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The Intranuclear Location of Simian Virus 40 Polypeptides VP2 and VP3 Depends on a Specific Amino Acid Sequence

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A cDNA fragment coding for poliovirus capsid polypeptide VP1 was inserted into a simian virus 40 (SV40) genome in the place of the SV40 VP1 gene and fused in phase to the 3′ end of the VP2-VP3 genes. Simian cells were infected with the resulting hybrid virus in the presence of an early SV40 mutant used as a helper. Indirect immunofluorescence analysis of the infected cells using anti-poliovirus VP1 immune serum revealed that the SV40/poliovirus fusion protein was located inside the cell nucleus. Deletions of various lengths were generated in the SV40 VP2-VP3 portion of the hybrid gene using BAL 31 nuclease. The resulting virus genomes expressed spliced fusion proteins whose intracellular location was either intranuclear or intracytoplasmic, depending on the presence or absence of VP2 amino acid residues 317 to 323 (Pro-Asn-Lys-Lys-Lys-Arg-Lys). This was confirmed by site-directed mutagenesis of the Lys residue at position 320. Modification of Lys-320 into either Thr or Asn abolished the nuclear accumulation of the fusion protein. It is concluded that at least part of the sequence of VP2 amino acids 317 to 323 allows VP2 and VP3 to remain stably located inside the cell nucleus. The proteins are most probably transported from the cell cytoplasm to the cell nucleus by interaction, with VP1 acting as a carrier.

Simian virus 40 (SV40) virions are made by the assembly of three structural proteins, VP1, VP2, and VP3, with M_rs of 46,000, 39,000, and 27,000, respectively (9, 23, 26). The three corresponding viral genes are organized in a partially overlapping manner on the SV40 genome, of which they constitute most of the late genes portion (31). Capsid proteins VP2 and VP3 are encoded by nucleotides 545 to 1601 and 899 to 1601, respectively, and both are read in the same frame. VP3 is therefore a subset of VP2, corresponding to the C-terminal two-thirds of the molecule. Capsid protein VP1 is encoded by nucleotide residues 1488 to 2574. The end of the VP2-VP3 open reading frame therefore overlaps by 113 nucleotides the beginning of the VP1 open reading frame, which is read in an alternative frame (31).

The three capsid polypeptides are synthesized during the late phase of the virus replication cycle and are transported rapidly after their synthesis into the nucleus of the infected cell (30), in which they assemble into SV40 particles (22).

The molecular mechanisms underlying the transport of proteins from the cytoplasm to the nucleus in mammalian cells are poorly understood. One approach used to determine the presence and location of possible migration sequences in nuclear proteins has involved the construction and expression of chimeric genes containing various regions of a nuclear protein fused to a cytosolic protein. Thus, the 13 NH2-terminal amino acids of the *Saccharomyces cerevisiae mat*α*2* protein were found to suffice to transport a *mat*α*2* β-galactosidase fusion protein to the nucleus (11). The amino-terminal portions of the yeast Gal4 (28) and L3 (19) proteins were sufficient to target β-galactosidase fusion proteins to the cell nucleus. Similarly, fusing the chicken pyruvate kinase molecule to the highly basic stretch of amino acids found in the SV40 T antigen (Pro-Lys-Lys-Lys-Arg-Lys-Val) resulted in efficient transport of the protein to the cell nucleus (13). Moreover, proteins coupled to a synthetic peptide with the sequence of these seven amino acids were found to accumulate in the cell nucleus (16).

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We have recently inserted into a SV40 genome the cDNA sequence of the poliovirus capsid polypeptide VP1, a cytosolic protein, in such a manner as to generate fusion proteins, with poliovirus VP1 at the COOH terminus and various amounts of SV40 VP1 at the NH2 terminus. We observed that all the fusion proteins could be recovered in the cell nucleus as long as they contained the first eight NH2-terminal amino acids of SV40 VP1. This led to the conclusion that a migration signal sufficient to target the fusion protein to the cell nucleus was present within that stretch of amino acids in the SV40 VP1 protein (35).

While screening the hybrid SV40/polioviruses thus generated, we noticed that many expressed proteins that were immunoprecipitated by anti-poliovirus VP1 immune serum showed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis a different M_r from that expected from a SV40 VP1/poliovirus VP1 fusion protein. As will be shown, these hybrid viruses correspond to SV40 recombinants in which the poliovirus VP1 sequence has been inserted in phase with, and downstream from, the SV40 VP2 and VP3 genes. These SV40 VP2-VP3/poliovirus VP1 fusion proteins accumulate inside the nucleus of the infected cell, provided that they contain the domain of VP2 covering amino acid residues 317 to 323 and are expressed in the presence of SV40 polypeptide VP1. A model is presented to account for these data.

MATERIALS AND METHODS

Enzymes and reagents. Restriction enzymes were purchased from Boehringer Mannheim Biochemicals, New England BioLabs, Inc., or Amersham Corp.; T4 DNA ligase, Klenow DNA polymerase, and polynucleotide kinase were purchased from Boehringer Mannheim; BAL 31 nuclease was purchased from New England BioLabs; and M13mp18 was purchased from P-L Biochemicals, Inc. All enzymes were used according to the instructions of the manufacturer. [γ-^{32}P]ATP at 3,000 Ci/mmol was purchased from Amersham. Oligodeoxynucleotides 5′-CCTTTTGTTTTTGTTGGGG-3′ (oligodeoxynucleotide 1), 5′-CCTTTTCGTTTTGTTGGGG-3′ (oligodeoxynucleotide 2), and 5′-CCTTTAC-

TTCTAGGCCTG-3′ (oligodeoxynucleotide 3) were synthesized by J. Igolen (Institut Pasteur). Oligodeoxynucleotides 1 and 2 were used for site-directed mutagenesis, and oligodeoxynucleotide 3 was used for plasmid sequencing according to the method of Zagursky et al. (37).

