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Transforming potential of a human protooncogene (c-*fps/fes*) locus

(cellular oncogenes/feline sarcoma virus/NIH 3T3 transfection/tyrosine kinase)

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ABSTRACT **The protooncogene c-*fps/fes* is a single vertebrate locus homologous to both the *fps* transforming gene of Fujinami avian sarcoma virus and the *fes* transforming gene of two feline sarcoma virus isolates. The human c-*fps/fes* locus has been previously cloned and characterized. We report that a recombinant gene in which 3′ human c-*fps/fes* sequences replace over 80% of the feline sarcoma virus *fes* sequences transforms NIH 3T3 mouse fibroblasts and encodes a protein kinase. Cells transformed by this recombinant possess increased phosphotyrosine levels. These observations demonstrate that a large carboxyl portion of a human c-*fps/fes* protein product can functionally complement a retroviral transforming protein.**

Several highly conserved cellular genes, called protooncogenes or c-*onc* genes, induce rapid malignancies in animals when transduced by retroviruses (1, 2). Some of these genes have been implicated in the genesis of a number of nonviral animal and human tumors (3). The mechanism of activation of these genes to a transforming state is of central interest in understanding neoplastic processes. Moreover, studies of human c-*onc* gene activation may be relevant to clinically important malignant disease.

The Fujinami avian sarcoma virus (FSV) and the Gardner–Arnstein and Snyder–Theilen feline sarcoma viruses (GA-FeSV and ST-FeSV) have transduced host cell sequences (denoted *fps* and *fes*, respectively) that are necessary for their transforming ability (4, 5). These sequences, fused to 5′ viral *gag* sequences, encode tyrosine-specific protein kinases (6–9). The carboxyl portions of the FSV and FeSV transforming products exhibit homology at the protein level with other retroviral tyrosine kinases and a cAMP-dependent protein kinase (10). Studies of deletion mutants have established the importance of the *fes* product's carboxyl half in transformation (4, 5).

The genome of vertebrates possesses a single locus that was transduced independently by FSV, ST-FeSV, and GA-FeSV (11). The entire human region (c-*fps/fes*) homologous to the transforming genes of these retroviruses is located within a 14-kilobase (kb) *Eco*RI DNA fragment (Fig. 1) (11, 13–15). The regions of homology in the human genome are discontinuous, suggesting an exon–intron structure with a minimum of six exons homologous to the FSV transforming gene (v-*fps*) (11).

Here we report the construction of a recombinant gene in which 3′ human c-*fps/fes* sequences replace the majority of FeSV *fes* sequences necessary for transformation of NIH 3T3 mouse fibroblasts. This gene fully transforms NIH 3T3 cells and encodes a tyrosine kinase. These observations demonstrate that a large carboxyl portion of a human c-*fps/fes* protein product can functionally complement a retroviral transforming protein.

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MATERIALS AND METHODS

Materials. Mycophenolic acid (MPA) was supplied gratis by Eli Lilly.

Construction of Recombinants. Subclones of the appropriate fragments of the 14-kb *Eco*RI human c-*fps/fes* clone were made in pBR322 derivatives. Restriction enzyme digestions were performed according to supplier's specifications and, in cases where partial digestions were required, in the presence of ethidium bromide. The integrity and in-phase nature of the v-*fes*/c-*fes* recombinant junctions were determined by DNA sequence analysis (18, 19).

NIH 3T3 Transformation Assays. NIH 3T3 transfections were performed as described (20). For tests of anchorage independence, transfected cells were treated with trypsin 3 days after transfection and seeded into medium supplemented with 10% calf serum in the presence of xanthine and MPA as described (16). Twenty randomly selected MPA-resistant colonies as well as mixtures of total MPA-resistant populations from each transfection were suspended in Dulbecco's medium/10% calf serum/0.25% agarose. Anchorage-independent colonies were scored at 12–14 days. Positive colonies reached 200–1000 cells at the time of scoring, whereas negative transfectants exhibited no colonies greater than 3–4 cells. Tumorigenicity in nude mice was assessed by subcutaneous injection of approximately 5×10^5 cells into the flanks of Swiss *nu/nu* males. Positive animals developed easily visible tumors within 2 weeks of injection and died due to tumor growth within 5 weeks after inoculation. Negative animals failed to develop palpable tumors within 8 weeks after injection.

