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The t-Unique Coding Domain Is Important to the Transformation Maintenance Function of the Simian Virus 40 Small t Antigen

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The small t antigen (t) of simian virus 40, a 174-amino-acid-containing protein, when present together with the other early viral protein, large T antigen (T), plays an important role in the maintenance of simian virus 40-induced neoplastic phenotype in certain cells. Indeed, each protein functions in a complementary manner in this process. The t coding unit is composed of two segments, a 5′ region of 246 nucleotides which is identical to that of the corresponding 5′ region of the T coding unit and a 3′ segment of 276 nucleotides which is unique. Two mutant, t-encoding genomes, one bearing a missense and the other a nonsense mutation at the same point in the t-unique coding region were constructed in vitro and found to be defective in their ability to dissolve the actin cytoskeleton of rat fibroblasts and to complement T in the growth of mouse fibroblasts in soft agar. Therefore, the unique segment of the t gene encodes a portion of the t molecule which is essential to its transformation maintenance function.

The transforming region of simian virus 40 (SV40) encodes two proteins, large T antigen and small t antigen (T and t, respectively) (25). The genomic organization of this ~2,500-base-pair region is such that the most 5′ coding segments of each gene are identical, and the remaining 3′ regions are unique. Specifically, T and t have a common, N-terminal 82-amino-acid sequence, and their respective C-terminal 626 and 92 residues are unique.

Both proteins can function in a complementary fashion in promoting the maintenance of a neoplastic phenotype in certain established rodent cell lines which have been infected under selected conditions (3, 4, 11, 13, 19, 28, 30, 31, 34). Hence, it might be suspected that their unique coding segments are important to their respective roles in this process. By contrast, T alone can sustain the neoplastic phenotype of other SV40 transformants, whereas t alone appears to be ineffective in this regard (6, 7, 26, 31). Given these findings, it is possible that, in some settings, T can perform its own function and that of t or that certain cells can supply a t-like function.

Little is known of how t or T operates in the viral transforming mechanism, and there is limited information on the in vitro functional properties of t. By immunofluorescent and biochemical extraction analysis, both the SV40 and the polyomavirus t protein appear to be located in both the nucleus and the cytoplasm of infected cells (10, 22, 40). When introduced into rat cells, the SV40 protein can promote the dissolution of the actin cytoskeleton (2, 14, 15, 26). Moreover, it can render certain cells relatively resistant to the DNA synthesis inhibition effect of theophylline, stimulate them to synthesize a new centriolar antigen(s), and, together with T, promote persistent cycling of G1-arrested cells (5, 15, 27, 29, 33). Whether any of these properties contributes to its transformation maintenance function is not yet clear, but this can eventually be tested by genetic means. In this regard, the technology of synthetic oligonucleotide-directed mutagenesis has made it possible to isolate a variety of specific and useful mutants of transforming genes. As the starting point for an analysis of certain t structure-function relationships, with the goal of learning which individual biologic and biochemical properties of t are linked to its transforming function, this approach was used to introduce mutations into the t gene. Given the possibility that at least part of the unique region of this protein is essential to its transforming activity, selected transversions were produced in the corresponding region of its coding sequence. Specifically, two point mutations were generated within the TGC triplet encoding Cys_{111}. The t-unique segment contains 10 of its 11 Cys residues. Six of them are organized into the structure Cys_{111} X Cys X X Cys X_{21} Cys X Cys X X Cys, which is also found in other papovavirus t antigens (8, 21). Because each of the α and β subunits of these mammalian, glycopeptide tropic hormones, which have been sequenced, contains a single Cys X Cys X X Cys cluster, Friedmann et al. (12) raised the possibility that there may be some functional homology between these proteins and t. This possibility and the knowledge that at least some of these hormones have growth-promoting activity provided additional impetus for asking whether selected mutations within this Cys-rich region affect the transforming function of t.

