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DNA Sequence Homology between the Terminal Inverted Repeats of Shope Fibroma Virus and an Endogenous Cellular Plasmid Species

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DNA hybridization experiments indicate that the genome of a tumorigenic poxvirus, Shope fibroma virus (SFV), possesses sequence homology with DNA isolated from uninfected rabbit cells. Southern blotting experiments, either with high-complexity rabbit DNA as probe and SFV restriction fragments as targets or with high-specific activity, ^{32}P-labeled, cloned SFV sequences as probes and rabbit DNA as target, indicate that the homologous sequences map at two locations within the viral genome, one in each copy of the terminal inverted repeat sequences. Unexpectedly, Southern blots revealed that the homologous host sequences reside in a rabbit extrachromosomal DNA element. This autonomous low-molecular-weight DNA species could be specifically amplified by cycloheximide treatment and was shown by isopycnic centrifugation in cesium chloride-ethidium bromide to consist predominantly of covalently closed circular DNA molecules. DNA sequencing of pSIC-9, a cloned 1.9-kilobase fragment of the rabbit plasmid species, indicated extensive homology at the nucleotide level over a 1.5-kilobase stretch of the viral terminal inverted repeat. Analysis of open reading frames in both the plasmid and SFV DNA revealed that (i) the N-terminal 157-amino acid sequence of a potential 514-amino acid SFV polypeptide is identical to the N-terminal 157 amino acids of one pSIC-9 open reading frame, and (ii) a second long pSIC-9 open reading frame of 361 amino acids, although significantly diverged from the comparable nucleotide sequence in the virus, possessed considerable homology to a family of cellular protease inhibitors, including α1-antichymotrypsin, α1-antitrypsin, and antithrombin III. The potential role of such cellular plasmid-like DNA species as a mediator in the exchange of genetic information between the host cell and a cytoplasmically replicating poxvirus is discussed.

Certain members of the poxvirus family have been known for many years to be the etiologic agents for a variety of tumors found in the wild (2, 28, 34). Unlike other DNA tumor viruses, the poxvirus genome replicates within virosomes or "factories" in the cytoplasm of infected cells, and it is believed to encode all or most of the enzymes required for its own replication. Thus, poxviruses are less dependent upon host cell functions than are any other animal virus group (for reviews, see references 10, 18, 25, 25a, and 43).

Shope fibroma virus (SFV), a member of the genus leporipoxvirus, is of special interest as a prototype of the tumorigenic poxviruses because it grows well in tissue culture, induces characteristic fibromas in rabbits and is amenable to analysis at the molecular level (15, 34). The physical map of the viral DNA has recently been deduced, and the complete genomic library in plasmid vectors is available (7, 11, 42). The SFV genome is 160 kilobases (kb) in size (11) and has a coding capacity for in excess of 100 proteins. It is of particular interest that different strains of SFV vary in tumorigenicity and that isolates can spontaneously lose their oncogenic potential without loss of infectivity (15), implying that the genetic information governing tumor induction is highly variable. This is strikingly reminiscent of observations with regard to members of the orthopoxvirus genus, such as vaccinia virus, in which spontaneous DNA rearrangements occur with high frequency at or near the terminal inverted repeat sequences (TIR) of the viral genome (1, 13, 14, 24, 26), and suggests that a relatively small region of the SFV genome may be responsible for the tumorigenic potential of this virus.

Reports that the genomes of several herpesviruses (herpes simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus) contain sequences homologous to mammalian cell DNA (27, 29, 30) prompted us to ask whether SFV DNA possesses detectable homology with host rabbit DNA and, if so, whether these sequences might also be implicated in the tumorigenicity of this virus. Although poxvirus morphogenesis is believed to be entirely cytoplasmic, and the mechanism by which a poxvirus could capture host genomic DNA is unclear, recent evidence that the thymidine kinase and a 19,000-molecular-weight protein of vaccinia virus share significant amino acid sequence homology with chicken thymidine kinase (22) and transforming growth factor α (5, 6), respectively, suggests that an exchange of genetic information between host and poxviruses is possible. Here we report that SFV possesses demonstrable DNA sequence homology with an endogenous cellular plasmid species and suggest that small polydisperse circular (spc) DNA molecules may potentially function as intermediates for the exchange of genetic information between poxviruses and their host cells.

MATERIALS AND METHODS

Cells and viruses. SFV (strain Kasza) was obtained from the American Type Culture Collection. The SIRC cell line (American Type Culture Collection) and primary rabbit

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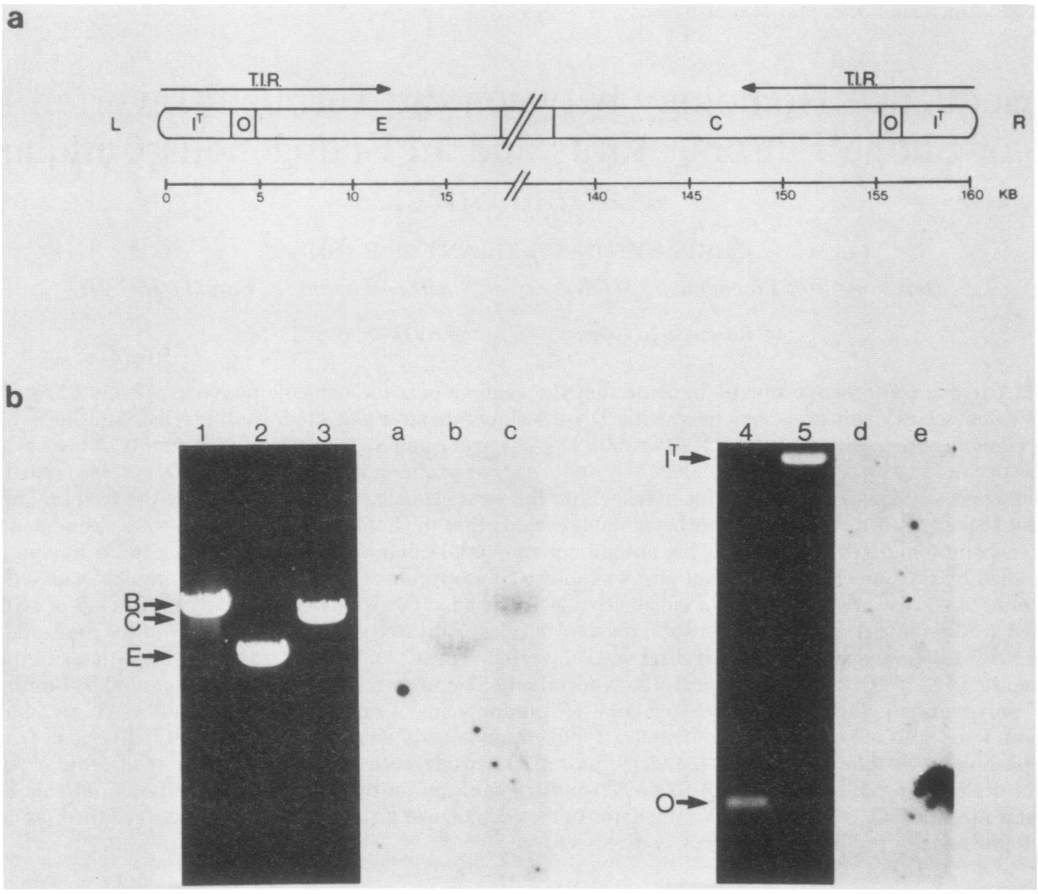


FIG. 1. Hybridization of total rabbit DNA probe to cloned *Bam*HI fragments of SFV DNA. (a) Abbreviated *Bam*HI restriction map of the SFV genome (11). (b) Lanes 1 through 5 contain 100 ng of purified cloned SFV *Bam*HI restriction fragments B, C, E, O and I^T, respectively, per kb, electrophoresed in 0.7% agarose. Fragment B maps in the center of the SFV genome and is included here as a high-molecular-weight negative control. Lanes a through e are the corresponding nitrocellulose blots probed with total rabbit DNA by using a modified Southern procedure (see Materials and Methods). All other SFV *Bam*HI fragments tested (not shown) gave negative results.

kidney fibroblasts (Flow Laboratories) were grown in Dulbecco minimal Eagle medium supplemented with 10% fetal calf serum.

