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v-*rel* Oncoproteins in the Nucleus and in the Cytoplasm Transform Chicken Spleen Cells

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The transforming protein encoded by the v-*rel* oncogene of the highly oncogenic avian retrovirus reticuloendotheliosis virus strain T (Rev-T) is a 59,000-dalton protein, p59$^{v\text{-}rel}$. The mechanism by which p59$^{v\text{-}rel}$ induces transformation of early lymphoid cells is unknown. As a step towards understanding the mechanism of v-*rel*-induced transformation, we sought to establish the subcellular site of action of p59$^{v\text{-}rel}$. In this report, we show that p59$^{v\text{-}rel}$ contains sequences that are necessary for its efficient localization in the nucleus of infected chicken embryo fibroblasts. These v-*rel* sequences when added to the normally cytoplasmic protein, beta-galactosidase, directed that protein to the nucleus. A mutation in the v-*rel* nuclear-localizing sequence did not affect the transforming function, although it did alter the nuclear-localizing function. The addition of a supplemental nuclear-localizing sequence from simian virus 40 large T-antigen to v-*rel* resulted in the expression of a transforming *rel* protein which was located exclusively in the nucleus of transformed spleen cells, in contrast to wild-type p59$^{v\text{-}rel}$, which was largely cytoplasmic in transformed spleen cells. Our results support the hypothesis that v-*rel* encodes a protein which can act either in the nucleus or in the cytoplasm to transform spleen cells.

Reticuloendotheliosis virus strain T (Rev-T) is a highly oncogenic avian retrovirus that transforms early lymphoid cells in vivo and in vitro (9, 15). However, Rev-T does not transform primary avian fibroblasts (11). The oncogene of Rev-T is v-*rel*; v-*rel* has no obvious sequence similarity to other known oncogenes (34, 41). The product of v-*rel* is a 59,000-dalton phosphoprotein designated p59$^{v\text{-}rel}$ (10, 11, 14, 28); p59$^{v\text{-}rel}$ has viral envelope-derived sequences at its amino and carboxy termini (34, 41). We are interested in the mechanism by which p59$^{v\text{-}rel}$ transforms cells and the basis for the cell type-specific transforming properties of p59$^{v\text{-}rel}$.

Previously, we showed that p59$^{v\text{-}rel}$ is expressed at equal levels in both infected nontransformed chicken embryo fibroblasts and in vitro-transformed spleen cells (11). However, although p59$^{v\text{-}rel}$ behaves primarily as a soluble cytoplasmic protein in transformed spleen cells (11, 33), it is largely a nuclear protein in fibroblasts (11). These results led us to propose that subcellular localization is an important determinant of the transforming potential of p59$^{v\text{-}rel}$ (11); that is, that p59$^{v\text{-}rel}$ could transform only cells in which it localized to the cytoplasm.

In this report, we have tested this hypothesis in the following two ways: by deleting or mutating sequences within v-*rel* that are important for the efficient nuclear localization of p59$^{v\text{-}rel}$ in fibroblasts and by adding heterologous subcellular-localizing sequences to p59$^{v\text{-}rel}$. Our results show that v-*rel*, like other genes encoding nuclear proteins (12, 16, 19, 29, 30), contains sequences important for nuclear localization of p59$^{v\text{-}rel}$ and that these sequences also can direct a normally cytoplasmic protein to the nucleus. A mutation that altered one of these nuclear-localizing sequences did not affect the transforming function of p59$^{v\text{-}rel}$. Furthermore, when the bulk of p59$^{v\text{-}rel}$ was maintained in the nucleus by the insertion of additional nuclear-localizing sequences, it still transformed spleen cells. These results demonstrate that p59$^{v\text{-}rel}$ need not be primarily in the cytoplasm to transform spleen cells, and they indicate that p59$^{v\text{-}rel}$ possesses a transforming function which can also act in the nucleus.

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MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEF) were prepared from SPAFAS eggs (SPAFAS, Inc., Norwich, Conn.) and were cultured as described previously (11). The Rev-T spleen cell clone, clone 9, used in these studies is the same clone that was used previously (11). Spleen cells were grown in Temin-modified Eagle medium supplemented with 10% fetal calf serum. Spleen cells and CEF were grown at 37°C.

The v-*rel*-transformed nonproducer cell line was isolated after infection of chicken spleen cells with a helper-free virus stock of CM103 (24) (see below). The helper-free virus stock of CM103 was made by transfecting a D17 dog helper virus cell line (39) with pCM103, isolating a clone of cells expressing v-*rel* (as determined by immunofluorescence), and harvesting virus from a culture of this clone of D17 helper cells.

To obtain virus stocks, CEF in 60-mm plates were transfected with 5 μg of defective virus DNA and 0.1 μg of Rev-A helper virus DNA (38) by the dimethyl sulfoxide-Polybrene method (17). Five days after transfection, virus was harvested. (None of the viruses used in this study induced morphological transformation of CEF.)

The transformation of spleen cells was assayed as described previously (15, 35) except the assays were performed at 40.5°C, since we found that that we obtained a greater number of transformed colonies at this temperature than at 37°C. Fresh spleen cells were infected with virus for 1 h and then were immediately plated in agarose. Colonies were scored 8 to 10 days after infection. Generally, Rev-T, BS102, or CM103 (all of which contain wild-type v-*rel* genes) gave 100 to 200 colonies per ml of virus per 3×10^7 spleen cells.

Plasmids. All plasmids were constructed by using conventional techniques (22). The exact details of the constructions will be provided upon request and will only be described

