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Specific expression of the human cellular *fps/fes*-encoded protein NCP92 in normal and leukemic myeloid cells

(myeloid differentiation/myelomonocytic leukemia/colony-stimulating factor/tyrosine kinase)

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ABSTRACT **We have found that both an antibody directed against a synthetic peptide representing an amino acid sequence of the conserved kinase domain of transforming protein P140 of Fujinami sarcoma virus and a regressing tumor antiserum recognized the products of the c-*fps/fes* genes of both avian and mammalian cells. The anti-peptide antibody also recognized a 94-kilodalton protein that was related to but distinct from the c-*fps/fes* product in structure and in tissue distribution. A 92-kilodalton protein, NCP92, was found to be the mammalian counterpart of the previously identified avian c-*fps/fes* protein NCP98 by its structural similarity to NCP98, its associated tyrosine kinase activity, and its similar tissue distribution. The highest levels of NCP92 were found in tissue macrophages and in bone marrow. In bone marrow NCP92 expression was restricted to cells of the monocyte/macrophage and granulocyte lineages. That the expression of NCP92 is limited to these cell types was confirmed by the analysis of murine and human hematopoietic tumors representing different cell lineages: NCP92 was positive in leukemic cells of granulocytic and monocytic origin but not in B-lymphocytic, T-lymphocytic, or erythroid tumor cells. The expression of NCP92 seems to be related to the capacity of myeloid cells to differentiate and to respond to certain colony-stimulating factors.**

Fujinami sarcoma virus (FSV) is an avian sarcoma virus that causes rapid transformation both *in vivo* and *in vitro*. The genome of FSV contains a part of the viral *gag* gene fused to unique cell-derived sequences called *fps* (1, 2). The Δ*gag–fps* sequence of the FSV genome encodes a transforming protein of 140 kilodaltons (P140) that is associated with a tyrosine-specific protein kinase activity (3, 4), which has been implicated as essential in transformation by FSV (4, 5). The transforming *fps* sequence was derived from c-*fps/fes*, a normal cellular gene that is highly conserved in the DNA of normal vertebrate cells (6, 7). In chickens, c-*fps/fes* is known to encode a normal cellular protein (NCP98) that is antigenically, structurally, and functionally related to FSV P140 (8) and is preferentially expressed in myeloid cells (8, 34).

In this paper we report the identification of the human and murine c-*fps/fes* gene product by using FSV-specific antisera broadly reactive with the gene products of avian and mammalian c-*fps/fes*. The expression of this gene is restricted to cells of the monocyte/macrophage and granulocyte lineages of both normal and tumor origin. The pattern of expression of this normal cellular protein in some tumor cell lines provides some possible clues regarding its biological role.

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MATERIALS AND METHODS

Chicken Cells and Viruses. Chicken embryo fibroblasts and FSV were prepared as described (2).

Cells Lines. *Murine.* The interleukin 3 (IL-3)-dependent cell lines LTBM and IO-3 (9, 10) were provided by Y. P. Yung and A. Oliff, respectively (Sloan–Kettering Institute). J774.1, PU5-1.8, and P388D1 (11) were provided by P. Ralph (Sloan Kettering Institute); RAW 264.7 (11) was obtained from the American Type Tissue Culture collection; WEHI-3B(D^+) and WEHI-3B(D^-) (11) are routinely carried in our laboratory. *Human.* THP-1 (11), NALM 12, and Jurkat (12) were provided by P. Ralph; HL-60, U937 (11), K562, and Daudi (12) are routinely carried in our laboratory; KG-1 and its variant KG-1a (13) were provided by E. Platzer (Sloan Kettering Institute); BALL-1, CCRF-CEM, MOLT-4, CCRF-HSB-2, HUT-78 (12), ARH.A 10 (14), and T-45 (15) were provided by M. Tanimoto (Sloan Kettering Institute). Cell lines derived from lung adenocarcinomas (SK-LC-1, -7, -9, -10, -11, -12, -15, -16, -20, -25) and squamous cell carcinoma (SK-LC-8) were provided by J. Feickert (Sloan Kettering Institute). The osteogenic sarcoma-derived cell lines Saos-2 (16) and U-2 OS (17) and the hepatoma cell line SK-HEP-1 (16) were provided by A. Houghton (Sloan-Kettering Institute).

