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cis-Active Elements from Mouse Chromosomal DNA Suppress Simian Virus 40 DNA Replication

MARKUS HARTL,† THOMAS WILLNOW, AND ELLEN FANNING*

Institute for Biochemistry, Karlstrasse 23, D-8000 Munich 2, Federal Republic of Germany

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Simian virus 40 (SV40)-containing DNA was rescued after the fusion of SV40-transformed VLM cells with permissive COS1 monkey cells and cloned, and prototype plasmid clones were characterized. A 2-kilobase mouse DNA fragment fused with the rescued SV40 DNA, and derived from mouse DNA flanking the single insert of SV40 DNA in VLM cells, was sequenced. Insertion of the intact rescued mouse sequence, or two nonoverlapping fragments of it, into wild-type SV40 plasmid DNA suppressed replication of the plasmid in TC7 monkey cells, although the plasmids expressed replication-competent T antigen. Rat cells were transformed with linearized wild-type SV40 plasmid DNA with or without fragments of the mouse DNA in *cis*. Although all of the rat cell lines expressed approximately equal amounts of T antigen and p53, transformants carrying SV40 DNA linked to either of the same two replication suppressor fragments produced significantly less free SV40 DNA after fusion with permissive cells than those transformed by SV40 DNA without a cellular insert or with a cellular insert lacking suppressor activity. The results suggest that two independent segments of cellular DNA act in *cis* to suppress SV40 replication in vivo, either as a plasmid or integrated in chromosomal DNA.

Mammalian cells transformed by simian virus 40 (SV40) harbor viral DNA sequences integrated in their chromosomal DNA and, in general, constitutively express SV40 large T antigen (for a review, see reference 48). T antigen is able to induce and maintain cell transformation, in part through its association with cellular proteins such as p53 and the retinoblastoma susceptibility gene product Rb105 (7; for a review, see references 24 and 48), although our understanding of the transformation process remains very incomplete. Clearly, however, activities of T antigen required for productive infection of monkey cells, such as initiation of viral DNA replication, are not essential for initiation or maintenance of cell transformation (48). The fact that many SV40-transformed cells express T antigens defective in their ability to initiate viral DNA replication has led to speculation that cell transformation by SV40 selects for expression of T antigens that maintain transformation but are no longer able to replicate SV40 DNA, perhaps thereby avoiding rearrangements of chromosomal DNA induced by T antigen (48). Alternatively, because T-antigen replication functions are not required to initiate or maintain cell transformation, replication-defective mutations in T antigen can accumulate in transformed cells (48).

Upon fusion of many SV40-transformed cell lines with permissive monkey cells, viral origins are activated by the resident T antigen, followed by amplification and recombination to yield free rescued SV40 DNA (for a review, see reference 48). This mobilization depends on the presence of an intact origin region and a replication-competent T antigen in the transformed cells (20, 41). In the event that the resident T antigen is replication defective, SV40 DNA can be rescued by fusion with COS1 monkey cells, which constitutively express a replication-proficient T antigen (20, 21). A number of replication-defective T-antigen mutants isolated by this method have proved useful in elucidating the biochemical activities of T antigen required for viral DNA replication, such as SV40 DNA binding and ATPase-helicase activities (for a review, see references 8 and 55).

The SV40-transformed mouse line VLM (66) expresses large T and super T antigens able to bind specifically to both major sites in the SV40 control region and to cleave ATP (33). Fusion of VLM cells with TC7 cells yielded little free SV40 DNA, whereas viral DNA was efficiently rescued from VLM-COS1 fusions, suggesting that the VLM T antigens were almost inactive in replication (33). Thus, it seemed likely that identification of the presumed mutation in VLM T antigen could provide genetic evidence for other biochemical activities possibly involved in SV40 replication.

We report here the characterization of a prototype clone rescued from VLM-COS1 fusions and compare it with genomic VLM DNA. Contrary to prediction, no defect in the origin function, early gene expression, or replication proficiency of the rescued T-antigen gene could be found. However, we demonstrate that mouse DNA sequences present in the SV40 late region of the rescued clone and derived from sequences flanking the SV40 integration site in VLM DNA suppress replication of SV40 plasmid DNA in uninfected monkey cells in an orientation-dependent manner. Furthermore, integrated viral DNA carrying the suppressor sequences in *cis* was rescued fivefold less efficiently from newly created transformed rat cell lines than viral DNA lacking the suppressor elements.

MATERIALS AND METHODS

Cells. All cells were cultured in Dulbecco modified Eagle medium (DMEM) (GIBCO-Bethesda Research Laboratories, Inc., Eggenstein-Leopoldshafen, Federal Republic of Germany) supplemented with 5% fetal calf serum (Hyclone; Greiner, Nürtingen, Federal Republic of Germany). VLM (67), TC7, a subline of CV1 African green monkey kidney cells (49), Rat2 (60), and COS1 (20) cells were described. VLM cells were cloned in soft agar prior to construction of genomic libraries. Seven of eight cell clones analyzed by genomic blotting with SV40 DNA probes revealed the same viral DNA integration pattern described previously (33) (data not shown).

* Corresponding author.
† Present address: Department of Microbiology, University of Southern California, Los Angeles, CA 90033-1054.

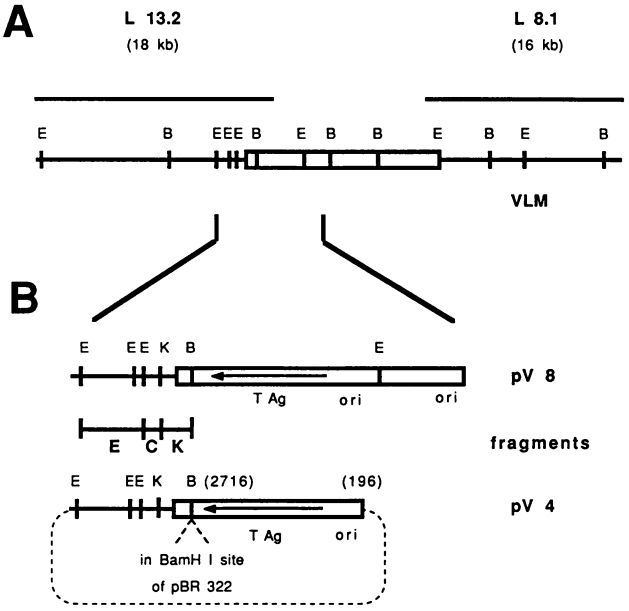

FIG. 1. Restriction maps of the SV40 integration site in VLM DNA and the rescued plasmid clones pV4 and pV8. (A) Diagram of the *Eco*RI (E) and *Bam*HI (B) cleavage sites in the genomic DNA of a representative clone of VLM cells. The map was deduced from Southern blotting of VLM DNA fragments separated by pulse-field gel electrophoresis and analysis of genomic clones (Willnow and Fanning, unpublished data). Mouse DNA is depicted as a black line and SV40 DNA as an open bar. Two genomic clones carrying the SV40-mouse DNA junction regions are indicated above. (B) Region rescued in pV4 and pV8. Both circular DNAs were inserted in the *Bam*HI site of pBR322, as indicated below. Open bars indicate SV40 sequences, horizontal lines indicate mouse DNA, and dashed lines represent plasmid vector DNA. The arrow under the early region shows the direction of transcription of the large-T-antigen (T Ag)-coding sequence. Mouse fragments E, C, and K, common to both rescued clones, were sequenced and characterized (cf. Fig. 2A and B). The diagrams show the cloned DNA linearized at the junction between viral and cellular DNA (SV40 nucleotide 196), as shown. ori, Origin of replication. kb, Kilobases.

