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The Mouse c-*rel* Protein Has an N-Terminal Regulatory Domain and a C-Terminal Transcriptional Transactivation Domain

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We have shown that the murine c-*rel* protein can act as a transcriptional transactivator in both yeast and mammalian cells. Fusion proteins generated by linking *rel* sequences to the DNA-binding domain of the yeast trascriptional activator GAL4 activate transcription from a reporter gene linked in *cis* to a GAL4 binding site. The full-length mouse c-*rel* protein (588 amino acids long) is a poor transactivator; however, the C-terminal portion of the protein between amino acid residues 403 to 568 is a potent transcriptional transactivator. Deletion of the N-terminal half of the c-*rel* protein augments its transactivation function. We propose that c-*rel* protein has an N-terminal regulatory domain and a C-terminal transactivation domain which together modulate its function as a transcriptional transactivator.

The immediate cellular response to external stimulus culminates in the induction of a large set of nuclear genes. Some of these early response genes are proto-oncogenes, and their products have been shown to be involved in the transcription of other genes (1, 9, 13, 17, 32, 33, 41, 42). The theme of oncogenes as transcriptional factors or cofactors has been well illustrated by the Fos-Jun paradigm (13). The two nuclear oncoproteins associate to form heterodimers which bind to their cognate DNA motif to promote transactivation (12, 50). More recently, the product of the proto-oncogene *myb* has also been shown to act as a transcriptional activator (42, 62). Interestingly, the BAS1 protein, which is required for activation of GCN4-independent (basal) *HIS4* transcription in yeast, shares homology to the N-terminal region of *myb* protein (58). The c-*rel* gene is a member of the immediate-early response gene family and is induced by serum and phorbol ester (TPA) (9). Its viral homolog, the v-*rel* protein, has been shown to function as a transcriptional activator in certain cell types (20, 25).

v-*rel* is the resident transforming gene of reticuloendotheliosis virus strain T, a highly oncogenic avian retrovirus that induces a rapidly fatal lymphoma in young birds (18, 57). Reticuloendotheliosis virus strain transforms only avian lymphoid cells, and despite high levels of expression in chicken embryo fibroblasts, no transformed phenotype is evident (10, 23, 54). The product of v-*rel* is a 59-kilodalton phosphoprotein that is located in the cytoplasm of transformed spleen cells and in the nucleus of the nontransformed fibroblasts (10, 19, 22, 54). However, subcellular localization appears to be irrelevant, because both cytoplasmic and nuclear forms of the v-*rel* protein can induce cellular transformation (23, 25). The v-*rel* protein has been shown to associate with a number of cellular proteins, including a closely associated serine-threonine protein kinase in both untransformed chicken embryo fibroblasts and transformed spleen cells (15, 53, 60). The c-*rel* protein is cytoplasmic, and the major c-*rel* mRNA species in avian cells is 4.5 kilobases, whereas in mouse and human cells it is 7.5 and 10 kilobases, respectively (8–10, 26). Although c-*rel* expression is seen in many cell types, high levels are observed only in lymphoid cells (6, 8).

The c-*rel* protein shares extensive homology with the *Drosophila dorsal* protein, encoded by a maternal effect locus essential for the establishment of dorsal-ventral polarity in the developing embryo (2, 55). The N-terminal 300 amino acids of avian, murine, and human *rel* proteins have nearly 45 to 50% identity with the N-terminus of the *dorsal* protein (55; Fig. 1), but the C-terminal halves of these proteins are quite dissimilar. The *dorsal* protein is uniformly distributed throughout the cytoplasm of early embryos, but 90 min after fertilization, the *dorsal* protein present in ventral, but not dorsal, regions is selectively transported to the nucleus. Thus, there is a graded distribution of the maternal morphogen *dorsal* initiated by nuclear transport (45, 46, 56). Because of the sequence homology between *dorsal* and *rel*, their apparent dual cytoplasmic and nuclear distribution in the cell, and their ability to activate transcription of certain promoters, we undertook a detailed analysis of the mechanism of transcriptional activation by c-*rel*. We show that the murine c-*rel* protein is a potent transcriptional activator in both *Saccharomyces cerevisae* and mammalian cells when linked to a sequence specific DNA-binding domain. We also demonstrate that transcriptional transactivation requires the C-terminal portion of the c-*rel* protein. Surprisingly, the full-length c-*rel* protein was 10- to 20-fold less active than the C-terminal fragment. Maximal transcriptional activity of the c-*rel* protein was revealed only if the N-terminal half of the protein was removed. We propose that c-*rel* has a regulatory domain and a transactivation domain which together modulate its function as a transcriptional activator.

MATERIALS AND METHODS

S. cerevisiae strains YM335:171, YM335:1Δ1, and YT6: LEXΔ1(0X2) have previously been described (29, 37). The yeast cells were grown in YPD (rich) or synthetic, defined media (39) at 30°C. The carbon sources for the defined media, where appropriate, were glucose, glycerol, or ethanol at 2% final concentration.

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                                                                      50                                                     100
mouse (PCR)  MASSGYNPYV EIIEQPRQRG MRFRYKCEGR SAGSIPGERS TDNNRTYPSV QIMNYYGKGK IRITLVTKND PYKPHPHDLV GKDCRDGYYE AEFGPERRPL
mouse        .......... .......... .......... .......... .......... .......... .......... .......... .....P.... ..........
human        ...GA....I .......... .......... ........H. .........I .......... V......... .......... .......... ....Q.....
chicken         ISE..I ..F....... .......... ........H. ....K.F..I ..L..F..V. ..T......E ....L..... .......... ........V.
turkey         GISE.CI ..F....... .......... ........H. ....K.F..I ..L..F..V. ..T......E .......... .......... ........V.
v-rel          GISE..I ..F....... T......... ........H. ....K.F..I ..L..F..V. ..T......E .......... ..G....... .......QV.
dorsal       TKNVRKK... K.T...AGKA L....E.... .......VN. .PE.K...TI E.VG.K.RAV .....VTK.. ..R....N.. ..EGCKKGVC TLEINSETMR

             101                                          150
mouse (PCR)  F FQNLGIRC VKKKEVKEAI ILRISAGINP FNVPEQQLLD IEDCDLNVVR LCFQVFLPDE HGNFTTA LP PIVSNPIYDN RAPNTAELRI  CRVNKNCGS
mouse        . ........ .......G.. .......... ...G...... .......... .V.MF..... D...... V. .......... .......... IA........
human        . ........ .......... .T..K..... .....K..N. .......... .......... ...L... .. .V........ .......... .........
chicken      S ......Q. ....DL..S. S....KK... .....EL.HN .DEY...... ....A..... ...Y.L. .. .LI....... ..........  .........
turkey       S ......Q. ....DL..S. S....KK... .....E..HN .DEY...... ....A..... ...Y.L. .. .LI....... ..........  .........
v-rel        S ......Q. ....DL..S. S....KK... .....E..HN .DEY...... ....A..... ...Y.L. .. .LI....... ..........  .........
dorsal       AV.S....Q. ....DIEA.L KA.EEIRVD. .KTGFSHRFQ PSSI...S.. ......MES. QKGRF.SP.. .V..E..F.K K.  MSD.V.  ..LCSCSAT

             200                                                   250
mouse (PCR)  VRGGDEIFLL CDKVQKDDIE VRFVLND     WEARGVFS QADVHRQVAI VFKTPPYCK  AILEPVTVKM QLRRPSDQEV SESMDFRYLP DEKDAYGNKS
mouse        .......... .......... .......     ........ .......... .........  .......... .......... .......... .....A....
human        .......... .......... .......     ...K.I.. .......... .........  ..T....... .......... .......... ....T....A
chicken      .K........ .......... .....DF     ...K.S.. .........S ..R...FLR  D.T..I.... .......... ..P....... ....P...FA
turkey       .K........ .......... .....DN     ...K.S.. .......... ..R...FLR  D.T..I.... .......... ..P....... ....S....A
v-rel        .K........ .......... .....GN     ...K.S.. .......... ..R...FLG  D.T..I.... ........A. ..PV...... ..E.PS...A
dorsal       .F.NTQ.I.. .E..A.E..S ...FEEKNGQ SV...F.D.Q HT...K.T.. T.K....HTL D.T..AK.FI .......GVT ..ALP.E.V. MDS.PAHL.R

                   300                                                  350
mouse (PCR)  KKQKTTLIFQ KLLQDCGHF  TEKPRTAPLG ST GEGRFIK KESNLFSHGT VLPEMPRSSG VPGQAEPYYS SCGSISSGLP HHPPAIPSVA HQPTSWPPVT
mouse        .......... .........  .......... .. ....... .......... .......... .......... .......... .......... ..........
