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Preferential Expression of the c-*fps* Protein in Chicken Macrophages and Granulocytic Cells

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We have studied the expression of the protein kinase activity of NCP98, the c-*fps* gene product, in several hemopoietic tissues of chickens as a function of the developmental stage of these organs. We found that in bone marrow, spleen, and bursa, maximum NCP98 kinase activity on a per-cell basis correlates with the peak of granulopoiesis in these organs. Furthermore, in a bovine serum albumin density gradient fractionation of bone marrow cells, granulocytic cells appeared to account for most of the NCP98 kinase activity. No correlation was found between the distribution of erythrocytic, lymphocytic, or thrombocytic cells and the distribution of the expression of NCP98 kinase activity. However, NCP98 protein and kinase activity were 10-fold higher in macrophages than in bone marrow. In addition, depletion by complement-mediated lysis of erythrocytic cells in bone marrow did not significantly reduce the total recovery of NCP98 kinase activity. These results argue for the specific expression of the c-*fps* gene product in granulocytic cells and macrophages.

Fujinami sarcoma virus is a replication-defective avian sarcoma virus whose genome contains a unique sequence of ca. 2,700 nucleotides called v-*fps* (10, 13, 19). This virus encodes a transforming protein of 1,182 amino acids, P130, which exhibits tyrosine kinase activity and is phosphorylated in vivo (6, 9).

A cellular sequence, c-*fps*, has been detected in the DNA of uninfected cells of various vertebrate species. In the avian system, this sequence was shown to be expressed in a tissue-dependent manner (20); c-*fps* gene product was identified as a phosphoprotein of 98,000 daltons, NCP98 (15). NCP98 has a tyrosine protein kinase activity and is structurally related to the viral transforming protein P130. The expression of NCP98 kinase activity is tissue specific, with a predominance in bone marrow. Moreover, NCP98 kinase activity is high in avian myeloblastosis virus-transformed myeloid cells but low in avian erythroblastosis virus-transformed erythroid cells (15). These observations suggest that c-*fps* might be differentially expressed in some hemopoietic cells. To identify hemopoietic cells that preferentially express NCP98, we further analyzed NCP98 expression in purified hemopoietic cell populations and in hemopoietic tissues during the development of chickens. We found the highest levels of NCP98 in granulocytic cells and macrophages.

MATERIALS AND METHODS

Preparation of bone marrow cells. Bone marrow cells were collected from tibias and femurs of chicken embryos and chickens by flushing the bone marrow cavity with Dulbecco modified medium (DEM) containing 10% fetal calf serum (FCS) (DEM–10% FCS). The cells were dissociated by several passages through a Pasteur pipette and spun down for 5 min at 300 × *g*. The cells were resuspended in DEM–10% FCS.

Separation of bone marrow cells by density centrifugation. Bone marrow cells were isolated after centrifugation for 30 min at 400 × *g* on Ficoll Paque (Pharmacia Fine Chemicals). The cells at the interface were collected, washed with DEM–10% FCS, and loaded onto a discontinuous bovine serum albumin (BSA) gradient (21 to 31% BSA) as described previously (7). The gradient was centrifuged for 30 min at 1,000 × *g*. The cells at the interfaces between each layer were collected and washed in DEM–10% FCS.

Immunolysis of bone marrow cells with antierythrocyte antisera. The antisera L19 and L16 were kindly provided by J. P. Blanchet (University of Lyon, France). They were raised against 1-day-old and adult chicken erythrocytes, respectively (1). The antisera L16 M6 and L16 M7 were derived from L16 after one and two cycles, respectively, of absorption on nonproducer avian myeloblastosis virus-transformed myeloblasts (clone BM2, given by C. Moscovici).