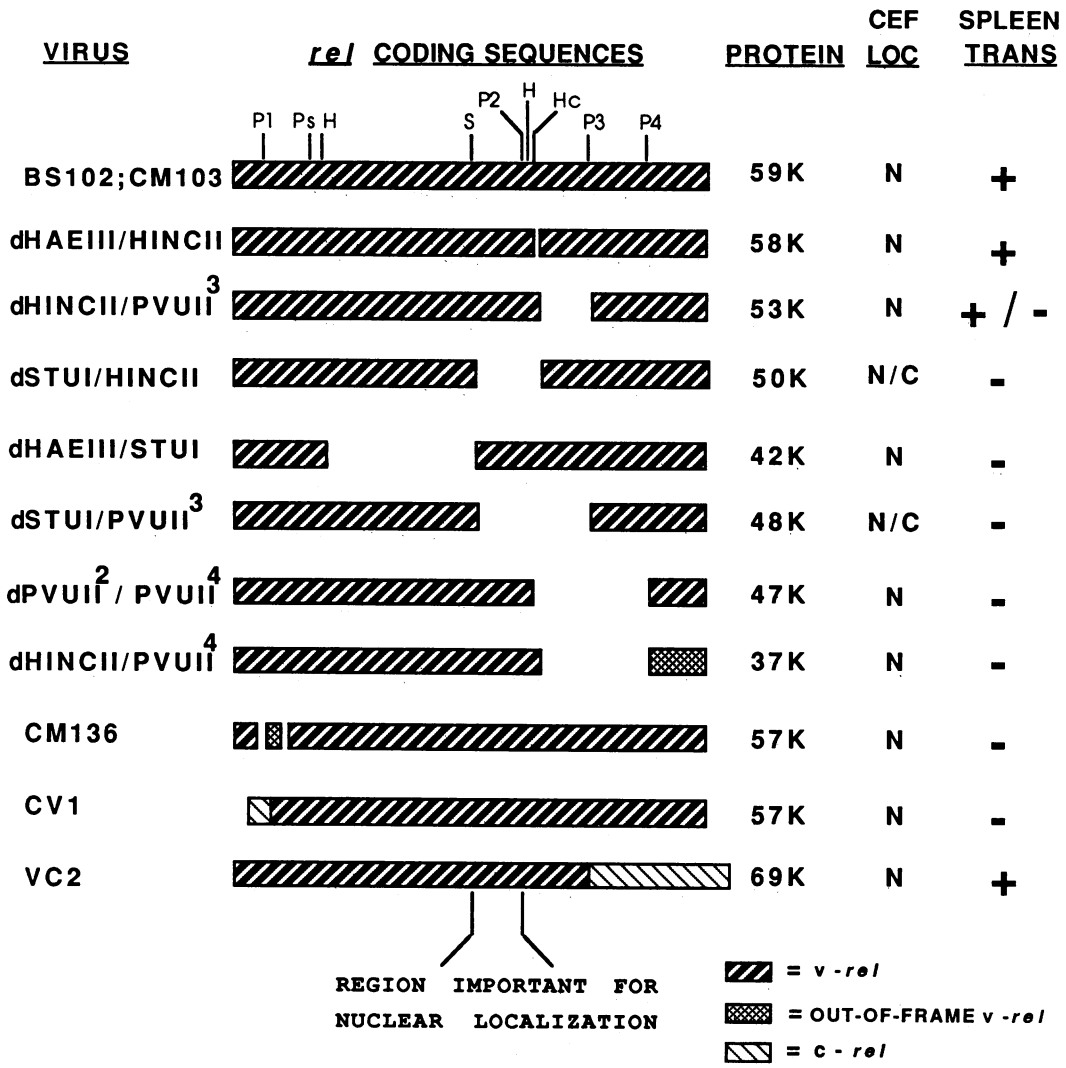


FIG. 1. Definition of a nuclear-localizing sequence within v-*rel*. CEF were transfected with the indicated viral plasmids in the presence of REV-A helper virus DNA. BS102 and CM103 contain a wild-type v-*rel* gene (24, 35); d*Hae*III/*Hin*cII through d*Pvu*II^2/*Pvu*II^4 have the indicated in-frame deletions in v-*rel*; d*Hin*cII/*Pvu*II^4 encodes a genuine v-*rel* polypeptide up to the *Hin*cII site and then has a short stretch of out-of-frame v-*rel*-encoded amino acids 3′ from the *Pvu*II^4 site; CM136 has two small deletions 5′ to the first *Pst*I site in v-*rel* (see Materials and Methods), and CV1 and VC2 are c-*rel*/v-*rel* recombinant viruses (35). In CV1, the first 28 amino acids of v-*rel* (including the virus envelope-derived amino-terminal amino acids) are deleted; in VC2, the carboxy-terminal c-*rel* sequences have been substituted for the v-*rel* sequences (such that the out-of-frame envelope sequences normally at the carboxyl end of v-*rel* are missing). The approximate positions of relevant restriction enzyme cleavage sites are indicated above the wild-type v-*rel* gene; P1 through P4, *Pvu*II (there are four *Pvu*II sites within v-*rel*, and they are distinguished in the viruses by superscripts 1 to 4, proceeding 5′ to 3′); Ps, *Pst*I; H, *Hae*III; S, *Stu*I; and Hc, *Hin*cII. CEF were analyzed for the production of v-*rel* protein by immunoprecipitation (PROTEIN), and the approximate sizes of the v-*rel* proteins are indicated in kilodaltons (K). The subcellular locations of the v-*rel* polypeptides in CEF (CEF LOC) were monitored by indirect immunofluorescence. Abbreviations: N, primarily nuclear; N/C, distributed both in the nucleus and cytoplasm. The region important for nuclear localization is located between the *Stu*I and *Pvu*II^2 sites in v-*rel*. Virus was harvested from transfected CEF and analyzed for its ability to transform chicken spleen cells (SPLEEN TRANS); +, transforming; −, nontransforming; +/−, abortive transformation (see text). Other symbols are as indicated at the bottom of the figure. The drawing is not precisely to scale.

briefly here. All plasmids have a p before the name (e.g., pTG15), whereas viruses derived from the plasmids do not (e.g., TG15).

The Rev-A helper virus DNA clone, pSW253, has been described previously (39).

The deletion mutants of v-*rel* shown in Fig. 1 were constructed by isolating subfragments of v-*rel* and substituting the appropriately ligated fragments for wild-type v-*rel* in either pBS102 or pCM103 (24, 35; these plasmids are designated pVV in reference 35). pBS102 and pCM103 are virtually identical recombinant virus plasmids that have v-*rel* inserted between two spleen necrosis virus long terminal repeats, such that v-*rel* is expressed from a full-length message in which the v-*rel* ATG is the first ATG in the message. The only difference between pBS102 and pCM103 is that pBS102 has an *Xba*I site in place of the *Avr*II site in pCM103 which is located approximately 400 base pairs (bp) downstream of the v-*rel* termination codon.

pCM136 is a v-*rel* variant which arose during cloning. pCM136 has two small deletions of 14 and 16 bp both 5′ to the first *Pst*I site in v-*rel* (S.-T. Lee, T. D. Gilmore, C. K. Miller, and H. M. Temin, unpublished data). CEF transfec-

ted with pCM136 express a 57,000-dalton v-*rel* polypeptide. pCM136 was kindly provided by C. Miller.

The construction of the c-*rel*/v-*rel* recombinants pCV1 and pVC2 is described elsewhere (35).

An approximately 3.1-kilobase-pair-*Hin*dIII-to-*Aha*III fragment containing a chloramphenicol acetyltransferase (CAT)/beta-galactosidase fusion gene was obtained from A. Panganiban (unpublished work) and was inserted into the SNV vector pME111 (8) which had been cut with *Hin*dIII and *Sma*I to create pTGIV. The CAT coding sequences encompass a region from the initiating ATG to the *Pvu*II site located 38 codons downstream. The beta-galactosidase sequence encodes approximately 1,020 amino acids, starting at a 5′ *Sma*I site in pMC1403 (4) and extending to the end of the gene. pTGIII was constructed by inserting the 174-bp *Stu*I-to-*Hin*cII fragment of v-*rel* at the CAT-beta-galactosidase junction of pTGIV.

pTGV was constructed by inserting a 624-bp *Hin*cII-to-*Hin*cII fragment (from positions 1,725 to 2,349) of the *lacZ* gene (32) into pd*Stu*I/*Hin*cII at the site of the deletion in v-*rel*.

In pTGVI, v-*rel* sequences up to the unique *Stu*I site in v-*rel* were joined to *lacZ* sequences starting at position 1,725 (32). In pTGVII, sequences up to the *Hin*cII site of TGIV (at position 1,725 in *lacZ* [32]) were joined to v-*rel* sequences, starting at the unique *Hin*cII site in v-*rel*.

To generate pTG15 and pTG18 through TG20, complementary oligonucleotides were synthesized (DNA Synthesizing Facility, University of Wisconsin, Madison) with the coding sequence Leu-Glu-Met-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro (corresponding to the simian virus 40 (SV40) large T-antigen nuclear-localizing sequence [16]). (The nucleotide sequence of the coding strand of the oligonucleotides was CTAGAGATGACGCCACCAAAGA AGAAAAGGAAAGTGGAGGACCCT.) This fragment was then inserted in the following places: at the amino terminus of v-*rel*, after deletion of the v-*rel* initiating codon to create pTG15, at the *Hin*cII site in the middle of v-*rel* to create pTG18, at the *Stu*I site to create pTG19, and at the *Stu*I-*Hin*cII junction of pd*Stu*I/*Hin*cII (Fig. 1) to create pTG20.

Site-directed mutagenesis was performed by the procedure of Kunkel (20). The unique *Bam*HI-to-*Hin*cII fragment of v-*rel* was inserted into M13mp18 RF and mutagenized (see Fig. 4) with a 22-bp synthetic oligonucleotide (DNA Synthesizing Facility). Mutant plasmids were confirmed by dideoxy DNA sequencing after both mutagenesis had occurred and reinsertion into the v-*rel*-containing viral plasmid was done.

Immunoprecipitation. Immunoprecipitations were performed essentially as described previously (11). CEF were labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) 6 days after transfection. Spleen cells were derived from transformed clones which were isolated in agarose and grown in liquid medium. Spleen cells were labeled as described elsewhere (11). Detergent lysates containing equal numbers of trichloroacetic acid-precipitable counts were immunoprecipitated with *rel* antiserum and were analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gels. Autoradiography was performed on gels which had been treated with sodium salicylate before drying.

Indirect immunofluorescence. Indirect immunofluorescence was performed as described previously (11). CEF were transferred onto cover slips 5 days after transfection and were analyzed on the following day; spleen cells were centrifuged onto cover slips before analysis. After methanol fixation, cells were incubated with either rabbit *rel* antiserum (11) or rabbit beta-galactosidase antiserum (a kind gift from V. Baichwal and B. Sugden) as indicated, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (Cappel Laboratories, West Chester, Pa.). The p59$^{v\text{-}rel}$ protein colocalized with small nuclear ribonucleoprotein antigens located throughout the nucleus. Its location was clearly different from that of lamin antigens (data not shown).