MATERIALS AND METHODS

Cells and viruses. All cells were grown on plastic surfaces in Dulbecco modified Eagle minimal essential medium at 37°C in a 10% CO_2 atmosphere. SV402 is a T^-, t^+ derivative of SV40 (26). It was routinely grown in Cos-1 cells and arose originally by transfection of its parental plasmid genome, pHR402, onto these cells. SVt-Trp_{111} and SVt-$STOP_{111}$ are mutant derivatives of SV402, bearing TGG and TGA substitutions, respectively, for the TGC triplet encoding Cys_{111} (see below). Like SV402, they were propagated in Cos-1 cells. The titers of all viruses were determined by VP_1 induction, as measured by specific immunofluorescence (26). SV40 and dl883 were also assayed by plaque formation on CV-1P cells. BALB/c 3T3 ClA31 and Rat-1 cells are immortal lines of murine and rat embryonic fibroblasts, respectively, which fail to display other known characteristics of a

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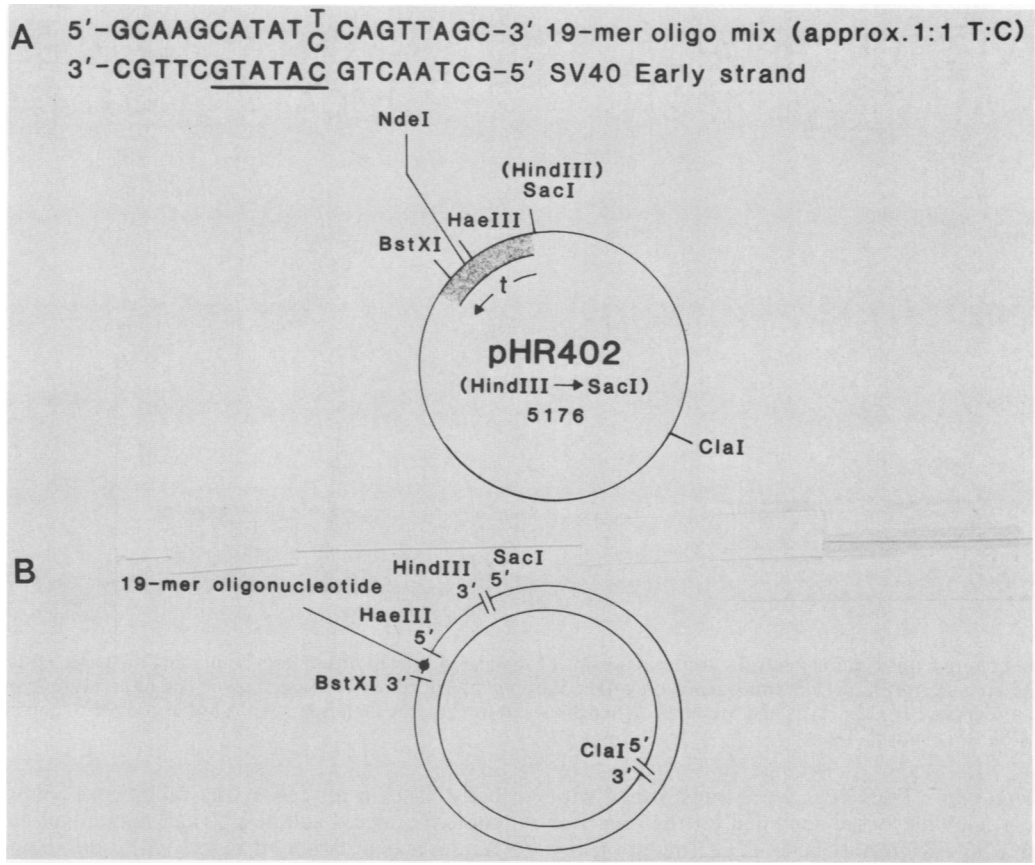

