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Expression of the Mammalian c-*fes* Protein in Hematopoietic Cells and Identification of a Distinct *fes*-Related Protein

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The avian c-*fps* and mammalian c-*fes* proto-oncogenes are cognate cellular sequences. Antiserum raised against the P140$^{gag\text{-}fps}$ transforming protein of Fujinami avian sarcoma virus specifically recognized a 92,000-M_r protein in human and mouse hematopoietic cells which was closely related in structure to Snyder-Theilen feline sarcoma virus P87$^{gag\text{-}fes}$. This polypeptide was apparently the product of the human c-*fes* gene and was therefore designated p92$^{c\text{-}fes}$. Human p92$^{c\text{-}fes}$ was associated with a tyrosine-specific protein kinase activity in vitro and was capable of both autophosphorylation and phosphorylation of enolase as an exogenous protein substrate. The synthesis of human and mouse p92$^{c\text{-}fes}$ was largely, though not entirely, confined to myeloid cells. p92$^{c\text{-}fes}$ was expressed to relatively high levels in a multipotential murine myeloid cell line, in more mature human and mouse granulocyte-macrophage progenitors, and in differentiated macrophagelike cells as well as in the mononuclear fraction of normal and leukemic human peripheral blood. p92$^{c\text{-}fes}$ was not found in erythroid cells, with the exception of a human erythroleukemia line which retains the capacity to differentiate into macrophagelike cells. These results suggest a normal role for the p92$^{c\text{-}fes}$ tyrosine kinase in hematopoiesis, particularly in granulocyte-macrophage differentiation. In addition, a distinct 94,000-M_r polypeptide, antigenically related to p92$^{c\text{-}fes}$, was identified in a number of hematopoietic and nonhematopoietic human and mouse cells and was also found to be associated with a tyrosine-specific protein kinase activity.

Proto-oncogenes (c-*onc*) are normal cellular genes that through a variety of genetic events can acquire the ability to induce neoplastic transformation (6). The dominant effects of activated oncogene expression on cellular proliferation, the relationship of some oncogene proteins to growth factors or their receptors (13, 58), and the extreme conservation of oncogene-related sequences during eucaryotic evolution (49) suggest that the usual function of proto-oncogenes may be in regulating cell growth. However, surveys of proto-oncogene expression in both simple eucaryotic organisms and mammalian species have revealed that many proto-oncogenes are transcribed in a tissue-specific or developmentally regulated fashion and may therefore also be involved in processes related to cell and tissue differentiation (11, 33, 41, 51). The proliferation of immature multipotential stem cells leads to self-renewal and is also a usual component of their differentiation to more developed cell types. Thus, the expression of specific c-*onc* proteins in cells of defined developmental lineages might be important in the process of self-renewal or commitment. In addition, some c-*onc* gene products are synthesized in both proliferating and terminally differentiating cells, for example, p60$^{c\text{-}src}$, which is synthesized to high levels in neuronal processes and in postmitotic neuroretinal cells (18, 53). Thus, the physiology of c-*onc* proteins apparently involves a complex interplay between proliferative and developmental functions.

During hematopoiesis a number of differentiated cell types of quite distinct function develop from a single pluripotent stem cell. Several proto-oncogenes are known to be transcriptionally regulated during this process (19, 39, 40, 61). The oncogenically active counterparts of c-*onc* genes, including a number of oncogenes which encode proteins with tyrosine-specific protein kinase activity (e.g., *abl*, *erbB*, *fps/fes*, *src*) (1, 6, 10, 12, 25, 44, 54, 56), can have disruptive effects on hematopoietic development. The avian c-*fps* gene is of particular interest in hematopoiesis since, in the chicken, c-*fps* transcription and synthesis of the p98$^{c\text{-}fps}$ protein it encodes are largely restricted to hematopoietic tissue such as bone marrow (36, 47). The v-*fps* oncogene is the transforming sequence of Fujinami avian sarcoma virus (FSV) (22, 32). The FSV genome encodes a 140-kilodalton (kDa) protein (P140$^{gag\text{-}fps}$) with an intrinsic tyrosine-specific protein kinase activity capable both of autophosphorylation and of phosphorylating exogenous protein substrates (43, 59, 60). Mammalian cells contain a gene equivalent to avian c-*fps* which was originally identified as the viral oncogene of Snyder-Theilen feline sarcoma virus (ST-FeSV) and hence is known as c-*fes*; despite this difference in nomenclature, c-*fps* and c-*fes* correspond to a single, common genetic locus (21, 48). A survey of proto-oncogene expression in the developing mouse embryo failed to detect c-*fes* transcripts, suggesting that in the mouse as in the chicken c-*fes* expression is restricted (51). A similar analysis of human neoplastic tissues has revealed a human c-*fes* RNA, although its expression was largely confined to hematologic malignancies (52). A normal 92-kDa protein proposed as the c-*fes* product has been identified in some primate fibroblast cell lines by using antiserum raised against the P87$^{gag\text{-}fes}$ transforming protein of ST-FeSV, but no corresponding human or murine proteins were found (3). Here we describe the identification of human and mouse c-*fes* gene products and of a distinct antigenically related protein.

MATERIALS AND METHODS

Cells and cell lines. The following human cell lines were used: HL-60, a promyelocytic leukemia line (9); KG-1, a line derived from a patient with acute myelogenous leukemia (26); HEL, an erythroleukemia cell line (35); K562, a chronic myelogenous leukemia (CML) cell line with erythroid characteristics (2, 5); MOLT-4, an immature T-cell line (38);