Electrophoresis, blotting and hybridization. Conditions of restriction enzyme digestions, agarose gel electrophoresis, nick translation, and standard Southern blotting have been described (11, 42). To probe cloned viral DNA fragments with total high-complexity rabbit DNA, a modified Southern blot procedure was used: calf thymus DNA was omitted from the prehybridization and hybridization solutions by adjusting these to contain 50% formamide, 7× Denhardt solution, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.35% sodium dodecyl sulfate (27). Hybridization with a total of 1.2×10^8 cpm of ^{32}P-labeled rabbit DNA probe was done at 42°C for 40 h, and washing was performed in 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C.

Isolation of high-molecular weight DNA. Rabbit cells were suspended (10^7/ml) in 10 mM Tris (pH 8.0)–1 mM EDTA–pronase (500 μg/ml; Calbiochem-Behring Corp.)–0.5% sodium dodecyl sulfate, incubated at 37°C for 2 h, and extracted three times with phenol-chloroform (1:1) and once with chloroform. Nucleic acid was precipitated with 2 volumes of alcohol, suspended in 10 mM Tris (pH 8.0) containing RNase (50 μg/ml), incubated at 50°C for 1 h, extracted once with phenol-chloroform and once with chloroform, and then precipitated again with alcohol.

Hirt precipitation of high-molecular-weight DNA. The method of DNA isolation described above was followed except that, after incubation at 37°C for 2 h, the solution was made 1.0 M NaCl, left 16 h at 0°C, and centrifuged at 15,000 rpm in a Sorvall SS-34 rotor to precipitate high-molecular-weight DNA (17). The resultant supernatant was then treated as further described above.

DNA cloning and sequencing. Cloning and mapping of the relevant SFV restriction fragments in bacterial vectors have been described previously (11, 42). For cloning of the endogenous rabbit plasmid species, rabbit SIRC cells were pretreated for 16 h with cycloheximide (50 μg/ml), the Hirt supernatant was prepared as described above, and the DNA was fractionated by preparative agarose gel electrophoresis. The open circular (OC) and covalently closed circular (CCC) DNA species which hybridized to the SFV TIR probe (see Fig. 2) were excised, purified, digested with *Bam*HI and cloned into the *Bam*HI site of pUC8, with JM83 as the host (41). Recombinants were screened with the viral probe, and six positive clones were isolated. One clone, pSIC-9, contained a 1.9-kb insert and was used for sequence analysis. DNA sequencing was performed by using the Sanger dideoxy-chain termination method and exonuclease III-

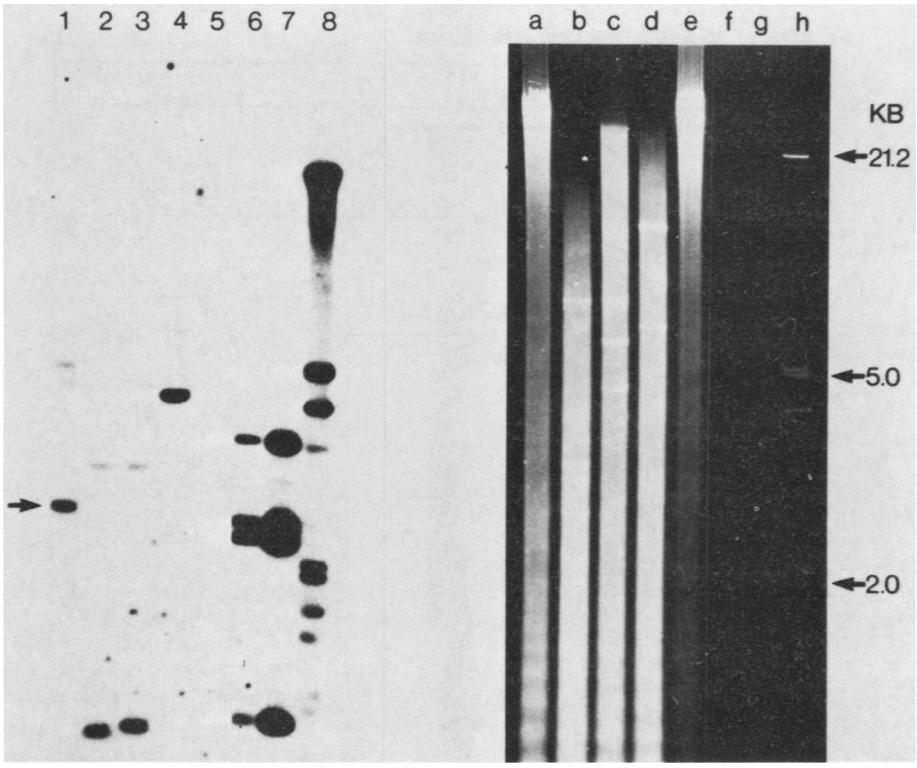


FIG. 2. Effect of cycloheximide treatment on rabbit plasmid copy number. Ethidium bromide-stained agarose gel (right panel, lanes a through h) of rabbit spc DNA and matching Southern blot (left panel, lanes 1 through 8) probed with 10^7 cpm of SFV DNA (*Bam*HI E fragment minus the E^B subclone; See Fig. 3a) plus 5×10^5 cpm of λ probe for size markers. All samples were from rabbit SIRC cells and prepared by Hirt precipitation (17) to remove most of the high-molecular-weight chromosomal DNA. Cycloheximide treatment (lanes 1 through 4 and a through d) was 50 μg/ml for 16 h before harvest. Lanes 1 through 5 and a through e each contain 3 μg of undigested DNA (lanes 1, 5, a, and e) or 3 μg of DNA digested with *Ava*I plus *Eco*RI (lanes 2 and b), *Ava*I (lanes 3 and c), or *Eco*RI (lanes 4 and d). Lanes 6, 7, f, and g contain 10 pg (lanes 6 and f) and 100 pg (lanes 7 and g) of the E^A, E^B, E^C, E^D, and E^E subclones of SFV *Bam*HI E fragment (see the map in Fig. 3). Lanes 8 and h contain 50 ng of λ DNA digested with *Eco*RI plus *Hin*dIII. The arrow in the panel to the left refers to the major plasmid species (shown in Fig. 4 to be CCC DNA) detected in the undigested lanes.

generated unidirectional deletions as described previously (16, 31, 41). Sequence manipulations and data base analysis were performed by using the core library programs of BIONET (IntelliGenetics, Inc.).