Fractionation of Bone Marrow and Peripheral Blood Cells. Human bone marrow cells were fractionated by centrifugation on Ficoll/Hypaque (density = 1.077 g/cm^3) followed by centrifugation on a continuous Percoll density gradient essentially as described (18). Peripheral blood cells were similarly fractionated by centrifugation on Ficoll/Hypaque and then fractionated into lymphocytes and monocytes by adherence to plastic culture dishes as previously described (19).

Peptide Synthesis. The dodecapeptide Lys-Gln-Ile-Pro-Val-Lys-Trp-Thr-Ala-Pro-Glu-Ala, corresponding to amino acids 1080-1091 in the sequence of the FSV protein (20), was synthesized by the stepwise solid-phase method of Merrifield (21, 22).

Antisera. Tumor-bearing rat antiserum specific for *fps*-coded sequences (anti-FST) was obtained by injection of FSV-transformed 3Y1 cells into syngeneic rats as described before (8). To prepare antiserum against the synthetic peptide, rabbits were immunized with peptide that was coupled to keyhole limpet hemocyanin as previously described (23).

Protein Analysis. The methods used for protein analysis have been described (3, 8, 24).

RESULTS

NCP92 Is the Gene Product of Human c-*fps/fes*. [^{35}S]Methionine-labeled extracts from FSV-transformed chicken

Abbreviations: FSV, Fujinami sarcoma virus; CSF, colony-stimulating factor; GM-CSF, granulocyte macrophage CSF; IL-3, interleukin 3.

embryo fibroblasts and from uninfected chicken and human bone marrow cells were immunoprecipitated with several antisera and analyzed by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. FSV P140 and its cellular homolog in chickens, NCP98, were precipitated from extracts of FSV-transformed fibroblasts and from uninfected chicken bone marrow cells, respectively (Fig. 1, lanes C and F), by using anti-FST serum, and these immunoprecipitates were associated with protein kinase activities that phosphorylated FSV P140 and NCP98 (Fig. 1, lanes L and O). The anti-peptide antiserum but not preimmune serum was also capable of precipitating FSV P140 and NCP98, and in addition, a 94-kilodalton cellular protein (NCP94) that was present both in FSV-transformed fibroblasts and in uninfected bone marrow cells (Fig. 1, lanes A, B, D, and E). As shown in Fig. 1, lanes K and N, immunoprecipitates by antipeptide antiserum were associated with protein kinase activities that phosphorylated FSV P140, NCP98, and NCP94.

When extracts of uninfected human (Fig. 1, lane H) or murine (data not shown) bone marrow cells were immunoprecipitated with anti-peptide antiserum, two proteins of molecular masses 92 kilodaltons (NCP92) and 94 kilodaltons (NCP94) were specifically recognized, and these immunoprecipitates were associated with protein kinase activities that phosphorylated NCP92 and NCP94 (Fig. 1, lane Q). Anti-FST serum precipitated NCP92 and its associated protein kinase activity but, unlike anti-peptide antiserum, it did not precipitate NCP94 (Fig. 1, lanes J and S). This was true for five different anti-FST sera that were tested (data not shown).

Fig. 2 shows that the V8 partial proteolysis maps of human bone marrow NCP92 and chicken bone marrow NCP98 labeled *in vitro* with [^{32}P]ATP are very similar, indicating that NCP92 is structurally related to NCP98. Analysis of the phosphoamino acid composition of [^{32}P]NCP92 labeled in the *in vitro* kinase reaction showed that the amino acid acceptor of phosphate in NCP92 is tyrosine (Fig. 3). Like NCP98 (8), NCP92 was found to be a cAMP-independent protein kinase with a marked preference for Mn^{2+} over Mg^{2+} and capable of using only ATP as a donor of γ-phosphate (data not shown).

The data presented above show that human NCP92 and chicken NCP98 are structurally and functionally related and that they are the only proteins specifically recognized by anti-FST serum. This strongly suggests that NCP92 and NCP98 are the products of cognate genes in different species. Moreover, the experiments described in the following sections show that NCP92 has the same tissue distribution as NCP98. NCP94 is a cellular tyrosine kinase (data not shown) encoded by a gene different from c-*fps/fes*, and it probably has a biological function distinct from that of NCP92: human NCP94 and NCP92 had different tryptic fingerprints and, unlike NCP92, NCP94 was found to be present in every tissue that was examined (data not shown). The characterization of NCP94 will be published elsewhere.