For the immunolysis reaction, bone marrow cells were first separated by centrifugation through a BSA gradient made of three layers of BSA of density 1.060, 1.065, and 1.068 g/cm^3. The cells retained at the interface between the densities 1.065 and 1.068 g/cm^3 were collected and washed in phosphate-buffered saline. Ten million cells were centrifuged in small test tubes, and the pellets were resuspended in 500 μl of antiserum diluted with DEM–10% FCS. As a control, cells were treated with 500 μl of DEM–10% FCS. The cells were incubated for 30 min on ice; then 50 μl of rabbit complement (Miles Laboratories) was added to each tube. The mixtures were further incubated for 15 min at 37°C. The reaction was then stopped by the addition of 500 μl of ice-cold DEM–10% FCS, and each suspension was loaded onto a cushion of BSA at a density of 1.068 g/cm^3. The tubes were centrifuged for 30 min at 1,000 × *g*. Intact cells were recovered at the surface of the BSA layer, whereas the nuclei of the lysed cells were pelleted at the bottom of the tubes. The cells were then washed with DEM–10% FCS.

In vitro culture of bone marrow cells. Bone marrow cells from 4-week-old chickens were isolated by centrifugation on Ficoll Paque and then on a BSA gradient. Cells of density 1.065 g/cm^3 and less were pooled and seeded into 35-mm tissue culture dishes at a final concentration of 9 × 10^5 in 2

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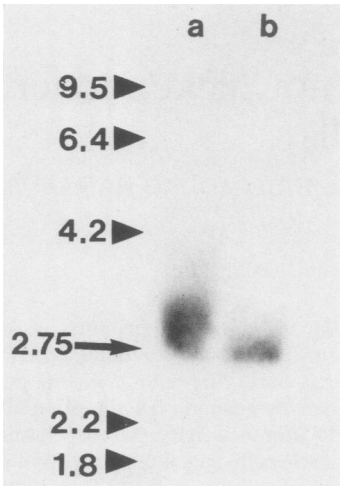

FIG. 1. Identification of the c-*fps* RNA transcript. Polyadenylated RNAs were isolated from total 6-day-old chicken embryos without their yolk sac membranes (lane a) and from 8-day-old chicken bone marrow (lane b). Sizes of the RNAs were estimated from the migration of denatured fragments of *Hin*dIII-digested lambda DNA.

ml of DEM–10% FCS containing 10% normal chicken serum (GIBCO Laboratories) and 20% chicken fibroblast conditioned medium (3). After various times of incubation, cells in suspension were collected. After 96 h of culture, cells adherent to the bottom of the dishes were also recovered separately after trypsinization.

Preparation of peripheral blood macrophages. Blood (10 ml) was collected on sodium citrate from the wing vein of an adult chicken. The cells were centrifuged through Ficoll Paque, and the floating leukocytes were recovered and seeded in a 100-mm tissue culture dish in BT88 medium containing 10% tryptose phosphate broth, 5% calf serum, and 5% chicken serum. Two hours later, the nonadherent cells were removed by several washes of the dish with culture medium. The adherent cells were then grown for at least 10 days before use. The secondary cultures were seeded after trypsinization of the adherent primary macrophages.

Cytology. The cells were centrifuged in a Cytospin cytocentrifuge (Shandon Southern) on glass slides and then stained with Wright Giemsa. The various cell classes were identified by the method of Lucas and Jamroz (14).

Protein biochemistry. Cell lysates were obtained as described previously (15), except that, in the case of whole tissues, a Dounce minihomogenizer was used instead of a mechanical potter. Lysates were normalized for either protein content or cell number. Immunoprecipitation was carried out with anti-FST antiserum, a tumor-bearing rat antiserum against Fujinami sarcoma virus-transformed 3Y1 cells, and analyzed on 8.5% polyacrylamide gels as described previously (15). [^{3}H]leucine labeling was done as follows. Cells (1×10^6 to 2×10^6) were starved for 1 h in leucine-free medium, 500 μCi of [^{3}H]leucine (New England Nuclear Corp., specific activity, 43.2 Ci/mmol) was added, and cells were incubated for another 4 h. Cells were harvested, washed, and lysed as described above. The protein kinase assay was performed as described previously (15).

RNA biochemistry. Total cellular RNA was obtained as described previously (16). Polyadenylated RNA was selected by oligodeoxythymidylate chromatography, fractionated on 1% agarose gels, transferred to a nitrocellulose filter, and hybridized to nick-translated probes as described previously (22).