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SU-DHL-4, an Epstein-Barr virus-negative, Fc receptor-negative, immunoglobulin-positive histiocytic B-lymphoma cell line (15); WAY-1, an Epstein-Barr virus-transformed B-lymphocyte cell line (D. Howard, personal communication); and NCI-H82, a small-cell lung carcinoma cell line (34). A number of murine cell lines were also used: P388AD-4, an adherent macrophagelike cell line (8); WEHI-3B, a myelomonocytic leukemia cell line (57); B6SUtA, an interleukin (IL)-3-dependent nontumorigenic cell line with some properties of a multipotential myeloid progenitor (20); EL-4, a thymoma line (16); P815, a mastocytoma cell line (14); P3-NSI/1-Ag4-1 (NSI), a nonsecreting myeloma cell line (27); MEL, a Friend virus-induced erythroleukemia line (17); P19 and O1A1, embryonal carcinoma cell lines (37); and Y1, a mouse adrenocortical tumor cell line (62). ST-FeSV-transformed and normal NIH 3T3 fibroblasts have been described previously (4). Cells were grown in Dulbecco modified Eagle medium or RPMI 1640 supplemented with 10% fetal bovine serum, horse serum, and IL-2 or IL-3 when appropriate. E26 virus-transformed chicken myeloblasts were grown as described previously (24). Normal mouse bone marrow cells were obtained from the femurs of CBA mice, and mouse erythroblasts were obtained from the spleens of mice treated with acetylphenylhydrazine (30). Peripheral blood and bone marrow cells from healthy donors and from patients with acute myelogenous leukemia (AML) were separated by buoyant density centrifugation over Ficoll-Hypaque (55).

In vivo radiolabeling and immunoprecipitation. Cells were radiolabeled either by incubation at 37°C for 16 h with [^{35}S]methionine (Amersham Corp.; >1,000 Ci/mmol) at 200 μCi/ml in Dulbecco modified Eagle medium lacking nonradioactive methionine and supplemented with 5% fetal bovine serum or by incubation for 4 h with $^{32}P_i$ (ICN; carrier free) at 1 mCi/ml in Dulbecco modified Eagle medium lacking phosphate and supplemented with 5% fetal bovine serum. Cells were harvested, washed, lysed, and subjected to immunoprecipitation with control or immune rat antiserum as described previously (24, 59). Immunoprecipitated proteins were subjected to electrophoresis through 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels of ^{32}P-labeled protein were exposed to X-ray film (XAR-5, Eastman Kodak) at −80°C in the presence of an intensifying screen. Gels of [^{35}S]methionine-labeled proteins were impregnated with En^{3}Hance (New England Nuclear Corp.) and exposed to film at −80°C.

Antisera. Anti-*fps* rat sera were obtained by injecting 4-week-old female Fischer rats with 5×10^6 FSV strain L5-transformed rat-1 cells as previously described (24). Anti-pEX-2-*abl* rabbit antiserum (28) was a gift of J. Konopka and O. Witte. Goat antiserum to ST-FeSV P87$^{gag\text{-}fes}$ (anti-ST_{AUT}) was a gift of M. Barbacid (3).

Immune complex kinase reaction. Samples of 5×10^7 to 1×10^8 cells were harvested, lysed in buffer containing 20 mM Tris hydrochloride, pH 7.5, 150 mM Nacl, 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of aprotinin per ml, and immunoprecipitated with rat antiserum as described previously (24, 59). The resulting immune complexes were incubated in 35-μl reaction mixtures with 10 mM $MnCl_2$–20 mM Tris-hydrochloride (pH 7.5)–5 μCi of [γ-^{32}P]ATP (2,000 Ci/mmol) at 20°C for 15 min as described previously (59). For tryptic phosphopeptide analysis, 20 μCi of label was added to each reaction mixture. The preparation and addition of acid-denatured rabbit muscle enolase to the immune complex kinase reaction has been described in detail elsewhere (60). After the immune complex kinase reactions, the immunoprecipitates were washed and then analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels, followed by exposure to X-ray film for 1 to 4 days in the presence of an intensifying screen at −80°C. Reaction mixtures containing enolase were analyzed immediately without washing.

Tryptic phosphopeptide analysis and V8 protease digestion. ^{32}P-labeled proteins were eluted from gel slices, precipitated, oxidized with performic acid, and digested with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin as detailed previously (43, 59). Tryptic digests were separated by electrophoresis at 1,000 V for 90 min at pH 2.1 on thin-layer cellulose (TLC) plates (thickness, 0.1 mm; E. Merck AG), followed by chromatography in *n*-butanol–acetic acid–water–pyridine (75:15:60:50) in the second dimension. Plates were exposed to film with intensifying screens for 7 days. V8 protease cleavage and analysis of the digestion products were done exactly as described previously (59). Digests were exposed to film with an intensifying screen for 8 h.

Phosphoamino acid analysis. ^{32}P-labeled proteins were eluted from gel slices, precipitated with trichloroacetic acid, and subjected to acid hydrolysis in 6 N HCl at 110°C for 90 min as described elsewhere (10, 43). The acid hydrolysates were then subjected to electrophoresis at pH 1.9 and 3.5 before the TLC plates were exposed to X-ray film at −80°C in the presence of an intensifying screen for 7 to 15 days. The identity of radiolabeled spots was confirmed by their comigration with nonradioactive phosphoamino acid markers.

RESULTS

Identification of a 92-kDa human c-*fes* protein. Antisera to FSV P140$^{gag\text{-}fps}$ obtained from Fischer rats bearing tumors induced by FSV-transformed rat-1 cells were previously shown to recognize the avian p98$^{c\text{-}fps}$ cellular protein in chicken bone marrow cells and in a chicken myeloblast cell line (24). Some of these antisera cross-reacted with the P87$^{gag\text{-}fes}$ transforming protein of ST-FeSV (24), and we concluded that they contained antibodies to antigenic determinants conserved between *fps*/*fes*-encoded proteins of different vertebrate species. We therefore investigated whether these antisera recognized any proteins in human hematopoietic cell lines phenotypically related to avian myeloblasts. Cells of the human promyelocytic leukemia cell line HL-60 were metabolically labeled with [^{35}S]methionine and subjected to immunoprecipitation with a cross-reactive anti-*fps* rat tumor serum. A protein with an apparent molecular weight of 92,000 was specifically precipitated from the radiolabeled HL-60 cell lysate by the anti-*fps* antiserum (Fig. 1). A labeled protein of identical mobiltiy was immunoprecipitated from a lysate of HL-60 cells which had been metabolically labeled with $^{32}P_i$, indicating that the 92-kDa polypeptide was a phosphoprotein (Fig. 1).

