

Integration, Loss, and Reacquisition of Defective Viral DNA in SV40-Transformed Mouse Cell Lines

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We have examined the state of viral DNA in a set of SV40-transformed mouse cell lines. Using restriction enzymes which cut SV40 DNA in one place, we demonstrate that anchorage-independent SV40-transformed mouse cells commonly contain one or more detectable defective monomers of integrated viral DNA. The defective viral DNA in one of these cell lines, SV101, was extensively mapped using single and double enzyme digests. The results of this analysis indicate that SV101 contains nondefective viral DNA as well as defective viral DNA of the following sizes: 5.0, 4.3, 3.7, 3.4, and 1.5 kb. Three of these defective monomers (4.3, 3.7, and 1.5 kb) preserve the amino terminal exon of large T antigen, and two monomers (4.3, and 3.7 kb) preserve the little t coding region. Anchorage-dependent subclones of SV101 preferentially lose the defective viral DNA, while retaining an intact SV40 early region and the ability to express lytic-sized large and small T antigens. Despite a considerable amount of viral DNA rearrangement which accompanies subcloning, anchorage-independent subclones of SV101 retain defective viral DNA, especially the 4.3- and 3.7-kb monomers. Also, when an anchorage-independent subclone is selected from an anchorage-dependent revertant of SV101, it reacquires defective viral DNA, although of a size not seen in SV101. We conclude that defective viral DNA plays a role in generating the anchorage-independent phenotype. In earlier studies, we have reported that anchorage-transformed mouse lines contain a variant (100kDa) T antigen. The possible role of defective viral DNA in generating this T antigen is discussed.

INTRODUCTION

SV40 is a small DNA tumor virus which is capable of transforming the growth behavior of mouse, rat, and hamster cells in culture. SV40-transformed cells have a more rapid doubling time, require less serum for proliferation, and have a much higher plating efficiency in semisolid media than do their untransformed precursors. Such a transformation requires the integration of viral DNA and the expression of viral tumor antigens (Topp *et al.*, 1980). It has been possible to select from SV40-transformed cell lines revertant subclones which no longer display any or part of the SV40-transformed phenotype (Pollack *et al.*, 1968; Vogel and Pollack, 1973; Steinberg *et al.*, 1978; Gurney and Gurney, 1979). Steinberg *et al.* selected revertants from the SV40-transformed rat

cell line, 14B, which contains one copy of the SV40 early region. Several of these revertants lost all or part of the integrated viral DNA, and therefore no longer express viral T antigens. Others retained the viral DNA, but nevertheless no longer express viral T antigens. These results provided an explanation for the mechanism of reversion in the case of the rat cell line, 14B: loss of viral gene expression (Steinberg *et al.*, 1978).

More confounding are a set of revertants isolated by Pollack and co-workers from an SV40-transformed mouse cell line, SV101 (Pollack *et al.*, 1968; Vogel and Pollack, 1973; Todaro *et al.*, 1964). These revertants differ from the rat revertants in two fundamental ways. First, they were isolated from a cell line which contains several copies of the SV40 early region. Second, all of the revertants still express both large and small T antigens (Chen *et*

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al., 1981). These revertants are the subject of the present study. For the purpose of this report, we have segregated the mouse cell lines into two phenotypic categories: anchorage dependent and anchorage independent. Anchorage independence is the *in vitro* cell growth property which correlates best with tumorigenicity (Shin *et al.*, 1975). We have recently shown that a nonlytic viral protein of 100kDa is directly correlated with anchorage independence in SV40-transformed mouse cell lines (Chen *et al.*, 1981). This result has obliged us to undertake an analysis of the integrated viral DNA present in SV101 and its subclones to determine if certain features of the integrated DNA are specific to anchorage independence.

It is well established that SV40 DNA integrated into mouse cells is unstable (Hiscott *et al.*, 1980; Sager *et al.*, 1981b; Bender and Brockman, 1981). Alterations in the arrangement of SV40 DNA have been observed after integration in clonally related mouse cell lines. New viral DNA insertions appeared when subclones of a tsA SV40-transformed cell line were selected by their ability to grow without anchorage at the restrictive temperature (Hiscott *et al.*, 1980). Also, Sager and colleagues have shown that large rearrangements of integrated viral DNA occur upon nonselective subcloning of SVT2, another SV40-transformed mouse cell line (Sager *et al.*, 1981b). We have been able to take advantage of this instability to identify integrated viral DNA which is specifically associated with anchorage independence above a background of nonessential SV40 DNA. We report that defective viral DNA is routinely present in mouse cells fully transformed by SV40, is largely lost in anchorage-dependent subclones of an SV40-transformed cell line, and is reacquired when anchorage-independent subclones are selected from an anchorage-dependent revertant of SV101.

MATERIALS AND METHODS

Cells. Cell culture techniques, and isolation and characterization of SV101 and its subclones have been described else-

where (Pollack *et al.*, 1968; Vogel and Pollack, 1973; Steinberg *et al.*, 1978). Mock revertants were isolated from recloned SV101 as colonies surviving negative selection in bromodeoxyuridine (BrdU) or fluorodeoxyuridine (FudR). All mock revertants grow rapidly in 1% serum with a plating efficiency of 10% and a doubling time of approximately 40 hr. The mock revertants also have the ability to grow efficiently in semisolid media with a colony forming efficiency of >3%. All other anchorage-independent cell lines also have similar plating efficiencies in semisolid media. Thawed vials of SV101 frozen in 1967 were used to initiate cultures for all subclonings. Thus, even though these lines were initially isolated over a period of more than a decade, all data are drawn from populations of approximately the same passage.

Blot hybridization and analysis. Cells on 12 150-mm plates were washed thrice with phosphate-buffered saline (PBS), scraped into centrifuge tubes, and pelleted at 250 *g* for 10 min. Cells were resuspended in 10 mM Tris, 140 mM NaCl, and 2 mM ethylenediaminetetraacetate (EDTA), pH 7.5. Sodium dodecyl sulphate (SDS) was added to the cell suspension to 0.5%. Proteinase K (Type XI proteinase: Sigma) was added to 50–100 μ g/ml. The solution was allowed to stand at 37° overnight. The DNA was then extracted once with 1 vol phenol (saturated with resuspension buffer):chloroform:isoamyl alcohol in the ratio 24:24:1, and once with 1 vol chloroform:isoamyl alcohol in the ratio 24:1. DNA was then precipitated with 300 mM NaCl and 2 vol of ethanol at 4°, spooled onto a pipette, and rehydrated in 1 mM Tris, 0.1 mM EDTA overnight at room temperature. Enzymes (New England Biolabs) were used according to vendor's instructions, except that digestions were carried out with a five-fold excess of enzyme. Digested DNA was electrophoresed through a 3.4-mm-thick, 14 × 15-cm vertical slab, 1.0 or 1.4% agarose gel. Running buffer was 40 mM Tris, pH 8.0, 10 mM sodium acetate, and 1 mM EDTA. Sample buffer was the same as running buffer except that sucrose and bromophenol blue tracking dye were added to

10% and 0.1% respectively. After electrophoresis, the gel was stained with 0.5 μ g ethidium bromide/ml in running buffer. Transfer of the digested DNA to nitrocellulose and hybridization of the [32 P]DNA to SV40 were carried out as described previously (Southern, 1975; Botchan *et al.*, 1976; Wahl *et al.*, 1979; Kelley *et al.*, 1970). Reconstruction lanes in Figs. 3, 4, 5, and 6 represent SV40 DNA at a concentration of 1-2 copies per diploid genome. Molecular weights were assigned to relevant fragments by plotting the size of marker DNA fragments obtained against distance migrated. Plotting was facilitated by a Fortran program. Size reproducibility from gel to gel in the size range of 3- to 6-kb SV40-containing bands from different preparations of restricted SV101 DNA was ± 0.05 kb (Schaffer and Sederoff, 1981).

