protein, with no appearance of a transformed phenotype (11). Rev-T does not immortalize primary rat fibroblasts, but it does cooperate with the polyomavirus middle-T protein in the transformation of established rat fibroblasts by increasing the levels of middle-T mRNA and protein from the polyomavirus late promoter (10). By use of a transient transfection assay, it was shown that expression of the v-*rel* protein in rodent fibroblasts results in activation of certain viral promoters, including polyomavirus early and late promoters, simian virus 40 (SV40) early promoter, and the long terminal repeats (LTRs) of both avian and murine retroviruses. The ability of the v-*rel* protein to transactivate expression from these promoters correlates with a cytotoxic effect of high levels of v-*rel* expression in rodent fibroblasts (10, 33).

The v-*rel* protein is a 59-kilodalton (kDa) phosphoprotein that is located in the cytoplasm of transformed spleen cells and in the nucleus of nontransformed CEF (9, 11, 19, 36, 44). The v-*rel* protein contains a sequence that is responsible for its nuclear localization in CEF, but amino acid substitutions within this sequence have no effect on the transforming property of the v-*rel* protein (12). Furthermore, the addition of a nuclear targeting sequence (NTS) from the SV40 large-T protein to the v-*rel* protein results in a protein that is located in the nucleus of transformed spleen cells (12). Thus, the cytological location of the v-*rel* protein is irrelevant to cell-specific transformation by Rev-T.

The v-*rel* protein is actually an *env-rel-env* fusion protein, containing 11 *env*-derived amino acids at its N terminus, 474 c-*rel*-derived amino acids, and 18 amino acids at its C terminus encoded by out-of-frame *env*-derived amino acids

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(37, 48). The v-*rel* protein has no known enzymatic activity, although it is associated with a cellular serine/threonine protein kinase in both CEF and transformed spleen cells (11, 30, 44). The v-*rel* protein is not homologous to any other oncogenic protein; however, the N-terminal 295 amino acids of the v-*rel* protein are approximately 47% identical with the N terminus of the *dorsal* protein of *Drosophila melanogaster* (38).

The portions of the turkey c-*rel* proto-oncogene that are homologous to the v-*rel* oncogene have been molecularly cloned and sequenced (48, 49). The c-*rel* proto-oncogene is encoded by at least nine exons distributed over more than 20 kilobases of chromosomal DNA (49). There are multiple nucleotide differences between the v-*rel* and c-*rel* genes, resulting in 14 single-amino-acid differences and three small deletions of one or three amino acids in the v-*rel* protein in comparison with the product of the c-*rel* gene (48). By using recombinant viruses that substituted turkey-derived c-*rel* exons for the corresponding regions of v-*rel*, it was shown that some of the internal amino acid differences between the v- and c-*rel* proteins are important for transformation and immortalization (40). The N and C termini of the c-*rel* protein are not known, although the c-*rel* protein has recently been identified as a 68-kDa protein located in the cytoplasm of avian lymphoid cells (34).

We have isolated a c-*rel* cDNA clone that encodes the C terminus of the chicken c-*rel* protein. To examine the role of C-terminal c-*rel* sequences in transformation and transactivation, we have constructed chimeric v/c-*rel* genes between the turkey-derived v-*rel* gene and the chicken-derived cDNA. Replacement of the C terminus of the v-*rel* gene with the C-terminal coding region of the chicken c-*rel* gene does not affect the transforming properties of the v-*rel* gene. However, the resultant v/c-*rel* protein is located in the cytoplasm of CEF and rat fibroblasts. Both nuclear and cytoplasmic chimeric v/c-*rel* proteins were able to activate gene expression from the polyomavirus late promoter in Rat-1 cells in a dose-responsive manner. Thus, transactivation, like transformation, is independent of cytological location. Replacement of an internal portion of the v-*rel* gene with the chicken c-*rel* cDNA results in a decrease in transformation efficiency and a loss of immortalization potential, although the resultant chimeric protein is still able to transactivate gene expression. These results indicate that transactivation by *rel* proteins is indirect, perhaps by affecting intracellular signaling pathways that regulate gene expression. Furthermore, transactivation does not correlate well with transformation, suggesting that transactivation may be necessary but is not sufficient for transformation by *rel* proteins.

MATERIALS AND METHODS

Recombinant DNA methodology. Standard techniques were used for screening a chicken spleen cDNA library with a v-*rel* probe and for constructing the various recombinant plasmids used (26). The chicken spleen cDNA library was a generous gift of J. H. Chen, of St. Jude Children's Research Hospital, Memphis, Tenn. Nucleotide sequence analysis of the chicken c-*rel* cDNA was performed on subclones (18) of the c-*rel* cDNA, using the dideoxy protocol of Sanger et al. (31). Nucleotide sequence analysis of exons 0, 1, and 2 of genomic chicken c-*rel* sequences was performed on plasmid subclones of chicken genomic DNA containing these exons (6). The nucleotide sequence of the 3′ end of the turkey c-*rel* gene was determined from plasmid subclones of this region (49). The replication-defective spleen necrosis virus (SNV)-derived virus vector pJD214 was used for the expression of the v-*rel* gene and the recombinant v/c-*rel* genes in CEF, chicken spleen, and Rat-1 cells (7). Plasmids that allowed for expression of the respective *rel* genes from the major immediate early promoter of cytomegalovirus (CMV) (39) or the early promoter of SV40 were also constructed. These plasmids contain the respective promoter elements, the respective *rel* coding sequences, and the SV40 late polyadenylation sequence on a pUC19 plasmid. Details of the plasmids will be provided upon request. The reporter plasmid, pPyLCAT, contains the polyomavirus late promoter, the coding sequences for the chloramphenicol acetyltransferase (CAT) gene, and the SV40 late polyadenylation sequence. This plasmid was a gift of J. Hassell, McMaster University, Hamilton, Ontario, Canada.

The vvc-*rel* gene contains C-terminal c-*rel* sequences coding for 171 amino acids substituted for C-terminal v-*rel* sequences coding for 53 *rel*-derived and 18 out-of-frame *env*-derived amino acids. The vcc-*rel* gene contains c-*rel* sequences coding for 358 C-terminal amino acids substituted for the corresponding C-terminal v-*rel* sequences. The vcv-*rel* gene contains internal c-*rel* sequences coding for 187 amino acids substituted for the corresponding v-*rel* coding sequences. The vvc18-*rel* is derived from pTG18 (12) and contains the coding sequence for the NTS of the large-T antigen of SV40 inserted at a unique *Hin*cII site (nucleotide 4211 of reference 48) of the vvc-*rel* gene. The vvc15-*rel* gene is derived from pTG15 (12) and contains the coding sequence for the NTS of large-T antigen of SV40 at the N terminus of the vvc-*rel* gene. The asx-*rel* gene contains an in-frame deletion of 509 nucleotides located between an *Apa*I site (nucleotide 3524) and a *Stu*I site (nucleotide 4040) in the vvc-*rel* gene.

Generation of virus stocks and transformation assays. Primary CEF were grown in Temin modified Eagle medium containing 20% tryptose-phosphate broth, 4% calf serum, 2% fetal bovine serum, and antibiotics. The cells were plated at 6×10^5 cells per 60-mm-diameter dish the day before transfection. The cells were cotransfected with 5 μg of plasmid constructs and 0.1 μg of plasmid DNA containing a replication-competent clone of reticuloendotheliosis virus strain A (46), using the dimethyl sulfoxide-Polybrene method (22). Virus was collected at 5 days posttransfection and was used immediately for infection of 3×10^7 freshly prepared primary chicken spleen cells (21). The infection was carried out in the presence of Polybrene (2 μg/ml) for 1 h at 37°C. The spleen cells were then plated in RPM 1640 containing 0.35% Bacto-Agar (Difco Laboratories), 15% fetal bovine serum, 1% chick serum, 1% beef embryo extract, and 0.028% sodium bicarbonate. Assays were carried out at 41°C and were scored after 14 days. Virus titers were determined by measuring the level of unintegrated viral DNA (20) after infection of CEF with the virus stock that was used in the transformation assay. The transforming titers of the virus stocks were normalized to the virus titers as determined by the level of unintegrated viral DNA in infected CEF and are expressed relative to the transforming titer of the wild-type v-*rel* virus. The titer of the wild-type v-*rel* virus in these assays ranged from 50 to 100 CFU/ml of virus.