We next investigated whether this *fps*-related 92-kDa protein was associated with a protein kinase activity. Nonradioactive HL-60 cells were lysed in buffer containing Nonidet P-40 and sodium deoxycholate and immunoprecipitated with anti-*fps* antiserum, and the immune complex was incubated with [γ-^{32}P]ATP and $MnCl_2$. The 92-kDa protein became radiolabeled during this immune complex kinase reaction (Fig. 1). Phosphoamino acid analysis of the in vitro-phosphorylated HL-60 92-kDa protein demonstrated that phosphorylation occurred principally at tyrosine and to a minor extent at serine and threonine residues (Fig. 2). Tryptic digestion of the HL-60 92-kDa

protein phosphorylated in an immune complex yielded two major ^{32}P-labeled tryptic phosphopeptides (Fig. 3), each of which yielded only phosphotyrosine when subjected to phosphoamino acid analysis (Fig. 2). Including 100 μM sodium orthovanadate, an inhibitor of phosphotyrosyl phosphatases, in all cell lysis and reaction buffers stimulated incorporation of ^{32}P into the 92-kDa protein during in vitro phosphorylation (data not shown), as expected for tyrosine phosphorylation and as previously noted for FSV P140$^{gag\text{-}fps}$ (Pawson, unpublished data). We interpret these data as suggesting that the 92-kDa protein recognized by the anti-*fps* antiserum is a tyrosine-specific protein kinase capable of autophosphorylation. We presume that the minor phosphorylation of the 92-kDa protein at serine and threonine in vitro results from the presence of a contaminating kinase. In contrast to the in vitro phosphorylation of p92$^{c\text{-}fes}$ at tyrosine in the immune complex, p92$^{c\text{-}fes}$ isolated from ^{32}P-labeled HL-60 cells was phosphorylated only at serine or threonine and contained no detectable phosphotyrosine (Fig. 2). Preincubating ^{32}P-labeled cells with 100 μM Na_3VO_4 and including Na_3VO_4 in all buffers had no apparent effect on phosphorylation of and did not reveal any phosphotyrosine in the 92-kDa protein (data not shown).

If the 92-kDa protein precipitated by anti-*fps* antiserum from HL-60 cells is indeed encoded by the human c-*fes* gene, it should be structurally similar to known v-*fes*-encoded proteins. To test this possibility, we compared the HL-60 92-kDa protein and ST-FeSV P87$^{gag\text{-}fes}$ by tryptic peptide analysis. The human protein and P87$^{gag\text{-}fes}$ were immunoprecipitated from HL-60 cells and ST-FeSV-transformed NIH 3T3 mouse fibroblasts, respectively, and were labeled by in vitro phosphorylation with [γ-^{32}P]ATP. The ^{32}P-labeled proteins were digested with trypsin and subjected to two-dimensional tryptic peptide mapping (Fig. 3). In each case two major tryptic phosphopeptides were identified which migrated with identical mobilities in the electrophoretic dimension at pH 2.1 and with similar but distinct mobilities in the chromatographic dimension. The tryptic phosphopeptides of P87$^{gag\text{-}fes}$ and the 92-kDa protein migrated differently from those of autophosphorylated FSV P140$^{gag\text{-}fes}$ (data not shown), reflecting the distinct amino acid sequences surrounding the autophosphorylation sites of the v-*fes* and v-*fps* gene products (46). Thus, in the vicinity of their autophosphorylation sites, P87$^{gag\text{-}fes}$ and the human 92-kDa polypeptide are more closely related to each other than to FSV P140$^{gag\text{-}fps}$. The structural comparison of the 92-kDa HL-60 protein and P87$^{gag\text{-}fes}$ was extended by performing comparative partial V8 protease digests of the in vitro-phosphorylated proteins. Since the N-terminal region of P87$^{gag\text{-}fes}$ is encoded by a sequence from a residual *gag* viral replicative gene, only its C-terminal *fes*-encoded kinase domain is potentially related to the 92-kDa protein. However, autophosphorylation of P87$^{gag\text{-}fes}$ selectively labels this C-terminal region because it contains the major autophosphorylation site. After the ^{32}P-labeled 92-kDa protein and P87$^{gag\text{-}fes}$ were digested with V8 protease, the majority of their proteolytic fragments comigrated during electrophoretic separation on an SDS-polyacrylamide gel (Fig. 4). The virtual comigration of the tryptic phosphopeptides from the 92-kDa protein and P87$^{gag\text{-}fes}$ and the similarity of their V8 protease digestion patterns provide compelling evidence that these two polypeptides are closely related in sequence. This finding, taken together with the specific precipitation of the 92-kDa protein with antiserum originally raised to the avian FSV P140$^{gag\text{-}fps}$, argues that the HL-60 92-kDa protein (referred to hereafter as p92$^{c\text{-}fes}$) is the human c-*fes* gene product.

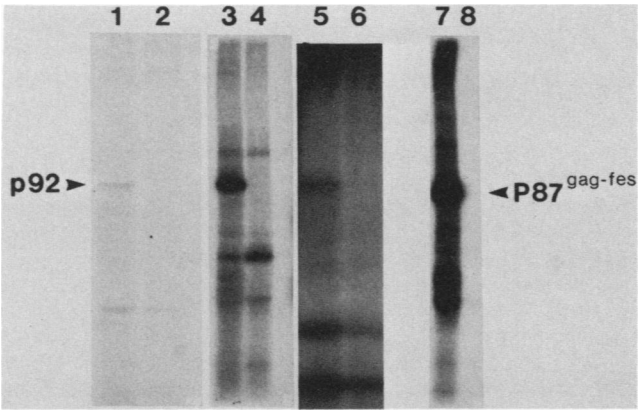

FIG. 1. Identification of p92$^{c\text{-}fes}$ in human HL-60 cells. Lysates of HL-60 cells (lanes 1 to 6) metabolically labeled in vivo with [^{35}S]methionine (lanes 1 and 2) or $^{32}P_i$ (lanes 5 and 6) were immunoprecipitated with anti-*fps* rat antiserum (lanes 1 and 5) or nonimmune rat serum (lanes 2 and 6). To assay for kinase activity, lysates of HL-60 cells (lanes 3 and 4) or ST-FeSV-transformed NIH 3T3 cells (lanes 7 and 8) were immunoprecipitated with anti-*fps* antiserum (lane 3), anti-ST_{AUT} antiserum (lane 7), or nonimmune rat serum (lanes 4 and 8), and the immune complexes were incubated in vitro with [γ-^{32}P]ATP in kinase reactions. The radiolabeled proteins in each case were analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels, followed by fluorographic detection. p92$^{c\text{-}fes}$ and P87$^{gag\text{-}fes}$ are indicated.