human        .......L.. ..C..HVN.  P.R..PGL.. .I ....YF. ..P.....DA .VR...  T. .SS...S..P .P.P.....S ..    A.M. PL.S..SS.A
chicken      .R.RS..AW. ..I....SAV ..R.KAT.IP TVNP..KL.. ..P.M.. P. LMLPGLGTLT SSS.MY.PC. QMPHQPAQ.G PGKQDTLPSC W.QLFSSSPS
turkey       .R.RS..AW. ..I....SAV ..R.KA..IP TVNP..KL.. ..P.M.. PT LMLPGLGTLA SSS.MY.AC. QMPTQPAQ.G LGKQDTLHSC W.QLYSPSPS
v-rel        .R.RS..AW. .PI....SAV ..R.KA..IP TVNP..KLI  ..P.M.. PT LMLPGLGTL  SSS.M.PAC. QMPTQPAQ.G PGKQDTLHSC W.QLY  SPS
dorsal       Q.TGGDPMHL L.Q.QQKQQL QNDHQDGRQT NMNCWNTQNI PPIKTEPRDT SPQPLGFLI. RLLSSH.RR. R.RHRATTTT TAR.RPTTWP PRSATNGQQQ

                  400                                                   450
mouse (PCR)  HPTSHPVSTN TLSTFSAGTL SSNSQGILPF LEGPGVSDLS    ASNSCLY  NPDDLARME TPSMSPTDLY SISDVNMLST RPLSVMAPST DGMGDTDNPR
mouse        .......... .......... .......... ..........    .......  ......... .......... .......... .......... ..........
human        ...PRSGN.. P..S..TR.. P......P.. .RI.VGN..N    .....I. N.A..IVG.. AS..PSA... G...P....N CSVNMMTT.S .S..E.....
chicken      ACC.LLSMHP HN. .T.EVP QP.A..SSS. .PAFHDNP.N WPDEKD.SF. R.FGSTNG.G AAMV.AADMQ .A.SNSIVHA THQASATAAS IVNME.NDMN
turkey       ASS.LLSMHS HN. .T.EVP QPGA..SSS. .PAYHDNP.N WPDEKD.SFY R.FGNTHG.G AALV.AADMQ .V.SSSIVQG THQASATAAS IMNME.NDMN
v-rel        ASS.LLSLHS HS. .T.EVP QPGA..SSS. .PAY  NP.N WPDEK..SFY R.FGNTHG.G AALV.AAGMQ .V.SSSIVQG THQASATTAS IMTMPRTPGE
dorsal       LMR.ATNGQQ Q.MSPNHPQQ QQQQ.QYGAT DL.SNYNPFA QQVLAQQQQH QQQQQQHQHQ HQQQHQQQQQ QQQQQQQQQS LQFHANPFGN P.GNSWESKF

                        500                                                   550
mouse (PCR)  LVSINLENPS CNARLGPRDL RQLHQMSPAS LSAGT SSSS VFVSQSDAFD RSNFSCVDNG LMNEPGLS   DDANNPTFVQ SSHYSSVNTL QSEQLSDPFT
mouse        .......... .......... .......... ..... .... .......... .......... ........   .......... .... ..... ..........
human        .L.M......  .SV...... .. ....SS. M...ANSNTT .........E G.D...A..S MI..SGP.NS TNP.SHG... D.Q..GIGSM .N.....S.P
chicken      CT.L.F.KYT  QVLNVSNHR Q....APA.C PPVAAPG.TP FSSQPNL.DT AVYSNSFLDQ EVISDSRLSS FPLQ.HQNSL TLDNLFYD.D GVHTDELYQS
turkey       CT.L.F.KYT  QMLNVSNHR Q....VPATC PPGSAWQHSL  SSQPNV.DR AVYSSSF
v-rel        VPRQQVGYR.
dorsal       SAAAVAAAAA TATGAAPANG NSNNLSNLNN PFTMHNLLTS GGGPGNANNL QW.LTTNHLH NQHTLHQQQQ LQQQQQQQYD NTAPTNN.AN LNNNNNNNNT

                 589
mouse (PCR)  YGFFKI*
mouse        ......*
human        .E..QV*
chicken      FQLDTNILQS YNH*

dorsal       A.NQADNNGP TLSNLLSFDS GQLVHINSED QQILRLNSED LQISNLSIST*
```

FIG. 1. Comparison of amino acid sequences of *rel* proteins. The deduced amino acid sequences of mouse PCR (this work), mouse (24), human clone 2 (7) chicken (25), turkey (63), v-*rel* (64), and *Drosophila dorsal* (46, 55) proteins are shown. The alignment was carried out by using the program of Smith and Wassermann (16). The amino acid numbers correspond to the mouse PCR clone. Dots indicate identity. The circled amino acids indicate the differences between the mouse PCR sequence and that of the mouse cDNA sequence (24). Notice that all of the altered amino acids in our sequence (mouse PCR) match with the human and avian c-*rel* sequences.

Plasmid constructions. All DNA manipulations were performed by standard procedures (40). The plasmid constructions were verified by restriction mapping, and the in-frame fusion and deletions were verified by double-stranded sequencing with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio).