RESULTS

Transformation Assays Using the Complete Human c-*fps/fes* Locus. The ability of the human c-*fps/fes* locus to transform cells was tested by transfection of NIH 3T3 fibroblasts with the 14-kb cloned DNA fragment inserted into plasmids that contained the selectable marker pSV_2gpt (Fig. 1) (16). Neoplastic transformation was assessed in two ways: observation of focus formation against a background of a contact-inhibited cell monolayer; or selection of colonies for the pSV_2gpt marker in MPA and xanthine and subsequent evaluation of anchorage-independent growth in soft agar. A 15-kb *Eco*RI fragment containing a complete integrated GA-FeSV provirus (12) served as a positive control in these assays.

Both the cloned 14-kb human c-*fps/fes* fragment alone and the same fragment positioned 3′ to strong transcriptional promoters (the SV40 early promoter and the GA-FeSV LTR) (Fig. 1, c-*fps/fes* and LTR c-*fps*) failed to transform NIH 3T3 fibroblasts. The inability to transform was not due to

Abbreviations: FSV, Fujinami avian sarcoma virus; FeSV, feline sarcoma virus; GA-FeSV, Gardner–Arnstein FeSV; ST-FeSV, Snyder–Theilen FeSV; SV40, simian virus 40; kb, kilobase(s); LTR, long terminal repeat; MPA, mycophenolic acid.

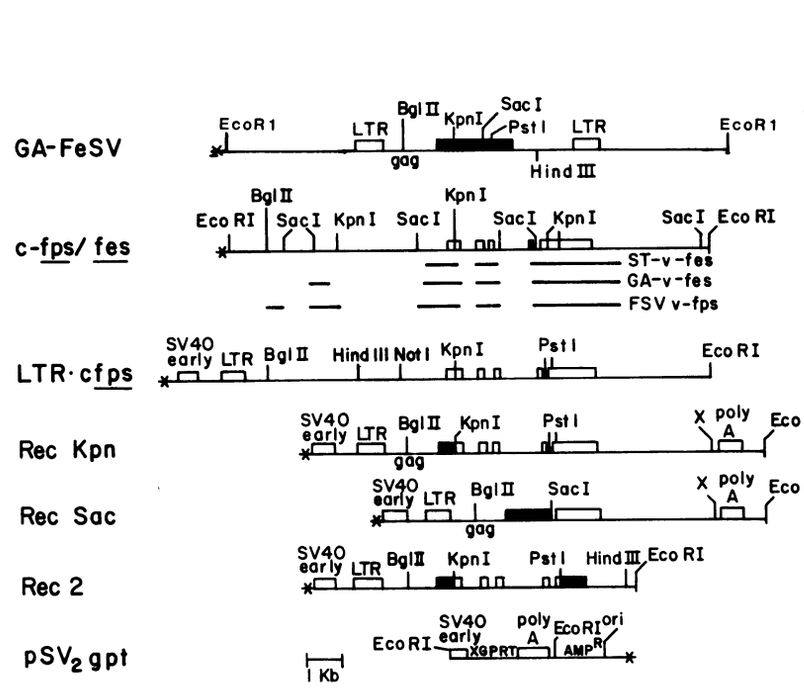

FIG. 1. Structures of recombinants and results of transformation assays. The structure of the cloned GA-FeSV provirus is shown along with relevant restriction endonuclease sites (12). Viral *fes* sequences are illustrated by the black box. The structure of the cloned 14-kb *Eco*RI fragment containing the entire human c-*fps/fes* locus is shown (11, 13–15). The maximal extent of regions with homology to the FSV, GA-FeSV, and ST-FeSV transforming regions as defined by Southern blots of the cloned c-*fps/fes* locus are designated by horizontal bars beneath the c-*fps/fes* restriction map. Regions of homology between v-*fes* and human c-*fps/fes* defined by DNA sequencing (our unpublished data) or heteroduplex analysis (15) are depicted as open boxes above the restriction map. The DNA sequences across the recombinant junctions are as follows:

	v-*fes*	*Kpn* I	c-*fes*
Rec-Kpn	CTG CAC GAG CAG CTG TGG TAC		CAC GGG GCC ATC CCG AGG
GA-FeSV	CTG CAC GAG CAG CTG TGG TAC		CAC GGG GCC CTC CCA CCG