RESULTS

Identification of c-*fps* RNA. Total RNA was extracted from entire 6-day-old chicken embryos without yolk sac membranes and from bone marrow cells of 8-day-old chickens. In each case, polyadenylated RNA was fractionated on an agarose gel and, after transfer to nitrocellulose paper, was hybridized to a v-*fps*-specific probe. A 2.75-kilobase RNA species could be detected in both preparations (Fig. 1). The diffuse pattern seen on lane a is likely due to overloading of polyadenylated RNA, but the possibility remains that more than one species of c-*fps* mRNA is expressed in 6-day-old embryos. Thus, the c-*fps* is expressed as early as 6 days of embryonic life, and its unique transcript has just enough information to encode the product NCP98 (15).

Separation of bone marrow cells by density centrifugation. Chicken bone marrow contains erythrocytic and granulocytic cells at various differentiation stages; these account for more than 80% of the cells. The minor populations are made of cells that belong mainly to lymphocytic, thrombocytic, and monocytic series and rare macrophages. After centrifugation of bone marrow cells through a discontinuous BSA gradient, the erythrocytic cells were recovered within one peak with a maximum at a density of 1.070 g/cm^3. The granulocytic cells were distributed in two peaks, one broad peak with a maximum at the densities 1.068 to 1.070 g/cm^3 and a small peak at the highest density that resulted from the sedimentation of aggregated granulocytic cells (Fig. 2). NCP98 kinase activity expressed on a per-cell basis showed a biphasic distribution that closely paralleled that of the granulocytic cells in all of the experiments. The other bone marrow cells including lymphocytic, thrombocytic, and monocytic cells were found at a very low frequency only in the fractions of density 1.060 to 1.065 g/cm^3. Since virtually no macrophages were found in the gradient, the profile for NCP98 kinase activity does not correlate with that of

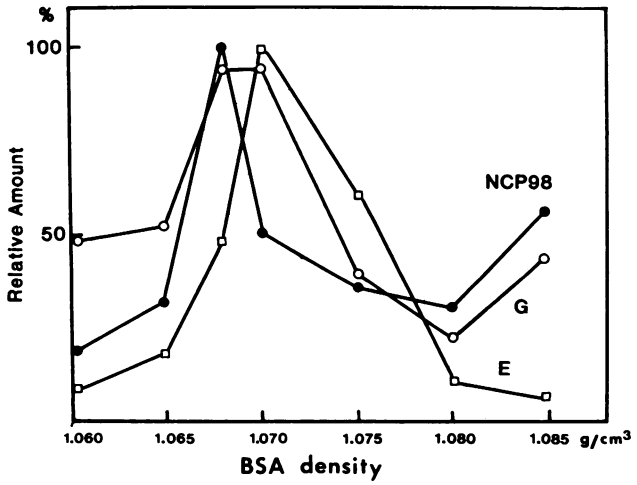

FIG. 2. Separation of bone marrow cells by density gradient centrifugation. The numbers of erythrocytic (□) and granulocytic (○) cells and NCP98 kinase activity (●) were measured in each fraction. The values were normalized and expressed as a percentage of the maximum. Each point represents the mean calculated from two experiments.

macrophages or monocytes but closely parallels that of granulocytic cells. The apparent dissociation between the two profiles observed at a density of 1.070 g/cm^3 may be due to the high predominance of erythroblasts in this fraction, which reduces NCP98 kinase activity expressed on a per-total-cell basis.

Immunolysis of bone marrow cells. Bone marrow cells recovered in the BSA fractions of density 1.068 g/cm^3 were treated with antibodies raised against chicken erythrocyte membrane antigens. The recovery of granulocytic cells and erythrocytic cells and NCP98 kinase activity after treatment with different antisera is shown in Fig. 3. The antiserum L19 removed almost all of the erythrocytic cells, whereas more than 50% of the granulocytic cells were preserved. Recovery of NCP98 kinase activity in intact cells was 50%. Treatment with antiserum L16 M6 gave a similar result, but this serum showed a high cytotoxicity against both erythrocytic and granulocytic cells. It was further absorbed on avian myeloblastosis virus nonproducer-transformed myeloblasts to give the antiserum L16 M7. Treatment with L16 M7 resulted in the killing of 75% of erythrocytic cells without affecting granulocytic cell population. In this case, the recovery of NCP98 kinase activity was more than 90%. The results show that the recovery of NCP98 kinase activity correlates with that of the granulocytic cells but not with that of erythrocytic cells.

