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Analysis of a Nuclear Localization Signal of Simian Virus 40 Major Capsid Protein Vp1

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The nuclear localization signal of the major structural protein, Vp1, of simian virus 40 was further defined by mutagenesis. The targeting activity was examined in cells microinjected with SV-Vp1 variant viral DNAs bearing either an initiation codon mutation of the agnoprotein or mutations in the Vp1 coding sequence or microinjected with pSG5-Vp1 and pSG5-Vp1 mutant DNAs in which Vp1 or mutant Vp1 is expressed from simian virus 40 early promoter. The Vp1 nuclear localization signal functioned autonomously without agnoprotein once the Vp1 protein was synthesized in the cytoplasm. The targeting activity was localized to the amino-terminal 19 residues. While replacement of cysteine 10 with glycine, alanine, or serine did not affect the activity, replacement of arginine 6 with glycine caused the cytoplasmic phenotype. When multiple mutations were introduced among residue 5, 6, 7, 16, 17, or 19, the targeting activity was found to reside in two clusters of basic residues, a cluster of lysine 5, arginine 6, and lysine 7 and a cluster of lysine 16, lysine 17, and lysine 19. The clusters are independently important for nuclear localization activity.

Once papovaviruses, a class of DNA tumor viruses, enter the cell nucleus, they spend most of their reproduction cycle there. During the late phase of infection, cytoplasmically synthesized structural proteins are transported to the nucleus, where they assemble into mature virions (24). While the nuclear localization signal (NLS) of each viral structural protein is usually responsible for the protein's nuclear localization (4, 5, 7, 10, 17, 26, 27), a localization-defective mutant Vp2/3 protein, $Vp2/3_{202T}$, of simian virus 40 (SV40) can localize to the nucleus if wild-type Vp1 is coexpressed in the same cell (13). This fact implies that some SV40 subvirion assembly must take place in the cytoplasm prior to the nuclear targeting of the structural protein. The SV40 Vp1 NLS has been mapped within the first 8 amino acid residues, which can promote nuclear localization of the poliovirus structural protein Vp1, a cytoplasmic protein (26). However, among the relatively well defined NLSs, neither 8 nor 11 amino acids of the amino terminus of SV40 Vp1 are able to promote nuclear entry of the reporter protein when the signal is chemically coupled to it (6). Chelsky et al. have argued that the SV40 Vp1 NLS may require the downstream cysteine residue 10 to maintain a specific conformation for its activity (6). As the Vp1 NLS is likely to be important not only for Vp1 nuclear targeting but also for virion nuclear targeting, we extended analysis of the Vp1 NLS by mutagenesis with an SV40 variant DNA, SV-Vp1, in which the coding segments for Vp2/3 are deleted and which expresses Vp1 (13). Our studies revealed that the Vp1 NLS is a bipartite signal composed of two tracts of basic residues between residues 5 and 19.