cDNA cloning by polymerase chain reaction (PCR). To molecularly clone the murine c-*rel* cDNA, we synthesized the following oligonucleotides as primers: (i) 5′ GCG-GCG-CGC-GGA-GCC-GAG-CC (nucleotides 381 to 400); (ii) 5′ ACT-TAT-TTA-AGT-CCT-ACA-AC (nucleotides 2187 to 2168); (iii) 5′ TCA-CTA-ACT-TCC-TGG-TCA-GAA-GG

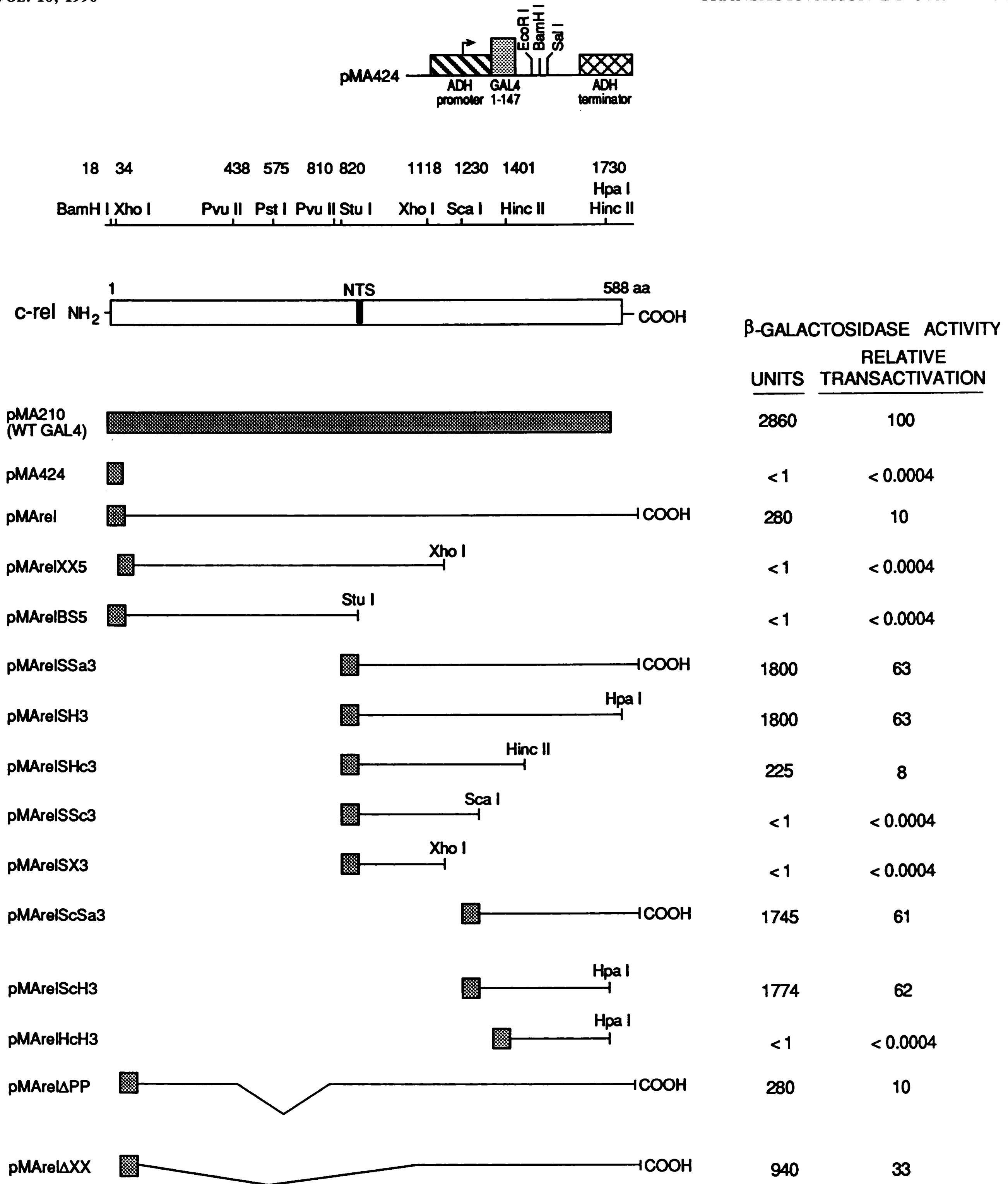


FIG. 2. The mouse c-*rel* protein is a transcriptional activator in yeast cells. A diagram of the parent effector plasmid (pMA424) in which all other fusion genes were constructed is shown. The restriction map of the region containing the c-*rel* coding sequence is also shown. The 588amino-acid-long c-*rel* protein is schematically represented. Symbols: ▮, the putative nuclear transport signal; ▩, pMA210, the wild-type (WT) GAL4 protein effector plasmids encoding various c-*rel* derivatives were linked in frame to the *GAL4* DNA-binding domain (amino acids 1 to 147, indicated as a stippled box) and were constructed by inserting the c-*rel* moiety into the polylinker of pMA424 (38). Plasmids expressing the *GAL4-rel* chimeras, the wild-type *GAL4*, or the *GAL4* DNA-binding domain were introduced into a yeast strain lacking a functional *GAL4* gene but bearing a *GAL80* gene and an integrated *GAL1*:*lacZ* fusion gene (YM335:RY171) and into a similar strain containing an integrated *GAL1*:*lacZ* fusion with a deleted UAS_G (YM335:RY1Δ1). β-Galactosidase activities were measured from cells grown in the presence of galactose, glycerol, and ethanol. Each result is the mean of at least three independent experiments. All constructs assayed in the strain YM335:RY1Δ1 gave no activity above background, demonstrating that transcriptional activation requires the UAS_G-binding site (data not shown).

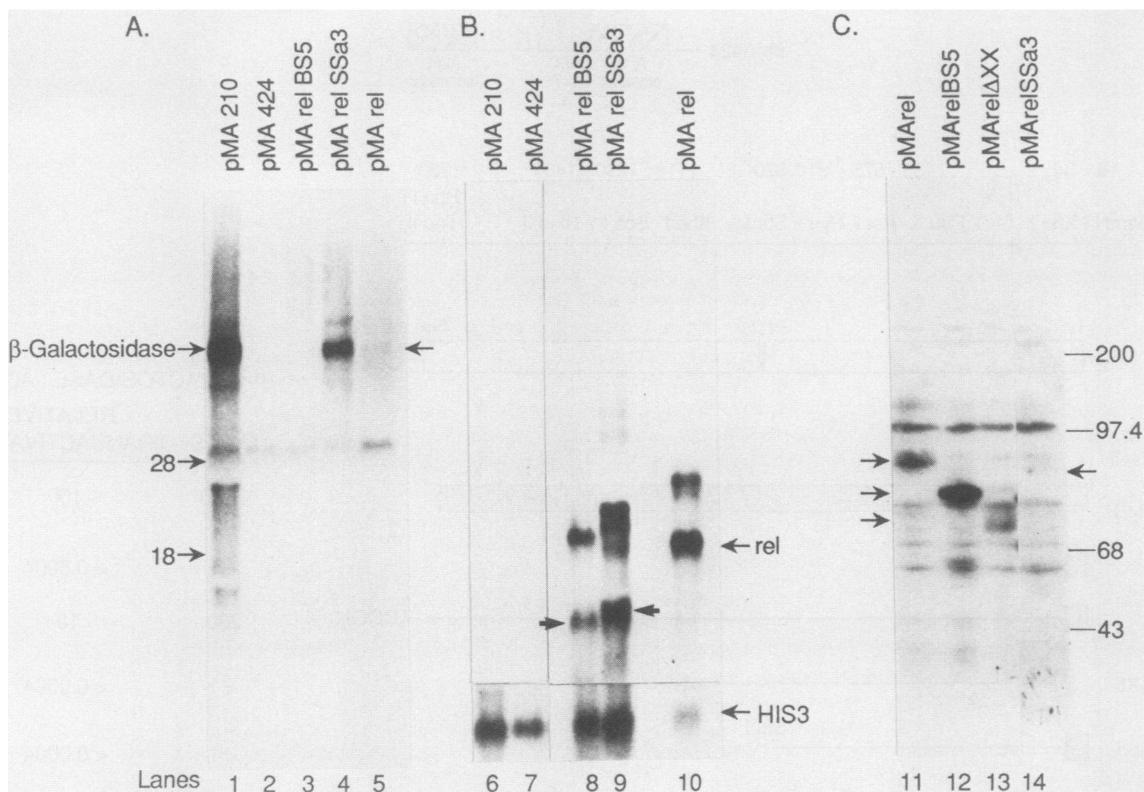


FIG. 3. Northern analysis. (A and B) Yeast cells (YM335:RY171) were transformed with the various effector plasmids and grown in histidineless selective medium with galactose, glycerol, and ethanol, and total RNA was isolated. RNA (10 μg) was separated by electrophoresis in formaldehyde-agarose gels, transferred by Hybond membranes and hybridized first to a β-galactosidase probe (A) and then stripped and rehybridized to *rel* and *HIS3* probes (B). The positions of β-galactosidase, c-*rel* fusion transcripts, and *HIS3* transcripts are shown by arrows. (C) Western blot. The positions of *GAL4*-full-length c-*rel*, *GAL4*-N-terminal c-*rel*, and *GAL4*-C-terminal c-*rel* fusion proteins are shown. Lanes 11 to 14 are from the same gel, and the background bands are the same intensity, indicating that equivalent amounts of protein were loaded in each lane.

(nucleotides 1221 to 1199); (iv) 5' CCT-TCT-GAC-CAG-GAA-GTT-AGT-GA (nucleotides 1199 to 1221). These oligonucleotides were synthesized on the basis of the sequence of mouse c-*rel* (24). We used mouse methylcholanthrene-transformed C3H 10T 1/2 cells (the Okayama-Berg cDNA expression library was a kind gift of H. Okayama). The oligonucleotide pair i and iii were used to amplify an 840-base-pair (bp) fragment containing the 5' portion of the c-*rel* cDNA, and the oligonucleotide pair ii and iv were used to amplify a 988-bp fragment containing the 3' portion of the c-*rel* cDNA. These fragments were labeled by nick translation and used to probe a Southern blot of mouse genomic DNA. The PCR-generated fragments were subcloned into the Bluescript SK$^-$ vector (Stratagene, La Jolla, Calif.), giving rise to rel-PCR-5 (840 bp) and rel-PCR-3 (988 bp). The full-length clone was generated by joining the 5' and 3' fragments at the *Stu*I site.