Distribution of NCP92 in Murine and Human Tissues. To gain some insight into the possible functions of NCP92, we first examined its distribution in normal murine tissues. Since the level of *in vitro* phosphorylation of NCP92 was proportional to the amount of tissue extract used for the immunoprecipitation when the antibody was in excess (data not shown), we used the *in vitro* kinase assay to compare the relative amounts of NCP92 present in different tissues. As shown in Table 1, the highest levels of NCP92 were found in resident peritoneal macrophages, followed by bone marrow. Low levels of NCP92 were also found in lung and spleen, but NCP92 was not detectable in any other tissue that we examined. Analysis of some normal human tissues showed that NCP92 was also present at elevated levels in human pulmonary macrophages (data not shown) and bone marrow cells (see below). The level of expression of NCP92 in macrophages was estimated to be about 3% of that of P140 in FSV-transformed cells.

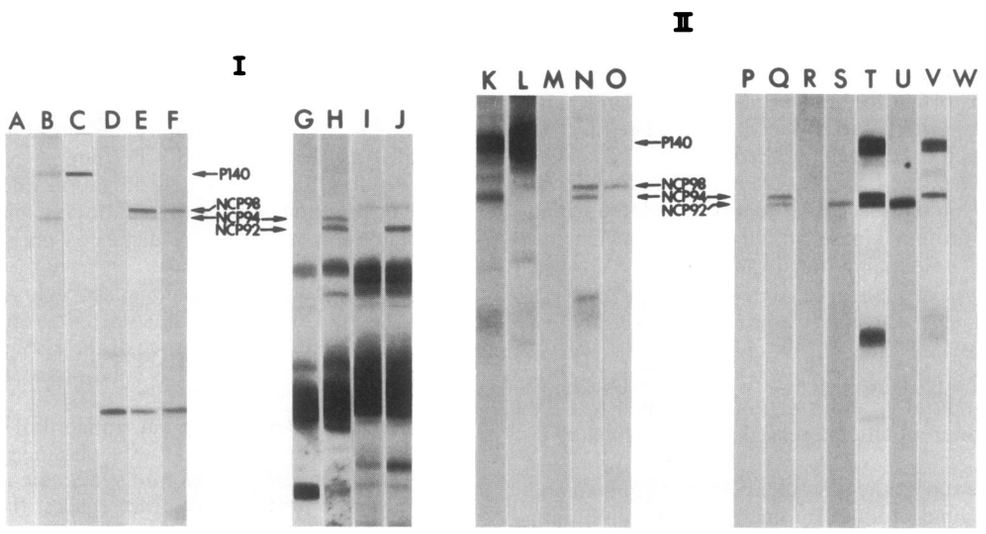

FIG. 1. Antisera directed against v-*fps* crossreact with avian and human c-*fps/fes*-encoded proteins. (*I*) [^{35}S]Methionine-labeled cell extracts prepared from FSV-transformed chicken embryo fibroblasts (lanes A–C), uninfected 5-day-old chicken bone marrow cells (lanes D–F), and normal adult human bone marrow cells (lanes G–J) were immunoprecipitated with the indicated antisera at a ratio of 200 μg of cell protein to 5 μl of serum. Conditions for immunoprecipitation were as described in ref. 8. Immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (8.5% acrylamide) followed by autoradiography. Lanes A, D, and G, preimmune anti-peptide serum; lane I, preimmune anti-FST serum; lanes B, E, and H, anti-peptide antiserum; lanes C, F, and J, anti-FST serum. (*II*) Unlabeled cell extracts prepared from FSV-transformed chicken embryo fibroblasts (lanes K and L), uninfected chicken bone marrow cells (lanes M–O), normal human bone marrow cells (lanes P–S), U937 (lanes T and U), and Jurkat (lanes V and W) cells were immunoprecipitated with the indicated antisera as above and then assayed for protein kinase activity as follows. Immunoprecipitates were incubated for 20 min at 28°C in 15 μl of a reaction mixture containing 50 mM Hepes at pH 7.4, 10 mM $MnCl_2$, and 0.3 μM [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq). After incubation, samples were analyzed by electrophoresis and autoradiography as above. Lanes M and P, preimmune anti-peptide serum; lane R, preimmune anti-FST serum; lanes K, N, Q, T, and V, anti-peptide antiserum; lanes L, O, S, U, and W, anti-FST serum.