Plasmids. pSVwt is wild-type SV40 DNA (strain SV-S) cloned in the *Bam*HI site of pAT153 (14, 62). pV4 and pV8 are prototype viral genomes rescued after VLM-COS1 cell fusion and cloned into the *Bam*HI site of pBR322 (Fig. 1B) (B. Huber, Ph.D. dissertation, Ludwig-Maximilian-University of Munich, Munich, Federal Republic of Germany, 1986). pK1 contains the SV40 genome cloned into the *Eco*RI site of pMK16/6 (21).

Genomic cloning. A genomic library was constructed with partially digested *Mbo*I fragments of genomic DNA from a subcloned line of VLM cells. DNA was cloned into the lambda replacement vector EMBL3 (15), as described previously (34). Bacteriophage clones were grown in *Escherichia coli* K803 (65).

Plasmid constructions. Standard cloning techniques were used for the constructions (40), and all plasmids were propagated in *E. coli* HB101.

The pV4 mouse DNA fragments E1 (1,028 base pairs [bp]), E2 (129 bp), C (254 bp), K (958 bp), Hi (484 bp), Ha (206 bp), and Sa (278 bp) (Fig. 2B) were subcloned in pUC vectors (66). For replication studies, fragments C, K, Hi, Ha, and Sa were cloned into the late region of pSVwt (Fig. 3).

All SV40 nucleotide numbers are given in the BBB system (59). The pV4 nucleotide numbering is simply continued from the viral-cellular DNA junction site (SV40 nucleotide 196) (Fig. 2A). Thus, the pV4 *Bam*HI site in the SV40 region of pV4 is at pV4 nucleotide 2716 rather than at 2533, as in the BBB system.

In pSV-KV4, the SV40 *Kpn*I-*Bam*HI fragment (nucleotides 294 to 2533) was replaced by the pV4 K segment (*Kpn*I-*Bam*HI; 1758 to 2716). pSV-CV4 harbors the C fragment (*Eco*RI-*Kpn*I; 1504 to 1758) in the opposite orientation to that of pV4, replacing the SV40 *Kpn*I-*Eco*RI (294 to 1782) sequences. In pSV-ΔN, the 1,482-bp *Ban*II fragment (778 to 2258) was simply deleted. The subcloned Sa segment (*Acc*I-*Hin*cII; 1964 to 2242) in pUC-SaV4 was excised by using the *Kpn*I and *Hin*dIII sites of the pUC18 polylinker and inserted in place of the SV40 *Kpn*I-*Hin*dIII fragment (294 to 1708) of pSVwt, resulting in pSV-SaV4.

pSV-sSaV4 contains the Sa fragment shifted 1.3 kilobases upstream of the origin-promoter region. For this purpose, the SV40 fragment of pSVwt *Acc*I-*Bam*HI (nucleotides 1628 to 2533) was replaced by the pV4 *Acc*I-*Bam*HI segment (1964 to 2716). pSV-HaV4 bears the Ha sequences *Kpn*I-*Acc*I (1758 to 1964) in pSVwt instead of the original SV40 segment (*Kpn*I-*Acc*I; 294 to 1628). In pSV-iHV4, the Hi fragment (*Kpn*I-*Hin*cII; 1758 to 2248) was inserted into plasmid pSV-ΔN in the opposite orientation to that in pSV-KV4 by replacing the SV40 *Kpn*I-*Pvu*II fragment (294 to 270).

Constructions derived from pV4 are pV-ΔE, from which the E1 and E2 fragments (*Eco*RI-*Eco*RI; nucleotides 293 to 1504) were deleted, and pV-Kwt, which contains pSVwt late-region sequences (*Kpn*I-*Bam*HI; 294 to 2533) instead of the pV4 K fragment (*Kpn*I-*Bam*HI; 1758 to 2716).

The Hi (*Kpn*I-*Hin*cII; 1758 to 2242) and Ha (*Kpn*I-*Acc*I; 1758 to 1964) segments were inserted into the *Kpn*I site (nucleotide 294) of pSV2Cat (22). Thus, pSV-HiV4Cat and pSV-HaV4Cat contain these fragments upstream of the SV40 enhancer repeats (Fig. 4). In pSV-pV4Cat, the entire origin-promoter region of pV-ΔE (*Hin*dIII-*Pvu*II; 5171 to 381) replaces the original sequences (*Hin*dIII-*Pvu*II; 5171 to 270) of pSV2Cat.

DNA replication. Semiconfluent TC7 cells (10^6 per 100-mm dish) were washed with Tris-buffered saline and transfected with precisely determined concentrations of uncut plasmid DNA (0.25 to 1.0 μg) in DEAE-dextran, as described previously (61). After 45 min at 37°C, the cells were washed again and supplied with fresh medium. Low-molecular-weight DNA (30) was isolated after 2 days.

As a control, input DNAs were linearized and analyzed by gel electrophoresis in parallel with the Hirt supernatant DNAs. SV40 plasmid DNA was detected by blot hybridization (54) by using ^{32}P-nick-translated (47) pBR322 or pAT153 DNA as a probe to ensure that all plasmids would yield a hybridization signal in proportion to their concentrations, regardless of the different SV40 and cellular sequences present in the variant late-region constructs.

DNA sequencing and analysis. Sequencing was performed by using the dideoxy-chain extension method with double-stranded DNA (6). The large fragment of *E. coli* DNA polymerase I (Pharmacia, Freiburg, Federal Republic of Germany) or chemically modified T7 DNA polymerase (U.S. Biochemicals-Renner, Dannstadt, Federal Republic of Germany) was applied as described previously (57).

