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The Human c-*fps/fes* Gene Product Expressed Ectopically in Rat Fibroblasts Is Nontransforming and Has Restrained Protein-Tyrosine Kinase Activity

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A 13-kilobase *Eco*RI genomic restriction fragment containing the human c-*fps/fes* proto-oncogene locus was expressed transiently in Cos-1 monkey cells and stably in Rat-2 fibroblasts. In both cases, human c-*fps/fes* directed synthesis of a 92-kilodalton protein-tyrosine kinase ($p92^{c\text{-}fes}$) indistinguishable from a tyrosine kinase previously identified with anti-*fps* antiserum which is specifically expressed in human myeloid cells. Transfected Rat-2 cells containing approximately 50-fold more human $p92^{c\text{-}fes}$ than is found in human leukemic cells remained morphologically normal and failed to grow in soft agar. Synthesis of $p92^{c\text{-}fes}$ in this phenotypically normal line exceeded that of the $P130^{gag\text{-}fps}$ oncoprotein in a v-*fps*-transformed Rat-2 line. Despite this elevated expression, human $p92^{c\text{-}fes}$ induced no substantial increase in cellular phosphotyrosine and was not itself phosphorylated on tyrosine. In contrast, $p92^{c\text{-}fes}$ immunoprecipitated from these Rat-2 cells or expressed as an enzymatically active fragment in *Escherichia coli* from a c-*fps/fes* cDNA catalyzed tyrosine phosphorylation with an activity similar to that of v-*fps/fes* polypeptides. Thus, $p92^{c\text{-}fes}$ is not transforming when ectopically overexpressed in Rat-2 fibroblasts. This lack of transforming activity correlates with a restriction imposed on the kinase activity of the normal c-*fps/fes* product in vivo which is apparently lifted for v-*fps/fes* oncoproteins, suggesting that regulatory interactions within the host cell modify *fps/fes* protein function and normally restrain its oncogenic potential.

fps/fes has been the most commonly isolated retroviral oncogene. *fps/fes* proteins encoded by avian viruses such as Fujinami sarcoma virus (FSV) or feline viruses such as Gardner-Arnstein feline sarcoma virus (GA-FeSV) are cytoplasmic protein-tyrosine kinases that associate with the cytoskeleton or plasma membrane of transformed cells (18, 19). The product of the normal avian c-*fps/fes* gene is a soluble 98-kilodalton (kDa) protein-tyrosine kinase whose expression is apparently limited to hematopoietic cells, notably granulocytes and macrophages (24, 31). Antisera raised against the FSV $P130^{gag\text{-}fps}$ oncoprotein or against a synthetic peptide corresponding to a segment of the v-*fps* kinase domain recognize proteins of 92 and 94 kDa in human and mouse cells, both of which display associated tyrosine kinase activity (9, 10, 23). The 92-kDa species shows partial structural homology with the $P87^{gag\text{-}fes}$ oncoprotein of Snyder-Theilen FeSV and is therefore predicted to correspond to the mammalian c-*fps/fes* gene product (23). This 92-kDa protein, tentatively distinguished as $p92^{c\text{-}fes}$, is expressed to significant levels only in hematopoietic cells (9, 23). Analysis of cell lines and fractionated bone marrow or peripheral blood has identified $p92^{c\text{-}fes}$ in immature myeloid progenitors as well as in macrophages.

The human genome contains a single human c-*fps/fes* locus that maps to chromosome 15. A 13-kilobase (kb) *Eco*RI fragment isolated from a human cosmid library on the basis of its nucleic acid homology with v-*fps/fes* sequences (13, 15, 16) apparently contains the entire c-*fps/fes* coding sequence in addition to 5′ and 3′ regulatory elements (27). DNA sequence analysis and comparison with avian and feline c-*fps/fes* genomic sequences suggests that the human gene comprises 19 exons, the first of which is noncoding (17, 26, 27). Although the promoter and mRNA cap site have not been formally identified, both the human and feline genes have three conserved CCGCCC motifs and a potential CATT box located a short distance upstream from the putative first noncoding exon. The human c-*fps/fes* product predicted from this sequence is closely related to v-*fps/fes* transforming proteins and is inferred to have a molecular size of 93 kDa.

The very restricted expression of the human c-*fps/fes* gene suggests a specific role in myelopoiesis. Furthermore, the frequent activation of *fps/fes* as a retroviral transforming element implies a readily uncovered oncogenic potential. Avian c-*fps/fes* incorporated into a retroviral vector is unable to transform chicken embryo fibroblasts, but it acquires transforming ability when retroviral *gag* coding sequences are fused in frame such as to encode a hybrid *gag*–c-*fps/fes* protein (11). Here we investigated the identity, biological function, and enzymatic activity of the normal human c-*fps/fes* gene product in comparison with transforming *fps/fes* genes.

MATERIALS AND METHODS

Cell culture and DNA transfections. Rat-2 fibroblasts (36) and Cos-1 cells (14) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Transfection of Rat-2 or Cos-1 cells was performed by calcium phosphate coprecipitation as described previously (35). Cotransfection of Rat-2 cells with pSV2neo (33) was followed by selection with the antibiotic G418 at 400 μg/ml. Isolated clones were subsequently maintained in G418 at 40 μg/ml. For transient expression in Cos-1

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cells, analysis was performed 72 h posttransfection. The following human leukemic lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. EM-2 was derived from a patient with chronic myelogenous leukemia and was obtained from A. Keating; HL-60 is a promyelocytic line (3); and KG-1 was derived from a patient with acute myelogenous leukemia (22).

DNA extractions and Southern blot hybridizations. Isolation of high-molecular-weight DNA, digestion with restriction endonucleases, agarose gel electrophoresis, and Southern blotting onto nitrocellulose were performed by standard procedures (32). After hybridization with nick-translated probes, blots were washed at a final salt concentration of 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate) at 65°C.

Construction of mammalian and bacterial expression vectors. Plasmids pEE3, pEE2, and pBE were constructed by standard recombinant DNA techniques. Human genomic c-*fps/fes* sequences were from a plasmid which contains a 13-kb *Eco*RI fragment corresponding to the c-*fps/fes* locus (15). In plasmids pEE3 and pEE2 this 13-kb fragment is cloned into the *Eco*RI site of the pECE mammalian expression vector (8) in either the 5′ or 3′ orientation, respectively, relative to the simian virus 40 (SV40) early promoter. In pBE the 5′ end of the c-*fps/fes* locus was deleted from pEE3 by digestion with *Bgl*II and religation of the cohesive *Bgl*II ends.

Bacterial expression plasmids were constructed in pATH vectors (provided by T. J. Koerner and A. Tzagoloff). Human c-*fps/fes* cDNA sequences were cloned from an HL-60 λgt10 library (obtained from D. Bentley) with GA-FeSV v-*fes* as a probe. The longest cDNA insert which contained a polyadenylated 3′ end was subcloned as an *Eco*RI fragment into pUC118. Unique *Sma*I, *Kpn*I, or *Bam*HI sites were used to generate nested cDNA fragments encoding the C-terminal 476, 362, or 214 residues of p92$^{c\text{-}fes}$ which were ligated into the appropriate pATH vectors to generate in-frame fusions with the partial *trpE* gene. The designations of these plasmids (c-*fes*346, c-*fes*460, c-*fes*608) identify the p92$^{c\text{-}fes}$ residue at which the fusion was made. Plasmids encoding *trpE* fusions with the C-terminal 453 [pTF(v-*fps*)729] or 360 [pTF(v-*fps*)822] residues of FSV P130$^{gag\text{-}fps}$ were provided by I. Sadowski (29). Similar plasmids encoding *trpE* fusions with 651 (v-*fes*306) and 362 (v-*fes*595) residues of C-terminal GA-FeSV P110$^{gag\text{-}fes}$ were also constructed, with pGABH (obtained from C. Sherr) as the source of P110$^{gag\text{-}fes}$-coding sequence.

