Antipeptide antiserum identifies a widely distributed cellular tyrosine kinase related to but distinct from the c-fps/fes-encoded protein.

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Antipeptide Antiserum Identifies a Widely Distributed Cellular Tyrosine Kinase Related to But Distinct from the c-fps/fes-Encoded Protein

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We raised antibodies directed against a synthetic peptide representing an amino acid sequence of the conserved kinase domain of the transforming protein of Fujinami sarcoma virus (FSV) (P140). The antiserum obtained specifically recognized FSV-P140 and its cellular homolog and in addition, it recognized a new cellular protein of 94,000 daltons (NCP94) in avian and mammalian cells. NCP94 was found to be associated with a cyclic nucleotide-independent protein kinase activity that was specific for tyrosine residues. Although NCP94 and FSV-P140 share antigenic determinants, NCP94 is not a cellular homolog of FSV-P140: NCP94 and the previously identified c-fps/fes product were different in their tryptic fingerprints and in their tissue specificities. Thus, the function of NCP94 in normal cells is probably different than that of the c-fps/fes product. NCP94 was expressed in every tissue and cell line that was examined. In chickens, NCP94 levels were highest during embryonic development and NCP94 expression was high in gizzard, brain, and spleen throughout embryonic and adult life. The universal expression of NCP94 suggests that this protein may be involved in an essential function of normal cells. NCP94 may be a new cellular tyrosine kinase of the src gene family.

DNA sequence analysis of cloned retroviral genomes suggests that a number of viral oncogenes originally thought to be unrelated were probably derived from a common ancestor gene (19, 50). This group of oncogenes is known as the src gene family and it includes the src, fps/fes, yes, ros, fms, abl, erbB, and fgr genes (3). The proteins encoded by these genes have a high degree of amino acid sequence homology (9, 18, 19, 25, 34, 35, 39, 40, 47, 50, 55, 61), and they are associated with tyrosine-specific protein kinase activities (1, 2, 8, 14, 15, 17, 21, 24, 26, 33, 37, 41, 43, 57) that are believed to be essential to the mechanism of cell transformation. Viral oncogenes were derived from cellular oncogenes by a process of recombination between viral and cellular sequences. The src-related cellular oncogenes are expressed at low levels in normal cells (51). Using cross-reacting antiserum, normal cellular proteins structurally related to their viral counterparts have been identified (1, 7, 15, 22, 26, 30, 36, 42, 58). Some of the cellular homologs of src-related oncogenes are associated with tyrosine-specific protein kinase activities (7, 13, 22, 26, 30, 36, 41), and there is mounting evidence that these proteins are involved in the control of cell proliferation and differentiation. For instance, the cellular homologs of v-erbB and v-fms are the receptors for epidermal growth factor (10) and the macrophage growth factor CSF-1, respectively (49). Because of the importance of this family of genes in normal cell function and in malignancy it is of great interest to identify all of its members. However, some of these may have escaped detection either because they have never been transduced by retroviruses or because their protein products are still unknown.

In this paper we describe an approach to identify src-related cellular proteins regardless of whether their encoding genes have been transduced by retroviruses or not. Because of the high degree of amino acid sequence homology between src-related transforming proteins we reasoned that antisera directed against amino acid sequences of their conserved kinase domains might cross-react with new members of this family. Accordingly, we prepared an antiserum directed against a synthetic peptide corresponding to a very conserved region in the transforming protein of Fujinami sarcoma virus (FSV), an acutely transforming avian sarcoma virus whose transforming gene is v-fps (20, 28). This antiserum specifically recognized the transforming protein of FSV (P140), it cross-reacted with the gene products of avian and mammalian fps/fes, and in addition, it recognized a new cellular protein of 94,000 daltons (NCP94) in avian and mammalian cells (13). NCP94 is associated with a tyrosine-specific protein kinase activity, it is structurally different than the cellular fps/fes product, and it appears to be present in all cells.

MATERIALS AND METHODS

Cell and viruses. Chicken embryo fibroblasts, FSV, and F36 virus, were prepared as described previously (16, 20).

Cell lines. The human monoblastic cell line U937 (52) and the human lymphocytic cell line MOLT 4 (32) were provided by J. L. Gabrilove, Sloan Kettering Institute, New York.

