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Simian Virus 40 Origin DNA-Binding Domain on Large T Antigen

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Fifty variant forms of simian virus 40 (SV40) large T antigen bearing point, multiple point, deletion, or termination mutations within a region of the protein thought to be involved in DNA binding were tested for their ability to bind to SV40 origin DNA. A number of the mutant large T species including some with point mutations were unable to bind, whereas many were wild type in this activity. The clustering of the mutations that are defective in origin DNA binding both reported here and by others suggests a DNA-binding domain on large T maps between residues 139 and approximately 220, with a particularly sensitive sequence between amino acids 147 and 166. The results indicate that the domain is involved in binding to both site I and site II on SV40 DNA, but it remains unclear whether it is responsible for binding to cellular DNA. Since all the mutants retain the ability to transform Rat-1 cells, we conclude that the ability of large T to bind to SV40 origin DNA is not a prerequisite for its transforming activity.

Simian virus 40 (SV40) large T antigen, the product of the viral *A* gene, is required for replication of the virus as well as for the transformation of nonpermissive cells in culture (59). A number of biochemical activities have been shown to be intrinsic to the protein including an ATPase activity (4, 58) and the ability to form complexes with the host phosphoprotein p53 (24, 26). Large T has also been shown to bind with high affinity to viral (8, 10, 31, 55–57) and cellular (2, 11, 41) DNA sequences. The role of the DNA-binding activity in both replicative and transforming functions of large T has been widely investigated. From analysis of a number of mutants isolated in several laboratories, it appears that the binding of large T to SV40 origin DNA is necessary (30, 32, 50, 54) though not sufficient (5, 29, 43) for replication, but not essential for efficient transformation of cells in culture (29, 43, 54).

In mapping the domain of large T involved in DNA binding, two types of activity have been measured in different studies: the ability to bind to cellular double-stranded (ds) DNA-cellulose (2, 11, 41) and the ability to bind to SV40 origin DNA sequences (8, 10, 31, 55–57). It is not yet clear whether these assays measure the activity of one or more domains. The variants used to map the DNA-binding domain include: (i) fragments of large T produced by in vitro translation of unspliced SV40 cRNA (42); (ii) various truncated forms of large T present in different SV40-transformed cell lines (3, 46); (iii) various chimeric proteins coded for by adeno-SV40 hybrid viruses containing defined portions of large T (33, 41, 57); (iv) defective large T species isolated from permissive or nonpermissive transformed cells (12, 28, 29, 43, 49, 54); (v) pseudorevertant forms of large T that are able to replicate from a defective origin sequence (30, 50); (vi) proteolytic fragments of wild-type large T (34); and (vii) large T variants encoded by constructed SV40 *A* gene deletion mutants (5, 39). Almost all of the data are consistent in suggesting that amino acids between 83 (corresponding to the splice site in the large T mRNA) and about 270 constitute part, if not all of a DNA-binding domain.

We have previously reported the isolation of over 50 large T variants, most of which carry mutations in the DNA coding for amino acids 106 to 158 of large T (19). Almost all of the mutants retain the ability to transform Rat-1 cells, but most are reduced or defective in their ability to replicate. Some of the mutations affect residues that are normally phosphorylated in vivo, and others are located within a sequence that is important in localizing large T to the nucleus (17, 18, 25). Some of the mutants isolated had a supertransforming activity in that they produced transformed foci in greater number and more rapidly than wild-type large T. A further series of mutants have been produced by introducing termination codons at different positions throughout the large T coding region (18; B. Roberts, unpublished data). Here we report the DNA-binding properties of the large T species present in cells transformed by the different large T variants we have isolated. The data show clearly that point, multiple point, deletion, and termination mutations within the target area used here can abolish the ability to bind to SV40 origin DNA sequences, as measured by an immunoprecipitation assay. This, together with data from other laboratories, allows us to define precisely those amino acids that constitute the amino-terminal portion of a DNA-binding domain on large T. The data also confirm that SV40 large T does not require the ability to bind to SV40 origin DNA to transform established cells. It remains to be seen whether the ability to bind to cellular DNA plays a part in this process.

MATERIALS AND METHODS

Cells. All cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The rat cells (RE52) transformed by microinjected *Taq-Bam*HI fragment of SV40 large T, called 70K, were the gift of A. Graessman. SVA31E7 cells, SV40-transformed mouse cells, have been described previously (13, 38). The generation of cell lines transformed by each of the mutant DNAs has been described in detail previously (19). The wild-type SV40-transformed rat line used in these experiments was generated as described (19) by transfection with the wild-type SV40-containing plasmid pPVU-0. Cell lines transformed by the termination

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mutants H23, dH, and HHpa and by the deletion mutants RL75-RL88 and RL12-S24 were also generated by transfection of Rat-1 cells with plasmid DNAs by the procedure previously described (19). The construction of the termination mutants is given in Kalderon et al. (18). The deletion mutants were created by ligating *Bam*HI-*Eco*RI-cut DNA from the appropriate pairs of linker insertion mutants (19).

Labeling and extraction of cells. The procedures for labeling and extraction of cells were carried out essentially as described previously (38, 51, 52). Cells were labeled with methionine for 5 h by replacing the medium on confluent monolayers of cells growing on 50- or 90-mm dishes (Nunc, Roskilde, Denmark) with growth medium minus methionine supplemented with 1% complete medium (containing 10% fetal calf serum) and 200 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham International) per ml. $^{32}PO_4$ labeling was for 2 h in growth medium minus phosphate containing 250 μCi of $^{32}P_i$ (Amersham International) per ml. After labeling, the cells were rinsed twice with ice-cold Tris-buffered saline and then lysed by the addition of 1 ml/90-mm dish or 0.5 ml/50-mm dish of cell extraction buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, and 0.5% Nonidet P-40 (NP-40). The samples were spun at 10,000 rpm in an Eppendorf microcentrifuge for 10 min at 4°C, and the supernatants were used directly or stored at −70°C.

Immunoprecipitation of cell extracts. Samples of lysates containing 3×10^6 trichloroacetic acid-precipiptable ^{35}S cpm (generally corresponding to 40 to 100 μl of extract) were mixed with 0.5 ml of buffer containing 20 mM Tris (pH 7.0), 100 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA and 0.5% NP-40. Portions (10 μl) of anti-SV40 tumor cell serum were added, and the mixture was incubated for 60 min at 18 to 20°C. A 10% suspension of washed *Staphylococcus aureus* bacteria (50 μl) was added, and incubation continued for 10 min. The bacterial pellet was washed twice in a buffer containing 20 mM Tris (pH 8.0), 2 mM dithiothreitol, 1 mM EDTA, 0.5% NP-40, and 0.5 M NaCl. After a third wash in the same buffer containing 0.1 M NaCl, the bound proteins were eluted with gel-loading buffer (0.0625 M Tris [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.1 M dithiothreitol, 0.01% bromophenol blue). Sample preparation and polyacrylamide gel electrophoresis were done as described previously (51, 52). Gels containing [^{35}S]methionine-labeled samples were soaked for 1 h in 10 volumes of 1 M sodium salicylate before being dried. Autoradiography was for 1 to 4 days at −70°C on Fuji Rx film with an Ilford fast tungstate intensifying screen.

SV40 origin binding. Four DNAs were used in these experiments. pSV328 (a gift from G. C. Grosveld) contains the larger *Bam*HI-*Eco*RI fragment of SV40 inserted between the *Bam*HI and *Eco*RI sites of pBR322. RL18 and O34 are both derivatives of pPVU-0 (16, 19), a plasmid that contains the *Bam*HI to *Pvu*II early-region fragment of SV40 (SVS strain) between the *Bam*HI and *Pvu*II sites in pBR328. RL18 has an *Eco*RI linker (CGGAATTCCG) inserted between positions 5194 and 5186, while O34 contains the linker in place of SV40 sequences between positions 5215 and 4739. These DNAs were digested with the restriction enzyme *Bst*NI, and in the case of RL18 also with *Eco*RI. The restricted DNAs were treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and then end-labeled with [γ-^{32}P]ATP (specific activity, 5 to 7,000 Ci/mmol; Amersham International) and T4 polynucleotide kinase by the method of Maniatis et al. (27). The specific activity of DNA obtained was generally between 1×10^7 and 3×10^7 cpm/μg. The DNA binding procedure, essentially that of McKay (31), has been described in detail previously (38). In these experiments, samples of cell extracts containing equivalent amounts of ^{35}S-labeled large T, as judged from experiments such as that shown in Fig. 1, generally 40 to 100 μl, were made up to 100 μl with extraction buffer and mixed with 0.4 ml of binding buffer (20 mM Tris [pH 7.0], 1 mM EDTA, 2.5 mM dithiothreitol, 125 μg of bovine serum albumin, 0.05% NP-40) and 10 ng of the appropriate labeled DNA. After incubation at 18°C for 40 to 60 min, 5 μl of a mixture of the monoclonal antibodies pAb419 and pAb413 was added. Incubation was continued for a further 40 to 60 min, and 25 μl of washed *S. aureus* bacteria was added. After an additional 5 to 10-min incubation, the pellets were washed three times in binding buffer without bovine serum albumin containing 0.2 M NaCl. The DNA was eluted in 15 μl of gel loading buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 2% sodium dodecyl sulfate) and loaded directly onto 2% agarose gels in Tris-acetate buffer (27). After electrophoresis, the gels were fixed in 2 volumes of ethanol before drying. Autoradiography was generally for 1 to 2 days.