RESULTS

Cell Lines

Figure 1 is a description of the origin of the cell lines used in this study. All anchorage-dependent cell lines have been placed on the left side of the figure and all anchorage-independent cell lines on the right side of the figure. The two sides are separated by the dashed line. Solid lines with arrowheads represent transformation of a cell line by SV40. Each arrowhead represents an independent SV40 transformation. Solid lines which do not have arrowheads connect subclones of SV101 and LS1. The assignment of anchorage dependence or anchorage independence is based on relative plating efficiencies in soft agarose and is detailed in the Materials and Methods section.

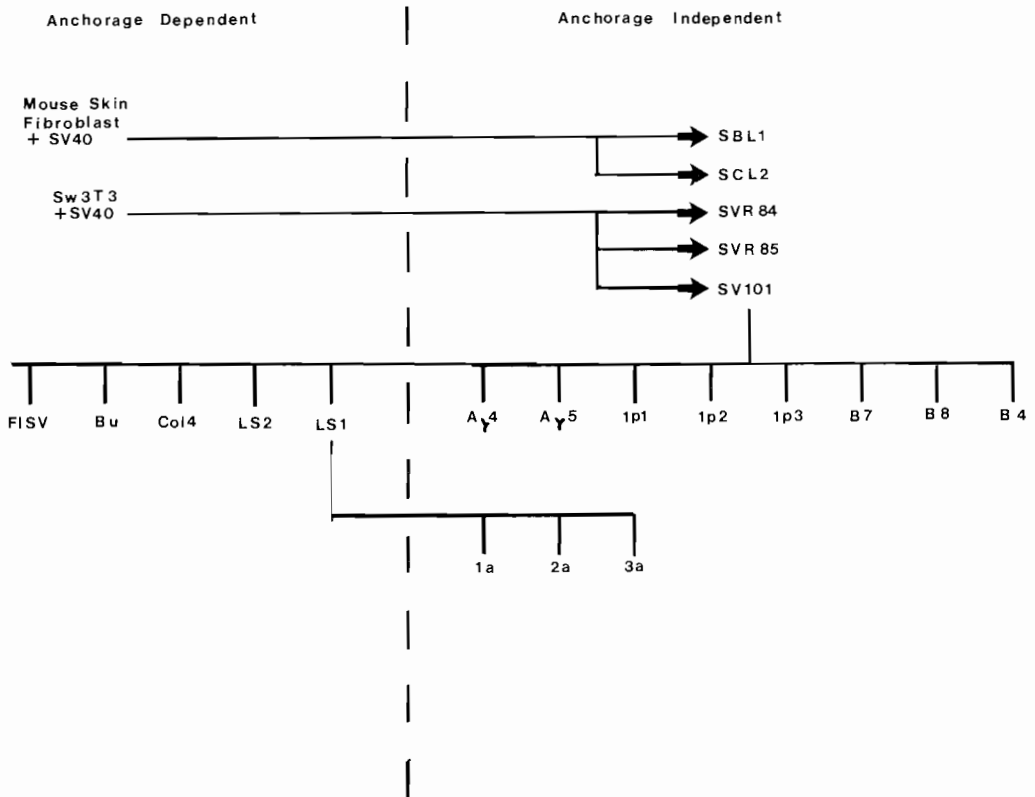
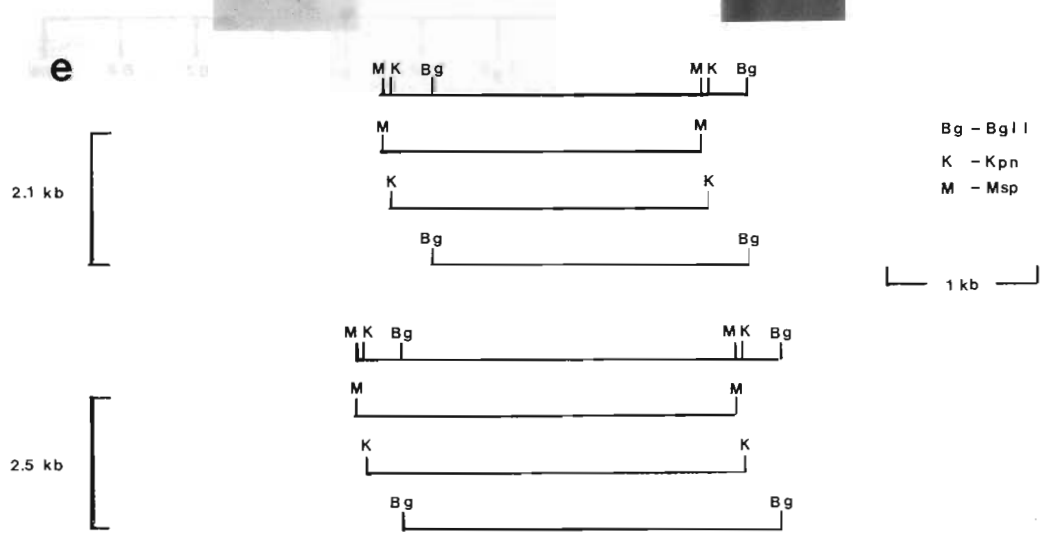
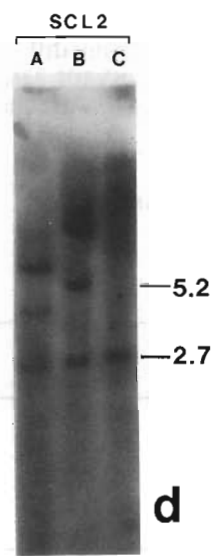
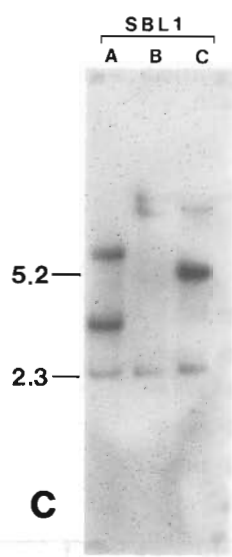
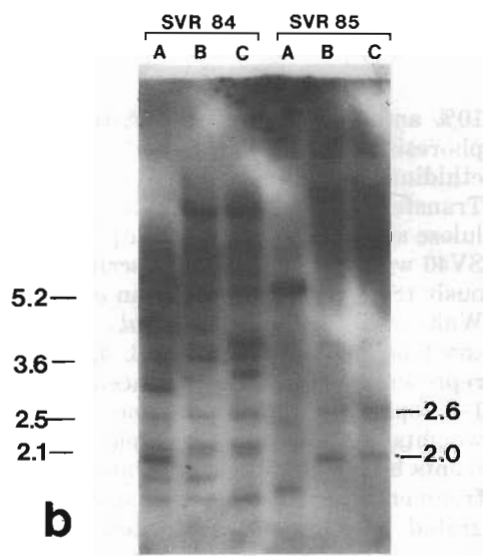
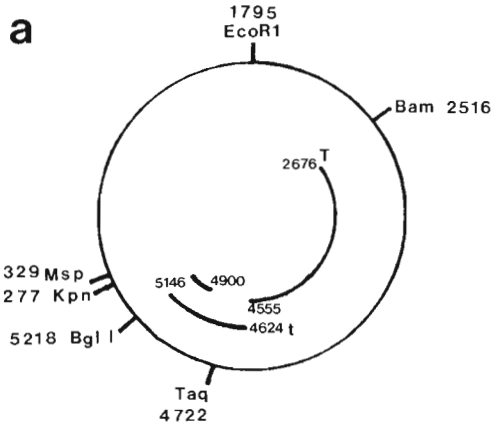


FIG. 1. Cell lines used in this report. The cell lines in this figure are described in the Results section of the text.