Growth and metabolic labeling of bacteria expressing *trpE-fps/fes* fusion proteins was done as described previously (30).

Metabolic labeling and immunoprecipitation of mammalian cell proteins. Monolayers were rinsed twice with Tris-buffered saline and labeled for 18 h in Dulbecco modified Eagle medium with 5% the normal concentration of methionine supplemented with 2% fetal bovine serum and 100 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml or for 4 h in Dulbecco modified Eagle medium without phosphate supplemented with 5% fetal bovine serum and 1 mCi of $^{32}P_i$ (carrier-free; New England Nuclear Corp., Boston, Mass.) per ml. For analysis of metabolically labeled proteins, cell lysis was with RIPA buffer (1% [vol/vol] Trition X-100, 150 mM NaCl, 1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 10 mM Tris hydrochloride [pH 7.5], 100 μg of leupeptin per ml, 100 μM sodium vanadate). Lysates were vortexed briefly and clarified by centrifugation at 14,000 × *g* for 30 min at 4°C. The supernatant was recovered, and portions were removed to determine protein content (Bio-Rad protein assay) and trichloroacetic acid-precipitable radioactivity. The clarified lysates were adjusted to contain equal amounts of protein before the addition of antiserum. Equal portions of clarified lysates were immunoprecipitated with either anti-*fps* or anti-GA$_{AUT}$ antisera or an appropriate nonimmune serum, as previously described (20, 38). After extensive washing with RIPA buffer and high-salt buffer (1 M NaCl, 1% [vol/vol] Triton X-100, 10 mM Tris hydrochloride [pH 7.5]), immunoprecipitates were prepared for analysis by SDS-polyacrylamide gel electrophoresis (PAGE) as described below.

Antisera. Goat antiserum to GA-FeSV P110$^{gag\text{-}fes}$ (anti-GA$_{AUT}$) was provided by M. Barbacid (1). Anti-*fps* rat serum which recognized both FSV P130$^{gag\text{-}fps}$ and human p92$^{c\text{-}fes}$ was obtained by injecting 4-week-old female Fischer × Wistar rats with 5 × 10^6 FSV-transformed Rat-1 cells as previously described (20). Polyclonal rabbit antisera were raised against the 37-kDa *trpE* product of the bacterial pATH expression vector as previously described (30).

Immune complex kinase reactions. Equal numbers of monolayer or suspension cells were washed twice with Tris-buffered saline containing 100 μM sodium vanadate and lysed on plates (Rat-2 or Cos-1 cells) or as pellets (HL-60, KG-1, or EM-2 cells) with kinase lysis buffer (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 100 μM sodium vanadate, 100 μg of leupeptin per ml).

Lysates were immunoprecipitated as described above. After extensive washing in kinase lysis buffer, the immunoprecipitates were washed in reaction buffer (100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 20 mM $MnCl_2$, 100 μM sodium vanadate), resuspended in reaction buffer, and incubated in aliquots prepared directly for SDS-PAGE or immune complex kinase assays as previously described (4, 39, 40). Samples were then analyzed on SDS-7.5% polyacrylamide gels. To estimate specific kinase activity, we labeled cells with [^{35}S]methionine and used aliquots of the immunoprecipitates to determine kinase levels by [^{35}S]methionine content or activity by the immune complex kinase assay. ^{35}S-labeled samples were detected by fluorography, while ^{32}P-labeled samples were analyzed by autoradiography. To quantitate ^{35}S or ^{32}P radioactivity, the bands of interest were excised from dried gels, solubilized at 65°C for 12 h in 30% hydrogen peroxide, and analyzed by liquid scintillation counting. Comigrating radioactivity in adjacent nonimmune control lanes was subtracted to give immune-specific values. After adjusting for specific activity of [^{35}S]methionine labeling and methionine content of the various *fps/fes* proteins, we calculated the in vitro specific kinase activities for both autophosphorylation and enolase phosphorylation. Bacterial lysates were prepared for immunoprecipitation of *trpE*–c-*fps/fes* fusion proteins by a modification of the procedure of Foulkes et al. (12). Induced cultures were pelleted, suspended in lysis buffer (50 mM NaCl, 1 mM EDTA, 100 mM sucrose, 0.015 [wt/vol] Brij 35, 20 mM Tris hydrochloride [pH 8.8]), sonicated (three times 10 s) and then clarified and immunoprecipitated as described above. Immunoprecipitations and in vitro kinase reactions were as described previously (29).

Phosphoamino acid analysis and tryptic phosphopeptide mapping. Whole-cell phosphoamino acids of ^{32}P-labeled cells were analyzed as previously described (6). Phosphoamino acid analysis and tryptic peptide mapping of ^{32}P-labeled *fps/fes* proteins have also been described previously (38, 40).

RESULTS

The human c-*fps/fes* gene encodes a 92-kDa protein-tyrosine kinase in Cos-1 monkey cells. The 13-kb *Eco*RI fragment previously reported to contain the human c-*fps/fes* gene was cloned into the pECE SV40-based mammalian expression vector to generate the plasmids pEE3 and pEE2. In pEE3, human c-*fps/fes* is inserted in a 5′ orientation such that transcription of c-*fps/fes* coding sequences is directed from the SV40 early promoter (Fig. 1). In pEE2, the orientation of c-*fps/fes* is reversed such that any transcription of c-*fps/fes* coding sequences will be from endogenous promoter elements. In pBE, a 1.5-kb *Bgl*II fragment was deleted from pEE3 which removes the 5′ end of c-*fps/fes* up to the *Bgl*II site in intron 2. c-*fps/fes* transcripts synthesized from the pBE template originate from the SV40 early promoter but lack the presumed first and second exons (Fig. 1). Cos-1 monkey cells were transfected with these plasmids, which contain an SV40 replication origin, and 3 days later were metabolically radiolabeled with [^{35}S]methionine followed by immunoprecipitation with anti-*fps* rat tumor serum. Alternatively, cells were lysed and immunoprecipitated without radiolabeling, and the immunoprecipitates were incubated with [γ-^{32}P]ATP and enolase. Cos-1 cells transfected with the pEE3 plasmid expressed a novel [^{35}S]methionine-labeled 92-kDa protein which was specifically recognized by the anti-*fps* antiserum (Fig. 2A, lane 1). This protein appeared capable of both autophosphorylation and the phosphorylation of enolase added as an exogenous substrate to the in vitro kinase reactions (Fig. 2B, lane 7). In both instances, phosphorylation was on tyrosine (Fig. 3D and E). Modest levels of phosphoserine and phosphothreonine (Fig. 3E) presumably result from contaminating kinases. In contrast, the pBE plasmid introduced into Cos-1 cells failed to induce the synthesis of any proteins immunoprecipitable with anti-*fps* antiserum (Fig. 2A, lane 3, and Fig. 2B, lane 9). Surprisingly, the pEE2 plasmid also induced the synthesis of a 92-kDa protein which was detectable at low levels by the immune complex kinase assay (Fig. 2B, lane 11) but not by metabolic labeling (Fig. 2A, lane 5). These data indicate that the 13-kb c-*fps/fes* sequence of pEE3 directly encodes a 92-kDa protein with associated protein-tyrosine kinase activity. The inability of cells transfected with pBE to synthesize such a polypeptide is consistent with the proposed location of the c-*fps/fes* translational initiation codon at the first in-frame ATG within the exon 2 sequences deleted from this construct. The low level of a 92-kDa protein in pEE2-transfected cells argues for the presence of endogenous c-*fps/fes* promoter elements which may be stimulated by the SV40 enhancer.

