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Species-specific differences in the mitogenic activity of heparin-binding growth factors in the sera of various mammals

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Abstract. Sera from different mammalian species displayed great differences in mitogenic activity, as measured by stimulation of DNA synthesis in BALB/c 3T3 cells (3T3 cells). Among the sera examined, fetal bovine serum was least active, and increasing activity was detected in calf serum, human serum, rat serum and mouse serum, in that order. Rat and mouse sera exhibited extremely high mitogenic activity with 3T3 cells, but when TIG-1 human fetal lung fibroblasts were used for the DNA assay instead, the activity levels of all of the sera were lower, and the differences between them were smaller. To determine the reasons for these differences, the heparin-binding growth factors in each serum were separated on a heparin affinity column. Five peaks of DNA-stimulating activity were obtained. Three of these were found in all sera examined, with both 3T3 cells and TIG-1 cells. Two other peaks were found only with 3T3 cells; one was peculiar to rat and mouse sera, with extremely high activity in the rat, and the other was specific to fetal serum. The dependence of the activity of these peaks on the cells used for the test was confirmed using normal rat lung fibroblasts and immortalized rat kidney cells. These findings adequately explain the species-specific differences in mitogenic activity of whole sera, and the variation in activity depending on the cells used for assay of DNA synthesis.
Key words. Heparin-binding growth factors; sera; mammals; species difference; BALB/c 3T3 cell; TIG-1 cell; DNA synthesis.

Many kinds of factors regulating cell growth, both stimulatory and inhibitory, have been discovered recently, and some have been purified and evaluated genetically[1-4]. Some of these factors have been found in serum, although in small quantities[5-7].

The proliferation of animal cells in tissue culture requires the presence of growth factors from serum. To fulfil this requirement fetal bovine serum (FBS) or calf serum (CS) is usually used, since these are commercially available in large volumes. It is not certain, however, whether FBS or CS is the more efficient in terms of stimulating mitogenic activity, because very few reports have been published of studies in which the sera from a variety of mammals have been compared[7-9]. In this paper, we examined the mitogenic activity of sera from different mammalian species by measuring their effect on DNA synthesis in 3T3 cells and TIG-1 cells. Extremely large species differences were discovered. Since there have been no reports explaining why serum mitogenic activity differs among species, we tried to determine the reason by analyzing growth factors

present in the various sera using several different kinds of cells: 3T3 cells, TIG-1 cells, normal rat lung fibroblasts, and immortalized normal rat kidney cells.

Since heparin affinity chromatography has been shown to be very useful in separating growth factors[10-12], we adopted this method and tried to isolate as many growth factors from serum as possible. Two distinct peaks, representing unknown factors, were found to be responsible for the differences.

Materials and methods

Cells and cultures: BALB/c 3T3 cells were cultured in standard growth medium of the following composition: 90% Dulbecco's modified Eagle's medium (DME) (Nissui Pharmaceutical Co. Ltd., Japan) supplemented with 15 mM N-2-hydroxyethylpiperazine-N′-2-ethansulfonic acid (HEPES), pH 7.3, antibiotics and 10% CS pre-treated at 56 °C for 30 min[13]. TIG-1 human fetal lung fibroblasts were cultured in Eagle's basal medium (BME) (GIBCO) supplemented with 10% FBS and antibiotics[14]. Primary cultures of rat fetal lung fibroblasts (RL cells) were prepared from the lung of a Wistar rat fetus (gestational age, 20 days) using the same method as for TIG-1 cells[14], and maintained in DME (pH 7.3) with 10% FBS and antibiotics. An immortalized rat cell line, NRK 940 F (NRK cells), was purchased from the American Type Culture Collection, Maryland, and cultured in DME (pH 7.3) supplemented with 5% FBS and antibiotics.

All of these cells were maintained in a humidified atmosphere of 5% CO_2 – 95% air at 37 °C, and all were free of mycoplasma contamination[15].

Preparation of sera: Three lots of FBS (Hyclone, Lot Nos. 1111650, 1111570 and 1111620) and CS (Hyclone Lot Nos. 2151682, 2111480 and 2111499) were purchased from Hyclone Laboratories, Inc., Utah. Individual human sera were obtained from three male volunteers (aged 30–49). Individual rat sera were prepared from 12-month-old male Wistar rats as described previously[9]. Individual mouse sera were obtained from 6-month-old male C57BL/6 mice as described for rat serum. All these sera were stored at – 70 °C and used in the experiments without prior heat-treatment.