The late region of pV4 containing the cellular insertion was subcloned in the sequencing vectors pUC18 and pUC19 (66). The sequence of the fragments was determined from

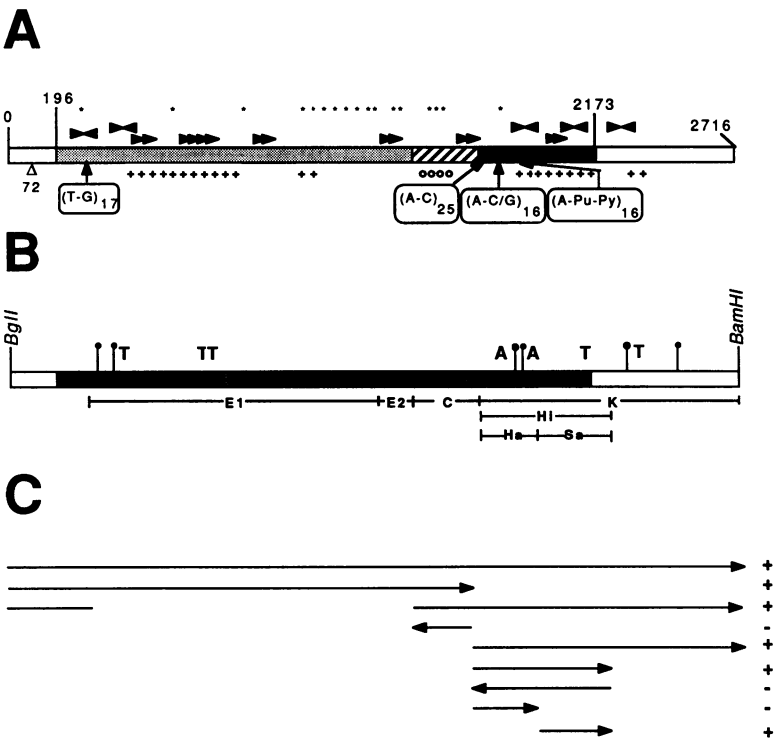

FIG. 2. Organization and sequence features of the pV4 late region. (A) ▭, SV40 DNA starting at the *Bgl*I site (nucleotide 0) and extending to the viral *Bam*HI site; ▩, ▨, and ■, mouse DNA sequences replacing the SV40 late region from nucleotides 196 to 2173. The different shading patterns indicate the fragments E1 plus E2, C, and the cellular segment of the K fragment. One copy of the 72-bp repeat is missing in pV4 (Δ72). ▼, Positions of di- and trinucleotide repeats; ⋈ and ➡, locations of inverted and direct repeated sequences of 11 to 19 nucleotides, respectively; ★, CpG dinucleotides; + and ○, AT- and GC-rich regions, respectively. (B) The same region as that in panel A is shown, indicating the restriction fragments analyzed in this report (below the bars). ▭, SV40 sequences; ■, mouse sequences with no detectable homology to SV40: ▩, mouse sequences with up to 25% homology with SV40 DNA; ▾, possible topoisomerase II cleavage sites (13 matches with the 15-nucleotide consensus) (51); A and T, A and T boxes, respectively, found in SAR sequences of *Drosophila* spp. (9 matches with a 10-nucleotide consensus sequence) (17). (C) SV40 DNA replication-suppressing activity of each of the mouse DNA segments tested is given at the right (+, suppression; −, no suppression). The orientation of each fragment tested is indicated (➡, ⬅).

both directions by using the pUC reverse primer (a gift from P. Heinrich) (5′-CAGGAAACAGCTATGAC-3′) and specific internal primers. Sequence analysis and homology searches were carried out by using previously described programs (18, 37, 45). The entire sequence will be made available upon request (GenBank accession no. M33654).

CAT assay. Calcium phosphate DNA precipitates were prepared with 15 μg of plasmid DNA (23) and applied to 60% confluent TC7 cells (100-mm dishes) by published methods (22).

After 4 h, the cells were shocked with 20% dimethylsulfoxide in DMEM for 2 min and incubated for another 48 h. Protein extracts were prepared as described previously (35), and 150 μl of clarified lysate corresponding to 300 μg of protein was mixed with 20 μl of 4 mM acetyl coenzyme A (Pharmacia) and 2 μl of [^{14}C]chloramphenicol (New England Nuclear, Dreieich, Federal Republic of Germany). After 1 h of incubation at 37°C, chloramphenicol and acetylated derivatives were analyzed by ascending thin-layer chromatography as described previously (35).

RESULTS

Isolation and characterization of SV40 genomes from VLM cells. A subcloned line of VLM cells was fused with COS1 monkey cells (20). Supercoiled SV40 DNA rescued from VLM-COS1 fusions was purified by centrifugation in equilibrium density gradients as described previously (21), linearized with *Bam*HI, and ligated into the *Bam*HI site of pBR322 to yield two major families of plasmid DNA (Huber, Ph.D. dissertation). Prototype plasmids pV4 and pV8 were chosen for further characterization. Restriction mapping and blot hybridization experiments (Fig. 1B) revealed that pV4 bears a subset of the SV40 sequences found in pV8 and that both clones carry the same 2-kilobase mouse DNA sequence in place of the SV40 late region (M. Hartl, Ph.D. dissertation, Ludwig-Maximilian-University of Munich, Munich, Federal Republic of Germany, 1989). pV4 contained an apparently intact SV40 early region and origin-promoter-enhancer region (Hartl, Ph.D. dissertation).

The structure of the rescued clones was then compared with that of the integrated SV40 DNA in VLM cells. A single integration site of tandemly repeated SV40 DNA was detected by detailed blot hybridization analysis of VLM DNA (33; Hartl, Ph.D. dissertation; Huber, Ph.D. dissertation). The mapping was confirmed and extended by restriction mapping analysis of genomic clones of VLM DNA bearing SV40 sequences (Fig. 1A) (T. Willnow and E. Fanning, unpublished data). Apparently, the rescued clones were created by recombination between cellular sequences adjacent to the E fragment and viral sequences adjacent to the