Correspondence of the human c-*fps/fes* gene product to a 92-kDa protein expressed in myeloid cells. The 92-kDa protein previously identified in human myeloid cells with anti-*fps* antiserum is abundantly expressed in the chronic myelogenous leukemia cell line EM-2 (Fig. 2B, lane 3). This line produces higher levels of the presumed p92$^{c\text{-}fes}$ than other human leukemic lines such as HL-60 and KG-1 (Fig. 2B, lane 1). The protein encoded by the human c-*fps/fes* insert of pEE3 in Cos-1 monkey cells comigrated with the polypeptide immunoprecipitated with anti-*fps* antiserum from EM-2 cells (Fig. 2B). The structural relationship between these proteins was investigated by comparative tryptic phosphopeptide analysis after their in vitro autophosphorylation on tyrosine. The tryptic phosphopeptide maps of the 92-kDa proteins from Cos-1 cells transfected with pEE3 and from EM-2 chronic myelogenous leukemia cells were identical (Fig. 3). These results suggest that the 92-kDa protein-tyrosine kinase expressed in human myeloid cells is indeed the product of the c-*fps/fes* locus on chromosome 15.

High-level expression of human p92$^{c\text{-}fes}$ in Rat-2 fibroblasts. The pEE3 plasmid was transfected into Rat-2 cells by calcium phosphate coprecipitation in conjunction with pSV2neo as a selectable marker. Transfected cells were cultured with the antibiotic G418, and G418-resistant clones were screened for the presence of the 13-kb human c-*fps/fes* fragment by Southern blot hybridization. One clone which

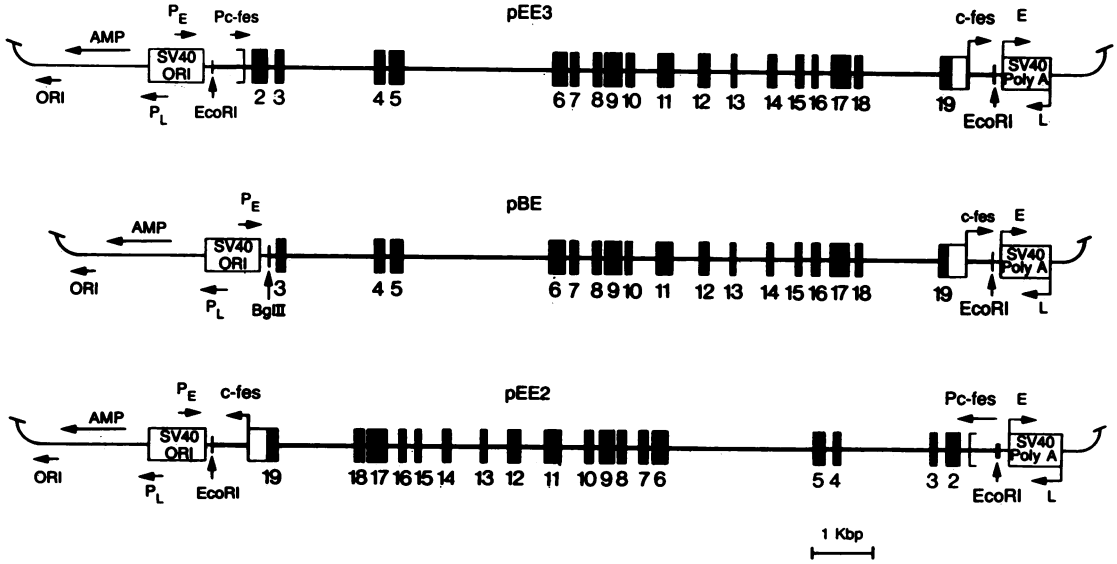

FIG. 1. Human c-*fps/fes* expression plasmids. Human c-*fps/fes* sequences are transcribed from the SV40 early promoter (P_E). *fps/fes* coding exons are numbered as in reference 27; poly(A) signals are indicated by the symbol ⌐►. The proposed position of the 3′ end of exon 1 is indicated as an open-ended box. The noncoding portion of exon 19 is indicated as an open box. Potential c-*fps/fes* promoter elements are indicated (Pc-fes). In pBE, 1.5 kb have been deleted from the 5′ end of the human DNA. In pEE2, the orientation of the *Eco*RI fragment has been reversed. Kbp, Kilobase pairs; ORI, origin.