Transfections of mammalian cells. Mammalian cells were grown in Temin modified Eagle medium containing 5% fetal bovine serum (for Cos-1 cells and D17 helper cells) or 5% calf serum (for Rat-1 cells). D17 helper cells (47) and Rat-1 cells were transfected by the dimethyl sulfoxide-Polybrene method, and Cos-1 cells (13) were transfected by using a

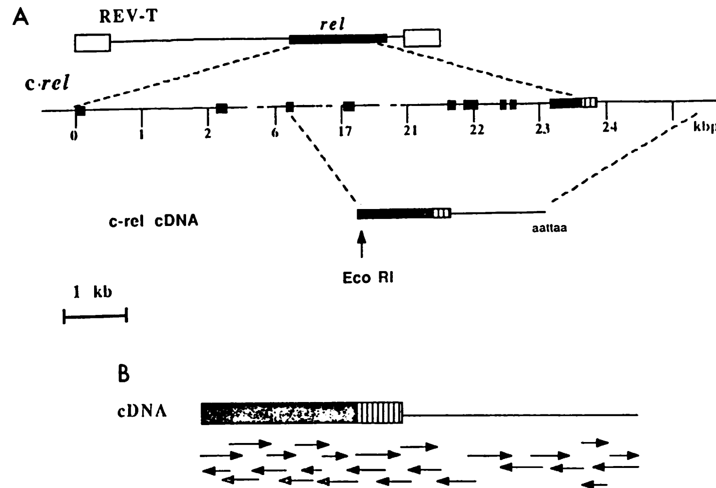

FIG. 1. (A) Structure of a partial c-*rel* cDNA. The structure of the c-*rel* cDNA is compared with the organization of the genomic c-*rel* sequences and with the structure of Rev-T. Symbols: ▭, LTRs of Rev-T; ■, v-*rel* gene and the regions of c-*rel* that are homologous to v-*rel*; ▥, C-terminal sequences of c-*rel* that are not present in the v-*rel* gene. The longest of the c-*rel* cDNA clones obtained from a chicken spleen cDNA library was truncated at an *EcoRI* site that is located in the middle of genomic exon 2. kb, Kilobase. (B) Sequencing strategy. Arrows show the direction of the sequence data obtained from sequence analysis of various subclones of the c-*rel* cDNA.

modified DEAE-dextran protocol (17). For CAT assays, Rat-1 cells were cotransfected with 10 μg of pPLCAT and 10 μg of the respective plasmids containing the v- and v/c-*rel* genes. Cell lysates were harvested after 48 h, and 50 or 100 μg of total cellular protein was used in CAT assays (14). Protein concentrations were determined by the method of Bradford (5). An internal control for plate-to-plate variation in transfection efficiency was not included because we found that the *rel* proteins examined in this study had differential effects on various promoters (this report and data not shown). In experiments in which identical samples were included in duplicate, the plate-to-plate variation within a single experiment was less than 20% (data not shown).

Immunoprecipitation and indirect immunofluorescence. Transfected Cos-1 cells were labeled with [^{35}S]methionine, and cell lysates were collected in RIPA buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 0.5% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Nonidet P-40). Immunoprecipitations using anti-*rel* antiserum were performed as described previously (11). For indirect immunofluorescence (11), either infected CEF or transfected Rat-1 cells were transferred onto cover slips the day before work-up. The cells were fixed and permeabilized in 100% methanol at −20°C for 10 min. The cells were then incubated with the anti-*rel* antiserum, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum. The cover slips were mounted in glycerol containing 10% 1 M Tris (8.0) and examined by epifluorescence.

Cytotoxicity assays. Rat-1 cells were cotransfected with 10 μg of the respective *rel*-containing plasmid and 1 μg of a plasmid expressing the gene for hygromycin from the SV40 early promoter. The cells were transferred into selective medium at a 1:3 dilution 2 days after transfection. The number of hygromycin-resistant colonies was determined after 2 weeks of selection.

RESULTS

Nucleotide sequence of the chicken c-*rel* gene. A partial cDNA clone of the chicken c-*rel* gene was obtained by screening a chicken spleen cell cDNA library with a v-*rel* probe (Fig. 1). The longest clone obtained terminated at an internal *EcoRI* site located within the third exon of the c-*rel* gene, presumably as a result of incomplete protection of internal *EcoRI* sites during library construction. The complete nucleotide sequence of the cDNA clone was determined, and the nucleotide sequence of the remaining chicken c-*rel* exons was determined from cloned genomic DNAs (6) containing exons 0, 1, and 2 (Fig. 2). The c-*rel* cDNA has a long, A+T-rich, 3′ nontranslated region of 1.7 kilobase pairs. The sequence AATTAA is located 34 nucleotides upstream of the end of the cDNA clone, and a G+T-rich block is present in the genomic DNA approximately 30 nucleotides downstream of the polyadenylation site (data not shown). These features suggest that the cDNA clone contains the authentic 3′ end of the c-*rel* mRNA (2).

Predicted amino acid sequence of the chicken c-*rel* protein and comparison with the turkey c-*rel* protein. The predicted amino acid sequence of the chicken c-*rel* protein is shown in Fig. 2. Initiation at the first ATG codon would result in the synthesis of a protein of 579 amino acids. However, there are several lines of evidence indicating that there is an additional coding exon of the c-*rel* gene located further upstream. First, the amino acid homology between c-*rel* and the *dorsal* protein of *D. melanogaster* continues for several amino acids 5′ of this ATG codon (38). Second, an in-frame TAA codon is located just upstream of the 5′ end of exon 0 of both turkey (49) and chicken c-*rel* genomic DNA (data not shown). A consensus splice acceptor sequence is also located immediately 5′ to exon 0 (49; data not shown). This putative upstream exon would be expected to encode only a few amino acids, including the authentic initiation codon for the c-*rel* protein, since the size of chicken c-*rel* protein has been reported to be 68 kDa (34).

The predicted amino acid sequence of the chicken c-*rel* protein is compared with the predicted amino acid sequence of the turkey c-*rel* protein (48) and the turkey-derived v-*rel* protein (48) in Fig. 3. The turkey and chicken c-*rel* proteins are more than 95% identical, differing in only 28 of 595 residues. The N-terminal regions of the two proteins are highly conserved, with differences at only 4 of the first 300

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        I  S  E  P  Y  I  E  I  F  E  Q  P  R  Q  R  G  M  R  F  R
   1    ATCTCAGAGCCCTACATTGAAATATTTGAACAACCCAGGCAAAGGGGCATGCGTTTCAGA    60

        Y  K  C  E  G  R  S  A  G  S  I  P  G  E  H  S  T  D  N  N
  61    TACAAATGTGAAGGAAGATCAGCAGGTAGCATTCCAGGAGAACACAGTACTGACAACAAC   120

        K  T  F  P  S  I  Q  I  L  N  Y  F  G  K  V  K  I  R  T  T
 121    AAGACATTCCCATCTATACAGATTCTGAACTATTTTGGAAAAGTCAAAATAAGAACTACA   180

        L  V  T  K  N  E  P  Y  K  P  L  P  H  D  L  V  G  K  D  C
 181    TTGGTAACAAAGAATGAACCCTACAAGCCTCTCCCTCATGATCTAGTTGGAAAAGACTGC   240

        R  D  G  Y  Y  E  A  E  F  G  P  E  R  R  V  L  S  F  Q  N
 241    AGAGATGGCTACTATGAAGCAGAGTTTGGGCCTGAACGTCGAGTCCTGTCTTTTCAGAAT   300