In vitro culture of bone marrow cells and macrophages. Low-density bone marrow cells containing granulocytic progenitors (7) were seeded in culture containing chicken embryo fibroblast-conditioned medium as a source of granulocyte-macrophage stimulating factors (3). Within 4 days of culture, the erythrocytic cells totally disappeared since the culture conditions did not promote erythropoiesis (Fig. 4). Conversely, the number of granulocytic cells in suspension increased with time. During the same time, a layer of

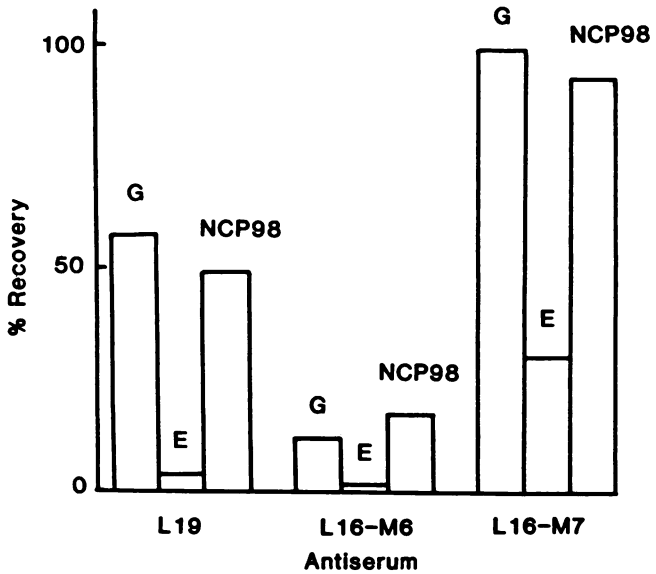

FIG. 3. Selective elimination of erythrocytic cells from bone marrow by in vitro immunolysis with antierythrocytic antisera. Bone marrow cells were incubated with each antiserum in the presence of complement. Control bone marrow was incubated without antiserum or complement. Numbers of granulocytic (G) and erythrocytic (E) cells and NCP98 kinase activity were measured after each treatment. Recovery after treatment by the antisera was calculated by reference to control bone marrow.

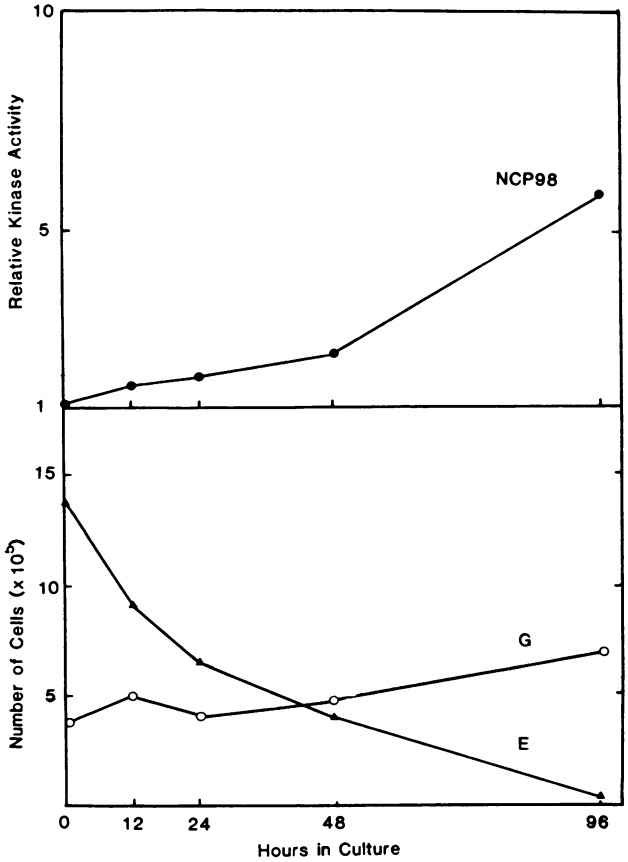

FIG. 4. In vitro culture of bone marrow cells. Fresh bone marrow cells were seeded in liquid culture. After various times, total numbers of floating erythrocytic (▲) and granulocytic (○) cells were measured. At the same time, NCP98 kinase activity was measured on constant numbers of floating cells. Values for NCP98 kinase activity (●) are given by reference to the value measured in bone marrow at the seeding time. At 96 h, the total number of adherent macrophages was 1.35 × 10^6 and the relative value of NCP98 kinase activity in these adherent cells was 5.9.

macrophages developed on the bottom of the culture dishes. At 96 h of culture, few macrophages could be observed floating in the culture medium. Those cells probably detached from the bottom of the culture dishes. However, their number never exceeded 10% of the cells in suspension.