Construction of recombinant plasmids. *Escherichia coli* 1106 (803 $r_k^-m_k^-$) (21) was used for the selection and propagation of recombinant plasmids. Plasmid pCW18 has already been described (36). Transformation of bacteria, extraction, and analysis of plasmids were as previously described (1, 17, 18).

Protruding 5′ ends of restricted DNA (20 μg/ml) were filled in by treatment with Klenow DNA polymerase (40 U/ml) in the presence of all four deoxynucleoside triphosphates (200 μM each). Ligation reactions were performed for 48 h at 4°C or for 18 h at 15°C in 60 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–10 mM $MgCl_2$–10 mM dithiothreitol–1 mM ATP–20 μg of bovine serum albumin per ml, by using 1 U of T4 DNA ligase per μg of DNA.

Before digestion with BAL 31 nuclease, pCW18 was linearized at the unique *Acc*I site of SV40. Digestion with BAL 31 nuclease was performed for different periods at 30°C in 120 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.6 M NaCl–12 mM $CaCl_2$–12 mM $MgCl_2$, by using an enzyme-to-DNA ratio of 0.08 U/μg (35). The reaction was stopped with EDTA, and the trimmed DNA was extracted with phenol and chloroform and precipitated with 2 volumes of ethanol before ligation. In the case of pCW18Δ202, the plasmid DNA was linearized at the *Acc*I site of SV40, digested with BAL 31 nuclease as described above, ethanol precipitated, and cleaved at the unique *Bgl*II site. The large fragment was then ligated with the small pCW18Δ0 *Bal*I-*Bgl*II fragment.

Site-directed mutagenesis. Synthetic oligodeoxynucleotides 1 and 2 (see above) were synthesized on an automated synthesizer and purified by high-performance liquid chromatography and electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Site-directed mutagenesis was performed directly on the DNA in the plasmids by the following protocol (20). The supercoiled plasmid DNA was purified by cesium chloride gradient centrifugation. Part of it was digested with *Bgl*II to linearize the plasmid, and another part was digested with two restriction endonucleases cleaving the DNA on either side of the region to be mutated (for example, *Bal*I and *Xba*I in the case of pCW18Δ0). The resulting molecules (0.03 pmol) were mixed together and added to 12.5 pmol of the synthetic oligodeoxynucleotide with the appropriate sequence that had been phosphorylated at its 5′ end by using polynucleotide kinase. The DNA mixture (in 8.6 μl of 0.1× TE buffer) was mixed with 2 μl of 10× concentrated polymerase-ligase buffer (1 M NaCl; 65 mM Tris hydrochloride [pH 7.5], 80 mM $MgCl_2$, 10 mM β-mercaptoethanol), dipped into a boiling-water bath for 3 min to denature the DNA molecule, and then gradually cooled by incubation for 30 min at 30°C. The tube was further incubated for 30 min at 4°C and then in an ice bath for 10 min. The mixture was then mixed with 4 μl of a solution of the four deoxyribonucleoside triphosphates (2.5 mM each): 2 μl of 10 mM ATP–3 μl of T4 DNA ligase (3 U)–0.4 μl of the Klenow fragment of DNA polymerase (2 U). The final mixture (20 μl) was incubated at 12.5°C overnight. The ligation mixture was then used to transform competent HB101 bacteria. Ampicillin-resistant colonies were selected and transferred to Schleicher & Schuell membrane filters, and those harboring a plasmid with the desired mutation were detected by colony hybridization with the [γ-^{32}P]ATP 5′ end-labeled oligonucleotide as a hybridization probe. Hybridization was done as described by Chesebro et al. (5) for 18 h at 10°C below the calculated T_m, using 2×10^6 cpm per filter. The temperature for the washing of the filters was determined experimentally by step-by-step increase.

DNA sequence analysis. The DNA of the plasmids to be sequenced was treated with *Stu*I (SV40 nucleotide 1446) and *Bgl*II. The resulting small fragment corresponding to the fused SV40/poliovirus VP1 gene was recovered from low-melting-temperature agarose gels (34) and digested by *Xba*I (poliovirus nucleotide 2546). The resulting fragments were inserted into M13mp18 replicative-form DNA molecules cleaved by *Hin*cII and *Xba*I. Sequence determination was performed on the M13 single-stranded DNA by using the four-dideoxynucleotide chain termination method (27). The mutation borne by plasmid pCW18Δ70 or by plasmid pCW18Δ71 was sequenced by the method of Zagursky et al. (37).

Preparation of virus stocks. The DNA from the recombinant plasmids was digested with *Bam*HI and *Bgl*II, recircularized by ligation, and transfected into CV1 cells by the method of Sompayrac and Danna (29). Briefly, transfection was done by using 10^6 CV1 cells, plated onto 60-mm-diameter petri dishes, and 0.4 ml of a 1-μg/ml mixture of the recombinant SV40 DNA and of the helper pDR404 DNA, in Dulbecco minimal essential medium containing 0.05 M Tris hydrochloride, pH 7.3 and 500 μg of DEAE dextran per ml. The cells were incubated at 37°C in a CO_2 incubator for 2 h, after which they were washed and fed with medium supplemented with 5% fetal calf serum. After 15 days, the cells were disrupted by freezing and thawing, cell debris was discarded by centrifugation, and the supernatant was titrated and used as a virus stock.

Preparation and immunoprecipitation of infected-cell extracts. Labeling of infected cells, preparation of cell extracts, and immunoprecipitation by using protein A Sepharose were as described previously (36).