Biochemical fractionation. Biochemical fractionation of transformed spleen cells was performed exactly as described previously (11, 42). Fractionations were performed in a low-salt (10 mM NaCl) buffer. Additionally, nuclear pellets were made by pelleting the homogenate at 1,000 × *g* for 5 to 8 min at 0°C. The anti-lamin antibody (23) was a kind gift from F. McKeon and M. Kirschner. Quantification of bands was performed either by scintillation counting of excised bands or by densitometric scanning of autoradiograms.

Tumorigenicity assay. Four-day-old chickens were injected intraperitoneally with 1 ml of a virus stock that had been harvested either from transformed spleen cells or transfected CEF. The virus stocks were mixtures of the replication-defective virus and are indicated by the name of the *rel*-containing virus followed by the name of the helper virus in parentheses, e.g., Rev-T(Rev-A). Dead animals were autopsied. A small fraction of the spleen and liver tumors were excised, sectioned, stained, and sent to a pathologist. (We thank Henry Pitot for kindly analyzing the slides of the tumor tissue.)

RESULTS

Definition of a nuclear-localizing sequence within v-*rel* by deletion analysis. Sequences important for the nuclear localization of a number of proteins have been determined (12, 16, 19, 29, 30, 40). To determine the sequences within v-*rel* that are important for the nuclear localization of p59$^{v\text{-}rel}$ in CEF, we constructed several deletion mutants of v-*rel* (Fig. 1). CEF were transfected with the viral plasmids containing these mutant v-*rel* genes in the presence of helper virus (Rev-A) DNA, and the predominant subcellular locations of the altered proteins were determined by indirect immunofluorescence. Protein expression was also monitored by immunoprecipitation of detergent lysates of [^{35}S]methionine-labeled CEF.

In all cases, we found that proteins of the expected sizes were synthesized in CEF (Fig. 1). In most of the cases, the v-*rel* polypeptides, as is the case with intact p59$^{v\text{-}rel}$ (11), were located in the nucleus of infected CEF (Fig. 1 and 2A and D). However, immunofluorescence revealed that viruses with deletions in v-*rel* between the unique *Stu*I site and the *Pvu*II site 132 bp downstream of it (either d*Stu*I/*Hin*cII or d*Stu*I/*Pvu*II[3]) directed the synthesis of polypeptides which were distributed approximately equally between the cytoplasm and nucleus in CEF (Fig. 1 and 2B and C). These results indicated that there was a sequence in this region which was important for the direction to or maintenance of p59$^{v\text{-}rel}$ in the nucleus of CEF.

v-*rel* nuclear-localizing sequence can function in beta-galactosidase. To determine whether the v-*rel* nuclear-localizing sequence defined by these deletions could direct a normally cytoplasmic protein to the nucleus, we constructed pTGIII and pTGIV (Fig. 3). TGIII encoded a fusion protein with 38 amino acids of bacterial CAT (to provide an initiator ATG), followed by 58 amino acids of v-*rel* (derived from v-*rel* between the unique *Stu*I and *Hin*cII sites), and ending with 1,020 amino acids of the bacterial beta-galactosidase gene.

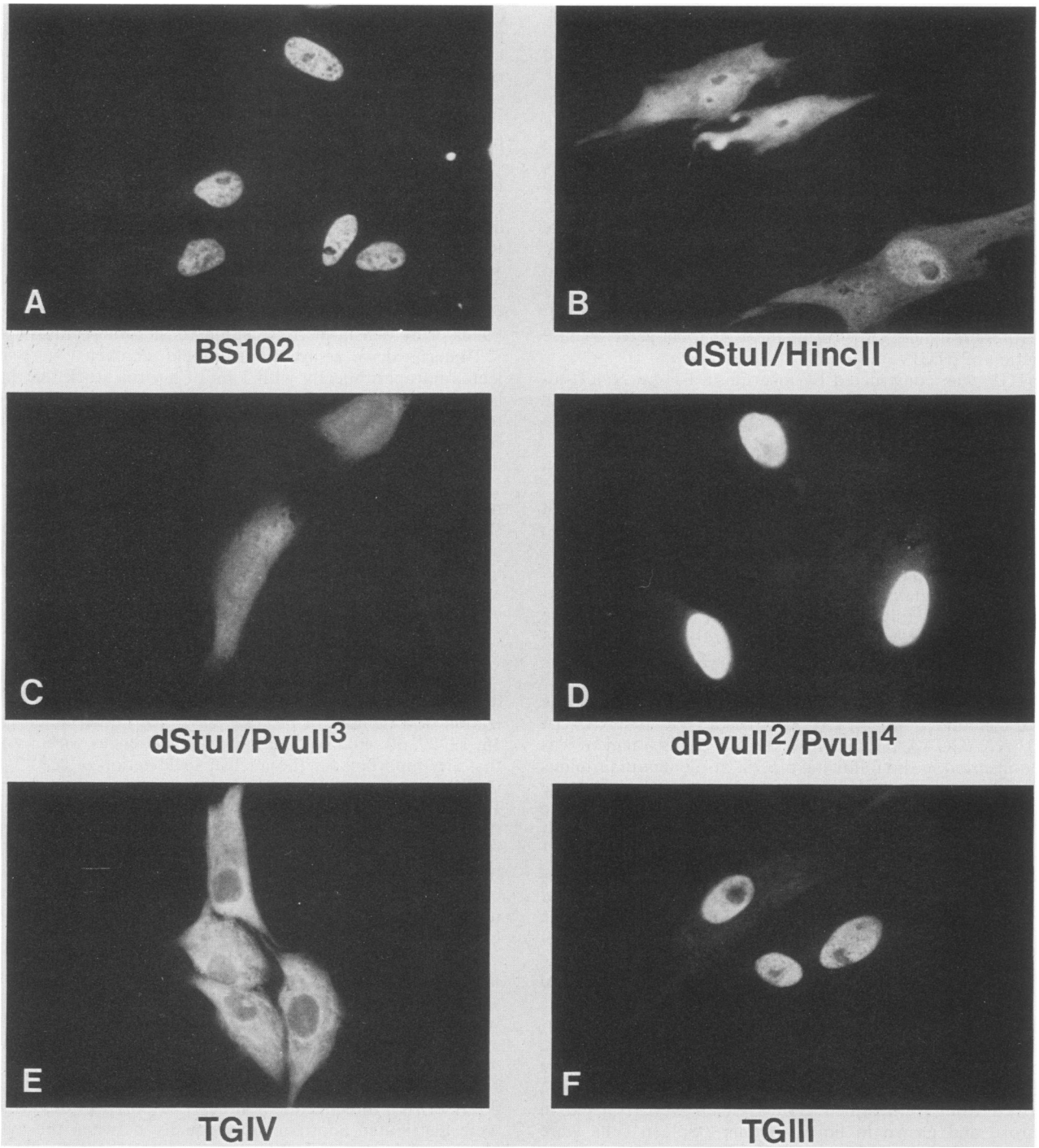


FIG. 2. Determination of sequences important for nuclear localization of $p59^{v\text{-}rel}$ by immunofluorescence. Infected CEF (panels A through J) or spleen cells (panels K and L) were analyzed by indirect immunofluorescence with anti-*rel* serum (panels A through D and G) or anti-beta-galactosidase serum (panels E, F, and H through L) primary antibody.

The v-*rel* sequences were not present in the otherwise identical pTGIV (Fig. 3).

CEF were transfected with pTGIII or with pTGIV in the presence of Rev-A helper virus DNA, and 6 days later, the cells were fixed and stained with a primary rabbit antibody against beta-galactosidase. Uninfected CEF showed no detectable staining with the anti-beta-galactosidase serum (data not shown). The polypeptide encoded by TGIV was exclusively cytoplasmic, whereas the polypeptide encoded by TGIII was primarily nuclear (Fig. 2E and F and 3). Occasionally, we detected some beta-galactosidase staining in the cytoplasm, as well as in the nucleus, in TGIII-infected CEF (note one cell in Fig. 2F). However, the overall pattern of nuclear staining in a population of TGIII-infected cells appeared to be identical to the generally nuclear staining that we see in CEF which express v-*rel*.