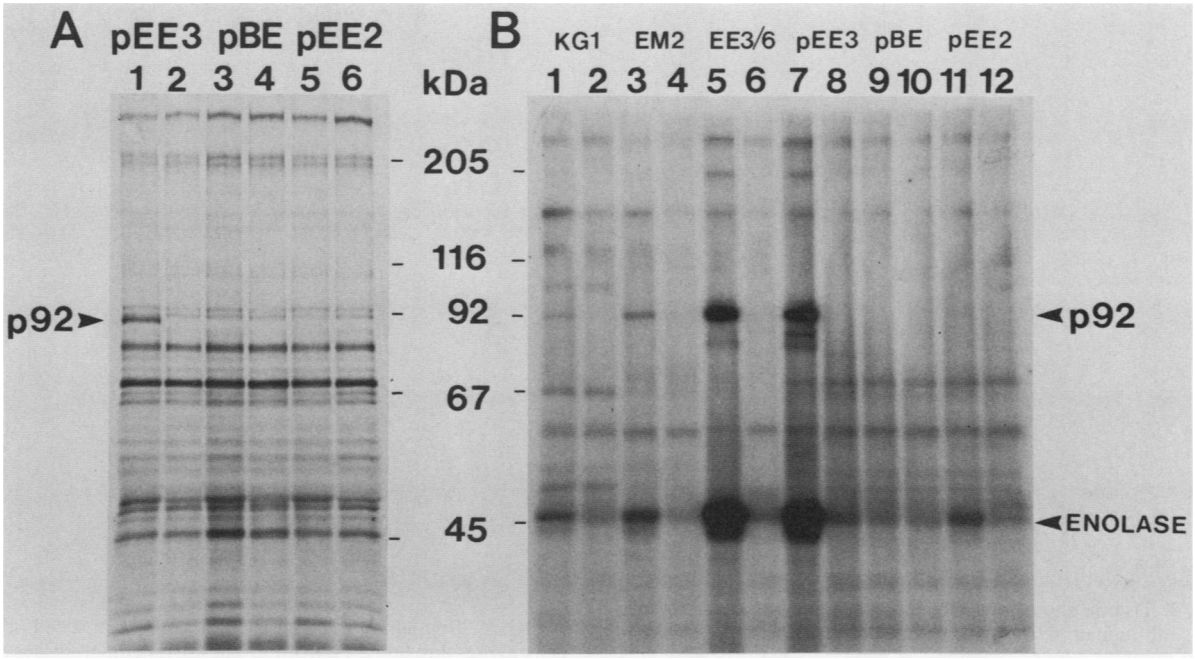

FIG. 2. (A) Identification of the human c-*fps/fes* protein transiently expressed in Cos-1 cells. The human c-*fps/fes* expression plasmids illustrated in Fig. 1 were transfected into Cos-1 cells as calcium phosphate-DNA precipitates (lanes 1 and 2, pEE3; lanes 3 and 4, pBE; lanes 5 and 6, pEE2). Seventy-two hours after transfection, the cells were labeled for 12 h with [^{35}S]methionine. Cell lysates were prepared and immunoprecipitated with anti-*fps* immune serum (lanes 1, 3, and 5) or with nonimmune serum (lanes 2, 4, and 6). Immunoprecipitates were washed extensively and analyzed by SDS-PAGE and fluorography. The 92-kDa human c-*fps/fes* protein is indicated. (B) In vitro kinase activity of human p92$^{c\text{-}fes}$. Human leukemic cells or Cos-1 and Rat-2 cells transfected with c-*fps/fes* expression plasmids were analyzed for in vitro p92$^{c\text{-}fes}$ kinase activity. Equal numbers of cells were lysed and immunoprecipitated with anti-*fps* antiserum (lanes 1, 3, 5, 7, 9, 11) or control nonimmune serum (lanes 2, 4, 6, 8, 10, 12). Immunoprecipitates were incubated with [γ-^{32}P]ATP and acid-denatured enolase, and the kinase reaction products were analyzed by SDS-PAGE and autoradiography. Human leukemic cells were KG-1 (lanes 1 and 2) and EM-2 (lanes 3 and 4). The EE3/6 cell line was derived by stable transfection of Rat-2 cells with the pEE3 construct (lanes 5 and 6). Cos-1 cells were transfected in a transient expression protocol with the c-*fps/fes* plasmids diagrammed in Fig. 1, including pEE3 (lanes 7 and 8), pBE (lanes 9 and 10), and pEE2 (lanes 11 and 12).

contained the intact human c-*fps/fes* *Eco*RI fragment, designated EE3/6, also expressed a high level of p92$^{c\text{-}fes}$ as judged by metabolic labeling with [^{35}S]methionine and immunoprecipitation with anti-*fps* antiserum (Fig. 4A, lane 3) or immunoprecipitation followed by an in vitro kinase reaction (Fig. 2B, lane 5, and Fig. 4B, lane 3).

Southern blot hybridization of EE3/6 DNA was undertaken with a cDNA probe corresponding to human c-*fps/fes* exons 6 through 19. This analysis indicated the presence of approximately 10 to 20 copies of the intact human c-*fps/fes* gene in EE3/6 cells, assuming 1 copy per haploid genome in HL-60 and EM-2 cells (Fig. 5). Digestion with *Eco*RI, *Bgl* II, or *Sac*I gave the same restriction fragment patterns for EE3/6, HL-60, and EM-2 DNAs; *Sac*I in particular cuts several times within the c-*fps/fes* locus and should have revealed any gross structural rearrangements.

Enzymatically active p92$^{c\text{-}fes}$ in EE3/6 cells was quantified by immunoprecipitation from cell lysates followed by immune complex kinase reactions. On the basis of ^{32}P incorporation into p92$^{c\text{-}fes}$ by autophosphorylation or into enolase as an exogenous substrate (Fig. 2B), EE3/6 cells were estimated to have approximately 50-fold more p92$^{c\text{-}fes}$ kinase activity than the EM-2 cell line. Comparable results were obtained by metabolic labeling with [^{35}S]methionine (data not shown). Since EM-2 has more p92$^{c\text{-}fes}$ than any other normal or leukemic human myeloid cells analyzed thus far, the amount of p92$^{c\text{-}fes}$ in EE3/6 Rat-2 cells is in large excess over the physiological levels observed in hematopoietic cells. As mammalian fibroblasts do not express readily detectable levels of p92$^{c\text{-}fes}$ (Fig. 4), it is not possible to estimate the fold increase in total mammalian p92$^{c\text{-}fes}$ in EE3/6 cells relative to that in the parental Rat-2 line.

Phenotypic comparison of Rat-2 cells expressing cellular and viral *fps/fes* genes. Despite the very high level of human p92$^{c\text{-}fes}$ expression in the EE3/6 cell line, these cells were morphologically indistinguishable from parental Rat-2 cells (Fig. 6). This contrasts with the transformed morphology of Rat-2 cells transfected with plasmids encoding the GA-FeSV (v-*fes*) or FSV (v-*fps*) transforming proteins (Fig. 6). In addition, EE3/6 cells failed to grow in soft agar under conditions in which Rat-2 cells containing the GA-FeSV or FSV genomes rapidly formed large colonies. Thus, by the criteria of morphology and anchorage independence, EE3/6 cells are phenotypically normal, suggesting that the human c-*fps/fes* gene is not activated as an oncogene by simple overexpression.

To investigate this possibility in more detail, we compared the relative level and kinase activity of human p92$^{c\text{-}fes}$ in EE3/6 Rat-2 cells with those of the v-*fps* and v-*fes* gene products in FSV- and GA-FeSV-transformed Rat-2 lines. Cells were radiolabeled with [^{35}S]methionine, immunoprecipitated with anti-*fps* or anti-GA$_{AUT}$ antisera, and divided into two aliquots. One sample was analyzed directly and served to measure *fps/fes* protein synthesis (Fig. 4A), while the second was introduced into an in vitro kinase reaction to estimate the relative enzymatic activities of the immunopre-

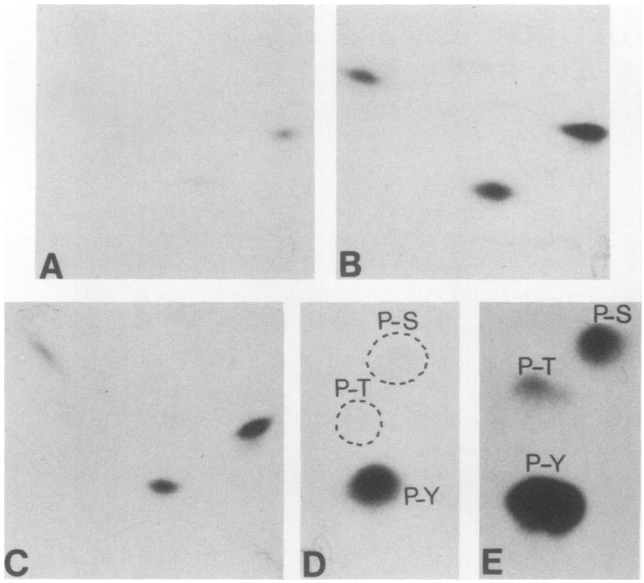

FIG. 3. Tryptic phosphopeptide mapping and phosphoamino acid analysis of human p92$^{c\text{-}fes}$. The 92-kDa proteins ^{32}P labeled in immune complex kinase reactions as shown in Fig. 2B were subjected to two-dimensional tryptic peptide mapping. p92 proteins were from EM-2 cells (A), from Cos-1 cells transfected with pEE3 (B), or from a mix (C). Electrophoresis was at pH 2.1 from left to right (anode to the left), and chromatography was from bottom to top. Phosphoamino acid analyses are of p92$^{c\text{-}fes}$ phosphorylated in vitro after immunoprecipitation from Cos-1 cells transfected with pEE3 (D) or of enolase added to the same kinase reaction (E). Phosphoamino acids were separated by two-dimensional electrophoresis at pH 1.9 from left to right (anode to the right) followed by electrophoresis at pH 3.5 from bottom to top (anode at the top). The positions of comigrating phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards were determined by reaction with ninhydrin.