In addition to the capsid proteins, the late region of the SV40 genome encodes a small protein, agnoprotein, which is a 61-amino-acid, basic protein that can bind DNA (14). The agnoprotein is expressed late in infection and is present predominantly in the cytoplasmic fraction (14, 19). It has been suggested to play a role in the assembly of virions in the SV40 lytic cycle (16, 18) and in Vp1 nuclear localization (3, 20). We first examined the effect of agnoprotein on Vp1 nuclear localization. pSV-Vp1ΔAgno was constructed, as outlined in Fig. 1, by exchanging the 1,452-bp *Kpn*I-*Sac*I fragment with a PCR-generated fragment in which the initiating ATG of the agnogene was changed to TAG (Fig. 1). For the PCR, the sense primer 5′-CCGCCTCAGAAGGTACCTAACCAAGTTCCT CTTTCAGAGGTTATTTCAGGCCtaGGTGCT-3′ (nucleotides 283 to 342) and the antisense primer 5′-CAAGAATTC GAGCTCGCCCCAACTTG-3′ (designated the *Sac*I antisense primer) were used along with the pSV-Vp1 template (lowercase letters in the PCR primer sequence represent the mutations). The subcellular distributions of large T antigen and agnoprotein (Fig. 2) and of Vp1 (Fig. 3) were examined following nuclear microinjection of SV-Vp1ΔAgno and were compared with those of SV-Vp1. Large T antigen localized to the nuclei in the SV-Vp1ΔAgno-injected cells in fashion similar to that in the SV-Vp1-injected cells (Fig. 2). The perinuclear and cytoplasmic localizations of agnoprotein in SV-Vp1-injected cells (Fig. 2) were the same as those reported for SV40-infected cells (14, 19). In contrast, no agnoprotein was observed in any compartment of the mutant-injected cells (Fig. 2). Vp1 expressed from SV-Vp1ΔAgno localized to the nucleus (Fig. 3), as it did when the agnogene-containing construct, SV-Vp1, was used (Fig. 3). Thus, the observation that Vp1 can localize autonomously is in contrast with the reported role of agnoprotein in Vp1 nuclear localization in the BSC-1 cell line (3, 20). The difference could be explained by differences in the cell lines, as proposed by others (3). As the protein coding sequences in the leaders of some late mRNAs may also function in late transcriptional regulation (1, 2, 11, 12, 23), subsequent analyses of the Vp1 NLS were performed with SV-Vp1.

Progressive amino-terminal-deletion mutants of Vp1, SV-Vp1 dl (3–7) (a deletion of residues 3 through 7), SV-Vp1 dl (3–12) (a deletion of residues 3 through 12), and SV-Vp1 dl

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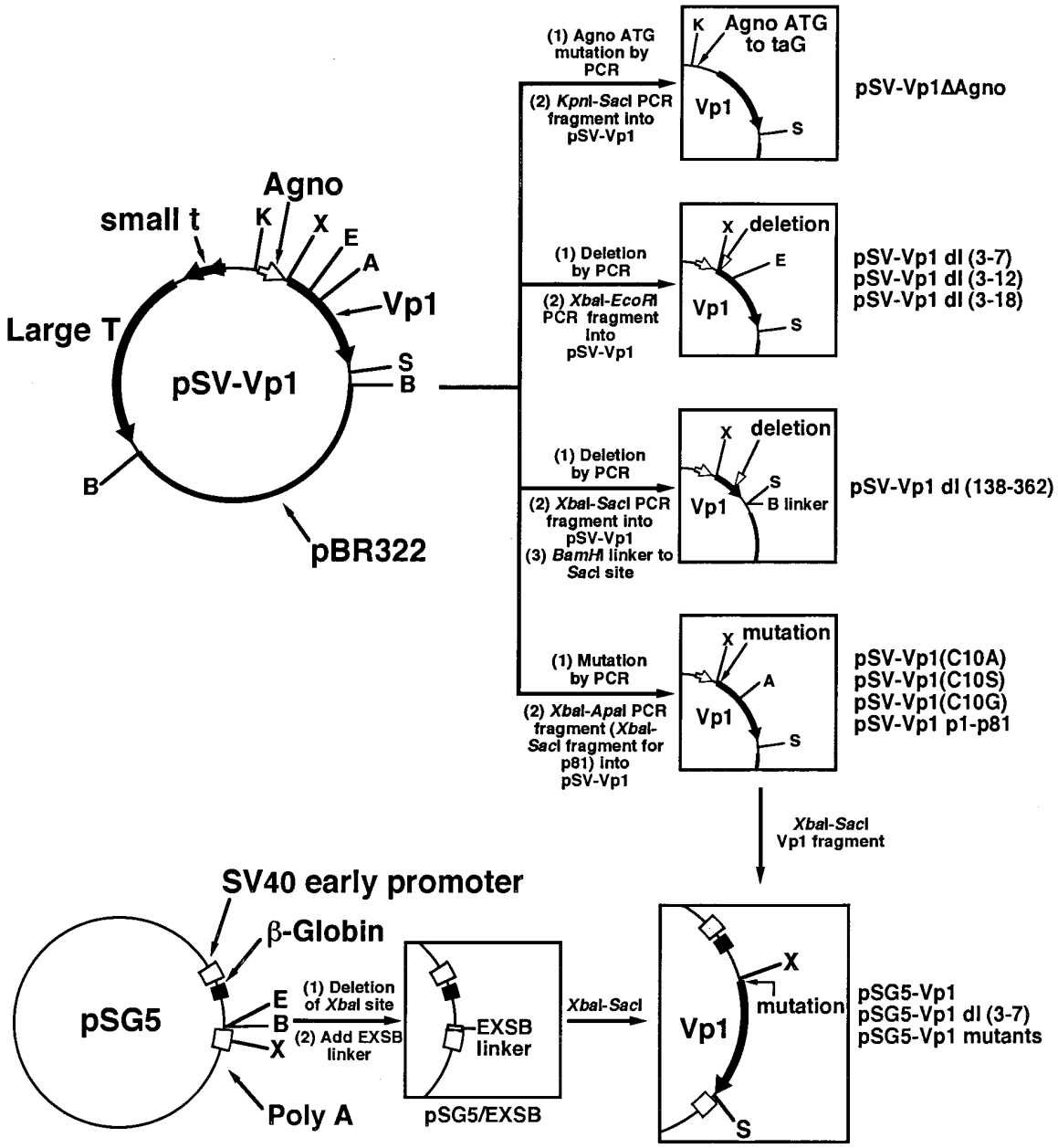


