

# Reacquisition of a Functional Early Region by a Mouse Transformant Containing Only Defective Simian Virus 40 DNA

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Viral DNA in simian virus 40-transformed mouse cells is capable of rearranging with passage. In this report, we show that such rearrangement can include an alteration in viral protein expression. SVT2, a simian virus 40-transformed mouse BALB/c 3T3 cell line, synthesizes only a super T antigen of molecular weight 100,000 without synthesizing the lytic-size large T or small t antigens with molecular weights of 94,000 and 17,000, respectively. Analyses of the integrated viral DNA revealed an early region of 4.4 kilobases instead of the lytic-size 2.7 kilobases. However, upon subcloning in either plastic or agarose or after being in culture for several passages, the appearance of lytic-size large T and small t antigens was detected. Concurrently, an early region of 2.7 kilobases, in addition to one of 4.4 kilobases, was observed.

Simian virus 40 (SV40) will grow in and lyse monkey cells and will stably transform the cells derived from several species into oncogenic lines. When SV40 transforms a cell, part of the viral DNA becomes covalently integrated with host DNA and is transcribed to produce tumor (T) antigen (15). After integration, the viral sequence can persist either as a replica of the viral early region or it can become altered in such a way as to create coding sequences for novel T antigens not seen in the lytic cycle. These two fates are not mutually exclusive, as both are compatible with successful transformation (2-4, 10). Many types of variant viral early regions are seen in transformed cells, including defective and duplicated viral sequences, as well as insertions of host DNA. However, since wild-type DNA can transform as well as, or more efficiently than, all known deleted or rearranged versions of SV40, the rearrangements and deletions within transformants must arise in a process which follows infection. The mechanism by which these variant sequences arise and the special functions, if any, of these variant T antigens remain unsolved problems.

Integrated viral DNA of SV40 has the capacity to undergo a considerable amount of subclonal rearrangement long after transformation (1, 1a, 8, 12). In some cases, delayed rearrangement of viral DNA is associated with a concomitant change in the growth behavior of cells. For example, we have recently shown that revertants of a transformed cell that regain the normal anchorage requirement lose variant SV40 sequences from the integrated SV40 array (1a). Also, others have shown that rearrangement of integrated viral sequences accompanies the transition from T-antigen-dependent to T-antigen-independent cell growth in agarose (8).

Subclonal variation in the arrangement of viral DNA also can be accompanied by subclonal variation in the expression of T antigen in SV40-transformed cells. Subclones of transformants may not express T antigen at all (14), may express it under certain conditions only (5), or may express electrophoretically abnormal as well as normal forms of T antigen (2). So far, however, a direct link between a subclonal change in T antigen expression and a subclonal change in viral DNA arrangement has not been demonstrated. In this

report we show that SVT2 (5), a single-copy SV40 transformant containing only a nonlytic arrangement of SV40 DNA and expressing only a variant T antigen, can simultaneously acquire a normal-sized T antigen and a normal arrangement of viral DNA.

## MATERIALS AND METHODS

**Cell lines.** All cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO Diagnostics) in a humidified incubator with 10% CO<sub>2</sub>. The parent SVT2 cells at passage 9 were plated at 10<sup>2</sup> per 60-mm petri dish, and subclones were picked with steel cloning rings 10 to 14 days later. For anchorage-independent subclones, SVT2 cells were plated out at 10<sup>3</sup> per 60-mm petri dish in 0.33% agarose on top of plates previously coated with 0.5% agarose. Subclones were picked 3 weeks later. Nine plastic subclones and 14 agarose subclones were isolated for further analysis.

**Immunoprecipitation.** Cells were labeled with [<sup>35</sup>S]methionine for 2 h, and cell extracts were prepared. Immunoprecipitated with either normal hamster serum (N) or hamster anti-SV40 tumor serum (T) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (2).

**Pulse and chase.** Cells were pulsed with [<sup>35</sup>S]methionine for 10 min, washed three times with medium containing excess methionine, and then fed with fresh medium with 10% fetal calf serum for the given chase periods. Cell extracts were then prepared, immunoprecipitated, and analyzed as described above.

