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Extensive Mutagenesis of the Nuclear Location Signal of Simian Virus 40 Large-T Antigen

W. H. COLLEDGE,[1] W. D. RICHARDSON,[1]† M. D. EDGE,[2] AND ALAN E. SMITH[1]‡*

Biochemistry Division, National Institute for Medical Research, London NW7 1AA,[1] and ICI Pharmaceuticals Division, Macclesfield, Cheshire SK10 4TG,[2] United Kingdom

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Site-directed mutagenesis was used to change Lys-128 of the simian virus 40 large-T nuclear location signal to Met, Ile, Arg, Gln, Asn, Leu, or His. Except for the large-T antigen of the Arg mutation, which was present in cytoplasmic and nuclear compartments, the resultant proteins were unable to enter the nucleus. By contrast, mutations at other sites within the signal were generally less severe in their effect. In some cases (Lys-128 to Gln, Asn, and His), the apparently cytoplasmic variants were able to support limited plasmid DNA replication, suggesting that low levels of large-T antigen undetectable by immunofluorescence were present in the nucleus. Such mutants did not support viral DNA replication. We conclude that there is a strong requirement for a basic residue at position 128 in the large-T nuclear location signal, with Lys the preferred residue.

The subcellular compartmentalization of eucaryotic cells has necessitated the evolution of mechanisms to localize specific proteins to their designated compartments (1, 18). Recently, a nuclear location signal was identified in the simian virus 40 (SV40) large-T antigen protein (6). The signal consists of a sequence of predominantly basic amino acids (Pro-Lys-Lys-128-Lys-Arg-Lys-Val) and is both necessary and sufficient to direct either large-T antigen (6) or chimeric cytoplasmic proteins (7) into nuclei. Alteration of Lys-128 to Thr (6) or Asn (10) abolishes nuclear accumulation, as judged by indirect immunofluorescence. To further characterize the sequence or structural requirements or both of the nuclear location signal, we conducted a systematic mutation analysis of this region. It is hoped that the information gained will enhance our ability to recognize functional signals in other nuclear proteins and provide insight into the type of interactions mediated by such signals. In the present study, we describe the isolation of several amino acid replacement mutations within the SV40 signal, particularly in residue Lys-128. The phenotypes of the mutants obtained emphasize the critical importance of Lys-128 in maintaining a functional nuclear location signal and also provide information about possible determinants in the signal sequence.

Mixed oligonucleotide and bisulfite mutagenesis were used as described previously (5, 6) to create mutations within the nuclear signal sequence of SV40 large-T antigen (Table 1). Plasmids pBi7, pBi16, pBi26, and pBi55 were produced by bisulfite treatment of looped heteroduplex molecules formed between d10 and pS11-S33 (5, 6). pBM10, pBM11, and pBM17 were derived by bisulfite mutagenesis of heteroduplex molecules formed by using pBi26 and pS11-S33. pA1, pA5, and pA8 were produced by mixed oligonucleotide mutagenesis (6) by using a synthetic oligonucleotide (GTTNTTNCTNCTNTTNCCAT; Oligo 1) partly complementary to nucleotides 4441 to 4422 of SV40 DNA. pMO4, pMO6, pMO7, and pHis128 were produced by using Oligo 2 (GTTTTGN$^{G}_{C}$TTCTCTTTCCAT), and pPK1 was formed by using Oligo 3 (TGAGGATTTTTTTGCTTCTC). N represents any of the four possible bases. Each mutated large-T gene was sequenced (11, 16) across the target region to deduce the position and nature of the mutation(s). A total of 15 different mutants were generated (Table 1). Nine possessed only single amino acid changes (seven of these were at position 128), four mutants had double changes, and two mutants possessed triple amino acid changes. Lysine-128 was altered to Met (pB126), Ile (pBM11), Arg (pA1), Gln (pMO4), Asn (pMO6), Leu (pMO7), or His (pHis128). The isolation of pMO6 (Lys-128 to Asn) was fortuitous, since the oligonucleotide used to generate this mutant (Oligo 2) was not designed to give this particular change.