FIG. 1. Site-directed mutagenesis of the C·G base pair at the *Nde*I site of pHR402 t. (A) Nucleotide sequence of the site. Two micrograms of the plasmid pHR402 (26) was partially digested with *Hin*dIII, and the products were separated by electrophoresis in a 1% agarose gel. Full-length linear DNA was isolated from this gel, and its overhanging 5′ ends were filled by incubation with 1 U of the Klenow fragment of DNA polymerase 1 and 2.5 mM of each of the four standard deoxyribonucleoside triphosphates in a 20-μl reaction mixture. A 5′-phosphorylated *Sac*I linker was then introduced at this site by incubation with 20 U of T4 DNA ligase. The DNA product was used to transform *E. coli* HB101, and colonies bearing plasmids containing a *Sac*I rather than a *Hin*dIII site at nt 5171 (denoted 5176 in this figure) were identified. The *Sac*I linker-containing derivative was denoted pHR402S. Five micrograms of pHR402S was (i) digested with *Cla*I, and the resulting ends were dephosphorylated with calf intestine alkaline phosphatase, phenol extracted, and ethanol precipitated as described in Materials and Methods; (ii) digested with *Sac*I (at nt 5176) and *Bst*XI (at nt 4759), and the resulting 4826-base-pair fragment was isolated; (iii) digested with *Sac*I (5176) and *Hae*III (4862), and the resulting 314-base-pair fragment was isolated. The fragments isolated from these digestions were mixed, denatured, and allowed to reanneal as described previously (23). (B) DNA fragments used to obtain gapped heteroduplex molecules for mutant construction. This protocol (23) was also followed to anneal the 1:1 mixture of synthetic oligodeoxyribonucleotides shown in the figure to the resulting gapped heteroduplex and to close the remaining gaps with the DNA polymerase I Klenow fragment and T4 DNA ligase. Mutagenized plasmids were used to transform *E. coli* HB101, and possible mutants were identified by parallel screening with end-labeled oligonucleotides of known sequence, as described in Materials and Methods (see also Fig. 2).

transformed phenotype. REF is the designation for a secondary culture of Fisher rat embryo fibroblasts.

Plasmid constructions. Standard techniques of plasmid isolation, restriction cleavage, and molecular cloning were used throughout (18). pMEAt is a deletion mutant derivative of pHR402. Unlike its 402 parent, it lacks all T-unique coding sequences, but retains an intact t cistron, early promoter, and polyadenylation site. It was constructed by selectively deleting all residual T-unique coding sequence in pHR402 between nucleotide(s) (nt) 4568 and 2666. In CV-1P cells, it led to the synthesis of full-size SV40 t but no detectable, truncated T species (I. Bikel and M. Agha, unpublished data).

pHR402-STOP$_{111}$ (pt-STOP$_{111}$) and pHR402-Trp$_{111}$ (pt-Trp$_{111}$) were constructed by the method of Oostra et al. (23). Specifically, a ~1:1 mixture of a 19-member oligodeoxyribonucleotide, synthesized in an Applied Biosystems 380A instrument and representing the antisense strand of the SV40 sequence 5′-d(GCAAGCATAT$^{T}_{C}$CAGTTAGC)-3′, was annealed to its complementary sequence in the 412-nt single-stranded gap of a circular heteroduplex structure, as described in Fig. 1B. The above-noted gap extended from the *Hin*dIII site at nt 5171 to the *Bst*XI site at nt 4759. A 314-base-pair fragment extending from nt 5176 (see legend to Fig. 1) to the *Hae*III site at nt 4862 was also annealed to this gap. The target nt for mutagenesis (G_{4827} in the antisense strand) was the complement of the 3′ base (i.e., C) of the SV40 E-strand triplet TGC encoding t Cys$_{111}$. It is also a component of an *Nde*I recognition site (CATATG) (Fig. 1A). After annealing the 5′ phosphorylated synthetic oligonucleotide mixture and the adjoining restriction fragment, as noted above, the single-stranded regions abutting these fragments were filled by incubation of the heteroduplex with the Klenow fragment of DNA polymerase I and four deoxynucleoside triphosphates as described previously (23). After ligation of the residual strand interruption with T4 DNA