Binding to ds calf thymus DNA immobilized on cellulose. Confluent monolayers of cells on 50-mm dishes (Nunc) were labeled with $^{32}P_i$ for 2 h as described above. The monolayers were washed with Tris-saline and lysed in 200 μl of buffer A (10 mM HEPES[*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1 mM $MgCl_2$, 0.5% NP-40, 0.3 mg of phenylmethylsulfonyl fluoride per ml) containing 0.45 M NaCl. The lysate was removed and immediately diluted by the addition of 2 volumes of buffer without salt. The pH was then adjusted to 6.5 by the addition of 0.01 M HCl, and the extract was mixed with 0.5 ml of packed native calf thymus DNA-cellulose (P-L Biochemicals, Inc., Milwaukee, Wis.) which had been equilibrated in buffer A at pH 6.5 with 0.15 M NaCl. The mixture was kept at 4°C for 1 h on a rotary mixer. The DNA-cellulose was then pelleted by centrifugation for 1 min at 4°C in an Eppendorf microcentrifuge, and the supernatant was removed. The DNA-cellulose pellet was washed three times with buffer A at pH 6.5 with 0.15 M NaCl and then incubated for 1 h at 4°C with 0.5 ml of buffer A at pH 8.5 with 0.15 M NaCl to elute bound protein. After three washes with this buffer, a second elution step was then carried out with buffer A at pH 8.5 with 1.0 M NaCl. Both of the eluted fractions and 20-μl samples of starting material and of the unbound fraction were adjusted to pH 8.5 and 0.5 M NaCl before overnight incubation with 10 μl of anti-SV40 tumor cell serum. Immunoprecipitates were then collected, washed, and analyzed as described above.

RESULTS

As part of our efforts to assign biochemical functions to domains of large T, we examined many of the large T variants we have isolated (19) for their ability to bind to SV40 origin DNA. The mutants used fall into two groups. The larger group consists of those variants carrying point mutations or small deletions in the DNA coding for amino acids 106 to 158 of large T. The sequence of the SV40 variants encoded by most of the mutants used has been reported in detail (19) and is summarized in Table 1.

The second group contains the so-called termination mutants which encode truncated large T protein (17, 19; Roberts, unpublished data). Three of these have been used in the studies reported here: H23, dH (previously referred to as Δ*Hin*dIII [18]), and HHpa, which encode amino-terminal fragments of large T consisting of 147, 272, and 362 amino

TABLE 1. DNA-binding activity of mutant SV40 large T protein[a]

Mutant	Sequence alteration																										
	106 Ser	107 Glu	108 Glu	109 Met	110 Pro	111 Ser	112 Ser	113 Asp	114 Asp	115 Glu	116 Ala	117 Thr	118 Ala	119 Asp	120 Ser	121 Gln	122 His	123 Ser	124 Thr	125 Pro	126 Pro	127 Lys	128 Lys	129 Lys	130 Arg	131 Lys	132 Val
C8	Phe																										
K79(K1)[c,d]		Lys																									
C37[c,d]			Lys																								
C22[d]		Lys	Lys																								
K7[d]			Lys				Asn																				
pPVU-1[d]	—	—	—	—	Tyr	—	—																				
W7																	Tyr	Phe									
D19																			Ser								
D20															Phe												
D29																		Ile									
T23-L7											—	—	—	Arg	Asn	Ser	Ala	—	—	—	—						
X46																						Thr					
X12[e]																							Thr				
δ1[f]																								Met		Thr	
S17-S29[e]																								Arg	Asn	Ser	Glu
S17-S36[e]																								Arg	Asn	Ser	Glu
W34																											
W38																											
U33																											
W67																											
W75																											
L27-S26																											
S1-S32																											
D111																											
D119																											
D120[g]																											
U24[g]																											
B9[c]																											
B11[g]																											
U19[g]																											
U20																											
W128[g]																											
W134																											
RL23[g]																											
D88[g]																											
D89[g]																											
D90																											
D94																											
D96[c]																											
D104[c]																											
D98																											

pPVU-2	Deletes Glu-Gln-Trp-Trp-Asn (92–96)
RL148	Tyr-Ser (179-180) → Ser-Glu-Phe-Arg
B18[c,d]	Ser 189 → Asn
RL75	Ser-Tyr-Asn-His-Asn (189–193) → Asn-Gly-Ile-Pro
RL115	Phe-Leu-Ile-Cys (220–223) → Arg-Asn-Ser-Gly
H23	Terminates at 147
dH	Terminates at 272
HHpa	Terminates at 362
70K	Transformed by microinjected *Taq-Bam* fragment

acid residues, respectively. As virtually all of the large T variants that we isolated gave rise, albeit to different extents, to foci of Rat-1 cells overgrowing a monolayer of normal cells, the transformed cell lines were used as a source of large T for the DNA binding studies. The biological properties of the cell lines transformed by these large T fragments will be reported elsewhere. We also tested the large T fragment produced in the so-called 70K cell line that was isolated after needle microinjection into rat cells of the smaller *Taq-Bam*HI fragment of SV40 which lacks the first exon of large T corresponding to amino acids 1 to 82 (13).

To establish the relative levels of large T present in the different cell lines, extracts were made from cells after labeling with [^{35}S]methionine for 5 h. This labeling period was chosen because it should be sufficient to label the cells to a reasonably high specific activity at something approaching steady state. When equal numbers of trichloroacetic acid-precipitable counts of [^{35}S]methionine-labeled material were immunoprecipitated with anti-T serum, the amount of

TABLE 1—*Continued*

133 Glu	134 Asp	135 Pro	136 Lys	137 Asp	138 Phe	139 Pro	140 Ser	141 Glu	142 Leu	143 Leu	144 Ser	145 Phe	146 Leu	147 Ser	148 His	149 Ala	150 Val	151 Phe	152 Ser	153 Asn	154 Arg	155 Thr	156 Leu	157 Ala	158 Cys	SV40 DNA-binding activity[b]
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		Leu																								++
Lys	Asn																									++
								Lys																		++
				Asn				Lys																		+
						Leu																				−
					Arg	Asn	Ser	Val																		−
	Ala	Glu	Phe	Arg																						+
															Tyr	Val										−
														Asn												−
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																Val										−
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																			Asn		Lys					−
																	Ile								Tyr	+
																							Phe			+
																	Met		Asn		Lys					−
														Pro	Glu	Phe	Arg									−
											Asn					Thr										(+)
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[a] The alterations predicted in the amino acid sequence of the mutant large T proteins are indicated. The superscripts refer to the following properties of the mutant plasmids as demonstrated by Kalderon and Smith (19). *c*, Mutant plasmids can replicate in CV1 cells as well as wild-type SV40, both as plasmids and as reconstructed virus. *d*, Plasmids are defective in transformation. Fewer foci are produced per microgram of DNA with a longer latent period than with wild-type DNA. *e*, Mutant large T is detected only in the cytoplasm by immunofluorescent staining. *f*, Mutant large T is found in both the cytoplasm and the nucleus of transformed cells. *g*, Mutant DNA produces more foci per microgram with a shorter latent period than does wild-type DNA. This phenotype has been called supertransforming (19).

[b] The ability of the mutant large Ts to bind SV40 origin DNA has been expressed as: ++, binding equivalent to wild type; +, decreased ability to bind; (+) weak binding detected with very small site I-containing DNA fragments; −, no detectable binding under any conditions with any DNA tested.

labeled large T detected was reasonably consistent between the different extracts, but some cells clearly contained significantly more than others (Fig. 1). Each of the cell lines was developed from a randomly picked dense focus after transformation by each of the mutant DNAs under equivalent conditions. Differences in the amount of large T between individual cell lines might therefore reflect their nonclonal nature. In addition, the stability of the mutant proteins or the extent to which the mutant proteins are expressed might vary between the different transformed lines. Further experiments would be required to distinguish between these possibilities for individual mutants. It is striking, however, that all of the large T variants that contain mutations within the sequence 127-Lys-Lys-Lys-Arg-Lys-Val-132 are overproduced 6 to 20-fold relative to wild type. We have already shown that these mutant large Ts are located entirely (X12, S17-S29, S17-S36) or partly (dl) in the cytoplasm of transformed or microinjected cells (17, 18). It is possible, therefore, that the observed overproduction might be caused by the inability of cytoplasmic large T to autoregulate its own production. Lack of autoregulation might explain why some of the other large T species appear to be present in large amounts.