Effector plasmids. To generate *GAL4-rel* fusion proteins, we used convenient restriction sites within the c-*rel* gene. The c-*rel* sequences were cleaved at the designated restriction sites (Fig. 2) and cloned in-frame in the polylinker sequences of the vector pMA424 (38). The plasmids are designated by the first letters of the restriction site followed by 5 (referring to the 5' fragment) or 3 (referring to the 3' fragment). The full-length *rel* plasmid has no suffix. The plasmid pMArelBS5 contains the 820-bp *Bam*HI-*Stu*I insert; pMASSa3 contains a 990-bp *Stu*I-*Sal*I insert; pMArelXX5 has the 1,080-bp *Xho*I fragment; pMArelSH3 contains the 910-bp *Stu*I-*Hpa*I insert; pMArelSHc3 has the 580-bp *Stu*I-*Hin*cII insert; pMArelSSc3 has the 410-bp *Stu*I-*Sca*I fragment; pMArelSX3 contains the 358-bp *Stu*I-*Xho*I insert; pMArelScSa3 has the 580-bp *Sca*I-*Sal*I fragment; pMAScH3 has the 500-bp *Sca*I-*Hpa*I fragment; and pMArelHcH3 contains the 329-bp *Hin*cII insert. The internal deletion construct pMArelΔPP contains an in-frame deletion of the sequences between the *Pvu*II sites. pMArelΔXX contains an in-frame deletion of the sequences between the *Xho*I sites. As a result of cloning, the fusion proteins contain an additional 5 amino acids (PEGIP) at the *GAL4-rel* junction. All fusion plasmids were sequenced across the fusion border. The LexA fusion constructs were made by inserting the full-length, 5', and 3' sequences into pLex (202+PL)' as stated above for the *GAL4* fusion constructs.

The effector plasmids generated for the mammalian transactivation studies contained c-*rel* DNA fragments subcloned into pSG424 (48) in the same manner stated above for the yeast effector constructs and had similar nomenclature (Fig. 3). For example, pSGrel was the sister construct of pMArel. As a result of cloning, the fusion proteins contained 7 additional amino acids (PELPGIP) at the *GAL4-rel* junction. The plasmid pSVrel5X was generated by removing the *GAL4* sequence with *Bam*HI-*Hin*dIII digest and replacing it with a *Bam*HI-*Hin*dIII fragment containing the 5' c-*rel* sequences including the nuclear translocation signal.

The reporter plasmid pG5BCAT (35) is a pSV72CAT-based construct that contains the bacterial chloramphenicol

acetyltransferase (CAT) gene whose transcription is driven by an E1b promoter containing five repeats of 17-mer consensus sequence for UAS_G. The plasmid pBCAT was generated by removing the *GAL4* DNA-binding sequences with a convenient restriction digest and religating the vector. The wild-type *GAL4* construct pRSVGALK2 (a gift from S. Hollenberg and R. Evans) contains a Rous sarcoma virus long terminal repeat driving the expression of the GAL4 protein.

Yeast transformation and β-galactosidase assay. The yeast cells were made competent for transformation by treatment with lithium acetate as described elsewhere (27). Liquid β-galactosidase assays were performed as previously described (65). All plasmid constructs were assayed for β-galactosidase activity at least three to five times from several independent transformants. The standard errors were within 20%.

Yeast RNA isolation and analysis. RNA from the different yeast transformants was isolated, and RNA samples were separated by electrophoresis in formaldehyde-agarose gels, transferred onto Hybond membranes, and cross-linked onto the membrane by a 5-min UV exposure. The blots were then prehybridized in a 0.2 M phosphate buffer at pH 7.2 containing 7% sodium dodecyl sulfate and 1% bovine serum albumin at 68°C for 30 min. The hybridization was performed 12 to 16 h at 68°C in the same buffer containing 10^6 cpm of a nick-translated probe per ml. The filters were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 68°C and exposed to X-ray film with an intensifying screen for 2 h at −70°C.

Western immunoblot analysis. Five-milliliter overnight cultures of the appropriate yeast transformants were grown in selective media at 30°C with vigorous shaking. The cells were harvested, and induction medium containing galactose, glycerol, and ethanol was added (5 ml). The cells were grown for 6 h at 30°C to a density of approximately 10^7 cells per ml. Then an equal number of cells from each transformant was pelleted by a 2-min spin at 10,000 rpm in a microcentrifuge. The cells were suspended in 50 μl of 2× GSB (50 mM Tris [pH 7.5], 4% sodium dodecyl sulfate, 24% glycerol, 0.5% 2-β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.01% bromophenol blue) and vortexed in the presence of glass beads for 2 min, followed by boiling for 3 min. The cell extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed as described by Towbin et al. (59). After transfer, the blot was processed with Blotto as described by Johnson et al. (28). The blot was then incubated at room temperature in Blotto containing antibody directed against GAL4 amino acids 1 to 147 (a gift of I. Sadowski and M. Ptashne). The bound antibody was visualized with ^{125}I-protein A. The blot was exposed to X-ray film at −70°C for 18 to 36 h.

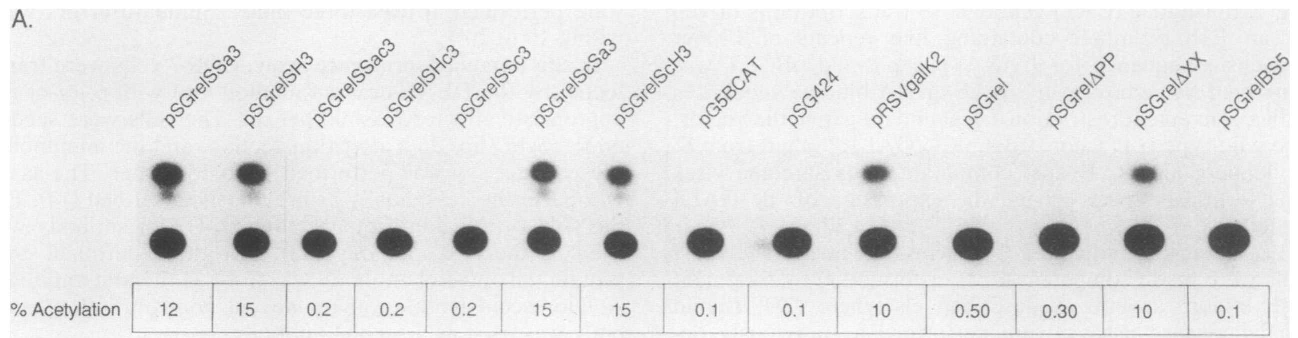
Mammalian transfection and CAT assays. NIH 3T3 cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum in a water-saturated atmosphere containing 5% CO_2. The cells were plated at a density of 5×10^5 cells per plate the night before transfection. The cells were transfected with calcium phosphate protocol with 3 μg of reporter plasmid and 6 μg of effector plasmid unless otherwise stated. The calcium phosphate precipitate was incubated with the cells overnight; the cells were then washed in phosphate-buffered saline, and 8 ml of Dulbecco modified Eagle medium containing 10% fetal calf serum was added to each plate. The cells were harvested and assayed for CAT activity 24 h later as previously described (50, 51). All assays were performed at least three times. Standard errors were within 10 to 20%.

In situ immunofluorescence assay. COS-7 cells were transfected by the DEAE-dextran method (36) with 6 μg of the appropriate effector plasmid per ml. The cells were seeded on to cover slips 36 h after transfection, and the immunofluorescence assay was performed 12 to 18 h later. The assay was performed essentially as previously described (14). For the *GAL4-rel* fusion proteins, the anti-GAL4 antibody was used as the first antibody (52). For the N-terminal c-*rel* protein, an anti-Rel antibody was used as the first antibody. A fluorescein isothiocyanate-labeled goat anti-rabbit antibody was used as a second antibody.

RESULTS

Murine c-*rel* protein. We have previously shown that murine c-*rel* is inducible with serum and TPA in fibroblasts, but the induced level of c-*rel* RNA transcripts is at least 10-fold lower than that of other early response genes (9). Therefore, rather than isolating a c-*rel* cDNA clone capable of encoding the c-*rel* protein by screening a large number of colonies in a cDNA library, we chose to amplify c-*rel* cDNA sequences by using the PCR based on the published mouse c-*rel* sequence (24) (see Materials and Methods). The nucleotide sequence of a 1,800-bp c-*rel* cDNA fragment was similar to the reported sequence of mouse c-*rel* cDNA (24), except for 21 nucleotide differences (13 point mutations, 4 insertions of one nucleotide, and 4 deletions of one nucleotide) resulting in 11 amino acid differences. A comparison of the c-*rel* protein from mouse (PCR), mouse (24), human (7), chicken (10, 25), turkey (63), v-*rel* (64), and *dorsal* (46, 55) is shown in Fig. 1. It is evident that the c-*rel* proteins from various species share extensive identities (47% identities) but only in the N-terminal half of the protein. The mouse and human proteins are more similar to each other in the C-terminal half than they are to the two avian c-*rel* proteins, which are nearly identical to each other in this region. Neither mammalian or avian c-*rel* proteins share identities with *dorsal* in the C-terminal region. When the c-*rel* cDNA obtained by PCR was transcribed in vitro and translated in a rabbit reticulocyte lysate, a 72-kilodalton protein similar in size to the human c-*rel* protein in Daudi cells could be identified (data not shown). Furthermore, the in vitro-synthesized c-*rel* protein was immunoprecipitated with avian anti-*rel* antibodies. We conclude that the cDNA clone we have obtained encodes full-length c-*rel* protein.

