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 $\alpha 1$ -antichymotrypsin, $\alpha 1$ -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 viroosomes 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 blot 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 ³²P-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 μ g/ml for 16 h before harvest. Lanes 1 through 5 and a through e each contain 3 μ g of undigested DNA (lanes 1, 5, a, and e) or 3 μ g 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 *Hind*III. 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*II, *Hind*III, *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 μ g/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 *Hind*III. 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, *Cl*aI; E, *Eco*RI; H, *Hha*I; S, *Sst*I; Sm, *Sma*I.

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X      10      20      30      40      50
GATCTTCTAAGTCCAACGCCGCTAGTTGATCTCTCGAATTCATCACCATA
.....
ACCAGTAACAGATCTTCTAAGTCCAACGCCGCTAGTTGATCTCTCGAATTCATCACCATA
9490      9500      9510      9520      9530

60      70      80      90      100     110
CTGGGCATAAGCCATCGGGTCTCGCTTCAACTCGGTCAGATCGTAGAGCTCGGAGAAT
.....
CTGGGCATAAGCCATCGGGTCTCGCTTCAACTCGGTCAGATCGTAGAGCTCGGAGAAT
9550      9560      9570      9580      9590

120     130     140     150     160     170
TTAAACAGATGTATACAACCTTCTTCGTTTACTATTTTGTAAACTCATGGGCACATTTT
.....
TTAAACAGATGTATACAACCTTCTTCGTTTACTATTTTGTAAACTCATGGGCACATTTT
9610     9620     9630     9640     9650

180     190     200     210     220     230
TTAATCAGGGGGTAAATCTGTAGATAGTGTCCCAATGAGAAAATGGATTTCGTTATTCCTT
.....
TTAATCAGGGGGTAAATCTGTAGATAGTGTCCCAATGAGAAAATGGATTTCGTTATTCCTT
9670     9680     9690     9700     9710

240     250     260     270     280     290
TTGTGTAATCTATCGATTCCGTTGACATGTAATAAATCACATCAAAATACGGTTTGTAA
.....
TTGTGTAATCTATCGATTCCGTTGACATGTAATAAATCACATCAAAATACGGTTTGTAA
9730     9740     9750     9760     9770

300     310     320     330     340     350
TCCGCCTCTAAGACAATCACGCTATGTTTTTTTCTACGAAGTCCCCATTAAACAGACTG
.....
TCCGCCTCTAAGACAATCACGCTATGTTTTTTTCTACGAAGTCCCCATTAAACAGACTG
9790     9800     9810     9820     9830

360     370     380     390     400     410
TAGAAGTATTTTGAATACGCGGAGAGCACCAACCGATGCGCTCGGATGCTTTTGCTTCC
.....
TAGAAGTATTTTGAATACGCGGAGAGCACCAACCGATGCGCTCGGATGCTTTTGCTTCC
9850     9860     9870     9880     9890

420     430     440     450     460     470
GCGACGATTTTCGACGTCGATAGTTTACCCTTCAAAAAGAGTTTATACAGAGGATACGAC
.....
GCGACGATTTTCGACGTCGATAGTTTACCCTTCAAAAAGAGTTTATACAAAGGATACGAC
9910     9920     9930     9940     9950

480     490     500     510     520     530
ATCATCTCGACCCCTCGTATCGTATATTACTTTTACCCTTTGTTATCGTTCCCATAAA
.....
ATCGTT-----CCCCGATTATAAGATATTATTTTACCCTTTAACAGTCTCA--ATAAT
9970     9980     9990     10000    10010

540     550     560     570     580
CAACACGGTCGTTGTAGGCTTGTGATAGATGAGAAACATAAACGGTTT-GTTCGCCACGA
.....
-AACACGGTAGTTGTGATTTTGTGATAGATGAGAAACATGACCGGTTTGTGTTACAACATA
10020    10030    10040    10050    10060    10070

600     610     620     630     640
TCGCCGTGAGGGCGTTCTCGGGATGAGGGTGATGGC----TGTGTCGCTCGACGCCGTC
.....
TCGATGTGT-GGCGGAGGTTGC-----GGGTCAATTCATTCTGTGTCATT-----GTC
10080    10090    10100    10110

650     660     670     680     690     700
GTTCCCCGTTTCGTCGCTCTATCTTGG-----ACGTCGTGAACACCTTCGTGACGTATA
.....
GTCATTTCGTTTCGTTTGAACACACAGATAGTTCACGGGG--AACTTATTCAT--CGTCA-
10130    10140    10150    10160    10170

710     720     730     740     750
GATCGTTTCGACGGGACGCTGACCGAAGTCC-----GCCCGGATGGATCGAACG--
.....
-ATCGATACAGTCTCTCTTTATCC--AGTCCCCAGGTAGCCCGGACGA--CGAACAC
10180    10190    10200    10210    10220