FIG. 1. Schematic diagrams for the construction of pSV-Vp1, pSG5-Vp1, and their mutant plasmid DNAs used in this study. All pSV-Vp1 constructs encode SV40 large T antigen, small t antigen, agnoprotein, and all the regulatory elements. Only the pertinent portion of each mutant plasmid is shown. All the mutations were verified by double-stranded-DNA sequencing with Sequenase version 2.0 (United States Biochemical). Oligonucleotides for PCR (sequences given in the text) and for sequencing were synthesized by the Oligonucleotide Preparation Laboratory of the UCLA Molecular Biology Institute. Following PCR-directed mutagenesis, the fragments were digested by two restriction enzymes and exchanged with those corresponding to the parent plasmids as described in the text. Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; X, *Xba*I. For the construction of the pSV-Vp1 dl (138–362) mutant, a *Bam*HI linker,

5′- CGGATCCATCGATGGATCCGAGCT-3′
3′-TCGAGCCTAGGTAGCTACCTAGGC -5′

was inserted at the *Sac*I site for excision of the pBR322 sequence and for circularization. Although SV40 nucleotide sequences 2533 to 2593 (part of the coding segment of Vp1) are present, they will not be translated, since three termination codons precede these sequences. The strategy for the construction of the pSG5-Vp1-NLS mutants is as follows. The unique *Xba*I site of the mammalian expression vector pSG5 (Stratagene) was destroyed by digestion with *Xba*I, followed by a fill-in reaction of the restriction site and religation. Into this construct, an EXSB linker,

5′-AATTCTCTAGAATCGTACGATAGAGCTCG -3′
3′- GAGATCTTAGCATGCTATCTCGAGCCTAG-5′

which includes *Xba*I and *Sac*I sites, was inserted at its *Eco*RI and *Bam*HI sites to yield pSG5/EXSB.

