A *fps* Gene Without *gag* Gene Sequences Transforms Cells in Culture and Induces Tumors in Chickens

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Received 8 July 1983/Accepted 30 August 1983

From molecularly cloned DNAs of Fujinami sarcoma virus (FSV) and the Schmidt-Ruppin-A strain of Rous sarcoma virus (SRA), viral DNA was constructed in which *fps*-specific sequences encoded in FSV replaced the *src* gene of SRA. A 3' fragment of FSV DNA, from an ATG methionine coding sequence 148 base pairs downstream from the *gag-fps* junction through the long terminal repeat, was joined to cloned SRA DNA at the translation start site for the *src* gene. The resultant DNA clone contained the splice acceptor site for *src* mRNA processing in SRA, but contained no *src* coding sequences from SRA nor any *gag* sequences from FSV. All genes for the replication of SRA were retained. Transfection of this cloned viral DNA genome into chicken embryo fibroblasts induced morphological transformation of the cells in culture. However, the morphology of the transformed cells was distinct from that observed in cells infected with wild-type FSV. The transformed cells produced a nondefective transforming virus called F36 which contained a hybrid FSV-SRA long terminal repeat. F36-infected cells produced a protein with the expected molecular weight of 91,000, which had an associated protein kinase activity and was immunoprecipitated by antibodies raised against *fps* gene determinants but not by antibodies raised against *gag* or *src* proteins. Injection of F36 virus into 8-day-old chicks produced tumors at the site of inoculation, detectable within 7 days. These results demonstrated that the *gag* portion of the *gag-fps* fusion protein of FSV is not required for transformation or tumorigenesis.

Several rapidly transforming retroviruses have been characterized in recent years (2). These viruses encode transduced cellular genes that transform cells in culture and induce tumors with a short latency (2). Most rapidly transforming retroviruses characterized to date contain hybrid genes that are fusions between the viral *gag* gene and cellular genes (2, 3, 16). These hybrid genes produce *gag*-linked fusion proteins that are responsible for the transforming capacity of the virus (2).

Fujinami sarcoma virus (FSV) encodes a 130,000-dalton *gag-fps* fusion protein (P130^{*gag-fps*}) (19, 25). P130^{*gag-fps*} contains a protein kinase activity specific for tyrosine residues that is similar to the products of several other related viral oncogenes (2, 3, 6, 10, 17, 32, 38). In an attempt to examine the role of *gag* determinants of *gag-fps* fusion proteins in cellular transformation, viral DNA was constructed so that the *src* gene of Rous sarcoma virus was replaced by greater than 90% of the *fps* sequences from FSV. The virus produced after transfection of this DNA, designated F36, contained the splice acceptor site for production of the *src*-specific message (7, 36, 44), so that the *fps* gene was

expressed as a spliced subgenomic message free of any *gag* gene sequences.

MATERIALS AND METHODS

Recombinant DNA and techniques. The cloned DNA used in the construction of F36 viral DNA was derived from pSRA2 (originally provided by J. M. Bishop) as described by DeLorbe et al. (8) and λ FSV-2 DNA described by Shibuya et al. (39). The two subclones of pSRA2 called pSR-XD2 and pSR-REP were obtained from F. Cross. The structures of these subclones are shown in Fig. 1. Neither is infectious by itself, but cotransfection of the two subclones gives rise to the infectious Schmidt-Ruppin-A strain of Rous sarcoma virus (SRA) (6a).

All restriction enzymes used were from either Bethesda Research Laboratories or New England Biolabs and were used in the buffers described by Maniatis et al. (27). T4 DNA ligase was obtained from New England Nuclear Corp.

Purification of DNA fragments. Restriction endonuclease-digested DNA fragments were electrophoresed on 0.8% agarose gels in 40 mM Tris-hydrochloride (pH 7.4)-5 mM NaOAc-1 mM EDTA. DNA bands were visualized by long-wave UV irradiation after staining with ethidium bromide. Gel slices were cut out and electroeluted in dialysis tubing for 2 h at 150 V in the electrophoresis buffer. The DNA fragments were collected after phenol extraction by ethanol precipitation.

FIG. 1. Strategy for construction of F36 virus from FSV and SRA DNA. pSR-XD2 and pSR-REP are subclones of pSRA2 which contains the SRA genome with a tandem LTR as described by DeLorbe et al. (8). λ FSV-2 DNA contains the entire FSV genome with tandem LTRs (39). Details of the construction are in the text. The strategy is represented schematically and is not drawn to scale.