**Isolation of poly(A)-containing mRNA.** All solutions were treated with diethylpyrocarbonate to destroy RNase. Cells from three confluent 150-mm petri dishes were washed twice with phosphate-buffered saline. The cells were then scraped in 10 mM Tris (pH 7.5)-125 mM NaCl-3 mM MgCl<sub>2</sub>, and cell pellets were obtained by sedimenting at 258 × g for 10 min. The pellets were washed twice in the same solution. The pellets were then lysed in the same buffer with 0.6% Triton X-100 and 10 mM vanadyl adenyate (Sigma Chemical Co.) for 10 min at 0°C. The nuclei were removed by centrifugation at 258 × g for 10 min. An equal volume of 10 mM Tris (pH 7.5)-125 mM NaCl-5 mM EDTA-1% SDS was added to the supernatant, which was then extracted twice with phenol-

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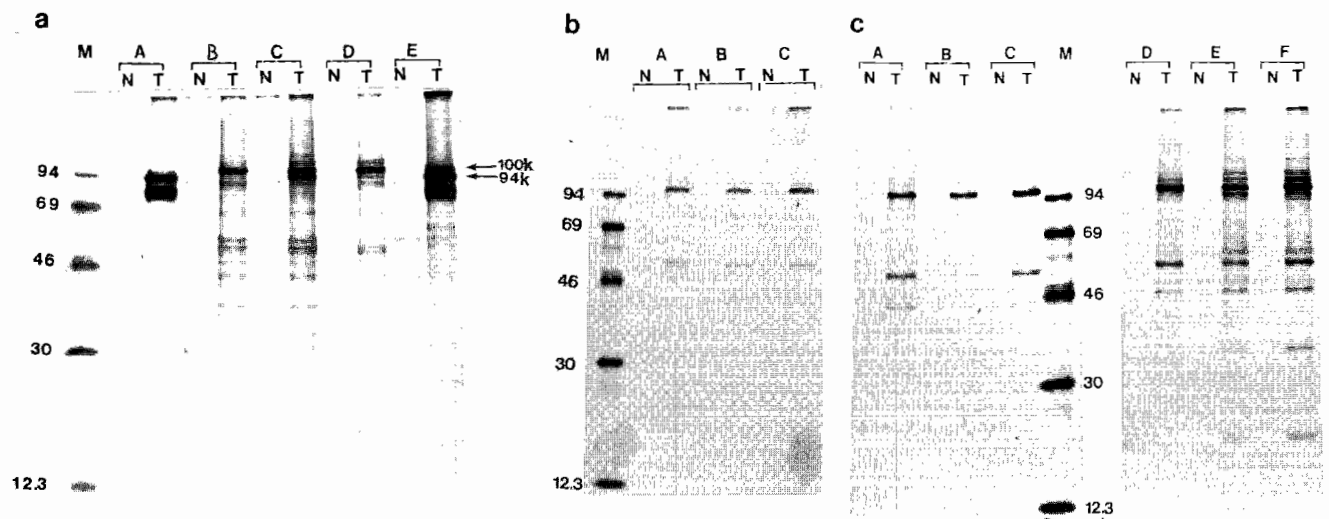


FIG. 1. (a) [ $^{35}$ S]methionine-labeled cell extracts were immunoprecipitated with normal hamster serum (N) or hamster anti-SV40 tumor serum (T) and analyzed by a 10 to 20% gradient SDS-polyacrylamide gel electrophoresis. Lane A is SV40-infected CV1 monkey cells; note the lytic 94K T antigen and its breakdown product. Lane B is SVT2 at passage 9; only the 100K super T antigen is present. Lane C is SVT2 at passage 34; in addition to the 100K super T antigen observed at passage 9, lytic-size 94K T antigen is also present now. Lane D subclone 9p also has both 94K and 100K T antigens. Lane E is a mixture of SV40-infected monkey cells and SVT2 at passage 9. (b) Subclones of SVT2 from agarose. Subclones 1p, 2p, and 3p only had the 100K super T antigen, similar to SVT2 at passage 9. (c) Subclones of SVT2 selected from agarose. Lanes A to C are subclones A2, A3, and A6, respectively; only the 100K super T antigen is present. Lanes D to F are subclones A7, A8, and A9; 94K T and 17K t antigens were also observed in addition to the 100K T antigen.