RESULTS

Construction of recombinant viruses with an SV40 VP2-VP3/poliovirus VP1 hybrid gene. We have previously reported the construction of plasmid pCW18 (Fig. 1), which carries an SV40 genome into which the cDNA coding for the poliovirus capsid polypeptide VP1 (poliovirus type 1 nucleotides 2472 to 3417) has been inserted between the unique *Eco*RI and *Bam*HI sites (36). Upon transfection of simian cells with the DNA of this plasmid cleaved at the *Bgl*II and *Bam*HI sites and recircularized, a hybrid SV40/poliovirus is generated, in which the sequence encoding SV40 VP1 amino acids 95 to 361 is replaced by that of the 302 amino acids of poliovirus VP1 flanked by a few additional amino acids from poliovirus VP3 and protein 2A. This hybrid virus is defective for capsid protein synthesis. It can be grown in a mixed infectious virus stock by cotransfection of the simian cells with the DNA of the SV40 T-antigen amber mutant *am*404 as a helper (24, 25).

To generate hybrid viruses expressing poliovirus polypeptide VP1 fused in phase with SV40 polypeptides VP2 or VP3 (or both), the DNA of plasmid pCW18 was cleaved at the unique *Acc*I site (SV40 nucleotide 1611), digested with BAL 31 nuclease for various lengths of time between 3 and 21 min, and then treated with *Eco*RI, filled in with Klenow enzyme, and recircularized (Fig. 1). Resulting deleted plasmids were cloned, and each clone was grown into an independent virus stock with *am*404 as a helper. The fusion protein expressed by each of the truncated SV40/polio virus hybrids was

analyzed by SDS-PAGE of [^{35}S]methionine-labeled infected-cell extracts after immunoprecipitation with an anti-poliovirus VP1 immune serum (2). When the poliovirus VP1 sequence was fused in frame with that of the SV40 VP1 polypeptide, the molecular weight (M_r) of the fusion protein was in the range of 37,000 to 50,000 (35, 36). When fusion occurred between SV40 VP2-VP3 polypeptides and poliovirus VP1, two bands of material with M_rs in the range of 62,000 to 76,000 were detected (Fig. 2; Table 1). Another band of material with an apparent M_r of 46,000 was also observed in all the gels. Part of this material could correspond to a cellular protein such as actin, as it was also present after immunoprecipitation of infected-cell extracts with a control non-immune rabbit serum (data not shown). However, another part of this material was probably SV40 VP1, as judged from the similarity of its electrophoretic migration with that of the material immunoprecipitated with an anti-SV40 immune serum (see below). A few additional minor bands of material were also observed, which could be either discrete degradation products or truncated translation products resulting from the abnormal use of internal initiation sites. These questions were not addressed further. The infected-cell extracts were also immunoprecipitated with a rabbit anti-SV40 immune serum (Fig. 2): two bands were observed, one with an apparent M_r of 46,000, SV40 VP1, and the other with an M_r of 92,000, probably SV40 T antigen. The anti-SV40 immune serum did not immunoprecipitate the SV40/poliovirus fusion proteins probably because it only poorly recognized SV40 polypeptides VP2 and VP3. The M_rs of the fusion proteins expressed by some of the defective recombinant viruses selected are summarized in Table 1.

Identification of an amino acid sequence responsible for the nuclear location of SV40 capsid polypeptides VP2 and VP3. To determine the intracellular location of the SV40 VP2-VP3/poliovirus VP1 fusion proteins expressed by these viruses,

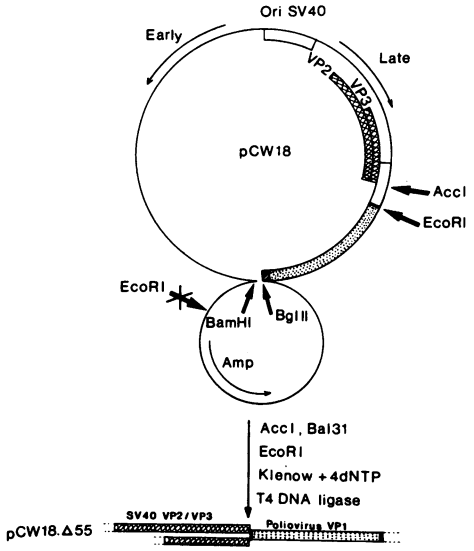

FIG. 1. Construction of SV40 genomes containing an SV40 VP2-VP3/poliovirus VP1 hybrid gene. Plasmid pCW18 (36) contains the poliovirus cDNA fragment (nucleotides 2472 to 3417) that encodes the COOH terminus of poliovirus VP3 (▬), the whole of poliovirus VP1 (▨), and the NH2 terminus of poliovirus polypeptide 2A (▨). ▭, The remaining 5′ terminal part of the SV40 VP1 gene; ▨, the SV40 VP2 and VP3 genes. The large circle corresponds to the SV40 genome, and the small circle corresponds to that of pML2, the *Eco*RI site of which has been destroyed.

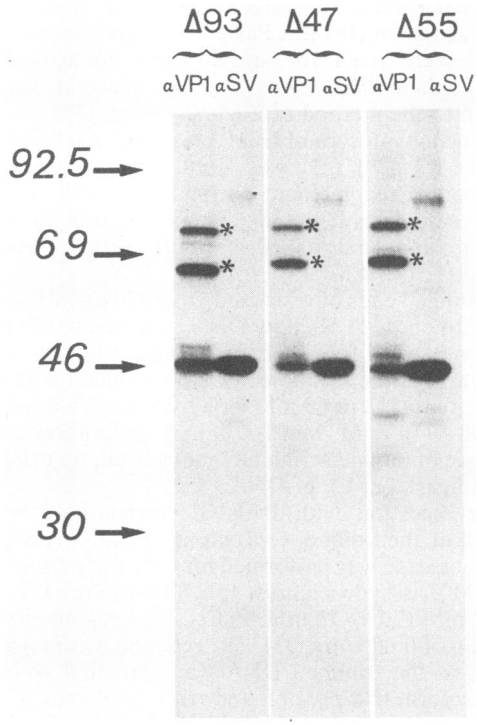

FIG. 2. SDS-PAGE analysis of immunoprecipitated SV40 VP2-VP3/poliovirus VP1 fusion proteins. Extracts from [^{35}S]methionine-labeled cells infected with the indicated virus were immunoprecipitated with anti-poliovirus VP1 rabbit immune serum or with anti-SV40 immune serum. The immune precipitates were analyzed by electrophoresis in a 10 to 18% polyacrylamide linear gradient gel (36) in the presence of SDS. Arrows on the left refer to the position (10^3) of ^{14}C-labeled markers. The asterisks correspond to the positions expected for SV40 VP2- and SV40 VP3/poliovirus VP1 fusion proteins, calculated from the size of the deletions generated into the genome of the hybrid viruses.