Isotopic labeling of cells. Monolayer cultures. (i) [35S]methionine labeling of uninfected and FSV-infected chicken embryo fibroblasts was carried out in 100-mm tissue culture plates. Cells were incubated with 3 ml of methionine-free minimal essential medium (GIBCO) containing 2% calf serum. After 1 h, the culture medium was changed and replaced with 3 ml of fresh medium containing 100 μCi of [35S]methionine (900 to 1,200 Ci/mmol; Amersham) per ml, and incubation was continued for 8 h, after which time the cells were harvested. For tryptic fingerprint analysis of uninfected or F36-infected chicken embryo fibroblasts, cells were labeled with 3 μCi of [35S]methionine. (ii) For 32P labeling, uninfected chicken embryo fibroblasts grown in 60-mm tissue culture plates were incubated with 2 ml of...
phosphate-free medium 199 (GIBCO) containing 2% dia-
lyzed calf serum. After 1 h the medium was changed and
replaced with 2 ml of fresh medium containing 1 mCi of
carrier-free 32P04 (Amersham) per ml, and incubation was
continued for 5 h, after which time the cells were harvested.

**Suspension cell cultures.** Chicken bone marrow cells or
human hematopoietic cells (107) were suspended in 1.5 ml of
methionine-free medium and incubated in a 60-mm tissue cul-
ture plate. After 1 h, 200 μCi of [35S]methionine per ml
was added, and incubation was continued for 8 h. At the end
of this period cells were harvested by centrifugation at 1,000
× g for 10 min at 4°C. For trypic fingerprint analysis, chink
bone marrow cells were labeled with 3 μCi of
[35S]methionine.

**Preparation of cell and tissue extract, immunoprecipitation,
and the kinase assay.** Preparation of cell and tissue extracts
was carried out as described previously (30). Briefly, labeled or
unlabeled monolayer and suspension cultures were lysed in
ice-cold modified Nonidet P-40 buffer (10 mM Na2HPO4 [pH 7.8],
150 mM NaCl, 25 mM EDTA, 10% glycerol, 2% Trasylol, 0.5% Nonidet P-40; 0.1% sodium
deoxylcholate, 0.2% 2-mercaptoethanol). Cell lysates were
centrifuged at 10,000 × g for 10 min at 4°C, and the resulting
clear supernatants were stored in liquid nitrogen until
use. Whole chicken tissues were homogenized in ice-cold
modified Nonidet P-40 buffer using a Dounce homogenizer.
Tissue lysates were then centrifuged and stored as described
above.

Immunoprecipitation of cell and tissue extracts was car-
ried out as previously described (30), using 5 μl of the
indicated antisera for 200 μg of cellular protein unless
otherwise indicated. Immunoprecipitates were analyzed di-
rectly by gel electrophoresis or first assayed for protein
kinase activity.

Protein kinase activity was assayed in immunoprecipitates
as previously described (30). Briefly, immunoprecipitates
were incubated in 15 μl of reaction mixture containing 50
mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesul-
fonic acid), (pH 7.4), 10 mM MnCl₂, and 3 × 10⁻⁷ M
[y-32P]ATP (3,000 Ci/mmole) (Amersham), unless otherwise
indicated. Reaction mixtures were incubated for 15 min at
28°C. After incubation the reaction was stopped by the
addition of 75 μl of electrophoresis sample buffer.

**Quantitation of 32P-radioactivity in gel bands.** Gel slices
containing 32P-labeled proteins were solubilized in 5 ml of
NCS tissue solubilizer (Amersham) for 6 h at 55°C before
counting.

**Antisera.** Rabbit antisera against Rous-associated virus-
2 virion proteins was obtained as described before (14).
FSV-specific regressing-tumor antisera (anti-FST) was ot-
tained by injection of FSV-transformed Y1 cells into
syngeneic rats as described before (30).

Antiserum against the synthetic peptide was prepared as
follows. Peptide was coupled to keyhole limpet hemocyanin
at a molar ratio of 32 to 1 with glutaraldehyde as described
previously (59). The conjugate was emulsified with complete
Freund adjuvant and injected subcutaneously and intramus-
cularly on day 1 and emulsified with incomplete Freund
adjuvant and injected subcutaneously on days 14 and 28.
Each rabbit was injected with 250 μg of coupled peptide each
time, and rabbits were bled on day 35. The work described
in this paper was carried out with serum obtained from one
rabbit.