The large T fragments immunoprecipitated from Rat-1 cells transformed by the termination mutants H23, dH, and HHpa are also shown in Fig. 1. The apparent molecular weights of the 147, 272, and 362 amino acid fragments are 14,000, 30,000, and 45,000, respectively. The large T found in 70K cells is shown in Fig. 1. Since these rat cells lack coding sequences corresponding to the first exon of large T, the large T variant expressed cannot include any of the residues 1 through 82. It is thought that translation begins at the first in-frame methionine of the large T gene, Met-109 (13). The predicted molecular weight of the resulting protein fragment is about 70,000; however, the mutant large T migrates with an apparent molecular weight of 84,000 (Fig. 1). Although large T species are known to migrate anomalously on sodium dodecyl sulfate-polyacrylamide gels, some doubt is cast on the exact nature of 70K large T as a result of the difference between the predicted and the observed molecular weights.

For the experiments described here we assumed that the total amount of large T present in the different transformed cells was proportional to that detected after a 5-h labeling period. Samples of extracts of the different cell lines containing equivalent amounts of large T were incubated with a mixture of ^{32}P-end-labeled fragments of SV40 DNA which had been produced by cleavage with the restriction enzyme *Bst*NI. Digestion with this enzyme produces among others an SV40 fragment of 311 base pairs (bp) which contains all three of the sites to which SV40 large T is known to bind (5, 8, 9, 35, 55–57). After incubation for 60 min, any large T bound to the SV40 DNA was recovered by immunoprecipitation with a mixture of the monoclonal antibodies PAb413 and PAb419 which recognize determinants localized at the carboxyl and the amino termini, respectively, of large T (15). The labeled DNA was then fractionated by agarose gel electrophoresis. Figure 2 shows the results of the so-called McKay assay (31), using extracts from the cells shown in Fig. 1. Some of the mutants coimmunoprecipitated wild-type levels of the 311-bp *Bst*NI fragment, whereas others had reduced levels of binding and some gave none. Addition of more extract from cells which were negative for binding in the experiment in Fig. 2 still failed to give any signal, emphasizing that the large T species present in these extracts were unable to recognize SV40 origin DNA in this assay. For such mutants, DNA-binding activity was consistently absent in all independently derived transformed cell lines examined.

These data, together with the predicted amino acid sequences of the different mutant large Ts, are summarized in Table 1. These data show that the mutations which affect the ability of large T to bind to SV40 origin-containing DNA are tightly clustered. Small deletions or point mutations up to and including amino acid 137 have little effect on the ability of the mutated large T to bind to SV40 origin sequences. This includes the region around the Lys-128 mutations which produces cytoplasmic large Ts (17, 25) and all of the phosphorylation sites mapped at the amino terminus of large T (Ser-106, Ser-111, Ser-112, Ser-123, and Thr-124 [48, 60]). Mutation of Pro-139, however, or deletions encompassing this residue result in large T which is defective in SV40 origin binding. Likewise, mutation of Ser-147 → Asn (D119) of Ala-149 → Val (U24) and of Ser-152+Arg-154 → Asn+Lys (U19) all abolish the ability of large T to bind to SV40 origin DNA. A number of multiple and deletion mutants encompassing these residues were also negative for DNA binding. Other mutants in the region between residues 140 and 160 had a severely reduced ability to bind to DNA. The clustering of all of these mutations suggests that they all lie within a functional domain whose amino-terminal end is defined by Pro-139.

There are few mutants in our collection that map downstream of residue 158; consequently, mapping of the extent of the domain is difficult. However, two mutants in the downstream sequences (RL148 and RL75) were reduced in their origin-binding activity, and the mutant RL115 (which has altered amino acids 220 to 223) was unable to bind origin DNA at all. This may mean that the domain extends at least as far as amino acid 223.

The data obtained with the other mutants tested is consistent with this interpretation. The 70K large T, which lacks at least amino acids 1 to 82, exhibited wild-type levels of origin binding (Fig. 2), indicating that the domain lies within the second exon of large T. The 147-amino acid fragment of large T coded for by the termination mutant H23 failed to bind DNA in this assay (data not shown). dH large T, which is 272 amino acids long, and therefore includes the sequences making up the region defined above, did bind to origin DNA, though weakly. HHpa large T bound DNA better, perhaps suggesting that the conformation of the 362-amino acid-long fragment more closely resembles that of wild-type large T than do the shorter forms. Taken together, these data support the notion that a region involved in binding to SV40 origin DNA begins at Pro-139 and extends at least as far as amino acid 223 of large T. Within this domain, a region between residue 147 and 154 appears to be particularly sensitive to mutation.

Ability of the mutants to bind to the SV40 site II origin region. The *Eco*RII G fragment of SV40 DNA contains three sites to which large T is known to bind. Site II spans the origin of SV40 DNA replication. Site I is located on the early side of the origin, and site III is on the late side. Binding of large T to site I is believed to block transcription of SV40 early mRNA resulting in autoregulation of large T synthesis (1, 9, 44). Binding to site II is presumably essential for the initiation of replication of viral DNA (8, 9, 50, 55, 56), while the consequences of site III binding are not yet understood. De Lucia et al. (8) have established that each site contains several copies of the consensus pentanucleotide 5′-GAGGC-3′ which are protected from methylation by dimethylsulfate when large T is bound. The sequence of the SV40 origin-