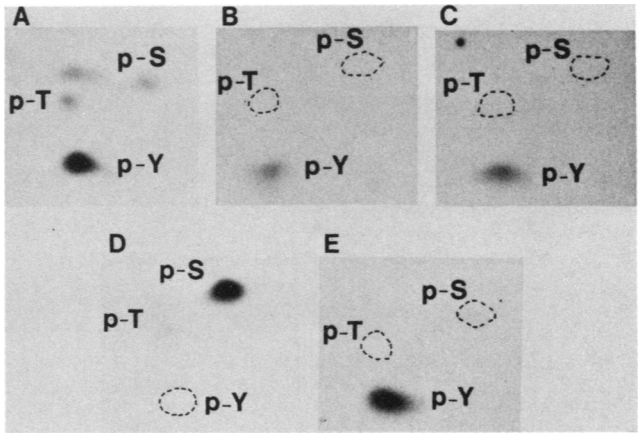

FIG. 2. Phosphoamino acid analysis of *fes* proteins. ^{32}P-labeled proteins were isolated from SDS-polyacrylamide gels and subjected to acid hydrolysis, followed by two-dimensional electrophoretic separation of phosphoamino acids. (A) HL-60 p92$^{c\text{-}fes}$ phosphorylated in an immune complex kinase reaction, (B) Tryptic peptide 1 of in vitro-phosphorylated HL-60 p92$^{c\text{-}fes}$, (C) Tryptic peptide 2 of in vitro-phosphorylated HL-60 p92$^{c\text{-}fes}$, (D) p92$^{c\text{-}fes}$ immunoprecipitated from HL-60 cells metabolically labeled with $^{32}P_i$, (E) K562 p94 phosphorylated in an immune complex kinase reaction. The positions of phosphotyrosine (p-Y), phosphoserine (p-S), and phosphothreonine (p-T) are indicated.

Phosphorylation of an exogenous protein substrate at tyrosine by p92$^{c\text{-}fes}$. To test whether an exogenously added protein substrate could be phosphorylated by the p92$^{c\text{-}fes}$-associated phosphotransferase activity, we included denatured rabbit muscle enolase in an HL-60 p92$^{c\text{-}fes}$ immune complex kinase reaction. Enolase is a substrate for phosphorylation at tyrosine by FSV P140$^{gag\text{-}fps}$ both in FSV-

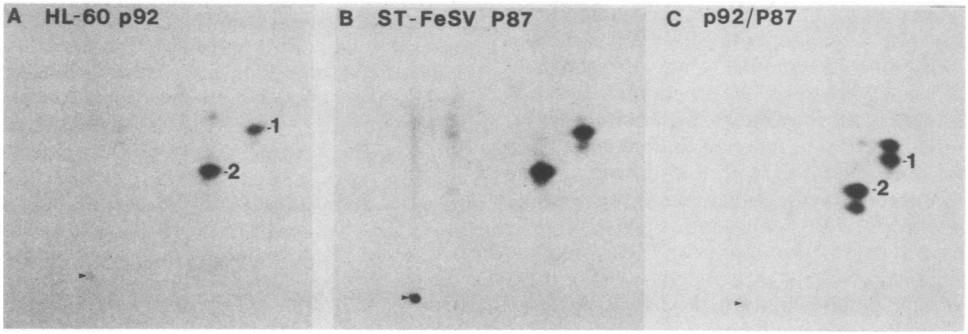

FIG. 3. Tryptic phosphopeptide analysis of human and viral *fes*-encoded proteins. Human and viral *fes* proteins, labeled in vitro with [γ-^{32}P]ATP (as in Fig. 1, lanes 3 and 7), were eluted from polyacrylamide gels and digested with trypsin. Tryptic digests were analyzed by electrophoresis at pH 2.1 on TLC plates, followed by chromatography in the second dimension. The resulting tryptic peptide maps were exposed to X-ray film in the presence of an intensifying screen. An equal amount of label was loaded onto each TLC plate. (A) p92$^{c\text{-}fes}$ from HL-60 cells; (B) P87$^{gag\text{-}fes}$ from ST-FeSV-transformed NIH 3T3 filbroblasts; (C) mixture of equal counts per minute of labeled p92$^{c\text{-}fes}$ and P87$^{gag\text{-}fes}$. The two major phosphopeptides obtained from p92$^{c\text{-}fes}$ are numbered 1 and 2. The sample origins are indicated by arrows.

transformed cells and in an in vitro kinase reaction (24, 60) and for avian p98$^{c\text{-}fps}$ in vitro (Fig. 5). Enolase was specifically phosphorylated by an immune complex containing the human p92$^{c\text{-}fes}$ protein (Fig. 5). Phosphoamino acid analysis revealed tyrosine as the predominant enolase phosphoacceptor (data not shown). Tryptic digestion of enolase phosphorylated in vitro showed that FSV P140$^{gag\text{-}fps}$, avian p98$^{c\text{-}fps}$, and human p92$^{c\text{-}fes}$ all induced phosphorylation of enolase within the same unique tryptic peptide fragment (data not shown).