**Peptide synthesis.** The dodecapeptide Lys-Gln-Ile-Pro-
Val-Lys-Trp-Thr-Ala-Pro-Glu-Ala, corresponding to amino
acids 1080 to 1091 in the sequence of the FSV-transforming
protein (50) was synthesized by the stepwise solid-phase
method of Merrifield (31) with the modifications of Tam et al.
(54). After purification by preparative reverse high-pressure
liquid chromatography, the peptide was 99% pure and it had
the expected amino acid ratio and sequence.

**Gel electrophoresis.** Immunoprecipitated proteins were
generated by sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) in 8.5% slab gels as described
by Laemmli (27).

**Tryptic peptide analysis of proteins.** (i) Electrophoretic
bands containing [33S]methionine-labeled proteins to be ana-
yzed were excised from dried gels and subjected to trypsin
hydrolysis as described by Rettenmier and Hanafusa (42).
Two-dimensional analysis of tryptic digests was carried out
by electrophoresis at pH 4.7 in the first dimension and
ascending chromatography in the second dimension as pre-
viously described (30), with the modification that the chro-
matography buffer contained 7% (wt/vol) 2,5 diphenyl
oxazole (44). Dried plates were exposed to Kodak XAR-5
film for fluorography. (ii) Excised gel bands containing
32P-labeled proteins were subjected to trypsin digestion as
described above. Two-dimensional analysis of tryptic di-
gests was carried out by electrophoresis at pH 1.9 followed
by ascending chromatography as described previously (14).
32P-labeled tryptic peptides were visualized by autoradiog-
raphy.

**Identification of phosphoamino acids.** 32P-labeled phos-
phoamino acids were identified by two-dimensional analysis
as previously described (14).

**RESULTS**

**Antiserum directed against a peptide sequence in FSV-P140
cross-reacts with a 94,000-dalton cellular protein.** The
antipeptide antisem was first tested for its ability to recog-
nize the transforming protein of FSV. Uninfected and
FSV-infected chicken embryo fibroblasts were labeled with
[33S]methionine and cell extracts were immunoprecipitated
with antisera against viral structural proteins or with antipeptide antisem. Immunoprecipitates were then ana-
yzed by SDS-PAGE and autoradiography. Antipeptide an-
tiserum was able to precipitate FSV-P140 from extracts of
FSV-transformed cells (Fig. 1A, lane 3). In addition to
FSV-P140, the antipeptide antisem recognized a 94,000-
dalton protein, called NCP94, that was present in extracts of
uninfected and FSV-infected cells (Fig. 1A, lanes 3 and 5).
Antipeptide preimmune serum precipitated neither FSV-
P140 nor NCP94 (Fig. 1A, lanes 2 and 4). The immunopre-
cipitation of FSV-P140 and NCP94 was completely blocked
by preincubation of the antipeptide antisem with the
synthetic peptide (Fig. 1B, lane 2). This suggests that FSV-
P140 and NCP94 were specifically precipitated by antibodies
directed against antigenic determinants in the synthetic
peptide and that therefore NCP94 and FSV-P140 share
amino acid sequences in the region of the peptide.

The antipeptide antisem was able to precipitate FSV-
P140 and NCP94 when cell extraction and immunoprecipita-

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tide antiserum would recognize the transforming proteins of other avian sarcoma viruses, namely pp60^v-src (4), Y73-P90 (24), and UR2-P68 (15). The antiserum did not recognize these proteins under any conditions that we tested. These included immunoprecipitation in non-SDS or SDS-containing buffers, boiling the samples in SDS before immunoprecipitation, and Western blot analysis (data not shown).

NCP94 is a phosphoprotein associated with a tyrosine-specific protein kinase activity. Antipeptide antiserum precipitated NCP94 from extracts of ^32P-labeled uninfected chicken embryo fibroblasts, indicating that NCP94 is a phosphorylated protein in vivo (Fig. 1A, lane 6). Since NCP94 was a phosphoprotein precipitable by an antiserum directed against an amino acid sequence that is very conserved in viral tyrosine kinases, we examined whether NCP94 was also associated with protein kinase activity. Unlabeled cell extracts from uninfected or FSV-infected chicken embryo fibroblasts were immunoprecipitated by antipeptide antiserum and immunoprecipitates were assayed for protein kinase activity. Immunoprecipitates of NCP94 were able to catalyze the transfer of ^32P from [γ^32P]ATP to NCP94 in an apparent autophosphorylation reaction (Fig. 2, lane 2). When immunoprecipitates from FSV-transformed cells were assayed for protein kinase activity, both FSV-P140 and NCP94 became phosphorylated (Fig. 2, lane 4). Preimmune serum did not precipitate any protein kinase activity from uninfected or from FSV-infected cells (Fig. 2, lanes 1 and 3).