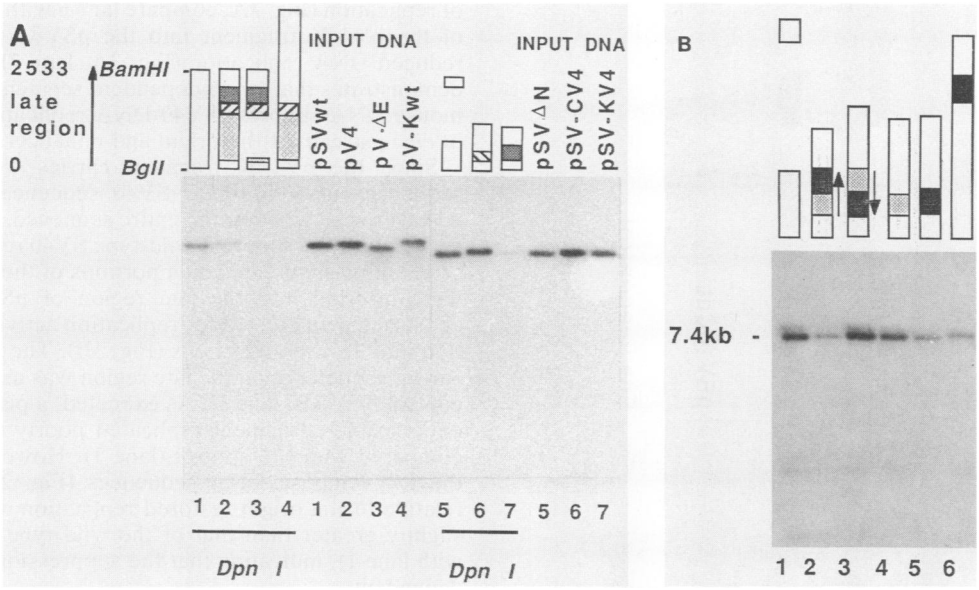

FIG. 3. Replication of SV40 plasmid DNA bearing different parts of the pV4 cellular sequence. (A) Late-region constructs are depicted by bars above each lane. Open bars represent wild-type SV40 DNA, and different shaded bars represent the various arrangements of pV4 fragments (note the reverse orientation of the C-V4 segment in lanes 6). Numbers at the bottom of each lane denote the corresponding samples of each Hirt supernatant and input DNA. Light shading indicates fragment E1 plus E2; hatching indicates fragment C and its orientation; dark shading indicates mouse sequences in fragment K. Supercoiled plasmid DNA was transfected into TC7 cells and low-molecular-weight DNA was isolated after 2 days, as described in Materials and Methods. One-fifth of the sample was digested with *Dpn*I prior to linearization with *Sal*I. A 3-ng sample of each type of *Sal*I-digested input DNA was analyzed in parallel by electrophoresis in a 1% agarose gel and blot hybridization against nick-translated pBR322 DNA (5×10^7 cpm/μg). Autoradiography was done for 6 h. The amount of newly replicated DNA was evaluated by microdensitometry and expressed as a percentage of the wild-type DNA. Lanes: 1, 100%; 2, 34%; 3, 18%; 4, <10%; 5, 100%; 6, 98%; 7, 8%. (B) Supercoiled DNA (250 ng) was transfected into semiconfluent TC7 cells, and low-molecular-weight DNA was isolated, digested with *Dpn*I and *Cla*I, and analyzed as in panel A. Lane 1, pSV-ΔN (7.4 kilobases); lane 2, pSV-KV4; lane 3, pSV-iHV4; lane 4, pSV-HaV4; lane 5, pSV-SaV4; lane 6, pSV-sSaV4. The smear visible above the bands in some lanes was caused by a slight displacement during blotting. Microdensitometry of the autoradiogram was used to compare the amounts of newly replicated DNA. Lane 1, 100%; lane 2, 25%; lane 3, 120%; lane 4, 70%; lane 5, 24%; lane 6, 27%. kb, Kilobases. Open bars represent SV40 DNA; light shading indicates the Ha fragment; solid bars show the Sa fragment (see Fig. 2B for a map).

late side of the enhancer region (Fig. 1B). Thus, the rescued clones pV4 and pV8 are colinear with VLM chromosomal sequences and span the mouse-SV40 junction region on one side of the integration site.

The origin region and late region of pV4 were characterized in greater detail by determination of the DNA sequence (Hartl, Ph.D. dissertation). Interestingly, much of the mouse sequence displayed up to 25% homology with SV40 sequences, whereas the remainder shared no sequence homology with SV40 DNA (Fig. 2B). Repetitive sequences were dispersed throughout most of the mouse-derived portion of the late region (Fig. 2A; Hartl, Ph.D. dissertation). Repetitive $(GT)_n$ and trinucleotide stretches were located at both ends of the mouse sequence (Fig. 2A). However, restriction fragment C (Fig. 2B) carried only single-copy mouse sequences, as shown by blot hybridization analysis of VLM and mouse L-cell DNAs (Hartl, Ph.D. dissertation). Extensive regions of direct and inverted repeats were found, particularly clustered in A+T-rich portions of the sequence (Fig. 2A). A large cluster of CpG dinucleotides was located in the central portion of the mouse sequence.

In the early region and control region of pV4, several nucleotide changes with respect to SV-S strain SV40 DNA were also noted. Nucleotide 4839 was changed from C to T, creating an *Acc*I site but no amino acid change in the T-antigen-coding sequence. Nucleotide 5209 was changed from C to T, and nucleotide 81 was deleted. All these sequence changes were also found in sequencing the Baylor SV40 strain used to derive the VLM line and thus are strain specific (67; Hartl, Ph.D. dissertation; R. Lanford, personal communication). In addition, one of the 72-bp repeats was deleted (nucleotides 179 to 250). Both the origin region of pV4 (Hartl, Ph.D. dissertation) and the enhancer (Fig. 4) were fully functional in TC7 cells.

Cellular DNA sequences suppress SV40 plasmid replication. Contrary to our expectations from the fusion experiments, the early region of pV4 was able to replace wild-type T-antigen-coding sequences of plasmid pK1 in DNA replication assays in TC7 cells, demonstrating that it was fully functional when placed in a wild-type SV40 background (Hartl, Ph.D. dissertation).

The apparent contradiction between this result and those from the cell fusion experiments (33) raised the question whether the mouse sequences in the late regions of pV4 and pV8 could affect SV40 DNA replication or mobilization upon cell fusion. To test this idea, SV40 plasmids carrying pV4 mouse DNA in whole or in part were constructed and assayed for replication in monkey cells. The following two groups of constructs were used: one set in which cellular sequences were deleted from pV4 or replaced by wild-type viral sequences and a second set in which pV4 cellular DNA was inserted into a wild-type SV40 plasmid. Each clone had an intact origin of replication and encoded a functional T antigen, derived from either pSVwt or pV4. All the clones expressed T antigen after transfection of TC7 cells, as judged