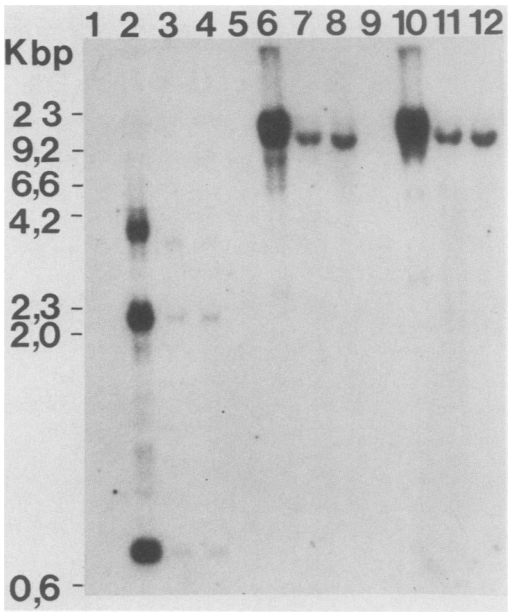

FIG. 5. Southern blot analysis of human leukemic cells and Rat-2 cells expressing human c-*fps/fes*. The pEE3 construct described in Fig. 1 was cotransfected into Rat-2 fibroblasts with pSV2neo as a selectable marker. EE3/6 is a G418-resistant clone found to express high levels of p92$^{c\text{-}fes}$ activity (Fig. 2B). High-molecular-weight DNA was prepared from parental Rat-2 cells (lanes 1, 5, and 9), EE3/6 cells (lanes 2, 6, and 10), and the human leukemic cell lines EM-2 (lanes 3, 7, and 11) and HL-60 (lanes 4, 8, and 12). DNA (10 μg per lane) was digested with restriction endonucleases *Sac*I (lanes 1 to 4), *Bgl*II (lanes 5 to 8), and *Eco*RI (lanes 9 to 12). After electrophoresis through agarose gels and blotting onto nitrocellulose membranes, the DNA was hybridized with a nick-translated human cDNA probe consisting of c-*fps/fes* exons 6 through 19. Under these conditions, the endogenous rat c-*fps/fes* gene was not detected. Kbp, Kilobase pairs.

cipitated *fps/fes* gene products (Fig. 4B). Expression of p92$^{c\text{-}fes}$ in EE3/6 cells was twofold higher than P130$^{gag\text{-}fps}$ in CL10 cells or one-fifth that of P110$^{gag\text{-}fes}$ in GA cells (Table 1). However, the in vitro specific kinase activities of all three *fps/fes* proteins as measured by autophosphorylation or the phosphorylation of enolase were similar (Table 1).

In vivo protein-tyrosine kinase activity of cellular and viral *fps/fes* products. One possible explanation for the inability of

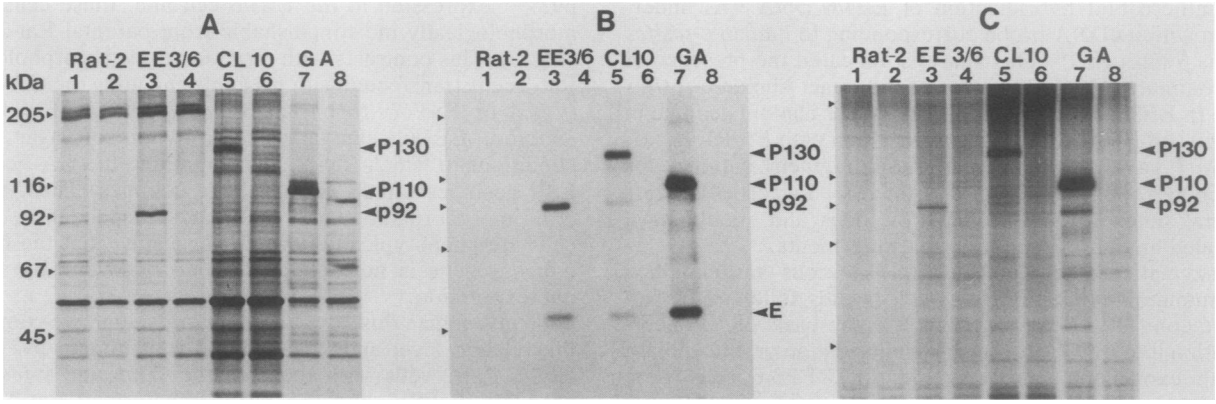

FIG. 4. Expression and activity of *fps/fes* proteins in Rat-2 cells. The parental Rat-2 line (Rat-2, lanes 1 and 2) or cell lines expressing p92$^{c\text{-}fes}$ (EE3/6, lanes 3 and 4), P130$^{gag\text{-}fps}$ (CL10, lanes 5 and 6), or P110$^{gag\text{-}fes}$ (GA, lanes 7 and 8) were metabolically labeled with [^{35}S]methionine (A and B) or $^{32}P_i$ (C) and immunoprecipitated with anti-*fps/fes* immune serum (lanes 1, 3, 5, and 7) or nonimmune serum (lanes 2, 4, 6, and 8). [^{35}S]methionine-labeled immunoprecipitates were either analyzed directly by SDS-PAGE and fluorography (A) or assayed for in vitro kinase activity including enolase as an exogenous substrate before gel analysis and autoradiography (B). For panel B, ^{35}S was shielded with four layers of tin foil. Immunoprecipitates from metabolically ^{32}P-labeled cells were analyzed directly by electrophoresis and autoradiography.