The identity of the amino acids serving as phosphate acceptors in the NCP94 kinase reaction was determined by two-dimensional analysis of partially acid-hydrolyzed ^32P-labeled products. The amino acid acceptor of phosphate in NCP94 was found to be tyrosine (Fig. 3A). Traces of phosphothreonine and phosphoserine were also observed. When in vivo-labeled ^3P-NCP94 was similarly analyzed, only phosphoserine was observed (Fig. 3B). This result did not change when sodium vanadate was included in the cell extraction buffer (data not shown).

Antipeptide antibody recognizes the gene product of chicken cellular fps. The gene product of chicken c-fps/fes is a

![FIG. 1. Antiserum directed against v-fps peptide cross-reacts with a 94,000-dalton cellular protein. (A) FSV-transformed chicken embryo fibroblasts labeled with [35S]methionine (lanes 1 to 3) and uninfected chicken embryo fibroblasts labeled with [35S]methionine (lanes 4 and 5) or with ^32P, (lane 6) were immunoprecipitated with the indicated antisera. Immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography. Lanes: 1, antiserum against total virion protein; 2 and 4, preimmune antipeptide serum; 3, 5, and 6, antipeptide antiserum. Autoradiography exposure time in lanes 1 to 5 was 14 days and in lane 6 exposure time was 6 h. (B) FSV-transformed chicken embryo fibroblasts labeled with [35S]methionine (lanes 1 and 2) were immunoprecipitated with the indicated antisera. Lane 1, antipeptide antiserum; lane 2, antipeptide antiserum that had been preincubated with 20 µg of synthetic peptide for 2 h at 4°C. Autoradiography exposure time was 12 days.]

![FIG. 2. NCP94 is associated with a protein kinase activity. Unlabeled cell extracts prepared from uninfected (lanes 1 and 2) and FSV-infected (lanes 3 to 5) chicken embryo fibroblasts were immunoprecipitated with the indicated antisera, assayed for protein kinase activity, and then analyzed by SDS-PAGE and autoradiography. Lanes: 1 and 3, preimmune antipeptide antiserum; 2 and 4, antipeptide antiserum; 5, antiserum against total virion protein. Autoradiography exposure time was 30 min.]

![FIG. 3. Analysis of the phosphoamino acid composition of NCP94 phosphorylated in vivo and in vitro. Partial acid hydrolysates of ^32P-labeled NCP94 from chicken embryo fibroblasts 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 phosphoamino acid standards are indicated. (A) NCP94 autophosphorylated in vitro; (B) NCP94 phosphorylated in vivo. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.]

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98,000-dalton protein (NCP98) that can be precipitated from extracts of bone marrow cells using regressing tumor antisera (anti-FST) specific for the unique sequence of FSV-P140. NCP98 is structurally related to FSV-P140, it is associated with a tyrosine-specific protein kinase activity, and is preferentially expressed in myeloid cells (30, 46). Since the antipeptide antibody was raised against an amino acid sequence in FSV-P140, we wanted to determine whether this antibody would also recognize NCP98. Unlabeled or [35S]methionine-labeled extracts from uninfected chicken bone marrow cells were immunoprecipitated with either anti-FST or antipeptide antiserum. Labeled and unlabeled immunoprecipitates were analyzed either directly or after assaying for protein kinase activity, respectively. Anti-FST precipitated NCP98 from chicken bone marrow cells (Fig. 4A, lane 3) but not from chicken embryo fibroblasts (Fig. 4A, lane 4). Anti-FST did not recognize NCP94, and this was true for five different anti-FST sera that we tested. On the other hand, the antipeptide antiserum was able to precipitate both NCP98 and NCP94 from bone marrow cells (Fig. 4A, lane 2). This serum also precipitated the protein kinase activities associated with NCP98 and NCP94 (Fig. 4B, lane 6).

We conclude from these results that the antipeptide antiserum is able to recognize both NCP94 and NCP98 and that these two proteins are differentially expressed in chicken embryo fibroblasts and chicken bone marrow cells. A study of the tissue specificity of NCP94 is presented below.