RESULTS

Detection of cross-hybridization between SFV and rabbit DNA. Although previous attempts to demonstrate DNA sequence homology between SFV and rabbit DNA were unsuccessful (19), the technologies then available limited investigators to the use of [^{3}H]DNA probes with relatively low specific activity. Thus, it seemed worthwhile to reevaluate the question by using cloned DNA in conjunction with [^{32}P]DNA probes of high specific activity. Our preliminary experiments were done by the method of Peden et al. (27), in which cloned viral DNA was exposed to high-complexity probe made from total rabbit DNA. Since the entire SFV genome had previously been cloned in bacterial plasmids (42) and mapped with respect to *Bam*HI, *Bgl*I, *Hin*dIII, *Pst*I, *Pvu*II, and *Sst*I restriction sites (11), it was possible to work with these cloned SFV fragments and, thus, to avoid the use of viral DNA isolated from infectious poxvirus virions, which can be contaminated with very small amounts of host DNA (18). The results of such hybridization studies with the *Bam*HI fragments of SFV DNA indicated that homology between viral and rabbit DNA could be detected and that only *Bam*HI fragments C and E hybridized to the rabbit DNA (Fig. 1). Examination of the *Bam*HI restriction map (Fig. 1A) reveals that the fragments C and E are located at opposite ends of the SFV genome and span the junctions between the unique internal sequences and each copy of the TIR. The position of these fragments is such that they have 6 kb in common within the TIR and 7.0 and 11.6 kb of unique internal sequences for E and C, respectively. The findings that only two fragments from the 160-kb SFV genome hybridized to the total rabbit DNA probe and that these contained 6 kb of identical DNA sequence strongly suggested that the homology with rabbit DNA resides within the TIR sequences shared by fragments C and E. Furthermore, this observation provided the impetus to use cloned viral TIR sequences as probes and total rabbit DNA as target. However, when purified *Bam*HI E fragment of SFV was used to probe samples of genomic rabbit DNA by Southern blot analysis, the majority of host DNA which hybridized to the viral probe migrated as a low-molecular-weight species, even for undigested control DNA. Since the amount of this low-molecular-weight DNA was rather variable from sample to sample, two techniques were used to amplify the hybridization signal. (i) Cells were pretreated for 16 h with cycloheximide (50 μg/ml) before DNA extraction. Smith and Vinograd (35) found that this treatment increased the num-

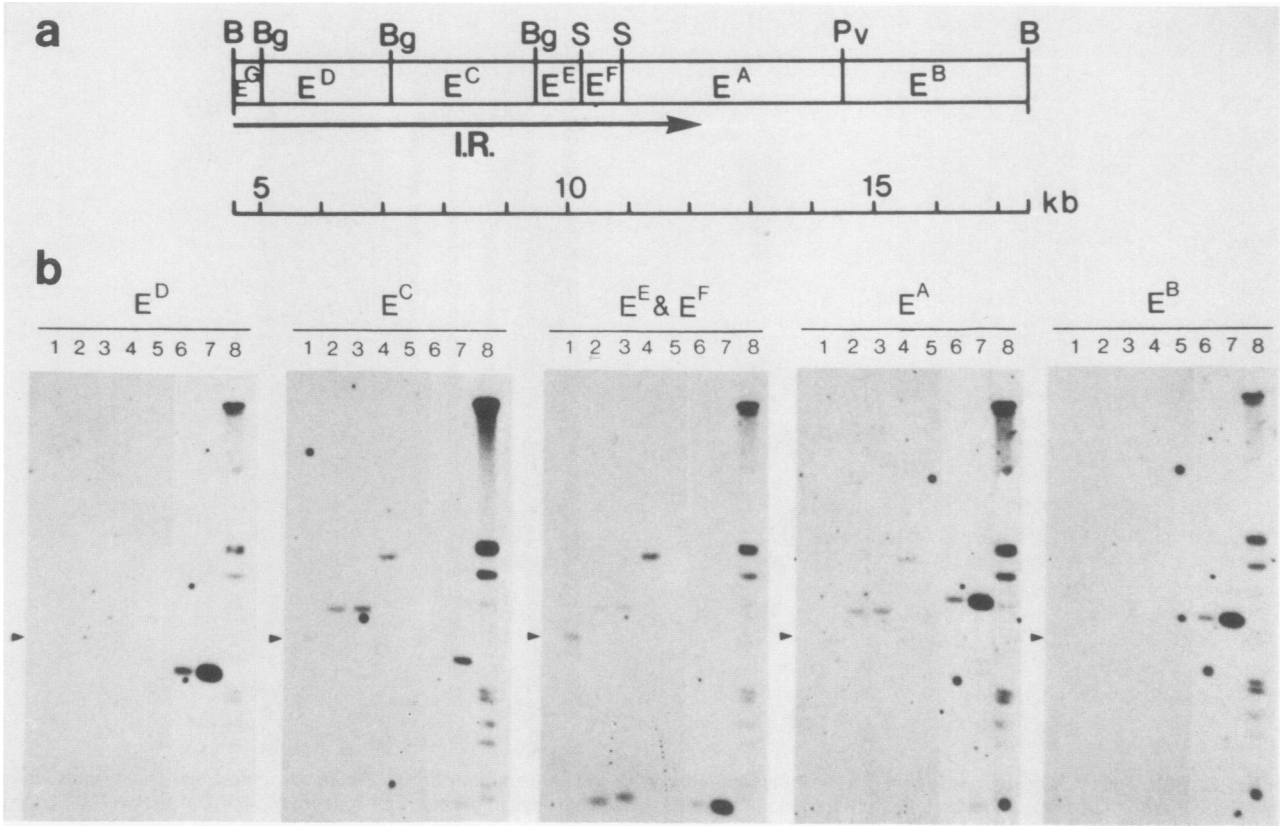


FIG. 3. Hybridization of SFV *Bam*HI E fragment subclones to the rabbit plasmid species. (a) Restriction map of the subclones of SFV *Bam*HI E fragment. The distance in kilobases from the left terminus of SFV and the extent of the inverted repeat of SFV (arrow) are also indicated. Abbreviations: B, *Bam*HI; S, *Sma*I; Pv, *Pvu*II. (b) Southern blots with subclones E^D, E^C, E^E plus E^F, E^A, and E^B as probes. Arrowheads refer to the mobility of the undigested plasmid species indicated by the arrow in Fig. 2. The DNA samples in lanes 1 through 8 are as described in the legend to Fig. 2.

ber of spc DNA molecules in eucaryotic cells by 20- to 30-fold. (ii) DNA was isolated from cells by the Hirt procedure (17) to precipitate most of the high-molecular-weight chromosomal DNA. Figure 2 demonstrates the effect of cycloheximide treatment on the amount of rabbit spc DNA present in Hirt supernatants which hybridizes to SFV DNA probe. The undigested DNA samples in lanes a and e of Fig. 2 were isolated by the Hirt procedure under identical conditions and with the same reagents except that the DNA in lane a was extracted from cells pretreated with cycloheximide. Although not visible in the photograph, faint bands were observed in lane 5 (untreated) of the original fluorogram at positions matching those seen in lane 1 (cycloheximide-treated DNA). Quantitation of the difference between these signals indicated that the cycloheximide treatment resulted in an approximately 50-fold amplification of this extrachromosomal DNA species without altering its apparent mobility. As will be shown further in the next section, the three bands in lane 1 of Fig. 2 represent the OC, linear, and CCC forms of the cellular extrachromosomal DNA elements. Digestion with a single-site restriction enzyme such as *Eco*RI (Fig. 2, lane 4) indicates the linearized plasmid size to be 4.8 kb. Since the sum of the molecular sizes of fragments produced by some restriction enzymes, such as *Ava*I (Fig. 2, lane 3), were often less than this, it can be concluded that only a fraction of the entire 4.8-kb species is homologous to the viral TIR. These results together suggest that the host sequences homologous to SFV are present on an endogenous rabbit plasmid-like element with a low and variable copy number. Reconstitution blots indicate a maximum copy number of one to five per cell in cycloheximide-amplified cultures but less than one per cell in the established SIRC cell line.

Mapping of the DNA homologies and characterization of the rabbit plasmid species. To determine the size and position of the relevant homologous sequences within the SFV TIR, DNA isolated from Hirt supernatants of cells that had been pretreated with cycloheximide was then hybridized sequentially with purified subclones of SFV *Bam*HI fragment E. These subclones of the SFV *Bam*HI E fragment are diagrammed and oriented with respect to the SFV inverted repeat (Fig. 3a). Subclones E^D and E^B did not hybridize to the rabbit plasmid, whereas the contiguous E^C and E^A, and E^E plus E^F subclones all gave positive hybridization signals (Fig. 3b). Control hybridizations with bacterial plasmid vector probes such as pBR322 failed to yield a signal (not shown). Digestion of the rabbit plasmid with *Ava*I (Fig. 3b, lane 3) produced two major fragments of 3.5 and 0.7 kb, both of which still hybridized to the SFV *Bam*HI-E probe but which could be distinguished by the fact that the E^C and E^A subclones only hybridized to the larger of these fragments, whereas the mixture of E^E plus E^F hybridized to both.