chloroform-isoamyl alcohol (1:1:20) and twice with chloroform. The total RNA was precipitated with ethanol and 250 mM NaCl. Polyadenylate [poly(A)]-containing RNA was isolated by oligodeoxythymidylic acid-cellulose column (Collaborative Research, Inc.), precipitated with ethanol, collected, redissolved in water, and stored at  $-80^{\circ}\text{C}$ .

**In vitro translation of mRNA.** Samples of mRNA were translated in vitro by using the nuclease-treated reticulocyte lysate system (11). Reticulocyte lysate was prepared from rabbits made anemic by multiple injections of acetyl phenylhydrazine as described previously (9). The translation assay consisted of 50% nuclease-treated lysate, 40  $\mu\text{g}$  of mRNA per ml, 1 mM ATP, 0.2 mM GTP, 12 mM creatine phosphate, 0.08 mg of creatine phosphokinase per ml, 30  $\mu\text{M}$  19 essential amino acids, 10 mM Tris (pH 7.4), 100 mM KCl, 2 mM magnesium acetate, 2 mM dithiothreitol, 1 mg of calf liver tRNA per ml, and 1 mCi of [ $^{35}$ S]methionine per ml. The translated products were then immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

**Analysis of the integrated SV40 DNA.** Genomic digests and Southern blotting were carried out according to methods described previously (1a, 16). Restriction enzymes were used according to instructions of the vendor. Bands representing consensus fragment lengths of DNA in cloned cell populations. Absence of a band means that 1 cell in 10, at the most, can contain a specific DNA fragment of that size. *BglI-Bam* SV40-specific fragments at 2.7 kilobases (kb) were identified by comigration with a lytic SV40 *BglI-Bam* fragment in all cases.

## RESULTS AND DISCUSSION

**Cell lines.** All of the lines were derived from SVT2, an SV40-transformed BALB/c mouse line (5). Before any subcloning was begun, SVT2 was itself cloned on plastic to

assure parental homogeneity. At passage 9 after cloning, SVT2 was in turn subcloned on plastic and on agarose for these studies.

**Analysis of viral proteins.** At passage 9, extracts were made from the parental SVT2 clone labeled with [ $^{35}$ S]methionine. These were immunoprecipitated with hamster anti-SV40 tumor serum or normal hamster serum and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1a, lane B). A major band is present at the 100,000-molecular-weight (100K) position, and no detectable protein is immunoprecipitated at the position of the wild-type 94K or 17K T antigens. Immunoprecipitates from SV40-infected CV1 monkey cells are shown in Fig. 1a, lane A. The lytic-size 94K large T is seen together with the breakdown product of large T. The 17K small t can be detected in longer exposure. A mixture of cell extracts from SVT2 passage 9 and SV40-infected CV1 is shown in Fig. 1a, lane E. Note that the 94K lytic and 94K transformed cell T are both smaller than the 100K T antigen in the parental SVT2 clone.

A 100K super T antigen observed previously by several groups (2, 6, 13) was recently shown to be correlated with anchorage-independent growth in a set of mouse transformants and revertants (2). At present, no other known property in addition to molecular weight distinguishes 94K T antigen from 100K T antigen, although circumstantial evidence (see below and reference 12) indicates that a duplication of viral DNA can be responsible for the 100K coding sequence (10). The 100K and 94K forms of T antigen are clearly closely related since Smith et al. (13) and others have shown that peptide maps of the two proteins are almost identical.