infected cells were assayed by immunofluorescence using anti-poliovirus VP1 immune serum. Strikingly different results were observed, depending on the hybrid virus studied (Fig. 3). The SV40/poliovirus proteins expressed by pCW18Δ55 and Δ47 were localized in the cell nucleus (Fig. 3A and B), whereas those expressed by pCW18Δ93 or Δ96 were located in the perinuclear region and the cytoplasm of the infected cells (Fig. 3C and D). The intracellular location

TABLE 1. Characterization of truncated SV40 VP2-VP3 proteins

Plasmid	Deletion of SV40 amino acids[a]		Apparent mol wt of fusion protein, 10^{3b}		Location[c]
	VP2	VP3	Poliovirus VP1/SV40 VP2	Poliovirus VP1/SV40 VP3	
pCW18Δ55	335–352	217–234	76	65	N
pCW18Δ47	330–352	212–234	75	64	N
pCW18Δ93	318–352	200–234	73	62	C
pCW18Δ96	310–352	192–234	73	62	C
pCW18Δ232	340–352	222–234	76	65	C
	317–323	199–205			

[a] Amino acid numbering according to Tooze (31).

[b] Apparent M_r was determined by SDS-PAGE analysis as described in the legend to Fig. 2.

[c] Intracellular location of the fusion proteins was determined by immunofluorescence staining of infected cells as described in the legends to Fig. 3 and 6. N, Nuclear; C, cytoplasmic.

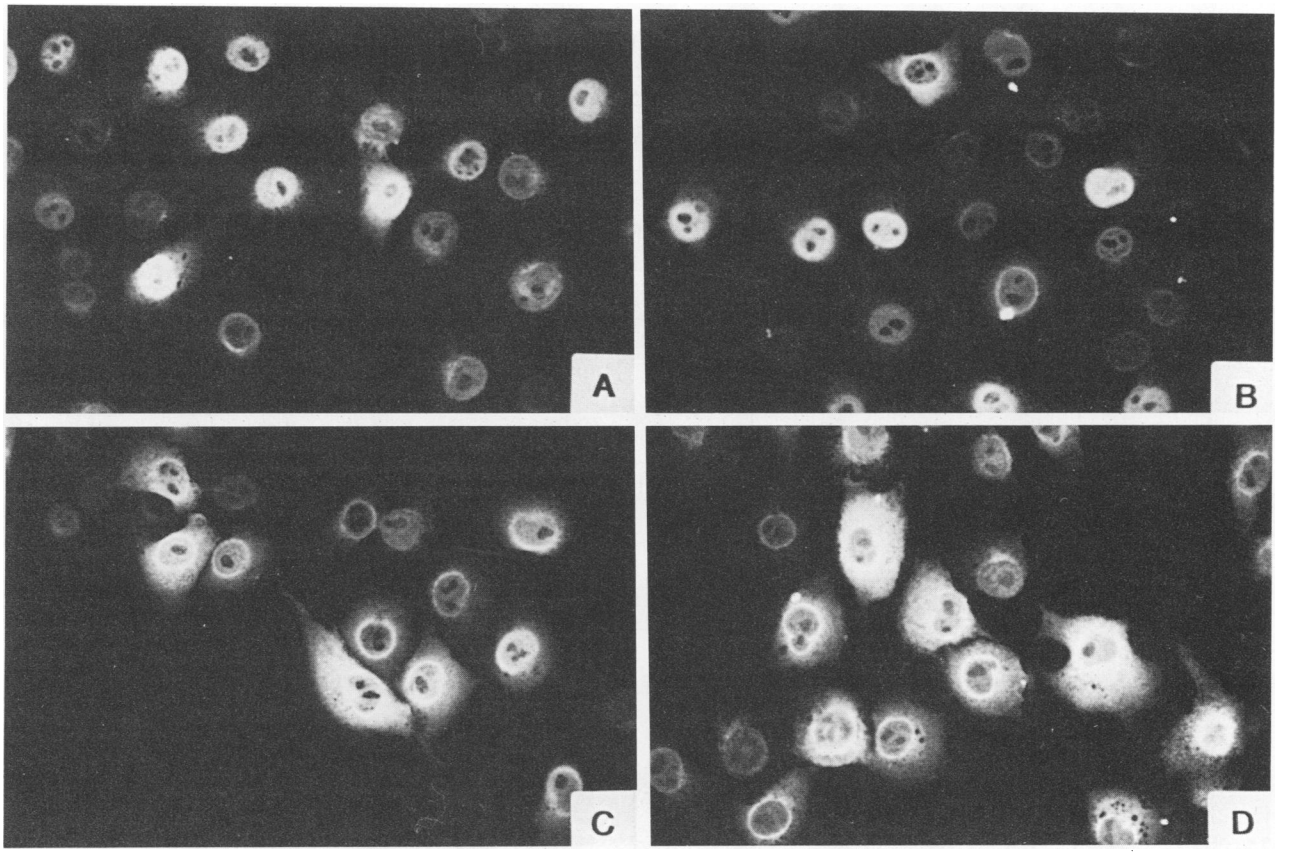

FIG. 3. Intracellular localization of the SV40/poliovirus fusion proteins. CV1 cells on cover slips were infected with 2 PFU of the following virus stocks per cell: A, SV40-Δ55-*am*404; B, SV40-Δ47-*am*404; C, SV40-Δ93-*am*404; D, SV40-Δ96-*am*404. Coverslips were fixed 40 h after infection and processed for immunofluorescence staining by using anti-poliovirus VP1 rabbit immune serum as previously described (36).

of the fusion proteins determined by immunofluorescence staining of infected cells is summarized in Table 1.