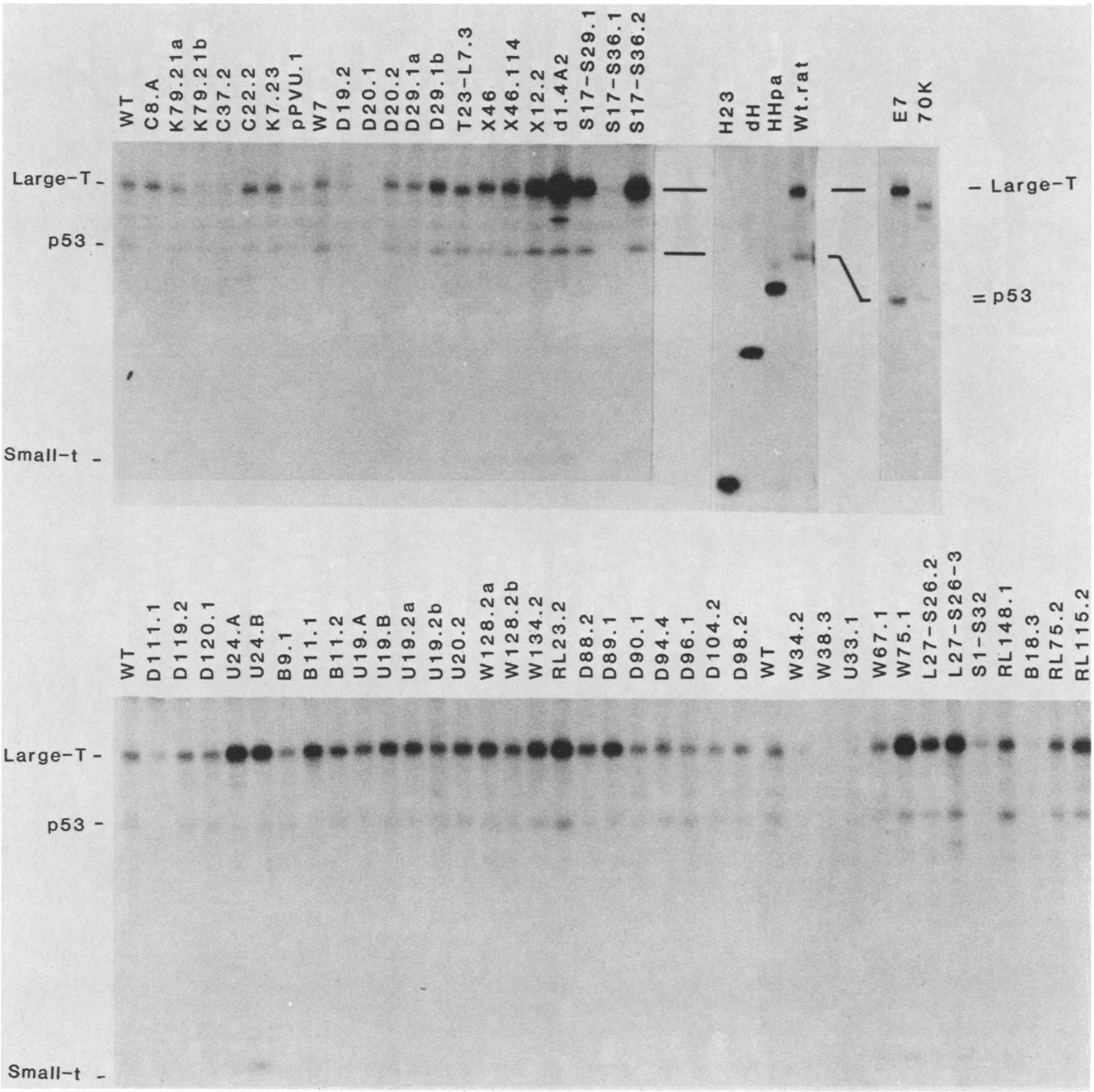
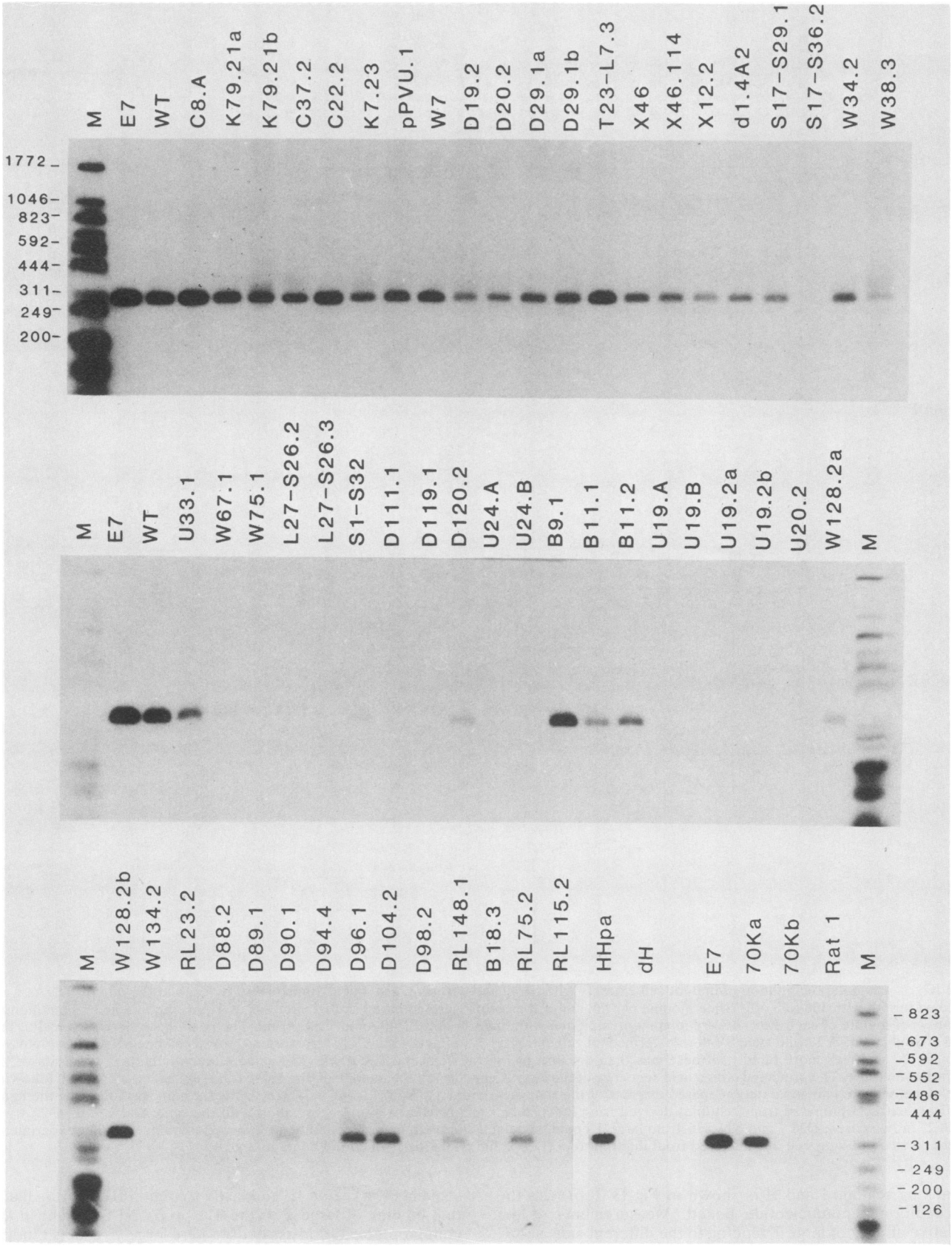


FIG. 1. Immunoprecipitation of mutant cell extracts with SV40 anti-T serum. Rat-1 cells transformed by each of the mutant DNAs were labeled for 5 h with 100 μCi of [^{35}S]methionine per ml, and cell extracts were prepared. Equal numbers of TCA-precipitable [^{35}S]methionine counts per minute of each extract were immunoprecipitated with hamster anti-SV40 tumor cell serum. The positions of large T, small t, and p53 are indicated. WT, wild-type SV40-transformed rat cell extract; E7, extract of the SV40-transformed mouse cell line SVA31E7. Note that mouse p53 migrates more rapidly in these gels than does rat p53 in the adjacent 70K track. The name of each mutant is given above the appropriate track. The predicted amino acid sequence of the large T encoded by the mutants is shown in Table 1. The letters a and b following the name indicate two separately prepared extracts of the same cell line, e.g., K79.21a and K79.21b. Different numbers following the name indicate extracts prepared from cell lines derived from independent foci produced by transformation with the same DNA, e.g., D20.1 and D20.2. In two cases, D20.1 and S17-S36.1, no large T could be detected upon immunoprecipitation of the cell extracts. Further experiments were carried out with cell lines derived from different foci (D20.2 and S17-S36.2) which did contain large T.

spanning regions I and II is shown in Fig. 3 (top) with the recognition pentanucleotide boxed. Measurements of the relative affinity of large T binding to the different sites at 4°C have led to the conclusion that the protein binds most tightly to the two perfect pentanucleotides in site I, then to the sequences in region II, and lastly to region III (8). It is likely that binding of large T to the 311-bp *Bst*NI fragment in the immunoprecipitation assay used here largely reflects binding to the highest-affinity site I (32). In an attempt to assess whether binding to site I and site II was affected in parallel in


M
E7
WT
C8.A
K79.21a
K79.21b
C37.2
C22.2
K7.23
pPVU.1
W7
D19.2
D20.2
D29.1a
D29.1b
T23-L7.3
X46
X46.114
X12.2
d1.4A2
S17-S29.1
S17-S36.2
W34.2
W38.3
1772
1046
823
592
444
311
249
200
M
E7
WT
U33.1
W67.1
W75.1
L27-S26.2
L27-S26.3
S1-S32
D111.1
D119.1
D120.2
U24.A
U24.B
B9.1
B11.1
B11.2
U19.A
U19.B
U19.2a
U19.2b
U20.2
W128.2a
M
M
W128.2b
W134.2
RL23.2
D88.2
D89.1
D90.1
D94.4
D96.1
D104.2
D98.2
RL148.1
B18.3
RL75.2
RL115.2
HHpa
dH
E7
70Ka
70Kb
Rat 1
M
823
673
592
552
486
444
311
249
200
126

the different mutants, we carried out immunoprecipitation assays with a number of different DNA fragments.

Initially, we separated site I and site II sequences by digesting the SV40 *Hin*dIII C fragment with *Dde*. Site I was present as a 57-bp fragment, and site II was included in a fragment of 302 bp (Fig. 3, bottom). The small site I-containing *Dde-Hin*dIII C fragment was preferentially recognized by wild-type large T, and recovery of the 302-bp site I fragment was poor (Fig. 4). This may have been a reflection of the lower affinity of T antigen for site II but could equally have been due to the position of the *Dde* site which is immediately adjacent to the large T-binding sequences on site II (shown in Fig. 3, bottom) at the extreme end of the 302-bp fragment. Therefore, two mutant plasmid DNAs were used, RL18 and O34, in which two or all three of the site I pentanucleotides were deleted. The DNA sequence of the origin region of these two mutants is illustrated in Fig. 3, bottom. The mutant DNAs were digested with *Bst*NI or *Bst*NI and *Eco*RI, end labeled, and used for DNA binding as before. Results obtained with RL18 are shown in Fig. 4, and some of the mutants tested with O34 are shown in Fig. 5. All of the results obtained followed the pattern observed in Fig. 2. Those mutants which bound the 311-bp wild-type *Bst*NI fragment also immunoprecipitated the origin-containing fragments of both mutant DNAs; those mutants which failed to bind to the wild-type origin fragment also failed to recognize the altered origins. In addition to the 858-bp SV40 origin-containing fragment bound by the different mutants in Fig. 5, the 1,772-bp plasmid DNA fragment was precipitated in some cases. Since that band was not detected in previous experiments using the same extracts under the same conditions, we believe this reflects nonspecific binding which can sometimes occur in immunoprecipitation assays and which has been seen by several other authors (32, 43). Nevertheless, these results suggest that the ability of large T to recognize site II sequences is lost in parallel with the ability to bind to site I. This observation must be tested more rigorously by using precise measurements of DNA binding such as dimethylsulfate protection assays. However, our initial results with a large number of mutants imply that the functional domain involved in binding to site I sequences also plays a role in recognizing sequences at the origin of replication.