The NCP98 kinase activity expressed on a per-cell basis increased sixfold within the 4 days of culture in cells in suspension. After 48 h in culture, NCP98 kinase activity in floating cells was almost exclusively due to granulocytic cells. If we express the kinase activity on a per-granulocytic-cell basis, then NCP98 kinase activity would show a nearly constant level during the 96 h of culture. This confirms the assignment of NCP98 kinase activity to the granulocytic cells and shows that this activity was not altered by culture conditions. However, NCP98 kinase activity was almost twice as high in the adherent layer (which consists largely of macrophages) as in the granulocytic cells found in suspension.

These results confirm that NCP98 kinase activity in bone marrow is not due to the presence of erythrocytic cells but is associated with some granulocytic cells. Moreover, they show that NCP98 is highly active in macrophages. To confirm this latter result, we developed cultures of macro-

TABLE 1. NCP98 kinase activity in peripheral blood leukocytes and macrophages[a]

Tissues	NCP98 kinase activity	
	Total cpm in NCP98	Relative activity
Peripheral blood leukocytes (old hen)	500	0.2
Whole bone marrow (1-day-old chick)	2,600	1.0
Macrophages in culture	13,350	10.5

[a] The same number of cells was lysed and used for immunoprecipitation in each test. Total radioactivity in ^{32}P-phosphorylated NCP98 was determined by cutting out the gel bands corresponding to NCP98 and counting them.

phages from peripheral blood leukocytes. NCP98 kinase activity was assayed in extracts derived from fresh peripheral blood leukocytes (mainly lymphocytes) and macrophages that developed from those cells after 10 days of culture (Table 1). Results were compared with those in total bone marrow from 1-day-old chickens. NCP98 kinase activity was very low in peripheral blood leukocytes compared with that found in whole bone marrow, whereas a 10-fold increase in activity was observed in macrophages on a per-cell basis. To rule out the possibility that the macrophage cultures were contaminated with some remaining granulocytes, we made secondary cultures from the primary macrophages. The same high NCP98 kinase activity was detected in those cultures (data not shown).

NCP98 kinase activity in developing hemopoietic tissues. To confirm the preferential expression of NCP98 in the granulocytic cells, we measured NCP98 kinase activity in various hemopoietic tissues during embryonic and postembryonic development. In adult chickens, granulocytic cells are mainly produced in bone marrow. However, the embryo, spleen, and bursa also show a transient granulopoiesis (18). In spleen, granulopoiesis remains active during the first week posthatching and declines thereafter, whereas in bursa, it ceases within a few days after hatching. Table 2 shows NCP98 kinase activity expressed in those various tissues at different times of development. Maximum NCP98 activity correlates in time with the peak of granulopoiesis in each organ. However, in spleen and bursa, the late basal NCP98 activity was shown, and this activity cannot be accounted for by the presence of granulocytic cells. At that time, both organs are mainly lymphopoietic in chickens, and granulopoiesis occurs mainly in bone marrow. Furthermore, NCP98 activity in spleen and bursa is higher than in bone marrow. This has not been found previously (15). The reason might be that in the present work, we used milder lysis conditions to better preserve NCP98 kinase activity. It is conceivable that this high basal activity detected in spleen and bursa might reflect the presence of numerous macrophages in these organs. However, we cannot rule out that spleen and bursa contain precursors of lymphoid cells which might express a high level of NCP98 kinase activity. In bone marrow, enhanced granulopoiesis takes place during the last days of embryonic life. The ensuing enrichment of granulocytic cells in bone marrow during that time is paralleled by a marked increase in NCP98 kinase activity. This activity declines rapidly thereafter and reaches a stable level up to 60 days after hatching (data not shown).