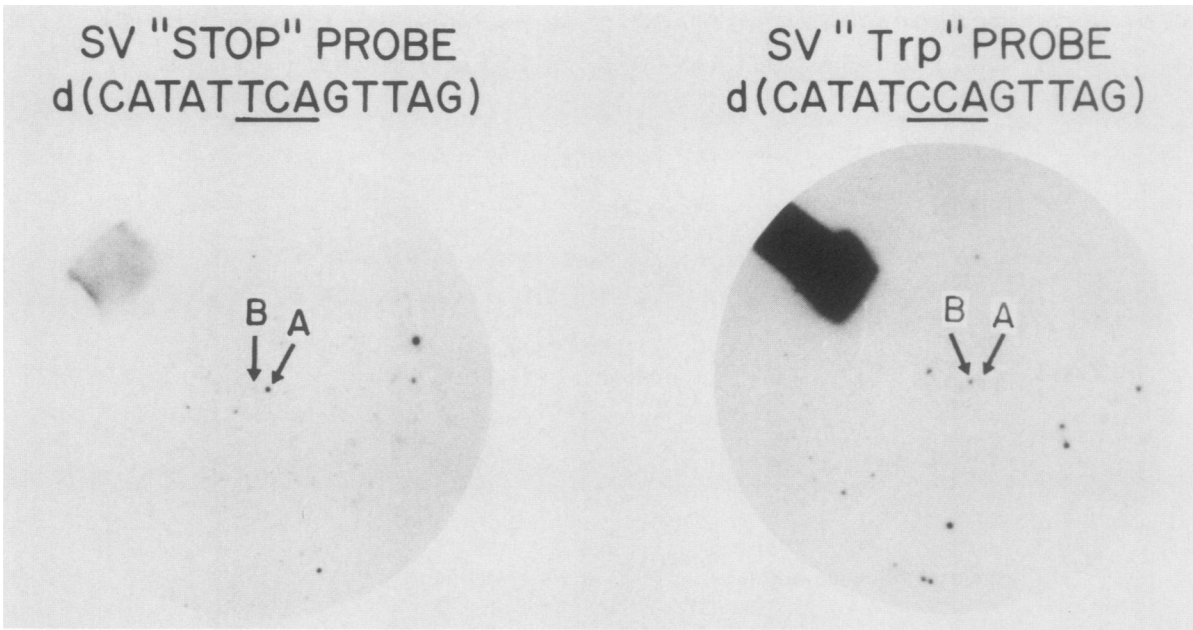

FIG. 2. Mutant colony hybridization analysis. Autoradiograms of replicate nitrocellulose filters containing the same initial *E. coli* HB101 ampicillin-resistant colonies resulting from transformation with in vitro mutagenized pHR402 are shown. The filters were hybridized with the indicated probes, as described in Materials and Methods. When analyzed further, the clones labeled A and B proved to contain the predicted mutations, i.e., pt-$STOP_{111}$ and pt-Trp_{111}.

ligase, *Escherichia coli* HB101 cells were transformed with the DNA product. Colonies were screened for the presence of the predicted TGC→TGA and TGC→TGG mutations by parallel hybridization of replicate, colony-imprinted nitrocellulose filters with two [^{32}P]-5′-end-labeled 13-member oligodeoxynucleotide probes: (i) 5′-d(CATATTCAGTTAG)-3′, T_m = 34°C (for the STOP mutant; Fig. 2, left filter), and (ii) 5′-d(CATATCCAGTTAG)-3′, T_m = 36°C (for the Trp mutant; Fig. 2, right filter) (specific activity of each was ~10^7 cpm/μg), with each probe bearing one of the above-noted third-base substitutions. Hybridization of groups of four filters was performed at 29°C in 8 ml of 0.1% Ficoll–0.1% polyvinylpyrollidone–0.1% bovine serum albumin–10% dextran sulfate, 0.4 M NaCl–0.09 M Tris hydrochloride (pH 7.5)–6 mM EDTA–0.5% sodium dodecyl sulfate (37). The filters were rinsed three times in 25 ml of 4× SSC (1× SSC is 0.01 M sodium citrate plus 0.14 M NaCl) at room temperature. These rinses were followed by progressive 4-h rinses at increasing temperatures to take advantage of the thermal stability of perfect oligonucleotide/single-stranded DNA duplexes compared to the relative instability of duplexes containing single-base-pair mismatches (38). All rinses were performed in a circulating water bath containing 3.5 liter of 4× SSC. Each filter was allowed to dry and then exposed to X ray film in the presence of an intensifying screen. Colonies hybridizing to one but not the other probe were picked (e.g., cf. Fig. 2) and replated for repeat analysis. Plasmid DNA from a suspected, clonal member of each mutant population was isolated, and the presence of the suspected mutation was tested first by assessing the sensitivity of the relevant region to digestion with *Nde*I, followed by direct DNA sequencing of the region from the *Bst*XI at nt 4759 to nt 4840 by the method of Maxam and Gilbert (20).

Immunofluorescence and anti-T/t immunoprecipitation analyses. Immunofluorescence and immunoprecipitation were performed by published methods on unlabeled and [^{35}S]methionine-labeled cells, respectively (2, 10). The antibody used in all cases was rabbit anti-SV40 t purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As described earlier, it reacts with both T and t in specific immunofluorescence and immunoprecipitation tests (2, 10).