***c-rel* is a transactivator in yeast cells.** Since it is not known whether the c-*rel* protein binds to DNA, we elected to use a transcriptional transactivation system based on a heterologous DNA-binding domain. Specifically, we have employed the DNA-binding domain (N-terminal 174 amino acids) of the yeast transcriptional activator *GAL4* as an anchor to which either the intact mouse c-*rel* protein or various fragments from its C-terminal region were fused to generate chimeric molecules capable of binding to promoters containing upstream activating sequences, UAS_G (37, 38). The *GAL4-rel* fusion gene was expressed from the alcohol dehydrogenase (*ADH1*) promoter and introduced into *GAL4* mutant yeast strains. One strain (YM335:RY171) contains an integrated *GAL1-lacZ* fusion construct, and β-galactosidase activity was used as a measure of the transcriptional activation function of each c-*rel* fusion protein. A second strain (YM335:RY1Δ1) also has an integrated *GAL1-lacZ* fusion construct but lacks the UAS_G sequences and serves as a control for binding site-dependent transcription activation.



B.

Effector Plasmid:

pSG424 — SV40 early promoter; GAL4 1-147; EcoR I; BamH I; Sal I; terminator SV40 pA

Reporter Plasmid:

5 GAL4 sites; E1bTATA; CAT

Construct	Relative Transactivation
pRSVGALK2 (WT GAL4)	100
pSG424	<1
pSGrel (COOH)	4.6
pSGrelBS5 (Stu I)	< 1
pSGrelSSa3 (COOH)	120
pSGrelSH3 (Hpa I)	150
pSGrelSSac3 (Sac II)	2
pSGrelSHc3 (Hinc II)	2
pSGrelSSc3 (Sca I)	2
pSGrelScSa3 (COOH)	150
pSGrelScH3 (Hpa I)	150
pSGrelΔPP (COOH)	3
pSGrelΔXX (COOH)	100

FIG. 4. c-*rel* transactivation in mammalian cells. (A) CAT assays. Plasmids were transfected into NIH 3T3 cells, and crude extracts were assayed for total protein and CAT activity. Equivalent amounts of proteins were assayed from each sample, and percent acetylation was determined by quantitating the amount of acetylated chloramphenicol as a fraction of unacetylated chloramphenicol. (B) pG5BCAT is the

The results in Fig. 2 show that the full-length c-*rel* fusion protein (pMArel) produces 10% of the wild-type GAL4 (pMA210) β-galactosidase activity. No detectable β-galactosidase activity was observed with either the N-terminal 147-amino-acid DNA-binding moiety of *GAL4* (pMA424) alone or with the N-terminal half of c-*rel* fused to it (pMArelXX5 and pMrelBS5). Surprisingly, if the C-terminal half (pMArelSSa3 and pMArelSH3) was fused with GAL4, over 60% of wild-type GAL4 activity was detected. No β-galactosidase activity was detected in YM335:RY1Δ1 transformants with either the wild-type *GAL4* or the *GAL4* fragment fused to the C-terminal fragment of c-*rel*, indicating an absolute requirement for the *GAL4* DNA-binding site. Thus, it appears that the N-terminal truncated form of the c-*rel* protein is a much more potent transactivator than the full-length c-*rel* protein. Furthermore, the N-terminal half of the c-*rel* protein has no intrinsic transcriptional activation activity and may be acting as an inhibitory domain, resulting in the low activity of the intact c-*rel* protein.

To delineate the transcriptional activation domain in the C-terminal half of the c-*rel* protein, a series of deletion mutants were generated and fused with the DNA-binding region of the *GAL4* protein. A fusion protein containing c-*rel* sequences encompassing *Sca*I to *Hpa*I (pMArelScH3) produced β-galactosidase activity equivalent to that observed with the entire C-terminal half of c-*rel* (pMArelSSa3) (Fig. 2). Since plasmids pMArelSSc3 and pMArelHcH3 had no activity and pMArelSHc3 had 12% of the maximal activity displayed by C-terminal *rel*, we deduce that the transactivation region lies between amino acids 403 and 568 (*Sca*I to *Hpa*I). Interestingly, all of the *GAL4*-c-*rel* fusion proteins capable of transactivation also lack the nuclear transport signal identified in c-*rel* protein (25).

Because the full-length c-*rel* protein displayed only 12% of the activity observed with the C-terminal half of the c-*rel* protein, we attempted to define the inhibitory sequences within the N-terminal half of c-*rel* protein. Deletion of a small region spanning between *Pvu*II sites (pMArelΔPP) caused no increase in transactivation, whereas a larger deletion (pMArelΔXX) increased activity to 50% of that observed with C-terminal fragments. Thus, it appears that sequences over a broad region in the N-terminal half of the *rel* protein act as negative regulators of the C-terminal transactivation domain.

The transactivation by c-*rel* fusion proteins occurs at the level of transcription, since Northern blot analysis of RNA extracted from yeast cells transformed with c-*rel* effector plasmids also showed increased levels of β-galactosidase transcripts (Fig. 3A). β-Galactosidase transcripts can be seen in the wild-type *GAL4* transformants (lane 1) and C-terminal fusion protein (lane 4) but not in the control (lane 2), N-terminal c-*rel* transformants (lane 3), or full-length c-*rel* fusion protein (lane 5). The steady-state levels of *HIS3* (the selectable markers on the pMA series of plasmids) RNA were used as an RNA loading control (Fig. 3B). The amount of RNA loaded from the full-length c-*rel* fusion gene transformants was 5- to 10-fold lower (HIS3, lane 10) than the corresponding transformants with the N- or C-terminal c-*rel* plasmids. When normalized, the increased β-galactosidase activity by c-*rel* fusion proteins closely paralleled increased levels of β-galactosidase transcripts, consistent with transcriptional activation.

c-*rel* fusion proteins in yeast transformants. The inability of N-terminal c-*rel* fusion proteins to transactivate transcription raised the possibility that some c-*rel* constructs in yeast cells may not make c-*rel* RNA or protein. Figure 3B shows that c-*rel*-specific transcripts were generated from the N-terminal, C-terminal, and full-length c-*rel* fusion genes. The multiple RNAs detected indicate multiple transcriptional start sites and readthrough in the effector plasmids, as has previously been observed (5, 37). Because N-terminal and full-length c-*rel* fusion proteins either are not active or have poor transactivation potential, we wanted to ensure that the c-*rel* fusion proteins are synthesized. Figure 3C shows immunoblot analysis of yeast lysates in which antibodies against the DNA-binding domain of *GAL4* were used to detect the various fusion proteins. Proteins of the expected size for the full-length, N-terminal, and C-terminal c-*rel* fusion proteins were detected (lanes 11 to 14). The C-terminal c-*rel* protein appears to be present at a lower level than either the full-length or N-terminal protein. We therefore believe that inability of the N-terminal half- and full-length c-*rel* proteins to transactivate transcription is not due to lack of their synthesis, but is an inherent property of these molecules.