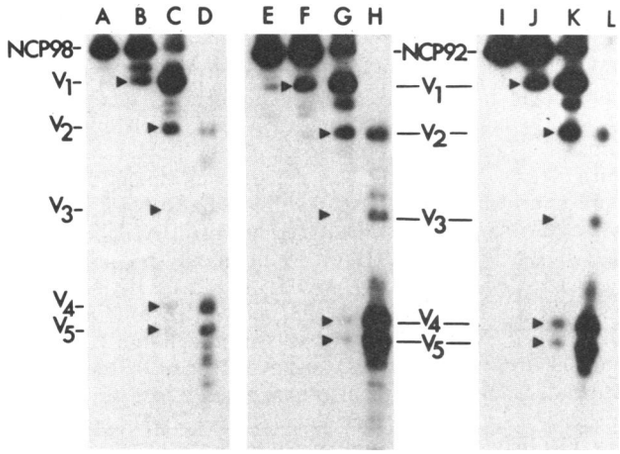

FIG. 2. Human NCP92 is structurally related to chicken NCP98. Excised gel bands of uninfected chicken bone marrow NCP98 (lanes A–D), normal human bone marrow NCP92 (lanes E–H), and HL-60 NCP92 (lanes I–L), labeled with [^{32}P]ATP in the *in vitro* kinase reaction, were subjected to partial proteolysis analysis with *Staphylococcus aureus* V8 protease. The concentrations of V8 protease used were as follows: lanes A, E, and I, no enzyme; lanes B, F, and J, 0.010 μg/ml; lanes C, G, and K, 0.200 μg/ml; lanes D, H, and L, 4.0 μg/ml. The positions of the V8 fragments V_1, V_2, V_3, V_4, and V_5, common to chicken NCP98 and human NCP92, are indicated by arrowheads. The chicken NCP98 and human NCP92 proteins used for V8 analysis were immunoprecipitated with anti-FST and anti-peptide antisera, respectively.

To further identify the hematopoietic cells that produce NCP92, human bone marrow was first fractionated by centrifugation on Ficoll/Hypaque. Three fractions were obtained: an erythrocyte fraction, a high-density fraction enriched in mature granulocytes, and a low-density fraction composed of immature monocyte, granulocyte, lymphocyte, and erythroid cells. The low-density fraction, in which the specific activity of NCP92 was twice as high as in the high-density fraction, was analyzed further by centrifugation in a Percoll density gradient as shown in Fig. 4. The highest

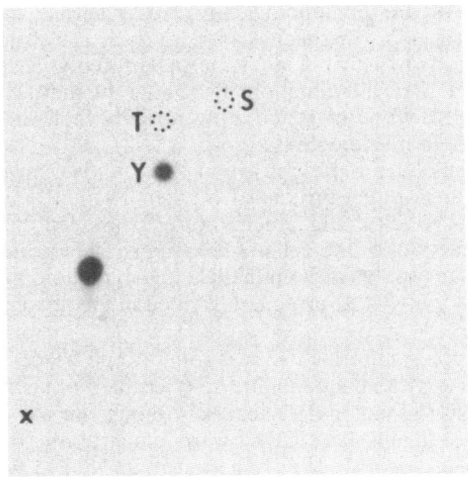

FIG. 3. Analysis of the phosphoamino acid composition of human bone marrow NCP92 phosphorylated *in vitro*. Partial acid hydrolysates of human bone marrow NCP92 labeled *in vitro* with [^{32}P]ATP were separated in two dimensions: electrophoresis at pH 1.9 was carried out from left to right, and electrophoresis at pH 3.5 was carried out from bottom to top. The positions of the internal phosphorylated amino acid standards are indicated: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The origin is marked ×. The serum used to immunoprecipitate NCP92 was anti-peptide antiserum.