Defective Viral DNA in Independent Transformants

When SV40 DNA integrates into a mouse cell, it frequently creates a tandem array of viral DNA, probably because of a spurt of viral DNA replication preceding integration (Chia and Rigby, 1981). Such an array can be detected by digesting cellular DNA with a restriction enzyme which cuts SV40 DNA in one place. This will create fragments containing both host and SV40 DNA, and fragments containing only SV40 DNA. The fragments which contain only intact SV40 DNA can be identified by their size, namely 5.2 kb. The existence of a 5.2-kb stretch of viral DNA uninterrupted by host DNA can be confirmed by digesting the cellular DNA with several one-cut enzymes. If two or more one-cut enzymes generate a fragment containing viral DNA at 5.2 kb, the probability that the fragment is something other than uninterrupted viral DNA is rather small.

If the restriction enzyme sites surround a piece of viral DNA which is lacking some of the DNA normally present in a complete SV40 molecule, a digest of the cellular DNA will produce a fragment smaller than the normal linear SV40 DNA. If that DNA is uninterrupted by host DNA and is part of a tandem array of SV40 restriction enzyme sites, then several different enzymes, each cutting the viral DNA on both sides of the point of deletion, will produce a fragment of the same molecular weight. In the case of simple deletions, the difference between linear SV40 DNA, 5.2 kb, and the smaller-sized fragment will represent the size of the deletion occurring between the restriction enzymes sites which were cut to generate the defective monomer.

Figure 2a is a diagram indicating the

positions of the restriction enzymes sites on intact SV40 DNA that were used in this study. We have used three of these enzymes to search the integrated viral DNA of independent transformants for defective viral DNA: *Msp*, *Kpn*, and *Bgl*I. These three closely linked enzymes provide the greatest opportunity to detect defective viral DNA because such a detection depends on the presence of restriction enzyme sites repeated in tandem. If the stretch of SV40 DNA which must be repeated in order for a set of restriction enzyme sites to be repeated is relatively small, then the odds of detecting a tandem arrangement of DNA are relatively high. Restriction enzyme sites which are far apart run a greater risk of being separated by host DNA during the integration event or by some rearrangement of viral DNA which creates a deviation from colinearity with lytic SV40 DNA.

Figure 2b-d are Southern blot (Southern, 1975; Botchan *et al.*, 1976; Wahl *et al.*, 1979) analyses of four independent SV40 transformants. High molecular weight DNA from each of these transformants was extracted and digested with *Msp* (lane A), *Kpn* (lane B), and *Bgl*I (lane C). The digested DNA was electrophoresed through an agarose gel, transferred to nitrocellulose paper, and probed with SV40 [³²P]DNA (Kelly *et al.*, 1970). First, it can be seen that SVR 84 DNA contains in each digest a band which migrates with linear SV40 DNA at 5.2 kb, indicating that this cell line contains a stretch of viral DNA which is uninterrupted by host DNA or any deviation from colinearity with SV40 DNA within the limits of the resolution of the gel (about ±50 base pairs). It can also be seen that SVR 84 contains a fragment of SV40 DNA in each of the three digests at 2.5 and at 2.1 kb. This result strongly

FIG. 2. Blotting analysis of independent SV40 transformants. a is a map of SV40 DNA showing the position of the restriction enzyme sites used in this study. The replication origin is at the *Bgl*I site (Buchman *et al.*, 1980). b, c, and d are *Msp* (A), *Kpn* (B), and *Bgl*I (C) digests of DNA from SVR 84, SVR 85, SBL1, and SCL2 as indicated in the figure. All lanes were probed with SV40 [³²P]DNA. The numbers indicate fragment size in kilo-base pairs. e is a schematic of the structure proposed for defective viral DNA in SVR 84. Each stretch of defective SV40 DNA is drawn in full, and beneath each the fragments released by successive enzymes are shown.