Transactivation by c-*rel* in mammalian cells. We next wanted to extend the results obtained in yeast cells to mammalian cells. Since GAL4 activates gene expression in mammalian cells with yeast UAS_G as the transcriptional enhancer, we used a strategy similar to the one employed above (30,, 61, 62). The reporter plasmid contains five copies of 17-mer UAS_G linked to the adenovirus E1b gene TATA box and the CAT gene (35). The effector plasmids contain simian virus 40 early promoter and the *GAL4* 147-amino-acid DNA-binding domain fused with the various c-*rel* coding domains. The effector and reporter plasmids were cotransfected into NIH 3T3 cells, and CAT activities were determined (Fig. 4A). The effector plasmid constructs containing portions of the c-*rel* gene and the relative transactivation activity based on CAT enzyme levels are tabulated in Fig. 4B. The transcription of the wild-type *GAL4* gene (pRSVgalK2) was driven by the Rous sarcoma virus long terminal repeat, and the resulting transactivation was arbitrarily fixed at 100 (10% acetylation, Fig. 4A). The parent plasmid containing only the DNA-binding domain of *GAL4* (pSG424) showed no activity. Like the results obtained in yeast cells, transactivation by full-length c-*rel* fusion protein (pSGrel) was at least 20- to 25-fold lower than that by wild-type GAL4, and no transactivation was observed with the N-terminal half of the c-*rel* protein (pSGrelBS5). On the other hand, the C-terminal half of the c-*rel* protein was a potent transactivator with the transactivation domain localized on a *Sca*I-to-*Hpa*I fragment. Furthermore, we showed that removal of N-terminal sequences from the full-length c-*rel* protein (pSGrelΔXX) increases the activity by at least 20-fold, to a level equal to that of wild-type GAL4. We therefore conclude that c-*rel* protein can act as a transactivator in both yeast and mammalian cells and that transactivation in both organisms is due to C-terminal transactivation domain and is regulated by a N-terminal inhibitory domain.

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reporter plasmid alone. The structures of all others are shown. The structure of the parent effector plasmid to which portions of the c-*rel* gene are linked and the structure of the reporter plasmid are shown. The full-length wild-type (WT) *GAL4* construct is shown (stippled box); all other effector plasmids containing amino acids 1 to 147 *GAL4* DNA-binding domain (stippled box) are shown. The relative amounts of transactivation are calculated from the percent acetylation shown in panel A.

To demonstrate that the transactivation by *GAL4-rel* fusion protein is binding site dependent, we deleted the UAS_G consensus sequences from the reporter plasmid G5BCAT. No transactivation was observed with either SGrel or SGrelSSa3 with the reporter BCAT (Fig. 5A), demonstrating that transcriptional activation by the fusion proteins is binding site dependent.

To rule out the possibility that the *GAL4* portion of the fusion protein could alter the conformation of the N-terminal half of the c-*rel* portion of the fusion protein and mask a possible transactivation function, we instead generated a c-*rel*-C-terminal fusion protein with the *GAL4* DNA-binding domain at the C terminus (pSrel3G). Results (Fig. 5B) show that like the *GAL4-rel* (pSGrel) fusion protein, the *rel-GAL4* (pSrelG) fusion protein is also a poor transcriptional activator. The location of the *GAL4* portion of the fusion protein did not influence the ability of the C-terminal half of the c-*rel* protein to transactivate transcription (Fig. 5B). We therefore conclude that inability of the full-length c-*rel* protein to transactivate transcription is not due to conformation changes caused by fusion with the GAL4 protein. In addition, we have tested the transactivation ability of c-*rel* fused to a *lexA* DNA-binding domain. LexA202 fusion proteins with the full-length, N- and C-terminal halves of the c-*rel* protein were also tested for transactivation in yeast cells containing a *lexA* operon upstream of *lacZ*. We obtained the same results with the *lexA* fusion constructs as were observed with the *GAL4* fusion constructs (K. Morley, unpublished results). We therefore conclude that we have isolated and characterized an authentic transactivation domain located in the C-terminal portion of c-*rel* protein.

One explanation of the lack of transactivation by full-length or N-terminal c-*rel* protein as compared with that by C-terminal c-*rel* protein may be competition between DNA-binding sites. If the N-terminal half of the c-*rel* protein has a DNA-binding domain, it is possible that the *GAL4-rel* fusion protein is directed to bind to as-yet-unidentified c-*rel*-specific DNA-binding sites. To examine this possibility, we cotransfected the full-length *GAL4-rel* fusion construct (pSGrel) with increasing amounts of N-terminal c-*rel* driven by a simian virus 40 early promoter. If the N-terminal half of the c-*rel* protein contained a c-*rel*-specific DNA-binding domain, one would predict that increasing concentrations of N-terminal half of the c-*rel* protein would compete with the *GAL4*-full-length c-*rel* protein for binding to c-*rel*-specific DNA-binding elements and thus increase transactivation of the *GAL4*-linked reporter gene. The results (Fig. 5C) show, however, that in a cotransfection assay in which the N-terminal half of c-*rel* protein was cotransfected with the pSGrel construct at a ratio of 3:1, there was no increase in CAT activity, a measure of transcriptional transactivation. The recombinant construct used to generate the N-terminal half of the c-*rel* protein contains a native nuclear transport signal (consequently, the protein is localized in the nucleus; Fig. 6) and therefore should compete with the *GAL4*-full-length c-*rel* fusion protein for sequence-specific binding activity. The c-*rel* construct encoding N-terminal half of the protein did not interfere with the transcriptional transactivation of the *GAL4*-3′ *rel* (pSGrelSSa3) fusion construct (Fig. 5C). We therefore conclude that the inability of the full-length *GAL4-rel* fusion protein to act as an efficient transcriptional activator is not due to competition for c-*rel*-specific DNA-binding sequences. It is, however, formally possible that a threefold excess of the N-terminal half of the c-*rel* protein is not sufficient to fully compete with *rel*-specific DNA-binding sites.

The inability of the *GAL4*-full-length c-*rel* fusion protein to be an efficient transcriptional activator can also be due to a lack of transport to the nucleus. Although presence of amino acids 1 to 147 of the *GAL4* DNA-binding portion of the GAL4 protein ensures nuclear localization, it is formally possible that cytoplasmic c-*rel* protein has signals which override nuclear transport. Furthermore, some cellular protein may associate with the full-length or N-terminal c-*rel* protein and consequently anchor it in the cytoplasm. To confirm nuclear localization of the full-length c-*rel* protein, we performed indirect immunofluorescence of cells transfected with *GAL4-rel* fusion constructs by using antibodies raised against the N-terminal portion of the GAL4 protein. Results (Fig. 6) demonstrate that all constructs containing *GAL4* (pSGrel, pSGrelBS5, and pSGrelSSa3) when transfected into COS-7 cells show nuclear localization. In contrast, cells transfected with non-*GAL4* fusion constructs showed no fluorescence. Similarly, staining with an antitubulin antiserum showed cytoplasmic staining (Fig. 6D). We therefore conclude that lack of efficient transactivation by full-length c-*rel* protein is not due to a lack of its nuclear localization. In addition, cells transfected with SVrel5X (to overproduce the N-terminal c-*rel* protein) were examined by immunofluorescence with an anti-Rel antibody. The native c-*rel* nuclear translocation signal present in the construction was sufficient to direct the protein to the nucleus (Fig. 6) and should therefore be able to act as an effective competitor for c-*rel*-specific DNA-binding sites within the genome.

DISCUSSION

It would be desirable to undertake transactivation studies with the authentic c-*rel* DNA-binding site, but none has yet been identified; therefore, we have explored the transactivation properties of the murine c-*rel* protein by using an assay in which the *GAL4* DNA-binding domain functions as a sequence-specific anchor (37). Full-length c-*rel* or portions of it were fused with the *GAL4* DNA-binding domain and assayed for transactivation in both yeast and mammalian cells. Our results show that the full-length c-*rel* protein is a weak transactivator, but if the N-terminal half is deleted, it becomes a strong transcriptional activator.

Regulatory and activation domains. Regulation of transcription by transcription factors can be either positive or negative. For instance, proto-oncogene *fos* can activate the transcription of promoters containing TPA-responsive elements (TGACTCA), but it represses the transcription of its own promoter (12, 50, 51). If sequences at the C terminus of the Fos protein are removed, it can act only as a transcriptional activator (51). Similarly, *junB*, a close relative of c-*jun*, can activate the transcription of genes containing a TPA-responsive element but suppresses the transcription of proto-oncogene *jun*, whose promoter also contains an AP-1 site (similar to a TPA-responsive element, TGACATCA) (11). On the other hand, proto-oncogene *jun* up regulates the transcription of its own promoter (3). Another transcription factor, CREB, the cyclic AMP response element-binding protein, acts as a potent transcriptional repressor of the c-*jun* promoter but activates it following phosphorylation by the catalytic subunit of protein kinase A, which presumably causes an allosteric change (32a). The transactivating protein E1a has also been shown to act as a positive or negative regulator of transcription of various genes (49).

