DNA Clone of Avian Fujinami Sarcoma Virus with Temperature-Sensitive Transforming Function in Mammalian Cells

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We have molecularly cloned an integrated proviral DNA of Fujinami sarcoma virus (FSV) into a lambda phage vector and further subcloned it into plasmid pBR322. The source of provirus was a quail nonproducer cell clone transformed by FSV. The FSV strain used is temperature sensitive in the maintenance of transformation of avian cells. The recombinant plasmid was shown to contain an entire FSV genome by fingerprinting the hybrids formed with ³²P-labeled FSV RNA. This analysis also revealed a previously undetected *env*-related sequence in FSV which represents the 3' end of the gp85 *env* gene. A physical map of cloned FSV DNA identifying sites of several restriction enzymes is described. Upon transfection, FSV DNA cloned in pBR322 transformed mouse NIH-3T3 cells, which proved to be temperature sensitive in maintaining transformation. Phosphorylation but not synthesis of p140, the only known gene product of FSV, was also temperature sensitive in these cells. The correlation between transformation and phosphorylation of p140 suggests that phosphorylation of p140 is necessary for transformation of mouse cells, as was shown previously for avian cells. These results provide direct genetic evidence that the mechanisms for maintaining transformation of mammalian and avian cells involve the same FSV gene product, p140. Homology was detected by hybridization between transformation-specific sequences of FSV DNA and certain restriction endonuclease-resistant fragments of cellular DNA of two avian species, chicken and quail. Under the same conditions homology was also detected with DNA of non-avian species, although apparently to a lower degree than with avian cells.

Fujinami sarcoma virus (FSV), originally isolated by Fujinami and Inamoto in 1914 (12), is a defective avian sarcoma virus having an RNA genome of 4.5 kilobases (kb) (20). This RNA contains a contiguous internal FSV-specific sequence of about 3 kb flanked by a 5'-terminal sequence of 1 kb and a 3'-terminal sequence of 0.5 kb related to the RNA of nondefective Fujinami-associated virus (FAV), the helper virus of FSV (20). A helper-virus-unrelated, specific sequence is considered to be the hallmark of the *onc* genes of acutely oncogenic retroviruses (10) and has been shown in other highly oncogenic viruses to be closely related to cellular sequence prototypes (27, 33, 35). The specific sequence of FSV has been reported to be closely related to that of avian sarcoma virus PRCII and also to the Synder-Theilen and Gardner-Arnstein strains of feline sarcoma virus by molecular hybridization (29). Together with the 5' helper virus *gag* gene-related region of FSV

RNA, the specific sequence encodes a *gag*-related protein of 140,000 daltons, termed p140 (16, 20). p140 is the only known gene product of FSV and is thought to have a transforming function (16, 19, 20). From studies of wild-type and temperature-sensitive mutants of FSV (15, 19), it was concluded that phosphorylation of p140 is necessary for initiating and maintaining transformation of infected avian cells (19, 24). Moreover, p140 obtained by immunoprecipitation from infected cells is associated with a kinase that phosphorylates p140 in vitro (2, 11). However, it is as yet uncertain whether p140 is associated with a kinase or has an intrinsic kinase activity, because most of this kinase activity is separated from p140 by sedimentation (2), p140 synthesized in vitro or purified by immunoaffinity chromatography does not have detectable kinase activity, and p140 in temperature-sensitive FSV is a temperature-sensitive substrate of phosphorylation (19; W.-H. Lee, Ph.D. thesis, University of California, Berkeley, 1981).

This paper describes the preparation and char-

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acterization of a molecular clone of proviral FSV DNA that represents a complete complement of FSV RNA and that upon transfection transforms mouse 3T3 cells. The clone was prepared from an FSV strain that is temperature sensitive in maintaining transformation of avian cells (19). As we show here, maintenance of the transformed phenotype of mouse 3T3 cells also proved to be temperature sensitive. Moreover, phosphorylation of p140 in the 3T3 cells transformed with cloned FSV DNA was temperature sensitive both *in vivo* and *in vitro*. It would appear that the mechanisms for maintaining transformation in avian and mammalian cells by FSV are similar and both involve a temperature-sensitive viral gene product, p140. Previous experiments reporting transformation of mammalian cells with defective avian tumor viruses have not been able to distinguish between direct transformation by a viral gene product and indirect transformation by positional effects of proviral integration, due to the low transforming efficiency of avian viruses (25) and proviral DNA (6, 18) on mammalian cells.

The availability of this clone should also allow a detailed investigation of the relationships between the transformation-specific sequence of FSV and those of other related viruses and of cellular prototypes in avian and mammalian cells. Our preliminary results indicate that under the same conditions of hybridization, the DNA of quail and chicken cells was found to have counterparts related to the FSV-specific sequence, but less homology was detected with DNAs of non-avian origin.