Abortive transformation assays. Abortive transformation assays were performed as described previously (26) by infecting replicate cultures of BALB/c 3T3 ClA31 cells with SV40 or the relevant mutant viruses at the indicated multiplicities. In each experiment, 10^4 cells per ml were seeded in medium containing 0.30% agar and 10% calf serum (Colorado Serum Co., Boulder, Colo.), and microcolonies of ≥16 cells were counted 7 to 12 days after infection. From each plate 400 to 600 cells or microcolonies were scored.

Needle microinjection of various cloned DNAs. Each DNA was dissolved in 0.01 M $NaPO_4$ (pH 7.4)–0.14 M NaCl at a concentration of 100 μg/ml, and 20 to 40 pl were injected into each target cell. The microinjection technique used in these experiments has been described previously (2, 26).

Actin cable analyses. Actin cable analyses were performed by UV fluorescence microscopy on HCHO-methanol-fixed cells, as described previously (2, 26), using rhodamine-conjugated phalloidin (a generous gift from S. Chen and R. Pollack) as the probe (36). Amounts of 25 to 200 injected cells from each experimental cover slip were scored.

RESULTS

Construction of mutants bearing substitutions at t codon 111. Following the in vitro recombination of a mixture of two defined-sequence oligodeoxynucleotides, one leading to the substitution of a guanine and the other of an adenine in the third position of the TGC triplet in the SV40 t gene encoding Cys_{111}, the relevant mutant plasmids were isolated. The genome carrying the TGC_{111}→TGG_{111} mutation was designated pt-Trp_{111} and that bearing the TGC_{111}→TGA_{111} mutation was designated pt-$STOP_{111}$. The presence of each mutation was confirmed by direct DNA sequencing (data not shown).

Mutagenesis was performed on a pHR402 template (26). This genome encodes an intact t molecule but has sustained a ~1.5-kilobase deletion in the second exon of the T cistron, retaining only 380 nt of T-unique coding sequence. It therefore fails to encode intact T (26), although under some conditions it can give rise to the synthesis of small amounts of an extremely unstable, ~25-kilodalton (kDa) truncated T species (X. Montano, D. Lane, M. Ellman, and D. Livingston, unpublished data).

pt-Trp_{111} and pt-$STOP_{111}$ were individually transfected onto Cos-1 cells and thereby reconstituted as infectious viruses. High-titer stocks of each were achieved by serial infection of these cells. The parental pHR402 plasmid was similarly treated and led to the development of a parallel stock of SV402 virus (26). The titer of each stock was determined by immunofluorescent analysis of VP-1 synthesis following infection of Cos-1 cells with serial dilutions of virus, as described previously (26).

In vivo and in vitro detection of mutant gene products. Replicate cultures of CV-1P cells were infected with SV402 and dl883 and with each of the two mutant t viruses. Others were microinjected with pMEAt (a t^+, T^- plasmid derivative of pHR402; see below) and with each of the two mutant t-encoding plasmids described earlier. After 24 h the cultures were either labeled with [^{35}S]methionine, extracted, and the lysate exposed to anti-t immunoprecipitation or they were fixed and incubated with the same anti-SV40 t serum (2), in preparation for immunofluorescent analysis. As shown in Fig. 3, both SV402- and SVt-Trp_{111}-infected cells synthesized a ~20-kDa t band, and, as expected, a ~12.5-kDa truncated species of t was detected in the lysate of SVt-$STOP_{111}$-infected cells. The relative yields of wild-type and mutant t proteins in such steady-state labeling experiments were minimally different, with, at most, two- to threefold variations in the concentrations of wild-type and mutant t antigen species in replicate experiments. Moreover, in all three cases, typical nuclear and cytoplasmic t fluorescence was noted (Fig. 4). In this regard, one of the two positive control DNAs in this experiment was pMEAt, a plasmid derivative of pHR402. This genome differs from its parent in having sustained a deletion of all residual T-unique coding sequences. It has retained a functional t gene, early viral promoter, and polyadenylation site. Like CV-1P cells infected with SV402, the same cells microinjected with pMEAt revealed intense nuclear and cytoplasmic fluorescence. Hence, this staining pattern, which was identical to that observed in SV402-infected CV-1P cells, cannot be an effect of the synthesis of a ~25 kDa truncated T species which can arise by transcription of the partially deleted T gene in pHR402. Moreover, the specific nuclear immunofluorescent signal noted in cells containing pt-$STOP_{111}$ and pt-Trp_{111} cannot be due to the presence of any contaminating, T-encoding recombinant genomes which might have arisen in Cos-1 cells because these mutant DNAs were introduced as clonal plasmids by needle microinjection. Given these results, it would appear that, by this criterion, the intracellular distribution of the two mutant t species is similar to that of wild-type t.