NCP94 and NCP98 have different primary structures. The fact that the antipeptide antiserum recognized FSV-P140, NCP98, and NCP94 raised the possibility that NCP94, like NCP98, is a cellular homolog of FSV-P140. To address this question, we compared the primary structures of chicken embryo fibroblast NCP94, chicken bone marrow NCP98, and the protein encoded by viral fps.

(i) Tryptic fingerprint analysis of [35S]methionine-labeled F36-P91, NCP98, and NCP94. F36 is a nondefective avian sarcoma virus that was constructed by replacing the src sequence in Schmidt-Ruppin A Rous sarcoma virus with viral fps sequences (16). The v-fps DNA fragment that was used contained the majority of the v-fps sequence of FSV except for about 150 base pairs corresponding to the 5' end of v-fps (16). F36 encodes a transforming protein of 91,000 daltons (P91) that has no gag sequences and also lacks about 50 amino acids encoded by the 5' portion of v-fps. We chose F36-P91 instead of FSV-P140 for peptide analysis because F36-P91 does not contribute any gag-coded peptides, thereby simplifying the comparison with the maps of NCP98 and NCP94.

The tryptic maps of F36-P91 and NCP98 are very similar, indicating that the two proteins are very closely related (Fig. 5). By contrast, the tryptic map of NCP94 is very different from that of NCP98 and F36-P91. NCP94 and NCP98 apparently have only one methionine-containing peptide in common (peptide number 8/24) (Fig. 5). These results indicate that NCP94 and NCP98 have different primary structures. While NCP98 is structurally related to F36-P91 and is the gene product of c-fps/fes (30), NCP94 is not structurally related to F36-P91 and therefore it is most likely encoded by a gene different than c-fps/fes.

(ii) Phosphopeptide analysis of NCP94 and NCP98. The structure of the tyrosine phosphorylation sites in NCP94 and NCP98 was compared by examining tryptic fingerprints of [32P]NCP94 and [32P]NCP98 labeled in the in vitro kinase reaction. NCP94 and NCP98 yielded two phosphopeptides each, and these four phosphopeptides had different mobilities suggesting that the amino acid sequences around the tyrosine phosphorylation site(s) in NCP94 and NCP98 are different (Fig. 6). This is also consistent with the idea that NCP94 and NCP98 are encoded by different genes.

Enzymatic properties of NCP94. To further characterize the protein kinase activity associated with NCP94, several variables of the NCP94 in vitro kinase reaction were examined. The kinase reaction was monitored by measuring 32P radioactivity in the NCP94 bands after the reaction products were separated by SDS-PAGE. The presence of cyclic AMP or cyclic GMP (1 to 100 μM) had no effect on the activity of NCP94 kinase. The enzyme had an absolute requirement for an appropriate divalent cation (Table 1). Mn2+ ions were found to be eight times more effective than Mg2+ ions, and Ca2+ ions did not support any detectable level of 32P incorporation. While NCP94 had a marked preference for [32P]ATP as a 32PO4 donor, some incorporation was also observed with [32P]GTP (Table 2).

Expression of NCP94 in embryonic and adult chicken tissues. To gain some insight into the possible biological role of NCP94, we examined its distribution in embryonic and adult chicken tissues. Since the level of in vitro 32P incorporation into NCP94 was proportional to the amount of tissue extract used for immunoprecipitation when the antibody was in excess, we used this assay to compare the amount of NCP94 in different tissues. Table 3 shows the tissue distri-
bution of NCP94 in 5-, 12-, and 20-day-old chicken embryos and in 5-day-, 14-day-, 2-month-, and 2-year-old chickens. In 5-day-old embryos, the earliest embryonic stage we examined, NCP94 was already present in the three developing organs that we could analyze, namely the brain, eyes, and heart. NCP94 levels were highest in the brain and eyes. At the next stages of embryonic development that we examined, NCP94 was preferentially expressed in gizzard, brain, and spleen. After hatching, these organs continued to exhibit the highest levels of NCP94. The lowest levels of NCP94 were found in muscle and in liver at all stages. In the embryo, NCP94 levels in these organs were five to ten times lower than in gizzard. Other tissues had intermediate levels of NCP94. In all tissues, NCP94 levels were highest in embryos between 12 and 20 days of age. NCP94 levels declined steadily after hatching, but after 2 years of age, NCP94 was still expressed.