MATERIALS AND METHODS

Cells and viruses. A quail cell nonproducer clone, infected by ts FSV L-15, was grown in medium F10 supplemented with 2% tryptose phosphate broth, 2% calf serum, 1% chicken serum, and 0.5% dimethyl sulfoxide. NIH-3T3 cells and one NIH-3T3 cell line transformed by transfection with cloned ts FSV L-15 DNA, termed F3B, were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Preparation of [32 P]cDNA. [32 P]cDNA from FSV-(FAV) was prepared by using either oligodeoxythymidylates 12 to 18 (oligo(dT) 12–18) or oligonucleotides prepared by random degradation of calf thymus DNA to prime reverse transcription of polyadenylic acid-selected 28–35S FSV(FAV) RNA (13). A typical 0.1-ml reaction mixture contained 0.002 mg of FSV(FAV) RNA, 1 mM each dATP, dGTP, and TTP, 0.5 mCi of [α - 32 P]dCTP (400 Ci/mmol), 0.001 mg of oligo(dT) 12–18, 20 mM Tris-hydrochloride, pH 8.0, 5 mM MgCl₂, 10 mM dithiothreitol, 0.01 mg of actinomycin D, and 40 U of reverse transcriptase. The reaction was carried out at 39°C for 2 h and terminated by adding 1% sodium dodecyl sulfate (SDS) and 0.1 N NaOH, and the mixture was then incubated at 39°C for 4 h to allow the RNA template to be degraded. After being neutralized with 0.1 N acetic acid–0.1 M Tris-

hydrochloride (pH 7.4), the reaction mixture was extracted with phenol and chromatographed on Sephadex G-50 with buffer containing 10 mM Tris-hydrochloride (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.1% SDS. Usually, 5×10^7 to 30×10^7 cpm of [32 P]cDNA was obtained from this reaction, with a specific activity of 2×10^5 cpm/ng of DNA.

Nick translation of DNA. Nick translation of DNA was performed essentially as described by Rigby et al. (26) with one 32 P-labeled deoxyribonucleotide triphosphate. Briefly, 0.1 ml of reaction mixture contained 0.001 mg of DNA, 0.0025 mM each dATP, dGTP, and TTP, 100 μ Ci of [α - 32 P]dCTP (400 Ci/mmol), 50 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 0.005 mg of bovine serum albumin, 0.2 ng of DNase I, and 5 U of DNA polymerase I. After being incubated at 10°C for 90 min, the reaction was terminated by the addition of 0.01 ml of 20% SDS and further incubation at 65°C for 10 min. The reaction mixture was then subjected to chromatography on Sephadex G-50. Usually, 0.001 mg of DNA with a specific activity of 2×10^4 to 10×10^4 cpm/ng of DNA was obtained. The DNA was denatured with 0.1 N NaOH at room temperature for 10 min and then neutralized with 1 N acetic acid before use in hybridization experiments.

Preparation of cellular DNA. FSV L-15 quail non-producer cell DNA was extracted as described previously (3). Cells (10^8) were lysed and digested in 10 ml of buffer containing 0.1 M EDTA (pH 7.5), 1% sarcosinate, and 0.01 mg of proteinase K at 56°C for 2 h. The mixture was then phenol extracted and dialyzed against TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 7.5). RNA was then digested by incubation with 0.36 mg of RNase A at 40°C for 4 h. After phenol extraction and dialysis, the DNA was quantitated by measuring absorbancy at 260 nm.

Hybridization of DNA fragments resolved by agarose gel electrophoresis. Electrophoretic DNA fragments contained in agarose gels were transferred to nitrocellulose filters by the method of Southern (31). The DNA in the agarose gel was denatured by soaking the gel in 0.5 N NaOH–1.0 M NaCl for 30 min and then neutralized with 1 M Tris-hydrochloride (pH 7.4)–1.5 M NaCl for another 30 min. The DNA was allowed to transfer to the nitrocellulose filter for 3 to 24 h with $10\times$ SSC (1 $\times$ SSC, 0.15 M NaCl–0.015 M sodium citrate). After being heated for 3 h at 80°C in vacuo, the filters were incubated for 4 to 12 h at 68°C in prehybridization buffer containing 6 $\times$ SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.25% SDS at pH 7.0. Hybridizations with 32 P-labeled DNA fragments or cDNA were carried out in the same buffer for 3 to 24 h at 68°C. After hybridization, the nitrocellulose filters were washed with 1 $\times$ SSC–0.1% SDS for 3 h at 68°C, dried, and subjected to autoradiography.

Construction and screening of a charon 27 phage library containing FSV-transformed quail cell DNA. FSV L-15 cell DNA (0.2 mg) was digested completely with 400 U of restriction endonuclease *Bgl*II at 37°C for 3 h and subjected to 5 to 20% NaCl (in TE buffer) gradient centrifugation in an SW41 rotor at 35,000 rpm for 3 h at 20°C. After being fractionated and analyzed by agarose gel electrophoresis, the fractions containing DNA fragments 6 to 9 kb long were pooled and ethanol precipitated. The *Bgl*II-digested cell DNA fragments (0.005 mg) were ligated with 0.02 mg of

charon 27 DNA which had been digested to completion by restriction endonuclease *Bam*HI. Ligation was performed by overnight incubation of the DNA in 50 mM Tris-hydrochloride (pH 7.4)–10 mM $MgCl_2$ –20 mM dithiothreitol–1 mM ATP–5 U of T4 DNA ligase at 10°C. The total DNA was then packaged in vitro into phage as described previously (3).