Subclones of SVT2 obtained by passage on plastic were studied next. One out of nine subclones (subclone 9p) expressed 94K T antigen as well as the 100K T antigen seen in parental SVT2 (Fig. 1a, lane D). The 17K t antigen can be seen in longer exposure. Other bands at higher molecular weight (115K and 130K) also appeared in this clone simulta-

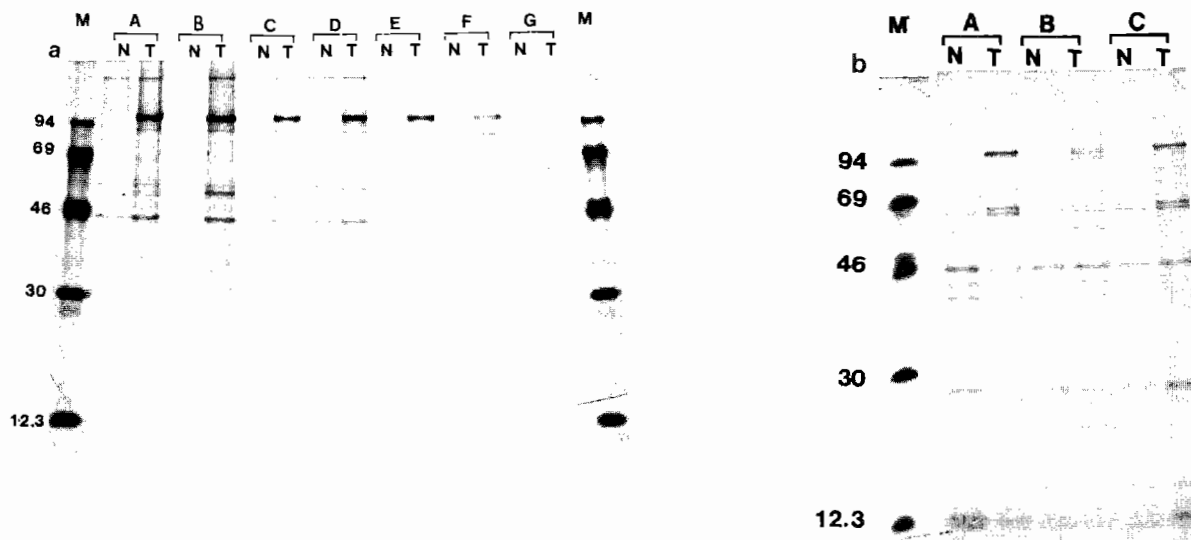


FIG. 2. (a) SVT2 cells at passage 9 labeled with [ $^{35}$ S]methionine for 2 h (lane A); SVT2 cells labeled for 10 min and chased for 2, 4, 18, 24, and 48 h, respectively. (b) Immunoprecipitate of in vitro translation of poly(A)-containing mRNA from SVT2 passage 9 (lane A), passage 34 (lane B), and subclone 9p (lane C).

neously with the appearance of the 94K T antigen. The other eight subclones were indistinguishable from SVT2 in viral protein expression. Immunoprecipitated 100K T antigen from subclones 1p, 2p, and 3p is shown in Fig. 1b, lanes A, B, and C, respectively. Note that the 100K protein travels above the 94K marker band.

Some SVT2 subclones obtained in agarose also were different from the parental SVT2 clone. Out of 14 subclones, 3 (clones A7, A8, and A9) were found to express the 94K and 17K T antigens as well as the 100K super T antigen. Immunoprecipitations of viral proteins of these subclones (A2, A3, A6, A7, A8, and A9) are shown in Fig. 1c, lanes A to F, respectively. In addition to the appearance of 94K and 17K T antigens in clones A7, A8, and A9, immunoreactive protein bands below 94K and at 115K and 130K were also observed.

Subcloning, that is, growth of colonies from single cells, was not necessary to generate cells with 94K and 17K T antigens. Passage of the parental SVT2 clone on plastic according to the culture conditions described previously (6) was sufficient to generate a significant amount of 94K and 17K protein in the culture (Fig. 1a, lane C).