The transforming potential of the mutant plasmids was tested by their ability to induce dense foci on monolayers of Rat-1 cells after calcium phosphate transfection by using 10 μg of mutant plasmid DNA per 50-mm dish of subconfluent cells (2). All the mutant large-T antigens could transform Rat-1 cells, and clones of these foci were able to grow in 0.5% fetal calf serum (Table 1). A number of the mutant plasmids appeared on first screening to have a reduced transforming potential compared with wild-type pPVU-0 (Table 1, pA1, pB17), but this result was not reproduced in subsequent experiments using fresh DNA preparations.

Transformed foci were expanded and examined by indirect immunofluorescence microscopy to ascertain the subcellular location of the mutant large-T antigens. The subcellular location of mutant proteins was also determined by immunofluorescence microscopy 16 h after microinjection of plasmid DNA into the nuclei of Vero cells (13). An internal control for the specificity of the staining procedure was provided by a proportion of untransformed cells in the cell population which did not stain for SV40 large-T antigen (compare Fig. 1C and D). Plasmid pPVU-0 encodes wild-type large-T antigen which accumulates in the nuclei of transformed (Fig. 1A) or microinjected cells (not shown). Large-T variants encoded by plasmids pA8 (Lys-131 to Met) and pA5 (Lys-129 to Arg) also accumulated in the nuclei of cells (Fig. 1B and D, respectively). In contrast, all but one of the large-T variants with mutations at residue 128 lost the ability to accumulate in nuclei (Table 1; Fig. 1G to H). The exception was the mutant large-T antigen encoded by pA1 which possessed the conservative change of Lys-128 to Arg. This variant was found in both the nucleus and the cyto-

* Corresponding author.
† Present address: Department of Zoology, University College, London WC1E 68T, United Kingdom.
‡ Present address: Integrated Genetics, Framingham, MA 01701.

TABLE 1. Properties of mutant SV40 large-T proteins[a]

PLASMID	Wt SV40 LARGE-T ANTIGEN AMINO-ACID SEQUENCE 125 Pro Pro Lys Lys Lys Arg Lys Val Glu Asp Pro 135	Transformation focus formation on Rat-1 cells % Wt ± S E M	Replication Plasmid DNA	Replication Virus	Subcellular location of Large-T
pPVU-0 (wt)		100 ± 13(7)	+	+	N
pBi7*	Thr Lys	38 ± 5(5)	-	-	C
pBi16*	Thr Ile	101 ± 17(3)	-	-	C
pBi26*	Met	125 ± 33(3)	-	-	C
pBi55*	Thr Lys Ile	100 ± 29(3)	-	-	C
pBM10*	Met Lys Ile	100 ± 27(3)	-	-	C
pBM11*	Ile	77 ± 17(3)	-	-	C
pBM17*	Met Lys	105 ± 11(3)	-	-	C
pA1	Arg	31 ± 2(4)	+	+	N/C
pA5	Arg	58 ± 19(3)	+	+	N
pA8	Met	64 ± 19(3)	+	+[L]	N>>C
pMo4	Gln	100 ± 25(3)	+	-	C
pMo6	Asn	64 ± 31(3)	+	-	C
pMo7	Leu	98 ± 33(3)	-	-	C
pPK1	Lys Thr	65 ± 2(3)	-	-	C
pHis128	His	80 ± 7(4)	+	-	C

[a] Transformation, plasmid replication, and virus replication assays using molecules incorporating the mutated large-T species were performed as described in the text. +, Plaques comparable to reconstructed wild-type virus visible 10 days posttransfection; −, no plaques visible up to 4 weeks later; L, plaque formation after more than 4 days delay compared with the wild type (wt). The locations of mutant large-T antigens were determined as described in the legend to Fig. 1. C, Cytoplasm; N, nucleus.

plasm of transformed or microinjected cells (Fig. 1E and F). Thus, for nuclear localization of SV40 large-T antigen, there is a strong requirement for a basic residue at position 128, with Lys the preferred amino acid. The less basic residue His is ineffective at this position (Fig. 1J), as are uncharged residues with similar steric properties to Lys, such as Gln (Fig. 1G and H) or Met (Table 1). Note that the mutant pK1 was made to test the hypothesis that the sequence Pro-Lys-Lys within a basic environment but including a mutated Lys-128 would be sufficient to act as a nuclear location signal. However, this mutant protein failed to enter the nucleus (Fig. 1I).