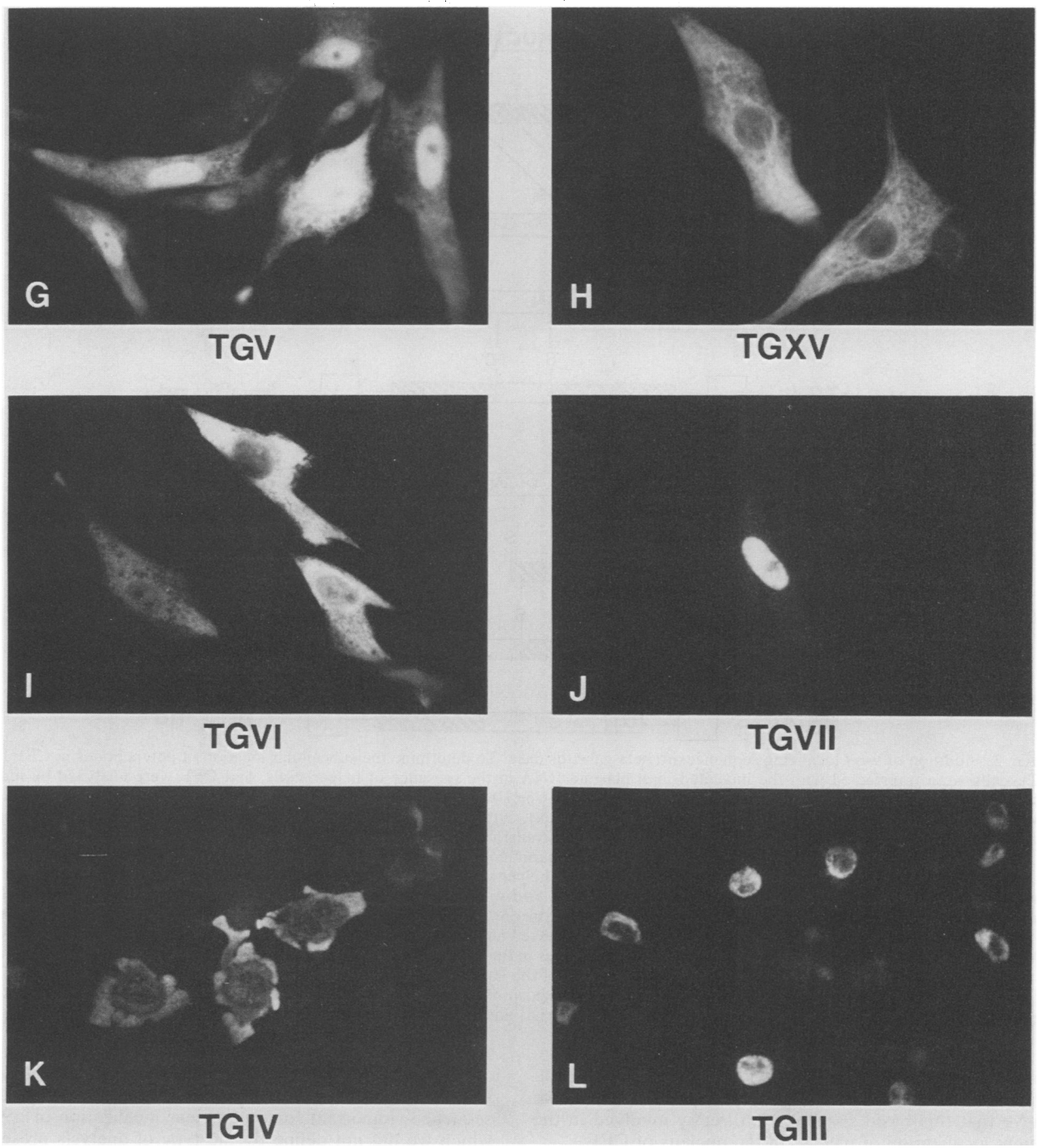


FIG. 2—*Continued.*

These results indicated that v-*rel* contains a sequence between the *Stu*I and *Hinc*II sites which encodes an autonomous nuclear-localizing sequence. Furthermore, this sequence appears to be sufficient to direct and maintain a normally cytoplasmic protein (CAT/beta-galactosidase) in the nucleus of CEF.

Mutation of v-*rel* nuclear-localizing sequence. In the region defined by deletion analysis as important for nuclear localization, there is a single stretch of basic amino acids; four of six amino acids are lysine or arginine (see Fig. 4 and references 34 and 41). This sequence is similar to previously described nuclear-localizing sequences (16, 30). Therefore, by using site-directed mutagenesis, we changed the internal lysine and arginine residues to asparagine and tryptophan and created virus NW-1 (Fig. 4). CEF infected with NW-1 virus encoding this mutated v-*rel* protein synthesized a polypeptide indistinguishable from $p59^{v\text{-}rel}$ by immunoprecipitation (data not shown). However, immunofluorescence of NW-1-infected CEF with anti-*rel* serum showed a nuclear and cytoplasmic distribution (Fig. 4 and 5A, panel B) similar to that seen with v-*rel* proteins with deletions in this region. Furthermore, when this mutated sequence was inserted into