The nucleotide sequence at the junction between the SV40 VP2 and VP3 genes and the poliovirus cDNA fragment in each of the hybrid SV40/poliovirus genomes was determined (Fig. 4). In the case of Δ55 and Δ47, the BAL 31 deletions extended upstream to SV40 nucleotides 1546 and 1531, respectively. The fusion proteins these viruses express therefore lack the 18 and 23 COOH-terminal amino acids from SV40 VP2-VP3 capsid polypeptides, respectively (Fig. 4). On the other hand, in the case of Δ93 and Δ96, the deletions generated by BAL 31 were found to extend to SV40 nucleotides 1495 and 1471, respectively. As a result, the fusion proteins these two viruses express lack the 35 and 43 COOH-terminal amino acids from SV40 VP2-VP3, respectively. The presence of COOH-terminal amino acid residues −35 to −23 (i.e., SV40 VP2 amino acids 317 to 329 or VP3 amino acids 199 to 211) therefore seems to be required for the fusion proteins to be targeted to the infected cell nucleus. It should be noted that in the case of Δ93, the BAL 31 deletion had also reached further downstream than the *Eco*RI site, removing the first 21 nucleotides of poliovirus cDNA.

The SV40 VP2-VP3 sequence identified above contains a stretch of amino acids (Asn-Lys-Lys-Lys-Arg-Lys; NKKKRK), which is reminiscent of that (Pro-Lys-Lys-Lys-Arg-Lys-Val) identified by Kalderon et al. (12, 13) in the SV40 T antigen and shown by those authors to be involved in the nuclear location of the protein. To ascertain that the ability to target the VP2-VP3 fusion protein to the cell nucleus was indeed due to this stretch of amino acids, we deleted their coding sequence from the SV40 VP2-VP3/poliovirus VP1 hybrid gene (Fig. 5).

This was done by starting from plasmid pCW18Δ202, which contains the poliovirus VP1 sequence fused in phase behind the sequence of SV40 VP1 amino acids 9 to 94 (35; Fig. 5). The sequence coding for SV40 VP1 amino acid residues 1 to 8, which has been deleted in pCW18Δ202, is

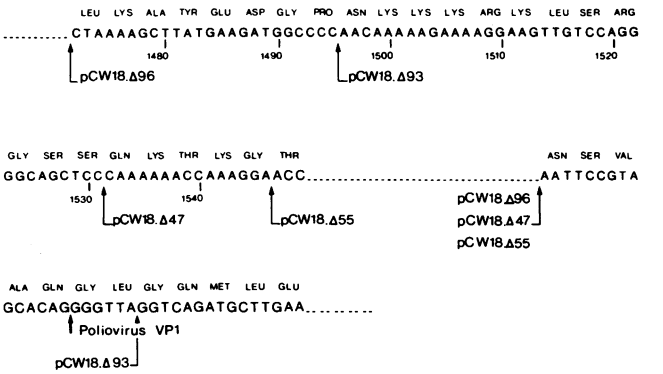

FIG. 4. Location and extent of the BAL 31-generated deletions. Plasmid pCW18 was digested with BAL 31 nuclease at the *Acc*I site, cut with *Eco*RI, treated with Klenow DNA polymerase, and recircularized (see the text). The extent of some of the deletions thus generated was determined by nucleotide sequencing (arrows). The beginning of the sequence of poliovirus VP1 is indicated by the short arrow.

also that which codes for the PNKKKRK sequence of VP2-VP3 (see Fig. 8). The DNA of pCW18Δ202 was cleaved at *Acc*I with BAL 31 nuclease for 1 min and then cleaved with *Bgl*II, and the resulting large fragment (7.02 kilobases) was ligated to the small *Bal*I-*Bgl*II fragment (0.95 kilodaltons) from pCW18Δ0 (35), which contains the sequence of the 2 COOH-terminal amino acids of poliovirus VP3, followed by the 302 amino acids of poliovirus VP1 and the 12 NH2-terminal amino acids of poliovirus protein 2A. A library of hybrid virus genomes was thus generated.

After conversion into individual virus stocks through transfection of CV1 cells in the presence of SV40 *am*404 helper DNA, extracts from cells infected by each of 20 such virus stocks were immunoprecipitated with anti-poliovirus VP1 antiserum and the SDS-PAGE pattern of the immunoprecipitates was examined. Six of the extracts analyzed showed a double band of material migrating with the apparent M_r expected for SV40 VP2-VP3/poliovirus VP1 fusion proteins. The location of these proteins was intracytoplasmic, as judged by immunofluorescence analysis of the infected cells (Fig. 6A).

The nucleotide sequence at the junction between the 3′ part of the SV40 VP2-VP3 sequence and the 5′ part of the poliovirus sequence was determined in four of the selected hybrid viruses. One of them, pCW18Δ232, showed a poliovirus VP1 cDNA sequence fused in frame to SV40 DNA at position 1561, i.e., 13 codons upstream from the termination codon of the VP2 and VP3 genes. Thus, the fusion protein expressed by pCW18Δ232 lacks the sequence PNKKKRK (amino acids 317 to 323 in VP2) together with the 13 last COOH-terminal amino acids from VP2-VP3 (not shown). This protein is located inside the cell cytoplasm. Comparison of the sequence of the pCW18Δ232 fusion protein with that of pCW18Δ55, which lacks the last 17 COOH-terminal amino acids of VP2-VP3 but has kept the internal PNKKKRK sequence, strongly suggests that the latter is indispensable