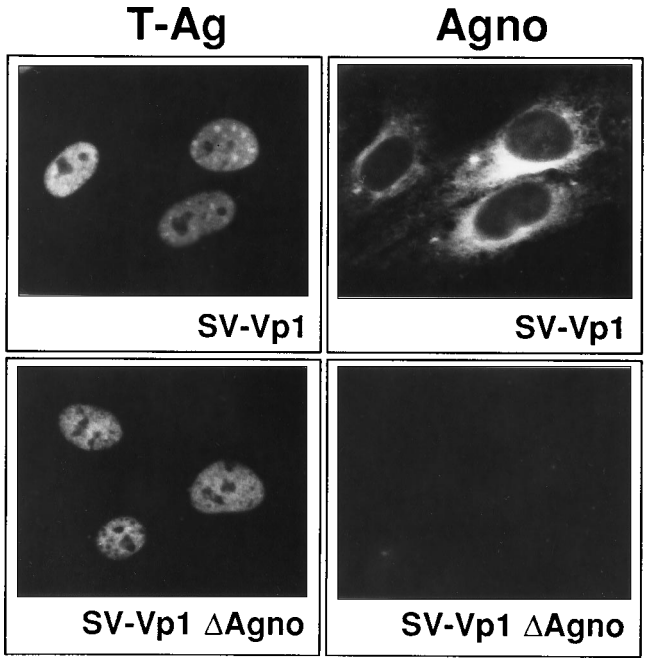


FIG. 2. Subcellular localization of large T antigen and agnoprotein. The variant viral DNAs SV-Vp1 and SV-Vp1ΔAgno were reconstituted by *Bam*HI digestion and recircularization. Culture conditions for TC7 cells, microinjection procedures, and indirect immunofluorescence have been described previously (13). DNAs (50 ng/μl) were microinjected into the nuclei of the cells, which were fixed after 24 h of incubation and reacted first with monoclonal mouse anti-SV40 T-antigen antibodies (Oncogene Science) (1:100) and rabbit antiagnoprotein antisera (1:50, kindly provided by G. Jay) and then with fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated goat secondary immunoglobulin G (1:50), respectively. Cells were microinjected with SV-Vp1 DNA or SV-Vp1ΔAgno. The same fields of cells are shown for each variant viral DNA.

(3–18) (a deletion of residues 3 through 18), as well as a carboxyl-terminal-deletion mutant, Vp1 dl (138–362), which has a deletion of the carboxy-terminal 225 residues, were constructed (Fig. 1), and the nuclear localization capability of each mutant protein was examined (Fig. 3). For the construction of the Vp1 amino-terminal-deletion mutants, the sense primers 5′-TGCTCTAGATGAAGATGGCCGGAAGTTGTCCAGG GGCA-3′ for pSV-Vp1 dl (3–7), 5′-TGCTCTAGATGAAGA TGGCCGCAGCTCCCAAAAAACCA-3′ for pSV-Vp1 dl (3–12), and 5′-TGCTCTAGATGAAGATGGCCAAGGAACC AGTGCAAGTG-3′ for pSV-Vp1 dl (3–18) were used; the *Sac*I antisense primer was also used. For the construction of carboxyl-terminal-deletion mutants, we used a 5′-CAGGTCC ATGGTCTAGAATGAAGATGGCC-3′ sense primer and a 5′-CAAGAATTCGAGCTCGCCCAACTTGTTTATTGCA GCTTATAATGGTTACAAATAAAGCAATAGCATCACA AATTTCACAAATAAAGCATTTTTTTCAATGAGTTTTT TGTGTCCCTGAAT-3′ antisense primer. The mutants were generated by exchanging the 1,180-bp *Xba*I-*Sac*I fragment of pSV-Vp1 with the corresponding fragments bearing deletions. The structure of each construct, together with the subcellular localization of each mutant Vp1, is summarized in Fig. 3A, and typical immunostaining patterns for the mutant Vp1 proteins are shown in Fig. 3B. Although the elimination of the carboxyl 225 residues of Vp1 did not affect the nuclear localization, the elimination of the amino-terminal 5, 10, or 16 residues caused mostly cytoplasmic phenotypes (Fig. 3). These results are in agreement with the reported observation that the SV40 Vp1 NLS is within the amino-terminal 11 residues of the protein (26).