The resulting phage, about 10^6 PFU, were mixed with 8 ml of a fresh overnight culture of *Escherichia coli* K802 and plated on two cookie sheets (42 by 24 cm) at a phage concentration of 5×10^5 PFU per sheet. After the phage had grown overnight at 37°C, duplicates of nitrocellulose filters moistened in 1 M NaCl were placed on each tray for 10 min. The filters were then removed from the trays, inverted, and processed in successive 10-min incubations by floating the filters on denaturing buffer (0.5 N NaOH, 1 M NaCl) and then on neutralizing buffer (1 M Tris-hydrochloride, pH 7.4, 1.0 M NaCl). The filters were air dried and baked in vacuo at 80°C for 2 h. One of two duplicate filters was hybridized with a *Bam*HI-resistant 1.3-kb DNA fragment of the *gag* gene of Schmidt-Ruppin Rous sarcoma virus (9, 29) labeled with ^{32}P by nick translation, and the other was hybridized with FSV(FAV) [^{32}P]cDNA. After autoradiography, positive plaques were picked, replated, and rescreened to purify the phage as previously described (1). Large-scale growth of recombinant lambda phage was carried out essentially as described previously (3).

Subcloning DNA fragments into *E. coli* plasmid pBR322. Typically, 500 ng of pBR322 DNA and 1,000 ng of the FSV-containing charon 27 DNA to be subcloned, respectively, were cleaved to completion by the appropriate restriction endonucleases. These DNA solutions were mixed, phenol extracted, and ethanol precipitated. The DNA was pelleted and ligated under the conditions described above.

The procedures used for transformation of *E. coli* strain HB101 with the plasmid pBR322 DNA were those described previously (22). The transformation-competent HB101 cells were harvested by sedimentation in a Sorvall GSA rotor at 5,000 rpm for 5 min at 2°C during the log phase of growth (absorbancy at 600 nm, 0.6) in Luria broth (10 g of tryptone [Difco], 5 g of yeast extract, and 5 g of NaCl per liter) and treated twice with 50 mM $CaCl_2$ for 20 min on ice. A sample of HB101 cells was used for transfection with the ligated DNA at a final concentration of 25 mM $CaCl_2$ –50 mM Tris-hydrochloride (pH 7.2) at 0°C for 15 min. The cells were heat shocked at 41°C for 2 min and then chilled at 0°C for another 30 min. Luria broth (1 ml) was added to the HB101 cells, and they were then incubated at 37°C for 30 min. Portions of transfected HB101 cells were plated on agar plates containing 0.015 mg of tetracycline or 0.03 mg of ampicillin per ml. Recombinant clones were purified from a cross-selection based on the drug-resistant or -sensitive phenotype (8).

The procedures used to prepare the plasmid DNA were mainly adopted from Davis et al. (8) with slight modifications. HB101 colonies which carried recombinant plasmids were picked, and each was grown in 3 ml of Luria broth overnight. Cells from 0.5 ml of each culture were pelleted, suspended in 0.1 ml of buffer I containing 50 mM Tris-hydrochloride (pH 8.5), 50 mM EDTA, 15% sucrose, and 1 mg of lysozyme per ml for 20 min on ice. Buffer II (0.2 ml) containing 0.2 N

NaOH and 1% SDS was then added, and the cells were left for 5 min on ice and then neutralized with 0.15 ml of 3 M sodium acetate, pH 4.8. The mixture was incubated on ice for 50 min and centrifuged in an Eppendorf Microfuge for 10 min. Plasmid DNA in the supernatant was ethanol precipitated, dissolved in 0.1 mM EDTA, and analyzed for FSV-related sequences by restriction enzyme cleavage and hybridization.

Transfection and transformation assays of mouse 3T3 cells with cloned FSV DNA. Calcium phosphate-DNA precipitates used for transfection of 3T3 cells were prepared as previously described (14). To about 20 μ g of DNA in 1 ml of $2\times$ HBS (280 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], and 1.5 mM Na_2HPO_4 at pH 7.05) we gradually added an equal volume of freshly prepared 0.25 mM $CaCl_2$ as air was bubbled through the solution. The mixture was blended briefly with a Vortex mixer and allowed to form precipitates for 30 min at room temperature. Three types of precipitates were prepared. One type was prepared with 20 μ g of quail cell DNA alone; others were prepared with 0.05 to 1 μ g of pFL-5 DNA with 20 μ g of quail cell DNA; and another type was prepared with 0.5 μ g of simian virus 40 DNA and 20 μ g of quail cell DNA. The precipitate (1 ml) was then added to recipient cultures of 3T3 cells in a 60-mm dish in 10 ml of medium. After 8 to 12 h, the medium was changed. The recipient cultures were prepared by seeding 2×10^5 3T3 cells and incubating the cultures overnight. One group of cells was kept in liquid medium, and the medium was changed every other day. Foci were scored after 7 to 10 days at 37°C. The other group of cells were reseeded in soft agar to allow formation of colonies. The method for agar colony formation was essentially that previously described (19), except that Dulbecco modified Eagle medium with 10% fetal calf serum was used.