Since each mutant plasmid possesses a functional SV40 origin of replication, they all have the potential to undergo large-T antigen-dependent episomal replication in permissive simian (CV-1P) cells. To test the ability of each mutant large-T antigen to support episomal plasmid replication, plasmid DNA was transfected into CV-1P cells using DEAE-dextran and low-molecular-weight DNA harvested 72 h later (4). The DNA was digested with *Mbo*I, electrophoresed on a 1% agarose gel, and probed after transfer by the Southern procedure (17). The results of such replication assays are shown in Fig. 2. The input plasmid DNA (I in Fig. 2) was methylated on adenine residues, whereas the replicated plasmid DNA (R) was unmethylated. This difference allowed *Mbo*I, a methylation-sensitive restriction enzyme, to be used to distinguish input from newly replicated DNA. Input plasmid DNA could not be digested with *Mbo*I (I in Fig. 2), whereas replicated plasmid DNA was cleaved by *Mbo*I (R). *Sau*3AI is an isochisomer of *Mbo*I which can cut both methylated and nonmethylated DNA and, therefore, can cleave both input and newly replicated DNA.

The large-T antigens produced by plasmids pA1 (Lys-128 to Arg), pA5 (Lys-129 to Arg), and pA8 (Lys-131 to Met) could all stimulate plasmid replication at a level slightly below that of wild-type large-T antigen (Fig. 2, compare lane pPVU-0 with pA1, pA5, and pA8). The deletion/insertion mutant pS11-S33 (3) and the majority of the other mutated plasmids could not replicate in CV-1P cells (Table 1). Interestingly, however, three of the large-T antigen mutants, pMO4 (Lys-128 to Gln), pMO6 (Lys-128 to Asn), and pHis128 (Lys-128 to His), could support limited episomal plasmid replication, although well below the level obtained with wild-type pPVU-0. It is unlikely that the *Mbo*I-digested DNA represents cleavage of demethylated input DNA since no such cleavage is observed for mutant pS11-S33 or a number of other mutant plasmids, including pBi26 (Lys-128 to Met), pBM11 (Lys-128 to Ile), and pMO7 (Lys-128 to Leu), which also produce cytoplasmic large-T antigens.

Mutated plasmid DNA was used to reconstruct viral DNA as described elsewhere (8). Viral replication was measured either by plaque formation or by isolation of low-molecular-weight DNA (4) followed by electrophoresis and Southern blotting (17). The plaque assay and Southern blot assay for viral replication gave identical results in all cases. Plaques could be observed with virus reconstructed from pA1, pA5, and pA8 (Table 1), although plaques formed by A8 virus appeared 4 to 5 days later than those formed by reconstructed wild-type virus. No plaques or viral DNA, as visualized by Southern analysis, could be observed with any other reconstructions, including pMO4, pMO6, and pHis128. How pMO4, pMO6, and pHis128 can support episomal plasmid replication and yet not produce viable virus is unknown. The observation may reflect a difference in the sensitivity of the two assays or, alternatively, may indicate a novel activity of large-T antigen required for viral but not plasmid replication. Keller and Alwine (9) demonstrated that large-T antigen can directly stimulate transcrip-

tion from the SV40 late promoter in the absence of viral DNA replication. If this property is important during productive infection by the virus, it is possible that the His, Asn, and Gln mutants represent SV40 variants that lack the ability or are not present in sufficient amounts to activate late-gene transcription, even though they are able partially to stimulate plasmid DNA replication.