We observed two distinct clusters of basic amino acids within the amino-terminal 20 amino acids: lysine-arginine-lysine (5 through 7) and lysine-lysine-proline-lysine (16 through 19). We have introduced a mutation(s) in the first or second cluster or both (Fig. 4) and have examined the nuclear localization capability of the mutant proteins for the following two reasons. First, many NLSs are composed of a dual signal or a bipartite signal, in which two clusters of basic residues are separated by various lengths of intervening amino acids (8, 9, 22). For example, polyomavirus large T antigen (21), *Xenopus* N1 (15), and nucleoplasmin (22) have two basic clusters, and mutation of either cluster partially impairs nuclear localization. However, simultaneous mutations of the two motifs eliminate the activity. In the case of the *Saccharomyces cerevisiae* ribosomal protein L29, either of the two clusters can target the fusion protein to the nucleus, and deletion of both clusters causes a greater loss of the nuclear localization activity (25). The two tracts of basic residues in the dual signals, therefore, are known to function in an interdependent manner. Second, the amino acid sequence of the amino terminus of SV40 Vp1 is substantially different from that of a related mouse polyomavirus Vp1 (24). Residues 5 to 19 of SV40 Vp1 have the characteristics of the bipartite NLS found in a large number of predominantly nuclear proteins, whereas polyomavirus Vp1 lacks the downstream cluster.

Plasmids bearing the amino-terminal point mutations pSV-Vp1 p6 (lysine 5 replaced with asparagine; herein designated 5N), p1 (6G), p55 (7N), p63 (16N), p26 (19N), p8 (5N6G), p33 (6G7N), p2 (17N19N), p16 (5N17N19N), p44 (6G16N19N), p46 (6G17N19N), p52 (7N16N17N), p81 (16N 17N19N), p19 (5N16N17N19N), p48 (6G16N17N19N), p39 (7N16N17N19N), p28 (5N7N16N17N19N), and p25 (5N6G 7N16N17N19N) were constructed (Fig. 1) by exchanging either the 765-bp *Xba*I-*Apa*I or the 1,179-bp *Xba*I-*Sac*I fragment of pSV-Vp1 with that of the mutation-bearing fragments. For the construction of mutants p1 to p63, the degenerate sense primers 5′-CAGGTCCAT GGTCTAGAATGAAGATGGC CCCAACAAA(A/c)(A/g)GAAA(A/c)GGAAGTTGTCCAG GGGCAGCTCCCAA(A/c)AA(A/c)CCAAA(G/c)GAAC CAGTGCAAGTGCCAAAGCTCG-3′ and the antisense primer 5′-CAAGGGCCCAACACCCTGCTC-3′ (nucleotides 2266 to 2246; designated the *Apa*I antisense primer) were used with the pSV-Vp1 template. For the p81 mutation, a 5′-CA GGTCCATGGTCTAGAATGAAGATGGCCCCAA CAAAAAGAAAAGGAAGTTGTCCAGGG-3′ sense primer and the *Sac*I antisense primer were used with the pSV-Vp1 p19 template. All point mutations are diagrammed in Fig. 4A, in which a summary of the subcellular localizations of the mutant Vp1s is given. Photographs of staining by mutants are shown in Fig. 4B.

When single point mutations were introduced into either cluster, most mutant Vp1 proteins localized to the nucleus (mutants p6, p55, p63, and p26) (Fig. 4A) except for the Vp1 mutant protein p1, in which arginine 6 was replaced with glycine and which was distributed mostly in the cytoplasm (mostly cytoplasmic phenotype). Double mutations in the upstream cluster (mutants p8 and p33) significantly affected the nuclear accumulation of the mutant proteins, while a double mutation in the downstream cluster (mutant p2) had very little effect on the localization. When three amino acids were replaced, with one mutation in the upstream cluster and two mutations in the downstream cluster (mutants p16, p44, p46, and p52), all of the mutant Vp1s except p16 showed the cytoplasmic phenotype. When all of the basic residues in the downstream cluster were