RESULTS

Construction of a recombinant DNA library of FSV-transformed quail cell DNA in phage lambda and isolation of FSV DNA-containing phage. A quail cell clone, nonproductively transformed by a strain of FSV (L-15) that is temperature sensitive in maintaining transformation of avian cells, was our source of proviral FSV DNA (19). The DNA from this cell clone was digested with restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Bgl*II. In parallel, DNA from uninfected quail cells was digested with the same enzymes. Fragments of each DNA were then compared by agarose gel electrophoresis, followed by hybridization with [^{32}P]cDNA from FSV(FAV). It was found that a *Bgl*II-resistant DNA fragment of FSV L-15 cell DNA of 8 to 9 kb which hybridized with the ^{32}P probe did not have a counterpart in *Bgl*II-digested uninfected quail cell DNA (Fig. 1). The other three restriction enzymes either generated too many fragments or gave unclear results. Thus, *Bgl*II was chosen to digest FSV L-15 DNA for ligation with the external DNA fragments (arms) of lambda phage charon

FIG. 1. FSV-related sequences in the DNA of normal quail cells in an FSV-transformed nonproducer quail cells. DNA from uninfected quail embryo fibroblasts (a) or from ts FSV L-15 quail nonproducer cells (b) was digested with *Bgl*II. After being electrophoresed on an 0.8% agarose gel, the DNA fragments were transferred to nitrocellulose filters and hybridized with [32 P]cDNA from FSV(FAV). Both cell types appear to share an FSV-related fragment of 13 kb. A fragment of 8 to 9 kb was detected only in the DNA from the FSV L-15 nonproducer cell clone.

27 (4). Since *Bgl*II produces DNA fragments with the same four-base, single-stranded ends as *Bam*HI does, the DNA fragments generated by *Bgl*II can be conveniently inserted into the single *Bam*HI site of charon 27 DNA by base pairing and ligation (F. Blattner, personal communication). However, both the *Bam*HI and *Bgl*II restriction sites would be lost after ligation. Roughly 10^6 phage were obtained by packaging in vitro 0.005 mg of *Bgl*II-cut FSV L-15 cell DNA and 0.02 mg of *Bam*HI-cut charon 27 DNA arms.

The resulting library was screened for recombinant phage containing FSV DNA by hybridizing DNA from about 10^6 lambda plaques with

two different 32 P-labeled DNA probes: a *Bam*HI-resistant 1.3-kb DNA fragment of the *gag* gene of Schmidt-Ruppin Rous sarcoma virus (RSV) that starts at about 0.5 kb from the 5' end of the RSV genome (9, 28) or cDNA from FSV(FAV) prepared by transcription of FSV(FAV) RNA with reverse transcriptase in the presence of an oligo(dT) 12–18 primer (see above). Only one autoradiographic spot coincided on two replicate filters of the same culture hybridized with the two different probes. The plaque that hybridized with both probes was picked, replated, and plaque purified three times until 95% of all daughter plaques were positive with both hybridization probes. The recombinant phage selected by the above procedure was termed CFL.

We prepared 0.5 mg of CFL DNA for analysis of its restriction pattern by growing the phage on a large surface of soft agar after infection at a multiplicity of about 0.01 PFU per bacterial cell. The CFL phage DNA was prepared and digested with *Bgl*II, an 8.4- and a 4.1-kb fragment hybridized with the FSV(FAV) cDNA probe, but only the 8.4-kb fragment hybridized with the *gag* DNA probe as well (Fig. 2). Since the insert was

FIG. 2. A recombinant lambda charon 27 phage carrying proviral FSV DNA. DNA from a recombinant phage was digested with *Bgl*II and electrophoresed on an 0.8% agarose gel. After being transferred to nitrocellulose, the DNA fragments were hybridized with three different 32 P-labeled DNA probes: (a) a combination of DNA from the original lambda charon 27 and FSV(FAV) cDNA prepared by transcription of FSV(FAV) RNA with reverse transcriptase and an oligo(dT) primer (see the text); (b) FSV(FAV) cDNA as in lane a; (c) a cloned *Bam*HI-resistant 1.3-kb DNA fragment from the *gag* gene of RSV (9, 28). A single 8.4-kb fragment was hybridized with both probes b and c.

prepared from FSV L-15 cell DNA digested with *Bgl*II, these particular *Bgl*II sites must have survived the original digestion. The 4.1-kb *Bgl*II DNA fragment may be a deleted variant of the 8.4-kb fragment or the product of another unknown sequence rearrangement. However, the DNA of the clone changed its restriction enzyme pattern, and much of the resulting phage lost FSV-related sequences when phage-infected cultures were grown in liquid broth. Some of the resulting phage had either no insert of FSV DNA sequences or only a 1- to 1.5-kb insert of FSV-related sequences. Therefore, we decided to subclone the 8.4-kb fragment that hybridized with both FSV(FAV) cDNA and *gag* DNA into plasmid pBR322.