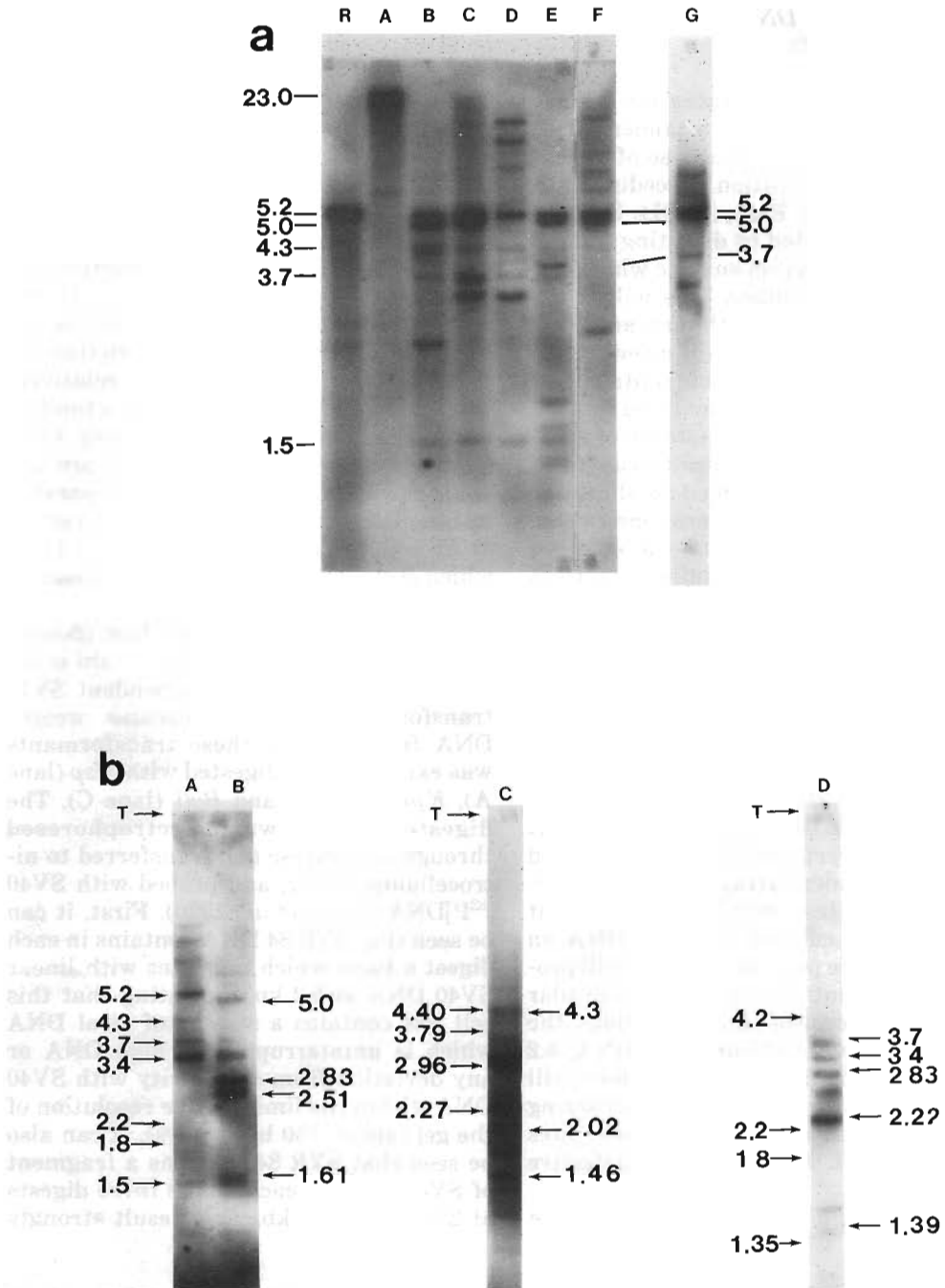


FIG. 3. Blotting analysis of SV101. In a, DNA from SV101 was extracted and digested with *Bgl*II (A), *Msp*I (B), *Kpn*I (C), *Bgl*I (D), *Taq*I (E), *Bam*HI (F), and *Eco*RI (G). Lane R is SV40 DNA digested with *Kpn*I. All lanes were probed with SV40^[32P]DNA. The numbers indicate fragment sizes in kilobase pairs. *Bgl*II does not cut SV40 DNA, and manifests a major band at 23.0 kb and a minor band at about 8 kb. Each of the other enzymes cut SV40 DNA in one place (Fig. 2). Certain fragments appear consistently in two or more one-cut digests. The 5.2-kb fragment appears in lanes B-G; the 4.3-kb fragment appears in lanes B-F, with varying intensities; the 3.7-kb fragment appears in

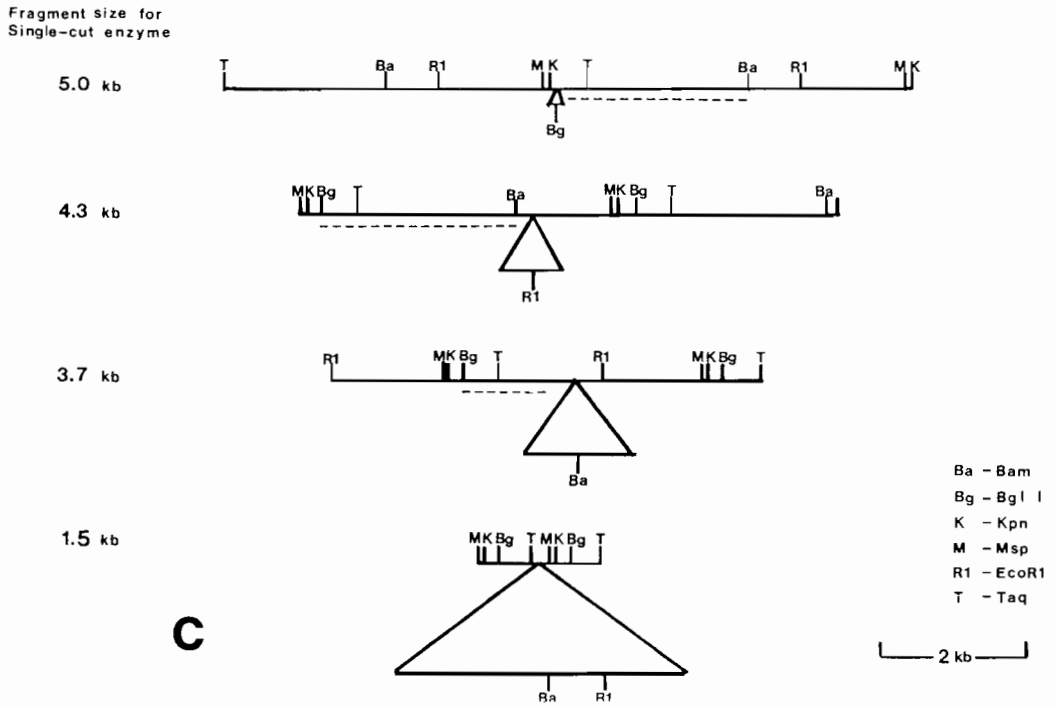


FIG. 3—Continued.

lanes A-E and G; the 3.4-kb fragment appears in lanes C and D; the 1.5-kb fragment in lanes B-E. In b SV101 DNA is digested with *Bgl*I (A), *Bgl*I plus *Bam* (B), *Taq* plus *Eco*RI (C) and *Kpn* plus *Bam* (D). The molecular weight assignments for lane B were based on a plot of the 4.3-, 3.7-, 2.2-, and 1.8-kb size standards shown in lane A, against distance migrated. The 5.0-kb fragment in lane B can be identified by a slightly faster mobility than the 5.2-kb fragment in lane A. For lanes C and D, the numbers on the left indicate the mobility of the size standard in kilo-base pairs used to assign molecular weights to the fragments in the lane. The fragments of interest are indicated by their molecular weights shown on the right of the lane. The 4.3-kb fragment in lane C is clearly visible in longer exposures of the lane. In c, the diagrams were produced by analyzing the data presented in this figure and Table 1. It should be noted that the ends of these structures may very well overlap in the integrated array of viral DNA of SV101. They are presented here to emphasize the position of the points of deletion with respect to neighboring SV40 restriction enzyme sites. The point of deletion has been arbitrarily placed midway between the restriction enzyme sites known to surround it. The dotted line represents early region coding sequences.

suggests a deletion of SV40 DNA occurring between one set of the three restriction enzyme sites and an adjacent set. A schematic representation of this proposal is shown in Fig. 2e. The size of the deletions are 2.7 kb in the case of the fragment produced at 2.5 kb, and 3.1 kb in the case of the fragment produced at 2.1 kb. A similar result is obtained with SBL1 and SCL2. Digestion of SBL1 DNA with *Msp*, *Kpn*, and *Bgl*I demonstrates a defective monomer at 2.3 kb and digestion of SCL2 DNA with the same enzymes demonstrates a defective monomer at 2.7 kb. The deleted viral

DNA in these two monomers as well as that deleted from the 2.5- and 2.1-kb monomers in SVR 84 must not include adjacent *Msp*, *Kpn*, or *Bgl*I sites, since each of these enzymes are capable of freeing the defective monomers from the integrated viral DNA.

Examination of digests of DNA from SVR 85 with the same enzymes shows a common band in only two of the three lanes. These are at 2.6 and 2.0 kb. It is likely that this result also indicates a defective monomer present in SVR 85. However, in this case, the deletions of 2.6 and

3.2 kb probably include an *Msp* site, because neither the 2.6- or the 2.0-kb fragment appear in the *Msp* digest. A similar interpretation can be made for the fragment at 3.6 kb in SVR 84 because it is also absent from the *Msp* digest.