From these results it is apparent that NCP94 is a widely distributed protein that is expressed throughout embryonic and adult life and that this protein seems to be preferentially expressed in the gizzard, brain, and spleen at all ages. We should note, however, that the tissue distribution of NCP94 determined using the in vitro kinase assay should be taken as preliminary. As several factors, such as post-translational modification, may affect the protein kinase activity of NCP94 differently in different tissues, this assay may not

![Image](https://mcb.asm.org/)

**FIG. 5.** Comparison of [35S]methionine-containing tryptic peptides of F36-P91, NCP98, and NCP94. Tryptic digests of [35S]methionine-labeled proteins were separated in two dimensions. Electrophoresis carried out in the horizontal dimension with the cathode at the right was then followed by ascending chromatography in the vertical dimension. Top panel: A, NCP98; B, F36-P91; C, mixture of NCP98 and F36-P91; D, schematic drawing of mixture of NCP98 and F36-P91. Closed circles, peptides common to NCP98 and F36-P91; open circles, peptide unique to F36-P91; half-closed circle, peptide unique to NCP98. Bottom panel: E, NCP98; F, NCP94; G, mixture of NCP98 and NCP94; H, schematic drawing of mixture of NCP98 and NCP94. Closed circles, peptides unique to NCP98; open circles, peptide unique to NCP94; half-closed circle, peptide common to NCP98 and NCP94. A through C and E through G are the results of two independent experiments. Peptides 1 to 9 in A and E designate peptides derived from NCP98 in A and E. Peptides 7' and 9' are NCP98 peptides observed only in E. Peptides 2 to 10 in B designate peptides derived from F36-P91. Peptides 11 to 24 in F designate peptides derived from NCP94. The origin is marked x.

![Image](https://mcb.asm.org/)

**FIG. 6.** Comparison of tryptic phosphopeptides of NCP98 and NCP94. Tryptic digests of [32P]NCP98 and [32P]NCP94 autophosphorylated in vitro were separated in two dimensions. Electrophoresis was in the horizontal dimension and chromatography was in the vertical dimension. (A) NCP98, (B) NCP94; (C) mixture of NCP98 and NCP94. a and b and c and d designate phosphopeptides derived from NCP98 and NCP94, respectively. The origin is marked x.

<table>
<thead>
<tr>
<th>Cation</th>
<th>[32P] (10^6 cpm) in NCP94</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.8</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>23.0</td>
</tr>
</tbody>
</table>

* Unlabeled cell extracts from chicken embryo fibroblasts were immunoprecipitated with antipeptide antiserum, and immunoprecipitates were assayed for protein kinase activity as described in the text, except that the cations used in the kinase buffer are indicated in the table. Kinase reaction mixtures were analyzed by SDS-PAGE, and the radioactivity in NCP94 gel bands was determined as described in the text.

* The cation concentration in the kinase buffer was 10 mM in all cases.
TABLE 2. Phosphate donors in the NCP94 kinase reaction*

<table>
<thead>
<tr>
<th>32P donor</th>
<th>Concentration (nM)</th>
<th>32P (10^4 cpm) in NCP94</th>
</tr>
</thead>
<tbody>
<tr>
<td>[γ32P]ATP</td>
<td>100</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>33.4</td>
</tr>
<tr>
<td>[γ32P]GTP</td>
<td>100</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* NCP94 immunoprecipitates were obtained as described in Table 1 and assayed for protein kinase activity as described in the text, except that [γ32P]ATP and [γ32P]GTP were present at the indicated concentrations. Kinase reaction mixtures were analyzed by SDS-PAGE and the radioactivity in NCP94 gel bands was determined as described in the text.

The gene product of human c-fps/fes is a 92,000-dalton protein called NCP92 that is associated with a tyrosine-specific protein kinase activity and is specifically expressed in normal and leukemic myeloid cells (13). NCP92 and its associated kinase activity can be precipitated by anti-FST serum from cell extracts of U937, a human monoblastic cell line (Fig. 7A, lane 2, and Fig. 7B, lane 6), but not from extracts of MOLT 4 (Fig. 7A, lane 4, and Fig. 7B, lane 8). Antipeptide antiserum can precipitate both NCP92 and NCP94 and their kinase activities from U937 cells (Fig. 7A, lane 1, and Fig. 7B, lane 5). The ability of this antipeptide antiserum to precipitate human NCP92 and NCP94 has been previously reported (13).

The antipeptide antiserum also recognized the corresponding murine proteins NCP92 and NCP94 (13). In normal murine tissues NCP94 levels were also high in the stomach and brain (data not shown), suggesting that the function of NCP94 has been conserved through vertebrate evolution.