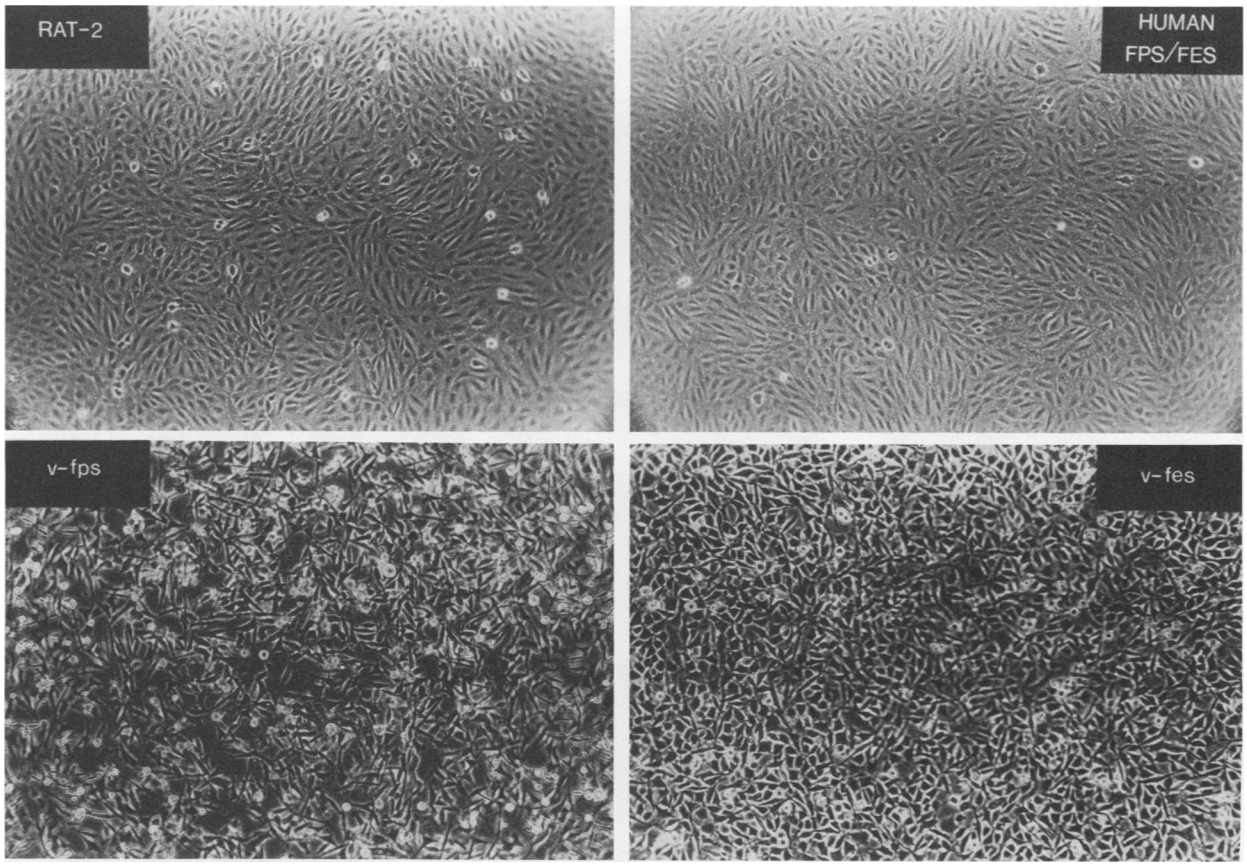

FIG. 6. Photomicrographs of Rat-2 cells expressing *fps/fes* genes. A Rat-2 cell line (EE3/6) expressing human c-*fps/fes* is compared with the parental cells (Rat-2) or with Rat-2 cells transformed with FSV (v-*fps*) or GA-FeSV (v-*fes*).

p92$^{c\text{-}fes}$ to transform mammalian fibroblasts despite its relatively high in vitro activity is that its activity in vivo is subject to intracellular restraints which are no longer effective on the activated v-*fps/fes* oncoproteins. The phosphotyrosine content of Rat-2 cells expressing human c-*fps/fes* or v-*fps/fes* oncogenes was therefore investigated to assess the relative in vivo kinase activities of p92$^{c\text{-}fes}$, P130$^{gag\text{-}fps}$, and P110$^{gag\text{-}fes}$.

In contrast to the substantial elevation in whole-cell phosphotyrosine seen in P130$^{gag\text{-}fps}$- and P110$^{gag\text{-}fes}$-transformed cells, the level of phosphotyrosine in p92$^{c\text{-}fes}$-expressing cells was only marginally higher than that in Rat-2 cells (Table 1). The cellular and viral *fps/fes* gene products were themselves all phosphorylated in vivo (Fig. 4C); however, phosphoamino acid analysis of metabolically labeled p92$^{c\text{-}fes}$ showed phosphoserine and phosphothreonine but no detectable phosphotyrosine (Fig. 7A). In contrast, P130$^{gag\text{-}fps}$ and P110$^{gag\text{-}fes}$ are also phosphorylated on tyrosine in vivo (Table 1) (see references 2, 25, and 37). Phosphorylation of p92$^{c\text{-}fes}$ at tyrosine in vivo could only be detected when EE3/6 cells were labeled with $^{32}P_i$ in the presence of the phosphatase inhibitor vanadate (data not shown).

Activity of human c-*fps/fes* polypeptides expressed in bacteria. To investigate whether the distinct activities of viral and cellular *fps/fes* proteins in Rat-2 cells are contingent upon interactions with regulatory proteins in vertebrate cells, we compared the kinase activities of human c-*fps/fes* and v-*fps/fes* polypeptides expressed in *Escherichia coli*. For this analysis, we isolated human c-*fps/fes* cDNAs that encode the carboxy-terminal two-thirds of p92$^{c\text{-}fes}$ from an HL-60 λgt10 cDNA library. DNA sequence analysis of these cDNAs showed them to contain human c-*fps/fes* exons 6 through 19 (P. Greer, unpublished observations). C-terminal human *fps/fes* fragments of decreasing size were then ex-

TABLE 1. Expression and activity of *fps/fes* proteins in rat fibroblasts[a]

Expression	Control (Rat-2)	Human genomic p92$^{c\text{-}fes}$ (EE3/6)	Avian FSV P130$^{gag\text{-}fps}$ (CL10)	Feline GA-FeSV P110$^{gag\text{-}fes}$ (GA)
Relative synthesis of *fps/fes* gene product	ND[b]	1.0	0.5	4.9
Relative in vitro kinase activity				
Autophosphorylation		1.0	2.0	0.9
Enolase phosphorylation		1.0	0.9	1.1
Phosphoamino acid analysis				
In vivo *fps-fes* protein (%)				
Phosphotyrosine		ND	67	24
Phosphoserine		82	32	55
Phosphothreonine		18	1	21
In vivo whole cell (%)				
Phosphotyrosine	0.09	0.14	0.58	0.71
Phosphoserine	92.6	91.0	87.5	92.0
Phosphothreonine	7.3	9.0	11.5	7.1

[a] Quantitations based on averages from three independent experiments.
[b] ND, Not detected.

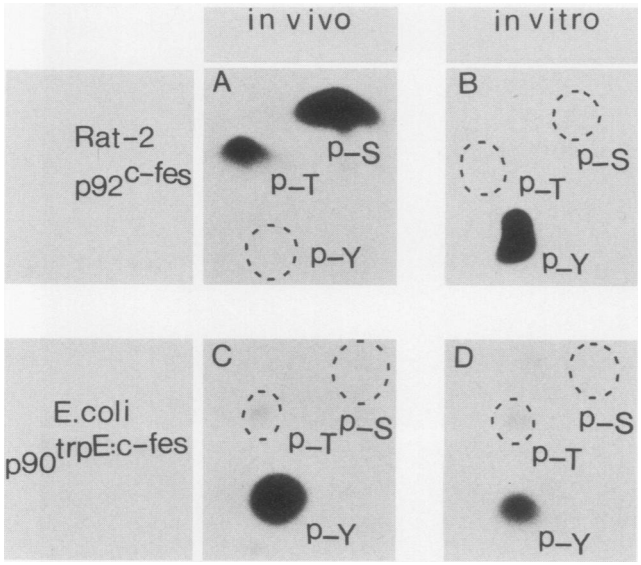

FIG. 7. Phosphoamino acid analysis of human c-*fes* proteins from Rat-2 cells or *E. coli*. Human p92$^{c\text{-}fes}$ from the EE3/6 Rat-2 line was either metabolically labeled with ^{32}P in vivo (A) or allowed to autophosphorylate in vitro with [γ-^{32}P]ATP (B). The p90 bacterial *trpE*–c-*fps/fes* fusion protein was metabolically labeled with $^{32}P_i$ in *E. coli* (C) or autophosphorylated in vitro in an immune complex kinase reaction (D). ^{32}P-labeled proteins were acid hydrolyzed and analyzed by two-dimensional electrophoresis. The positions of comigrating phosphoamino acid standards (phosphoserine [P-S], phosphothreonine [P-T], and phosphotyrosine [P-Y]) were determined by reaction with ninhydrin.

pressed in *E. coli* as *trpE-fps/fes* fusion proteins (Fig. 8). The products of the bacterial expression plasmids share 323 N-terminal *trpE* residues linked to the C-terminal 476 (p90), 362 (p75), or 214 (p50) residues of human p92$^{c\text{-}fes}$. The *fps/fes* kinase region is composed of a catalytic domain joined to an N-terminal regulatory region (SH2) (30). p90 encodes all these domains, p75 lacks part of SH2 but retains all sequences required for kinase activity (29), whereas p50 lacks the predicted ATP-binding site located at the start of the catalytic region.