The results from Fig. 2 suggest that anchorage-transformed mouse cell lines typically carry multiple deletions of SV40 DNA. One such line, SV101, is the parent and grandparent of a set of anchorage-dependent revertants (Fig. 1). SV101 also carries deletions of SV40 DNA (Fig. 3).

Defective Viral DNA in SV101

SV101 grows in low serum, to a high saturation density and without anchorage. It expresses both the 94- and 17-kDa tumor antigens as well as a 100-kDa variant of the 94-kDa protein which has been correlated with the ability to grow without anchorage (Chen *et al.*, 1981). Fig. 3a, lane A is a Southern blot of SV101 DNA digested with *Bgl*II, an enzyme which does not cut SV40 DNA. The DNA was probed with SV40^[32P]DNA. There is one major band in the high molecular weight fraction of the gel, indicating that SV40 DNA has integrated into the mouse cell DNA. Since the resolving power in the high molecular weight range is poor, it is impossible to tell whether the band in the *Bgl*II digest represents overlapping fragments.

In order to determine whether SV101 contained defective viral DNA, we digested its DNA (Fig. 3a) with *Msp* (lane B), *Kpn* (lane C), *Bgl*II (lane D), *Taq* (lane E), *Bam* (lane F), and *Eco*RI (lane G). Each of these enzymes cuts SV40 DNA in one place (Fig. 2). Each of these digests reveals a fragment at 5.2 kb, which represents intact SV40 DNA stretching between enzyme sites repeated in tandem. Below the 5.2-kb fragment, there are a series of bands which appear in two or more enzyme digests: 5.0, 4.3, 3.7, 3.4, and 1.5 kb. As discussed above, these are likely to represent stretches of SV40 DNA which have undergone a deletion between enzyme sites in tandem. In order to verify this and to localize the deletions, we have undertaken a series of double enzyme digests. The re-

sults of three of these digests are shown in Fig. 3b, and a total of six are summarized in Table 1.

Just below the 5.2-kb fragment in each of the single-cut digests (Fig. 3a) except *Bgl*II (lane D) is a fragment at 5.0 kb. This molecular has apparently suffered a deletion of about 200 base pairs which includes the *Bgl*II site. If this were so, doubly digesting DNA from SV101 with *Bam*, which frees the 5.0-kb monomer, plus *Bgl*II, should leave the 5.0-kb monomer intact. Figure 3b, lanes A and B demonstrate that this prediction is born out. Alternatively, digesting SV101 DNA with *Taq* plus *Eco*RI should free a 2.07-kb piece from the 5.0-kb monomer. 2.07 kb is 200 base pairs less than the nondefective SV40 DNA which normally lies between the *Taq* and *Eco*RI sites, and includes the *Bgl*II site. Likewise this prediction is born out, within the resolution of the gel (Fig. 3b, lane C). Several other double digests are consistent with a 200 base pair deletion in the region of the *Bgl*II site. *Kpn* plus *Eco*RI yields a 3.54-kb fragment (Table 1); *Bam* plus *Msp*, a 2.74-kb fragment (Table 1); and *Kpn* plus *Bam*, a 2.83-kb fragment (Table 1 and Fig. 3b, lane D). Each of these is about 200 base pairs smaller than the normal (*Bgl*II site containing) SV40 DNA fragment produced by these enzyme pairs.

Below the 5.0-kb fragment in some of the single enzyme digests of SV101 DNA is a fragment at 4.3 kb, which appears (with varying intensities) in each single-cut digest except for *Eco*RI (Fig. 3a, lane G). This monomer is likely to have suffered a deletion of SV40 DNA which includes the *Eco*RI site. This proposal is supported by the appearance of a 1.61-kb fragment in the *Bgl*II plus *Bam* digest (Fig. 3b, lane B). The normal size of the SV40 DNA fragment liberated by these two enzymes and including the *Eco*RI site is 2.51 kb. The 1.61-kb fragment accounts for about 900 base pairs deleted from a normal 5.2-kb stretch of SV40 DNA to create the 4.3-kb monomer. Other double digests listed in Table 1 support this interpretation of the 4.3-kb fragment as well.

Similar analyses have been carried out for the 3.7-, 3.4-, and 1.5-kb defective

TABLE 1
SV40 FRAGMENTS OBSERVED IN DOUBLE RESTRICTION ENZYME DIGESTS OF SV101

Enzymes used	5.2		5.0 (-0.2)		4.3 (-0.9)		3.7 (-1.5)		3.4 (-1.8)		1.5 (-3.7)	
	e	o	e	o	e	o	e	o	e	o	e	o
<i>Bgl</i> I plus <i>Bam</i>	2.70	2.83	<u>5.0</u>	<u>5.0</u>	NC	—	<u>3.7</u>	<u>3.7</u>	<u>3.4</u>	<u>3.4</u>	<u>1.5</u>	<u>1.5</u>
	2.52	2.51	NP	—	1.62	1.61	NP	—	NP	—	NP	—
<i>Taq</i> plus <i>Eco</i> RI	2.96	2.95	NC	—	<u>4.3</u>	<u>4.3</u>	1.46	A	NP	—	<u>1.5</u>	A
	2.27	2.30	2.07	2.02	NP	—	NC	—	NP	—	NP	—
<i>Kpn</i> plus <i>Bam</i>	2.99	UA	2.79	2.83	NC	—	<u>3.7</u>	<u>3.7</u>	<u>3.4</u>	<u>3.4</u>	<u>1.5</u>	<u>1.5</u>
	2.24	2.22	NC	—	1.34	1.39	NP	—	NP	—	NP	—
<i>Bam</i> plus <i>Msp</i>	3.04	3.04	2.84	2.74	2.14	UA	<u>3.7</u>	<u>3.7</u>	NP	—	<u>1.5</u>	<u>1.5</u>
	2.19	2.18	NC	—	NC	—	NP	—	NP	—	NP	—
<i>Kpn</i> plus <i>Eco</i> RI	3.79	3.74	3.59	3.54	<u>4.3</u>	<u>4.3</u>	2.29	2.07	3.4	UA	<u>1.5</u>	A
	1.44	A	NC	—	NP	—	NC	—	NP	—	NP	—
<i>Kpn</i> plus <i>Taq</i>	4.40	4.47	NC	—	3.50	3.54	2.90	2.92	<u>3.4</u>	UA	0.7	A
	0.83	A	0.63	A	NC	—	NC	—	NP	—	NC	—

Note. e, expected fragment size for restriction enzyme digestions indicated; o, observed fragment size; NP, no prediction of fragment size possible; NC, no change from normal size SV40 DNA fragment expected; A, ambiguity created by two or more fragments with unresolvable electrophoretic mobilities. The underscore indicates defective monomers expected to be insensitive to one member of the enzyme pair indicated. UA = unexplainably absent.

monomers. Schematic diagrams representing these results are shown in Fig. 3c. A representation of the 3.4-kb fragment has not been attempted because it is likely to represent a stretch of viral DNA which has suffered deletions in two or more places. This fragment is insensitive to both *Taq* and *Msp*, which are separated from one another by about 4.4 kb in a normal SV40 DNA molecule. However, only 1.8 kb of DNA is missing from the 3.4-kb fragment. Hence, it is a likely possibility that the 3.4-kb defective monomer is more complex than the other four discussed above. An examination of Fig. 3c reveals that only one of these stretches of SV40 DNA, the one which yields a 4.3-kb monomer, could contain an intact early region at the level of resolution provided by this analysis. The other four stretches of DNA cannot contain an intact SV40 early region. Only one other stretch, that yielding the 3.7-kb monomer should contain an intact little t coding region. And finally, one additional stretch, that yielding a 1.5-kb monomer,

should contain an intact large T, amino-terminal exon. These interpretations are summarized in Table 2.