Chicken cells, virus infection, and DNA transfection. Chicken embryo fibroblasts (CEFs) were prepared as described previously (18). The virus infection and the focus assay were performed as described by Hanafusa (18). Secondary cultures were seeded at 5×10^5 cells per 60-mm plate. Between 16 and 24 h later, transfection of DNA into the cells was performed as described by Wigler et al. (51). A 1- μ g sample each of pSR-REP DNA and pSR-F36 DNA cut with *SalI* was ligated for 45 min with 1 U of T4 DNA ligase. This DNA was then precipitated directly in a calcium phosphate suspension (51), added to the cell culture medium, and allowed to sit for 6 h.

Analysis of proviral DNA. Chromosomal DNA was prepared from transformed CEFs that had undergone at least one passage as described previously (13). DNA (15 μ g) was digested with restriction enzymes, run on an 0.8% agarose gel, and transferred to nitrocellulose by the method of Southern (43). Prehybridization was for 6 to 24 h in 50% formamide-3 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (27)-50 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA-20 μ g of yeast tRNA per ml-20 μ g of sheared denatured salmon sperm DNA per ml-1 $\times$ Denhardt solution (27) at 37°C. Hybridization was with 5×10^6 cpm of nick-translated DNA in fresh prehybridization buffer for 48 h at 37°C. The filter was washed twice in 0.1 $\times$ SSC-0.1% sodium dodecyl sulfate at 50°C for 1 h.

Analysis of RNA. Polyadenylated RNA was purified from transformed cells, run on a 1% agarose gel, and transferred to nitrocellulose as described previously (49). Hybridization to 2×10^6 cpm of nick-translated DNA was done using the same conditions used for analysis of proviral DNA. The DNAs used as hybridization probes were a 500-base-pair (bp) *EcoRI*-*BamHI* fragment from the long terminal repeat (LTR) and *gag* regions of SRA (8) and a 700-bp *PvuII* fragment from the *fps* gene of FSV (39).

Analysis of proteins. [35 S]methionine-labeled and unlabeled cell lysates were prepared in RIPA buffer as described previously (10, 11). Preparation of antisera and immunoprecipitations were as previously described (28). Analysis of immunoprecipitates on 8.5% acrylamide-sodium dodecyl sulfate gels and the protein kinase assay have been described elsewhere (10, 12, 28).

RESULTS

Construction of viral DNA in which *fps* is substituted for *src*. We wanted to construct a viral DNA that was free of all *gag* gene sequences since *gag* precursor proteins are functionally modified in their amino terminus (21, 31, 33, 35, 37). The strategy for construction was to

FIG. 2. Focus formation on CEFs by FSV and F36. CEFs were infected with low concentrations of FSV (A) or F36 (B), and foci were observed 7 days after infection. Typical foci from each culture are shown.

join a 3' portion of FSV DNA containing greater than 90% of the *fps* sequence, but lacking all *gag* sequences, to a 5' portion of cloned DNA from SRA that lacked all *src* sequences. The amino-terminal methionine coding sequence of *src* in Rous sarcoma virus is included in an *Nco*I site which recognizes the sequence CCATGG. Downstream 148 bp from the *gag-fps* junction of FSV, there is another *Nco*I site that also includes a methionine coding region. A viral DNA

genome was constructed by ligating the two viral genomes together at this point (Fig. 1).

Both pSR-XD2 and λ FSV-2 were cut with *Sst*I which cuts once at a site between the LTR and *gag* gene of both DNAs. This was followed by partial digestion with *Nco*I to generate the linear fragments shown in Fig. 1. The 3.6-kilo-base-pair (kbp) FSV DNA fragment containing the *fps* gene and two tandem FSV LTRs was then ligated into the pSR-XD2 vector in the

TABLE 1. Tumor formation by F36^a

Virus	Tumor size in mm ^b at indicated days after inoculation				
	4	7	9	11	15
FSV	0	3	20	>20	>20
F36	0	1	8	20	>20

^a Samples of media (5×10^4 focus-forming units) from transformed CEF cultures were injected into the wing web of 7-day-old chicks.