Proto-oncogene *rel* provides a novel mode of action for transcription regulators. A unique feature of c-*rel* is that in the full-length protein, the negative regulatory domain is

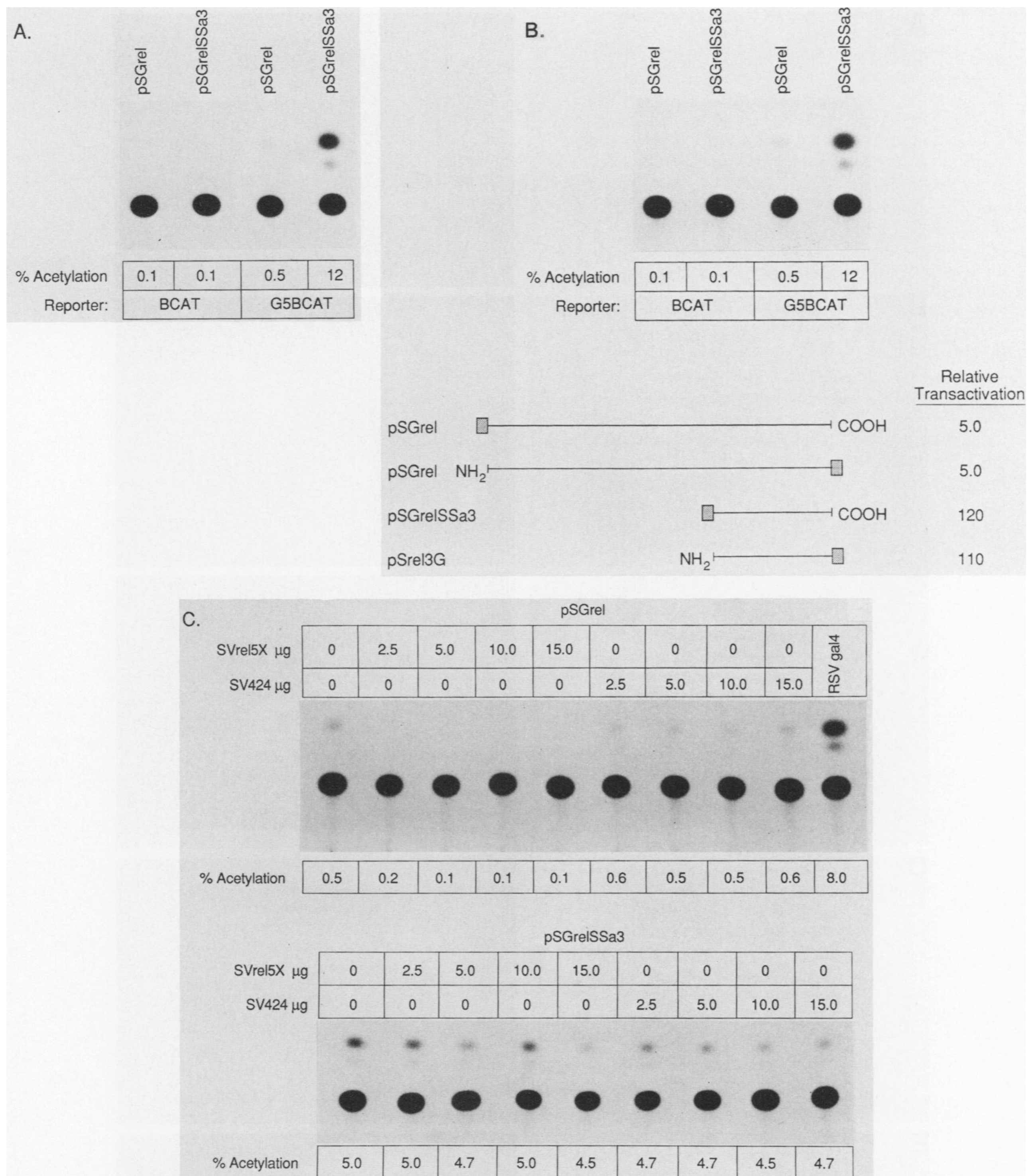
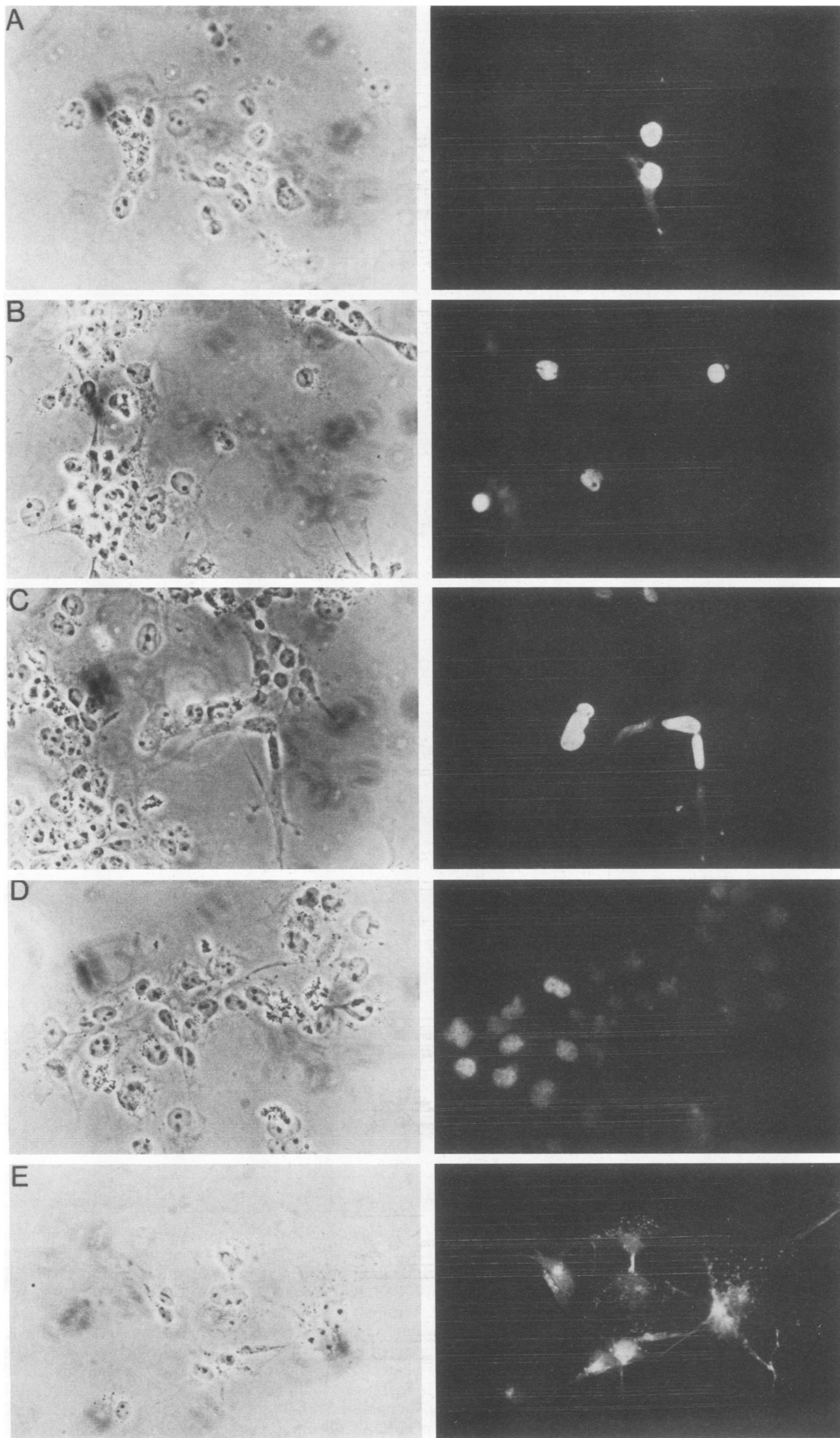


FIG. 5. Transactivation by c-*rel*. Plasmids were transfected into NIH 3T3 cells, and crude extracts were assayed for total protein and CAT activity. Equivalent amounts of protein were assayed, and percent acetylation was determined by quantitating the amount of acetylated chloramphenicol as a fraction of unacetylated chloramphenicol. (A) Binding site dependence of transactivation. Plasmids pSGrel and pSGrelSSa3 were cotransfected with two different reporter plasmids: one containing the UAS_G sequences (pG5BCAT) and the other containing a deletion of the UAS_G sequences (pBCAT). (B) Effect of C-terminal positioning of the *GAL4* DNA-binding domain. The effector plasmids were cotransfected with the reporter pG5BCAT and assayed for CAT activity. The positions of the GAL4 amino acids 1 to 147 (▢) are shown relative to the c-*rel* sequences. The relative amounts of transactivation are calculated from the percent acetylation. (C) Effect of overexpression of the N-terminal c-*rel* protein. Expression of the N-terminal c-*rel* protein was driven from the simian virus 40 early promoter and was designated pSVrel5X. Plasmid pSVrel5X was cotransfected in increasing amounts with 5 μg of pSGrel (top panel) or pSGrelSSa3 (bottom panel) and 2.5 μg of pG5BCAT. As a control for promoter competition, the vector backbone of pSVrel5X (pSV424) was also cotransfected in increasing amounts with pSGrel or pSGrelSSa3 and pG5BCAT. The percent acetylation is shown.