Binding to cellular DNA. In addition to binding to sequences at the viral replication origin, SV40 large T is known to bind tightly to cellular DNA sequences (2, 10, 11, 41). It has not yet been established whether one or two functional domains are responsible for these activities. From experiments using a series of truncated large T molecules, Chaudry et al. (3) tentatively mapped a region on large T between amino acids 109 and 272 that was sufficient for binding to ds cellular DNA. This region includes the sequences which we have shown here are involved in binding specifically to SV40 origin DNA. In a further attempt to determine whether these sequences play a role in binding to cellular DNA, we tested a number of mutants carrying alterations within the domain for their ability to bind to ds calf thymus DNA immobilized on cellulose.

Of the mutants we have constructed, we chose to examine the point mutants U24 (Ala-149 → Val) and U19 (Ser-152 → Asn and Arg-154 → Lys), both of which had lost the ability to bind to the SV40 origin, and B11 (Ala-149 → Thr and Val-150 → Met), which could bind origin but not replicate. To probe the region on the carboxyl side of these point mutants, we used the small deletion mutants RL148 (Tyr-179 and Ser-180 → Ser-Glu-Phe-Arg) and RL115 (Phe-220-Cys-223 → Arg-Asn-Ser-Gly) since we have few point mutants in that area. RL148 bound origin DNA weakly in an origin immunoprecipitation assay, while RL115 showed no detectable binding. Because we anticipated that the so-called nonspecific binding to ds DNA might be less sensitive to point mutation than was binding to origin-specific sequences, we also examined mutants carrying larger deletions. This ensured that the protein domain under scrutiny was tested in a severely altered form. The mutant RL75-RL88 has four amino acids (Arg-Asn-Ser-Asp) inserted in place of the sequence between amino acids 188 and 212. The other mutant, RL12-S24, has Gly-Ile-Pro replacing amino acids 230 to 251. Both of these mutants are capable of producing dense foci on Rat-1 cells, and DNA binding experiments like those shown in Fig. 1 and 2 established that both mutants are defective in origin binding. We also used the large T fragment of 272 amino acids coded for by the termination mutant dH.

Cells transformed by each of the mutants were labeled with ^{32}P, and cell extracts were prepared. The extracts were mixed with calf thymus ds DNA-cellulose at pH 6.5, and bound proteins were eluted at pH 8.5, first with a low-salt buffer and then in buffer containing 1 M NaCl. Samples of the starting material, of the unbound fraction, and of the entire sample of both low-salt and high-salt eluates were then immunoprecipitated as described. Some representative results are shown in Fig. 6. A fraction of the population of large T of each mutant tested was capable of binding tightly to calf thymus DNA-cellulose as judged by elution under conditions of high pH and high salt concentration (tracks 4).

Our results with the mutant dH confirm that the amino-terminal 272 amino acids of large T are sufficient for binding to cellular DNA. However, these results show that there is no correlation between the ability to bind with high affinity to SV40 origin DNA and the ability to bind to cellular DNA. When considered together with the observation that large deletions within the putative origin-binding domain apparently do not affect the ability of the mutant protein to bind to cellular DNA, these results might imply that there is a second site on large T that is capable of functioning independently in binding to cellular DNA.

DISCUSSION

SV40 origin region DNA-binding domain. Earlier data indicating that a DNA-binding domain on SV40 large T lies

FIG. 2. Immunoprecipitation of mutant large T bound to SV40 origin-containing DNA. Samples of each cell extract containing equivalent amounts of ^{35}S-labeled large T (Fig. 1) were incubated with 10 ng of ^{32}P-labeled *Bst*NI-digested DNA from the plasmid pSV328, which contains the entire early region of SV40. Large T was immunoprecipitated with a mixture of the monoclonal antibodies pAb419 and pAb413 which recognize amino- and carboxyl-terminal determinants, respectively, on large T (15). The immunoprecipitates were separated on 2% agarose gels. Tracks M: 0.5 ng of *Bst*NI-digested pSV328 DNA. The sizes of the different fragments are given in base pairs. E7, extract of SVA31E7 cells; WT, extract of wild-type SV40-transformed rat cells. The names of the different mutants are given above the appropriate tracks. 70Ka, 70K cell extract immunoprecipitated with pAb413; 70Kb, 70K cell extract immunoprecipitated with pAb419; Rat-1, extract of untransformed Rat-1 cells. The tracks shown on the bottom right were analyzed in a separate gel which was electrophoresed for a longer time, to achieve better separation of the marker fragments.

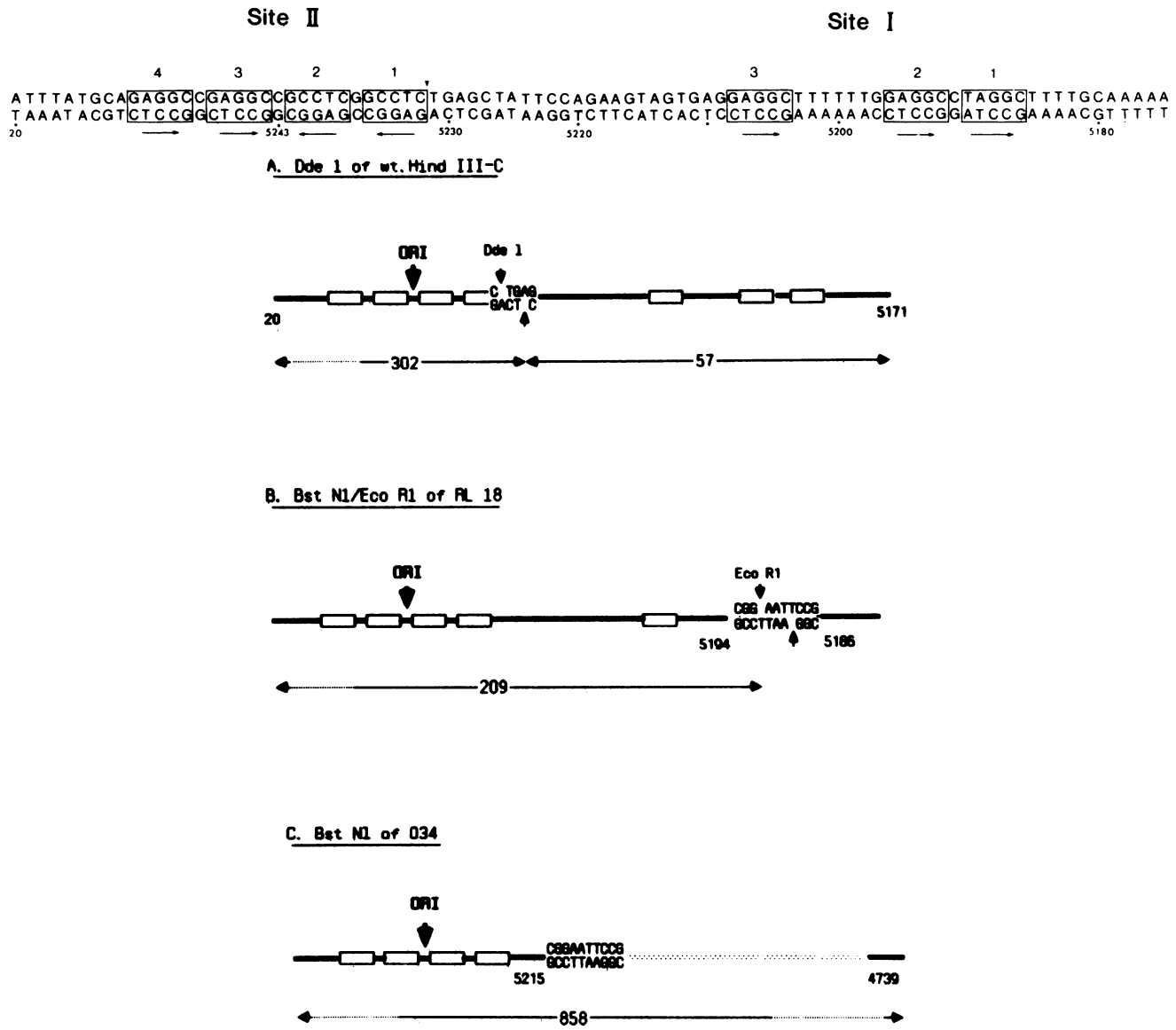


FIG. 3. (Top) Sequence of SV40 DNA about the origin of replication. The nucleotides are numbered according to Tooze (59). The consensus pentanucleotide recognition sequence which De Lucia et al. (8) have shown to be protected from methylation by the binding of SV40 large T are boxed and numbered as described by those authors. Arrows beneath the boxes indicate the 5′ → 3′ polarity of the pentanucleotides. (Bottom) Structure of the origin-containing DNA fragments produced by digestion of wild-type and mutant SV40 DNAs with different enzymes. The sequence between nucleotides 20 and 5171, shown in the top panel, is presented here in a diagrammatic form with the boxes representing the recognition pentanucleotide. The position of the origin of replication is indicated. (A) Fragments produced by digestion of the purified wild-type *Hin*dIII C fragment with *Dde*I. (B) Fragments produced by digestion of RL18 DNA with both *Bst*NI and *Eco*RI. RL18 has an *Eco*RI linker (CGGAATTCCG) inserted between positions 5194 and 5186 of wild-type SV40. (C) Fragments produced by digestion of O34 with *Bst*NI. Mutant O34 has the SV40 sequences between positions 5215 and 4739 deleted and replaced with the *Eco*RI linker.