Expression of p92$^{c\text{-}fes}$ in normal and leukemic human and mouse hematopoietic cells and cell lines. The HL-60 promyelocytic leukemia cell line corresponds phenotypically to a committed myeloid cell which can be induced to differentiate into both granulocytes and macrophages (9, 61). We examined other cell lines which have been characterized as belonging to this hematopoietic lineage for expression of p92$^{c\text{-}fes}$ (Table 1). KG-1 human myelobast cells were isolated from a patient with AML, possess granulocyte markers, and form myeloid colonies (26). KG-1 cells also expressed a p92$^{c\text{-}fes}$ protein which after autophosphorylation displayed a tryptic phosphopeptide map identical to that of p92$^{c\text{-}fes}$ from HL-60 cells (data not shown). Peripheral blood leukocytes (PBLs) isolated from a patient with AML and from healthy donors also contained p92$^{c\text{-}fes}$, which was identified by immunoprecipitation with anti-*fps* antiserum and autophosphorylation (Fig. 6) and by metabolic labeling with [^{35}S]methionine (data not shown). The expression of p92$^{c\text{-}fes}$ in primary human hematopoietic tissue suggests that its synthesis in HL-60 and KG-1 cells is not an artifact of their

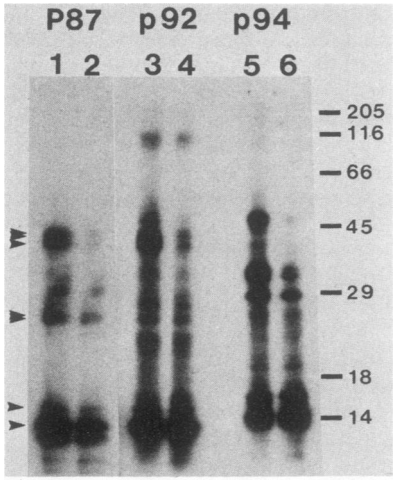

FIG. 4. V8 protease digestion of viral and cellular *fes* proteins. P87$^{gag\text{-}fes}$ was immunoprecipitated with anti-*fps* antiserum from ST-FeSV-transformed NIH 3T3 cells, p92$^{c\text{-}fes}$ from HL-60 cells, and p94 from K562 cells. Precipitated proteins were phosphorylated in immune complex kinase reactions, and the resulting ^{32}P-labeled polypeptides were separated on a 7.5% SDS-polyacrylamide gel, identified by autoradiography, excised, and applied to the wells of a new 12.5% SDS-polyacrylamide gel. The proteins digested in situ in the gel and the amounts of V8 protease used were: (lane 1) P87$^{gag\text{-}fes}$, 50 ng; (lane 2) P87$^{gag\text{-}fes}$, 200 ng; (lane 3) p92$^{c\text{-}fes}$, 50 ng; (lane 4) p92$^{c\text{-}fes}$, 200 ng; (lane 5) p94, 50 ng; and (lane 6) p94, 200 ng. Comigrating cleavage fragments are marked by arrows. The size markers with their molecular weights (in thousands) are indicated to the right.

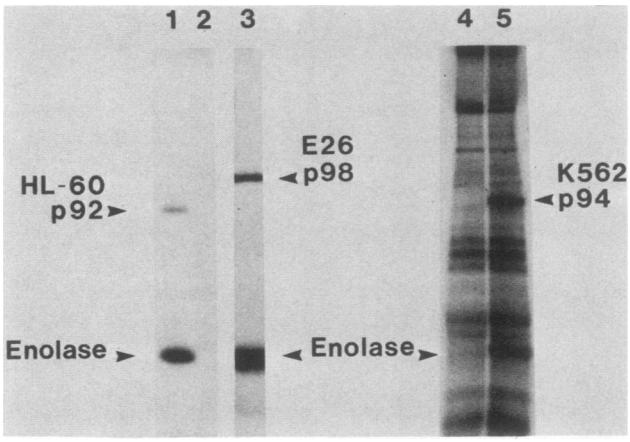

FIG. 5. In vitro phosphorylation of enolase by p92$^{c\text{-}fes}$, p98$^{c\text{-}fps}$, and p94. Lysates of human HL-60 cells (lanes 1 and 2), avian E26-transformed myeloblasts (lane 3), and K562 cells (lanes 4 and 5) were immunoprecipitated with rat anti-*fps* antiserum (lanes 1, 3, and 5) or nonimmune rat serum (lanes 2 and 4). Each immune complex was resuspended in a 30-μl kinase reaction mixture containing 5 μg of acid-denatured rabbit muscle enolase. Proteins phosphorylated in the kinase reaction were analyzed by electrophoresis in a 7.5% SDS-polyacrylamide gel, followed by fluorography.

TABLE 1. Expression of p92$^{c\text{-}fes}$ and p94 in human and mouse cell lines and in normal mouse tissues

Species	Tissue cell line (cell type)	Expression	
		p92$^{c\text{-}fes}$	p94[a]
Human	HL-60 (promyelocyte)	+	±
	KG-1 (myeloblast)	+	ND
	K562 (erythroid, CML patient)	−	+
	HEL (erythroleukemia)	+	+
	MOLT-4 (T-cell leukemia)	−	+
	SU-DHL-4 (B-lymphoma)	+	+
	WAY-1 (B-lymphocyte)	+	+
	PBLs (normal adult)	+	+
	PBLs (AML patient)	+	+
	NCI-H82 (small-cell lung carcinoma)	−	ND
Mouse	B6SUtA (myeloid multipotential cell)	+	ND
	WEHI-3B (myeloblast)	+	+
	P388AD-4 (macrophagelike)	+	ND
	P815 (mastocytoma)	+	ND
	MEL (erythroleukemia)	−	+
	NSI (myeloma)	−	+
	EL-4 (thymoma)	−	ND
	P19, O1A1 (embryonal carcinoma)	−	+
	NIH 3T3 (fibroblast)	±	+
	Y1 (adrenal cortex)	−	+
	Bone marrow	+	ND
	Spleen (normal)	+	ND
	Spleen (phenylhydrazine-treated mice, early and late erythroblasts)	−	+
	Heart	−	ND
	Liver	−	ND

[a] HL-60 cells express a low level of p94 only detected by some antisera. ND, Not tested for low level of p94 expression.

extensive passaging in culture but reflects their hematopoietic phenotype.