		Sac I	
Rec-Sac	ATC TAC ATC GTC ATC GAG CTC		CAA CTA GGG CGC GCA GCT
GA-FeSV	ATC TAC ATC GTC ATC GAG CTC		CTG CAG GGG GGC GAC TTC.

In addition to the sequences shown, each circular plasmid contains pBR322 sequences from the unique *Eco*RI site to the *Pvu* II site as well as the pSV_2gpt construction (see bottom structure) (16). These have been inserted at *Eco*RI sites (designated by asterisks) into the plasmids used for transfection. The simian virus 40 (SV40) early region promoter and origin of replication [positions 5107 to 208 of the map of Reddy *et al.* (17)] are denoted "SV40 early." The GA-FeSV long terminal repeat (LTR) and untranslated sequences 5′ to the *Bgl* II site of the provirus are designated "LTR." The SV40 small tumor antigen splice acceptor and polyadenylylation signal derived from the pSV_2gpt vector (16) are designated "poly A." "XGPRT" indicates the position of the *Escherichia coli* xanthine/guanine phosphoribosyltransferase gene in pSV_2gpt. "ori" marks the pBR322 origin of replication and "AMP^R" the ampicillinase gene. Focus-forming efficiency equals the number of foci per 3×10^5 cells per μg of tested DNA. The percentage of mycophenolic acid (MPA)-selected clones that were anchorage independent is noted. Tumorigenicity in nude mice represents the number of animals that developed tumors/number of inoculated animals. ND, not determined.

unsuccessful transfection, because Southern blot analysis of MPA-resistant colonies revealed the presence of integrated intact DNA from these constructs (Fig. 2, lanes 3 and 4); however, only LTR c-*fps* transfectants produced detectable levels of *fps/fes*-related polyadenylylated RNA (Fig. 3, lanes 3 and 4). The RNA produced in these cells was polyadenylylated, probably from a signal within the human c-*fps/fes* locus itself, since no extrinsic signals of this sort were provided in the construction of the vector. The RNA was also spliced, as it was 3.3 kb in length yet could be detected by using probes from both the 5′ and 3′ ends of the v-*fes* gene (data not shown). This RNA was of a size consistent with that expected if v-*fes* homologous regions corresponded to exons. Limited DNA sequence analysis of some of the boundaries between the v-*fps* homologous and nonhomologous regions has revealed the presence of consensus sequences for eukaryotic splice donor and acceptor sequences at appropriate sites (unpublished observations). Thus, the RNA produced by this construction in NIH 3T3 cells appears to be consistent with a properly spliced and polyadenylylated message. These observations indicate that the human c-*fps/fes* fragment used in our studies is not transforming, even when transcriptionally activated. An assessment of the transforming potential of the complete human c-*fps/fes* gene requires further study, because our cloned 14-kb *Eco*RI fragment may not contain the entire gene.

Construction of Recombinants Between Human c-*fps/fes* and GA-FeSV. To determine if portions of the human c-*fps/fes* locus can complement regions of the viral *fes* gene, recombinants between the c-*fps/fes* locus and GA-FeSV were constructed. Recombinants were made at apparently conserved restriction sites (Fig. 1). DNA sequence analysis demonstrated that the Rec-Kpn and Rec-2 recombinants possess in-phase protein-reading sequences across the