To demonstrate conclusively that the rabbit sequences homologous to SFV were present in the host cells as CCC

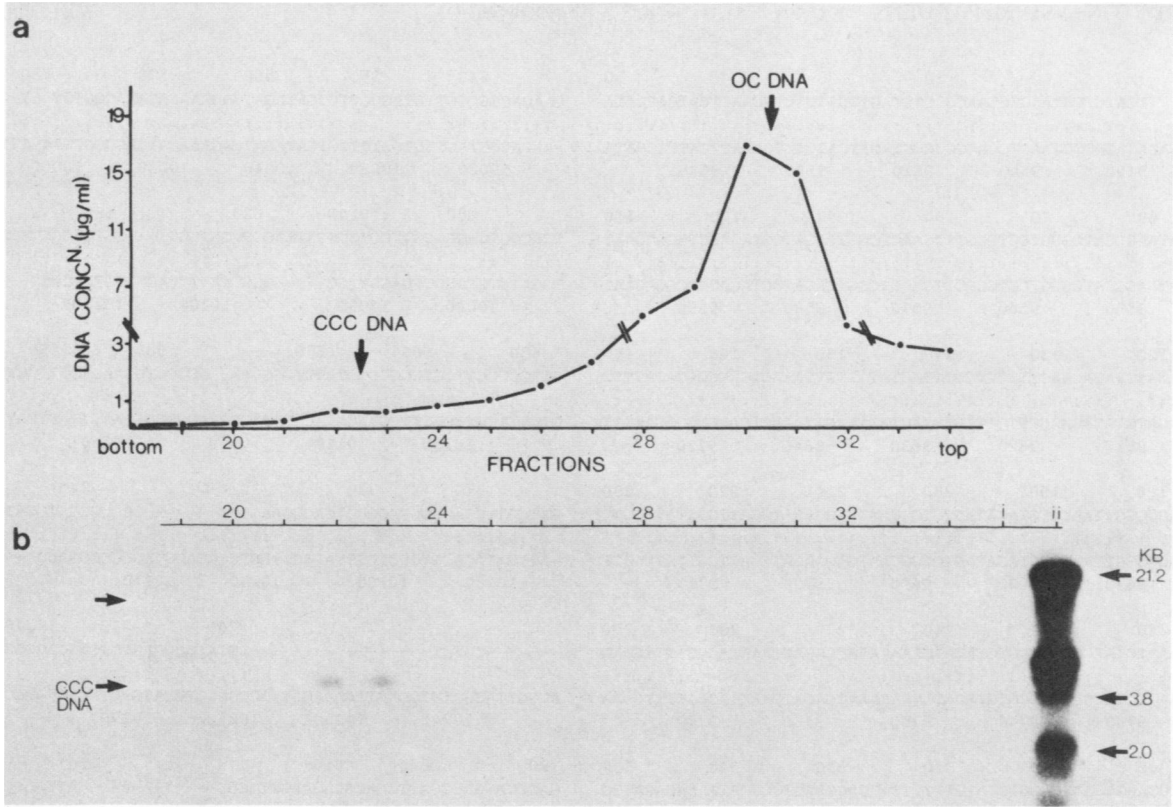


FIG. 4. Isopycnic centrifugation of rabbit plasmid DNA. (a) DNA (10 μg) from a Hirt supernatant of cycloheximide-treated (50 μg/ml, 16 h) primary rabbit kidney fibroblasts was subjected to isopycnic centrifugation in 5.0 M CsCl plus 250 μg of ethidium bromide per ml. The gradient was fractionated, and the DNA concentration was determined by fluorimetry. (b) Aliquots of each fraction (undigested) were electrophoresed in 0.7% agarose, transferred to nitrocellulose, and probed with 5×10^6 cpm of SFV *Bam*HI E fragment plus 5×10^5 cpm of λ DNA. Lane i contains 10 pg of the SFV *Bam*HI E fragment digested with *Pvu*II plus *Sma*I (Fig. 3). Lane ii contains 50 ng of λ DNA digested with *Eco*RI plus *Hin*dIII. Upper arrow refers to migration position of OC plasmid DNA which bands with rabbit chromosomal DNA (lanes 29, 30, and 31).

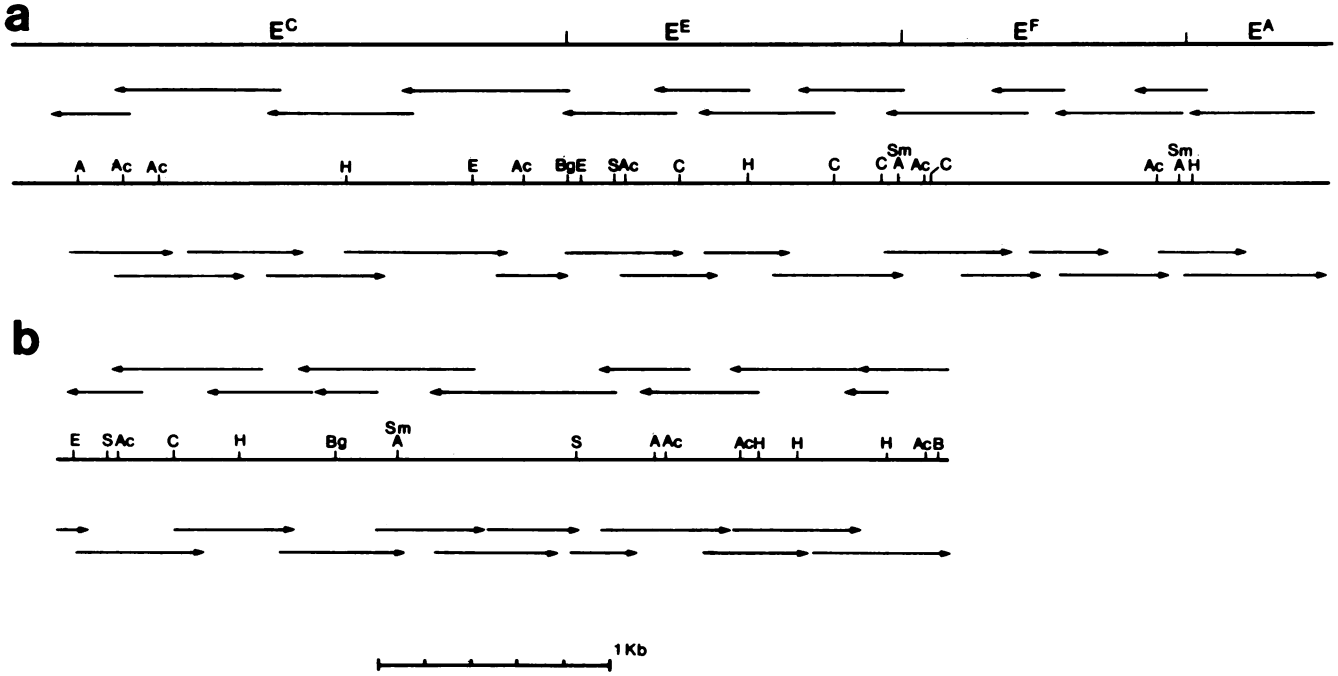
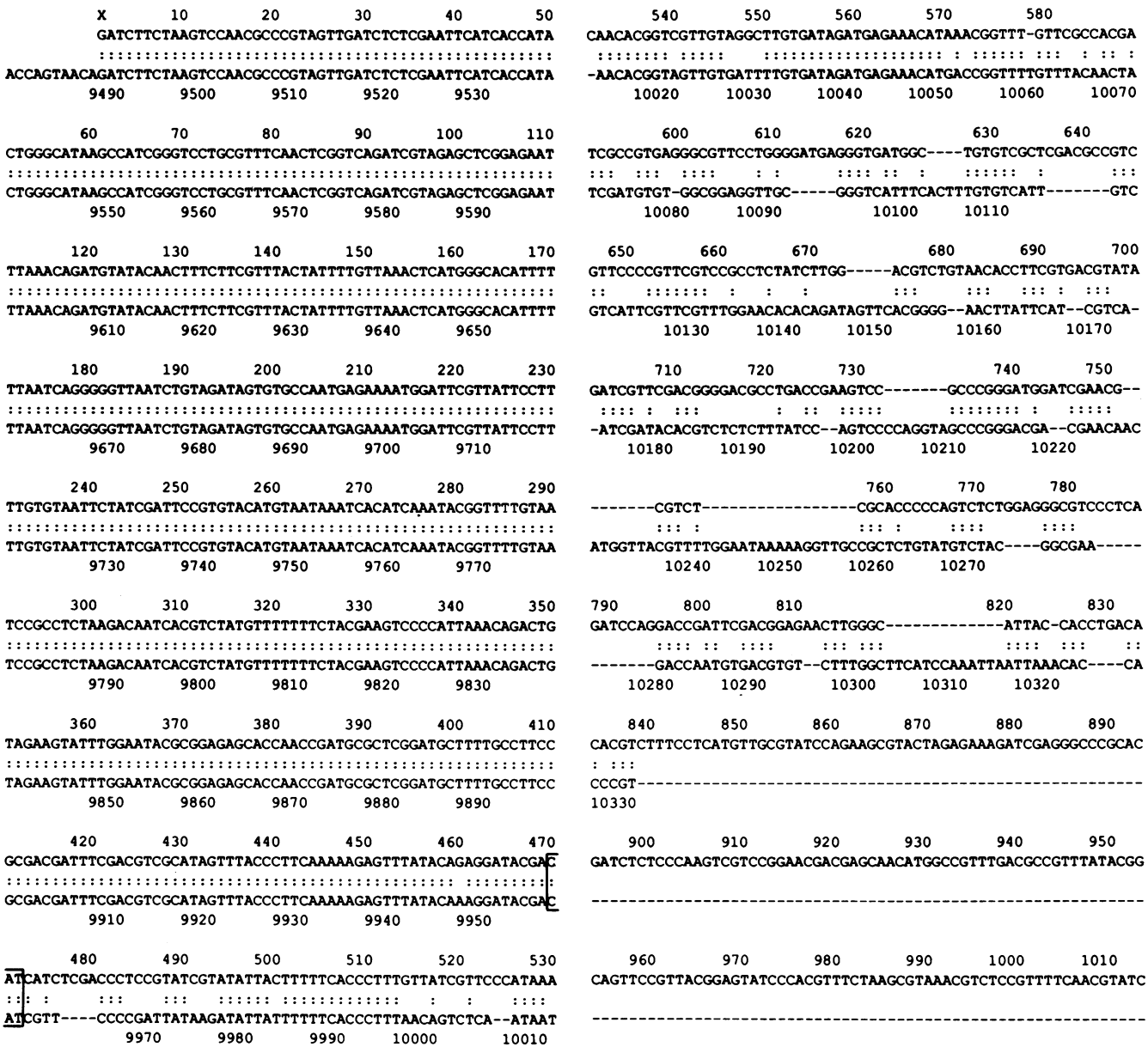