        L  G  I  Q  C  V  K  K  K  D  L  K  E  S  I  S  L  R  I  S
 301    TTGGGAATTCAATGTGTGAAGAAGAAAGACCTGAAAGAATCAATTTCTTTGCGAATCTCA   360

        K  K  I  N  P  F  N  V  P  E  E  Q  L  H  N  I  D  E  Y  D
 361    AAGAAGATCAACCCCTTTAATGTGCCTGAAGAACAGCTGCACAACATCGATGAATATGAT   420

        L  N  V  V  R  L  C  F  Q  A  F  L  P  D  E  H  G  N  Y  T
 421    CTCAACGTTGTCCGCCTCTGTTTCCAAGCTTTCCTCCCTGATGAACATGGCAACTACACA   480

        L  A  L  P  P  L  I  S  N  P  I  Y  D  N  R  A  P  N  T  A
 481    TTAGCTCTTCCTCCTTTGATTTCCAACCCAATCTATGACAACAGAGCTCCCAACACAGCA   540

        E  L  R  I  C  R  V  N  K  N  C  G  S  V  K  G  G  D  E  I
 541    GAACTGAGAATTTGCCGTGTGAATAAGAACTGTGGAAGTGTAAAGGGAGGAGATGAAATT   600

        F  L  L  C  D  K  V  Q  K  D  D  I  E  V  R  F  V  L  D  N
 601    TTTCTTCTGTGTGATAAAGTTCAAAAAGATGACATAGAAGTCAGATTTGTCTTGGACAAC   660

        W  E  A  K  G  S  F  S  Q  A  D  V  H  R  Q  V  A  I  V  F
 661    TGGGAGGCAAAAGGCTCCTTCTCCCAAGCTGATGTTCATCGCCAGGTTGCAATTGTGTTC   720

        R  T  P  P  F  L  R  D  I  T  E  P  I  T  V  K  M  Q  L  R
 721    AGAACACCGCCATTCCTCAGAGACATCACAGAACCCATCACGGTGAAGATGCAGTTACGA   780

        R  P  S  D  Q  E  V  S  E  P  M  D  F  R  Y  L  P  D  E  K
 781    AGACCTTCAGACCAGGAAGTCAGTGAACCAATGGATTTCAGATACCTACCAGATGAAAAG   840

        D  P  Y  G  N  K  A  K  R  Q  R  S  T  L  A  W  Q  K  L  I
 841    GATCCATATGGTAACAAAGCAAAAAGGCAAAGATCAACGCTGGCCTGGCAAAAGCTCATA   900

        Q  D  C  G  S  A  V  T  E  R  P  K  A  T  P  I  P  T  V  N
 901    CAGGACTGTGGATCAGCTGTGACAGAGAGGCCAAAAGCCACTCCAATCCCCACTGTCAAC   960

        P  E  G  K  L  I  K  K  E  P  N  M  F  S  P  T  L  M  L  P
 961    CCTGAAGGAAAGCTGATTAAGAAAGAACCAAATATGTTTTCACCTACACTGATGCTGCCT  1020

        G  L  G  T  L  T  S  S  S  Q  M  Y  P  P  C  S  Q  M  P  H
1021    GGGCTAGGAACGCTGACGAGCTCCAGCCAGATGTACCCTCCATGCAGCCAGATGCCCCAC  1080

        Q  P  A  Q  L  G  P  G  K  Q  D  T  L  P  S  C  W  Q  Q  L
1081    CAGCCTGCGCAGCTTGGCCCTGGAAAGCAGGACACACTCCCTTCCTGCTGGCAGCAGCTG  1140

        F  S  S  S  P  S  A  S  S  L  L  S  M  H  P  H  N  S  F  T
1141    TTCAGCTCCTCCCCTTCAGCCAGCAGCCTGCTCAGCATGCACCCGCACAACAGCTTCACA  1200

        A  E  V  P  Q  P  N  A  Q  G  S  S  S  L  P  A  F  H  D  N
1201    GCAGAAGTGCCTCAGCCCAATGCTCAGGGCAGTAGCTCTCTCCCAGCTTTCCACGATAAC  1260
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FIG. 2. Nucleotide sequence of the chicken c-*rel* gene. The sequence shown is a composite of genomic exon 0 (nucleotides 1 to 141), genomic exon 1 (nucleotides 142 to 290), a portion of genomic exon 2 (nucleotides 291 to 310), and the cDNA clone (nucleotides 311 to 3583). The predicted amino acid sequence of the chicken c-*rel* protein in single-letter code is shown above the nucleotide sequence.

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      P  L  N  W  P  D  E  K  D  S  S  F  Y  R  N  F  G  S  T  N
1261  CCACTGAACTGGCCTGATGAGAAGGATTCCAGTTTTTACAGGAATTTTGGCAGCACAAAT  1320

      G  M  G  A  A  M  V  S  A  A  D  M  Q  S  A  S  S  N  S  I
1321  GGGATGGGAGCAGCGATGGTGTCAGCTGCGGATATGCAGAGTGCTTCCAGTAACAGCATC  1380

      V  H  A  T  H  Q  A  S  A  T  A  A  S  I  V  N  M  E  T  N
1381  GTCCATGCCACTCATCAGGCCAGTGCCACTGCTGCGAGCATCGTGAACATGGAGACCAAT  1440

      D  M  N  C  T  S  L  N  F  E  K  Y  T  Q  V  L  N  V  S  N
1441  GACATGAACTGCACTAGTCTCAACTTTGAAAAGTATACTCAGGTGTTAAATGTAAGCAAC  1500

      H  R  Q  Q  L  H  Q  A  P  A  A  C  P  P  V  A  A  P  G  S
1501  CACAGGCAGCAGCTCCATCAGGCACCTGCAGCATGTCCACCTGTGGCAGCCCCTGGCAGC  1560

      T  P  F  S  S  Q  P  N  L  A  D  T  A  V  Y  N  S  F  L  D
1561  ACTCCCTTCAGTTCACAACCAAATTTAGCTGATACAGCAGTTTACAACAGCTTTCTAGAC  1620

      Q  E  V  I  S  D  S  R  L  S  T  N  P  L  Q  N  H  Q  N  S
1621  CAAGAAGTTATAAGTGATTCAAGACTATCAACCAACCCTCTCCAGAACCATCAGAACAGC  1680

      L  T  L  T  D  N  Q  F  Y  D  T  D  G  V  H  T  D  E  L  Y
1681  CTTACCCTTACAGATAACCAGTTCTATGACACCGATGGTGTCCACACTGATGAGCTCTAT  1740