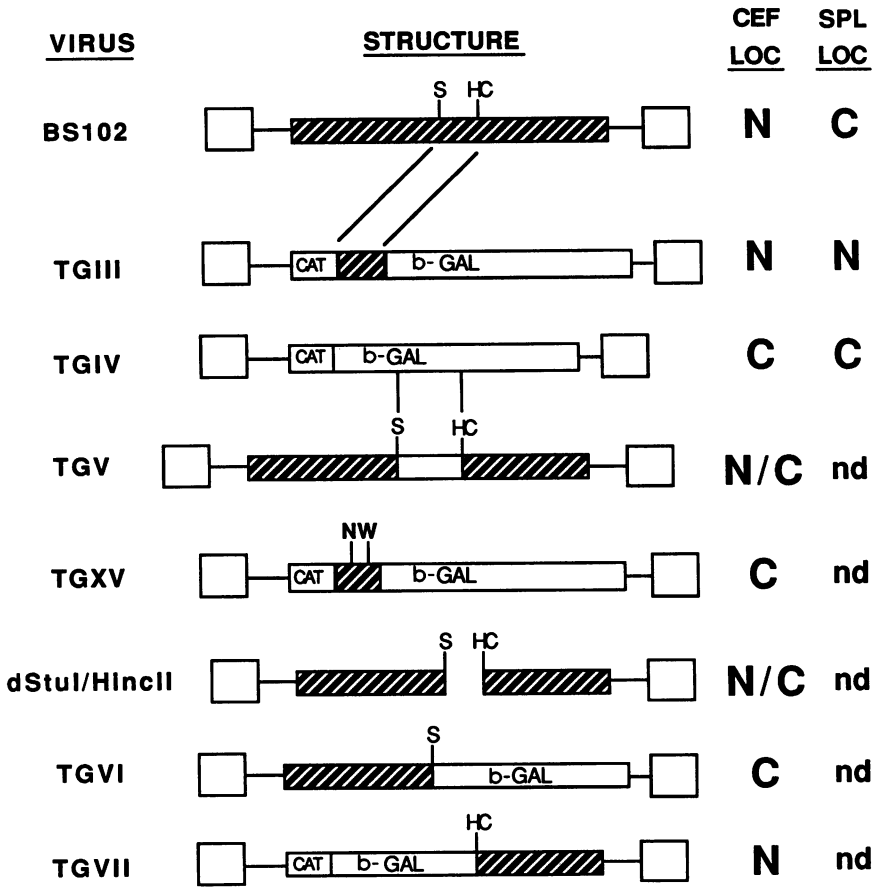


FIG. 3. Addition of v-*rel* localizing sequences to beta-galactosidase. To determine the subcellular location of polypeptides in CEF (CEF LOC), cells were transfected with the indicated viral plasmid DNA in the presence of helper virus, and CEF were analyzed by indirect immunofluorescence with anti-*rel* serum (BS102, TGV, and d*Stu*I/*Hin*cII) or anti-beta-galactosidase serum (TGIV, TGIII, TGXV, TGVI, and TGVII). The location of the protein in CEF was either nuclear (N), cytoplasmic (C), or nuclear and cytoplasmic (N/C). To determine the location of the polypeptides in spleen cells (SPL LOC), a CM103-transformed nonproducer spleen cell line was superinfected with stocks of the indicated viruses and analyzed 7 days later by indirect immunofluorescence with anti-beta-galactosidase serum. (The location of the protein in BS102-infected spleen cells was determined for both a helper virus-containing cell line and helper virus-free cell line with anti-*rel* serum with identical results.) In TGIII, v-*rel* sequences from the *Stu*I-to-*Hin*cII sites in v-*rel* were inserted at the junction of the CAT and beta-galactosidase (b-GAL) sequences of TGIV. TGV is derived from d*Stu*I/*Hin*cII and has a 624-bp insertion of beta-galactosidase sequences (□) into the site of the v-*rel* deletion in d*Stu*I/*Hin*cII. TGV expressed an approximately 70,000-dalton hybrid polypeptide in infected CEF. TGXV is the same as TGIII except the lysine and arginine residues in the v-*rel* nuclear-localizing sequence have been changed to asparagine (N) and tryptophan (W), respectively. Symbols: □ (at the ends of the structures), viral long terminal repeats; ▨, v-*rel* sequences. Other sequences are as indicated in the figure. S and HC indicate the positions of the unique *Stu*I and *Hin*cII sites, respectively, in v-*rel*. The diagrams in the figure are only approximately to scale (see Materials and Methods for precise descriptions). nd, Not determined.

beta-galactosidase in TGXV, it no longer directed beta-galactosidase to the nucleus (Fig. 2H and 3). Thus, we believe that this basic sequence is directly involved in the efficient localization of p59$^{v\text{-}rel}$ in the nucleus of CEF.

v-*rel* nuclear-localizing sequence can function in spleen cells. To determine the subcellular location of the TGIV- and TGIII-encoded beta-galactosidase polypeptides in v-*rel*-transformed spleen cells, we superinfected a CM103-transformed nonproducer spleen cell line and performed indirect immunofluorescence with a primary antibody against beta-galactosidase. The results were indistinguishable from those seen in CEF: in TGIV-infected spleen cells, the immunofluorescent staining was exclusively cytoplasmic, and in TGIII-infected spleen cells, the staining was primarily nuclear (Fig. 2K and L and 3). These results indicated that the nuclear-localizing sequence of v-*rel* within the context of the CAT/beta-galactosidase fusion protein could be efficiently used in both CEF and v-*rel*-transformed spleen cells.

v-*rel* contains other sequences important for nuclear localization of p59$^{v\text{-}rel}$. It appears that there are also other sequences important for the nuclear localization of p59$^{v\text{-}rel}$ which we did not define by the type of analysis presented above. Since the abbreviated v-*rel* polypeptides encoded by d*Stu*I/*Hin*cII and d*Stu*I/*Pvu*II[3] were 48,000 to 50,000 daltons and therefore small enough that they may have been able passively to enter the nucleus of infected CEF (3, 21, 25, 26), we constructed pTGV. In pTGV, beta-galactosidase sequences encoding approximately 20,000 daltons of protein were substituted for the v-*rel* sequences deleted in pd*Stu*I/*Hin*cII (Fig. 3) to create a coding sequence for a protein too large to enter the nucleus passively. TGV-infected CEF expressed a 70,000-dalton v-*rel*/beta-galactosidase polypeptide (data not shown) which was distributed approximately equally between the nucleus and the cytoplasm in CEF, as determined by immunofluorescence with anti-*rel* serum (Fig 2G and 3). The pattern of immunofluorescence in TGV-