A
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E

dominant, as judged by poor transcriptional activation. When the N-terminal half of the protein is removed, the C-terminal half of the protein functions as a potent transcriptional activator. Previous results have also indicated that the v-*rel* protein, which is missing 118 residues at the C terminus as compared with the c-*rel* protein, can act as a weak transcriptional activator without fusion to a DNA-binding domain. However, the extent of transactivation depended upon the type of the promoter and the cell type used (20, 25). Furthermore, in chimeric constructs containing portions of v- and c-*rel*, it appears that when the C-terminal half originated from c-*rel*, there was greater transactivation (25).

Transactivation domain is not conserved. We have localized the transactivation domain to the C-terminal one-fourth of the murine c-*rel* protein (amino acids 403 to 568) (Fig. 1). Further delineation of this region delimits the transactivation domain to amino acids 434 to 546. It is possible that this region contains multiple interacting domains. This region is negatively charged (18 acidic residues, 6 basic residues) and is rich in prolines and serines (11 prolines, 26 serines). Despite the fact that this region of the protein is not well conserved among various species, the acidic amino acid residues are largely conserved between mouse and human c-*rel* proteins, and some are even conserved between mouse and avian c-*rel* proteins (Fig. 1). The *dorsal* protein of drosophila is unrelated to vertebrate c-*rel* protein in the transactivation domain, but *dorsal* has another type of transactivation motif in its C-terminal half, which is rich in glutamine residues (some stretches of 10 to 15 consecutive glutamines; Fig. 1) (46). The precise mechanism of transactivation by transcription factors is not known, but one popular view is that negatively charged domains interact with DNA motifs or other cellular factors required to initiate transcription (38). Thus, the transactivation domains of various transcription factors may not be identical but do still perform similar function. In fact, despite the differences between the mouse and chicken c-*rel* C-terminal sequences, the C-terminal region of the chicken c-*rel* is also a more potent transactivator than the intact protein (T. Gilmore, personal communication).

In contrast to the transactivation domain, the regulatory (inhibitory) domain whose excision increases transactivation appears to be relatively well conserved (47% identities) among the various species (Fig. 1). This suggests that the function of the c-*rel* protein is modulated by cellular factors which interact with the N-terminal half to either suppress transactivation or activate transcription by exposing the C-terminal transactivation domain. At present, we do not know whether the factors interacting with the N-terminal domain act in *cis* or *trans*. However, since threefold overexpression of the c-*rel* N-terminal half did not cause an increase in transactivation, it is unlikely that negative factors are acting in *trans*.

Parallels between c-*rel*, NF-κB, and *dorsal*. The c-*rel*, NF-κB, and *dorsal* proteins share the common property of being localized in either the cytoplasm or the nucleus (4, 25, 46). However, unlike NF-κB, it is not known whether c-*rel* and *dorsal* bind to DNA directly. c-*rel* and *dorsal* further share the common feature that their C-terminal regions may be required to localize these proteins in the cytoplasm (10, 25, 46). Retention of nuclear proteins in the cytoplasm may be a consequence of their association with other cytoplasmic proteins. For instance, when associated with the product of *hsp90*, the glucocorticoid receptor is cytoplasmic, but upon addition of the ligand, the receptor migrates to the nucleus. This notion is strengthened by the observation that a glucocorticoid receptor with a C-terminal truncation is constitutively present in the nucleus even in the absence of the hormone ligand (44). Similarly, the tissue-specific transcription factor NF-κB is located in an inactive form in the cytoplasm in association with another protein, IκB (inhibitor of NF-κB) (34). Following the addition of phorbol ester, IκB is phosphorylated and dissociates from NF-κB and the latter is translocated to the nucleus (21, 34). The catalytic subunit of type II cyclic AMP-dependent protein kinase is also translocated to nucleus from the cytoplasm following increased intracellular levels of cyclic AMP (43).

It will be interesting to define conditions under which c-*rel* moves to the nucleus in fibroblasts. The mechanism of translocation of *dorsal* to the nucleus from the cytoplasm is not know, but it appears that C-terminal truncation leads to nuclear localization and higher transactivation. Nuclear translocation of c-*rel* may involve proteins interacting with the C terminus or perhaps with the negative regulatory domain which interacts with the C terminus. It is possible that a portion of the c-*rel* protein is cleaved and translocated to the nucleus. Presently available *rel* antibodies may not detect the cleaved *rel* protein transported to the nucleus. Alternatively, translocation to the nucleus may be regulated by factors that act at the N-terminal domain of the c-*rel* protein. It will be interesting to determine whether linking of the c-*rel* regulatory domain to other transactivators will also exert an inhibitory effect. The regulatory domain of the c-*rel* protein may be an additional control to allow transcriptional transactivation only in specific cell types. The high degree of conservation of the N-terminal domain suggests that it interacts specifically with other proteins or DNA. Clearly the sequence requirements for transactivation are less stringent.

Oncogenes as transcriptional factors. There is a growing network of oncogene products which can act as transcriptional factors or cofactors. In addition to *fos*, *jun*, and E1a, *myb* has also been shown to be a potent transcriptional activator (13, 35, 42; I. M. Verma, *Human Encyclopedia*, in press). Furthermore, genes like *fos* and *jun* have multiple members in their families and can act combinatorially to affect the transcriptional machinery. Addition of c-*rel* to this repertoire expands the scope of oncogenes as transcriptional regulators. In the case of c-*rel*, it appears that transactivation and transformation may be independent events (25). It is noteworthy that the v-*rel* protein is truncated in the middle of the c-*rel* transactivation domain. Certainly transformation appears to be independent of cellular localization of *rel* proteins (23, 25). It is, however, formally possible that either c-*rel* or a truncated form is transiently transported to the nucleus and is sufficient to influence the transcription of other genes by acting as a bridging protein between DNA-binding proteins and components of the RNA polymerase

FIG. 6. In situ immunofluorescence assay. COS-7 cells were transiently transfected with the effector plasmids pSGrel, (A), pSGrelBS5, (B), pSGrelSSa3, (C), pSVrel5X (D) or with no plasmid (E). Phase and fluorescence photographs are shown. The primary antibody used in panels A to C was the anti-GAL4 antibody, that in panel D was an anti-Rel antibody and that in panel E was an anti-β-tubulin antibody. A fluorescein isothiocyanate-labeled goat anti-rabbit antibody was used as the secondary antibody in all cases. The left panels show the Nomarski image of the cells, while the right panels display immunofluorescence.

machinery. Availability of transcriptional assay systems in which a DNA-binding anchor is fused to a candidate protein will no doubt lead to the discovery of other oncogenes with properties of transcriptional modulators.

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ADDENDUM

While the manuscript was in its final stages of preparation, Kamens et al. (31) published a paper describing that nearly full length avian *rel* protein is a potent transactivator in yeast cells using LexA fusion constructs. However, subsequent studies carried out with the same constructs in mammalian cells showed that *lexA*-full-length *rel* protein is a weak transactivator as compared to the C-terminal half of the *rel* protein (T. Gilmore, personal communication), in agreement with the results presented here.

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