between amino acids 83 and 250 was summarized in the Introduction. The data presented here allow a more precise map to be deduced. These data, together with relevant information from other laboratories, are summarized in Fig. 7. The most amino-terminal of the point mutations we examined that is negative in the DNA binding assay is Pro-139 → Leu (W75). Deletion of the surrounding region also produces SV40 origin DNA-binding-negative large T (L27-S26). Mutations at residues 147 (Ser → Asn; D119), 149 (Ala → Val; U24), and 152 plus 154 (Ser → Asn, Arg → Lys; U19) also abolish origin DNA binding as do a number of multiple point and deletion mutations in the same region (D111, W134, RL23, D89, D98; listed in Table 1). Several of the point mutations in this region are reduced in their binding activity relative to wild type (W67, D120, B11, U20, D88, D90, D94). It is striking that mutation of Ala-149 to Thr (D120) retains some binding activity, whereas mutation to Val (U24) does not. Even more noticeably, conversion to the adjacent residue Val-150 → Met (B9) generates a virus that is able to replicate and has a wild-type SV40 origin DNA-binding phenotype.

The close clustering of mutations with a common defect

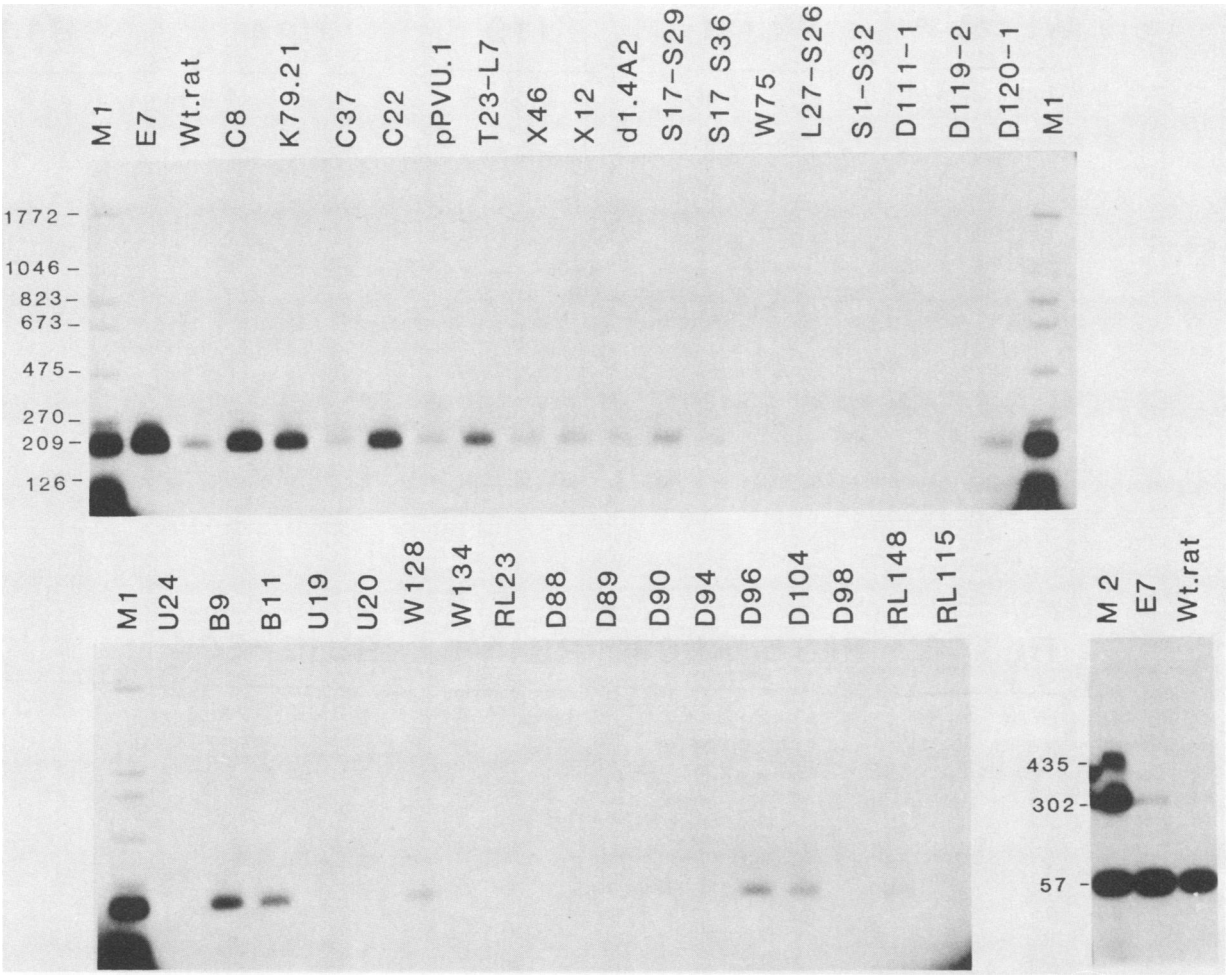


FIG. 4. Binding of large T to SV40 origin DNA containing a deleted site I. (Upper panel and lower left panel) Samples of different cell extracts containing equivalent amounts of ^{35}S-labeled large T were incubated with 10 ng of RL18 DNA which had been digested with *Eco*RI and *Bst* NI and then end labeled with ^{32}P. Large T bound to DNA was immunoprecipitated and analyzed as described in the text. Tracks M1, 0.5 ng of *Eco*RI-*Bst*NI-digested RL18 DNA. The sizes of the different fragments are given in base pairs. (Lower right panel) Extracts were incubated with 2.5 ng of purified *Hin*dIII-C which had been digested with *Dde*I before labeling.

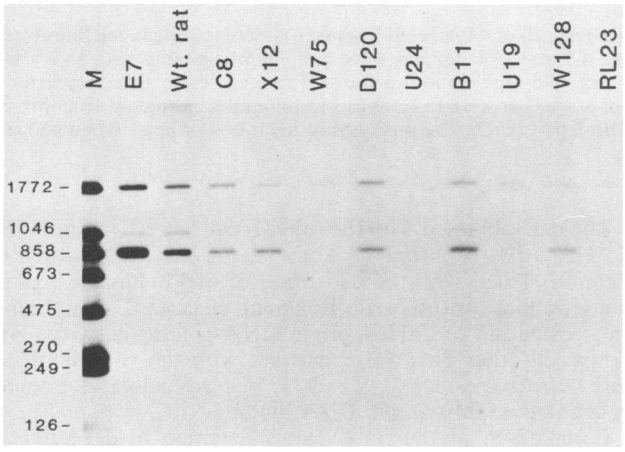


FIG. 5. Binding of large T to SV40 origin DNA lacking site I. Samples of the different cell extracts were incubated with 10 ng of *Bst*NI-cut, end-labeled O34 DNA. Track M, 0.5 ng of starting DNA. The sizes of the different fragments are given in base pairs.

strongly suggests that they lie within a functional domain. This is made even more likely by findings from two separate studies. By recovering the integrated SV40 DNA from transformed permissive monkey cells and sequencing the early regions, the defects that prevent the expected productive infection of these cells by the virus have been identified (12). In the case of the C6-2 mutation, the defect appears to be in SV40 origin binding (43), and the lesion maps to residue 153. Similarly, the SV40 insert coding for the large T in human SV80 cells has been isolated and found to be replication defective. Again, the protein appears to be reduced in its SV40 origin DNA-binding activity (14). The lesion in this case maps to amino acid 147 (W. Gish and M. Botchan, personal communication). Using a totally different approach, Nathans and colleagues (50) have isolated pseudorevertant (sr) forms of SV40 large T that are able to replicate SV40 virus containing a defective origin of replication. Presumably, the pseudorevertant large T species are altered in those amino acid residues intimately involved in the sequence-specific interaction with origin DNA. sr-3 maps to amino acid 157, and sr-2 maps to amino acid 166 (30). We believe, therefore, that the amino-terminal end of the origin DNA-binding domain on large T can now be