FIG. 5. Sequencing strategy for 2.5 kb of the SFV TIR and the cloned endogenous rabbit plasmid pSIC-9. DNA was cloned into appropriate M13 vectors, unidirectional deletions were constructed as described in Materials and Methods, and sequencing was performed by the Sanger dideoxy method (31). (a) SFV TIR between 8.4 and 10.9 kb from the termini. (b) The 1.9-kb insert from the cloned rabbit plasmid pSIC-9. Abbreviations: A, *Ava*I; Ac, *Acc*I; B, *Bam*HI; Bg, *Bgl*I; C, *Cla*I; E, *Eco*RI; H, *Hha*I; S, *Sst*I; Sm, *Sma*I.



molecules, DNA present in the Hirt supernatant prepared from cycloheximide-treated primary rabbit kidney cells was subjected to isopycnic centrifugation in CsCl gradients containing ethidium bromide. Undigested DNA samples from each gradient fraction (Fig. 4a) were electrophoresed in an agarose gel, blotted, and probed with the *Bam*HI E fragment of SFV DNA (Fig. 4b). Mitochondrial DNA and the rabbit plasmid banded at almost the same buoyant density in the CsCl gradient but were separated on the basis of their size during electrophoresis in agarose; note that mitochondrial DNA (17 kb) did not hybridize to the SFV probe (the mitochondrial DNA bands are visible on the stained gel in Fig. 2). The rabbit plasmid isolated from primary rabbit kidney cells (Fig. 4) was very similar to that detected in the SIRC cell line, and both displayed comparable homology to the SFV *Bam*HI E fragment. In the original fluorogram, faint bands were also visible in fractions 29, 30, and 31 of the gradient, corresponding to the OC form of the plasmid. Thus, we conclude that the bulk of the endogenous plasmid species detected by the SFV probe, at least in the case of the cycloheximide-amplified samples, exists as unnicked circles.

Cloning and DNA sequencing of the rabbit DNA plasmid. Two types of potential artifact could, in principle, account for the above observations. Firstly, in a recent report by Jones and Hyman (21), specious hybridization between herpes simplex virus DNA and human cellular DNA was shown to be caused by guanine-rich sequences which bind nonspecifically to pyrimidine-rich tracts on single-stranded DNA (40). Secondly, the possibility of low-level microbial plasmid contamination in the cultured cells or reagents (33) must be discounted. To rule these possibilities out, experiments to clone the endogenous rabbit plasmid sequences in pUC vectors were done. Control blots indicated that the rabbit plasmid could be linearized by several convenient