Recombinant pBR322 plasmid containing FSV DNA (pFL-5) and its restriction endonuclease map. To obtain stable FSV recombinant DNA, we subcloned the *Bgl*II-resistant 8.4-kb DNA fragment from the recombinant lambda phage described above into the bacterial plasmid pBR322. Several recombinant plasmids were obtained by the subcloning procedure. One, termed pFL-5, contained the *Bgl*II-resistant 8.4-kb DNA fragment which hybridized with both the *gag* DNA and FSV(FAV) cDNA probes. pFL-5 proved to be stable when grown in large quantities. To establish a physical map of FSV DNA, we analyzed the pFL-5 DNA by cleavage with restriction endonucleases *Bam*HI, *Kpn*I, *Pvu*I, *Sma*I, and *Sst*I. Restriction enzymes such as *Eco*RI, *Hind*III, *Bgl*II, *Sal*I, and *Xba*I had no cleavage site in the FSV DNA (Fig. 3). The

restriction enzyme map resulting from these digestions is shown in Fig. 4. Two long terminal repeat (LTR) sequences of FSV were identified by hybridization with 32 P-labeled DNA from a recombinant plasmid, p53, carrying the LTR of RSV (23). Like the LTRs of MC29 virus (18) and Rous-associated virus-0 (17), but unlike the LTRs of RSV (7, 9, 23; D. Schwartz, R. Tizard, and W. Gilbert, Cell, in press), the LTR of FSV did not contain an *Eco*RI restriction enzyme cleavage site. In accord with previous analyses of FSV RNA (20), the *gag* gene-related sequence element of FSV was located adjacent to the left-hand LTR of FSV (Fig. 4). The relative locations of the *gag*-related and FSV-specific elements in the clone (Fig. 4) and in viral RNA (20), and the coincidence of the 2.7-kb *Bam*HI-resistant fragment in the clone and in the DNA of FSV-transformed quail cells detectable in a genomic blot (data not shown), signaled that the pFL-5 clone was probably colinear with authentic proviral DNA. The biological activity of pFL-5, described below, confirmed this notion. The 5' and 3' junctions between the FSV provirus and quail cell DNA in pFL-5 have not as yet been clearly determined.

pFL-5 contains the entire FSV genome. To determine what portion of the FSV genome was present in the plasmid pFL-5, we annealed 32 P-labeled FSV(FAV) 50-70S RNA with alkali-degraded pFL-5 DNA to form RNA-DNA hybrids as described previously (21). After RNase T1 digestion, the hybrid was isolated, and the [32 P]RNA of the hybrid was fingerprinted (Fig.

FIG. 3. Electrophoretic analysis of recombinant pBR322 plasmid pFL-5 carrying proviral FSV DNA and quail cell DNA after digestion with restriction enzymes. Lanes: 1, 16, and 31, *Bam*HI; 2, 17, and 32, *Kpn*I; 3, 18, and 33, *Sma*I; 4, 19, and 34, *Sst*I; 5, 20, and 35, *Hind*III/*Bam*HI; 6, 21, and 36, *Hind*III/*Kpn*I; 7, 22, and 37, *Hind*III/*Sal*I; 8, 23, and 38, *Hind*III/*Sma*I; 9, 24, and 39 *Hind*III/*Sst*I; 10, 25, and 40, *Hind*III/*Bgl*II; 11, 26, and 41, *Bam*HI/*Kpn*I; 12, 27, and 42, *Bam*HI/*Sal*I; 13, 28, and 43, *Bam*HI/*Sma*I; 14, 29, and 44, *Bam*HI/*Sst*I; 15, 30, and 45, *Bam*HI/*Xba*I. Electrophoresis was done in 0.8% agarose gels as described in the text. Panel A: DNA fragments stained with ethidium bromide. Panels B and C: Hybridizations of these fragments transferred to nitrocellulose by the method of Southern (31) with FSV(FAV) [32 P]cDNA and 32 P-labeled *gag* DNA (see the legend to Fig. 2 and the text).

FIG. 4. Restriction endonuclease map and organization of the FSV proviral DNA insert in plasmid pFL-5. The size of the FSV DNA provirus was deduced from the sizes of the respective FSV-related fragments shown in Fig. 3 and is in accord with the known size of FSV RNA (4.5 kb) (20). A sequence element related to the *gag* gene of avian tumor viruses, Δ *gag*, was identified near the left LTR, and a sequence element related to the *env* gene, Δ *env*, was identified near the right LTR of the FSV genome. The locations of the junctions between the LTRs of FSV and cell DNA are tentative. pFL-5 was prepared as follows: a *Bgl*II digest of cellular DNA carrying FSV provirus was initially cloned into the *Bam*HI site of lambda charon 27. A *Bgl*II-resistant fragment of 8.4 kb was then cut out of the charon DNA at the *Bgl*II site and at an additional, previously undigested *Bgl*II site of the cellular DNA insert (see the text). This 8.4-kb *Bgl*II-resistant fragment was then cloned into the *Bam*HI site of pBR322 to generate pFL-5. Hence, the 79 base pairs of charon DNA that separate the *Bgl*II site from its original *Bam*HI site (F. Blattner, personal communication) define the left-hand border of the 8.4-kb FSV proviral insert of pFL-5. The distance between the 79-base-pair sequence and the left-hand LTR of FSV must be short but is not known exactly (question mark). Since the *Bgl*II-resistant insert of the recombinant charon phage was cloned into the *Bam*HI site of pBR322, the ligation sites of pFL-5 are no longer sensitive to *Bam*HI or *Bgl*II (see text).