Other biological properties of the t species encoded by pt-$STOP_{111}$ and pt-Trp_{111} were tested (Tables 1 and 2). First, in analyses of the t actin cable effect, Rat-1 cells and primary rat embryo fibroblasts (REF) were microinjected with pHR402, the T^-, t^+ parental plasmid, as positive control. In keeping with prior observations (2, 26), significant perturbation of the actin cytoskeleton was noted. By contrast, neither mutant plasmid was active in this assay in either cell line at the concentrations used (Table 1). In addition, the abortive transforming property of the three viruses was measured (Table 2). Specifically, replicate cultures of growing BALB/c 3T3 ClA31 cells (1) were infected with similar multiplicities of SV402 and each of the two mutant viruses in the presence and absence of a T^+, t^- virus, dl883 (28). In keeping with prior results (4, 26, 31), mixed infection by pHR402 and dl883 led to much more efficient agar microcolony formation than was observed with either virus alone. In agreement with this observation, neither mutant virus was active in this assay when tested alone; mixed infection by dl883 and either SVt-Trp_{111} or SVt-$STOP_{111}$ also failed to induce significantly greater agar microcolony formation than was observed with dl883 alone. Hence, both t mutants are relatively defective in their ability to promote Rat-1 and REF actin cable dissolution and to complement T in the acute transformation of an established mouse cell line.

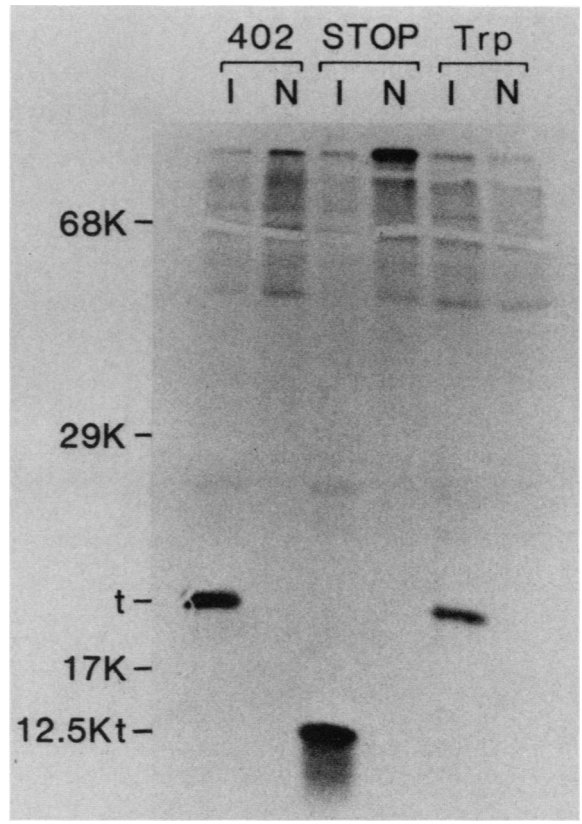

FIG. 3. Immunoprecipitation of [^{35}S]methionine-labeled CV-1P cell extracts 24 h after infection with SV402, SVt-$STOP_{111}$, and SVt-Trp_{111}. Parallel cultures of subconfluent CV-1P cells ~75% confluent, 100-mm dishes) were infected by each of the above-noted viruses at a multiplicity of ~10. After 24 h each culture was labeled with [^{35}S]methionine (100 μCi/ml) for 4 h. After lysis of each culture, anti-t immunoprecipitation was performed as described previously (2, 10). Sodium dodecyl sulfate-gel electrophoresis was performed in a 15% polyacrylamide gel, and a fluorographic exposure (3 days) of the dried and stained gel is shown. Half of each extract (700 μl) was reacted with rabbit antiserum raised against gel-band-purified t antigen (I), and the other half was reacted with preimmune rabbit serum (N).