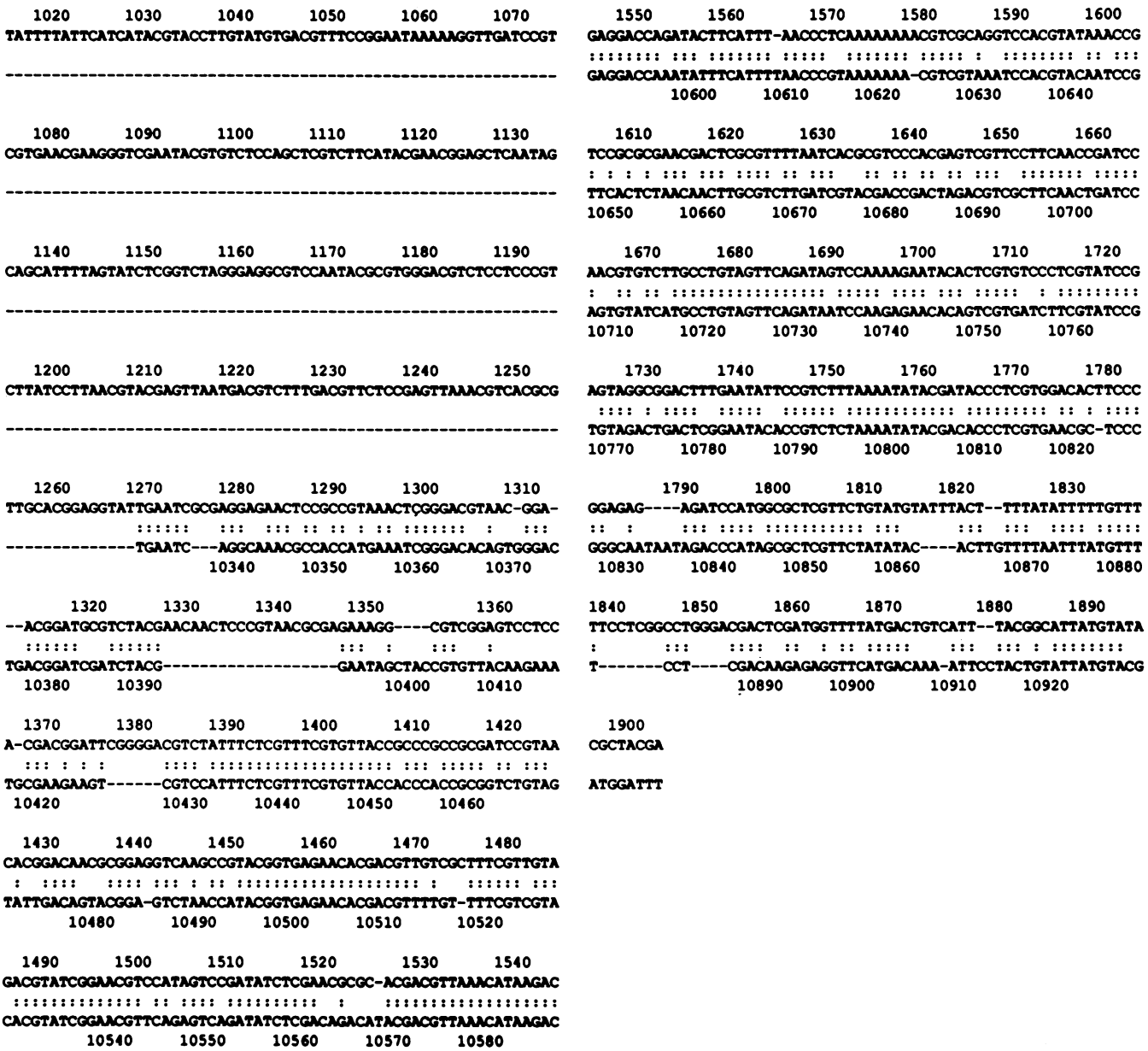


FIG. 6. DNA sequence homology between pSIC-9 and the SFV TIR. The 1.9-kb plasmid sequence from pSIC-9 (upper line) is displayed relative to the homologous 1.5-kb stretch of the SFV TIR (lower line). The SFV nucleotide numbers are from the terminus. The boxed triplet designates the putative initiator codons for the SFV ORF-T8 and the pSIC-9 ORF-2 (see Fig. 8).

restriction enzymes, including *Hin*dIII, *Sal*I, and *Bam*HI (not shown); thus, cloning experiments were done with the *Bam*HI site of pUC8. Plasmids from cycloheximide-treated SIRC cells were purified as either OC or CCC species by preparative gel electrophoresis of DNA from Hirt supernatants, linearized with *Bam*HI, and ligated to *Bam*HI-digested pUC8. Transformation was performed into *Escherichia coli* JM83, and recombinant clones were screened with SFV *Bam*HI E probe. All positive clones, however, were found to contain inserts smaller than the expected 4.8 kb representing the entire plasmid species, suggesting that at least some of the endogenous plasmid sequences are unstable in *E. coli* JM83. The clone with the largest insert (1.9 kb), pSIC-9, was selected for sequence analysis. Control Southern blots, in which the pSIC-9 insert was used as probe, indicated that both the correct plasmid species and the appropriate region of the SFV TIR hybridized as expected to the cloned fragment. Furthermore, the deduced restriction site profile of the pSIC-9 insert turned out to be consistent with the Southern blotting data (see below).

The 1.9-kb insert of pSIC-9 was sequenced by the dideoxy-chain termination method (Fig. 5) and compared with the homologous region of the SFV TIR. The entire 12-kb SFV TIR has now been sequenced and will be presented elsewhere, but the relevant 1.4 kb extending between 9.5 to 10.9 kb from the viral terminus is shown in Fig. 6. The SFV sequence from 9.49 to 9.95 kb from the terminus was identical to 0.46 kb at one end of the pSIC-9 insert. This was

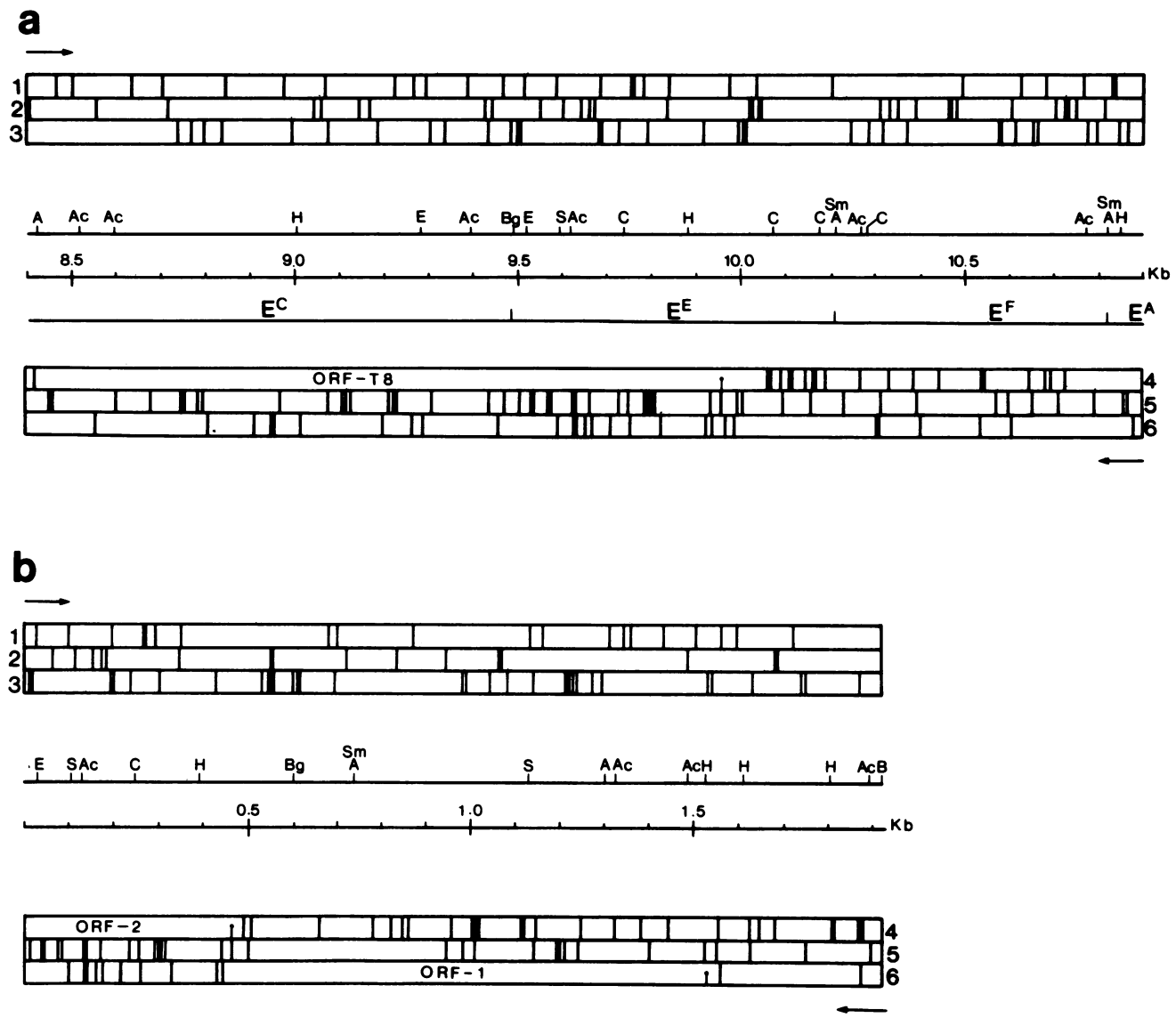


FIG. 7. Genomic organization of pSIC-9 and the homologous region of the SFV TIR. The six reading frames deduced from the DNA sequencing analysis of SFV TIR from 8.4 to 10.9 kb from the terminus (a) and pSIC-9 (b). Arrows refer to the direction of transcription. Restriction enzyme abbreviations are defined in the legend to Fig. 5. The first ATG codon ( ) in the designated open reading frames is indicated.

followed first by 0.39 kb of detectable sequence homology but with demonstrable divergence and then a large gap of 0.43 kb for which no pSIC-9-related sequences could be found in the SFV DNA. However, sequence homology reappeared at this point, and the remaining 0.63 kb of pSIC-9 sequences were extensively homologous to SFV sequences. If this region of the SFV TIR had been originally derived from the host rabbit cell via the pSIC-9 plasmid species, then these results suggest that (i) at least 0.46 kb has been highly conserved in the virus, (ii) 0.5 kb has been deleted, and (iii) almost 1 kb of pSIC-9-like sequences remains in the virus but has significantly diverged, although DNA cross-hybridization can still be detected.