The 94K T antigen detected in passage 34 of SVT2 and some of the plastic (9p) and agarose (A7, A8, A9) clones is not a breakdown product of the 100K super T antigen. This was shown by both pulse-chase and in vitro translation experiments. SVT2 cells at passage 9 were labeled with [ $^{35}$ S]methionine for 10 min and then chased for various time periods as described above. The results are shown in Fig. 2a. Normally, the cells are labeled for 2 h (lane A). However, after a 10-min pulse, the majority of the immunoprecipitable protein is labeled (lane B). Lanes C through G are cell extracts after a 10-min pulse and 2-, 4-, 18-, 24-, and 48-h chases, respectively. The 100K T antigen remained at the 100K position throughout the chase. It did not break down to the 94K protein. After a 48-h chase, almost none of the 100K T remained. Since equal trichloroacetic acid-precipitable total protein was used in each immunoprecipitation, the half-life of this 100K T antigen is around 24 h. An identical pulse-chase experiment was carried out with SVT2 at passage 34.

The intensities of both 100K and 94K T antigens were measured by densitometry as described previously (2). The ratio of amount of 94K to 100K is shown in Table 1. These results also indicated no breakdown from 100K T to 94K T antigen.

In vitro translation of poly(A)-containing mRNA from SVT2 passage 9, SVT2 passage 34, and SVT2 plastic subclone 9p revealed 100K super T alone in SVT2 passage 9 (Fig. 2b, lane A) and revealed 94K and 100K T antigens in both SVT2 passage 34 and SVT2 9p (Fig. 2b, lanes B and C). Again, these data support the conclusion that the 100K super T and the 94K T antigen are distinct proteins.

**Analysis of integrated viral DNA.** Either the 94K and 17K tumor antigens in subclones of SVT2 are encoded by an SV40 early region that is silent in parental SVT2 or they are encoded by an early region sequence that is acquired during cell passage and was absent in the parental SVT2 clone. To distinguish between these two possibilities, we examined the structure of the integrated viral DNA in SVT2 and its subclones. Figure 3 is a *Bgl*I plus *Bam* sequential digest of SVT2 DNA probed with  $^{32}$ P-labeled pSV*Bgl*-*Bam*, a plasmid containing the *Bgl*I-*Bam* early region of SV40 DNA. SVT2 contains only one fragment of 4.4 kb (Fig. 3, lane B). This is ca. 1.7 kb larger than the lytic-size early region. Reconstruction experiments with known quantities of SV40 DNA indicate that this fragment occurs about once per diploid genome (Fig. 3, lane C). The absence of a fragment at 2.7 kb indicates that the majority of the cells of the SVT2 parental population do not contain an intact early region. However, a

TABLE 1. Ratio of 94K T antigen to 100K super T antigen

Pulse	Chase	94K/100K
2 h		0.90
10 min		0.83
10 min	2 h	1.20
10 min	4 h	1.10
10 min	18 h	1.05
10 min	24 h	1.07