Table 1. Distribution of NCP92 in normal murine tissues

Tissue	NCP92, %
Peritoneal macrophages	100
Bone marrow	13.9
Spleen	4.1
Lung	1.8
Thymus	<0.1
Liver	<0.1
Kidney	<0.1
Brain	<0.1
Muscle	<0.1
Heart	<0.1
Stomach	<0.1

Extracts from different tissues of normal 8-week-old BALB/c mice were prepared and aliquots containing the same amount of protein were immunoprecipitated with excess anti-peptide antibody. Immunoprecipitates were assayed for protein kinase activity and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography, and radioactivity in NCP92 gel bands was determined. Values given are percentages of the amount of radioactivity in resident peritoneal macrophages.

levels of NCP92 coincided with the peak of immature monocytes (fraction 4) and with the peak of immature granulocytes (fraction 6), which had 42% and 47%, respectively, of the total NCP92 of the gradient. The specific activity of NCP92 in fraction 4 was 60% higher than in fraction 6 (data not shown). No NCP92 was detected in fractions 1 and 2, which were enriched in lymphoid cells, or in fraction 7, which was entirely composed of normoblasts.

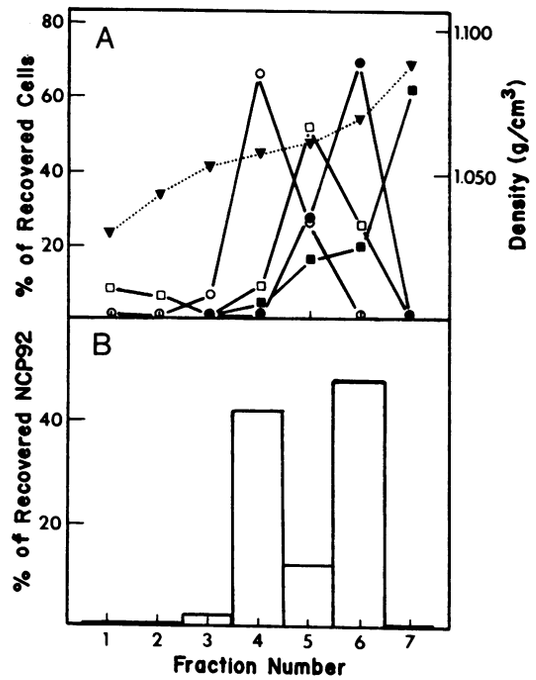

FIG. 4. Distribution of NCP92 in human bone marrow fractions. Normal human bone marrow cells were first fractionated by centrifugation on Ficoll/Hypaque. The low-density fraction thus obtained was further fractionated by centrifugation in a continuous Percoll density gradient and seven fractions were obtained. Each fraction was analyzed for its cellular composition by Wright/Giemsa staining of slide preparations and for NCP92 contents as described in the legend to Table 1. The total numbers of cells of each type recovered in the gradient were as follows: monocytic cells, 7.4 × 10^5; granulocytic cells, 1.1 × 10^6; lymphocytic cells, 4.8 × 10^5; erythroid cells, 5.5 × 10^5. (*A*) Distribution of monocytic (○), granulocytic (●), lymphocytic (□), and erythroid (■) cells across the gradient. ▼, Percoll density. (*B*) NCP92 distribution across the gradient.

Fractionation of peripheral blood cells on Ficoll/Hypaque followed by adherence depletion of monocytes showed that NCP92 was present in mature monocytes and granulocytes but was not detectable in lymphocytes or erythrocytes. The specific activity of NCP92 in peripheral blood fractions enriched in mature monocytes and granulocytes was about 50% lower than in bone marrow fractions enriched in monocyte and granulocyte precursors (data not shown).

Expression of NCP92 in Murine and Human Hematopoietic Tumors. To further investigate the biological role of NCP92, we extended our study of NCP92 expression to cells derived from murine and human hematopoietic tumors. These cells are representative of the different lineages and they are considered to be arrested at different stages of cell maturation. For many of the cell lines described below the presence or absence of NCP92 was assessed by analysis of [^{35}S]methionine-labeled cells, in addition to the *in vitro* kinase assay. In every case examined, the results obtained by both methods were in agreement (data not shown). As shown in Fig. 1, lanes T and U, NCP92 was readily detectable in U937, a monoblast/monocyte cell line that was derived from a patient with a true histiocytic lymphoma. The levels of NCP92 found in U937 and in other cell lines that were positive for NCP92 were approximately 5 times higher than in unfractionated normal bone marrow, but NCP92 was not abnormally elevated when compared to NCP92 levels in normal bone marrow fractions enriched in immature monocytes or when compared to NCP92 levels in macrophages (data not shown). NCP92 was also present in the promyelocytic leukemia cell line HL-60. As seen in Fig. 2, the V8 protease map of HL-60 NCP92 was identical to that of bone marrow NCP92, suggesting that no gross structural change in the NCP92 molecule has occurred.