Genomic organization of the rabbit plasmid and the homologous SFV sequences. To assess whether the highly conserved regions between pSIC-9 and SFV could be in biologically relevant regions, the open reading frames of the pSIC-9 insert and the region of SFV between 8.4 and 10.9 kb from the viral terminus were diagrammed (Fig. 7). The viral sequence contained one long open reading frame, designated ORF-T8, in the strand which is transcribed towards the viral terminus (Fig. 7a). This is the only strand that was found to be transcribed efficiently (C. Macaulay and G. McFadden, manuscript in preparation), suggesting that ORF-T8 may, in fact, express a relevant viral protein. Note that the SFV reading frames from 9.49 to 9.95 kb are identical to those of the pSIC-9 sequences from 0 to 0.46 kb (Fig. 7B). It appears that the pSIC-9 clone was truncated in the cloning procedures at a point 157 amino acids from the N terminus of the putative conserved protein. Figure 8 illustrates the entire 514-amino acid sequence of the putative viral ORF-T8 protein, and the underlined region indicates this conserved 157-amino acid stretch at the N terminus of the plasmid ORF-2 protein.

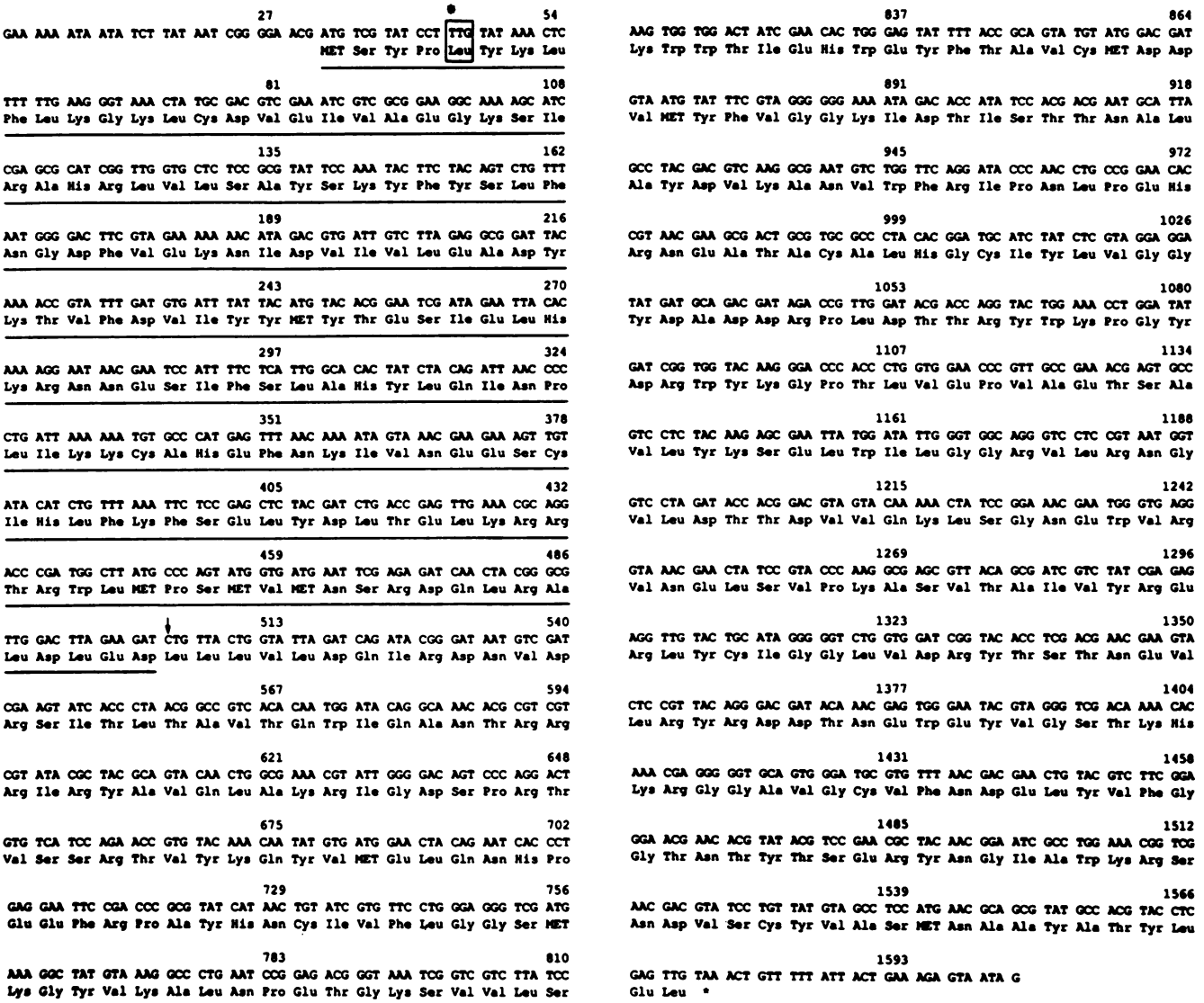


FIG. 8. Comparison of the deduced SFV ORF-T8 amino acid sequence with the pSIC-9 ORF-2 sequence. The SFV nucleotide sequence encompassing the viral ORF-T8 sequence is displayed. Nucleotide 1 here corresponds to nucleotide 9989 of Fig. 6, and the displayed sequence extends to nucleotide 8406. The underlined region corresponds to the area of identity with the pSIC-9 ORF-2 amino acid sequence. The box with the asterisk contains the single-nucleotide difference (SFV nucleotide 9947) between ORF-T8 and ORF-2, although the amino acid (leucine) is unchanged. The arrow indicates the breakpoint for the cloned pSIC-9 sequence.

A second long major open reading frame, designated ORF-1, was detected in pSIC-9 (Fig. 7) but had no counterpart in the virus because of the previously mentioned 0.5-kb deletion and the pronounced sequence divergence in this region between the plasmid and the virus. ORF-1 potentially encodes for a 361-amino acid protein which may once have been acquired by SFV but has now been discarded from the virus genome as a functional genetic entity.