      Q  S  F  Q  L  D  T  N  I  L  Q  S  Y  N  H  *
1741  CAGTCTTTCCAGTTAGATACAAACATATTACAAAGCTATAACCATTGAGCCGGCACTAGG  1800

1801  CTGAGGTAGGCACACAGAGCTTTACGAAGGAGTAACTCACCCTTCTGCTTTCTCTTTCAG  1860

1861  AATGCTACTGTGTAAATCTCACGGTGTAACTTAAAGTTTTTATATATATATATATTGTC  1920

1921  AGCCCCCAAACTGTTGCCCTTGAAGAAGCATTTAGTGTGTACCTTCAAAGCTCTTAAACA  1980

1981  TTTTTTATGTGTTGGAAAAAGGAATTACTTAAAGCATTGGGAAGAAAAGTGTGTATCTTC  2040

2041  AGAAGGTCAAATTTTCAATAATTCACAGGTTAATACTGTTTGGAAATAAAACATTTTGCT  2100

2101  TATGGAAATTAAATTAACATTTCAAAAGACAAACTAGGAAAAAACTGCATGTGGGCACTC  2160

2161  TAGTTCAGACTCACCTAACATTTATGTTTTATTCAACTTGCTTGAAAGATTTAGGTTTCT  2220

2221  TACCCTCTTTCGCATAAAATATTTTTCTCCTAAAACAAAATCATGCATTGATTTTTTTTT  2280

2281  CTATATTTCACCGCATTCCATTTGCTTTCAAGGCAAATTTACTCTAAAGAAATTTAATGA  2340

2341  CTATGTCATTGTTAGATATGTTTTAAAGACCGGAAGGTTTGAGTTTTAAATAGTCCAGCT  2400

2401  CTCTTGTAAATGCATCACTGCACAAAAGAATGAGCAGAAAGTAAGGACTTCTCTCGCATC  2460

2461  ACCCAAGTGCACTATTTTAAATGAGTAAAACCCTGTTGCTTTAGTAATGGAGCATGCTGT  2520

2521  TATTTCTCTGGAATTTGTTTTTTTTCTTTGTGTGTGTGTGTGTGTGTGTGTAGCCATG  2580

2581  ATGTGCATTTTATTTTGAACTGCAGAAATGTATTGAGCCGAAGCACCTACCTGGTCCTTC  2640

2641  CTCAGATGGCCCAGCAGTTTCCTGCTATGCTTGTGACTGCTGAAGGGCTCAGGTCTGGCT  2700

2701  CCCTAGCTCCCCTCACAGGAGGAGGTGAAATGCAGCCCACTCCCAGTGCGTTGTATGAAC  2760
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FIG. 2—*Continued.*

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2761  AGTGTGAGGCTGTTCCAATCAGTGTCAATTAAAAACAGCCTCCAGCCGCGGATATCAAGA  2820
2821  ACCAGAACTTCCTTCTTCTGCTAGCTTCCCAGTCTCCACTCTGAGGCCTGAGGTGTGAGA  2880
2881  TGTCCAGTGACAGCCTCGCATAGGTATCTGGCTAGGTAAGGCACTCAAGTCTGCATTATA  2940
2941  GGGCTTTGCTATTTTATTACTAATGTACGAAGCAACACAGCAAGAAACATACTGGTGTAT  3000
3001  TTATTTATACAGTGGCAGATAAGGCTTAAGGCTTAAGAACCTTATTTTTACTGTCTGGCT  3060
3061  ATCTAAAAATGCACACTTGGAGACATAGGGAACAGAAACCGCAACACAAGACAAAAAGCC  3120
3121  ACTTGAGATGTACAGTCATATACCAGATAGAAAGGAACACCAAAAACCACCGAGGTTTAA  3180
3181  GGGAAAGCAGTTAATTTACTCAAATGCAGTAATTCACTGCATGCAGAGCAATGAGGTGCT  3240
3241  GCAGCTGTAATGCATTATATCAATTACACGGGTCTGACAGAATGGGCTCTTCCACACTGT  3300
3301  ACAAATGAAGTCAGGAAAACTGTTCCTGTAACCCCATGTACCCAACTCCACACAGCAGTT  3360
3361  TTTCCTCACTTCTGCAGCTGCAGCACACTTTCCAGAAGCATGAAAGAGATACAGAGAACG  3420
3421  CTTGCAATGTGTCGTTTATTCAGTTCTTCCTTTTAAGTTCTCAATGTTTAAGTTTATTGA  3480
3481  ATGTAAACATTTTCTTTATAGAAGGCTCTTTATAGCACAATTTGTTTTTACAGTATAATT  3540
3541  AAATATTTTCAAAATTCTGTTTTTCTTTGTAAAAAAAAAAAAAA  3583
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FIG. 2—*Continued.*

amino acid positions. This region includes the homology to *dorsal* (amino acid residues 4 to 292). The C-terminal regions of the two c-*rel* proteins are more divergent, with 24 of 295 residues differing between the chicken and turkey c-*rel* proteins. The recombination with *env* sequences during the formation of v-*rel* occurred at amino acid 478, with the result that the v-*rel* protein lacks 118 residues at its C terminus that are present in both the turkey and chicken c-*rel* proteins.

Expression of chimeric v/c-*rel* proteins in fibroblasts. Several fusions were constructed between the turkey-derived v-*rel* gene and the chicken c-*rel* cDNA (Fig. 4). The chimeric v/c-*rel* genes encoded proteins of the expected size, as determined by immunoprecipitation of ^{35}S-labeled cell lysates (Fig. 5). The v-*rel* protein and the vcv-*rel* proteins were both 59 kDa, whereas the vvc-*rel* and vcc-*rel* proteins were 67 kDa.

The chimeric v/c-*rel* genes were inserted into a retrovirus vector, and CEF were transfected in the presence of helper virus DNA. To determine the cytological location of the proteins in CEF, the transfected cells were examined by indirect immunofluorescence, using antiserum directed against the v-*rel* protein (Fig. 6). The v-*rel* protein was located predominantly in the nucleus of infected CEF, as previously determined (11), as was the protein encoded by the vcv-*rel* virus. However, the proteins encoded by the vvc-*rel* and vcc-*rel* viruses were found to be located in the cytoplasm of more than 90% of the CEF that were positive for *rel* protein expression. A similar result was obtained when cells of dog retroviral helper cell lines (47) expressing the recombinant v/c-*rel* proteins were examined by indirect immunofluorescence (data not shown). The previously identified NTS of the v-*rel* gene (12) was found to be conserved in the chimeric v/c-*rel* proteins; however, its presence was not sufficient to cause the vvc- and vcc-*rel* proteins to be localized to the nucleus in CEF. These results indicated that the c-*rel* protein contains sequences at its C terminus that are important for cytoplasmic localization in CEF and suggested that these sequences might function as a cytoplasmic retention signal.

Deletions at either the N or the C terminus of the c-*rel* protein are sufficient to allow nuclear localization. To determine whether the C-terminal c-*rel* sequences function as a cytoplasmic retention signal, we first determined whether the C-terminal c-*rel* sequences could also prevent nuclear transport of proteins that contained other well-characterized NTSs. Previous studies demonstrated that the presence of the NTS from SV40 large-T antigen efficiently targets the v-*rel* protein to the nucleus (12). We therefore inserted the coding sequence for the NTS of SV40 large-T antigen into the vvc-*rel* gene, creating vvc18-*rel* (Fig. 4). Virus stocks of vvc18-*rel* were made in CEF, and the cellular location of the 69-kDa vvc18-*rel* protein was determined by indirect immunofluorescence. The vvc18-*rel* protein was predominantly nuclear, although a low level of cytoplasmic staining could be detected in 30 to 50% of the cells expressing the vvc18-*rel* protein (Fig. 6). Another virus, vvc15-*rel*, encoded a protein in which the NTS of SV40 large-T antigen was located at the N terminus instead of internally as in the vvc18-*rel* protein. The vvc15-*rel* protein was exclusively nuclear in location (data not shown). These results indicate that the C-terminal c-*rel* sequences do not function as a dominant cytoplasmic

FIG. 3. Comparison of the predicted amino acid sequences of the chicken (top line) and turkey (middle line) c-*rel* proteins and the turkey-derived v-*rel* protein (bottom line). The predicted amino acid sequence of the turkey c-*rel* protein is given in single-letter code, and the dashed lines above and below indicate that identical residues are present in the chicken c-*rel* protein and the turkey-derived v-*rel* protein, respectively. Symbols: x, deletions of one and three amino acids that are present in the v-*rel* protein; *, C terminus. The *env*-derived amino acids at the N and C termini of the v-*rel* protein are also shown.

retention signal in the presence of the nuclear targeting signal of SV40 large-T antigen.