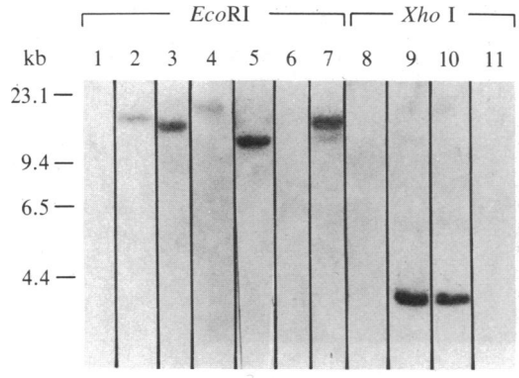

FIG. 2. Southern blots of transfected cells. Ten micrograms of high molecular weight DNA of the transfected cells was Southern blotted and probed (21) with a nick-translated (22) *Bgl* II/*Hin*dIII *gag–fes* fragment from GA-FeSV. Filters were washed as described (13). DNA in lanes 1–7 was digested with *Eco*RI, that in lanes 8–11, with *Xho* I. With the exception of cells derived from a Rec-Kpn focus (lane 10), all NIH 3T3 cells were cloned and selected in MPA and xanthine. The sources of the DNA are NIH 3T3 cells transfected with pSV_2gpt alone (lane 1); GA-FeSV (lane 2); c-*fps/fes* (lane 3); LTR·c-*fps* (lane 4); Rec-Sac (lane 5); Rec-Kpn, untransformed, but MPA resistant (lanes 6 and 8); Rec-Kpn, transformed (lanes 7 and 9); and Rec-Kpn, complete revertant (lane 11).

recombinant junctions (see legend to Fig. 1). The reading frame was not conserved in the Rec-Sac construct, as ligation occurred at a nonconserved *Sac* I site (within a putative c-*fps/fes* intron).

Transfection Assays Using the Recombinants. The recombinant plasmids containing pSV_2gpt were transfected into NIH 3T3 cells. As noted in Fig. 1, pSV_2gpt alone and the Rec-Sac construction failed to transform NIH 3T3 cells. Southern blot analysis revealed that MPA-resistant Rec-Sac-transfected colonies contained the entire integrated recombinant locus (Fig. 2, lane 5). Thus, the viral sequences in Rec-Sac alone are not sufficient for transformation of NIH 3T3 cells, consistent with other studies of GA-FeSV deletion mutants (4, 5).

The Rec-Kpn plasmid was able to induce foci on NIH 3T3 cells, though at a lower efficiency than occurs upon transfection with the GA-FeSV provirus. The foci induced by this recombinant consisted of refractile fusiform cells. Three of

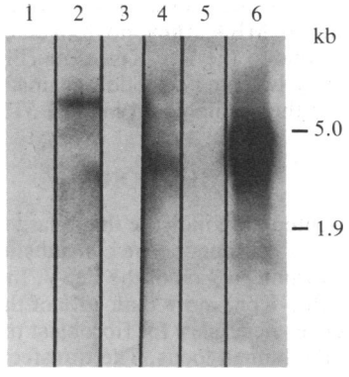

FIG. 3. Blot hybridization of RNA from transfected cells. Cells from MPA-resistant clones shown by Southern blotting to contain intact plasmids were used for total RNA extraction by the guanidine hydrochloride technique (23). Two to 10 μg of polyadenylylated RNA (24) was blotted (25), probed with a nick-translated *Bgl* II/*Hin*dIII v-*fes* fragment, and washed as described (13). Sources of RNA were NIH 3T3 cells transfected with pSV_2gpt alone (lane 1); GA-FeSV (lane 2); c-*fps/fes* (lane 3); LTR·c-*fps* (lane 4); Rec-Kpn, untransformed but MPA resistant (lane 5); Rec-Kpn, MPA selected and transformed (lane 6).

18 of the Rec-Kpn MPA-resistant clones were anchorage independent, as were all the foci tested. These anchorage independent cells formed tumors that were easily visible in nude mice 2 weeks after subcutaneous injection of less than 10^6 cells (Fig. 1). Injection of 10^6 untransformed NIH 3T3 cells did not produce tumors in control experiments after 8 weeks of observation.

Nucleic Acid Analysis of the Transformed Cells. To understand why only some of the Rec-Kpn transfectants exhibited a transformed phenotype, the structure of the Rec-Kpn sequences in the MPA-resistant colonies was examined. A 14-kb *Eco*RI fragment and two 4.0-kb *Xho* I fragments, expected from integration of the intact recombinant locus, were detected in the DNA of transformed cells (Fig. 2, lanes 7 and 9). Analysis of the integrated DNA of these clones by using several other restriction endonucleases indicated that no detectable rearrangements had occurred in the Rec-Kpn construct during transfection and integration (data not shown).