**DISCUSSION**

This paper describes the identification and initial characterization of a new cellular protein that is associated with a tyrosine-specific protein kinase activity and is antigenically related to but distinct from the gene product of c-fps/fes. This protein was identified by using an antiserum directed against an amino acid sequence in the conserved kinase domain of FSV-P140. The antipeptide antiserum that we obtained recognized FSV-P140, it was broadly reactive with the gene products of avian and mammalian c-fps/fes, and it cross-reacted with avian and mammalian NCP94.

Part of the peptide sequence used for immunization is highly conserved among tyrosine kinases (35). In pp60^c-src^ (55) and in Y73-P90 (25), the seven C-terminal amino acids of this sequence are conserved and the rest of the sequence is relatively divergent. The lack of reactivity of the antipeptide antiserum with these proteins might suggest that the antipeptide antibodies that we obtained were directed primarily against the less conserved N-terminal portion of the peptide. However, an alternative explanation is that although the serum may contain antibodies directed against the conserved C terminus of the peptide, pp60^c-src^ and Y73-P90 have a conformation in which the antigenic sites are either not accessible or cannot be recognized by the antibodies.

Immunoprecipitates of FSV-P140, NCP98, NCP92, and NCP94 by antipeptide antiserum were associated with protein kinase activity. Thus, binding of the antipeptide antisem to antigenic sites in the peptide domain did not inhibit protein kinase activity. Since at least some of the amino

**TABLE 3. Distribution of NCP94 in embryonic and adult chicken tissues^a^**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age of Chicken</th>
<th>% of NCP94 ^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>Embryo (days)</td>
<td>Adult</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>70.9</td>
</tr>
<tr>
<td>Gizzard</td>
<td>12</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
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<td></td>
<td>14 days</td>
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<td></td>
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<td>12</td>
<td>77.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>74.2</td>
</tr>
<tr>
<td>Brain</td>
<td>12</td>
<td>81.3</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>72.4</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>69.6</td>
</tr>
<tr>
<td></td>
<td>2 mo</td>
<td>46.5</td>
</tr>
<tr>
<td></td>
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^a^ Samples of tissue extracts containing the same amount of protein were immunoprecipitated with an excess of antipeptide antiserum. Immunoprecipitates were assayed for protein kinase activity, analyzed by SDS-PAGE, and radioactivity in NCP94 gel bands was determined.

^b^ Values are percentages of radioactivity in NCP94 gel band obtained from 12-day-old embryo gizzard.
acids in this sequence can be blocked by antibody binding without affecting phosphotransferase activity, these amino acids are not likely to be directly involved in a catalytic function. On the other hand, it has been reported that in Rous sarcoma virus pp60c-src, single amino acid substitutions involving the four C-terminal amino acids of this sequence nearly abolished pp60c-src protein kinase activity (5). Although the results of experiments involving in vitro mutagenesis and our results with the antipeptide antiserum cannot be directly compared, taken together, these results might be pointing out that the highly conserved C-terminal sequence of the peptide region is essential for protein kinase activity because it serves an important structural function rather than a catalytic function.

In addition to FSV-P140 and its normal cellular homolog, the antipeptide antiserum recognized a new cellular protein called NCP94 in avian and mammalian cells. The immunoprecipitation of NCP94 was completely blocked by the synthetic peptide. This suggests that NCP94 was specifically precipitated by antibodies directed against the immunizing peptide and that therefore NCP94 shares amino acid sequences with FSV-P140 in the region of the peptide. Although NCP94 and FSV-P140 share these amino acid sequences, NCP94 is most likely encoded by a gene different than c-fps/fes. This conclusion is based on the fact that NCP94 is structurally different from the products of viral and cellular fps/fes: while NCP98 and F36-P91 had very similar tryptic fingerprints, NCP94 had a tryptic fingerprint very different from F36-P91 and NCP98. At most, NCP94 and NCP98 share one tryptic peptide, suggesting that these two proteins may not be closely related. Similarly, while the tyrosine phosphorylation sites in NCP98 and FSV-P140 are the same (30), the tyrosine phosphorylation sites in NCP94 and NCP98 are different. Furthermore, while NCP98 follows the tissue distribution of c-fps/fes mRNA (30, 51), the distribution of NCP94 follows a different pattern. This is also in agreement with the idea that NCP94 is not encoded by c-fps/fes and suggests that NCP94 and NCP98 have different biological functions as well. The results of tryptic fingerprint analysis suggest that NCP94 may not be closely related to the gene product of c-fps/fes. On the other hand, it has been recently reported that a polyclonal anti-fps tumor serum cross-reacted with a 94,000-dalton protein (p94) that was associated with tyrosine kinase activity and had a wide cell type distribution (29). Although the identity of this protein to the NCP94 protein described in this paper will require further characterization, if these two proteins are indeed the same, then NCP94 may be more related immunologically to the c-fps/fes products than the tryptic fingerprint analysis suggests.