We also constructed an in-frame deletion of 507 nucleotides in vvc-*rel*, forming asx-*rel*. This deletion resulted in the removal of 169 amino acids from the vvc-*rel* protein (Fig. 4). The asx-*rel* virus encodes a *rel* protein of approximately 54 kDa (Fig. 5B). This protein migrated slower in sodium dodecyl sulfate-polyacrylamide gels than expected from its calculated molecular mass. The cellular location of this protein was examined in CEF. It was exclusively nuclear in location (Fig. 6). This result indicates that deletions at either the C terminus (v-*rel* and vcv-*rel* proteins) or in the N-

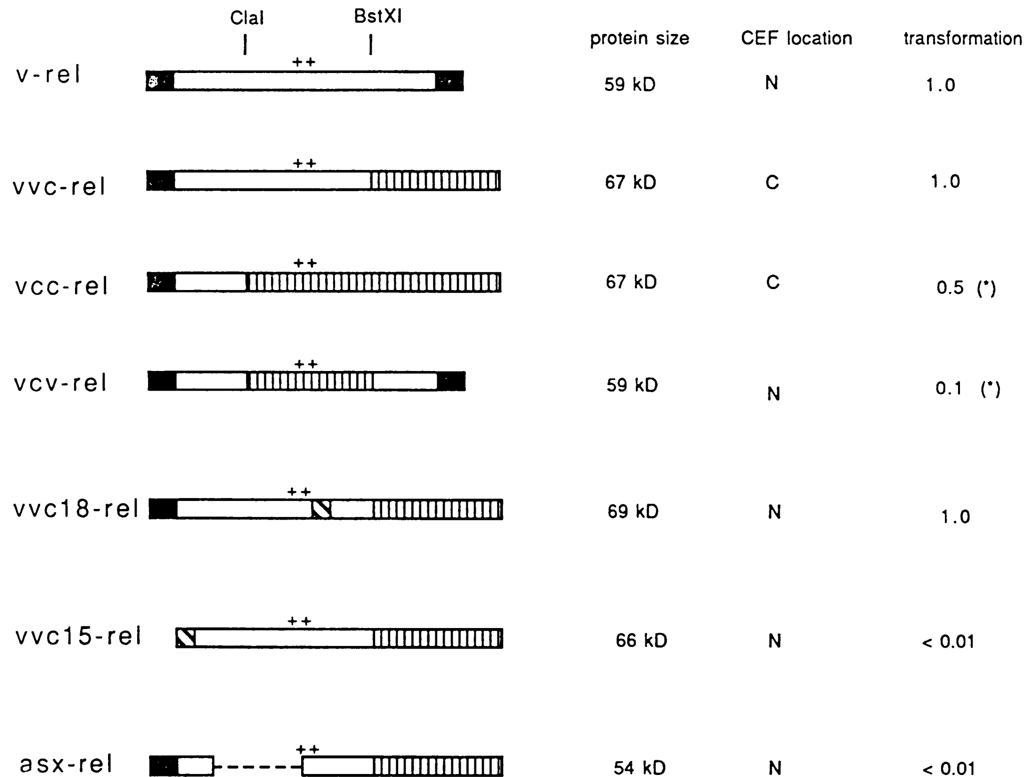

FIG. 4. (A) Structures of v/c-*rel* chimeric proteins used. Symbols: □, v-*rel* protein; ▥, sequences encoded by the c-*rel* gene; ■, *env*-derived sequences that are present at the N and C termini of the v-*rel* protein; + +, NTS of the v-*rel* protein (12); ▧, short stretch of amino acids corresponding to the NTS of SV40 large-T antigen; – – –, deletion of 169 amino acids in the asx-*rel* protein. The drawing is not exactly to scale. Locations of the *Cla*I and *Bst*XI restriction sites used to construct the chimeric v/c-*rel* genes are indicated. (B) Abbreviations and symbol: N, nuclear; C, cytoplasmic; (*) spleen cells obtained in the transformation assays were not capable of immortalized growth in liquid culture. Transforming activity of the chimeric v/c-*rel* proteins is relative to that of the wild-type v-*rel* protein.

terminal region (asx-*rel*) allow nuclear localization of the respective proteins. This result indicates that the *rel* nuclear localization sequence is very context dependent and suggests that the *rel* nuclear localization sequence may in fact be cryptic and revealed only by deletions within the protein.

Transforming potential of chimeric v/c-*rel* proteins. The transforming potential of the recombinant v/c-*rel* proteins was determined by infection of primary chicken spleen cells with virus stocks generated in CEF. Both the wild-type v-*rel* and the vvc-*rel* viruses were able to transform spleen cells efficiently, as measured by colony formation in soft agar. Colonies that arose in the transformation assays could be expanded into permanent cell lines at a frequency of 25 to 50%. However, the vcv-*rel* and vcc-*rel* viruses had a reduced transformation efficiency, to approximately 50 and 10% of wild-type v-*rel* for the vcc-*rel* and vcv-*rel* viruses, respectively. No permanent cell lines could be established from the colonies obtained after infection of spleen cells with the vcc-*rel* or vcv-*rel* viruses in 25 attempts. Indirect immunofluorescence of spleen cells from colonies soon after transfer into liquid culture demonstrated that the wild-type v-*rel* protein and the chimeric v/c-*rel* proteins were located in the cytoplasm (data not shown).

Transactivation properties of chimeric v/c-*rel* proteins. The v-*rel* protein has recently been shown to transactivate gene expression of the polyomavirus late promoter in Rat-1 cells (10). Plasmids were constructed that expressed the v-*rel* and v/c-*rel* proteins from the major immediate early promoter of human CMV to provide for high levels of *rel* expression in Rat-1 cells. The localization of the v-*rel* and v/c-*rel* proteins in Rat-1 cells was determined by indirect immunofluorescence. Rat-1 cells were transfected with the respective plasmids, transferred the next day onto cover slips, and processed for indirect immunofluorescence 2 days after transfection. Whereas the v-*rel* and vcv-*rel* proteins were located in the nucleus, the larger vvc-*rel* and vcc-*rel* proteins were located in the cytoplasm (Fig. 7). Addition of the NTS from SV40 large-T antigen to the vvc-*rel* protein (vvc18-*rel*) resulted in efficient transport of the protein to the nucleus (data not shown). The location of the *rel* proteins in Rat-1 cells paralleled their location in CEF and indicates that the size of each *rel* protein determines its location in fibroblasts, even in the presence of the NTS present in the v-*rel* protein.

To determine the transactivating properties of the chimeric v/c-*rel* proteins, plasmids expressing the chimeric v/c-*rel* proteins from the CMV promoter were cotransfected into Rat-1 cells with a plasmid expressing the CAT gene from the polyomavirus late promoter. Expression of the *rel* proteins was confirmed by immunoblotting of protein extracts from the transfected cells (data not shown). All of the chimeric v/c-*rel* proteins were able to transactivate expression from the polyomavirus late promoter (Table 1). Chimeric proteins that contained the C terminus of the c-*rel* protein (vvc-*rel*, vvc18-*rel*, and vcc-*rel*) gave the highest levels of transactivation of the polyomavirus late promoter (approximately 20-fold activation), whereas the vcv-*rel* protein gave consistently lower levels of transactivation (approximately 10-fold activation). Surprisingly, the lowest level of transactivation of the polyomavirus late promoter

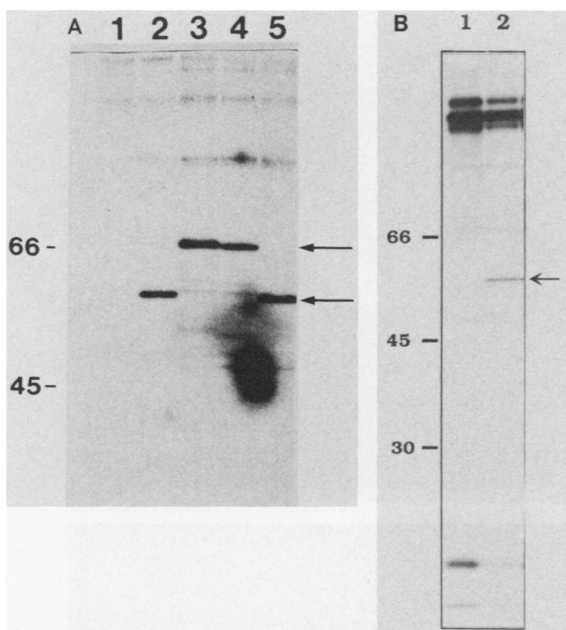

FIG. 5. Immunoprecipitation of v- and v/c-*rel* proteins (A) Cos-1 cells transiently expressing the various v/c-*rel* genes were labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with anti-*rel* antiserum. The immunoprecipitates were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel, and the immunoprecipitated proteins were visualized by autoradiography. Arrows indicate the respective v/c- or v-*rel* proteins. Lanes: 1, mock-transfected cells; 2, vcv-*rel*-transfected cells; 3, vcc-*rel*-transfected cells; 4, vvc-*rel*-transfected cells; 5, v-*rel*-transfected cells. Molecular size markers (shown on the left): bovine serum albumin, 68 kDa; ovalbumin, 45 kDa. (B) Labeled cell lysates of CEF infected with the indicated viruses analyzed as described above. The arrow indicates the 54-kDa asx-*rel* protein in lane 2. Size markers (shown on the left): bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa.