On the other hand, substantial alterations in the structure of the transfected Rec-Kpn plasmid were detected in the MPA-resistant clones that were not transformed. In most cases, no *fps/fes*-homologous DNA (except for faint hybridization to the endogenous mouse c-*fps/fes* locus at 6.0 kb) was detected (Fig. 2, lanes 6 and 8). In some cases substantial interruptions of the Rec-Kpn construct had occurred during integration or transfection in the untransformed clones.

Examination of the anchorage-independent Rec-Kpn transfectants for *fes*-related RNA species revealed the presence of a 4.5-kb RNA transcript that was detected by hybridization to DNA probes prepared from the GA-FeSV *gag–fes* region (Fig. 3, lane 6). No *fps/fes*-related transcripts were observed in untreated NIH 3T3 cells or in MPA-resistant, untransformed clones that had been transfected with the Rec-Kpn DNA (Fig. 3, lanes 1 and 5).

Protein Product of the Transforming Recombinant. To characterize the protein product of the Rec-Kpn gene, total cell protein lysates from transfected cells were immunoprecipitated by using monoclonal antibodies prepared against the p15 *gag*- and the v-*fes*-specific regions of the GA-FeSV p110 transforming protein (Fig. 4) (26). A protein that migrates slightly slower on sodium dodecyl sulfate/polyacrylamide gels than does the p110 GA-FeSV transforming gene product was precipitated by anti-p15 *gag* and one of the anti-*fes* region monoclonal antibodies (F113) in the cells that were transformed by the Rec-Kpn construct. No such proteins were precipitated in extracts prepared from untreated NIH 3T3 cells or MPA-resistant cells that were not transformed but that had also been transfected with the Rec-Kpn construct. An additional monoclonal antibody prepared against the GA-FeSV p110 protein that recognizes *fes*-specific determinants (F123) did not immunoprecipitate a protein from the Rec-Kpn-transformed cells (26). These data demonstrate that the Rec-Kpn chimeric product possesses *fes*-encoded antigenic determinants, some similar to and some different from those of the GA-FeSV p110.

***In Vitro* Protein Kinase Activity.** Immunoprecipitated GA-FeSV p110 possesses the ability to phosphorylate a tyrosine residue near its carboxyl terminus *in vitro* (9, 27). This function depends on intrinsic kinase ability and a seven amino acid recognition sequence near the phosphorylated tyrosine that is conserved in retroviral transforming proteins with tyrosine kinase activity (10, 28, 29). The phosphorylation of p110 is readily seen after immunoprecipitation with anti-p15 *gag* monoclonal antibodies, but the activity is markedly reduced when anti-*fes* monoclonal antibodies are used (26). To test whether the Rec-Kpn product exhibited kinase activity *in vitro*, [^{32}P]ATP was added to immunoprecipitates of the Rec-Kpn-transformed cells formed by using anti-p15 *gag* (F72) and anti-*fes* (F113) monoclonal antibodies, both of which recognize the Rec-Kpn protein. As is seen in Fig. 4,