After induction of the *trpE* operon with indoleacrylic acid, bacteria carrying these plasmids expressed novel proteins of the predicted sizes (data not shown). Metabolic labeling of the induced bacteria with $^{32}P_i$ revealed p75 and p90 as the major cellular phosphoproteins (Fig. 9A, lanes 3 and 4, respectively). Phosphorylation of these *trpE-fps/fes* proteins was primarily at tyrosine (Fig. 7C), indicating that they were active in autophosphorylation. To assay their kinase activities in vitro, we immunoprecipitated soluble bacterial extracts with an antiserum to the N-terminal *trpE* sequence, and the immune complexes were incubated with [γ-^{32}P]ATP and enolase. The p75 and p90 proteins phosphorylated enolase and rabbit immunoglobin and autophosphorylated in vitro (Fig. 9B, lanes 3 and 4, respectively) at tyrosine (Fig. 7D). p50 was not phosphorylated in vivo (Fig. 9A, lane 2) and was inactive in vitro (Fig. 9B, lane 2), as expected from the absence of elements deemed essential for ATP binding and phosphotransfer.

trpE–v-*fps* proteins containing C-terminal FSV P130$^{gag\text{-}fps}$ sequences have been previously expressed in bacteria (29, 30). As a result of restriction sites conserved between v-*fps* and human c-*fps/fes*, constructs homologous to p90 and p75 are available but with c-*fps/fes* sequences replaced with corresponding v-*fps* fragments. In addition, two constructs containing GA-FeSV P110$^{gag\text{-}fes}$ sequences were produced, one of which was comparable to p75, while the other contained all the GA v-*fes* sequences (Sadowski et al., manuscript in preparation). After correction for differences in relative expression and solubility of these fusion proteins, we could find no substantial difference in the kinase activities of homologous cellular and viral polypeptides as measured by either metabolic labeling with $^{32}P_i$ (Fig. 9A) or in vitro immune complex kinase assays (Fig. 9B).

DISCUSSION

Product of the human c-*fps/fes* locus. Transfection of the human c-*fps/fes* gene into Cos-1 monkey cells or Rat-2 fibro-

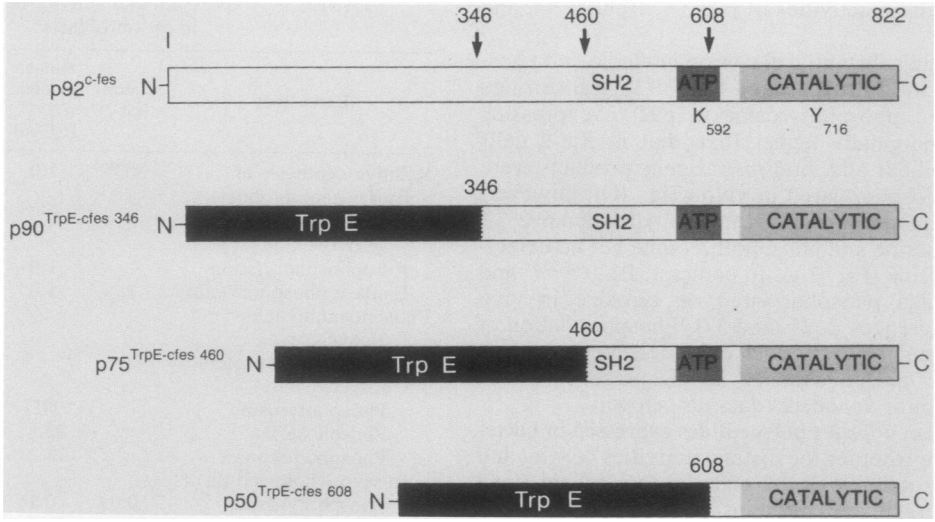

FIG. 8. Bacterial expression of human c-*fes* polypeptides. *trpE*–c-*fps/fes* fusion proteins are compared diagrammatically with human p92$^{c\text{-}fes}$. A conserved lysine (K_{592}) in the presumed ATP-binding region and a conserved tyrosine (Y_{716}) in the catalytic domain are indicated. The adjacent noncatalytic SH2 region is homologous with other members of the cytoplasmic protein-tyrosine kinase family (30). Positions 346, 460, and 608 correspond to the p92$^{c\text{-}fes}$ residues at which the *trpE* fusions have been made to give polypeptides p90, p75, and p50.

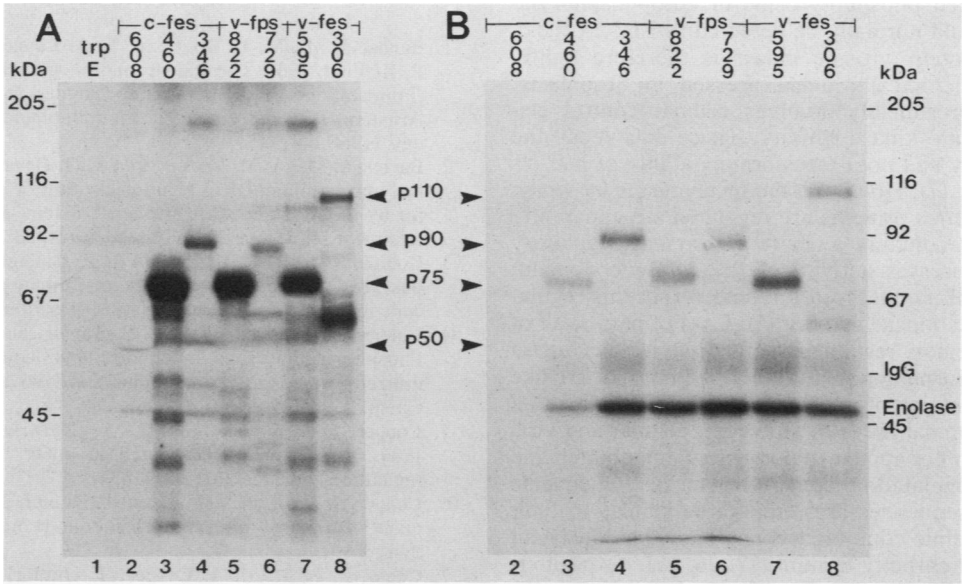

FIG. 9. Expression and activity of bacterial *trpE-fps/fes* fusion proteins. Bacterial cultures expressing *trpE-fps/fes* fusion proteins were metabolically labeled with $^{32}P_i$ (A). Alternatively, *trpE-fps/fes* proteins were immunoprecipitated from bacterial lysates with anti-*trpE* antiserum and incubated in immune complex kinase reactions containing enolase as an exogenous substrate (B). In addition to the three *trpE*–c-*fps/fes* fusions described in Fig. 8, four viral *trpE-fps/fes* fusions and a 37-kDa *trpE* control were analyzed. The 37-kDa *trpE* protein (A, lane 1) and the c-*fes*608 p50 protein (lane 2) were detectable by Coomassie blue staining (not shown). The p75 proteins c-*fes*460, v-*fps*822, and v-*fes*595 (lanes 3, 5, and 7, respectively) contain approximately 360 homologous C-terminal residues of the *fps/fes* domain. The p90 proteins c-*fes*346 and v-*fps*729 (lanes 4 and 6, respectively) contain approximately 475 homologous C-terminal residues. The p110 protein v-*fes*306 (lane 8) contains all the v-*fes*-specific sequence of the GA-FeSV oncoprotein and 43 amino acids of mammalian *gag*. The positions of the Coomassie blue-stained fusion proteins are indicated. IgG, Immunoglobulin G.

blasts induced the expression of a 92-kDa protein ($p92^{c\text{-}fes}$) with associated protein-tyrosine kinase activity. Formal proof that the human c-*fps/fes* product is a protein-tyrosine kinase is given by the enzymatic activity of *fps/fes* polypeptides expressed from human cDNA sequences in *E. coli*. In conjunction with previous work (23), these experiments show that the human c-*fps/fes* proto-oncogene encodes a 92-kDa protein-tyrosine kinase whose synthesis is limited to hematopoietic cells, particularly mature myeloid cells and their progenitors.