To determine whether any of the nucleotide or putative protein sequences determined here have any counterparts in the current databases, homology searches were carried out with the available GenBank (National Institutes of Health) nucleotide and NBRF protein data bases. These searches revealed that the SFV and rabbit plasmid sequences were unrelated to any other known plasmid or viral sequences, thereby arguing against a close relationship between pSIC-9 sequences and any of the commonly known infectious agents. However, one interesting homology involving the pSIC-9 ORF-1 amino acid sequence was highlighted during the data base analysis. A series of mammalian protease inhibitors, including α1-antichymotrypsin (Fig. 9), α1-antitrypsin, and antithrombin III, were found to display significant homology with the putative ORF-1 protein. In the important region from amino acids 367 to 399 of the α1-antichymotrypsin precursor, which contains the reactive site (underlined in Fig. 9), 18 of the 33 amino acids were identical. This compares favorably with the values of 11 of 33 for α1-antitrypsin versus α1-antichymotrypsin and 18 of 33 for antithrombin III versus α1-antichymotrypsin (9). While these data do not prove that the putative pSIC-9 ORF-1 protein is a serine protease inhibitor, they do suggest that the plasmid sequences described here are, in fact, bona fide cellular sequences derived from uninfected rabbit cells. The organization of these rabbit ORF-1 and ORF-2 se-

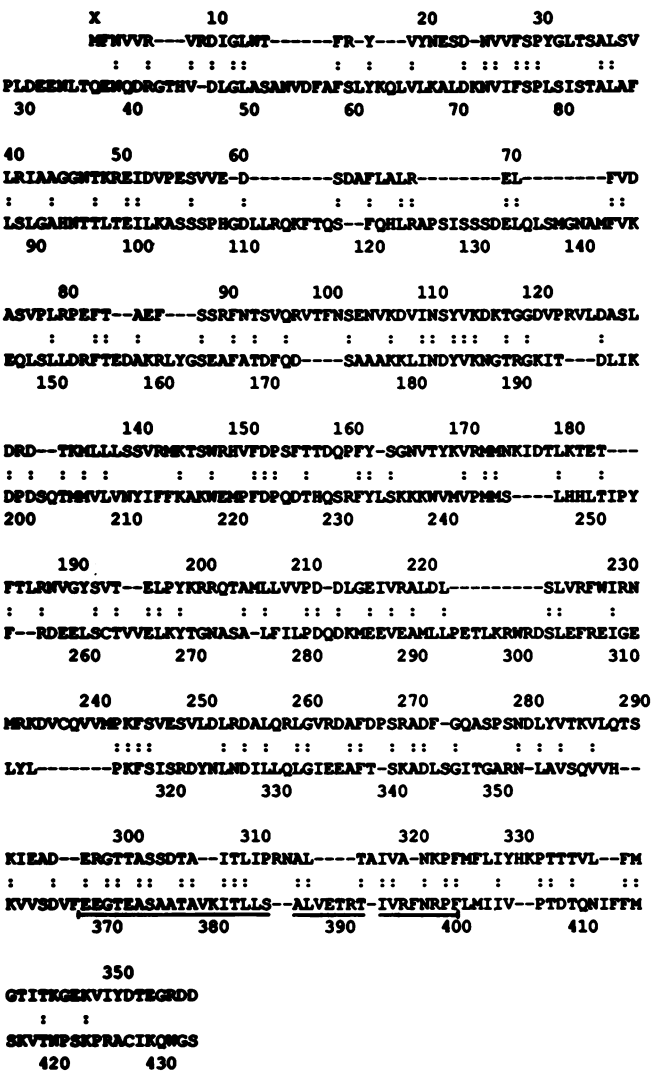


FIG. 9. Comparison of the deduced amino acid sequence of the rabbit plasmid ORF-1 with that of human α1-antichymotrypsin. The published sequence of human α1-antichymotrypsin (9) is displayed below the deduced sequence of pSIC-9 ORF-1. The underlined region represents the region flanking the α1-antichymotrypsin active site.

quences with respect to the rabbit genome, as well as the more general question of how they came to reside within CCC DNA molecules, is currently being investigated.

DISCUSSION

Although first detected in HeLa cells more than a decade ago (35), the presence of spc DNA in a broad variety of mammalian cells has become a widely documented but poorly understood phenomenon. The copy number of these plasmid-like DNA species can vary enormously from cell type to cell type and can be markedly influenced by such factors as growth rate, state of differentiation, or aging in culture. They have been detected in a variety of cell lines, including *D. melanogaster* (36, 38), HeLa (20, 35), Chinese hamster ovary (37), and monkey (3, 32) cells, and also in a number of tissues (8, 12). Frequently, more than 100 spc DNA molecules per cell have been detected (35), but the copy number of an individual spc DNA can vary widely; values of under 0.1 per cell have been reported (37). The function, if any, of these molecules in eucaryotic cells is unknown, but pertinent to this discussion is the fact that there is evidence that at least some of these plasmid species can at times have a cytoplasmic location (35, 37). Therefore, it is possible to rationalize how a poxvirus such as SFV, which replicates exclusively in the cell cytoplasm, could encounter and recombine with such extrachromosomal host sequences. The recent demonstration that at least two vaccinia virus polypeptides, thymidine kinase and a 19,000-molecular-weight early gene product, appear to be related to host polypeptides (5, 6, 22) may, in fact, be a reflection of a more generalized mechanism by which poxviruses can acquire host sequences.

In this paper, we have shown that DNA probes from a limited 2- to 3-kb region of the SFV TIR region cross-hybridized with a novel endogenous plasmid-like DNA species detected in uninfected rabbit cells. This extrachromosomal DNA species was shown to be amplified 20- to 50-fold by treatment of the cells with cycloheximide and could be detected predominantly as CCC molecules, consistent with observations made for spc DNA in other eucaryotic cells. A cloned 1.9-kb fragment of the rabbit plasmid from SIRC cells was sequenced and compared with the homologous 2.5-kb region of the SFV genome. Interestingly, one of the two plasmid open reading frames, ORF-2, was identical to the analogous stretch of one SFV open reading frame, ORF-T8. If the first methionine codon was utilized as an initiator in each case, then the N-terminal 157 amino acids of the putative 514 residue SFV protein would be identical to the N-terminal 157 amino acids encoded in the plasmid ORF-2, suggesting that the encoded protein was conserved by the virus. On the other hand, a second plasmid open reading frame, ORF-1, diverged considerably in the viral genome and no longer exists in SFV as a defined reading frame, although significant DNA sequence homology was still in evidence. Data base analysis revealed that the putative plasmid ORF-1 protein is closely related to a series of proteins from the serine protease inhibitor superfamily. For example, 135 of the 361 amino acids of the putative ORF-1 gene product are identical to the published human α1-antichymotrypsin sequence (9). Although the origin and function of the rabbit plasmid sequences remain to be determined, it is intriguing that the genes for serine protease inhibitors, at least as determined to date (e.g., see reference 23), contain multiple intervening sequences, and yet the rabbit plasmid ORF-1 represents a single continuous 361-amino acid sequence. The possibility that the rabbit plasmid species described here may have been originally generated by reverse transcription of rabbit mRNA into CCC DNA molecules will be more readily assessed once the genomic organization of these putative rabbit genes is determined.

The localization of this plasmid homology within 2 to 3 kb of the SFV inverted repeats closest to the unique internal sequences has significance for another reason. Recently Strayer et al. (39) isolated a novel tumorigenic poxvirus of rabbits, designated malignant rabbit virus (MRV), which possesses a number of biological features reminiscent of both SFV and myxoma virus, which is a related leporipoxvirus of rabbits and the agent of myxomatosis. Of particular interest is the fact that MRV induces, at early times of infection, fibromas in rabbits that are indistinguishable from those of SFV, but later these tumors spread throughout the body of the rabbit with an invasive profile similar to that of the lesions of myxomatosis (39). The

genomic structure of MRV DNA indicates that this virus is indeed a bona fide recombinant between SFV and myxoma virus and that the only difference between MRV and myxoma virus is the replacement of 4 to 6 kb of myxoma sequences with a SFV DNA sequence of equivalent size (4). This 4 to 6 kb of SFV DNA inserted into the myxoma genome is derived from the SFV terminal repeat region closest to the unique internal sequences (4). Thus, the stretch of DNA that we identified in the SFV TIR which is homologous to the endogenous cellular plasmid species is a subset of those sequences donated by SFV to the myxoma virus genome in the creation of the recombinant MRV. In fact, the SFV ORF-T8, which is identical in sequence to the truncated pSIC-9 ORF-2, has been transferred in toto to MRV and provides suggestive evidence that it may play a critical role in SFV tumorigenesis.

Regardless of the precise genetic function of the SFV sequences mapped in this paper, it seems likely that endogenous cellular plasmids can mediate the transposition of biologically important genes. Further analysis of the origin of these plasmids may not only reveal how cytoplasmically replicating viruses such as poxviruses can acquire cellular genes but also shed light on the origin of tumorigenic poxviruses in general.

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