Amount of NCP98 in various tissues. To see whether differences in NCP98 kinase activity in macrophages, bone marrow, spleen, and bursa reflected steady-state levels of the protein in these tissues, we labeled in vitro cultures of these tissues with [^{3}H]leucine. Because a steady-state labeling would have required an incubation period during which significant cell death in some cell populations would have occurred, we labeled the cells for only 4 h in vitro with [^{3}H]leucine. Cells were then harvested, washed, and lysed. Immunoprecipitates obtained from the cell extracts were separated by gel electrophoresis. After fluorography, the intensity of NCP98 bands was measured with a scanning spectrophotometer. We found roughly comparable amounts of NCP98 protein (on a per-cell basis) in bone marrow and spleen and ca. 10 times more in macrophages. These results are consistent with those obtained by measuring NCP98 kinase activity in those tissues. We were unable to detect any labeled NCP98 protein in bursa, leaving the possibility that in this tissue the specific kinase activity of NCP98 is much higher than in the other tissues. Conversely, it is also possible that the protein turnover of NCP98 in bursa is much lower or that the specific population of bursal cells expressing NCP98 had died during the incubation of the cells with [^{3}H]leucine.

DISCUSSION

The idea that c-*onc* gene products play a crucial role in cell growth, development, and differentiation has been substantiated by two lines of evidence. First, the products of v-*sis* and v-*erb B* genes have been shown to be closely related or identical to the β-chain of platelet-derived growth factor and epidermal growth factor receptor, respectively (4, 5, 12, 21). Second, the amounts of c-*onc* transcripts and their products have shown quantitative differences among normal tissues or in cell cycle stages (11). However, little attempt has been made thus far to identify specific cells within a given tissue which might be responsible for the expression of a c-*onc* gene. Coll et al. (2) have analyzed the RNAs extracted from chicken bone marrow cells isolated on density gradient and found higher levels of c-*myb* and c-*myc* transcripts in cells banding in low-density fractions (immature cells). Moreover, using the in situ hybridization technique on bone marrow smears, they detected c-*erb* transcripts in a few bone marrow cells that were not cytologically identified. In this study, we found that c-*fps* kinase activity is exclusively detected in myelocytic cells in chickens. Furthermore, we have identified specific hemopoietic cells in bone marrow that express a high level of NCP98 kinase activity. Bone marrow contains various hemopoietic cells and progenitors. Mainly these are granulocytic and erythrocytic cells, as well as minor populations of lymphocytic and thrombocytic cells, monocytes,

TABLE 2. NCP98 kinase activity in different hemopoietic tissues during embryonic and postembryonic development[a]

Stage of development	Tissues			
	Bone marrow	Spleen	Bursa	Thymus
17-day embryo	ND[b]	1.66	ND	ND
19-day embryo	0.97	4.40	1.60	ND
Hatching chicken	1.00	3.10	1.50	ND
5-day chicken	0.26	2.60	3.50	0.30
14-day chicken	0.22	1.70	1.90[c]	ND

[a] The same amount of protein obtained from each of the different tissues was immunoprecipitated. Results are expressed as relative NCP98 kinase activity compared with that measured in hatching bone marrow. Results represent the average of three experiments.

[b] ND, Not done.

[c] Measured on 11-day-old chickens.

and macrophages. Although macrophages are very rare in fresh bone marrow and absent from peripheral blood, they can develop in culture from monocytes or earlier progenitors present in bone marrow or peripheral blood. The highest level of NCP98 kinase activity was found in cultures of chicken macrophages derived from bone marrow or peripheral blood macrophage progenitors. Granulocytic cells also express high NCP98 kinase activity, albeit at comparatively lower levels. However, in bone marrow, granulocytic cells account for most of the observed NCP98 kinase activity. This is based on the fact that removal of adherent cells such as macrophages and monocytes from fresh bone marrow did not decrease total NCP98 kinase activity (data not shown). In addition, the kinase distribution of NCP98 correlated with the distribution of granulocytic cells in a BSA density fractionation from which no significant numbers of monocytes or macrophages were recovered.