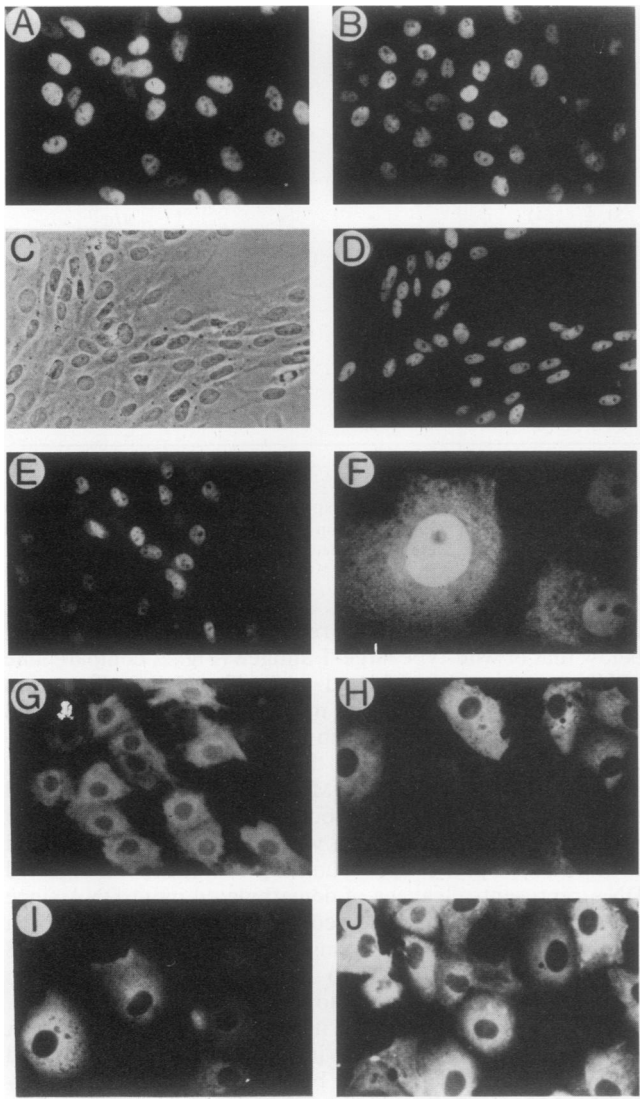

FIG. 1. Subcellular location of mutant SV40 large-T antigens in transformed Rat-1 cells and in Vero cells microinjected with plasmid DNA. Cells derived from transformed foci of Rat-1 cells were grown on glass cover slips, fixed in 4% formaldehyde in phosphate-buffered saline (20 min at 25°C), permeabilized with 1% Nonidet P-40 (5 min at 25°C), and incubated with anti-SV40 large-T monoclonal antibodies pBA423 and pAB419 (3). The cells were then incubated with tetramethylrhodamine isothiocyanate-labeled Fab (2) fragments from a rabbit anti-mouse antibody followed by incubation with similarly labeled sheep anti-rabbit antibody. Vero cells were examined by indirect immunofluorescence microscopy 18 h after manual microinjection of about 200 copies of plasmid DNA into the nuclei of semiconfluent cells growing on glass cover slips (13). Indirect immunofluorescence of large-T antigen encoded by pPVU-0 (A), pA8 (B), pA5 (phase contrast) (C), pA5 (D), pA1 (E), pA1 (microinjection) (F), pMO4 (G), pMO4 (microinjection) (H), pPK1 (microinjection) (I), and pHis128 (microinjection) (J).