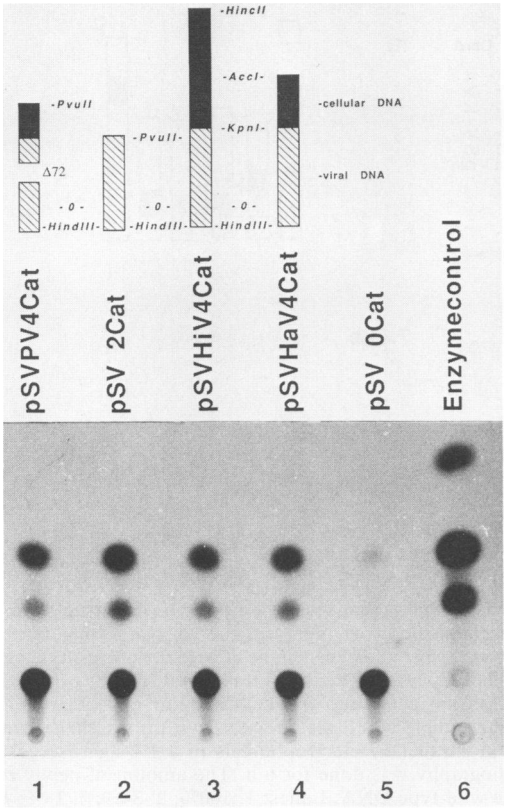

FIG. 4. CAT activity of constructs containing cellular sequences linked to the SV40 early promoter. The structure of the CAT clones is depicted above each lane. The negative control pSV0Cat lacks the entire SV40 early promoter (36). Transfection and CAT assays were performed as described in Materials and Methods. Acetylated products were detected by thin-layer chromatography followed by autoradiography for 48 h.

by immunoperoxidase staining with an antibody specific for T antigen (data not shown).

The clones were tested for replication by transfection into TC7 cells, followed 2 days later by isolation and analysis of Hirt supernatant DNA (Fig. 3A). Replication of pV4 was significantly reduced compared with pSVwt, as demonstrated by the smaller amount of *Dpn*I-resistant pV4 (Fig. 3A, lane 2) compared with pSVwt (lane 1) DNA. Comparison of the input DNAs (Fig. 3A, lanes 1 and 2) demonstrated that equal amounts of DNA were transfected. To identify the sequences responsible for the observed reduction in replication efficiency, the two *Eco*RI fragments were deleted from pV4 DNA. Replication of the resulting plasmid dropped to an even lower level than that of pV4 (Fig. 3A, lane 3), indicating that the distal part of the cellular region (fragment K) was sufficient to suppress replication. Conversely, the E fragment (E1, E2, and C) was also sufficient to suppress plasmid replication (Fig. 3A, lane 4) in the absence of the K fragment.

The clones of the second set are almost identical in size and bear the early region and origin-promoter region of pSVwt DNA. The plasmid pSV-ΔN (Fig. 3A, lane 5), from which viral sequences in the late region were deleted, was used as a control, demonstrating that the reduced replication observed with pV4 and its derivatives was not caused by the absence of the late region. Insertion of the pV4 C fragment into pSVwt DNA did not cause any detectable suppression of replication (Fig. 3A, compare lane 6 with lane 5). Insertion of the pV4 K fragment into the pSVwt plasmid severely reduced DNA replication (Fig. 3A, lane 7). Figure 3A thus demonstrates that two independent segments of pV4 cellular mouse DNA suppress SV40 DNA replication when located in *cis* upstream of the origin and enhancer.

Since the pV4 K fragment carries 484 bp of mouse sequences in addition to SV40 sequences (Fig. 2B), we wished to test whether the entire sequence, or only part of it, was required to suppress wild-type SV40 replication. A third series of plasmids, in which portions of the pV4 K fragment were inserted into the late region of pSVwt DNA, was constructed and tested for replication activity after transfection into TC7 monkey cells (Fig. 3B). The plasmid pSV-ΔN carrying a deletion in the late region was used as a wild-type control (Fig. 3B, lane 1). As expected, a plasmid bearing the entire pV4 K fragment replicated poorly (Fig. 3B, lane 2) compared with the control (lane 1). However, inversion of the K-V4 mouse DNA sequences (Fig. 2B, fragment Hi) relative to the origin restored replication activity to a level slightly greater than that of the wild type (compare lane 3 with lane 1), indicating that the suppression was orientation dependent.

The cellular sequences of the K fragment were dissected into two portions, the Ha (206 bp) and Sa (278 bp) fragments (Fig. 2B), and each was inserted into pSVwt DNA. The plasmid bearing the Ha fragment replicated with nearly wild-type efficiency (Fig. 2B, lane 4), whereas the Sa-bearing plasmid (lane 5) replicated more weakly than the wild type but better than pSV-KV4. Insertion of the Sa fragment 1.3 kilobases more distal to the origin-enhancer region resulted in the same reduction in replication observed with the origin-proximal Sa plasmid (Fig. 2B, compare lanes 5 and 6). These results demonstrate that a 278-bp segment of the pV4 K fragment is sufficient to suppress replication of wild-type SV40 DNA, in a manner independent of distance but dependent on orientation relative to the origin-enhancer region.

SV40 promoter-enhancer activity is not suppressed by pV4 mouse DNA sequences. Although the pV4 origin and early regions were shown to be functional (Hartl, Ph.D. dissertation), it was conceivable that the expression of replication-competent T antigen could be limited by the presence of the pV4 cellular sequences upstream of the promoter-enhancer region. To address this question, the pV4 origin-promoter-enhancer region was placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene and CAT enzyme activity was assayed after transfection of plasmid DNA into TC7 cells. The CAT activity expressed from the pV4Cat construct was comparable to that expressed from the wild-type control pSV2Cat (Fig. 4, lanes 1 and 2). The Hi fragment bearing the entire cellular portion of the pV4 K fragment which showed replication suppression activity, or the suppressor-negative Ha fragment (Fig. 3A and B), was placed upstream of the enhancer in pSV2Cat. CAT activity measured with both these constructs was indistinguishable from that measured with the pSV2Cat control plasmid (Fig. 4, compare lanes 3 and 4 with lane 1). These results indicate that the replication suppression activity of the pV4 mouse DNA sequences is unlikely to be due to their effect on promoter-enhancer activity.