NCP92 was detected in human primary myeloid leukemias such as acute promyelocytic leukemia, acute myelogenous leukemia, and chronic myelogenous leukemia in accelerated phase/blast crisis (Table 2). NCP92 was also present in leukemic human cell lines that represent different maturation stages of the granulocyte (KG-1 and HL-60) and the monocyte/macrophage lineages (THP-1 and U937). The exception was KG-1a, which is a variant derived spontaneously from KG-1. The presence of NCP92 in KG-1 and its absence in KG-1a correlated with the capacity of KG-1 but not of KG-1a cells to differentiate and to respond to human granulocyte/macrophage colony-stimulating factor (GM-CSF) (13) and/or pluripotent CSF (ref. 25; unpublished results), a factor with biological activity similar to that of murine IL-3. No NCP92 was detected in human cell lines representing different stages of cell maturation of B lymphocytes: NALM 12 (pre-B), Daudi (B blast I), BALL-1 (B blast II), ARH.A 10 (B blast II), or T lymphocytes: T-45 (T blast I), CCRF-CEM (T blast I), MOLT-4 (T blast II), Jurkat (T blast II) (Fig. 1, lanes V and W), CCRF-HSB-2 (T blast III), and HUT-78 (T blast IV). The erythroid cell line K562 was also negative for NCP92. Murine cell lines of the monocyte/macrophage lineage also expressed c-*fps/fes*. NCP92 was also positive in the murine cell lines LTBM and IO-3, a mast cell line and a myeloblast cell line, respectively, which have an absolute dependence on IL-3 and/or GM-CSF for their growth (9, 10, 26).

Table 2. Expression of NCP92 in human and murine myeloid leukemias

Human		Murine	
Tumor/cell line	NCP92	Cell line	NCP92
APL (1)	+	WEHI-3B(D$^+$) (Myelo/Mono)	+
AML (2)	+	WEHI-3B(D$^-$) (Myelo/Mono)	+
CML-AP (2)	+	P388D1 (Mono/Macro)	+
KG-1 (Mybl/ProM)	+	J774.1 (Mono/Macro)	+
KG-1a (Mybl)	−	RAW 264.7 (Mono/Macro)	+
HL-60 (ProM)	+	PU5-1.8 (Mono/Macro)	+
THP-1 (MonoB/Mono)	+	LTBM (Mast)	+
U937 (MonoB/Mono)	+	IO-3 (Mybl)	+

Lysates from the indicated primary tumors cells or cell lines were divided into two portions. One portion was immunoprecipitated with anti-peptide antiserum and the other portion was immunoprecipitated with anti-FST serum. Immunoprecipitates were assayed for protein kinase activity and analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography. The results obtained with both antisera were in agreement in every case. + and − indicate the presence or the absence, respectively, of NCP92. Numbers in parentheses indicate the number of different primary tumors that were examined. APL, acute promyelocytic leukemia; AML, acute myelogenous leukemia; CML-AP, chronic myelogenous leukemia in accelerated phase/blast crisis; Mybl, myeloblasts; ProM, promyelocytes; Myelo, myelocytes; MonoB, monoblasts; Mono, monocytes; Macro, macrophages; Mast, mast cells.

NCP92 Expression in Other Tumor Cells. NCP92 expression was also examined in a number of cell lines derived from lung adenocarcinomas (SK-LC-1, -7, -9, -10, -11, -12, -15, -16, -20, -25) and squamous cell carcinoma (SK-LC-8), from an adenocarcinoma of the liver (SK-HEP-1), and from two osteogenic sarcomas (Saos-2 and U-2 OS). No NCP92 was detected in any of these cell lines.