^b Tumor size represents the average tumor diameter from two chicks injected in each wing.

position formerly occupied by the *src* gene and two tandem SRA LTRs. The resultant DNA clone was designated pSR-F36. A complete viral DNA genome (Fig. 1) could be generated by cutting pSR-F36 with *Sall*, which liberates viral sequences from the vector, and ligating it to *Sall*-cut pSR-REP (another subclone of SRA that contains the LTR, *gag*, *pol*, and remaining *env* gene sequences) (6a). This DNA contains all replication genes and the splice acceptor site for the *src* gene (7, 36, 44) but contains *fps* rather than *src* coding regions. The construct thus would produce a *fps* message free of *gag* gene sequences. As constructed, tandem LTRs at the 5' end are derived from SRA, and tandem LTRs at the 3' end are derived from FSV.

Transfection of CEFs with viral DNA and recovery of virus. The constructed DNA was transfected into CEFs as described above by using the calcium phosphate method (51). Three days after transfection, the cells were split 1:3, and 4 to 5 days later a change in cell morphology could be detected, characterized by an increased refractility that distinguished transformed cells from flat nontransformed cells. Medium from transformed cultures was collected as a source of virus which was called F36. Fresh CEF cultures were infected with low concentrations of F36, and single colonies were selected for growth in soft agar as described previously (23). All 12 clones picked produced virus; a result consistent with F36 virus being nondefective as constructed. Infection of CEFs with low concentrations of F36 resulted in formation of foci which consisted of cells whose morphological changes were more subtle than those formed by FSV (Fig. 2). However, when the entire cultures were transformed by F36, morphological changes were quite evident in comparison with uninfected cells (data not shown).

Tumor formation by F36 virus. Although F36 virus induced colony formation in soft agar, the different morphologies of FSV- and F36-transformed cells raised the possibility that the ability to induce tumors in chickens might be different as well. Both FSV and F36 virus were injected

into the wing web of 7-day-old chicks. Table 1 shows that both viruses induced large tumors with approximately the same latency.

Analysis of the *fps* gene product of F36 virus. Transformed cultures of CEFs were labeled with [³⁵S]methionine and lysed as described previously (10, 19). Cell lysates were immunoprecipitated with tumor-bearing rabbit antiserum raised against tumors induced by the Schmidt-Ruppin-D strain of Rous sarcoma virus (TBR) (4) and from tumor-bearing rat antiserum raised against tumors induced by FSV (anti-FST) (28). TBR serum recognized *gag* determinants but not *fps* determinants. Anti-FST serum recognized both *gag* and *fps* determinants. These two sera were used to distinguish between *fps* gene products that were linked or unlinked to *gag* gene determinants. Immunoprecipitates were electrophoresed on sodium dodecyl sulfate-acrylamide gels and analyzed by fluorography. FSV-infected cells produced a protein with a molecular weight of 130,000 (P130^{*gag-fps*}) that was recognized by both TBR and anti-FST sera (Fig. 3). F36-infected cells produced a protein with the expected molecular weight of 91,000 (P91^{*fps*}) that was precipitated only by anti-FST serum. These results established that significant portions of *gag* gene determinants were not associated with P91^{*fps*}.

Unlabeled cell extracts were immunoprecipitated and subjected to an in vitro protein kinase assay (11). The results (Fig. 4) demonstrated that anti-FST serum precipitates a protein kinase activity in extracts from F36-infected cells

FIG. 3. Analysis of F36-specific proteins. [³⁵S]methionine-labeled cell lysates from an uninfected control culture and FSV- or F36-transformed cells were immunoprecipitated by TBR antisera (a) or by anti-FST antisera (b). Immunoprecipitated proteins were electrophoresed on an 8.5% acrylamide-sodium dodecyl sulfate gel and observed by fluorography (13). Pr180 is the *gag-pol* precursor product; Pr76 is the *gag* precursor product.

and that P91 is phosphorylated very efficiently. TBR serum failed to precipitate a kinase activity from this extract. The other major phosphorylated species observed near the bottom of the figure are immunoglobulin molecules which serve as good substrates in this assay (10, 12).

Analysis of the genome of F36 virus produced in culture. The structure of F36 virus produced upon transfection was examined in two ways. The structure of the integrated provirus in infected cells was examined by restriction enzyme digestion, and the size of RNA transcripts from infected cells was compared with the size of those from SRA- and FSV-infected cells.