5B). By comparing this fingerprint with that obtained from the 4.5-kb FSV RNA (Fig. 5A), it can be seen that all of the unique, specific (numbers 2, 3, 6, 7, 8, 9, 10, 11, 12, and 13), and group-specific oligonucleotides of FSV (numbers 1, 4, 5, cap, and probably 14) were recovered from the DNA-RNA hybrid. In the fingerprint of the DNA-RNA hybrid (Fig. 5B), the autoradiographic intensity of the group-specific oligonucleotides was higher than that of FSV-specific oligonucleotides. This reflects the presence of FSV and helper FAV-derived group-specific oligonucleotides in hybrids formed by annealing FSV and FAV RNA with an excess of pFL-5 DNA (Fig. 5). The identity of all of the FSV oligonucleotides in the hybrid was verified by analyzing their RNase A-resistant fragments (20). We conclude that pFL-5 contains a DNA equivalent of the entire FSV genome.

It was surprising that FAV oligonucleotide 115, which was previously found to be part of the *env* region in FAV RNA but not in FSV RNA (20), was also hybridized by cloned FSV DNA. This result indicates that FSV probably contains a small region of *env*-related sequences which were not so identified previously (20). Since FSV RNA lacks oligonucleotide 115, the

env-related region of FSV appears to differ in its primary structure from that of FAV. Oligonucleotide 115 maps at sequence positions 6,198 to 6,212 of Prague RSV subgroup C cDNA, which is near the 3' end of the gp85 *env* gene (Schwartz et al., in press). Since the *env* gene encodes a 5' product, gp85, and a 3' product, gp37, the oligonucleotide sequence we found is located approximately in the middle of the *env* gene (Schwartz et al., in press). It would appear that FSV contains an internal fragment of the *env* gene of avian retroviruses.

Biological activity of cloned FSV DNA. (i) **Maintenance of mouse cell transformation controlled by a temperature-sensitive gene product of FSV.** To test its biological activity, in particular the transforming function of FSV DNA, we transfected pFL-5 DNA into mouse NIH-3T3 cells by the calcium phosphate procedure (14). Simian virus 40 DNA served as a positive control, and quail cell DNA was used as the carrier. pFL-5-transfected 3T3 cells formed foci (Fig. 6A) which were similar to those induced by simian virus 40 after 7 to 10 days in culture at 36°C (data not shown). The number of foci induced by pFL-5 DNA was roughly proportional to the amount added; e.g., 8 foci per 100 ng, 12

foci per 200 ng, and 32 foci per 800 ng of pFL-5 DNA. The cells of a majority of these foci reverted to a normal phenotype on propagation into mass cultures and did not contain detectable FSV p140 (see below). Since the number of molecules of cloned proviral DNA per cell was very high under our conditions for transfection (about 10^4 molecules per cell), it is possible that most of the cells were initially infected by multiple copies of cloned FSV DNA. Such tandemly integrated FSV DNAs are subsequently readily lost by homologous recombination. As a consequence, cells initially transformed by multiple FSV copies may revert to normal (G. M. Cooper, personal communication). It is also possible that the abortive transformation by FSV DNA could have been caused by failure to integrate stably and subsequent loss of FSV.

3T3 cells transfected by pFL-5 DNA (100 to 800 ng) also formed colonies in soft agar suspension after 17 to 20 days (Fig. 6B). A stably transformed cell line, termed F3B, was obtained

from such a colony. The transformed 3T3 cells had a fusiform morphology and grew into criss-cross patterns (Fig. 6C) similar to those of the FSV-transformed avian cells (19), but different from 3T3 cells transformed by other retroviruses.

To determine whether F3B cells were transformed directly by expression of the transforming gene of FSV or indirectly, as for example by integration at a specific cellular site, we asked whether expression of the transformed phenotype of the 3T3 cells was temperature sensitive, as was the phenotype of avian cells transformed by the FSV from which the pFL-5 DNA was derived. For this purpose, F3B cells were grown at 36°C and then shifted to 39°C for 12 h (Fig. 6C and D). During this process, the cell morphology changed from an initially transformed to a flat phenotype that was indistinguishable from normal 3T3 cells grown at 36 or 39°C (Fig. 6E and F). F3B cells grown at 39°C reverted to the transformed phenotype overnight after being

FIG. 5. RNase T1-resistant oligonucleotides of FSV RNA and FSV and FAV RNA hybridized by molecularly cloned FSV DNA. (A) Electrophoretically purified 4.5-kb FSV RNA was prepared and fingerprinted as described previously (20). (B) About 0.015 mg of alkali-degraded pFL-5 DNA, cloned in plasmid pBR322, was annealed with 2×10^6 cpm of FSV(FAV) 50-70S [32 P]RNA. After the unhybridized RNA was digested with RNase T1, the hybrid was purified and fingerprinted as described previously (21). The oligonucleotide sequences recovered from this fingerprint were identified by their RNase A-resistant fragments and their chromatographic locations and were numbered as described previously (20).

FIG. 6. Biological activity of cloned FSV DNA. A focus induced in NIH-3T3 cells (A) and a colony formed 17 to 20 days after transfection with pFL-5 DNA (B). The morphology of an FSV DNA-transformed 3T3 cell line, derived from a colony like that shown in B, maintained at 36°C (C) and 12 h after a shift from 36 to 39°C (D). Normal 3T3 cells maintained at 36°C (E) or shifted to 39°C (F).

shifted back to 36°C. We conclude that transformation of 3T3 cells is maintained by a temperature-sensitive gene product of FSV.