Analysis of mouse bone marrow by immunoprecipitation with anti-*fps* antiserum followed by an immune complex kinase reaction revealed a murine protein which comigrated with human p92$^{c\text{-}fes}$ (Fig. 7). An IL-3-dependent multipotential murine cell line, B6SUtA, capable of differentiation along erythroid, neutrophil-granulocyte, and basophil-mast cell pathways (20), also expressed p92$^{c\text{-}fes}$ (Fig. 7). In addition, we identified murine p92$^{c\text{-}fes}$ in the granulocyte-macrophage precursor cell line WEHI-3B, in a differentiated macrophagelike Ia$^+$ adherent murine cell line (P388AD-4) capable of presenting soluble antigen to primed T-cells, and in a mastocytoma cell line (P815). Screening several mouse tissues with anti-*fps* antiserum identified p92$^{c\text{-}fes}$ only in organs with hematopoietic involvement (Table 1).

We examined a number of human and mouse lymphocyte lines for expression of p92$^{c\text{-}fes}$. Two human B-lymphocyte lines contained readily detectable levels of p92$^{c\text{-}fes}$ (Table 1), but transformed human and mouse T-lymphocyte lines and

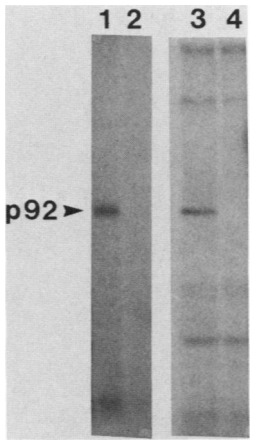

FIG. 7. Identification of p92$^{c\text{-}fes}$ in mouse hematopoietic cells. Bone marrow cells from CBA mouse femurs (lanes 1 and 2) and B6SUtA cells (lanes 3 and 4) were lysed and immunoprecipitated with anti-*fps* antiserum (lanes 1 and 3) or nonimmune rat serum (lanes 2 and 4). Immune complexes were incubated with [γ-^{32}P]ATP, and in vitro-phosphorylated proteins were identified by electrophoretic separation and fluorography.

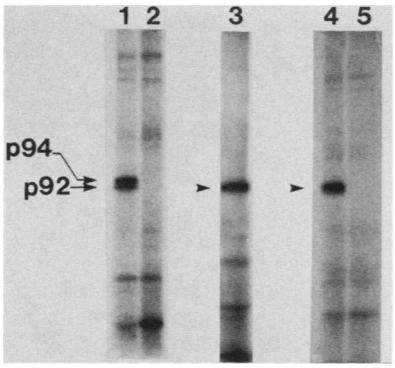

FIG. 6. Immunoprecipitation of p92$^{c\text{-}fes}$ and p94 from normal and leukemic human PBLs. Lysates of fresh PBLs from a normal donor (lanes 1 and 2) and a patient with AML (lanes 4 and 5) and a lysate of HL-60 cells (lane 3) were immunoprecipitated with anti-*fps* antiserum (lanes 1, 3, and 4) or nonimmune rat serum (lanes 2 and 5). Immunoprecipitates were incubated with [γ-^{32}P]ATP in in vitro kinase reaction mixtures and analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels, followed by fluorography.

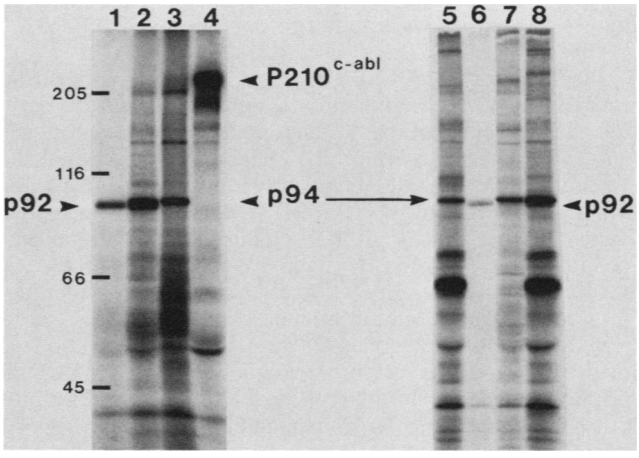

FIG. 8. Identification of a p94 *fes*-related protein in human and mouse cell lines. HL-60 (lanes 1 and 6), HEL (lane 2), K562 (lanes 3, 4, and 7), Y1 (lane 5), and NIH 3T3 mouse fibroblast (lane 8) cells were lysed and immunoprecipitated with anti-*fps* antiserum (lanes 1 to 3 and 5 to 8) or anti-pEX-2-*abl* antiserum (lane 4). Immune complexes were incubated with [γ-^{32}P]ATP, and in vitro-phosphorylated proteins were identified by electrophoretic separation and fluorography. The size marker molecular weights (in thousands) are indicated to the left.