(approximately twofold) was observed with the wild-type v-*rel* protein.

Transactivation by the chimeric v/c-*rel* proteins is dose responsive. To determine whether levels of transactivation correlated with the relative levels of *rel* protein, similar experiments were performed, using the SNV LTR to express the *rel* proteins. The SNV LTR is approximately 10-fold less active than the CMV promoter as a promoter in Rat-1 cells (data not shown). Expression of *rel* proteins at the lower level obtained from the SNV promoter in Rat-1 cells also resulted in transactivation of expression from the polyomavirus late promoter, although the levels of transactivation were markedly lower (Table 1). In these experiments, expression of the vvc-*rel* protein was found to result in the highest level of transactivation (approximately sixfold), whereas expression of the other v/c-*rel* proteins and the wild-type v-*rel* protein gave lower levels of transactivation (two- to fourfold). The low activity of the v-*rel* protein in these experiments is in contrast to the higher levels (up to 25-fold) that were previously reported for transactivation by the v-*rel* protein of the polyomavirus late promoter (10). The most likely explanation for this discrepancy is differences in transfection efficiency between the respective experiments. However, as indicated by the standard deviations reported in Table 1, the results that we obtained in this study are reproducible. These results indicated that there is a dose-response relationship between the level of expression of the chimeric v/c-*rel* proteins and the level of transactivation obtained. However, transactivation of the polyomavirus late promoter by the wild-type v-*rel* protein was not affected by the level of v-*rel* protein expression. Since high levels of expression of the wild-type v-*rel* protein have been shown to be cytotoxic to Rat-1 cells (10), this result raises the possibility that transactivation by the wild-type v-*rel* protein is masked by its cytotoxic effect.

Expression of both v-*rel* and v/c-*rel* proteins is cytotoxic to Rat-1 cells. To examine the possibility that expression of only the v-*rel* protein is cytotoxic to Rat-1 cells, the plasmids expressing the v- and v/c-*rel* proteins from the CMV promoter were transfected into Rat-1 cells along with a plasmid expressing the hygromycin resistance gene. After selection in the presence of hygromycin for 2 weeks, a reduction in the number of surviving colonies was obtained in the presence of plasmids expressing either the v-*rel* protein or the chimeric v/c-*rel* proteins (Table 2). The surviving colonies did not express detectable levels of *rel* protein, as determined by indirect immunofluorescence of pooled surviving cells (data not shown), whereas transiently transfected cells expressed easily detectable levels of *rel* proteins (Fig. 7). These results demonstrate that expression of a high level of either the v-*rel* protein or the v/c-*rel* proteins is cytotoxic to Rat-1 cells. Since expression of all of the *rel* proteins could be detected in Rat-1 cells at 2 days after transfection by both indirect immunofluorescence (Fig. 7) and immunoblotting (data not shown), these results indicate that cytotoxicity of the v-*rel* protein cannot account for the observed differences in transactivation of the polyomavirus late promoter by the v-*rel* or the v/c-*rel* proteins in these transient assays.

DISCUSSION

Our results indicate that *rel* proteins which are located in either the cytoplasm or the nucleus are effective transactivators of gene expression from the polyomavirus late promoter. Thus, cytological location (cytoplasmic versus nuclear) is unimportant for both transformation (12) and transactivation by *rel* proteins. Our results suggest that transactivation by *rel* proteins is not through the direct action of *rel* proteins as transcription factors but is indirect and mediated by other cellular factors. Two recent reports have suggested that the v-*rel* protein is complexed with several other proteins in transformed spleen cells, including a serine/threonine protein kinase (35, 43). An attractive hypothesis for the mechanism of transformation by v-*rel* is that association of the v-*rel* protein with this protein kinase modulates its activity. The *rel*-induced modulation of this cellular protein kinase activity then results in alteration of gene expression and eventual transformation. It will be of interest to determine whether *rel* proteins are associated with a protein kinase in rodent fibroblasts and whether this association is required for transactivation of gene expression.

An alternative explanation of our results is that a low level of nuclear *rel* protein is, in all cases, responsible for the observed activation of gene expression. Although we cannot eliminate this possibility, comparison of the vvc-*rel* and vvcl8-*rel* proteins provides a strong argument against this explanation. If only the nuclear form of the *rel* protein can transactivate gene expression, then the vvcl8-*rel* protein, located in the nucleus, should transactivate much more efficiently than the vvc-*rel* protein that is located in the cytoplasm. This should be particularly noticeable when these proteins are expressed at low levels from the SNV