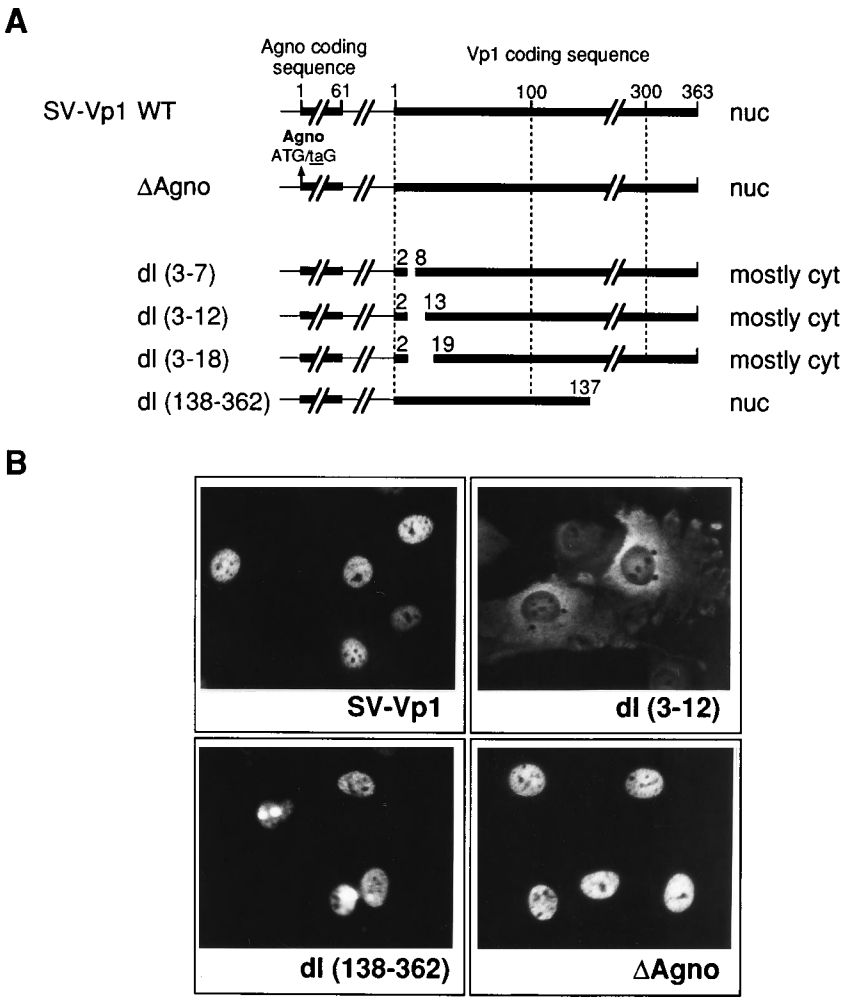
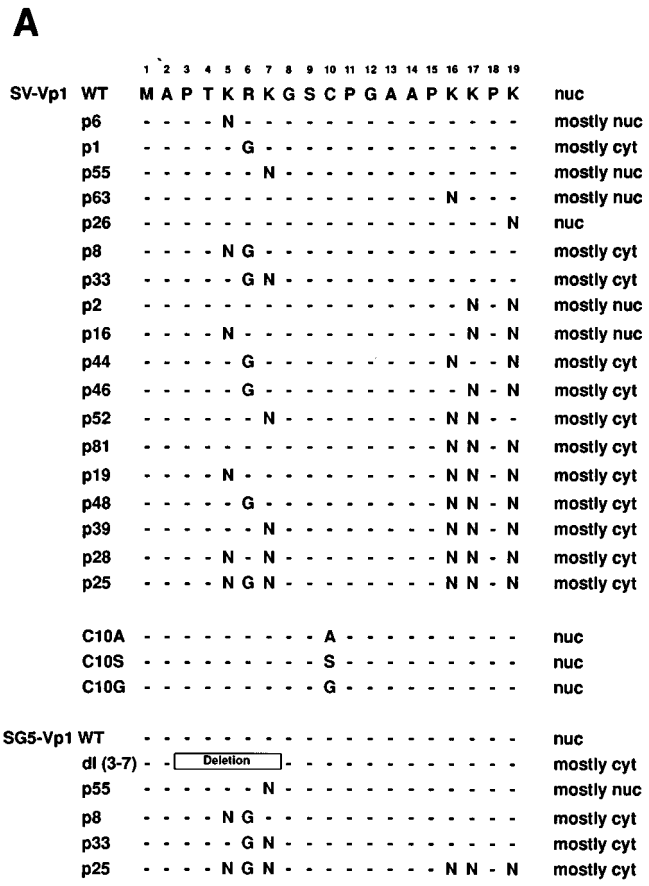