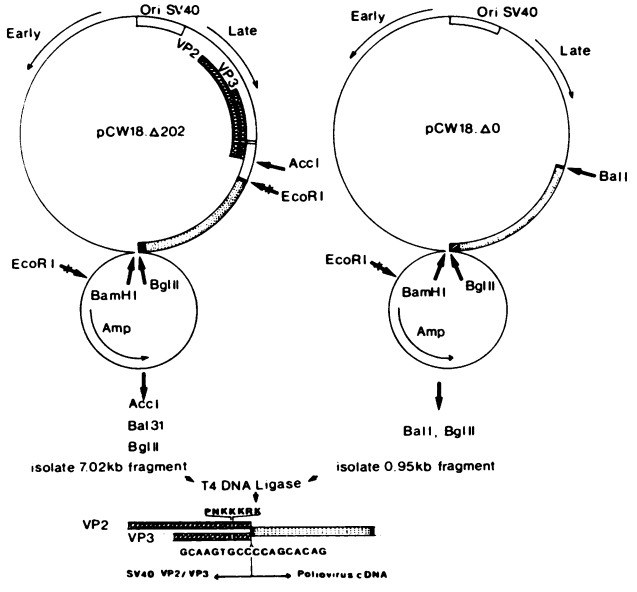

FIG. 5. Deletion of the sequence PNKKKRK from the SV40 VP2-VP3/poliovirus VP1 hybrid gene. Plasmid pCW18Δ232 was constructed as indicated (see the text for details). The sequence at the junction of SV40 VP2-VP3 and of the poliovirus cDNA was determined and is indicated at the bottom of the figure. The same symbols are used as in Fig. 1. kb, Kilobase.

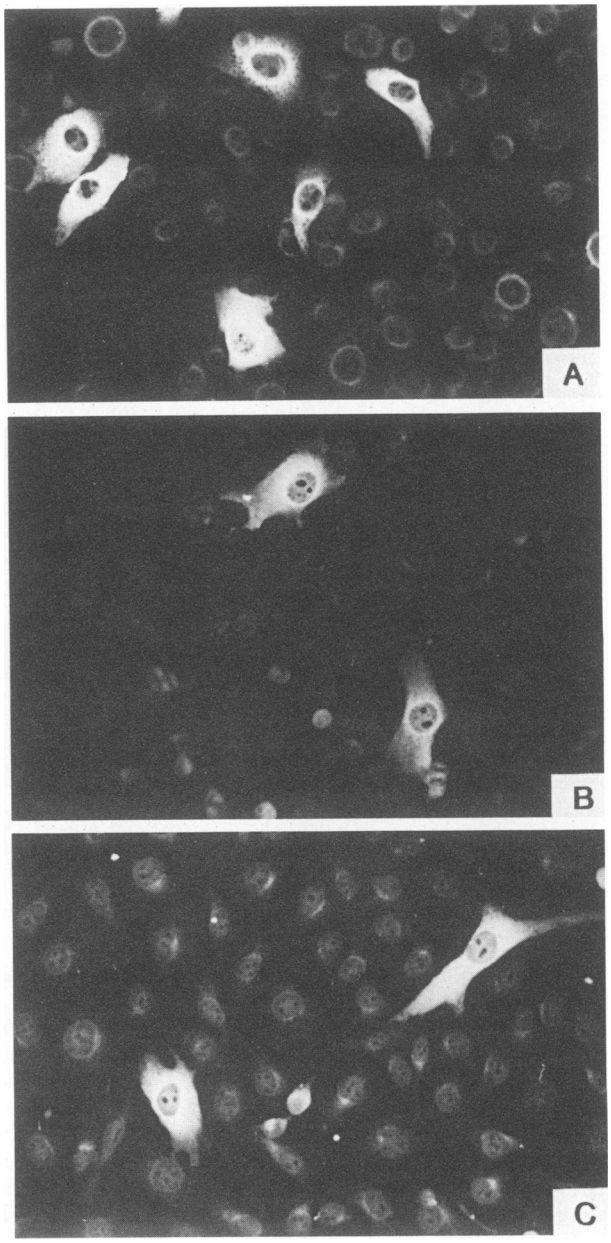

FIG. 6. Intracellular localization of the SV40/poliovirus fusion protein. CV1 cells on cover slips were infected with: (A) SV40-Δ232-*am*404 at 3 PFU per cell; (B) SV40-Δ55-*am*404; and (C) SV40-Δ47-*am*404 at 10^{-4} PFU per cell. Cells were fixed at 40 h after infection and processed for immunofluorescence staining by using anti-poliovirus VP1 rabbit immune serum.

for the location of SV40 VP2-VP3 capsid polypeptides in the cell nucleus. No information is available, however, on the possible role of the last 13 amino acids of SV40 VP2-VP3. The size of the deletions of SV40 VP2-VP3 in the fusion proteins is summarized in Table 1.

Mutation of SV40 VP2 Lys-320 alters the intracellular location of the polypeptide. As an additional proof of the role played by the PNKKKRK sequence in the migration of SV40 VP2-VP3 polypeptides to the cell nucleus, we undertook the site-directed mutagenesis of the SV40 VP-2/poliovirus VP1 gene in pCW18Δ55 through the use of