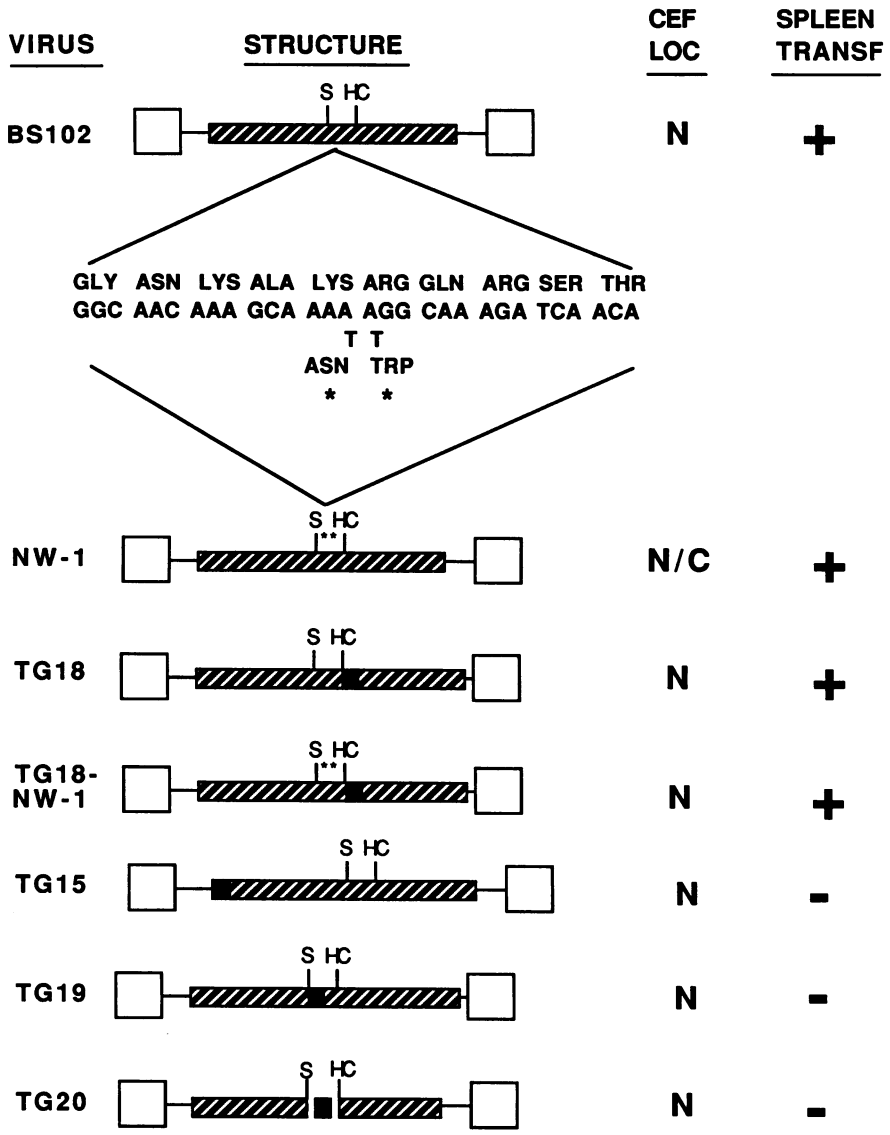


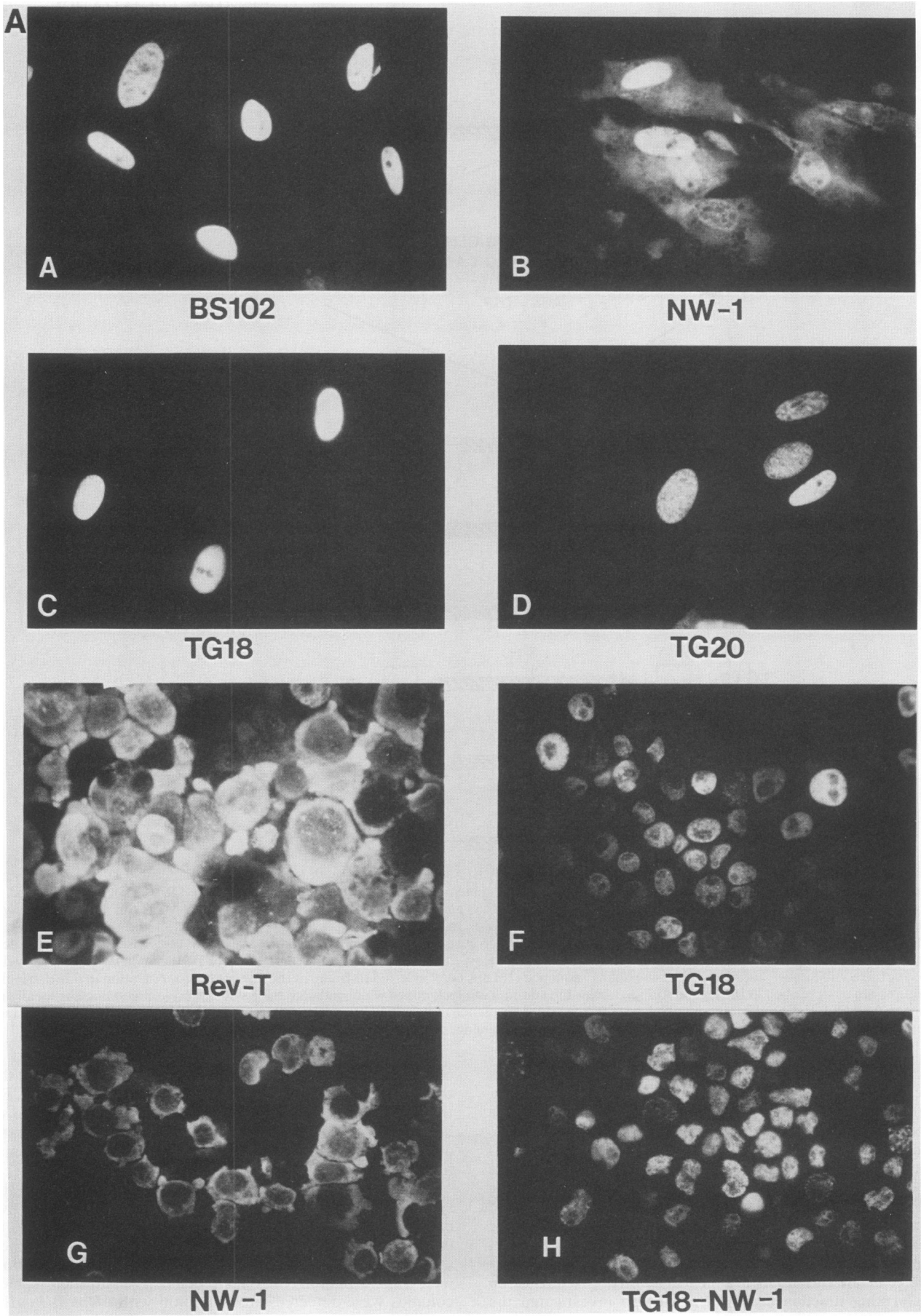
FIG. 4. Addition of heterologous localizing sequences to v-*rel*. CEF were transfected with the indicated viral plasmid DNAs, and protein (data not shown), subcellular locations in CEF (CEF LOC), and spleen cell-transforming abilities (SPLEEN TRANSF) were analyzed as described in the legend to Fig. 1. The relative positions of the *Stu*I (S) and *Hin*cII (HC) sites are indicated. NW-1 has the mutations shown; these mutations are indicated by asterisks for NW-1 and TG18-NW-1. In NW-1, the final A residue of a Lys codon and the adjacent first A residue of an Arg codon in v-*rel* were changed to T residues, which resulted in Asn and Trp codons, respectively. Three-letter amino acid designations are as conventionally used and are shown above and below the nucleic acid coding sequences for BS102 and NW-1, respectively. In TG20, the SV40 large T-antigen nuclear-localizing sequence (16) has been inserted in-frame at the site of the v-*rel* deletion in d*Stu*I/*Hin*cII. Structures are as described in the legend to Fig. 3, with the addition of black boxes which indicate the SV40 large T-antigen nuclear-localizing sequence (see Materials and Methods). The sizes of the added sequences are roughly to scale but are generally larger for visual clarity (see Materials and Methods for the exact sizes of the added coding sequences).

infected CEF was indistinguishable from that seen in d*Stu*I/*Hin*cII-infected CEF. Therefore, v-*rel* contains sequences, other than those between the *Stu*I and *Pvu*II[2] sites, with a limited capacity to direct proteins to the nucleus in CEF.

These sequences appear to be towards the carboxy terminus of p59$^{v\text{-}rel}$. In TGVI, sequences upstream of the *Stu*I site in v-*rel* were attached to beta-galactosidase; the resulting fusion protein was cytoplasmic in infected CEF (Fig. 2I and 3). In TGVII, sequences downstream of the *Hin*cII site were attached to beta-galactosidase; this protein was located in the nucleus of CEF (Fig. 2J and 3).

Effect of alterations in the nuclear-localizing sequence on transforming function of p59$^{v\text{-}rel}$. We also investigated the effect of the various deletions in v-*rel* shown in Fig. 1 on the transforming ability of v-*rel*. Virus was harvested from CEF transfected with recombinant plasmids in the presence of helper virus DNA, and this virus was used to infect fresh chicken spleen cells, which are target cells for transformation by v-*rel*.

In most cases, deletion of v-*rel* sequences inactivated the spleen cell-transforming function (Fig. 1). There were two exceptions: d*Hae*III/*Hin*cII, which has a 27-bp deletion in the middle of v-*rel*, still transformed with the same efficiency as wild-type v-*rel* did, and d*Hin*cII/*Pvu*II[3] induced an abortive transformation of spleen cells. Transformed spleen colonies were detected after infection with d*Hin*cII/*Pvu*II[3].


A
A
BS102
B
NW-1
C
TG18
D
TG20
E
Rev-T
F
TG18
G
NW-1
H
TG18-NW-1

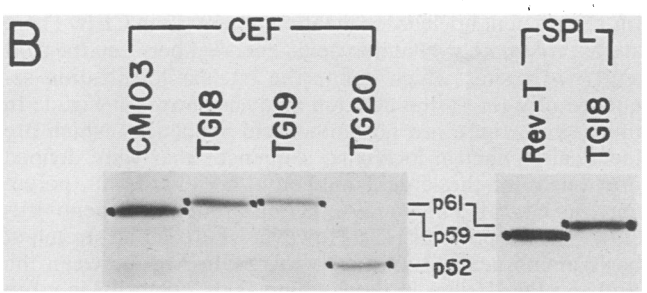


FIG. 5. Analysis of v-*rel* hybrid proteins. (A) Infected CEF (panels A through D) or spleen cells (panels E through H) were analyzed by indirect immunofluorescence with an anti-*rel* primary antibody as described in the legend to Fig. 2. (B) [^{35}S]methionine-labeled CEF (CEF) or spleen cells (SPL) were analyzed by immunoprecipitation as described in Materials and Methods. The positions of the specifically immunoprecipitated bands are indicated in the figure by black dots at the sides of the bands and by lines at the sides of the figure. CM103 and Rev-T encode wild-type v-*rel* proteins, TG18 and TG19 encode 61,000-dalton proteins, and TG20 encodes a 52,000-dalton protein (Fig. 4 and text).

However, these colonies could not be propagated indefinitely in liquid culture. This limited growth potential appears to be a property of the altered protein encoded by d*Hin*cII/*Pvu*II[3], since when virus was harvested from d*Hin*cII/*Pvu*II[3]-transformed spleen cells before they died, it induced the same abortive transformation in fresh spleen cells (data not shown). We have not yet been able to obtain enough of these abortively transformed cells to perform immunoprecipitation or immunofluorescence on d*Hin*cII/*Pvu*II[3]-transformed spleen cells. These results indicate that amino acid sequences near the *Hin*cII site of v-*rel* are not of major importance for the transforming function of v-*rel*. (This point is important for some of the analysis presented below.)

The v-*rel* protein encoded by NW-1 which contains the mutated nuclear-localizing sequence still transformed spleen cells (Fig. 4) with the same efficiency as wild-type p59$^{v\text{-}rel}$ did. Furthermore, the protein encoded by NW-1 was located exclusively in the cytoplasm of NW-1-transformed spleen cells, as determined by indirect immunofluorescence (Fig. 5A, panel G). This result indicates that the spleen cell-transforming function of p59$^{v\text{-}rel}$ can be separated from the nuclear-localizing function that we have defined as being active in CEF.

Addition of subcellular-localizing sequences to v-*rel*. Since wild-type p59$^{v\text{-}rel}$ has different subcellular locations in spleen cells and CEF, that is, primarily cytoplasmic in spleen cells and nuclear in CEF, we decided to test the effect of the addition of heterologous subcellular-localizing sequences on the transforming potential of v-*rel*.