Heparin-sepharose chromatography: Each sample, after being thawed, was centrifuged at 12,000 rpm for 15 min to remove precipitates. CHAPS (3-[(3-Cholamidopropyl) dimethylammonio] 1-propanesulfonate, Sigma) was added to the supernatant to a final concentration of 0.1% in order to prevent the growth factors in samples from adhering to the equipment[16]. Aliquots (0.5–1.0 ml) were then applied directly at a flow rate of 12.5 ml/h to a heparin-sepharose CL-6B (Pharmacia) column (4 ml bed volume, 1 × 6 cm) equilibrated with 10 mM Tris-HCl-0.1% CHAPS, pH 7.3 (TCB), containing 0.1 M NaCl. Growth factors were eluted at the same rate with 10 ml of the same buffer at first, then with 8 ml of 0.3 M NaCl in TCB, followed by 0.3–2.0 M NaCl (8.5 ml 0.3 M NaCl, 8.5 ml 2.0 M NaCl) linear gradient in TCB. Thirty-drop (0.56 ml) fractions were collected in polypropylene tubes, cooled immediately to 4 °C, and then stored at – 70 °C until DNA synthesis was assayed.

Assay of DNA synthesis: DNA synthesis in the four kinds of cells (3T3 cells, TIG-1 cells, RL cells and NRK cells) was determined as described previously[13] with slight modification. In the case of 3T3 cells, cells were trypsinized and suspended in DME (pH 7.3) containing 3% CS. One ml of the cell suspension (2×10^4 cells) was delivered to each well of a 12-well cluster dish (Corning Multiwell Plate). Five hours later the cells were washed once with DME (pH 7.3), and the medium was replaced by 1 ml serum-deficient medium (0.2% CS). After 24 h, 10–40 µl/well test samples were added to the cultures, and 16 h later the cultures were pulsed for 3 h with [^{3}H]thymidine (5.1×10^{-9} mmole, 14.8 KBq/well, Amersham). This was followed by measurement of the incorporation of radioactivity into trichloroacetic acid-precipitable materials. (See figure legends for details for TIG-1 cells, RL cells and NRK cells). One unit of mitogenic activity for all culture cells was defined as the amount of FBS (Hyclone, Lot No. 1111650) which elicited a half-maximal increase in activity in the same assay procedure[17]. To determine the mitogenic activity of whole serum, the sera were sterilized by passage through a Millipore membrane (MILLEX-GV) with a pore size of 0.22 µm.

Chemicals: Epidermal growth factor (EGF), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were purchased from Collaborative Research, Inc., Lexington, Mass., Takara Shuzo Co. Ltd., Kyoto, Japan and R & D Systems, Inc., Minneapolis, respectively. Acidic fibroblast growth factor (aFGF) was purified using the method described by Gospodarowicz et al.[18] with slight modifications[19].

Results

Mitogenic activity of sera from different species of mammals in BALB/c 3T3 cells and TIG-1 cells. Sera from different mammalian species were examined for their ability to stimulate DNA synthesis in BALB/c 3T3 cells. An extremely large species-specific difference in mitogenic activity was found (fig. 1 A). FBS and CS had similar low levels of activity, whereas human serum was slightly more active. Mouse and rat sera, on the other hand, displayed very high activity; the mouse serum was most active. The ratio of the activity of each of the sera to that of FBS was 1.3 for CS, 2.3 for human serum, 10.4 for rat serum and 19.6 for mouse serum.

To determine whether this pattern is different when cells of different species are used in the DNA-synthesis assay, the effect of the sera was tested in human fetal lung fibroblasts (TIG-1 cells). As in the case of 3T3 cells, FBS and CS exhibited low stimulating activity, while mouse serum had the highest (fig. 1 B). In contrast to the findings in 3T3 cells, human serum displayed higher mito-