Viral DNA in Revertants

Figure 1 shows that seven revertants have been isolated from SV101. The revertants were isolated by a negative selection protocol devised by Pollack and co-

TABLE 2
CODING REGIONS PRESENT IN DEFECTIVE
VIRAL DNA IN SV101

Defective viral DNA	Large T amino-terminal exon	Little t coding region	Complete early coding region
5.0 kb	Unknown	Unknown	Unknown
4.3 kb	+	+	+
3.7 kb	+	+	—
3.4 kb	Unknown	—	—
1.5 kb	+	Unknown	—

Note. The coding regions present in the various regions of defective viral DNA were determined by data presented in Fig. 3 and Table 1.

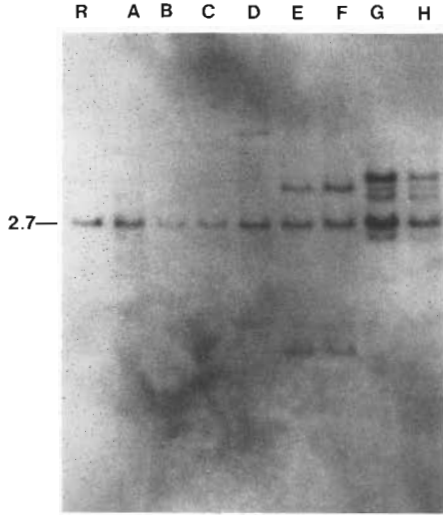


FIG. 4. *BglI*-*Bam* digest of revertants' DNA. DNA from SV101 (A), F1SV (B), Bu (C), Col4 (D), LS1 (E), LS2 (F), A γ 4 (G), and A γ 5 (H) was digested sequentially with *BglI* plus *Bam*. Lane R is SV40 DNA similarly treated. All lanes were probed with [³²P]pSVBgl-*Bam*, a plasmid containing the fragment of SV40 DNA between the *BglI* site and the *Bam* site, and including the early transcription unit. Fragments not seen in lane A, but present in Fig. 3b, lane B, are absent from lane A because not all the fragments in Fig. 3b, lane B anneal to early region sequences and because lane A is a shorter exposure. The 2.83-kb fragment in Fig. 3b, lane B is equivalent of the 2.7-kb fragment in lane A above (Table 1).

workers (Vogel and Pollack, 1973; Pollack *et al.*, 1968). F1SV, Bu, Col4, LS1, and LS2 have reverted to the anchorage-dependent phenotype. A γ 4 and A γ 5 have retained the anchorage-independent phenotype, but have reverted to a high serum dependence. Also shown in Fig. 1 are a group of six "mock revertants," which have been so termed because they have been passaged through the revertant enrichment protocol, but have retained the entire transformed phenotype, including anchorage independence (Chen *et al.*, 1981). The mock revertants 1p1, 1p2, 1p3, B4, B7, and B8, and the anchorage-independent serum revertants A γ 4 and A γ 5 serve as control groups to the anchorage-dependent revertants.

Each of the subclones of SV101, including the anchorage-dependent revertants,

synthesize the 94- and 17-kDa SV40 tumor antigens (Chen *et al.*, 1981; Chen, unpublished results). Therefore, each of the anchorage-dependent revertants should contain an intact SV40 early region, which is supported by the digestion of the revertants DNA with *BglI* plus *Bam* shown in Fig. 4. An examination of the figure reveals that the anchorage-dependent revertants F1SV, Bu, Col4, LS1, and LS2, as well as the anchorage-independent revertants A γ 4 and A γ 5, contain a 2.7-kb fragment which anneals to a probe of SV40 DNA early region sequences between base pairs 5218 and 2516. The predicted size is 2.7 kb for a normal SV40 *BglI*-*Bam* fragment containing the early region coding sequences.

We next determined whether anchorage-dependent revertants retained the defective viral DNA present in SV101 with an equally high frequency. Figure 5 shows *Kpn*, *BglI*, and *Bam* digests of DNA from the five anchorage-dependent revertants and the two serum revertants that have retained the anchorage-independent phenotype. Clearly, most of the defective DNA is absent from the DNA prepared from the anchorage-dependent revertants. In the *Kpn* analysis, F1SV and Bu retain only the 3.4-kb fragment; Col4 retains only the 5.0-kb fragment; LS1 retains the 5.0- and 1.5-kb fragments; and LS2 retains only the 1.5-kb fragment.

The inability of an enzyme to demonstrate defective viral DNA could be due to the loss of only one of two restriction enzyme sites surrounding the monomer in question, rather than due to an absence of the entire stretch of defective DNA. However, an examination of the *BglI* and *Bam* digests reveals that F1SV and Bu have lost all the defective viral DNA demonstrable in SV101 by these enzymes as well, except for the 3.4-kb monomer in the *BglI* digests. Col4 still manifests only the 5.0-kb monomer, as seen in the *Bam* digest. The 5.0-kb monomer in the Col4 *BglI* digest cannot be the 5.0-kb defective monomer in SV101, since the SV101 monomer lacks the *BglI* site. LS1 and LS2 show only the 1.5-kb monomer. The 5.0-kb monomer present in the *Kpn* digest of LS1 is not present in the

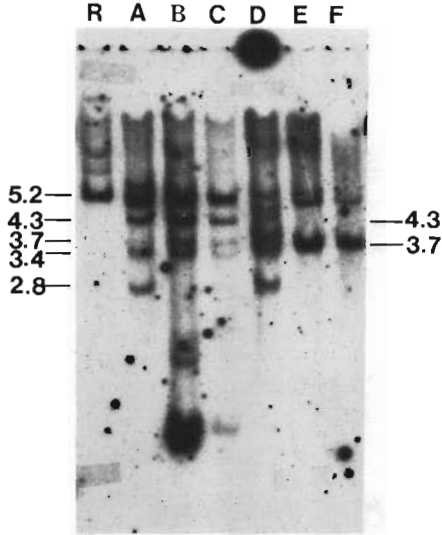


FIG. 6. *Kpn* analysis of DNA from mock revertants. DNA from each of the mock revertants was extracted and digested with *Kpn* for blotting analysis. Lane assignments are as follows: 1p1 (A), 1p2 (B), 1p3 (C), B7 (D), B8 (E), and B4 (F). Lane R is SV40 DNA digested with *Kpn*. Lanes were probed with SV40[³²P]DNA. The numbers indicate fragment sizes in kilo-base pairs.