The DNA used to generate F36 contained 5' tandem LTRs derived from SRA and 3' tandem LTRs derived from FSV (Fig. 1). Upon the recovery of virus and reinfection of CEFs, proviral DNA would be expected to contain hybrid SRA-FSV LTRs with the U3 portion contributed by FSV and the U5 portion contributed by SRA (9, 40, 45, 47). This was tested by analyzing proviral DNA in infected cells. *PvuI* cuts once in the U3 portion of the SRA LTR but not in the U3 portion of FSV DNA contained in F36. *EcoRV* cuts FSV DNA in the U3 portion of the LTR and cuts SRA DNA once in the *pol* gene at a distance 4 kbp downstream from the 5' LTR (Fig. 5a). Chromosomal DNA from SRA- and F36-infected cells was digested with *PvuI* or *EcoRV* or both, followed by agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to a nick-translated 3,800-bp *SmaI* fragment from the *pol* and *env* regions of SRA (Fig. 5a). The results of this analysis are shown in Fig. 5b. The DNA probe hybridized to several endogenous viral bands as shown in the uninfected control panel of Fig. 5b. Digestion of DNA from SRA-infected cells with *PvuI* gave a discrete 10-kbp band that hybridized with the probe, and digestion with

FIG. 4. In vitro kinase activity of F36-transformed cell extracts. Immunoprecipitates of extracts from F36- or FSV-infected cells were subject to an in vitro kinase assay and electrophoresed as in Fig. 3. (a) TBR, (b) anti-FST.

FIG. 5. Analysis of proviral DNA. (A) Cleavage sites for *PvuI* and *EcoRV* in proviral DNA from F36- and SRA-infected cells. (B) Southern blot analysis of integrated proviral DNA. Chromosomal DNAs (15 μ g) were digested with *PvuI* (a), *PvuI* + *EcoRV* (b), and *EcoRV* (c). The DNAs were then run on an 0.8% agarose gel, transferred to nitrocellulose, and hybridized to the nick-translated DNA fragment shown in (A). The markers (M) indicated by bars between the different DNAs are restriction fragments from *HindIII*-digested λ -DNA and are presented in kbp.

both *PvuI* and *EcoRV* gave two bands of 4 and 6 kbp, representing the 5' and 3' regions of the SRA genome, respectively (Fig. 5a and 5b). Digestion with *EcoRV* alone, which cuts once in the SRA genome, yielded no discrete bands. Presumably this was because the infected cells used here were not clonal, and the integration of proviral DNA was random (22, 26).

Digestion of DNA from F36-infected cells with *PvuI* yielded no discrete bands (Fig. 5b), which indicated that *PvuI* sites are not present in the LTRs of F36 proviral DNA. Digestion with *EcoRV*, however, gave two bands of 4 and 7.1 kbp. These data are consistent with F36 provirus having FSV-derived U3 regions at both 5' and 3'

FIG. 6. Analysis of polyadenylated RNA from cells infected with SRA, F36, and FSV. Polyadenylated RNA was obtained from SRA-infected cells (lane a), F36-infected cells (lane b), and FSV-infected cells (lane c) and was run on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated LTR-*gag* DNA fragment (lane a) or an *fps*-specific DNA fragment (lanes b and c). The DNA probes were described in the text. The markers (m) used were denatured DNA fragments from a *Hind*III digest of λ -DNA, and the sizes are presented in nucleotides $\times 10^{-3}$.

LTRs (Fig. 5a). These results demonstrated that integrated proviral DNA from F36-infected cells has the structure that was expected for the nondefective virus as constructed and that a hybrid LTR containing the U3 region of FSV is functional in virus production and provirus integration. Hybrid LTRs have been shown to be functional in other recombinant viruses as well (9).

To further verify F36 viral structure and to examine the expression of viral RNAs, an analysis was performed on polyadenylated viral RNAs from F36-infected cells, and they were compared with those from SRA- and FSV-infected cells. SRA-infected cells produce three viral messages: a genome length message used for *gag* and *pol* gene expression; a spliced subgenomic message for *env* gene expression that also contains *src* sequences; and a spliced subgenomic *src* message (20, 50). These three SRA messages of 10,000, 5,400, and 3,000 nucleotides in length were observed in Fig. 6, lane a. Genomic length FSV RNA of 4,600 nucleotides in length (38) was observed in lane c of Fig. 6 by using a *fps*-specific probe. F36-infected cells produced three bands (11,100, 6,500, and 4,100 nucleotides in length) that were about 1,100 nucleotides in length larger than the three corresponding bands of SRA (Fig. 6, lane b). This represents the size difference between SRA and F36 virus as constructed. Additional bands observed in F36-infected cells may represent specific degradation products or deletion mutants generated during infection. There is evidence

suggesting that insertion of DNA fragments of this size or larger into the *src* gene region of RSV may lead to the generation of spontaneous deletion mutants (42). However, analysis of proviral DNA did not show significant amounts of such deleted proviral DNA (Fig. 5b).