DISCUSSION

Specific mutations have been introduced into the SV40 t gene at a cysteine codon which serves as the N-terminal

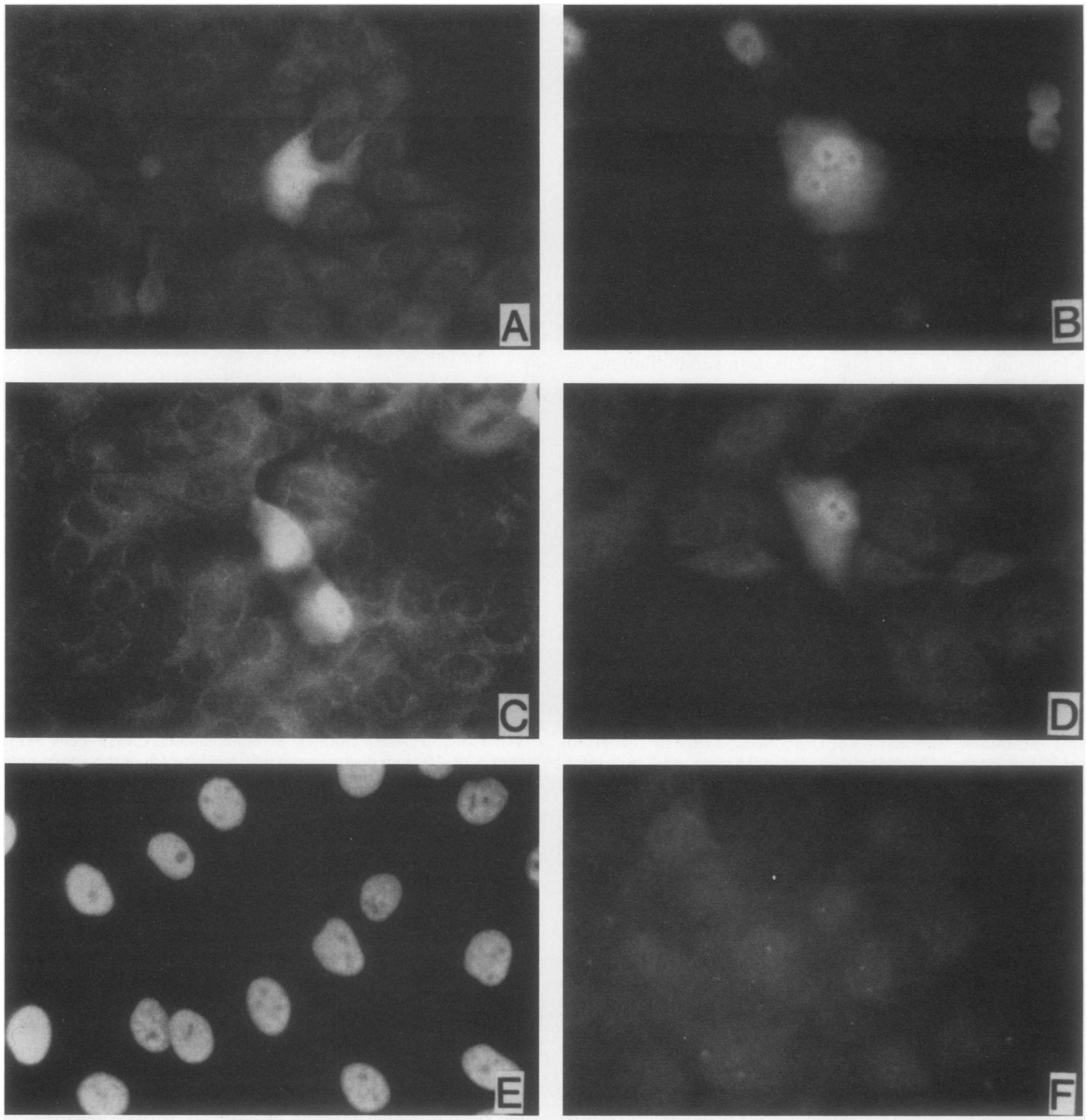

FIG. 4. Anti-t immunofluorescence of CV-1P cells 48 h after microinjection or infection with the following plasmids or viruses: pMEAt (A), SV402 (B), pt-STOP$_{111}$ (C), pt-Trp$_{111}$ (D), dl883 (E). Cells mock infected with an extract of Cos-1 cells.

boundary of a six-residue, canonical sequence which is twice repeated in the t gene of multiple papovaviruses and widely represented in another growth-promoting protein family, the glycopeptide tropic hormones (12). This residue also lies within the t-unique sequence segment of the antigen, a region suspected of contributing to the transformation maintenance function of the protein (30).