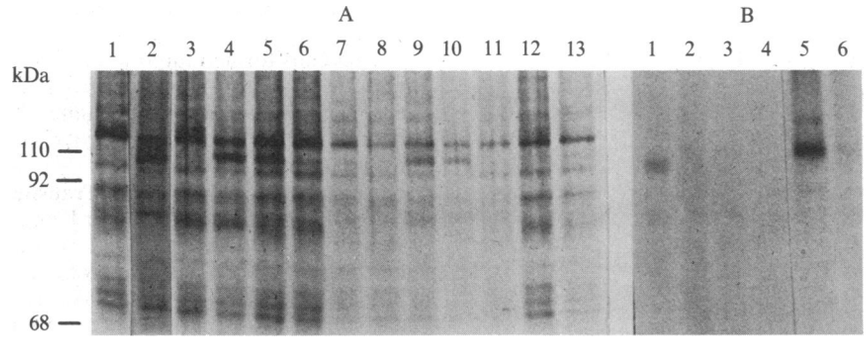

FIG. 4. Immunoprecipitations and *in vitro* kinase assay. (*A*) For immunoprecipitation of transfected cells, [^{35}S]methionine-labeled cells were lysed and incubated at 4°C overnight with monoclonal antibodies directed against feline leukemia virus or GA-FeSV determinants (gift of J. R. Stephenson), goat anti-rat immunoglobulin G, and staphylococcal protein A-Sepharose (Pharmacia) as described (26). Precipitates were washed and subjected to electrophoresis on sodium dodecyl sulfate/polyacrylamide gels as described (26). kDa, Kilodaltons. Immunoprecipitates formed by using monoclonal antibodies F113 (anti-v-*fes*) (lanes 1–6), F72 (anti-p15 *gag*) (lanes 7–11), F40 (which recognizes a p30 *gag* determinant not found in the GA-FeSV polyprotein (lane 12), and F123 (anti-v-*fes*) (lane 13) are shown. Cell lysates were derived from NIH 3T3 cells transfected with pSV_2gpt alone (lanes 1 and 7); GA-FeSV (lane 2); Rec-Kpn, MPA resistant, but untransformed (lanes 3 and 8); Rec-Kpn, MPA resistant and transformed (lanes 4, 9, 12, and 13); Rec-Kpn focus (lanes 5 and 10); Rec-Kpn, partial revertant (lane 6); and Rec-Kpn, complete revertant (lane 11). (*B*) For *in vitro* kinase assays, unlabeled cell lysates were immunoprecipitated with monoclonal antibodies and incubated with [^{32}P]ATP as described (26). Reactions were carried out for 15 min at 30°C, and the products were washed, electrophoresed in sodium dodecyl sulfate/polyacrylamide gels, and autoradiographed (26). *In vitro* kinase assays using monoclonal antibodies F72 (anti-p15 *gag*) (lanes 1, 3, and 5) and F113 (anti-v-*fes*) (lanes 2, 4, and 6) are shown. Cell lysates were derived from MPA-resistant NIH 3T3 cells transfected with GA-FeSV (lanes 1 and 2); Rec-Kpn, MPA resistant but not transformed (lanes 3 and 4); and Rec-Kpn, MPA resistant and transformed (lanes 5 and 6).

the Rec-Kpn product is phosphorylated in the transformed, but not in control, cell lysates, indicating that the chimeric product possesses *in vitro* kinase ability. This kinase reaction is also diminished somewhat when anti-*fes* antiserum is used for the immunoprecipitation.

Phosphotyrosine Content of the Transformed Cells. The level of total cellular phosphotyrosine is increased in cells transformed by a number of sarcoma viruses, including FSV and FeSV (6–9, 28, 29). To determine whether the Rec-Kpn product might possess tyrosine kinase activity in cells, total phosphotyrosine in the cells transformed by this recombinant was examined. Fig. 5 demonstrates that whereas only minute quantities of total phosphotyrosine were detected in the transfected cells that had lost the Rec-Kpn gene, higher levels are observed in cells transformed by Rec-Kpn or GA-FeSV. We conclude that the Rec-Kpn product is responsible for this phosphotyrosine increase.

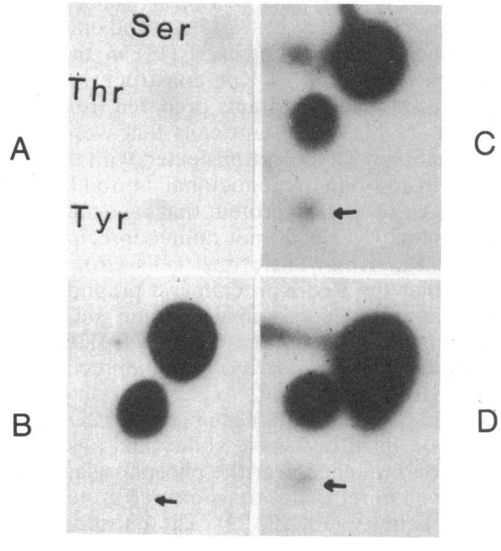

FIG. 5. Phospho amino acid analysis of transfected cells. Lysates from ^{32}P-labeled cells were hydrolyzed and electrophoresed in two dimensions as described (27). Ninhydrin-stained phospho amino acid markers (Ser, phosphoserine; Thr, phosphothreonine; Tyr, phosphotyrosine) are shown in *A*. The remaining frames demonstrate phospho amino acid content of cells transfected with Rec-Kpn, MPA resistant but untransformed (*B*); GA-FeSV (*C*); and Rec-Kpn, MPA resistant and transformed (*D*).