FIG. 3. Subcellular localization of Vp1 expressed from Vp1 deletion and agnoprotein mutants. Cells microinjected with each of the mutant DNAs were reacted first with the mouse anti-SV40 T antibody (1:100) and guinea pig anti-Vp1 antisera (1:50), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (1:50) and tetramethylrhodamine isothiocyanate-conjugated goat anti-guinea pig immunoglobulin G (1:50). (A) The Vp1 coding sequence, amino acids 1 to 363, and those of the deletion mutants are shown as horizontal bars. The agnoprotein coding segment is upstream of the Vp1 gene in the SV-Vp1ΔAgno construct. The subcellular localization of each individual mutant Vp1 is at the right: nuc, distinct nuclear localization (example shown in panel B for SV-Vp1); mostly cyt, predominantly cytoplasmic localization [example shown in panel B for dl (3–12)]. (B) Immunofluorescence photographs of the cells. Cells were microinjected with DNA from SV-Vp1, dl (3–12), dl (138–362), or ΔAgno.

changed to asparagine (p81), or the p81 mutation was combined with an additional mutation in one of the upstream basic residues (p19, p48, and p39), the mutant proteins were found in the cytoplasm. Mutant proteins with more than one alteration in the upstream cluster in addition to the downstream mutations (p28 and p25) were also cytoplasmic. Within a set of mutants examined, the most influential basic residue that caused a change in the Vp1 subcellular localization was arginine 6, regardless of whether it was in the context of a single alteration (p1) or multiple alterations (p8, p33, p44, p46, p48, and p25). Lysine 5 and lysine 7 each contributed to the localization if the downstream cluster was simultaneously mutated. The simultaneous alterations of the three downstream lysines were sufficient to make Vp1 cytoplasmic.

As cysteine 10 of Vp1 has been suggested to be involved in the protein's nuclear localization (6), we next investigated the effect of the replacement of the cysteine with alanine, serine, or glycine in SV-Vp1-C10A, SV-Vp1-C10S, and SV-Vp1-C10G, respectively. For the alterations, the degenerate sense primer 5′-TGGTCTAGAATGAAGATGGCCCCAACAAAAAGAAAAGGAAGT(g/T)(G/c)TCCAGGGGCAGCTCCCAAA-3′ (lowercase letters represent mutations) and the *Apa*I antisense primer were used for PCR, and the 765-bp *Xba*I-*Apa*I fragment bearing the mutations was exchanged for that of the wild-type counterpart in pSV-Vp1. The mutation of the cysteine to alanine, serine, or glycine did not alter the nuclear localization activities of the mutant proteins (Fig. 4). It does not appear that the replacement of the cysteine with serine caused a conformational change that affected the nuclear localization capability of the conjugate protein.

In the SV-Vp1 variant viral genome, Vp1 is expressed from the late promoter, following the expression of the large T antigen (13). In an independent experiment, we placed some of the mutant Vp1s under the control of the early promoter by replacing the mutation-bearing *Xba*I-*Sac*I fragments of the pSV-Vp1s dl (3–7), p55, p8, p33, or p25 with that of pSG5/EXSB (Fig. 1). Of all mutant Vp1s examined, SG5-Vp1 dl (3–7), SG5-Vp1-p8, SG5-Vp1-p33, and SG5-Vp1-p25 showed subcellular distributions similar to those shown by SV-Vp1 dl (3–12) (Fig. 3) or SV-Vp1-p33, -p52, and -p19 (Fig. 4). A