DISCUSSION

Seven independent isolates of *fps*-containing transforming retroviruses (FSV, PRCII, PRCIV, URI, S-T FeSV, G-A FeSV, and 16L) all contain *gag*-linked *fps* genes coding for *gag-fps* fusion gene products that are responsible for the transforming properties of the viruses (2, 3, 14, 19, 25, 29, 30, 41, 48). This study attempts to clarify the role of *gag* gene determinants in the *gag-fps* fusion protein encoded in FSV. Data obtained with F36 virus demonstrate that *gag* gene determinants are not required for the transforming capacity, tumor-inducing ability, or protein kinase activity of the *fps* gene product of FSV. Although the data presented here show that *gag* gene sequences are not required for transformation, the fact that all characterized *fps*-containing transforming retroviruses express *gag*-linked *fps* fusion proteins suggests that the association is more than fortuitous.

Two alternative explanations for the *gag*-linkage of *fps*-carrying retroviruses are that *gag* gene determinants replace the amino terminus of the *c-fps* gene product which blocks the transforming potential of this protein and that linkage to the *gag* gene replaces cellular control elements with viral control elements. These two possibilities are not mutually exclusive and either or both could be important for the acquisition of a *c-fps* gene with transforming potential.

The chicken *c-fps* gene encodes a protein with a molecular weight of about 98,000 (NCP98) (28). FSV contains *fps*-specific coding sequences for a protein with a molecular weight of 96,000 (38). Thus, it is possible that 2,000-daltons worth of amino-terminal *c-fps* sequences may have been replaced by *gag* sequences in FSV. If this portion of NCP98 blocks the transformation potential, then substitution with *gag* gene sequences would permit transformation and provide a viral translation start signal to replace the cellular one lost upon recombination with a retrovirus. This would also put expression of the *fps* gene under viral control.

Several viral transforming genes (*myc*, *myb*, *sis*, and *ras*) are translated into both *gag*-linked and *gag*-unlinked transforming gene products (1, 2, 5, 15, 24, 34). These viral oncogenes are expressed as subgenomic messages when not linked to *gag* gene sequences. The splice acceptor site for the generation of subgenomic messages is usually provided by cellular sequences.

although in some cases (*mos* and *myb*) the use of the *env* gene splice acceptor site has not been ruled out (2, 24, 46). The subgenomic *fps* message expressed by F36 utilized the *src* gene splice acceptor site and generated a gene product that was translated from an internal AUG start signal. This kind of virus could not easily have been generated naturally unless the cellular *fps* gene had a splice acceptor site at an appropriate distance upstream from a comparable in-phase AUG translation start signal. If such a site does not exist, then the most likely way that *c-fps* gene expression could come under viral control is via the formation of a hybrid gene such as the *gag-fps* fusion gene of FSFV.

Recently it was shown that a deletion of *gag* sequences from the transforming gene of Abelson MuLV (*abl*) abolishes induction of B-cell lymphomas in mice by this virus (32a). These authors also point out that although a deletion in the *gag* portion of the viral *abl* gene abolishes the ability to induce lymphomas, it does not abolish the ability to transform fibroblasts in culture. These results may point out important differences between sarcoma- and lymphoma-generating systems or between the *abl* and *fps* genes, both of which possess a tyrosine kinase activity (10, 52).

ACKNOWLEDGMENTS

We express gratitude to Fred Cross for providing the pSR-XD2 and pSR-REP clones and for helpful suggestions in cloning, to Bernard Mathey-Prevot for providing anti-FST and TBR antisera, and to Lu-Hai Wang for help in the RNA analysis. Thanks are also extended to David Baltimore for communicating results before publication. We would also like to thank Fred Cross, Bernard Mathey-Prevot, Ricardo A. Feldman, and George Q. Daley for critically reading the manuscript.

This work was supported by Public Health Service grants CA14935 and CA18213 from the National Cancer Institute. D.A.F. is a recipient of National Research Service Award F32 CA07141 from the National Cancer Institute.

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