The structure of the human c-*fps/fes* gene has been inferred from sequence analysis of the 13-kb *Eco*RI fragment used here for transfection and by comparison of the human sequence with cellular and viral *fps/fes* genes of both avian and feline origin (26, 27). The synthesis of authentic $p92^{c\text{-}fes}$ from this restriction fragment confirms that it contains the entire *fps/fes* coding sequence. High levels of $p92^{c\text{-}fes}$ were detected when the SV40 early promoter was used to direct human c-*fps/fes* transcription in either Cos-1 or Rat-2 cells. Weak expression was seen in Cos-1 cells transfected with a pECE plasmid containing the *fps/fes* fragment in a 3′ transcriptional orientation with respect to the SV40 early promoter. The 13-kb *Eco*RI fragment contains 5′ sequence elements characteristic of mammalian promoters; however, even if sufficient for c-*fps/fes* transcription, these elements might be expected to be functionally restricted to hematopoietic cells. $p92^{c\text{-}fes}$ expression seen in Cos-1 cells containing the c-*fps/fes* gene in a 3′ transcriptional orientation may be partially due to the effect of the SV40 enhancer acting on the *fps/fes* promoter. The identity of elements that normally confer myeloid-specific expression on c-*fps/fes* is under investigation.

Overexpression of human $p92^{c\text{-}fes}$ does not induce fibroblast transformation. To determine whether $p92^{c\text{-}fes}$ has transforming activity when overexpressed in mammalian fibroblasts, we isolated a Rat-2 cell line containing high levels of the human c-*fps/fes* gene product. Since fibroblasts do not generally produce $p92^{c\text{-}fes}$ the expression of the endogenous human gene in this Rat-2 line was correlated to $p92^{c\text{-}fes}$ in a human chronic myelogenous leukemia line and to $P130^{gag\text{-}fps}$ in an FSV-transformed Rat-2 line. Expression of human $p92^{c\text{-}fes}$ in the EE3/6 Rat-2 cells was approximately 50-fold greater than in human EM-2 leukemic cells and about 2-fold greater than that of $P130^{gag\text{-}fps}$ in transformed Rat-2 cells. Despite this abundant synthesis, human $p92^{c\text{-}fes}$ had no obvious effect on the phenotype of Rat-2 cells. The apparent lack of $p92^{c\text{-}fes}$ transforming ability implies that its oncogenic potential is restrained within the cell. Such a restriction might operate at the level of enzymatic activity or might prevent access of $p92^{c\text{-}fes}$ to cellular targets involved in transformation.

Different regulation of cellular and viral *fps/fes* kinase activities in Rat-2 cells. The in vitro kinase activity of $p92^{c\text{-}fes}$ in immune complex assays was similar to that of $P130^{gag\text{-}fps}$ or $P110^{gag\text{-}fes}$. The sensitivity of this reaction may be compromised by the use of an anti-*fps* antiserum known to contain antibodies to the *fps/fes* catalytic domain (29). Binding of such antibodies might stimulate the c-*fps/fes* gene product or inhibit v-*fps* kinase activity. Furthermore, the validity of comparing the normal human protein with oncogenic feline or avian *fps/fes* products may be questioned. Nonetheless, the results indicate that $p92^{c\text{-}fes}$ and $P130^{gag\text{-}fps}$ or $P110^{gag\text{-}fes}$ activities are not remarkably different in vitro. Similarly, the kinase activities of *trpE-fps/fes* bacterial fusion proteins containing cellular or viral *fps/fes* catalytic sequences were equivalent in vitro or in vivo. In contrast, the kinase activity of $p92^{c\text{-}fes}$ in Rat-2 cells was repressed in comparison with viral *fps/fes* oncoproteins. Indeed, no in-

crease in whole-cell phosphotyrosine or autophosphorylation of $p92^{c\text{-}fes}$ could normally be detected in EE3/6 cells.

The $p92^{c\text{-}fes}$ protein-tyrosine kinase is therefore tightly regulated when ectopically overexpressed in fibroblasts. This regulation presumably involves cellular control elements that modulate kinase activity. These data recall the low kinase activity and poor transforming ability of $p60^{c\text{-}src}$ relative to $p60^{v\text{-}src}$ (7). However, the mechanisms by which the c-*src* and c-*fps/fes* proteins are regulated are apparently different. $p60^{c\text{-}src}$ contains a short C-terminal regulatory sequence that represses activity of the catalytic domain; phosphorylation of a conserved tyrosine within this regulatory region inhibits kinase activity (5). Loss of part or all of the C-terminal region results in activation of *src* kinase activity and transforming potential (21). A family of *src*-like genes encodes proteins of approximately 60 kDa which all contain this C-terminal tail (19). However, cellular and viral *fps/fes* proteins do not appear to possess such a domain and are presumably regulated by some other means. Oncogenic v-*fps/fes* coding sequences are remarkably similar to their normal avian or feline counterparts and show no consistent variations at their carboxy termini (17, 26, 27). Two differences are observed between normal and transforming retroviral *fps/fes* proteins. v-*fps/fes* oncoproteins are membrane or cytoskeleton associated or both, perhaps as a consequence of their N-terminal *gag* sequences, whereas avian $p98^{c\text{-}fps}$ is apparently soluble at physiological salt concentrations (41). In addition, v-*fps/fes* transforming proteins are highly phosphorylated at tyrosine and are apparently activated by autophosphorylation, whereas c-*fps/fes* proteins are not phosphorylated at tyrosine in vivo (25, 40; this study). These differences in phosphorylation and location might have a critical effect on $p92^{c\text{-}fes}$ kinase function.

The lack of transforming ability exhibited by genes such as c-*fps/fes* and c-*src* that encode cytoplasmic protein-tyrosine kinases is in contrast to proto-oncogenes encoding growth factor receptors such as the colony-stimulating factor-1 and epidermal growth factor receptors. Thus, human c-*fms* readily transforms mouse fibroblasts in the presence of exogenous human colony-stimulating factor-1 (28). Similarly, coexpression of epidermal growth factor and its receptor in fibroblasts results in autocrine growth stimulation and neoplastic transformation (34). If a physiological stimulus that normally activated human $p92^{c\text{-}fes}$ kinase activity in hematopoietic cells could be identified, it is possible that it would induce EE3/6 cells to transform. Determination of the role of *fps/fes* in normal hematopoiesis or cellular transformation will likely require an understanding of the molecular basis for regulation of the c-*fps/fes* kinase activity. Cell lines expressing high levels of human $p92^{c\text{-}fes}$ will be essential to these investigations.

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