Mouse DNA sequences suppress mobilization of chromosomal SV40 DNA. The ability of pV4 mouse DNA sequences to suppress replication of wild-type SV40 plasmid DNA (Fig. 3A and B) suggested that these sequences might also affect the efficiency of rescue of SV40 genomes from transformed rodent cells by cell fusion. This idea was tested by construct-

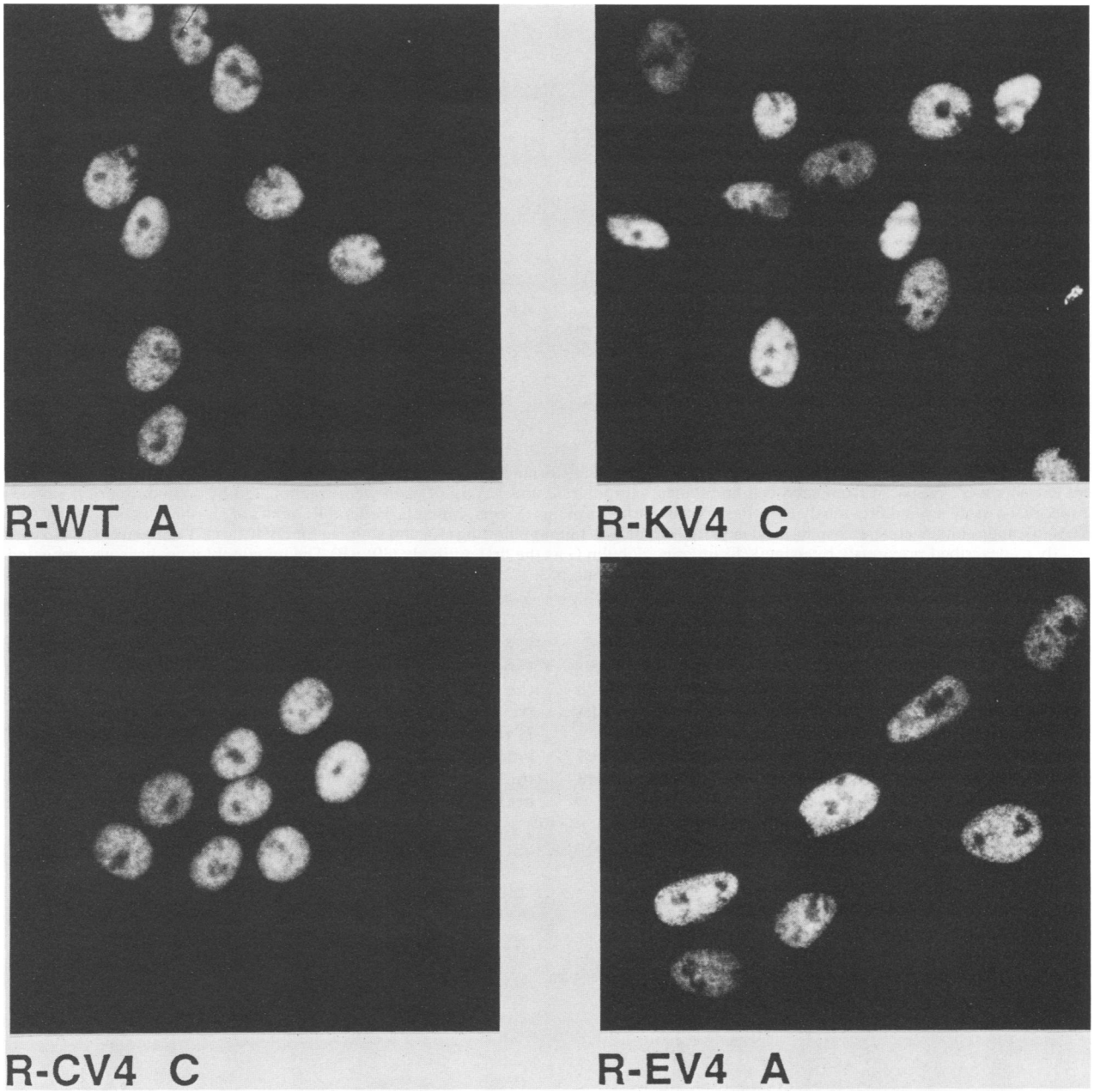

FIG. 5. Transformation of Rat2 cells by SV40 DNA carrying mouse DNA sequences in *cis*. Rat2 cells were transfected with *Sal*I-*Cla*I–linearized SV40 plasmid DNA carrying the K, C, or E fragment of pV4 or without an additional fragment (Fig. 1B). Foci, picked after 20 days, were purified in soft agar as described previously (52). Single colonies were taken to establish the cell lines R-WT, R-CV4, R-KV4, and R-EV4. Several colonies of each type (lettered A and C) were characterized in detail. Semiconfluent dishes (2×10^5 cells per 60-mm dish) were fixed by using 3% paraformaldehyde–0.1% Triton X-100 in phosphate-buffered saline. T antigen was visualized by indirect immunofluorescence staining (53) using 4 μg of Pab416 and Pab 419 (27) per ml as the first antibody. Cells were photographed by using a Zeiss IM35 microscope equipped with a Plan-Neofluar63 oil immersion objective.

ing a series of SV40-transformed Rat2 cell lines with or without pV4 mouse sequences in *cis*. Wild-type SV40 plasmid DNA or SV40 plasmids carrying insertions of the pV4 E (E1 + E2 + C), C, or K fragments in the late region were transfected into Rat2 cells. The plasmid DNAs showed nearly equal transformation activity, and all four types of transformants grew in soft agar. Colonies isolated from soft agar were expanded into cell lines and characterized. All the cells expressed nuclear T antigen (Fig. 5). Immunoblot analysis of protein extracts from four representative transformed lines demonstrated that all of the lines expressed similar amounts of T antigen of wild-type size and similar amounts of p53 (Fig. 6A and B).

Blot hybridization analysis of genomic DNA from each transformed line digested with *Bgl*II, which does not cleave any of the transfected DNAs, revealed that one of the lines

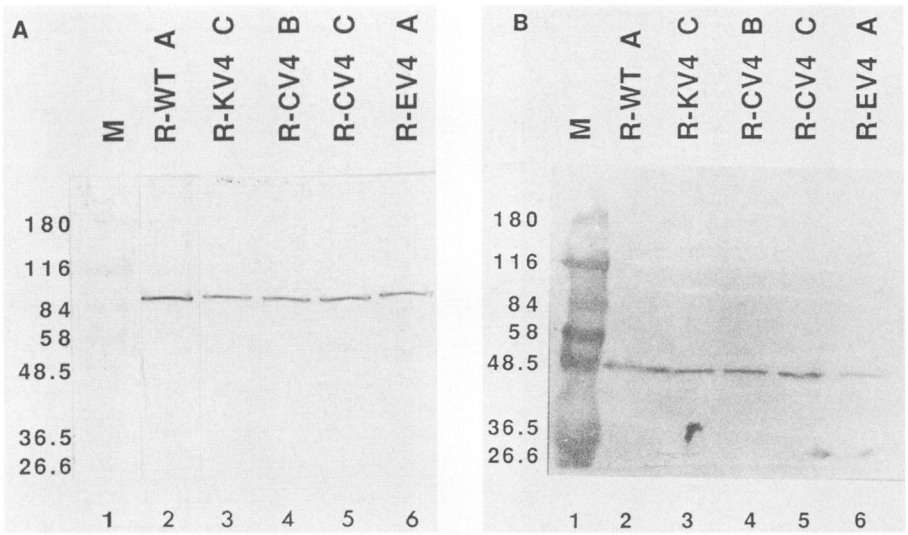

FIG. 6. Immunoblots of protein extracts from rat cell lines transformed by SV40 DNA with and without pV4 mouse DNA in *cis*. (A) Protein extracts were prepared from each cell line (lanes 2 through 5), and 125 μg of each were fractionated by sodium dodecyl sulfate-gel electrophoresis, as described previously (53). Prestained marker proteins (Sigma, Munich, Federal Republic of Germany) of known M_r (listed at the left in kilodaltons) were electrophoresed in parallel (lane M). Immunoblotting (12) and staining for SV40 large T antigen (53) were carried out exactly as described previously by using KT3 immunoglobulin G as the first antibody (39). (B) The immunoblot analysis was repeated by using Pab 122 culture supernatant (25) as the first antibody to stain p53.