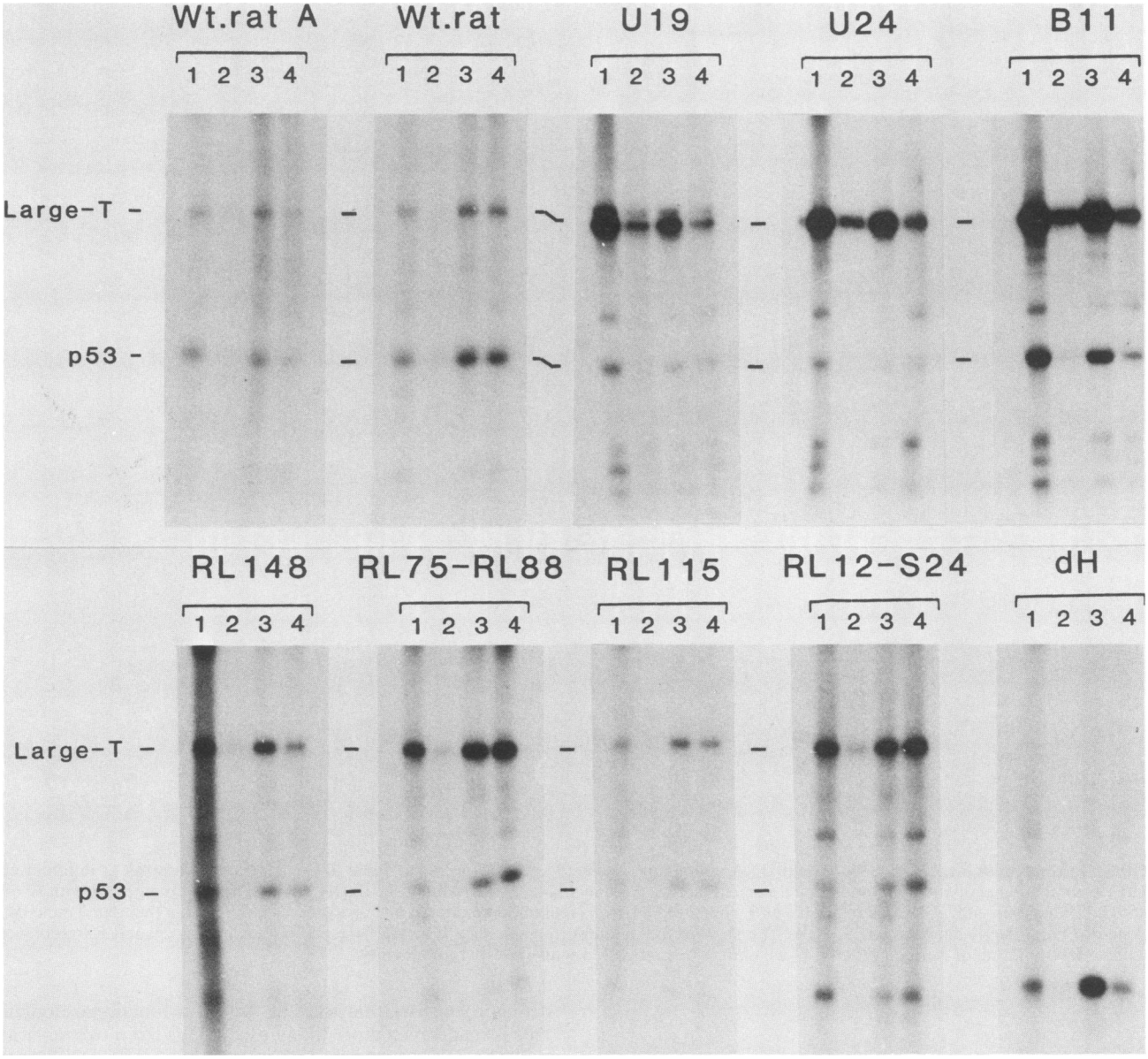


FIG. 6. Binding of mutant large T to calf thymus DNA-cellulose. Samples (500 μl) of ^{32}P-labeled lysates of different mutant cell lines were mixed with calf thymus DNA-cellulose, washed, and eluted as described in the text. All samples were immunoprecipitated with anti-SV40 T serum before analysis on SDS-containing 15% acrylamide gels. Extracts of wild-type SV40-transformed rat cells were also analyzed on underivatized cellulose as a control (WT rat A). Lanes: 1, 20-μl sample of starting material; 2, 20 μl of unbound supernatant fraction; 3, material eluted at pH 8.5 with 0.15 M NaCl; 4, material eluted at pH 8.5 with 1.0 M NaCl. The positions of migration of large T and p53 are indicated.

localized to a region beginning at Pro-139 with a particularly important sequence between amino acids 147 and 166.

We do not know how far the putative domain extends to the carboxy-terminal side because we have not made many mutants mapping to this region. However, the mutant RL115 which affects residues 220 to 223 is origin-binding defective, and two other SV40 point mutants recovered from transformed permissive or nonpermissive cells that map to residue 203 (T22) (29) and 214 (SVR 9D) (54) also lack origin DNA-binding activity. Since domains mapped on procaryotic DNA-binding proteins are usually of the order of 70 to 90 residues (37), these mutations may lie at the extreme carboxyl end of the same domain.

The data obtained with the other SV40 large T variants (70 K, H23, dH) reported here are also consistent with this location. Thus, with the exception of one published report claiming that a proteolytic fragment of large T comprising only residues 1 to 130 has origin DNA-binding activity (34), almost all other data are consistent with the site identified here between residues 139 and 223 being a major component in defining origin-specific DNA binding.

Comparison of the SV40 large T amino acid sequence between residues 135 and 225 with the region between amino acids 289 and 379 of large T of the related papovavirus, polyomavirus, is shown in Fig. 7. The underlined amino acids are conserved between the two viruses. It is striking