760     770     780
-----CGTCT-----CGCACCCCCAGTCTCTGGAGGGCGTCCCTCA
.....
ATGGTTACGTTTGGGAATAAAAAGTTGCGCTCTGTATGCTAC--GGCGAA-----
10240    10250    10260    10270

790     800     810     820     830
GATCCAGGACCGATTTCGACGAGAACTTGGGC-----ATTAC-CACCTGACA
.....
-----GACCAATGTGACGTGT--CTTTGGCTTCATCCAAATTAATTAACAC-----CA
10280    10290    10300    10310    10320

840     850     860     870     880     890
CACGCTTTCTCTCATGTTGCGTATCCAGAAGCGTACTAGAGAAAGATCGAGGGCCCCGAC
.....
CCCGT-----
10330

900     910     920     930     940     950
GATCTCTCCCAAGTCGTCCGGAACGACGAGCAACATGGCCGTTTGACGCCGTTTATACGG
.....

960     970     980     990     1000    1010
CAGTTCGGTTACGGAGTATCCACGTTTCTAAGCGTAAACGTTCTCCGTTTCAACGTATC
.....

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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), spurious 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 *Hind*III, *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.

27	54	837	864
GAA AAA ATA ATA TCT TAT AAT CGG GGA ACG ATG TCG TAT CCT <u>TTG</u> TAT AAA CTC		AAG TGG TGG ACT ATC GAA CAC TGG GAG TAT TTT ACC GCA GTA TGT ATG GAC GAT	
MET Ser Tyr Pro <u>Leu</u> Tyr Lys Leu		Lys Trp Trp Thr Ile Glu His Trp Glu Tyr Phe Thr Ala Val Cys MET Asp Asp	
81	108	891	918
TTT TTG AAG GGT AAA CTA TGC GAC GTC GAA ATC GTC GCG GAA GGC AAA AGC ATC		GTA ATG TAT TTC GTA GGG GGG AAA ATA GAC ACC ATA TCC ACG ACG AAT GCA TTA	
Phe Leu Lys Gly Lys Leu Cys Asp Val Glu Ile Val Ala Glu Gly Lys Ser Ile		Val MET Tyr Phe Val Gly Gly Lys Ile Asp Thr Ile Ser Thr Thr Asn Ala Leu	
135	162	945	972
CGA GCG CAT CGG TTG GTG CTC TCC GCG TAT TCC AAA TAC TTC TAC AGT CTG TTT		GCC TAC GAC GTC AAG GCG AAT GTC TGG TTC AGG ATA CCC AAC CTG CCG GAA CAC	
Arg Ala His Arg Leu Val Leu Ser Ala Tyr Ser Lys Tyr Phe Tyr Ser Leu Phe		Ala Tyr Asp Val Lys Ala Asn Val Trp Phe Arg Ile Pro Asn Leu Pro Glu His	
189	216	999	1026
AAT GGG GAC TTC GTA GAA AAA AAC ATA GAC GTG ATT GTC TTA GAG GCG GAT TAC		CGT AAC GAA GCG ACT GCG TGC GCC CTA CAC GGA TCC ATC TAT CTC GTA GGA GGA	
Asn Gly Asp Phe Val Glu Lys Asn Ile Asp Val Ile Val Leu Glu Ala Asp Tyr		Arg Asn Glu Ala Thr Ala Cys Ala Leu His Gly Cys Ile Tyr Leu Val Gly Gly	
243	270	1053	1080
AAA ACC GTA TTT GAT GTG ATT TAT TAC ATG TAC ACG GAA TCG ATA GAA TTA CAC		TAT GAT GCA GAC GAT AGA CCG TTG GAT ACG ACC AGG TAC TGG AAA CCT GGA TAT	
Lys Thr Val Phe Asp Val Ile Tyr Tyr MET Tyr Thr Glu Ser Ile Glu Leu His		Tyr Asp Ala Asp Asp Arg Pro Leu Asp Thr Thr Arg Tyr Trp Lys Pro Gly Tyr	
297	324	1107	1134
AAA AGG AAT AAC GAA TCC ATT TTC TCA TTG GCA CAC TAT CTA CAG ATT AAC CCC		GAT CCG TGG TAC AAG GGA CCC ACC CTG GTG GAA CCC GTT GCC GAA ACG AGT GCC	
Lys Arg Asn Asn Glu Ser Ile Phe Ser Leu Ala His Tyr Leu Gln Ile Asn Pro		Asp Arg Trp Tyr Lys Gly Pro Thr Leu Val Glu Pro Val Ala Glu Thr Ser Ala	
351	378	1161	1188
CTG ATT AAA AAA TGT GCC CAT GAG TTT AAC AAA ATA GTA AAC GAA GAA AGT TGT		GTC CTC TAC AAG AGC GAA TTA TGG ATA TTG GGT GGC AGG GTC CTC CGT AAT GGT	