Initially we added sequences such as the plasma membrane-directing sequence of v-*src* (27) and the nuclear-directing sequence of the SV40 large T-antigen (16) to the amino terminus of v-*rel* (pTG15; Fig. 4). These sequences had the predicted effect on the v-*rel* protein localization; that is, the v-*src*/v-*rel* protein was located in the cytoplasm of CEF (data not shown) and the T-antigen/v-*rel* protein was in the nucleus of CEF (Fig. 4). These hybrid *rel* proteins did not transform spleen cells. However, the results were somewhat difficult to interpret with respect to transforming ability, since the addition of some presumably nonlocalizing sequences (for example, from the amino terminus of c-Ha-*ras* and v-Ha-*ras*) to the amino terminus of v-*rel* also abolished the transforming function (data not shown). These results are consistent with previous experiments indicating that the transforming function of v-*rel* is sensitive to changes at the amino terminus (35).

Therefore, we constructed pTG18, pTG19, and pTG20 (Fig. 4); pTG18 contains a 17-amino acid addition of SV40 T-antigen nuclear-localizing sequences (see Materials and Methods and reference 16) at the *Hin*cII site of v-*rel* (a region that we had determined may be permissive for amino acid changes; see above and Fig. 1), pTG19 contains these same T-antigen sequences inserted into the *Stu*I site in v-*rel*, and pTG20 contains the T-antigen sequences substituted for the deleted sequences of pd*Stu*I/*Hin*cII (Fig. 1), a plasmid that lacks the v-*rel* nuclear-localizing sequence (see above). Again CEF were transfected with plasmid DNA and helper virus DNA, cells were analyzed for protein synthesis by immunoprecipitation and immunofluorescence, and virus derived from these plasmids was tested for its ability to transform spleen cells. In each case, proteins of the expected sizes were synthesized in CEF (Fig. 5B), and these cells invariably showed nuclear fluorescence (Fig. 4 and 5A, panels C and D and data not shown) which was indistinguishable from that seen in TG15-infected CEF. However, only TG18 transformed spleen cells.

Spleen cells transformed by TG18 synthesized a protein that was the same size as that seen in TG18-infected CEF (Fig. 5B), and, most surprisingly, spleen cells transformed by TG18 showed a uniformly nuclear pattern of immunofluorescent staining in contrast to wild-type v-*rel*-transformed spleen cells, which showed primarily cytoplasmic staining (Fig 4 and 5A, panels E and F; 11, 33). This result indicated that v-*rel* encodes a protein with a transforming function which can act in the nucleus. More specifically, the ability of TG18 to transform spleen cells indicated that the addition of the SV40 T-antigen nuclear-localizing sequences to v-*rel* at the 5′ end (pTG15) or at the *Stu*I site (pTG19) disrupted the transforming function of v-*rel* by insertional mutagenesis and not as a result of an effect on the subcellular localization of the encoded polypeptide.

The results obtained with TG20 indicated that the SV40 T-antigen nuclear-localizing sequence could substitute for the v-*rel* nuclear-localizing sequence in CEF. However, there must be other sequences between the *Stu*I and *Hin*cII sites of v-*rel* needed for the transforming function which could not be simply replaced by providing a heterologous nuclear-localizing sequence (Fig. 4 and 5A, panel D).

TABLE 1. Subcellular distribution of v-*rel* oncoproteins after biochemical fractionation of transformed spleen cells[a]

Virus	Protein	Distribution of fraction		
		N	P	C
Rev-T	p59$^{v\text{-}rel}$	18%	3%	79%
TG18	p61^{TG18}	17%	2%	81%
Rev-T	Lamin	97%	3%	0%
TG18	Lamin	93%	7%	0%

[a] v-*rel*-transformed spleen cells were labeled with [^{35}S]methionine for 6 h at 37°C. Cells that had been swollen in a hypotonic buffer were homogenized in a tight-fitting Dounce homogenizer and separated into nuclear (N), particulate (P), and cytosolic (C) fractions. These fractions were then immunoprecipitated with an excess of anti-*rel* or anti-lamin antibody, and samples were subjected to electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. Gels were dried and subjected to fluorography. Bands were quantified either by scintillation counting of excised bands (for *rel* proteins) or by densitometric scanning of autoradiograms (for lamin proteins). Values correspond to the amount of the polypeptide in the indicated fraction as a percentage of the total of the polypeptide in all three fractions (N plus P plus C). Values are the average of two experiments.

To determine whether the SV40 T-antigen nuclear-localizing sequence could function in the presence of a mutated v-*rel* nuclear-localizing sequence, we constructed pTG18-NW-1. TG18-NW-1 is identical to TG18 except the v-*rel* nuclear-localizing sequence has been mutated, as in NW-1. TG18-NW-1 transforms spleen cells with the same efficiency as TG18 does (Fig. 4). Furthermore, the protein encoded by TG18-NW-1 is the same size on polyacrylamide gels as $p61^{TG18}$ and shows a pattern of staining after indirect immunofluorescence of transformed spleen cells with anti-*rel* serum that is indistinguishable from the nuclear staining seen in TG18-transformed spleen cells (Fig. 5A, panel H). Therefore, the T-antigen nuclear-localizing sequence can function in the presence of the mutated v-*rel* nuclear-localizing sequence.

Tumorigenicity of viruses containing altered v-*rel* sequences. To test the in vivo transforming function of viruses encoding altered *rel* proteins, 4-day-old chickens were injected intraperitoneally with either Rev-T(Rev-A), BS102 (Rev-A), TG18(Rev-A), NW-1(Rev-A) or TG18-NW-1(Rev-A) virus stocks. In each case, all (5 of 5 for Rev-T, 4 of 4 for BS102, 16 of 16 for TG18, 6 of 6 for NW-1, and 6 of 6 for TG18-NW-1) of the birds died within 12 days of a malignant lymphoproliferative disease characteristic of Rev-T (36) (data not shown). Autopsies revealed the presence of multiple spleen and liver tumors in all birds.

Distribution of *rel* oncoproteins after biochemical fractionation of transformed spleen cells. Although by immunofluorescence, the distributions of wild-type $p59^{v\text{-}rel}$ and $p61^{TG18}$ in transformed spleen cells were dramatically different, biochemical fractionation showed a similar distribution of protein. Spleen cells were labeled with [^{35}S]methionine; swollen in hypotonic buffer; broken by homogenization; and separated into nuclear, membrane, and cytosolic fractions by differential centrifugation. Both $p59^{v\text{-}rel}$ and $p61^{TG18}$ were largely (80%) localized in the cytosolic fraction (Table 1). The results obtained for $p59^{v\text{-}rel}$ are similar to those obtained previously (11, 33). The results with $p61^{TG18}$ indicate that the association of this protein with the nucleus is relatively unstable. It did not appear that we were grossly disrupting the nuclei during fractionation, since a known nuclear protein, lamin (23), was located primarily in the nuclear fraction (Table 1).

DISCUSSION

In this report, we demonstrated that the v-*rel* oncogene of Rev-T contains a nuclear-localizing sequence and that a mutation in this sequence does not affect the transforming function of $p59^{v\text{-}rel}$. Furthermore, the addition of the nuclear-localizing sequence of SV40 large T-antigen in a region of v-*rel* not essential for transformation resulted in the expression of a hybrid v-*rel*-transforming protein which appeared to be uniformly nuclear in transformed spleen cells by indirect immunofluorescence. These results support the hypothesis that v-*rel* encodes a protein which can act in either the nucleus or the cytoplasm to transform spleen cells.

Nuclear-localizing sequence in v-*rel*. Recombinant viruses CV1 and VC2 that have c-*rel* sequences in place of viral envelope-derived sequences at the amino and carboxy termini, respectively, of v-*rel* encode polypeptides which localize to the nucleus in CEF (35) (Fig. 1). This result indicates that the envelope-derived sequences in v-*rel* are not involved in nuclear localization of $p59^{v\text{-}rel}$.