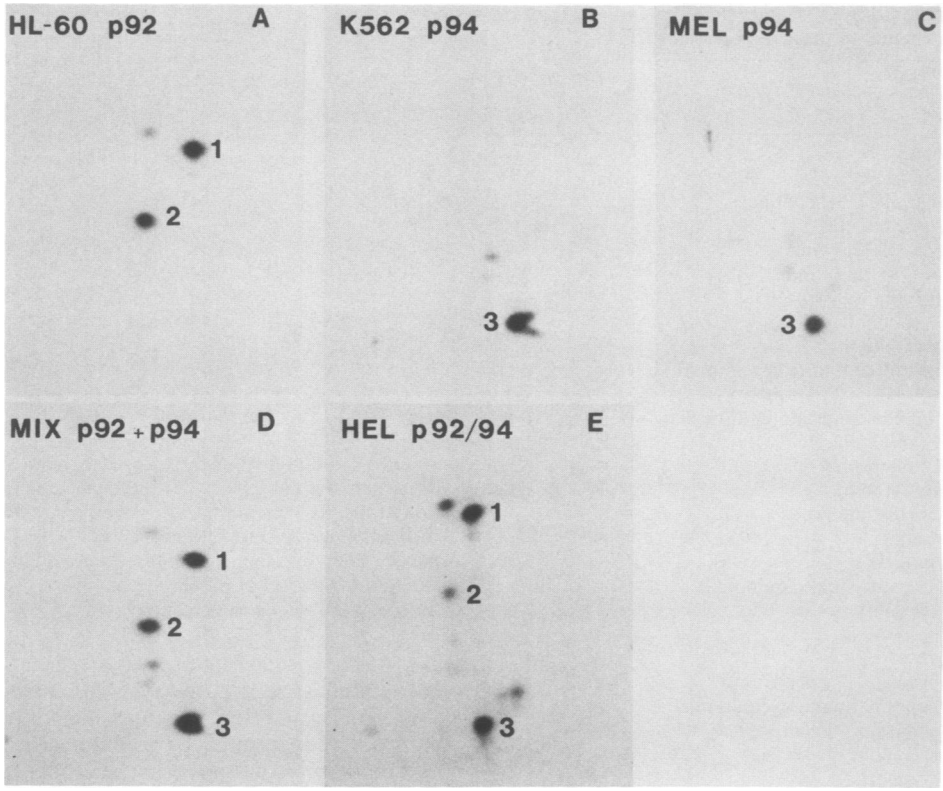

FIG. 9. Comparative tryptic phosphopeptide analysis of $p92^{c\text{-}fes}$ and p94. Lysates of human and murine cells were immunoprecipitated with anti-*fps* antiserum, and the immune complexes were incubated with [γ-^{32}P]ATP. In vitro-phosphorylated proteins were isolated from SDS-polyacrylamide gels, digested with trypsin, and subjected to two-dimensional tryptic phosphopeptide analysis. Panel D shows a mixture of equal counts per minute of labeled HL-60 $p92^{c\text{-}fes}$ and K562 p94. Panel E shows the HEL p92/p94 doublet. The phosphopeptide obtained from p94 is numbered 3.

mouse antibody-producing cells expressed no $p92^{c\text{-}fes}$. $p92^{c\text{-}fes}$ was not present in a variety of human and mouse erythroid cells (Table 1, Fig. 8), with the exception of the human erythroleukemia cell line HEL (Fig. 8). Of a number of nonhematopoietic cell types examined, only NIH 3T3 mouse fibroblasts were found to contain detectable $p92^{c\text{-}fes}$ (Table 1, Fig. 8).

Identification of a 94-kDa mammalian protein antigenically related to $p92^{c\text{-}fes}$. Examination of the cells discussed above for the presence of $p92^{c\text{-}fes}$ by immunoprecipitation with anti-*fps* antiserum and in vitro phosphorylation revealed in several instances a ^{32}P-labeled polypeptide with an apparent molecular weight of 94,000 (p94) (see, for example, normal human PBLs, Fig. 6). K562, a Philadelphia chromosome-positive CML cell line with some erythroid characteristics (2, 5), expressed relatively large amounts of p94, identified by phosphorylation in an immune complex kinase reaction (Fig. 8), but no detectable $p92^{c\text{-}fes}$. A similar observation was made for a mouse erythroleukemia cell line (MEL) and primary mouse erythroblasts which expressed p94 but little or no $p92^{c\text{-}fes}$ (Table 1). Phosphoamino acid analysis of in vitro-phosphorylated K562 cell p94 revealed that phosphorylation occurred predominantly at tyrosine residues (Fig. 2). Enolase added to an immune complex kinase reaction containing K562 p94 became specifically phosphorylated (Fig. 5), although less efficiently than in kinase reactions containing $p92^{c\text{-}fes}$. p94 might be a more highly modified form of $p92^{c\text{-}fes}$, or it might correspond to a distinct but antigenically related gene product. Several lines of evidence suggest that the 94-kDa protein (p94) is a separate protein. Tryptic phosphopeptide analysis of p94 from K562 and MEL cells labeled by in vitro phosphorylation revealed a single major peptide which migrated quite differently from those of HL-60 $p92^{c\text{-}fes}$ and ST-FeSV $P87^{gag\text{-}fes}$ (Fig. 9). Furthermore, V8 protease digestion of in vitro-phosphorylated p94 yielded a set of cleavage products distinct from those observed after digestion of $p92^{c\text{-}fes}$ and ST-FeSV $P87^{gag\text{-}fes}$ (Fig. 4). The recognition of p94 by anti-*fps* antiserum and its associated protein kinase activity argue that it is a tyrosine-specific protein kinase related to $p92^{c\text{-}fes}$, but its unique tryptic phosphopeptide map and V8 protease cleavage products suggest that it is a different protein.

K562 CML cells possess an amplified and translocated c-*abl* gene (23). Thus, in addition to p94, K562 cells synthesize an aberrant $P210^{c\text{-}abl}$ protein with greater tyrosine-specific protein kinase activity than the normal human $P145^{c\text{-}abl}$ (29). The anti-*fps* antiserum did not recognize $P210^{c\text{-}abl}$ in K562 cells, suggesting that it does not indiscriminately cross-react with any tyrosine-specific protein kinase. This implies that p94 possesses structural determinants shared with *fes*- and *fps*-encoded proteins but absent from other tyrosine-specific protein kinases. $P210^{c\text{-}abl}$ was positively identified in a K562 cell lysate by immunoprecipitation with anti-*abl* antiserum and in vitro phosphorylation (Fig. 8) and was shown to contain phosphotyrosine (data not shown).

In contrast to K562 and MEL cells, the HEL cell line expressed high levels of both $p92^{c\text{-}fes}$ and p94 (Fig. 8). Tryptic phosphopeptide analysis of the HEL 92/94-kDa doublet after in vitro phosphorylation demonstrated that

peptides characteristic of both p92$^{c\text{-}fes}$ and p94 were present (Fig. 9). The identification of cells which express principally p92$^{c\text{-}fes}$ (i.e., HL-60), of cells which express only p94 (i.e., K562) and of cells which express both species in similar amounts (i.e., HEL) provides further evidence that the two polypeptides are not differently modified forms of the same primary translation product. p94 was found in a number of myeloid, erythroid, and lymphoid cell lines (Table 1). Examination of a fibroblastic mouse cell line, NIH 3T3, and an epithelial mouse adrenal cortex cell line, Y1, revealed that both contained p94 (Fig. 8). Thus, the expression of p94 is apparently more widespread than that of p92$^{c\text{-}fes}$.