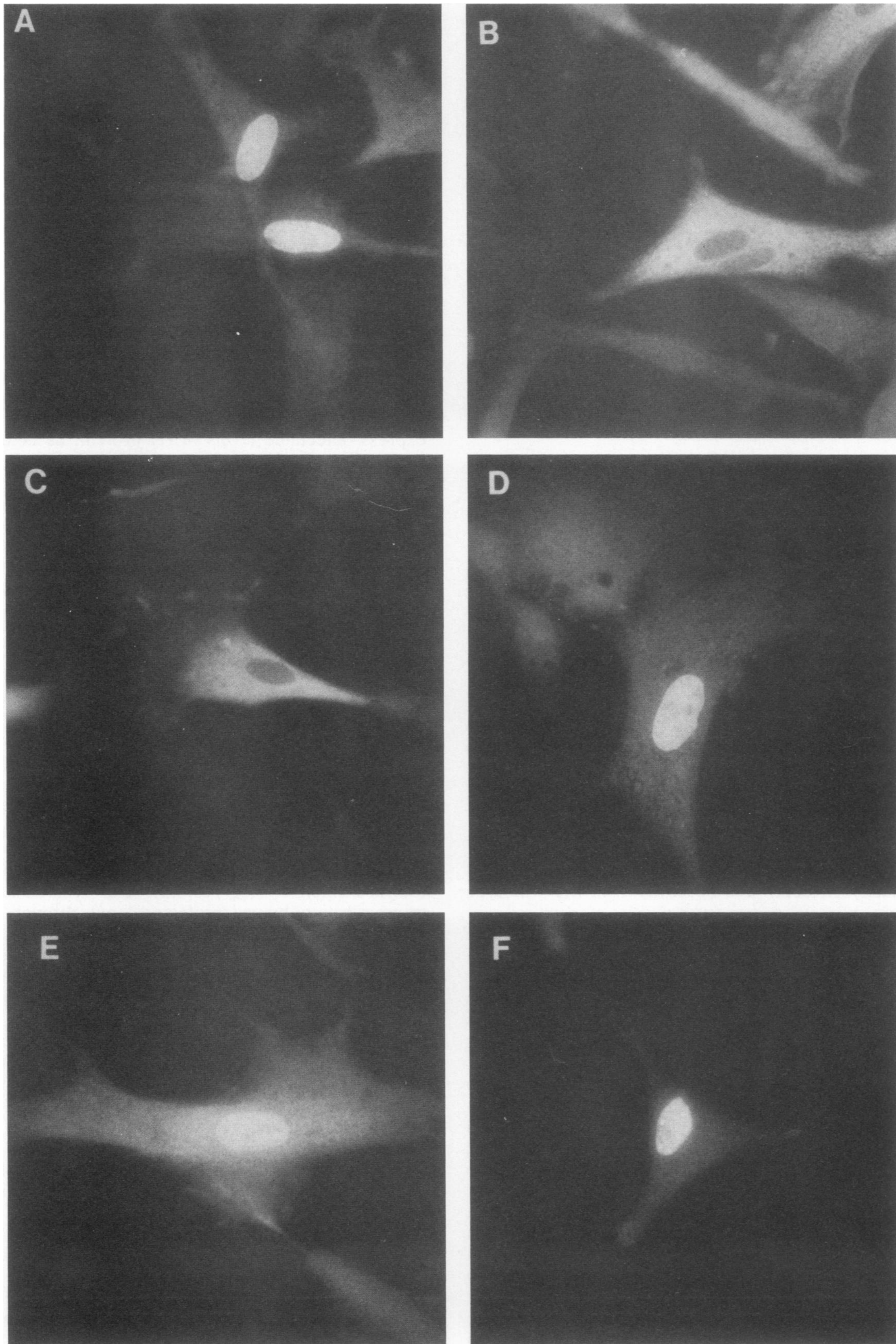

FIG. 6. Indirect immunofluorescence of *rel* proteins in CEF. CEF were transfected with retroviral vectors expressing the various *rel* proteins along with helper virus DNA. The cells were transferred onto cover slips 5 days after transfection and processed for immunofluorescence the next day. (A) Nuclear localization of the vcv-*rel* protein; (B) cytoplasmic localization of the vcc-*rel* protein; (C) cytoplasmic localization of the vvc-*rel* protein; (D) nuclear localization of the v-*rel* protein; (E) nuclear localization of the vvc18-*rel* protein; (F) nuclear localization of the asx-*rel* protein. The results shown are representative of more than 90% of the cells that were positive for *rel* expression.

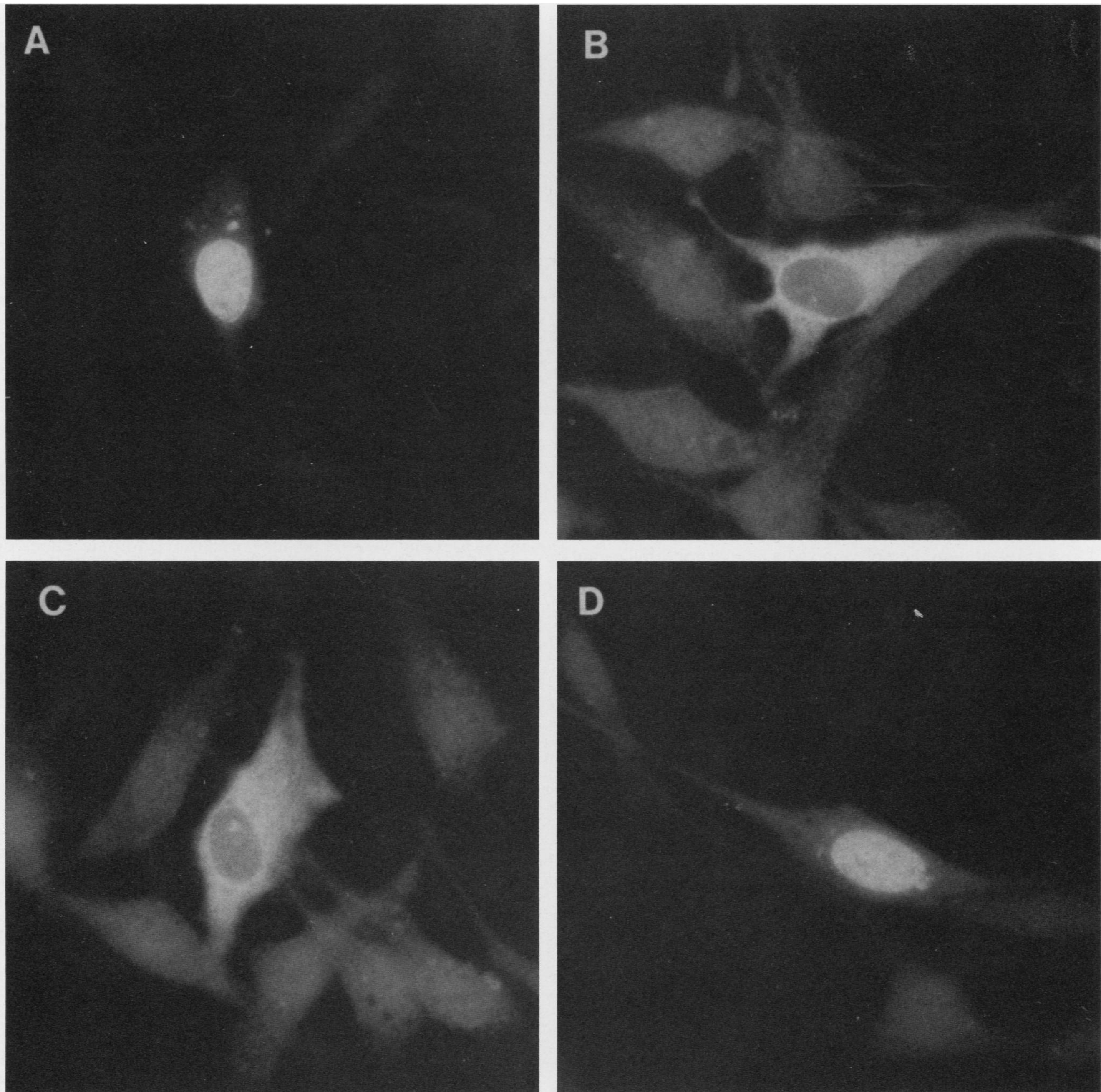

FIG. 7. Indirect immunofluorescence of *rel* proteins in Rat-1 cells. Rat-1 cells were transfected with recombinant plasmids in which the CMV promoter was used for expression of the various *rel* proteins. The cells were transferred onto cover slips the day after transfection and processed for immunofluorescence the next day. (A and D), Nuclear location of the vcv-*rel* and v-*rel* proteins, respectively; (B and C) cytoplasmic location of the vcc-*rel* and vvc-*rel* proteins, respectively. The results shown are representative of more than 50 cells that were positive for *rel* expression examined for each plasmid in at least two independent experiments.

LTR (when the putative cellular target of these proteins is not saturated). This is not the case (Table 1). When these proteins are expressed at low levels from the SNV LTR, a low level of transactivation is obtained that is similar for the respective proteins. Increasing the level of expression of these *rel* proteins also increases, in a parallel manner, the level of transactivation observed. These results indicate that the cytoplasmic vvc-*rel* protein is as effective as the nuclear vvc18-*rel* protein in transactivation of gene expression. Transactivation correlates with the total amount of *rel* protein that is present in the cell, not with the level of *rel* protein that is present in the nucleus.

In this study, we have examined changes in the level of CAT activity from the polyomavirus late promoter as a measure of transactivation of gene expression by *rel* proteins. Although we have not examined message levels directly, a previous study provided evidence that expression of the v-*rel* protein does result in an increase in the steady-state level of RNA from the polyomavirus early promoter (10). Those experiments did not allow determination of the

TABLE 1. Transactivation of gene expression from the polyomavirus late promoter by *rel* proteins[a]

Gene	Rat-1 location[b]	Transactivation[c]	
		CMV *rel*	SNV *rel*
vcv-*rel*	N	10.5 (±4.2)	2.4 (±0.8)
vcc-*rel*	C	17.3 (±8.6)	2.9 (±1.0)
vvc-*rel*	C	18.3 (±6.3)	5.7 (±1.8)
vvc18-*rel*	N	15.5 (±2.5)	4.2 (±0.5)
v-*rel*	N	2.1 (±0.9)	2.2 (±0.9)

[a] Rat-1 cells were cotransfected with plasmids in which the v-*rel* or the v/c-*rel* proteins were expressed from either the CMV promoter or the SNV promoter along with a plasmid in which the CAT gene was expressed from the polyomavirus late promoter. Cell lysates were collected 2 days after transfection, and the level of CAT activity present in the cell lysates was determined.