Leu Ile Lys Lys Cys Ala His Glu Phe Asn Lys Ile Val Asn Glu Glu Ser Cys		Val Leu Tyr Lys Ser Glu Leu Trp Ile Leu Gly Gly Arg Val Leu Arg Asn Gly	
405	432	1215	1242
ATA CAT CTG TTT AAA TTC TCC GAG CTC TAC GAT CTG ACC GAG TTG AAA CGC AGG		GTC CTA GAT ACC ACG GAC GTA GTA CAA AAA CTA TCC GGA AAC GAA TGG GTG AGG	
Ile His Leu Phe Lys Phe Ser Glu Leu Tyr Asp Leu Thr Glu Leu Lys Arg Arg		Val Leu Asp Thr Thr Asp Val Val Gln Lys Leu Ser Gly Asn Glu Trp Val Arg	
459	486	1269	1296
ACC CGA TGG CTT ATG CCC AGT ATG GTG ATG AAT TCG AGA GAT CAA CTA CGG GCG		GTA AAC GAA CTA TCC GTA CCC AAG GCG AGC GTT ACA GCG ATC GTC TAT CGA GAG	
Thr Arg Trp Leu MET Pro Ser MET Val MET Asn Ser Arg Asp Gln Leu Arg Ala		Val Asn Glu Leu Ser Val Pro Lys Ala Ser Val Thr Ala Ile Val Tyr Arg Glu	
513	540	1323	1350
TTG GAC TTA GAA GAT CTG TTA CTG GTA TTA GAT CAG ATA CGG GAT AAT GTC GAT		AGG TTG TAC TGC ATA GGG GGT CTG GTG GAT CCG TAC ACC TCG ACG AAC GAA GTA	
Leu Asp Leu Glu Asp Leu Leu Leu Val Leu Asp Gln Ile Arg Asp Asn Val Asp		Arg Leu Tyr Cys Ile Gly Gly Leu Val Asp Arg Tyr Thr Ser Thr Asn Glu Val	
567	594	1377	1404
CGA AGT ATC ACC CTA ACG GCC GTC ACA CAA TGG ATA CAG GCA AAC ACG CGT CGT		CTC CGT TAC AGG GAC GAT ACA AAC GAG TGG GAA TAC GTA GGG TCG ACA AAA CAC	
Arg Ser Ile Thr Leu Thr Ala Val Thr Gln Trp Ile Gln Ala Asn Thr Arg Arg		Leu Arg Tyr Arg Asp Asp Thr Asn Glu Trp Glu Tyr Val Gly Ser Thr Lys His	
621	648	1431	1458
CGT ATA CGC TAC GCA GTA CAA CTG GCG AAA CGT ATT GGG GAC AGT CCC AGG ACT		AAA CGA GGG GGT GCA GTG GGA TGC GTG TTT AAC GAC GAA CTG TAC GTC TTC GGA	
Arg Ile Arg Tyr Ala Val Gln Leu Ala Lys Arg Ile Gly Asp Ser Pro Arg Thr		Lys Arg Gly Gly Ala Val Gly Cys Val Phe Asn Asp Glu Leu Tyr Val Phe Gly	
675	702	1485	1512
GTG TCA TCC AGA ACC GTG TAC AAA CAA TAT GTG ATG GAA CTA CAG AAT CAC CCT		GGA ACG AAC ACG TAT ACG TCC GAA CGC TAC AAC GGA ATC GCC TGG AAA CGG TCG	
Val Ser Ser Arg Thr Val Tyr Lys Gln Tyr Val MET Glu Leu Gln Asn His Pro		Gly Thr Asn Thr Tyr Thr Ser Glu Arg Tyr Asn Gly Ile Ala Trp Lys Arg Ser	
729	756	1539	1566
GAG GAA TTC CGA CCC GCG TAT CAT AAC TGT ATC GTG TTC CTG GGA GGG TCG ATG		AAC GAC GTA TCC TGT TAT GTA GCC TCC ATG AAC GCA GCG TAT GCC ACG TAC CTC	
Glu Glu Phe Arg Pro Ala Tyr His Asn Cys Ile Val Phe Leu Gly Gly Ser MET		Asn Asp Val Ser Cys Tyr Val Ala Ser MET Asn Ala Ala Tyr Ala Thr Tyr Leu	
783	810	1593	
AAA GGC TAT GTA AAG GCC CTG AAT CCG GAG ACG GGT AAA TCG GTC GTC TTA TCC		GAG TTG TAA ACT GTT TTT ATT ACT GAA AGA GTA ATA G	
Lys Gly Tyr Val Lys Ala Leu Asn Pro Glu Thr Gly Lys Ser Val Val Leu Ser		Glu Leu *	

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 α 1-antichymotrypsin (Fig. 9), α 1-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 α 1-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 α 1-antitrypsin versus α 1-antichymotrypsin and 18 of 33 for antithrombin III versus α 1-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-

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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