Visual inspection of numerous fluorescing cells indicated both cytoplasmic and nuclear mutant t distribution, as was the case for the wild-type protein (Fig. 4). Figure 4 shows three such mutant t-containing cells. Thus, neither mutation seemed to effect a gross alteration in the normal intracellular location of t, although subtle changes remain possible. Moreover, the data suggest that, if a specific t nuclear location signal analogous to that in SV40 T (16, 17) exists, it must lie between residues 1 and 111.

That both mutants are defective in their ability to complement the acute transforming activity of T is apparent from the results of the abortive transformation experiment described in Table 2. Hence, more than the N-terminal 111 residues are essential for this function, and the t-unique region and, possibly, one or both of the canonical Cys-rich clusters noted above, play a significant contributing role in this regard.

TABLE 1. Effect on Rat-1 and REF actin cable structures of wild-type and mutant t antigens[a]

DNA injected	T/t	% Cells with:			
		Intact cables		Dissolved cables	
		Rat-1	REF	Rat-1	REF
pHR401	T, t	39	ND	61	ND
pHR402	t	30	19	70	81
pt-STOP$_{111}$	t-STOP$_{111}$	83	81	17	19
pt-Trp$_{111}$	t-Trp$_{111}$	86	89	14	11
pBR322		88	77	12	23

[a] Growing Rat-1 cells or REF were injected with the various cloned DNA's and the cells were incubated at 37°C for 24 h and then stained for actin cables as described in Materials and Methods. ND, Not determined.

It is well known that SV40 t can promote either directly or indirectly the loss of actin cable structures in rodent fibroblast cells (2, 14, 15, 26). Prior to this observation, others noted that a variety of transformed cells are characterized by a depleted or disordered actin cytoskeleton (9, 24, 35, 36). These observations suggest that the cytoskeletal perturbation effect of t is linked to the mechanism of transformation maintenance in the relevant cell lines. The absence of both actin cable perturbing and acute transforming activity of pt-Trp$_{111}$ and pt-STOP$_{111}$ is consistent with this notion. These results do not, however, indicate that the actin cable effect is an integral part of the biochemical change which results in the development of a neoplastic phenotype. Rather, it is equally possible that this cytoskeletal perturbation is an effect of the development of transformed behavior and is, therefore, a post facto marker of this phenotypic change.

Cys$_{111}$ is the most N-terminal of six such residues which constitute the twice repeated canonical sequence Cys X Cys X X Cys. If this 33-amino-acid segment functions as a semi-independent unit, individual substitutions of each cysteine or one or more of the non-cysteinyl intervening residues of this group might lead to the same biological effect: for example, a perturbation of the abortive transforming activity of the protein and a concomitant alteration of the interaction of t with itself (2, 32) or with one or more functionally important cellular proteins (39; C. Murphy, I. Bikel, and D. Livingston, unpublished data). Experiments to test this possibility are in progress.

TABLE 2. Abortive transformation of T-containing BALB/c3T3 ClA31 cells by wild-type and mutant t antigens[a]

BALB/c 3T3 ClA31 cells infected with:	% Microcolonies
Control	<0.5
SV40	12
dl883	1.8
SV402	<0.5
SVt-Trp$_{111}$	<0.5
SVt-STOP$_{111}$	<0.5
dl883 + SV402	10.8
dl883 + t-STOP$_{111}$	2.6
dl883 + t-Trp$_{111}$	2.5

[a] Growing cells (~1.3 × 10^5 per 35-mm dish) were infected with the various viruses. Eight serial 45-min absorption periods at 37°C with 1.5-ml portions of the indicated virus stock(s) were used. After a 24-h incubation at 37°C, the cells were trypsinized, seeded in agar, and incubated at 37°C for up to 2 weeks. Similar infection multiplicities (200 per absorption) were used in all single virus infections. In coinfections (with dl883) the same amount of each virus tested in the singly infected cultures was used.

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