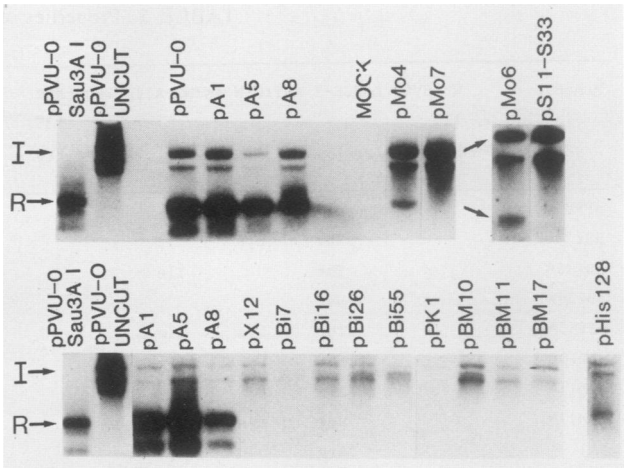

FIG. 2. Replication of mutant plasmids in permissive CV-1P cells. Plasmid DNA (200 ng) was transfected into 90% confluent CV-1P cells by using 400 μg of DEAE-dextran per ml. After 72 h, low-molecular-weight DNA was isolated (4), digested with *Mbo*I, and electrophoresed on a 1% agarose gel. Ethidium bromide staining of mitochondrial DNA was used as a rough indicator of the efficiency of low-molecular-weight DNA isolation. After transfer to nitrocellulose (17), the filters were hybridized to nick-translated pPVU-0 (14) and washed at 65°C in 0.6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before autoradiography. Mock transfections were performed by using only salmon sperm DNA. pS11-S33 is a replication-defective derivative of pPVU-0 used as a negative control. I, Input plasmid DNA; R, replicated plasmid DNA.

The results presented here demonstrate the importance of Lys-128 in maintaining a viable nuclear location signal in SV40 large-T antigen. Altering Lys-128 to Met, Ile, Gln, Asn, Leu, or His abolishes the ability of large-T antigen to accumulate in the nucleus, whereas single amino acid changes adjacent to position 128 (e.g., pA5 or pA8) modulate but do not abolish nuclear accumulation. pA1 is the only mutant with a lesion in codon 128 that can produce a large-T antigen capable of accumulating in the nucleus, in keeping with the conservative nature of the change introduced (Lys-128 to Arg). The mixed phenotype of pA1 may be due to a reduced rate of transport into the nucleus. This seems to be true for the previously described mutant d1 (6), which contains two amino acid replacements (Lys-129 to Met, Lys-131 to Thr) and produces a protein with a mixed phenotype. By microinjecting d1 plasmid DNA into Vero cells and assaying the location of large-T antigen by immunofluorescence either 4 or 18 h later, the ratio of nuclear to cytoplasmic fluorescence was found to increase with time (data not shown). This observation suggests that the effect of mutations within the signal sequence may be to alter the rate of accumulation of the protein in the nucleus rather than the final extent of accumulation.

Episomal plasmid replication is generally regarded as an entirely nuclear event. If this is the case, then the ability of pMO4, pMO6, and pHis128 to replicate episomally in CV-1P cells (Table 1 and Fig. 2) suggests that a low concentration of large-T antigen may be present in the nucleus despite the fact that by immunofluorescence assay the vast majority is visualized in the cytoplasm (Fig. 1G, H, and J). This is an important result, for although it is not unreasonable to propose that a nonnuclear form of SV40 large-T antigen can transform Rat-1 cells, the data indicate that we cannot rule out the alternative possibility that a very low level of nuclear

large-T antigen, undetectable by immunofluorescence, is sufficient for transformation.

The experiments described here probe in detail the sequence requirements of a nuclear location signal. Together with the mutants that first defined the signal, the mutants isolated here demonstrate that changes throughout the sequence are relatively inconsequential, except in the critical Lys-128 residue. The reason for this remains unclear, but it appears not to be related simply to the need for a sequence Pro-Lys-Lys within a basic context, since a mutant (pPK1) created with this sequence remains cytoplasmic. It appears from the data that if similar nuclear location signals are present in other proteins, they need not conform exactly to the SV40 large-T sequence. Indeed, this was shown to be the case for polyomavirus large-T antigen, which contains two signals of similar charge to, but different sequence from, the SV40 large-T prototype (12). It will be interesting to discover whether other nuclear location signals are predominantly basic sequences with a critically important residue equivalent to Lys-128 or whether the lysine residue is a peculiarity of large-T antigen.

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