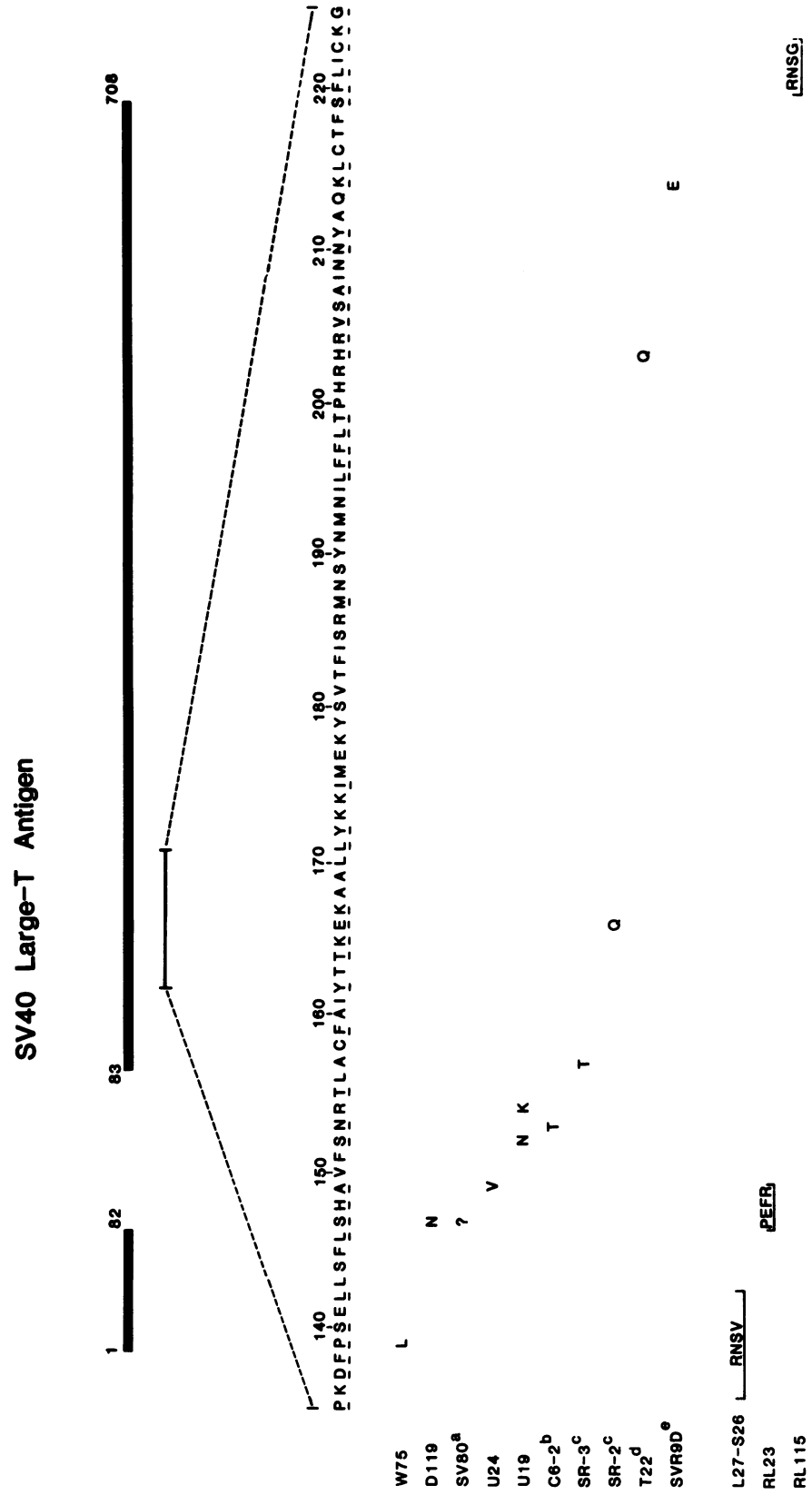


FIG. 7. Location of mutants which fail to bind SV40 origin DNA. The predicted amino acid sequence of SV40 large T between residues 135 and 225 is given in single letter code. The bar above indicates the approximate location of that sequence within the large T polypeptide. Those residues underlined are conserved in polyomavirus large T between amino acids 289 and 379. The single-letter amino acid code is: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; Y, tryosine; V, valine. The data summarized in this figure are taken from this publication and the following references: a, W. Gish and M. Botchan, personal communication; b, 12; c, 30; d, 29; e, 54; f, C. Cole, personal communication.

that all the mutants defective in origin binding shown in Fig. 7 map to residues shared with polyomavirus large T. The high degree of conservation of amino acids suggests that the region is essential to each virus and has a similar function. It has recently been shown (40) that polyomavirus large T can bind to the same pentanucleotide recognized by SV40 large T.

DNA-binding domains in procaryotic proteins have been characterized in some detail (37). For example, they comprise 66, 73, and 92 residues, respectively, for the Cro, CAP, and λ repressor proteins. Although the domain described here could be of approximately this size our preliminary searches have failed to find sequence homology between the SV40 amino acid sequences and those of the 20-residue helix-bend-helix motif characteristic of the procaryotic proteins.

Site I, site II, and dsDNA binding domains. The data reported here do not allow us to distinguish whether the domain has different specificities for binding sites I and II in the SV40 origin. As far as it is possible to tell, the site II binding can be detected in such an immunoprecipitation assay by using DNA which lacks site I sequences appears to parallel the site I binding results, suggesting that the domain has activity for both sites. Furthermore, the pseudorevertant large T antigens are presumably effective in origin, i.e., site II, binding since they are capable of initiating viral DNA replication and yet they map to the site I domain defined here. Thus, it seems likely that the region between residues 139 and 223 recognizes both sites I and II in the SV40 origin region, but this conclusion must be tested more rigorously before being regarded as established.

It remains to be seen whether other regions on large T form another DNA-binding domain. One report suggests that a second domain with a specificity for non-SV40 DNA lies between residues 245 and 325 (42). The results reported here are consistent with that hypothesis since mutations within the SV40 origin binding region, including some large deletions, do not seem to affect the ability of the mutant proteins to bind to ds cellular DNA. These results must be interpreted with some caution, however, because of the severe limitations of the DNA-cellulose binding assay itself. In our hands, large T binds efficiently to underivatized cellulose (compare Fig. 6, panel WT rat A, tracks 1 and 2) although apparently with low affinity as the bulk of the protein can be eluted under low-salt conditions (Fig. 6, WT rat A, track 3). Furthermore, we can only recover 10 to 20% of the ^{32}P-labeled protein bound to calf thymus DNA-cellulose in the elution steps. Any conclusions reached on DNA binding may, therefore, apply to only a small fraction of the entire protein population. The existence of a separate domain capable of binding independently to cellular DNA thus remains an open question.

SV40 origin binding and replication. Very few (5 of 50) of the mutants described in this study are capable of viral DNA replication. All others are severely defective or deficient in that function. Those mutants which can replicate (K79, C37, B9, and D96/D104) bind efficiently to SV40 origin DNA as expected. The only exception to this rule is mutant B18, which can replicate but shows weak DNA binding in these assays. B18 transforms cells very poorly, so the possibility exists that the large T found in the B18-transformed line used here was modified in some way, perhaps by a second site mutation or by gene rearrangement. It is easy to rationalize the inability to replicate of those mutants whose origin binding activity is severely impaired. Similarly, the replication defect in those mutants around Lys-128 which produce a cytoplasmic large T could perhaps be explained by an inability to accumulate functional large T in the nucleus. This idea is supported by the apparent failure of these mutants to autoregulate the levels of large T synthesized. It is more difficult to understand why the remaining mutants fail to replicate, even though they appear to bind well to SV40 origin DNA, site II as well as site I, as judged by the immunoprecipitation assays reported here. Perhaps virtually any mutation in this region of large T, even a point mutation 40 to 50 residues removed from the DNA-binding domain, disrupts the structure sufficiently to affect the intimate association reported to exist between the protein and SV40 site II DNA (6). Such mutations might prevent replication but still allow an interaction measureable by the McKay assay.

SV40 origin binding and transformation. All of the mutants described here were able to transform Rat-1 cells regardless of their ability to bind to SV40 origin DNA. In fact, some mutants which cannot bind DNA produced foci more rapidly and in greater numbers than wild-type virus. Kalderon and Smith (19) suggested that this supertransforming phenotype might be caused by an overproduction of large T since all mutants showing this property displayed brighter than normal large T fluorescence. Although not consistently reflected in the amount of large T immunoprecipitated from individual cell lines, a modest overproduction of most mutants displaying the supertransforming phenotype (B11, U19, U20, U24, W128, RL23, D88, D89) is seen here. More significantly, all of the supertransforming mutants show a discernible defect in SV40 origin DNA binding, with the mutants U19 and U24 displaying the most extreme phenotype in both respects. Furthermore, mutants mapping to the same region (B9, D96, D104) that are able to bind to SV40 origin DNA and to replicate do not discernibly overproduce or transform with an efficiency greater than wild type. Thus, the hypothesis that the observed supertransforming activity of some of the mutants can be explained in terms of an overproduction of protein is consistent with the deficiencies observed in this study in binding to site I, the site of transcriptional control by large T.

The work described here supports the conclusion that large T does not need to be able to interact with SV40 origin DNA to transform established Rat-1 cells (29, 43, 54). However, any suggestion that transformation by large T does not involve an interaction with cellular DNA must be tempered by the finding that all of the mutants retain the ability to bind to cellular ds DNA-cellulose. Scheller et al. (49) have already described a similar phenotype for the C6-2 mutant. If transformation of established cell lines by SV40 does not involve binding to DNA, it might imply that this aspect of transformation is a cytoplasmic event catalyzed by the membrane-associated forms of large T detected by a number of workers (7, 20, 21, 47 [and references therein]; 53). This would be consistent with the emerging view that transformation, as opposed to immortalization, is catalyzed by cytoplasmic events (22, 23, 36, 45). It would also explain why several of our mutant large T species that appear unable to localize to the nucleus of cells still retain the ability to transform established cells (17).

Domain structure of large T. From previous studies on the biological properties of the many mutants we have constructed and from the data reported in this paper, it has become apparent that the linear amino acid sequence of large T can be divided into discrete blocks each of which is associated with a distinct function or property.

All of the phosphorylation sites in the amino-terminal half

of large T are clustered between amino acids 106 and 124: Ser-106, Ser-111, Ser-112, Ser-123, and Thr-124 (48). Our studies have shown that mutants carrying alterations in amino acids 106 to 114 generally produced foci on Rat-1 cells more slowly and in reduced numbers than did wild-type large T (19). These mutants, therefore, define a domain which must play a role in the transformation process. We do not yet know whether mutations within the putative transformation domain alter the phosphorylation patterns of large T or whether some other function is responsible for their reduced transforming ability. The next domain includes the sequence 125-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-132 which we showed constitutes a nuclear location signal. Mutations within this region result in large Ts which are wholly or partially located in the cytoplasm (17, 18, 25). These mutants retain the ability to transform Rat-1 cells to continuous growth. Finally, in this publication, we defined a DNA-binding region which begins at Pro-139 and which may extend as far as Cys-223. Whether each of these functional domains corresponds to a structural domain remains to be determined. Nevertheless, the existence of mutations within each of the different functional domains facilitates the systematic analysis of large T functions and the role they play in its biological activities, particularly in transformation.

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