(ii) Phosphorylation of the nonstructural FSV protein p140 is temperature sensitive in F3B cells. The result discussed above implied that a viral gene product rather than a positional effect of FSV DNA integration was responsible for the transformation of mouse cells. In view of this possibility, we tested whether p140 was synthesized in F3B cells by labeling F3B cells with [35 S]methionine. The p140 was isolated from cell lysates by immunoprecipitation with anti-avian myeloblastosis virus (AMV) p27 serum. p140 with the same serological properties and electrophoretic mobility as p140 of FSV L-15 quail nonproducer cells was synthesized in the F3B cells (Fig. 7A). FSV-transformed 3T3 cells produced about two to three times less [35 S]methionine-labeled p140 per cell than did FSV-transformed quail cells. No p140 was precipitated from F3B cells with normal serum, nor was p140 precipitated from 3T3 cells with

anti-AMV p27 serum (Fig. 7A). Two proteins, p90 and p50, were occasionally observed in the immunoprecipitates of p140 in F3B cells (Fig. 7). Similar proteins were previously observed to be associated with p140 from avian cells infected with FSV (T. Gilmore and G. S. Martin, personal communication).

It was previously shown that synthesis of p140 in FSV L-15 is not temperature sensitive; however, phosphorylation of p140 both in vivo and in vitro is temperature sensitive (19). To test the possibility that pFL-5 cloned DNA possessed the same characteristics, we labeled F3B cells with [35 S]methionine or 32 PO $_4^{3-}$ for 2 to 3 h at 36 and 39°C. Cellular lysates were immunoprecipitated with anti-AMV p27 serum and then analyzed by SDS-polyacrylamide gel electrophoresis. p140 was synthesized equally well in F3B and in ts FSV L-15 quail nonproducer cells at either 36 or 39°C (Fig. 7B and C). It follows that synthesis of p140 is not temperature sensitive. However, phosphorylation of p140 in vivo was temperature sensitive, as detected by labeling

cells with $^{32}\text{PO}_4^{3-}$ (Fig. 7C). It is recognized that p140 is phosphorylated at the nonpermissive temperature (Fig. 7C) and that the difference between the extent of p140 phosphorylation at the permissive and nonpermissive temperatures is smaller in 3T3 mouse cells than it is in avian cells (Fig. 7C). The higher background phosphorylation of p140 at the nonpermissive temperature of 39°C in mouse cells is thought to reflect only partial inhibition of p140 phosphorylation at this temperature as compared with complete inhibition at 41.5°C in avian cells (19). It is also possible that the threshold level of phosphorylated p140 necessary to transform NIH-3T3 cells at the permissive temperature is lower than that required to transform avian cells. Phosphorylation at the nonpermissive temperature has been noted here (Fig. 7C) and previously in avian cells (19). The temperature-independent phosphorylation has been assigned previously to phosphorylation of the *gag*-related portion of p140 (19, 24).

As we showed previously with p140 immunoprecipitates from avian cells (19, 24), we also observed here that in vitro labeling of immune precipitates of p140 from F3B mouse cells with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 7D) was temperature sensitive.

The correlation between morphological transformation and phosphorylation of p140 and its

association with kinase suggests that fully phosphorylated p140 is necessary for transformation of mammalian cells infected by temperature-sensitive FSV. This is in accord with similar observations on the transformation of avian cells by temperature-sensitive FSV (19, 24).

Cellular DNA homologous to the specific sequence of FSV. Based on helper virus-unrelated, transformation-specific sequences, the known avian sarcoma viruses fall into several subgroups (10). The specific sequences of prototypes of two subgroups, RSV and avian sarcoma virus Y73, were shown to have homologous sequence counterparts in avian cells (32, 33, 35), and the specific sequence of RSV, the *src* gene, also showed homology with other vertebrate chromosomes (32). To test whether the specific sequence of FSV which is the prototype of a third subgroup of avian sarcoma viruses, the FSV subgroup, also has a sequence counterpart in avian and mammalian cells, we cleaved DNA from several animal species with the restriction enzyme *Bam*HI, and, after electrophoretic fractionation, the DNA fragments were hybridized with FSV-specific DNA, i.e., the *Pvu*I-*Sma*I fragment of pFL-5 DNA (Fig. 4). A single size class of *Bam*HI-resistant DNA fragments, measuring 12 kb in chicken and 8 kb in quail cells, hybridized with FSV-specific DNA (Fig. 8).