(Fig. 7, lane 5) had one major integration site of viral DNA, whereas the others had two insertions of viral sequences (Fig. 7, lanes 2 through 4). Blot hybridization analysis with *Bam*HI-cleaved genomic DNAs confirmed these results (data not shown).

Each of the transformed lines was fused with TC7 monkey cells to rescue the integrated viral DNA (Fig. 8). Abundant low-molecular-weight DNA was generated from the wild-type-transformed line (Fig. 8, lane 1) and the line carrying the pV4 C fragment (lane 2). The amount of low-molecular-weight SV40 DNA rescued from the lines bearing the K and E fragments was reduced by more than 80 to 90% (Fig. 8, lanes 3 and 4), as shown by microdensitometric scanning of the autoradiograms. Similar results were obtained with several independent lines of each type of transformant (Hartl,

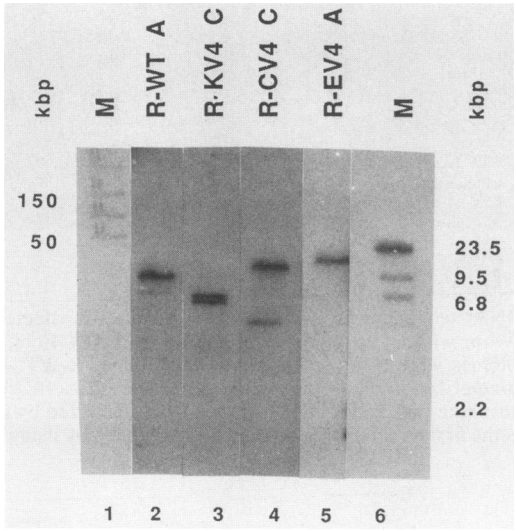

FIG. 7. Genomic blots of rat clones transformed by SV40 DNA with and without pV4 mouse DNA in *cis*. Genomic DNA (20 μg) isolated as described previously (56) from cloned rat lines was digested with *Bgl*II and fractionated by pulse-field gel electrophoresis. Marker DNAs (lanes M) were electrophoresed in parallel (lane 1, ligated lambda DNA; lane 6, *Hin*dIII-digested lambda DNA). Lanes 2 through 5 were blotted and hybridized with random-primed (Amersham, Braunschweig, Federal Republic of Germany) ^{32}P-labeled pSVwt DNA. kbp, Kilobase pairs.

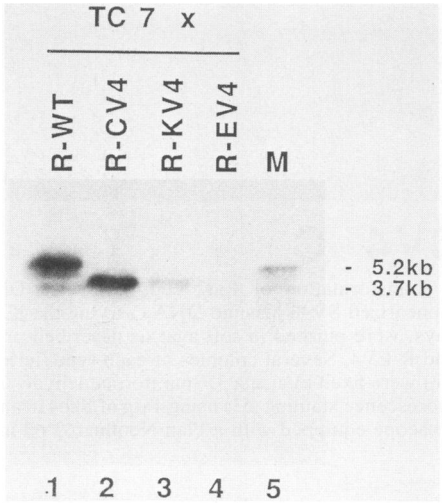

FIG. 8. Rescue of SV40 DNA from transformed rat cell lines by fusion with TC7 monkey cells. TC7 cells (2×10^6) were fused (×) with each of the indicated rat lines (10^6) as described previously (33). Half the low-molecular-weight DNA obtained from each fusion was digested with *Bam*HI and analyzed by blot hybridization with nick-translated pSVwt DNA (specific activity, 10^8 cpm/μg) (lanes 1 through 4). *Bam*HI-digested pSVwt DNA served as a size marker (lane M). The blot was autoradiographed for 30 h. kb, Kilobases.

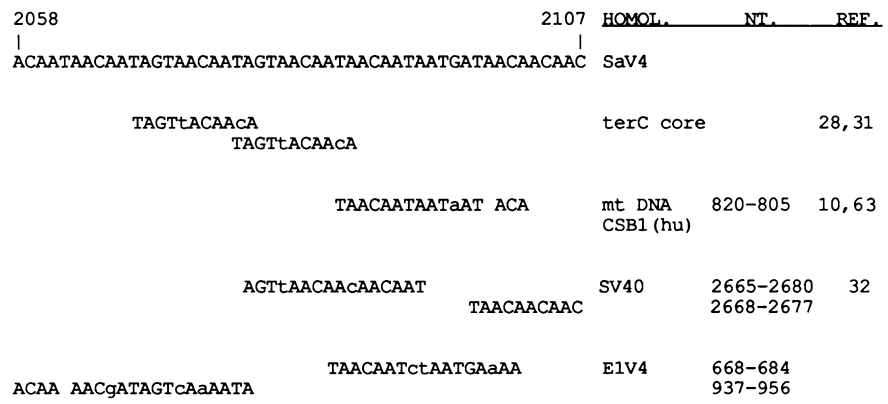

FIG. 9. Replication suppressor fragment Sa (209 bp) carries sequences homologous to the pV4 E1 fragment, human (hu) mitochondrial (mt) D loop DNA, *E. coli* replication terminator core sequence, and SV40 termination region. Nucleotide matches are given in uppercase letters, and mismatches are given in lowercase letters. For details, see text. Homol., Homology; NT., nucleotides; REF., reference.

Ph.D. dissertation), indicating that the presence of a plasmid replication suppression element flanking the integrated SV40 DNA is correlated with inefficient mobilization of the viral DNA after cell fusion and suggesting that these elements may be responsible for the inefficient rescue observed.

DISCUSSION

Mouse DNA sequences rescued together with SV40 DNA from the SV40-transformed cell line VLM have been shown to severely suppress viral DNA replication in *cis* in TC7 monkey cells (Fig. 3) and the mobilization of integrated SV40 DNA upon fusion with TC7 cells (Fig. 8). A comparable degree of suppression was observed in both approaches.