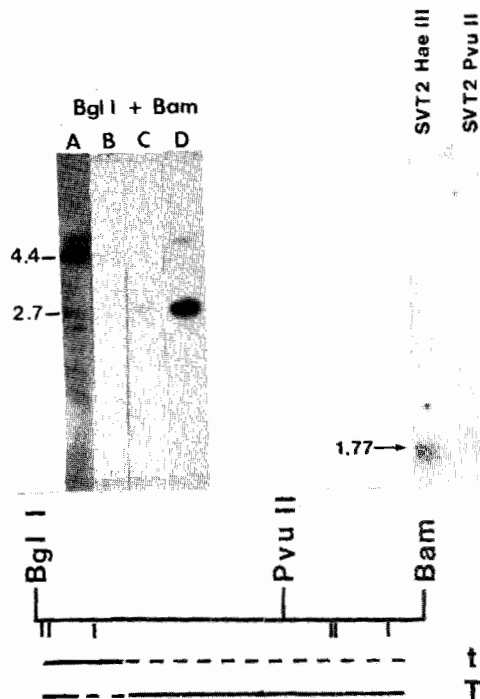


FIG. 3. SV40 DNA in SVT2. High-molecular-weight DNA from SVT2 (parent population) was extracted and digested with the restriction enzymes indicated above each lane. All lanes were probed with pSVBgl-Bam, a plasmid containing only SV40 early region sequences. Fragment sizes are in kb pairs. Below the lanes is a partial map of the SV40 early region. *Bgl*I, *Pvu*II, and *Bam* sites are indicated. Small vertical lines below the restriction map represent *Hae*III sites. The horizontal lines below the restriction map indicate early mRNAs, with the solid portions representing the coding portions of the mRNAs. Lane A, SVT2 DNA, 7-day exposure; lane B, SVT2, 12-h exposure; lane C, 1 copy of SV40 DNA per diploid genome for a 12-h exposure; lane D, 25 copies of SV40 DNA per diploid genome for a 12-h exposure.

long exposure of the *Bgl*I plus *Bam* digest reveals a faint band at 2.7 kb. Since this band is well below the intensity of a fragment which occurs in one or more copies per cell, we conclude that only a minority of cells (no more than 1 in 10) in this population can contain an intact early region (Fig. 3, lane A). It is therefore likely that the subclones described above which express both the 94K and 100K proteins are derived from a subset of cells in the parent population. This subset must have acquired an intact early region sometime after cloning of the parental line.

Our SVT2 clone is derived from the SVT2 culture which is also the parent cell line of SVT2/S, a cell line whose integrated viral DNA was examined in considerable detail by Sager and colleagues (12). SVT2/S does not contain an intact SV40 early region but rather has a stretch of SV40 DNA which includes a tandem repeat of about 1.75 kb. This repeat falls between, but does not include, the *Taq* and *Bam* restriction enzyme sites on the lytic SV40 map (Fig. 3). The repeat includes a *Pvu*II site and an *Hae*III site. Thus, liberation of a 1.7-kb fragment upon digestion of genomic DNA with these two enzymes is diagnostic for the presence of the SVT2/S repeat. We find this repeat in the 4.4-kb fragment of SVT2, the parent of the subclones in the present study (Fig. 3).

DNA samples from the plastic subclones (1p, 2p, and 3p)

of SVT2 which expressed the same 100K viral protein as the parent (Fig. 1b) were analyzed next. The only *Bgl*I-*Bam* region in these subclones is identical in size to that of the SVT2 parent. No rearrangements of early region sequences occurred in more than 1 in 10 of the cells upon the subcloning that generated these three lines (Fig. 4). In contrast, DNA samples from subclone 9p and from SVT2 passage 31 when digested with *Bgl*I plus *Bam* and probed with <sup>32</sup>P-labeled pSVBgl-Bam showed a 2.7-kb early fragment, which comigrates with the *Bgl*-*Bam* fragment from lytically produced SV40 DNA, as well as the 4.4-kb fragment (Fig. 4). The appearance of 94K and 17K antigens in this set of SVT2 subclones is specifically accompanied by the appearance of a wild-type SV40 early region. In the case of subclone 9p and the SVT2 population at late passage, the relative intensities of the 4.4-kb early region and the 2.7-kb early region indicate that not all members of these cell populations contain an intact early region. This is not surprising in the case of SVT2 at late passage. As we demonstrated above, a minor component of the normal SV40 early region can be detected in the SVT2 population. In the case of subclone 9p, the small number of cells containing the intact early region indicates that the acquisition of this coding sequence occurred after subcloning.

DNA from subclone 9p and SVT2 at passage 34 was digested with *Taq* and *Bam*. These digestions revealed the presence of a lytic size *Taq*-*Bam* fragment in 9p and late passage SVT2, which confirms the interpretation of the above described 2.7-kb fragment as an intact SV40 early region (data not shown).

All three agarose subclones expressing 94K and 17K T antigens (A7, A8, A9) have also acquired a fragment at 2.7 kb (Fig. 4). Since selection for anchorage independence increases the frequency of 94K-expressing SVT2 subclones and those agarose subclones which do express 94K T antigen reflect a greater number of intact early regions per cell than does the cloned 9p population, it is possible that 94K antigen supplements the role of 100K antigen in growth without anchorage.