Bam digest of LS1, indicating that one of the *Bam* sites bordering the point of deletion has been lost. Since the 5.0-kb stretch from *Kpn* site to *Kpn* site in LS1 is intact, the missing *Bam* site is likely to lie outside the *Kpn* to *Kpn* stretch (Fig. 3c). Thus the anchorage-dependent revertants described here have lost the defective viral DNA indicated rather than simply restriction enzyme sites bordering this DNA.

The results seen in the *Kpn*, *Bgl*II, and *Bam* digests of DNA isolated from the anchorage-dependent revertants contrast with those obtained with DNA from the anchorage-independent revertants. The *Kpn* analysis demonstrates that A γ 4 and A γ 5 lose the 5.0-kb monomer, but retain the 4.3-, 3.7-, 3.4-, and the 1.5-kb monomers (Fig. 5, lanes F and G). The *Bgl*II digests of DNA from A γ 4 and A γ 5 manifest the 4.3- and 3.4-kb monomers, and the *Bam* digest, the 4.3 monomer. The results are consistent with the surrounding structure of the 4.3-, 3.7-, and 3.4-kb defective viral DNA having remained similar to what it is in the parent population, SV101.

In order to determine the frequency with which defective viral DNA is retained in a larger sample of anchorage-independent subclones of SV101, we examined the DNA of the mock revertants (Fig. 1). DNA isolated from these cell lines was digested with *Kpn* and analyzed with Southern blotting as described above. Figure 6 shows that mock revertant 1p1 retains the 5.0-, 4.3-, and 3.4-kb defective monomers, 1p2 retains the 5.0-, 4.3-, 3.7-, and 3.4-kb monomers. 1p3 retains the 4.3-, 3.7-, and 3.4-kb monomers. B7 retains only the 4.3-kb monomer. B8 retains the 5.0- and 3.7-kb monomers. Finally, B4 retains the 5.0- and 3.7-kb monomers. It should also be noted that the mock revertants retain the 5.2-kb linear SV40 molecule with a greater frequency than do the anchorage-dependent subclones of SV101. This may reflect a greater overall stability of the stretch of SV40 DNA in the mock revertants.

We digested the DNA of three mock revertants, 1p2, B7, and B8 with *Bgl*II and with *Bam*. These results are shown in Fig. 7. They indicate that restriction enzyme sites close to the point of deletion in many cases have been lost. For example, although B8 does not contain a 3.4-*Kpn* fragment, it does contain a 3.4-kb *Bgl*II fragment. This indicates that the defective monomer is still present, and indeed is likely to contain a *Kpn* site between its two *Bgl*II sites, but an adjacent *Kpn* site has been lost. 1p2 retains the 4.3-kb fragment, but a nearby *Bam* site has been lost. This is indicated by the fact that the 4.3-kb fragment is seen in the *Bgl*II and *Kpn* digest of 1p2 DNA, but not in the *Bam* digest of 1p2 DNA. Thus, despite a large amount of viral DNA rearrangement in the neighborhood of the defective DNA in SV101, anchorage-independent subclones retain larger amounts of this DNA than do the anchorage-dependent revertants. The correlation of retained defective viral DNA with anchorage-independence is statistically significant (Table 3).

Defective Viral DNA in Anchorage-Independent Subclones of an Anchorage-Dependent Revertant

In order to learn whether the anchorage-dependent revertants were able to

generate additional defective SV40 DNA, we placed LS1 into agarose and picked three large colonies after 3 weeks. These colonies were grown to mass culture, and their plating efficiency in soft agarose was determined. They exhibited a 400-fold greater anchorage-independent plating efficiency than the parent cell line. DNA from these three subclones, LS1-1a, LS1-2a, and LS1-3a was isolated, digested with *Msp*, *Bgl*I, and *Kpn*I, and analyzed via Southern blotting as above. The results are shown in Fig. 8. The digest pattern for each enzyme is quite different from those obtained with the parent, LS1 (Fig. 5, lane D) but the digest patterns do not vary from subclone to subclone. The similarity of the digest pattern of these three clones makes it highly likely that they were all derived from the same cell. Since these three clones were picked from separate dishes, the

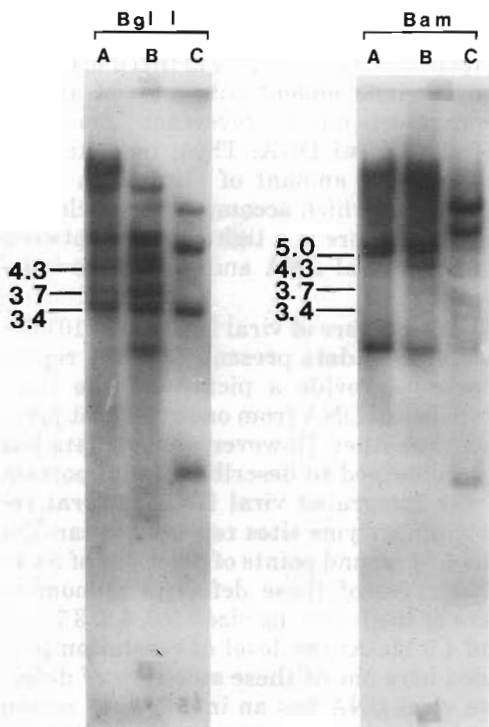


FIG. 7. *Bgl*I and *Bam*I analysis of DNA from mock revertants. DNA from the following mock revertants was extracted and digested with *Bgl*I or *Bam*I, as indicated in the figure, for blotting analysis: 1p2 (A), B7 (B), and B8 (C). Lanes were probed with SV40(³²P)DNA. The numbers indicate fragment sizes in kilo-base pairs.

TABLE 3
DEFECTIVE VIRAL DNA IN SUBCLONES OF SV101

Cell line	Defective monomer				
	5.0	4.3	3.7	3.4	1.5
F1SV				+	
Bu				+	
Col4	+				
LS1	+				+
LS2					+
—					
Aγ4		+	+	+	+
Aγ5		+	+	+	+
1p1	+	+		+	
1P2	+	+	+	+	
1p3	+		+	+	
B7		+			
B8	+		+		
B4	+		+		

Note. This table is a list of which defective monomers are retained by which subclones of SV101. It is possible to determine the statistical significance of the difference in the amount of defective viral DNA retained by the anchorage-dependent subclones as opposed to the anchorage-independent subclones using Student's *t* test. The anchorage-independent set has eight members retaining a total of 23 defective stretches of viral DNA, while the anchorage-dependent set has five members retaining a total of 6 stretches of defective viral DNA. This gives a *t* value of 3.13. There are 11 degrees of freedom, which results in a probability of about 99% that the difference in the retention of defective viral DNA is significant. The 5.0-kb defective monomer is retained by Col4 and LS1, as well as by some of the mock revertants. If it were eliminated from consideration in performing the *t* test because it does not have an intact large T, amino-terminal exon, the *t* value becomes 2.48. With 11 degrees of freedom, the probability that a significant difference exists between the anchorage-dependent and -independent subclones of SV101 is greater than 95%.

rearrangement of the viral DNA must have occurred before plating LS1 in agarose.