Two separate segments of the mouse DNA were shown to have replication suppression activity, as assayed by plasmid replication and by viral DNA rescue from cell fusions. Control experiments ruled out an effect of the mouse replication suppressor elements on the activity of the SV40 early promoter-enhancer in transient assays (Fig. 4) and on the steady-state level of T antigen in the transformed rat lines used for viral DNA rescue (Fig. 5 and 6A). The supply of replication-competent T antigen in the cell fusion experiments could be limited by complex formation with p53, which has been shown to inhibit SV40 replication (3, 16, 58, 64). However, all the transformed rat lines contained similar steady-state amounts of p53 (Fig. 6B), thus excluding this explanation for the rat cell fusion results.

The supply of active T antigen could also be limiting if it was sequestered by the cellular DNA sequences. A similar mechanism has been proposed to explain the limited SV40 plasmid replication observed in cells transformed by SV40-bovine papillomavirus hybrid plasmids (50). If a similar mechanism was operating in the experiments presented here, we would expect the cellular sequences to suppress plasmid replication in a manner independent of orientation relative to the origin and also when located in *trans*. However, inversion of one cellular suppressor element restored wild-type replication (Fig. 3B, lane 3) and no inhibition of plasmid replication was detected when the suppressor elements were cotransfected in *trans* with the SV40 plasmid (Hartl, Ph.D. dissertation). Thus, T-antigen sequestration by the cellular suppressor elements can be ruled out as an explanation for their suppression activity.

In summary, then, the presence of pV4 cellular sequences in *cis* appears to be directly responsible for the reduced replication of SV40 plasmid DNAs in TC7 cells and the inefficient mobilization of viral DNA in transformed rat cells after fusion.

DNA sequences in the pV4 replication suppressor elements. The general features of pV4 mouse DNA are depicted in Fig. 2A and B. Those regions of the sequence tested for replication suppression activity in both the plasmid and viral DNA rescue assays are summarized in Fig. 2C, which shows the orientation of each sequence tested. In general, the fragments with suppression activity are rich in A and T (Fig. 2A). The shortest segment with suppressor activity (Sa) contains 278 bp, of which 209 bp are mouse sequences. Although it is not yet clear which of the 209-bp sequences are responsible for the suppressor activity, we searched for homology between these sequences and the other regions of the pV4 late region. Two sequences with good homology to the Sa fragment were identified in the E1 fragment, within the replication-suppressing E fragment (Fig. 9). Several other homologies with sequences in the replication termination regions of SV40 (32), R6K, and *E. coli* chromosomal DNA (28, 31) as well as a highly conserved sequence in the D loop of mammalian mitochondrial DNA (10, 63) (Fig. 9) were identified.

On the basis of these homologies, it is tempting to speculate that the replication suppressors may act as terminators or pause sites for replication forks. The sequences responsible for replication fork pausing in vivo and the mechanisms involved are not well understood in eucaryotic cells (8). In contrast, in procaryotic chromosomes, not only the sequences required for replication termination but also the proteins involved and their function have been elucidated (28, 29, 31, 34a). Like the pV4 replication suppressor in the Hi fragment (Fig. 3B), the *E. coli* chromosomal terminators are inactive in the inverted orientation (29). Moreover, preliminary evidence suggests that a *trans*-acting factor in monkey cells is required to observe the Hi suppressor activity (M. Hartl and E. Fanning, unpublished data). Termination regions of mammalian chromosomal replication were shown to overlap with matrix or scaffold attachment regions (9, 26), characterized in many cases by the presence of potential topoisomerase II cleavage sites and sequences known as A and T boxes (17, 43). Such sequences are clustered at both ends of the pV4 mouse sequences (Fig. 2B). Two-dimensional gel electrophoresis of replicating plas-

mid DNA (4) should reveal whether the suppression is in fact caused by termination or pausing of replication forks.

Possible implications for chromosomal replication and recombination. The colinearity of the rescued plasmids with VLM chromosomal DNA suggests, but does not prove, that VLM cells express a wild-type T antigen. Assuming that they do, the question arises why the VLM-TC7 cell fusions yield so little rescued viral DNA relative to the amounts from VLM-COS1 fusions (33). A similar result was reported for cell fusions with MKS-A cells, another SV40-transformed mouse cell line (11, 33). The SV40 genomes rescued from these cells also encoded replication-competent T antigen (11), suggesting that a common mechanism may be responsible for the inefficient rescue observed.

It seems unlikely that rodent cells or even mouse cells are generally unable to permit efficient SV40 rescue after fusion with monkey cells, since more mouse cells mobilize SV40 DNA equally well after fusion with COS1 and other monkey cells (19, 33). Dora et al. (11) suggested that murine p53, present in elevated amounts in MKS-A cells and capable of inhibiting SV40 replication (3, 16, 58, 64), could explain the paradox, an explanation that would also apply to VLM-TC7 fusions. The improved mobilization observed in COS1 fusions would then be attributed to the excess of T antigen not complexed to p53 or more weakly associated with monkey p53 (13; D. Lane, personal communication). Another plausible explanation for the poor rescue from VLM-TC7 fusions comes from the presence of super T antigens in VLM cells, which in other cell lines have always been found replication defective (for a review, see reference 48). If any of these aberrant VLM T antigens interfered with the activity of the functional T antigen (68 and references therein), one might expect to overcome the interference by supplying an excess of replication-competent T antigen through a VLM-COS1 fusion, in agreement with the observations reported previously (33).

The results in this paper suggest that a third mechanism may contribute to the efficiency of SV40 rescue from cell fusions. The nature of the host DNA flanking the SV40 integration site has been demonstrated to influence SV40 rescue from transformed rat lines with equivalent levels of T antigen and p53 (Fig. 6 through 8). However, it is not yet clear whether this mechanism is involved in the different rescue efficiencies in VLM-TC7 and VLM-COS1 fusions.

Nevertheless, the pV4 suppressor elements may play a role in the regulation of mouse chromosomal DNA replication. For example, rat DNA flanking the integrated polyomavirus DNA in transformed rat cells harbors sequences that terminate polyomavirus amplification in chromosomal DNA and, like the pV4 elements, reduce the level of SV40 DNA replication in monkey cells (1, 2, 46). Moreover, the role of the pV4 mouse DNA sequences may not be restricted to chromosomal replication. The remarkable frequency with which the pV4-like DNA was rescued suggests that recombination may be favored in this region of the mouse DNA (5, 38, 42). Unusual recombination events associated with the mobilization of integrated polyomavirus DNA from transformed mouse cells have also been noted (44).

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