One of the agarose subclones (A6) does not express 94K or 17K T antigen but does have an early region fragment

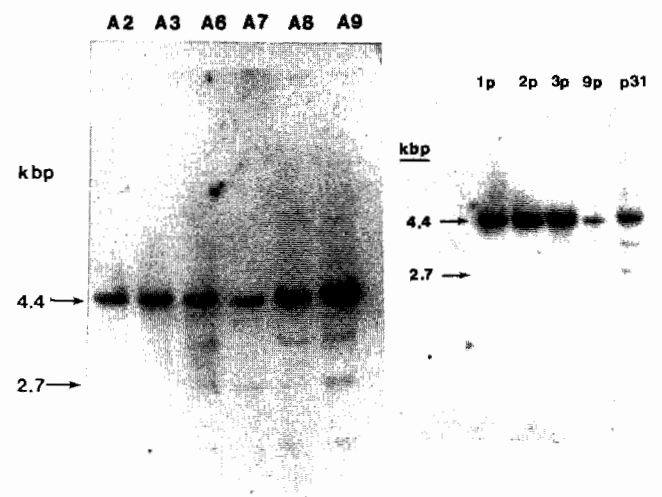


FIG. 4. SV40 *Bgl*-*Bam* fragments in SVT2 subclones. DNA from subclones of SVT2 was digested with *Bgl*I and *Bam* and probed with pSVBgl-Bam. Fragment sizes are in kb pairs.

migrating at 2.7 kb. We do not know why the presence of this early fragment does not result in the appearance of 94K and 17K proteins in this subclone. Subclones A2 and A3, which make only 100K T antigen, had only a 4.4-kb early fragment as seen in parental SVT2 (Fig. 4). Thus, the subcloning of SVT2 has demonstrated the heterogeneity of the SVT2 line with regard to the normal SV40 early region. Since SVT2 was itself cloned before the picking of the subclones described above, the normal early region must have been derived by DNA rearrangement from the 100K coding sequence in SVT2, presumably via loss of the 1.7-kb repeat (12).

In this report, we have shown that it is possible for an SV40-transformed cell line which contains only defective early viral DNA to reacquire an intact, expressing early region. Reacquisition of an intact early region leads to expression of both large and small T antigens. In no case were 94K or 17K T antigen found exclusive of one another. A previous study reported the reacquisition of an intact early region by one subclone of SVT2/S, a cell line related to SVT2, but protein expression was not studied (12).

Transition from the expression of a 100K super T antigen to expression of 100K super T plus 94K and 17K antigens conveniently links rearrangement of integrated viral DNA to variation in viral protein expression. A transition in the opposite direction would require a tedious amassing of DNA and amino acid sequences or transfection studies with isolated DNA fragments encoding variant T antigens (4) to firmly establish the connection between a newly expressed protein and its newly acquired coding sequence, especially if several variant coding sequences or proteins were involved. Since the size of the coding sequence for the 94K T antigen is already known, Southern blot data are sufficient to confirm its presence. Also, the regeneration of a lytic early region is confirmed by the presence of the 17K protein in SVT2 subclones which express the 94K T antigen. Both pulse-chase and in vitro translation experiments further confirm that 94K and 100K T antigens are both synthesized independently of each other. The 94K T antigen we have detected in late passage SVT2 and some of its subclones is not a breakdown product. Acquisition of lytic-size 94K T antigen in the subclones and late passages of SVT2 was accompanied by the generation of immunoprecipitable proteins of around 115K and 130K. These proteins may be encoded by other variant early regions (Fig. 4), but we have not yet tested this.

It is not yet known whether other variant SV40 sequences are capable of generating an intact coding region. It is interesting to note that the loss of the 1.75-kb repeat from the 100K coding region is frequently precise enough to reestablish the expression of two normal proteins. It is possible that the regeneration of the intact coding region is dependent on the occurrence of a repeat in the variant coding sequence.

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