DISCUSSION

92-kDa protein with associated tyrosine kinase activity is encoded by the human c-*fes* gene. We have identified a 92-kDa protein (p92$^{c\text{-}fes}$) in human and mouse hematopoietic cells which is apparently encoded by the c-*fes* proto-oncogene. The p92$^{c\text{-}fes}$ protein was specifically precipitated by antiserum raised to the avian FSV P140$^{gag\text{-}fps}$ transforming protein. Comparative tryptic phosphopeptide analysis and V8 protease digestion of human p92$^{c\text{-}fes}$ and ST-FeSV P87$^{gag\text{-}fes}$ indicated that they shared closely related sequences. p92$^{c\text{-}fes}$ became phosphorylated at tyrosine in an immune complex kinase reaction, suggesting that it is a tyrosine-specific protein kinase capable of autophosphorylation. The human p92$^{c\text{-}fes}$ protein was also able to induce phosphorylation of enolase in vitro at the same tyrosine residue as was phosphorylated by the normal avian p98$^{c\text{-}fps}$ protein and FSV P140$^{gag\text{-}fps}$.

In addition to their antigenic relationship and similar enzymatic properties, human p92$^{c\text{-}fes}$ and avian p98$^{c\text{-}fps}$ are phosphorylated in vivo only at serine and threonine residues in those cells examined thus far, despite their ability to autophosphorylate at tyrosine in vitro.

Expression of p92$^{c\text{-}fes}$ is largely restricted to cells capable of myeloid differentiation. What might be the role of p92$^{c\text{-}fes}$ in normal cells? The prominent expression of p92$^{c\text{-}fes}$ is apparently confined to hematopoietic cells. p92$^{c\text{-}fes}$ has been identified in a number of cells characterized as belonging to, or capable of differentiating along, the granulocyte-macrophage pathway. These include an IL-3-dependent multipotential murine cell line, committed human and mouse granulocyte-macrophage precursors, and mature macrophagelike cells. In addition to these cell lines, most of which are leukemic, p92$^{c\text{-}fes}$ could be identified in the mononuclear fraction of normal human peripheral blood and in mouse bone marrow and spleen. The expression of p92$^{c\text{-}fes}$ may not be an exclusive property of myeloid cells, as we have also found it in a mouse mastocytoma line, in two human B-lymphoid lines, and in mouse NIH 3T3 fibroblasts.

One particular observation suggests that p92$^{c\text{-}fes}$ might be actively involved in granulocyte-macrophage differentiation. Mouse erythroid cells such as primary mouse erythroblasts and the MEL cell line apparently fail to express p92$^{c\text{-}fes}$. Similarly, p92$^{c\text{-}fes}$ was absent from the human erythroid line K562 but was surprisingly present in the human erythroleukemia cell line (HEL). The HEL cell line, unlike K562 and MEL cells, retains a dramatic capacity for myeloid differentiation and responds to treatment with phorbol ester by expressing a macrophagelike phenotype (42). In this case there is an intriguing correlation between the ability of the HEL cell line to acquire a monocytic phenotype and the synthesis of p92$^{c\text{-}fes}$.

Possible function of p92$^{c\text{-}fes}$ and tyrosine kinases in hematopoiesis. The data presented here suggest that tyrosine kinases are relatively abundant in hematopoietic cells and presumably play some functional role in normal hematopoiesis. It is apparent that some tyrosine-specific protein kinases are involved in relaying mitogenic and developmental hormonal signals from the plasma membrane (13). The identification of p92$^{c\text{-}fes}$ in normal myeloid cells suggests that some biochemical activity of this tyrosine-specific protein kinase may provide a signal involved in the control of granulocyte-macrophage differentiation. It is possible that the kinase activity of p92$^{c\text{-}fes}$ (or p98$^{c\text{-}fps}$) might be transiently stimulated in response to one or more growth factors or colony-stimulating factors involved in the proliferation and concomitant differentiation of myeloid cells.

In this context it is interesting that v-*fps*/*fes* genes have striking effects on hematopoietic cell proliferation and differentiation (1, 25, 44). For example, FSV P140$^{gag\text{-}fps}$ can relieve avian myeloblasts and macrophages of their dependence on chicken myelomonocytic growth factor by inducing autocrine production of this hematopoietic regulator (1). The delivery of an unregulated phosphorylation signal by the oncogenically activated v-*fps*/*fes* genes might alter both the proliferative capacity and differentiation status of infected hematopoietic cells by mimicking a transient, functionally activated form of p92$^{c\text{-}fes}$ or p98$^{c\text{-}fps}$.

Identification of a 94-kDa *fes*-related protein. The immunoprecipitation with anti-*fps* antiserum of a 94-kDa protein distinct from p92$^{c\text{-}fes}$ suggests that mammalian cells may encode a second *fes*-related protein. We cannot entirely exclude the possibility that p94 is a posttranslationally modified form of p92$^{c\text{-}fes}$ although this seems unlikely. Our data indicate that this protein may also be a tyrosine kinase, although its expression in fibroblastic, epithelial, and hematopoietic cells suggests that it has a separate physiological function from p92$^{c\text{-}fes}$. Formally, p94 might be encoded at a cellular locus distinct from c-*fes* or might be formed by differential splicing of a c-*fes* transcript. In considering p94, it is interesting that two independent c-*src* loci have been identified within the human genome (31). In addition, the cellular *ras* proto-oncogenes are known to comprise a multigene family (50), and more recently it has been suggested that proto-oncogenes such as c-*myc* and c-*fos* are members of gene families (7, 45). The precise function of both *fes*-related mammalian proteins remains to be determined.

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