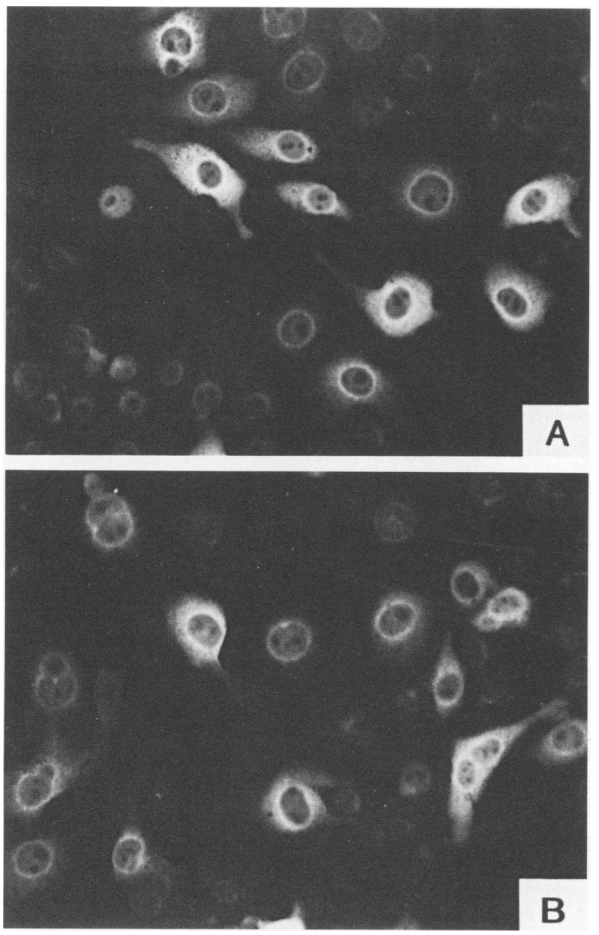

FIG. 7. Intracellular localization of the SV40-poliovirus fusion protein from two hybrid viruses mutated at VP2 Lys-320 in the accumulation sequence. CV1 cells were infected (A) with SV40-Δ70-*am*404 or (B) with SV40-Δ71-*am*404, and processed for immunofluorescence staining as described in the legend to Fig. 6.

custom-made oligodeoxynucleotides (see Materials and Methods). The sequence of the oligonucleotides (see Materials and Methods) was such as to generate a mutation of VP2 amino acid Lys-320 to Thr or Asn. Among several clones of putative mutants obtained, two were selected and sequenced: pCW18Δ70, in which the AAG codon of Lys-320 was replaced by ACG (Thr), and pCW18Δ71, in which the same AAG codon was replaced by AAC (Asn). Both plasmids were grown independently into mixed virus stocks by transfection of CV1 cells in the presence of pDR404 DNA, after which cells were infected with the resulting virus stocks and processed for immunofluorescence by using an anti-poliovirus VP1 immune serum. In both cases, the SV40 VP2-VP3/poliovirus VP1 fusion proteins were found to be located in the cytoplasm and the perinuclear region of the infected cells (Fig. 7). Therefore, modification of Lys-320 to Thr or Asn abolished the nuclear accumulation of SV40 VP2-VP3. The role of the adjacent amino acids is presently being determined by additional site-directed mutagenesis experiments.

The intranuclear accumulation of the fusion proteins depends on the presence of SV40 VP1. Successful infection and virus production in the system described above depend on the *am*404 helper virus supplying the three SV40 capsid polypeptides required for packaging of both its genome and that of the hybrid SV40/poliovirus. The question was raised of whether these proteins could play a role in the targeting of the SV40/poliovirus fusion proteins into the nucleus.

To examine the possibility that the fusion proteins were transported to the nucleus as a complex in association with the *am*404 helper virus capsid proteins, cells were infected with dilutions of the Δ47 and Δ55 virus stocks, i.e., at an approximate multiplicity of infection of 10^{-4} PFU per cell. Under these conditions, the probability for a cell to be simultaneously infected by both a recombinant virus and a helper virus could be considered negligible. In each instance, the SV40/poliovirus fusion protein expressed by the diluted recombinant virus stock was detected inside the cytoplasm of the infected cell, as judged by immunofluorescence staining using an anti-poliovirus VP1 antiserum (Fig. 6B and C). These results indicate that the accumulation of the SV40 VP2-VP3/poliovirus VP1 protein in the cell nucleus depends on the participation of a factor provided by the helper SV40 virus, probably SV40 VP1 (14). The PNKKKRK sequence present at the COOH terminus of SV40 VP2-VP3 seems therefore to be necessary, but not sufficient, to ensure the effective transport and accumulation of the fusion proteins inside the cell nucleus.

DISCUSSION

Translocation of proteins across cellular membranes is a key step in the intracellular sorting and secretion of proteins. For proteins targeted for import into mitochondria or export through the endoplasmic reticulum, the targeting is given by the leader amino acid sequence at the amino acid terminus of the polypeptide. Thus, mitochondrial precursor polypeptides have a leader peptide that is generally highly basic, contains no acidic residues, and has a high percentage of leucine, serine, and arginine (32), whereas excreted proteins carry at their NH2 terminus a signal peptide that is made up of a stretch of hydrophobic residues, often flanked by basic residues (33). At present, more than 200 procaryotic and eucaryotic signal peptides have been sequenced (33). Both leader peptides and signal peptides are cleaved from the precursor polypeptide at the time of translocation by membrane-associated processing proteases (signalases).

It is still, however, totally unclear what targets a protein to the cell nucleus. The entry of proteins into the nucleus cannot be entirely explained by diffusion through the pore complexes because some of these proteins are of very high M_r (7) and many proteins with low M_r enter the nucleus with much faster kinetics than expected from molecular diffusion only (3, 4). Nuclear proteins do not appear to be synthesized in the form of precursor proteins, with added sequences playing the part of a leader peptide or a signal peptide. It has been suggested that the specificity of the transport to the nucleus can be determined by an amino acid migration sequence located within the sequence of the protein itself (7). This sequence could be involved in the interaction with a nuclear membrane receptor or nuclear membrane translocation machinery. Nuclear migration sequences have indeed been identified in the SV40 T antigen (12, 13), in the SV40 capsid polypeptide VP1 (35), in the estradiol receptor (10), and in the yeast *matα2* protein (11) and ribosomal protein L3 (19). Several of these sequences are characterized by the fact that they contain a proline residue followed by a stretch of several basic amino acid residues. Another type of sequence has been identified in the estradiol receptor (10)

and in the yeast *matα2* protein (11). There is some dispute over the actual significance of the yeast *matα2* protein migration sequence, because several migration sequences appear to actually exist in the protein.