It can also be seen that all the subclones have gained defective viral DNA at 3.3, 2.6, and 0.9 kb. Defective viral DNA of this size is not seen in SV101, indicating that LS1-1a, LS1-2a, and LS1-3a are not SV101 contaminants of LS1. This result strongly links defective viral DNA to the anchorage-independent phenotype. It also demonstrates that the LS1 subclone of SV101 is

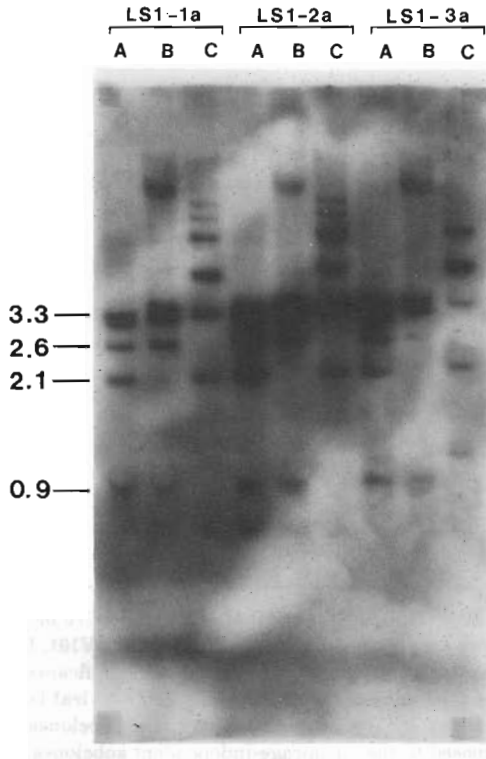


FIG. 8. Defective viral DNA in anchorage-independent subclones of an anchorage-dependent revertant. DNA from LS1-1a, LS1-2a, and LS1-3a was extracted and digested with *Msp* (A), *Kpn* (B), and *Bgl*I (C) for blotting analysis. Lanes were probed with SV40³²P]DNA. The numbers indicate fragment sizes in kilo-base pairs.

not inherently unable to acquire defective viral DNA.

DISCUSSION

Two major techniques are available to study the arrangement of integrated SV40 DNA in transformed cell lines: Southern blotting (Botchan *et al.*, 1976; Ketner and Kelly, 1976; Steinberg *et al.*, 1978) and molecular cloning (Botchan *et al.*, 1980; Clayton and Rigby, 1981; Stringer, 1981; Sager *et al.*, 1981a). Molecular cloning offers the advantage of examining DNA within the resolution of one base pair. Southern blotting affords less resolution, but provides an opportunity to study many cell lines simultaneously.

We have shown here that defective viral DNA commonly occurs in independent

SV40 mouse transformants. Defective viral DNA has been observed in the cell line SVT2/S by Sager and colleagues (Sager *et al.*, 1981b) and in the cell line J78 by Defendi and colleagues (Hiscott *et al.*, 1980). Clayton *et al.* (Clayton and Rigby, 1981) have reported partial repeats of viral DNA in two independently transformed Balb/c 3T3 cells, which they uncovered by molecular cloning of integrated viral DNA. A partial repeat is equivalent to a defective monomer in tandem with other SV40 DNA. It would appear that defective viral DNA in SV40-transformed mouse cells is the rule rather than the exception.

We have mapped the arrangement of this DNA in one SV40 transformant, SV101. An analysis of 16 different subclones of SV101 (including LS1-1a, LS1-2a, and LS1-3a) has shown that anchorage-dependent revertants of SV101 lose a large portion of defective viral DNA, despite retaining a transcriptionally active early region, while anchorage-independent subclones retain defective viral DNA. Furthermore, anchorage-independent subclones of an anchorage-dependent revertant reacquire defective viral DNA. Thus, in spite of a considerable amount of viral DNA rearrangement which accompanies subcloning of SV101, there is a tight linkage between defective viral DNA and anchorage independence.

The structure of viral DNA in SV101 determined by data presented in this report does not provide a picture of the integrated viral DNA from one viral host junction to another. However, enough data has been obtained to describe a large portion of the integrated viral DNA as viral restriction enzyme sites repeated in tandem which surround points of deletions of SV40 DNA. Five of these defective monomers were of the following sizes: 5.0, 4.3, 3.7, 3.4, and 1.5 kb. At the level of resolution provided here one of these stretches of defective viral DNA has an intact early region (4.3 kb), two have intact little t coding regions (4.3 and 3.7 kb), and three have intact origin proximal exons (4.3, 3.7, and 1.5 kb). The 3.4-kb monomer is missing the *Taq* site, the nearest point to the large T, amino-terminal exon detectable in this study. The 5.0-kb monomer is missing

about 200 base pairs including the *Bgl*I site. It is impossible to lose 200 base pairs in this region of the DNA without losing either the early region associated TATAA box or the initial portion of the coding region for large T antigen. A loss of a portion of the large T antigen would be identifiable as a truncated protein in an immunoprecipitation, electrophoresis analysis of the viral proteins in SV101. Since no such truncated protein is seen, it seems likely that the 5.0-kb monomer is transcriptionally inactive (Chen *et al.*, 1981).

It has been shown that little t antigen increases the efficiency with which rat cells grow in agar (Bouck *et al.*, 1978; Sleigh *et al.*, 1978; Steinberg and Pollack, 1979). None of the anchorage-dependent subclones of SV101 retain the 4.3- and 3.7-kb defective monomers, the only two defective regions of viral DNA shown to have intact little t coding regions. Also, each anchorage-independent subclone retains at least one of these two regions. However, analysis of virally encoded proteins in these and other mouse cell lines failed to reveal a correlation of the amount of small t with anchorage independence (Chen *et al.*, 1981). Therefore, it is unlikely that the extra little t coding capacity in anchorage-independent subclones of SV101 is wholly responsible for the anchorage-independent phenotype.

This same study showed that SV40 anchorage transformation in mouse cells is accompanied by the occurrence of a 100-kDa variant T antigen, not seen to a great extent in mouse cells incompletely transformed by SV40. It is possible that an arrangement of defective viral DNA in tandem specifically codes for this T antigen. There is a significant correlation between a large amount of 100-kDa T antigen and cell lines known to contain defective viral DNA, including the anchorage-independent subclones of LS1 (S. Chen, unpublished data). Sager and colleagues have shown that SVT2/S contains only defective viral DNA, including a partial duplication of viral DNA (Sager *et al.*, 1981b). Examination of the parent of SVT2/S, SVT2, in our laboratory has revealed that its integrated viral DNA is similar to that seen in SVT2/S in that it also does not

contain an intact SV40 early region. SVT2 does, however, produce a 100-kDa T antigen, but not the 94-kDa (lytic-sized) T antigen. Furthermore, when some subclones of SVT2 are picked or when SVT2 is passaged under certain culture conditions, as described by Hayflick (Hayflick and Moorhead, 1961), it is possible to observe a 94-kDa T antigen in addition to the 100-kDa T antigen. SVT2 descendants which acquire the 94-kDa T antigen also acquire the 17-kDa small t antigen (not seen in the parent cell line) and an intact early region. Therefore, it is likely that the 100-kDa T antigen of SVT2 is encoded by a stretch of viral DNA which does not include an intact early region, but does include a partial duplication of viral DNA (S. Chen *et al.*, in preparation).

Molecular cloning and expression of cloned viral DNA from SV40-transformed cells is bound to elucidate the way in which defective viral DNA codes for proteins (A. Levitt, in preparation).

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