[b] N, Nuclear; C, cytoplasmic.

[c] Results are averages from at least four independent experiments and are presented as the fold induction of CAT expression in the presence of the respective *rel* protein as compared with cotransfection with a plasmid that contained either the CMV or SNV promoter but did not contain any *rel* coding sequences. Numbers in parentheses are standard deviations.

level (transcription, transport, or stability) at which the v-*rel* protein acts to increase steady-state levels of message. Thus, our use of the term "transactivation" to describe the effects of *rel* expression on the polyomavirus late promoter is meant to be a general term indicating an increase in expression of a recorder gene without specifying that *rel* proteins act at the level of transcription.

The difference in levels of transactivation between the wild-type v-*rel* protein and the v/c-*rel* proteins is puzzling. Whereas expression of the v/c-*rel* proteins clearly activates gene expression from the polyomavirus late promoter in a dose-dependent manner, expression of the wild-type v-*rel* protein results in a low level of activation of gene expression from the polyomavirus late promoter and is not dose dependent. A possible explanation is that the putative cellular target that is responsible for the *rel*-induced activation of gene expression is easily saturated by expression of the wild-type v-*rel* protein, with the result that only a low level of activation is obtained. This putative cellular target would be less easily saturated by the chimeric v/c-*rel* proteins, with the result that a dose-response effect is observed. Other explanations, including inhibitory effects of the v-*rel* protein on gene expression, cytotoxicity of the v-*rel* protein (see below), or both are also possible, and we are currently attempting to distinguish among these possibilities.

Another explanation for the observed dose-independent activation of gene expression by the v-*rel* protein is that the v-*rel* protein is more cytotoxic than the v/c-*rel* proteins, such

TABLE 2. Cytotoxicity of the *rel* proteins in Rat-1 cells[a]

Gene	Cytotoxicity[b]	
	Expt 1	Expt 2
vcv-*rel*	90	140
vcc-*rel*	40	60
vvc-*rel*	40	60
v-*rel*	20	25
Control	407	295

[a] Rat-1 cells were cotransfected with plasmids expressing the various *rel* genes from the CMV promoter and a plasmid expressing the hygromycin resistance gene from the SV40 early promoter (pSVHy) or with pSVYy alone (control).

[b] Number of surviving hygromycin-resistant colonies obtained after 2 weeks of selection. Results are from two independent experiments.

TABLE 3. Summary of comparison of *rel* proteins[a]

Gene	Transformation	Immortalization	Transactivation	Cytotoxicity	Location
vcv-*rel*	+	−	++	+	N
vcc-*rel*	++	−	+++	++	C
vvc-*rel*	+++	+	+++	++	C
v-*rel*	+++	+	+	+++	N

[a] Structures of the *rel* proteins are shown in Fig. 4. Transformation refers to the ability of SNV-derived viruses expressing each *rel* protein to cause chicken spleen cells to form colonies in soft agar. Immortalization refers to the ability of the colonies obtained in the soft agar assay to grow indefinitely in liquid culture. Transactivation refers to the ability of each *rel* protein to transactivate gene expression from the polyomavirus late promoter. Cytotoxicity refers to the decrease in the number of drug-resistant colonies obtained in the presence of each *rel* protein compared with the number of drug-resistant colonies obtained with the selective marker alone. Location refers to the cellular location (nuclear [N] or cytoplasmic [C]) of each *rel* protein in both CEF and rat fibroblasts. Plus and minus signs indicate the extent to which each *rel* protein possesses the indicated property.

that the activation of gene expression by v-*rel* is masked by its cytotoxic effect. However, since expression of the wild-type v-*rel* protein could be detected in Rat-1 cells 2 days after transfection, the cytotoxic effect of v-*rel* expression is not the result of loss of cells during the transient assay that are expressing v-*rel*. Furthermore, the cytotoxic effect of expression of the v-*rel* protein in Rat-1 cells is dose responsive (10). Therefore, the cytotoxic effect of v-*rel* expression cannot solely account for the dose-independent activation of gene expression by the v-*rel* protein.

The basis for the cytotoxic effect of *rel* expression in rodent fibroblasts is not known but probably is the result of *rel*-induced alterations in gene expression that result in growth arrest of the cell. Thus, a protein that is highly transforming in one cell type by virtue of presenting a strong stimulatory growth signal to the cell is cytotoxic in another cell type as a result of a growth-inhibitory signal. A similar relationship exists between the transforming and cytotoxic properties of the *src* protein, for which alteration of the level of expression of the *src* gene (28, 41), or its association with the plasma membrane (29, 41), is sufficient to convert a transforming protein into a cytotoxic protein. The latent membrane protein of Epstein-Barr virus is also transforming when expressed at a low level in rodent fibroblasts but cytotoxic when its level of expression is increased (15a). These results, and the recent characterization of tumor suppressor genes (24), indicate a fundamental overlap between growth-stimulatory and growth-inhibitory pathways that is just beginning to be appreciated.

Transactivation of gene expression in Rat-1 cells does not correlate well with cellular transformation of chicken spleen cells by *rel* proteins (Table 3). The v/c-*rel* proteins containing internal chicken c-*rel*-derived sequences (vcv-*rel* and vcc-*rel*) transformed spleen cells at a lower efficiency than did the proteins containing internal v-*rel*-derived sequences (v-*rel*, vvc-*rel*, and vvc18-*rel*). In addition, the colonies that did arise in the spleen assays with the vcv-*rel* and vcc-*rel* viruses did not have the potential for immortal growth in liquid culture. The importance of internal amino acid alterations in the v-*rel* protein for immortalization has previously been described in recombinants between the turkey-derived c-*rel* gene and the v-*rel* gene (40), and our results for the chicken-derived c-*rel* cDNA are consistent with those. However, all of the chimeric v/c-*rel* proteins were able to transactivate gene expression in Rat-1 cells. Furthermore, both the wild-type v-*rel* and the vvc-*rel* proteins are able to transform spleen cells efficiently, yet the vvc-*rel* protein is able to

transactivate expression of the polyomavirus late promoter much more efficiently than can the wild-type v-*rel* protein.

One explanation is that the transformation assay and the transactivation assay measure unrelated activities of *rel* proteins. According to this viewpoint, the activity of *rel* proteins that results in alterations of gene expression in Rat-1 cells is irrelevant to the activity of *rel* proteins that leads to transformation of chicken spleen cells. However, the fact that expression of *rel* proteins in Rat-1 cells has both a biochemical effect (alteration of gene expression) and a biological effect (cytotoxicity) argues against this viewpoint, although this possibility cannot be ruled out at present. We suggest instead that the activity of the *rel* protein detected in the transactivation assay is a subset of the activities of the *rel* protein that are involved in the transformation process. In this view, expression of the *rel* protein in spleen cells results in the occurrence of certain cellular events, one (or a few) of which is reflected in the transactivation assay. Thus, transactivation of gene expression by *rel* proteins may be necessary but is not sufficient for transformation. In either case, alterations in gene expression are clearly a cellular response to expression of both the viral and cellular *rel* proteins, and elucidation of the mechanism by which *rel* proteins affect gene expression will contribute to our understanding of intracellular signaling mechanisms.

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ADDENDUM IN PROOF

The sequence reported in this manuscript has been deposited in the Genbank database and has been assigned the accession number M26381.

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