Reduced Transforming Efficiency of the Rec-Kpn Gene. Some of the factors potentially responsible for the lower transforming efficiency of Rec-Kpn compared to the cloned GA-FeSV provirus were examined. In-phase substitution of Rec-Kpn c-*fps/fes* sequences 3′ to a conserved *Pst* I site with analogous GA-FeSV sequences (Fig. 1, Rec-2) did not increase transformation efficiency. Thus, some of the c-*fps/fes* sequences determining lower transformation efficiency lie between the *Kpn* I and *Pst* I sites used to construct Rec-2.

One observation suggesting that genetic instability or lethality of Rec-Kpn may contribute to low transformation efficiency is the high rate of reversion to the untransformed state compared to that of GA-FeSV-transformed cells. Analysis of reverted subclones revealed that whereas they remained resistant to MPA, they no longer contained sequences homologous to the Rec-Kpn gene (Fig. 2, lane 11; Fig. 4). There may be some selection against the presence and expression of the recombinant in NIH 3T3 cells.

DISCUSSION

The results presented here indicate that a large 3′ portion of the human c-*fps/fes* protooncogene can substitute functionally for the transforming region of the FeSV. In the oncogenic recombinant (Rec-Kpn) more than 80% of the *fps/fes*-homologous sequences necessary for fibroblast transformation are supplied by the human locus. The transfected cells possess a fully transformed phenotype, including focus-forming ability, anchorage independence, and tumorigenicity in nude mice. In addition, the transformants exhibit increased cellular phosphotyrosine levels. The Rec-Kpn product has associated protein kinase activity that is probably supplied by human determinants, which replace v-*fps/fes* regions exhibiting homology at the protein level to other retroviral tyrosine kinases (10).

In our transforming recombinants, three types of structural changes in the human c-*fps/fes* locus were introduced,

any combination of which may be critical to transforming activity:

(*i*) Introduction of transcription initiation signals 5′ to the gene that function efficiently in NIH 3T3 fibroblasts. Surveys by RNA blot hybridization of human hematopoietic and solid tumor lines have failed to detect c-*fps/fes* transcripts (ref. 30 and unpublished data). The cloned 14-kb human fragment used in these studies is also transcriptionally silent when introduced into a murine cellular environment. This could be due to intrinsic properties of the human c-*fps/fes* promoter or to the absence of the promoter from our cloned fragment. Transcriptional activation is likely to be necessary for transformation. To determine whether it is sufficient will require more detailed characterization of the LTR c-*fps* construction and potential products translated from the 3.3-kb message observed in transfected cells.

(*ii*) Deletion of 5′ portions of the protooncogene, including probable exon regions. Functional exclusion of protooncogene sequences, either by deletion or mutation, is a feature of their activation to a transforming state in many instances of retroviral transduction. Exclusion of protooncogene exons from the transforming v-*onc* gene may circumvent regulatory functions of the encoded domains on the c-*onc* product, allowing transformation.

(*iii*) Substitution of the 5′ end of the protooncogene with viral translational initiation signals and AUG codon, *gag* determinants, and a small part of viral v-*fes*. Deletion of *gag* sequences does not appear to affect fibroblast transformation by the v-*fps* (31) or the Abelson murine leukemia virus v-*abl* gene, but in the latter case, *gag* sequences may be necessary for lymphocyte transformation (32). The importance of viral *gag* and v-*fes* sequences to the activity of the transforming Rec-Kpn gene could be assessed by substitution of those sequences with an in-phase initiation codon. Further studies should delineate which of the structural changes in the human c-*fps/fes* gene are necessary for its conversion to a transforming state.

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