NCP94 was associated with a protein kinase activity specific for tyrosine residues. This enzymatic activity had several properties in common with other src-related tyrosine kinases: it was cyclic nucleotide-independent, it had a marked preference for Mn²⁺ over Mg²⁺ ions, and it was capable of phosphorylating itself in addition to exogenously added substrates. Although the enzymatic properties of NCP94 are similar to those of NCP98, they differ in that unlike NCP98 (30), NCP94 can use [³²P]GTP as a phosphate donor. As has been reported for NCP98 (30), the only phosphoamino acid detected in vivo-labeled [³²P]-NCP94 was phosphoserine. Although this result did not change by including phosphatase inhibitors in the extraction buffers, it cannot be ruled out that the lack of detection of phosphotyrosine in vivo was due to technical problems. The low ratio of phosphotyrosine to phosphoserine in NCP94 may be of significance in the regulation of NCP94 function in normal cells.

NCP94 was found to be universally expressed in every tissue and cell line that we have examined. In chickens, NCP94 was detectable at the earliest stages of embryonic development. In fact, the highest levels of NCP94 were found in embryonic tissues. The levels of NCP94 decreased after hatching, but they remained relatively high throughout adult life. In our preliminary study of the tissue distribution of NCP94, this protein followed a characteristic pattern that started in the embryo, persisted in the adult, and was also observed across species. The highest levels of NCP94 were found in the gizzard, brain, and spleen. The levels of NCP94 were lowest in muscle and the liver and intermediate in other tissues. The particular tissue distribution exhibited by NCP94 may reflect some aspect of its function in normal tissues, but at present we have no clues as to what this function could be. Because of the ubiquitous distribution of NCP94 it is likely that this protein plays a role in the function of all living cells. The presence of high levels of NCP94 in embryonic tissues, which are engaged in rapid development, might be an indication that NCP94 is related to the proliferative or differentiation capacity of cells. In this connection, it is worth pointing out that two src-related cellular tyrosine kinases are in fact growth factor receptors (10, 49). Since NCP94 seems to be a src-related tyrosine kinase there is a possibility that NCP94 might also be involved in the cellular

**FIG. 7.** Antipeptide antiserum cross-reacts with human NCP94 and NCP92. (A) U937 (lanes 1 and 2) and MOLT 4 (lanes 3 and 4) cells labeled with [³²P]methionine were immunoprecipitated with the indicated antisera. Immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography. Lanes: 1 and 3, antipeptide antiserum; 2 and 4, anti-FST. Autoradiography exposure time was 15 days. (B) Unlabeled cell extracts prepared from U937 (lanes 5 and 6) and MOLT 4 (lanes 7 and 8) cells were immunoprecipitated with the indicated antiserum, assayed for protein kinase activity, and then analyzed by SDS-PAGE and autoradiography. Lanes: 5 and 7, antipeptide antiserum; 6 and 8, anti-FST. Autoradiography exposure time was 1 h.
response to growth or differentiation factors. However, at the present time this is only a matter of speculation, and elucidation of the biological role of NCP94 will require further characterization of this protein.

In addition to the known viral tyrosine kinases and their cellular homologs, other cellular tyrosine kinases have been identified (6, 12, 23, 38, 45, 48, 53, 60), and at least two of them have amino acid sequence homology to the src product (6, 11, 56). This suggests that there may still be more unidentified members of this family. Because of the importance of these proteins in normal cell function and because of their oncogenic potential, it is desirable to identify all of them. The immunological approach described in this paper allowed the identification of a new cellular protein that may belong to the src gene family. Although the gene encoding NCP94 has apparently not been transduced by retroviruses and no transforming counterpart of NCP94 may yet exist, NCP94 may have oncogenic potential.

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LITERATURE CITED


CHARACTERIZATION OF NCP94 TYROSINE KINASE