FIG. 7. Synthesis, phosphorylation, and association with kinase of p140 in F3B cells at the permissive and nonpermissive temperatures. (A) Serological and electrophoretic identification of ^{35}S -methionine-labeled p140 in FSV L-15 quail nonproducer cells (lane 1) and F3B mouse cells (lane 2). p140 (unlabeled arrows) was isolated from cell lysates by immunoprecipitation with anti-AMV p27 serum and electrophoresed as described previously (19). Control experiments testing the synthesis and specificity of the immunoprecipitation are shown in lanes 3–5; 3, immunoprecipitates of ^{35}S -labeled F3B mouse cell lysates incubated with normal goat serum; 4, immunoprecipitates of normal 3T3 cells incubated with anti-AMV p27 serum as used in lanes 1 and 2; 5, immunoprecipitates of normal 3T3 cells incubated with normal goat serum. (B) Comparison of p140 synthesis in FSV L-15 quail nonproducer cells (lanes 6 and 7) and F3B mouse cells (lanes 8 and 9) at the permissive (lanes 6 and 8, 36°C) and nonpermissive temperatures (41.5°C for quail cells, lane 7; 39°C for F3B cells, lane 9). p140 was labeled with ^{35}S -methionine and analyzed as described for A. (C) In vivo phosphorylation of p140 in FSV L-15 quail nonproducer cells (lanes 10 and 11) and F3B mouse cells (lanes 12 and 13) at the permissive (lanes 10 and 12) and nonpermissive (lanes 11 and 13) temperature. p140 was labeled with $^{32}\text{PO}_4^{3-}$ and analyzed. (D) In vitro test of kinase activity associated with p140 isolated from FSV L-15 quail nonproducer cells (lanes 14 and 15) and from F3B mouse cells (lanes 16 and 17). p140 was immunoprecipitated from the cells grown at the permissive (lanes 14 and 16) and nonpermissive (lanes 15 and 17) temperature, and autophosphorylation activity was assayed with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as previously described (19).

FIG. 8. Electrophoretic analysis of restriction enzyme-resistant DNA fragments of various animal species (genomic blots) related to FSV-specific DNA. Mouse cell DNA (10 μ g) and chicken, quail, and *Drosophila* cell DNA (5 μ g each) were digested completely with *Bam*HI and electrophoresed on 0.8% agarose gels. After being blotted (31), the nitrocellulose filter was prehybridized in 50% formamide, 6 $\times$ SSC, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin overnight and then hybridized with the *Puv*1-*Sma*I [32 P]DNA fragment of cloned FSV DNA (see Fig. 4) at 40°C for 36 h. The filter was then washed for 1 h with 2 $\times$ SSC-0.1% SDS and for 1 h with 1 $\times$ SSC-0.1% SDS at 68°C. The blot was autoradiographed for 3 days with an intensifying screen at -70°C.

These results are consistent with the view that possibly only one cellular locus is homologous to the FSV-specific sequence. Under the same conditions, a 20-kb fragment of *Drosophila* DNA and two fragments, 5.4 and 6 kb, of mouse DNA were also hybridized with less intensity.

DISCUSSION

The molecular DNA clone of FSV described here appears to represent the entire viral genome, based on its ability to hybridize a complete complement of viral RNA and to express, in transformed mouse 3T3 cells, the protein p140 which corresponds to most or all of the coding capacity of FSV (20).

All of the previous experiments in transformation of mammalian cells by defective avian tumor viruses have been unable to distinguish

between indirect transformation (as, for example, by positional effects of integrated proviral DNA) and direct transformation via a gene product of the transforming virus. In the absence of a genetic marker, such as the temperature-sensitive transforming gene of the FSV strain used here, this distinction is difficult to make due to the low specific transformation activity of proviral DNAs from defective avian tumor viruses on mammalian cells (6) and the failure to identify proteins with proven transforming function in such cells. Likewise, the low efficiency of avian tumor viruses in transforming mammalian cells (25) did not allow a distinction to be made between direct transformation by a viral gene product and indirect transformation by a positional effect of integrated proviral DNAs.

Our result, that the morphology of mouse 3T3 cells transformed by transfection with DNA from a temperature-sensitive strain of FSV is temperature sensitive for a transformation, provides direct evidence that a viral gene product was responsible for maintaining transformation of these mammalian cells. It follows that the mechanisms of maintaining transformation in both avian and mammalian cells by the avian FSV must be very similar or the same, i.e., both probably involve the same viral gene product(s). The FSV gene product that appears to be necessary for maintenance of transformation in mouse 3T3 cells is the phosphorylated form of p140, the only known gene product encoded by FSV (19, 20). This is consistent with previous evidence linking phosphorylated p140 with transformation of avian cells (19, 24).

The *onc* genes of acutely transforming retroviruses have homologous sequence counterparts in normal animal cells (27, 32, 33, 35). However, the degree of homology between a given viral *onc* gene and its cellular counterpart measured by nucleic acid hybridization seems to vary, greatly in some cases and in others very little, with cellular species. It was observed that the *onc* gene of avian reticuloendotheliosis virus has a closely related sequence counterpart in turkey cells but less homology with other mammalian cells (5). It was recently observed that several viral *onc* genes have homologous sequences in *Drosophila* cell DNA, although this was observed under less stringent conditions of hybridization (30). Here we found that DNAs from two avian species and from mice and *Drosophila* contain sequences homologous to the FSV-specific sequence. By screening a genomic library of chicken chromosomal DNA, we found that a single 16-kb fragment of cellular DNA contains about 2 kb of sequences homologous to the specific sequence of FSV (Lee and Duesberg,

unpublished data). Thus, both lines of experimentation suggest the existence of a single FSV-related locus in the chicken chromosome.

Under more stringent conditions of hybridization, the sequences homologous to the FSV-specific DNA region were only detected in avian cell DNA (unpublished data). Further work involving more direct sequence comparisons is necessary to determine the extent of homology between viral and cellular FSV-related sequences, since blot hybridizations are not readily quantitated.

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