Erythrocytic cells were ruled out as candidate cells for high expression of NCP98 kinase activity for several reasons. When bone marrow was depleted of erythrocytic cells by in vitro immunolysis or physical separation, the recovery of NCP98 activity was unaffected and comparable to that in whole bone marrow. Furthermore, in conditions that did not support erythrocytic cell growth but allowed myelocytic cells to proliferate, increasing levels of NCP98 kinase activity were observed with increasing time of culture. No significant expression of NCP98 kinase activity could be detected in lymphocytic cells. On a per-cell basis, NCP98 kinase activity was 50-fold higher in macrophages than in peripheral blood leukocytes from which the macrophage culture was derived. Also, no good correlation was observed, after density fractionation of bone marrow, between the distribution of lymphocytic and thrombocytic cells and that of NCP98 kinase activity. Bone marrow also contains hemopoietic progenitor cells that cannot be identified cytologically since their frequency is too low. These progenitor cells can usually be recovered on BSA gradient in fractions of density lower than 1.065 g/cm^3 (7, 8). Maximum NCP98 kinase activity was detected in higher density fractions, ruling out the possibility that these progenitors might account for the whole NCP98 kinase activity in bone marrow.

However, the direct assignment of NCP98 kinase activity to granulocytic cells in bone marrow was not only from negative inference. We have been unable to dissociate by physical separation the distributions of NCP98 kinase activity and of granulocytic cells. Furthermore, NCP98 kinase activity increased in suspension culture in concert with the specific development of granulocytic cells. However, we are at present unable to determine whether NCP98 kinase activity is expressed differentially at various stages of granulocyte differentiation. Also, we have no data on the relative NCP98 kinase activity in the various classes of granulocytic cells, i.e., heterophiles, eosinophiles, and basophiles (14). Direct assignment of NCP98 expression to specific subsets of granulocytic cells will require the use of monoclonal antibody against NCP98 to allow the cytological detection of NCP98 in isolated cells or hemopoietic clones developed in semisolid cultures.

We also studied NCP98 kinase activity in spleen and bursa. These two organs show some granulopoietic activity in late embryo and newly hatched chickens. Concomitant with granulopoiesis in these organs, we observed an increase in NCP98 kinase activity during that period. However, in both tissues, the specific activity of NCP98 was much higher than in bone marrow, even in older chickens, in which spleen and bursa are mainly lymphoid organs. However, we cannot rule out NCP98 kinase activity expression in some specific subtypes of lymphoid cells not present in peripheral blood. It is more likely that the high residual activity observed in spleen and bursa results from their infiltration by macrophages. Indeed, we observed the development of many macrophages in liquid cultures of spleen cells from 8-day-old chickens (data not shown).

One possible flaw in comparing the kinase activities of NCP98 in various tissues is that its specific activity might differ in each tissue and might be influenced by the differentiation stage of the cells. Therefore, estimation of the total amount of NCP98 in different tissues was also made by isotopic labeling of various tissues in culture with [^{3}H]leucine. We observed that the differential NCP98 kinase activity in bone marrow, macrophages, and spleen closely correlated with the respective amounts of NCP98 in those tissues. However, NCP98 could not be labeled in bursa, suggesting that in this tissue the turnover of the protein is different or that the cells expressing NCP98 were inactivated during the labeling period. Although originally we found an excellent correlation between the level of NCP98 kinase activity and the amount of c-*fps* transcripts in various tissues (15), we consistently observe higher expression of NCP98 kinase activity in bursa and spleen compared with bone marrow, which is in slight disagreement with the RNA data (20). It is possible that RNAs in bursa and spleen are more susceptible to degradation or that translational controls are responsible for these discrepancies.

NCP98 is expressed at a high level in macrophages and granulocytic cells. Thus, we may imagine that this protein is involved in some functions specific to these cells. Both kinds are phagocytic. In mice and humans, granulocytes and macrophages are closely related since they derive from a common progenitor cell (17). Although there is no evidence for a similar relationship between the two lineages in chickens, we might imagine that NCP98 plays some role in the differentiation of these cells. Alternatively, NCP98 may have some relation to growth factors or their receptors specifically required for these hemopoietic cells. Fujinami sarcoma virus has been described essentially as an agent inducing sarcomas in vivo and transforming fibroblasts in vitro. In light of the results presented here, the effects of the v-*fps* protein on macrophages and granulocytic cells, in vivo and in vitro, should be reinvestigated. Such studies could yield pertinent information on the function of NCP98 during the differentiation of those cells.

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