A genetic analysis of v-*rel* (Fig. 1) indicated that there were sequences in the middle of v-*rel* which were important for the efficient nuclear localization of $p59^{v\text{-}rel}$ in CEF. There are approximately 44 amino acids encoded between the *Stu*I and *Pvu*II[2] sites which define the nuclear localization sequence of v-*rel* by the deletion analysis shown in Fig. 1. In this region, there are no amino acid sequences which are identical to nuclear-localizing sequences that were defined previously for the large T-antigen of SV40 (16, 40), polyomavirus large T-antigen (29), the E1A protein of adenovirus (19), or yeast proteins (12). However, there is one stretch of basic amino acids (Lys-Ala-Lys-Arg-Gln-Arg) between the *Stu*I and *Pvu*II[2] sites in v-*rel* (amino acids 298 to 303 in v-*rel*; [34, 41]) which we propose to be the core sequence responsible for the nuclear localization of $p59^{v\text{-}rel}$ in CEF, since the nuclear-localizing sequences described previously have short stretches of basic residues. Furthermore, mutation of the internal Lys and Arg residues in this short sequence altered the ability of $p59^{v\text{-}rel}$ to be localized efficiently to the nucleus in CEF and destroyed the ability of these v-*rel* sequences to direct beta-galactosidase to the nucleus in CEF.

Further analysis will be necessary to map precisely the limits of this nuclear-localizing sequence of v-*rel*. However, insertion of coding sequences from v-*rel* for the 10 amino acids shown in Fig. 4 into beta-galactosidase (at a site downstream of the CAT-beta-galactosidase junction in TGIV) was not sufficient to direct beta-galactosidase to the nucleus in CEF (data not shown). Presently, it is unclear whether this insufficiency is because these 10 amino acids are only part of a $p59^{v\text{-}rel}$ nuclear-localizing signal or whether these v-*rel*-encoded amino acids cannot function within this region of beta-galactosidase. For example, the nuclear-localizing sequence of the SV40 large T-antigen is not functional at all sites within pyruvate kinase (31).

Deletion of sequences from the region of v-*rel* between the *Stu*I and *Pvu*II[2] sites resulted in v-*rel* immunofluorescence which was distributed approximately equally between the nucleus and the cytoplasm (Fig. 1). This result could indicate that there are two functions required for the localization of v-*rel* to the nucleus, one which is involved in entry of the protein into the nucleus and one which is involved in the maintenance of the protein within the nucleus. However, the insertion of the v-*rel* sequences between *Stu*I and *Hin*cII sites into the normally cytoplasmic CAT/beta-galactosidase fusion protein resulted in a protein which was as efficiently localized to the nucleus as $p59^{v\text{-}rel}$ was itself (Fig. 2F). For this reason, we believe that there is a single function in the region that we have defined which is responsible for both the entry and maintenance of $p59^{v\text{-}rel}$ in the nucleus of CEF. We cannot, however, rule out the possibility that there are separate functions important for entry and maintenance in the amino acids encoded by the sequence between the *Stu*I and *Hin*cII sites of v-*rel*.

With the large T-antigen of SV40, the deletion or alteration of the nuclear localization signal results in a protein which is wholly cytoplasmic (16). Whereas the altered large T-antigen of SV40 (as well as the CAT/beta-galactosidase fusion protein) is too large to enter the nucleus by passive diffusion, the d*Stu*I/*Pvu*II[3] and d*Stu*I/*Hin*cII truncated forms of the v-*rel* protein probably can still passively enter the nucleus (3, 21, 25, 26). We therefore thought that the sizes of the deleted v-*rel* polypeptides were the reason for the approximately equal distribution of v-*rel* between the cytoplasm and the nucleus in d*Stu*I/*Pvu*II[3] and d*Stu*I/*Hin*cII-infected CEF. However, the 70,000-dalton v-*rel*/beta-galactosidase polypeptide encoded by TGV also showed a nuclear and cytoplasmic distribution (Fig. 2G and 3). Data from other cell

types (3, 21, 25, 26) indicate that a polypeptide larger than 67,000 daltons cannot enter the nucleus without selective transport. The results obtained with TGV, TGVI, and TGVII therefore indicate that p59$^{v\text{-}rel}$ contains other signals located in the carboxy-terminal half of the protein that affect nuclear localization of p59$^{v\text{-}rel}$ in CEF. However, we believe that these other signals are not as efficient as the one located between the *Stu*I and *Pvu*II[2] sites in v-*rel*. The large T-antigens of polyoma and SV40 viruses and the v-*myc* protein also contain secondary nuclear localization signals which are less efficient than the primary localizing sequence is (16, 29, 40; W. Lee and C. Dang, personal communication).

If p59$^{v\text{-}rel}$ contains an autonomous nuclear-localizing sequence which is functional in CEF, why is it largely a cytoplasmic protein in Rev-T-transformed spleen cells (11, 33; Fig. 5A, panel E)? This result could indicate that the nuclear-localizing sequence of p59$^{v\text{-}rel}$ is not efficiently recognized in spleen cells. However, we have shown that p59$^{v\text{-}rel}$ can efficiently enter the nucleus in the D17 osteosarcoma dog cell line (unpublished data), in E26 virus-transformed avian myeloblasts, and in MSB-1 cells (11), a Marek's disease virus-transformed chicken T-cell line. Furthermore, the CAT/v-*rel*/beta-galactosidase fusion protein which was encoded by TGIII was as efficiently localized to the nucleus in transformed spleen cells as it was in CEF (Fig. 2F and L and 3). This result indicates that the nuclear-localizing sequence of v-*rel* encoded between the *Stu*I and *Hin*cII sites can be efficiently used to transport a heterologous protein to the nucleus in v-*rel*-transformed spleen cells and, therefore, that there must be other sequences in v-*rel* which cause p59$^{v\text{-}rel}$ to behave like a primarily cytoplasmic protein in spleen cells (11, 33). We are currently attempting to identify these sequences.

Transformation of spleen cells by p59$^{v\text{-}rel}$. Previously we showed that there is a correlation between the subcellular location of p59$^{v\text{-}rel}$ and its ability to transform spleen cells (11). That is, p59$^{v\text{-}rel}$ is a cytoplasmic protein in Rev-T-transformed spleen cells (11, 33) and a nuclear protein in Rev-T-infected nontransformed CEF (11). We no longer believe that this difference is significant, since TG18, which has the additional nuclear-localizing sequences of SV40 large T-antigen, was fully transforming and was located exclusively in the nucleus of transformed spleen cells, as judged by immunofluorescence. Moreover, the mutation of the v-*rel* nuclear-localizing sequence in NW-1 did not affect the transforming function of v-*rel*, even though it altered the nuclear localizing function in CEF. Furthermore, none of the mutant or hybrid *rel* proteins that were observed were found in the cytoplasm of CEF-transformed CEF.

We cannot rule out the possibility that a residual amount of v-*rel* protein in the cytoplasm of TG18-transformed spleen cells was responsible for transformation. However, we grossly altered the subcellular distribution of the v-*rel* polypeptide in spleen cells (compare Fig. 5A, panels E, F, G, and H) and did not affect its transforming function. This result, along with the identification of an autonomous nuclear-localizing sequence in v-*rel*, provides strong evidence that v-*rel* encodes a protein with a transforming function which can act in the nucleus.

Our results indicate that v-*rel* may possibly be classified with viral oncogenes defined by the nuclear location of their gene products. This group includes v-*myc* (1, 7, 13), v-*myb* (18), v-*myb-ets* (18), v-*ski* (2), v-*jun* (37), and v-*fos* (6). No clear function has been defined for any of these oncoproteins. Although p59$^{v\text{-}rel}$ can become phosphorylated in an immune complex kinase assay (11, 28, 33), it is not clear that this kinase activity is specifically associated with p59$^{v\text{-}rel}$ (11). Nonetheless, both p59$^{v\text{-}rel}$ and the 61,000-dalton v-*rel* protein encoded by TG18 were phosphorylated in the immune complex (data not shown). If p59$^{v\text{-}rel}$ is specifically associated with a kinase activity important for its transforming ability, our results indicate that substrates for this kinase activity may also be in the nucleus of spleen cells. Alternatively, p59$^{v\text{-}rel}$ may affect gene expression in a more direct way to induce transformation of spleen cells. It has been proposed that p59$^{v\text{-}rel}$ has sequence similarity with the bacterial transcriptional activator protein encoded by the *fnr* gene (5), and in certain cell types, v-*rel* can transactivate transcription from certain viral promoters (C. Gelinas and H. M. Temin, manuscript in preparation).

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