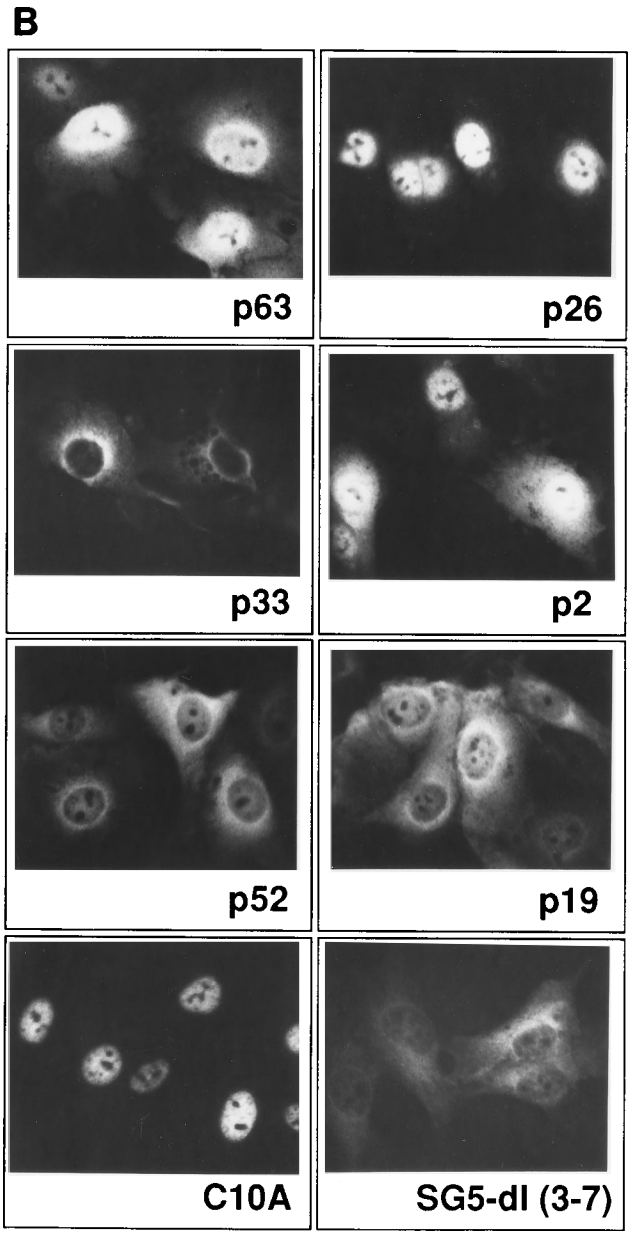


FIG. 4. Vp1 point mutants and subcellular localization of mutant Vp1s. Cells microinjected with each DNA were processed as described in the legend to Fig. 3. (A) The amino-terminal 19 residues of Vp1 are shown in single-letter code. Only amino acid alterations are indicated, by letters in the corresponding mutants. The subcellular localization of each mutant Vp1 is summarized at the right. Distinct nuclear localization in all cells observed is indicated (nuc). While Vp1 staining in most cells was observed in the nucleus (mostly nuclear staining [mostly nuc]) or in the cytoplasm (mostly cytoplasmic staining [mostly cyt]), it was also noted in both compartments in a fraction of the stained cells. (B) Immunofluorescence photographs of selected mutant Vp1s. Cells were microinjected with SV-Vp1-p63 (p63), SV-Vp1-p26 (p26), SV-Vp1-p33 (p33), SV-Vp1-p2 (p2), (5) SV-Vp1-p52 (p52), SV-Vp1-p19 (p19), SV-Vp1-C10A (C10A), and SG5-Vp1 dl (3–7) [SG5-dl (3–7)].

representative photograph is shown in Fig. 4B [results for SG5-Vp1 dl (3–7)]. The subcellular localization of SG5-Vp1-p55 was similar to those of SV-Vp1-p63 and SV-Vp1-p2, which are shown in Fig. 4B.

In summary, by using a number of SV40 Vp1 mutants, we further characterized the NLS of Vp1. The Vp1 NLS functions autonomously once the protein is synthesized in the cytoplasm, and agnoprotein does not play a role in Vp1 nuclear localization in the TC7 cell line. While the first eight amino acids of SV40 Vp1 are important for nuclear localization activity, as originally demonstrated by Wychowski et al. (26), our present findings revealed that the downstream basic residues 16 through 19 have an independent targeting activity. In SV40 Vp1, two clusters of basic residues (a cluster of lysine 5, arginine 6, and lysine 7 and a cluster of lysine 16, lysine 17, and lysine 19) are important for localization activity, the results of which contrasts with those of polyomavirus Vp1 (4).

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