DISCUSSION

The anti-peptide and the anti-FST sera used in our study were very specific for *fps/fes*-coded proteins. The two antisera recognized FSV P140 but not the transforming proteins of other avian sarcoma viruses, namely p60^{src} (27), Y73-P90 (28), and UR2-P68 (29) (data not shown), and they were broadly reactive with the gene products of avian and mammalian c-*fps/fes*, suggesting a high degree of structural conservation in vertebrate c-*fps/fes* proteins. The anti-peptide antiserum but not anti-FST serum also precipitated NCP94, a cellular tyrosine kinase that is probably encoded by a gene related to but distinct from c-*fps/fes*.

Several lines of evidence support our conclusion that NCP92 is the human counterpart of chicken NCP98. NCP92 and NCP98 are immunologically crossreactive with two different types of *fps*-specific antisera, they are structurally related as shown by their V8 partial proteolytic cleavage maps, and they are functionally similar in their tyrosine-specific protein kinase activities and in their restricted expression in myeloid cells.

There has been a previous report that antiserum specific for the transforming protein of Snyder–Theilen Feline sarcoma virus (P85) could precipitate a 92-kilodalton protein from extracts of feline cells (30). Because this protein was detected in fibroblastic, epithelial, and lymphoid cells, its relation to the NCP92 protein described in the present study is unclear.

The expression of NCP92 was found to be tissue specific and restricted to hematopoietic cells of the monocyte/macrophage and granulocyte lineages. Among normal tissues, the highest levels of NCP92 were consistently found in macrophages, followed by bone marrow. NCP92 was also detected in mature peripheral blood monocytes and granulocytes, but NCP92 levels in these cells were lower than in their bone marrow precursors, suggesting that NCP92 levels decrease during cell maturation. While low levels of NCP92 were also detected in lung and spleen, this may be due to the presence of macrophages in these organs. In fact, NCP92 was not expressed in any lung cell lines that we tested.

NCP92 was positive in primary myeloid leukemias and cell lines but not in any cell lines belonging to the B- or the

T-lymphocyte lineages or in the erythroid cell line K562. NCP92 exhibited the same specificity of expression in hematopoietic tumor cells as it did in normal hematopoietic cells. In addition, no abnormal elevation or gross structural alteration in NCP92 of tumor cells could be detected. Therefore, it is likely that the presence of NCP92 in leukemic myeloid cells reflects their lineage of origin rather than a direct role of NCP92 in maintenance of the transformed state. The detection of c-*fps/fes* mRNA in myeloid leukemic cells (31, 32) is consistent with our findings. The pattern of expression of NCP92 in some of the cell lines that we examined provides some clues regarding the possible biological role of NCP92. For many of the myeloid cell lines used in this study, in particular those of human origin, information regarding their response to particular CSFs is incomplete. However, from the information that is available, an interesting correlation becomes apparent. Unlike KG-1, KG-1a did not express NCP92 and, concomitantly, KG-1a has lost the capacity of KG-1 to differentiate and to respond to GM-CSF and/or pluripotent CSF (refs. 13 and 25; unpublished results). In addition, it is of interest to note that the presence of NCP92 in LTBM and IO-3, two cell lines that have an absolute dependence on IL-3, GM-CSF, or both for their growth (9, 10, 26), would be consistent with a similar relation between NCP92 and IL-3 and/or GM-CSF. Although this analysis is limited to a few cell lines, these correlations might well be implicating NCP92 in the cellular response to CSFs that promote growth, differentiation, or both in myeloid cells. Several growth factor receptors have tyrosine kinase activities, and one such receptor has been shown to be homologous to the product of the v-*erbB* oncogene (33), which shares amino acid sequence homology with the product of v-*fps*. Because the c-*fps/fes* product is a tyrosine kinase and because NCP92 expression seems to correlate with the capacity of myeloid cells to respond to certain CSFs, it is plausible that NCP92 kinase could have some functional association with myeloid CSF receptors. However, in the absence of direct evidence for this hypothesis, a role for NCP92 in some other specific function of myeloid cells is at the present time equally possible.

The availability of homogeneous populations of hematopoietic cells that express NCP92 and can respond to differentiation and growth signals provides good systems to study the precise biological role of NCP92. Because viral *fps/fes* transforming proteins may exert some of their biological effects by sharing common pathways with NCP92, the elucidation of the biological role of NCP92 may also shed light on the mechanism of cell transformation by c-*fps/fes*-derived oncogenes.

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