In the case of the SV40 capsid polypeptides, our data show that the intranuclear location of SV40 VP2 and VP3 depends on the presence of VP2 amino acids 317 to 323 (VP3 amino acids 199 to 205). Fusion proteins containing this stretch of seven amino acids with the sequence PNKKKRK readily migrated into the nucleus of the infected cell, whereas those in which this sequence had been deleted remained inside the cytoplasm of the infected cell. Different authors have shown that the modification of Lys-128 to Thr (6, 12) or Asn (15) abolishes nuclear accumulation of SV40 T antigen. Our results show that when VP2 Lys-320 was changed to Thr or Asn (by analogy with Lys-128 of the T antigen), the nuclear accumulation of SV40 VP2 and VP3 polypeptides was abolished.

However, this situation was observed only in cells doubly infected with both the hybrid SV40/poliovirus and a helper SV40. When the multiplicity of infection was greatly reduced, to ensure that cells were infected with the hybrid virus (or the helper virus) alone, all the SV40 VP2-VP3/poliovirus VP1 fusion protein detected was seen to remain intracytoplasmic. It has been suggested that SV40 VP1 alone is actively translocated into the cell nucleus, VP2 and VP3 being passively transported through interaction with VP1 (14). This conclusion was based on the observation that mutations within the coding sequence of VP1 could alter the transport of VP2 and VP3 to the nucleus. The results reported in this report strengthen the hypothesis that SV40 VP2 and VP3 migrate into the cell nucleus only in association with SV40 VP1. A possible explanation is that SV40 polypeptide VP1, which possesses an endogenous nuclear migration sequence within the sequence of its first eight NH2 terminal amino acids (35), is responsible for the active transport of polypeptides VP2-VP3 into the cell nucleus. The PNKKKRK sequence identified here could play a role either in the interaction of VP2-VP3 with VP1 or in the binding of the polypeptides to a receptor or a matrix in the cell nucleus. Alternatively, VP2 amino acids 317 to 323 could be simply required for the proper folding of the protein in order to correctly interact with VP1 before transport to the nucleus. The fact that a band of 46 kilodaltons, most probably SV40 VP1 could be immunoprecipitated from all infected cell extracts with an anti-poliovirus VP1 immune serum (Fig. 2) suggests the existence inside the infected cell of a poliovirus VP1/SV40 VP2-VP3-SV40 VP1 protein complex. This complex was observed independent of the extent of the deletion affecting the SV40 VP2-VP3/poliovirus VP1 fusion proteins, thus suggesting that the presence or absence of VP2 amino acids 317 to 323 was irrelevant to the formation of the complex. This implies that the PNKKKRK sequence in VP2-VP3 is not involved in the interaction with VP1 but rather plays the role of a nuclear accumulation signal.

Paradoxically, the sequence PNKKKRK shows a striking homology with the nuclear migration sequence identified by Kalderon et al. (12, 13) in the T antigen of SV40. It is rather puzzling that such similar sequences would be involved in two quite different mechanisms: direct translocation into the cell nucleus in the case of T antigen, and accumulation in the nucleus or specific interaction with SV40 VP1 (or both) in the case of VP2-VP3. Indeed, if the nuclear migration sequence of T antigen was sufficient to target T antigen to the cell nucleus, there should be no reason why a very similar sequence should not be sufficient also to target VP2-VP3.

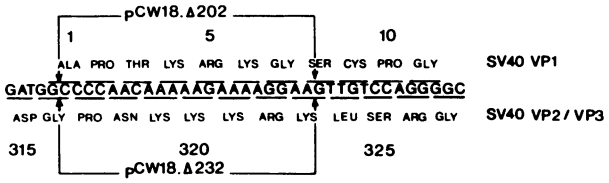

FIG. 8. The same region of the SV40 genome encodes the VP1 nuclear migration sequence and the VP2-VP3 nuclear accumulation sequence. pCW18Δ202 expresses a SV40 VP1-poliovirus VP1 fusion protein in which the first eight VP1 NH2-terminal amino acids are deleted (arrows). pCW18Δ232 expresses SV40 VP2-VP3/poliovirus VP1 fusion proteins deleted for VP2 amino acids 317 to 323 (arrows) and the 13 VP2 COOH-terminal amino acids (340 to 352). The deleted fusion proteins expressed by both plasmids remained located inside the cell cytoplasm. The reading frames used for the synthesis of SV40 VP1 and of SV40 VP2-VP3 are indicated by the bars above or below each codon, respectively. Amino acid numbering is after Tooze (31).

Perhaps tertiary and quaternary structures of the proteins are responsible for this difference.

In agreement with our conclusions, it was reported recently that in the absence of its 35 COOH-terminal amino acids, SV40 VP3 remains limited to a cytoplasmic and perinuclear location (8). The authors of that report concluded that the 35 COOH-terminal amino acids shared by VP2 and VP3 are required for the nuclear localization of both proteins and are likely to contain the karyophilic signal needed for their nuclear targeting. They also showed that nuclear localization of VP3 could be observed in the total absence of VP2 or the SV40 agnoprotein. Interestingly, quasi-normal transport of VP3 to the nucleus (and presumably of VP2 also) was observed when only one-third of the amino-terminal sequence of VP1 was present. This VP1 fragment contains the nuclear migration signal (35). The intracellular localization of the truncated VP1 protein was unfortunately not reported by those authors.

The sequence responsible for the migration of SV40 VP1 and that responsible for the accumulation of SV40 VP2-VP3 into the cell nucleus are encoded by the same portion of the SV40 genome (nucleotides 1492 to 1513), which is read in two different frames (Fig. 8). This leads us to predict that mutations in this region of the genome should profoundly affect the viability of the virus, because they would prevent the nuclear location of all three SV40 capsid polypeptides VP1, VP2, and VP3.

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