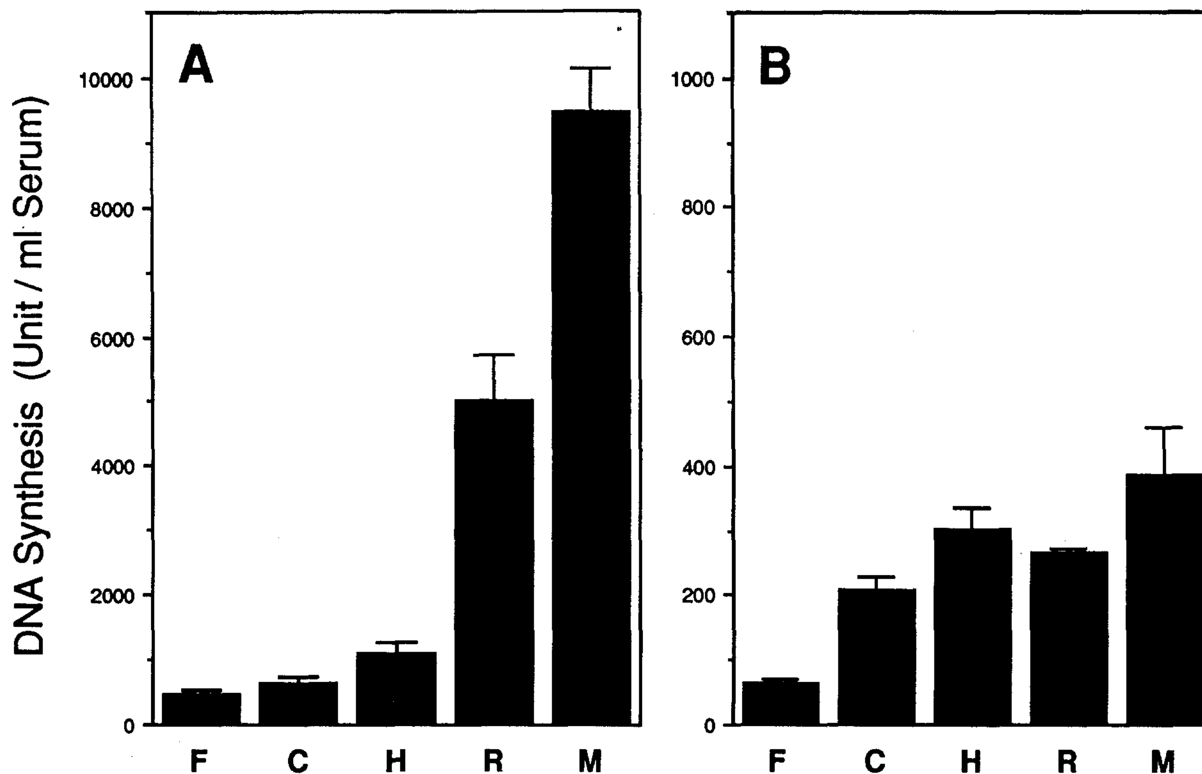

Figure 1. Mitogenic activity of sera from different mammalian species. Sera (0.2–2.0 μl) were assayed for DNA synthesis-promoting activity in BALB/c 3T3 cells (*A*) and TIG-1 cells (*B*) as described in 'Materials and methods'. The columns show the mean value, expressed as units/ml serum, of activity determined in serum from three lots for FBS and CS or three individuals for humans, rats and mice, and vertical bars represent standard deviations. F, fetal bovine serum; C, calf serum; H, human serum; R, rat serum; M, mouse serum.

genic activity than rat serum in TIG-1 cells. On the whole there was little quantitative difference in activity among the sera.

Heparin-sepharose chromatography of sera from different mammalian species. In order to simultaneously separate as many growth factors as possible from serum, a new NaCl-gradient elution system was devised. Our system started with 0.1 M NaCl to wash out unadsorbed substances (0.1 M peak). Then growth factors which had bound to the column weakly were eluted with 0.3 M NaCl (0.3 M peak). Gradient elution with 0.3–2.0 M NaCl followed the 0.3 M NaCl elution, yielding good separation of three peaks at 0.6 M NaCl (0.6 M peak), 1.1 M NaCl (1.1 M peak) and 1.4 M NaCl (1.4 M peak). For comparison, known growth factors were subjected to our chromatography system. As shown in figure 2, EGF was not adsorbed, and appeared in the 0.1 M peak. PDGF, aFGF and bFGF were subsequently eluted with 0.6 M NaCl, 1.2 M NaCl and 1.5 M NaCl, respectively. These results suggested that the 0.1 M peak in our previous experiments contained EGF and the 0.6 M peak PDFG. However, the mitogenic factors present in the other peaks remained unknown.

The typical patterns of DNA-synthesis stimulating activity after heparin-Sepharose chromatography for the five kinds of sera, using 3T3 cells, are shown in figure 2. The elution patterns of FBS and CS were almost identical, except that FBS contained a factor(s) that eluted at 1.4 M NaCl. The elution profile for human serum had three peaks, like CS, but with higher activity than those obtained from FBS and CS. The elution pattern of rat serum was very different from the other three; it had a large, sharp peak of activity which eluted at about 1.1 M NaCl. The NaCl concentration at which this peak was eluted was different from those for known growth factors, so it is not yet known what factor(s) the peak contains. Mouse serum also showed a 1.1 M peak, and the unadsorbed fractions, the 0.1 M peak, had an extremely high mitogenic activity. These results show that sera from different mammalian species contain qualitatively and quantitatively different growth factors, although the difference in total protein content among the sera is small (fig. 2).

Figure 3 shows the elution patterns of stimulating activity in the five sera when TIG-1 cells were used for the DNA assay. They are quite different from those shown in figure 2 for 3T3 cells: the individual profiles of all of the sera exhibit only three peaks, and there is little difference in activity either between peaks or between sera, although the protein content of each peak was almost the same as found with all of the sera using 3T3 cells.

The mitogenic activity of each peak was calculated by summing up the activity values of all fractions the peak contained, and the results are listed in the table. The

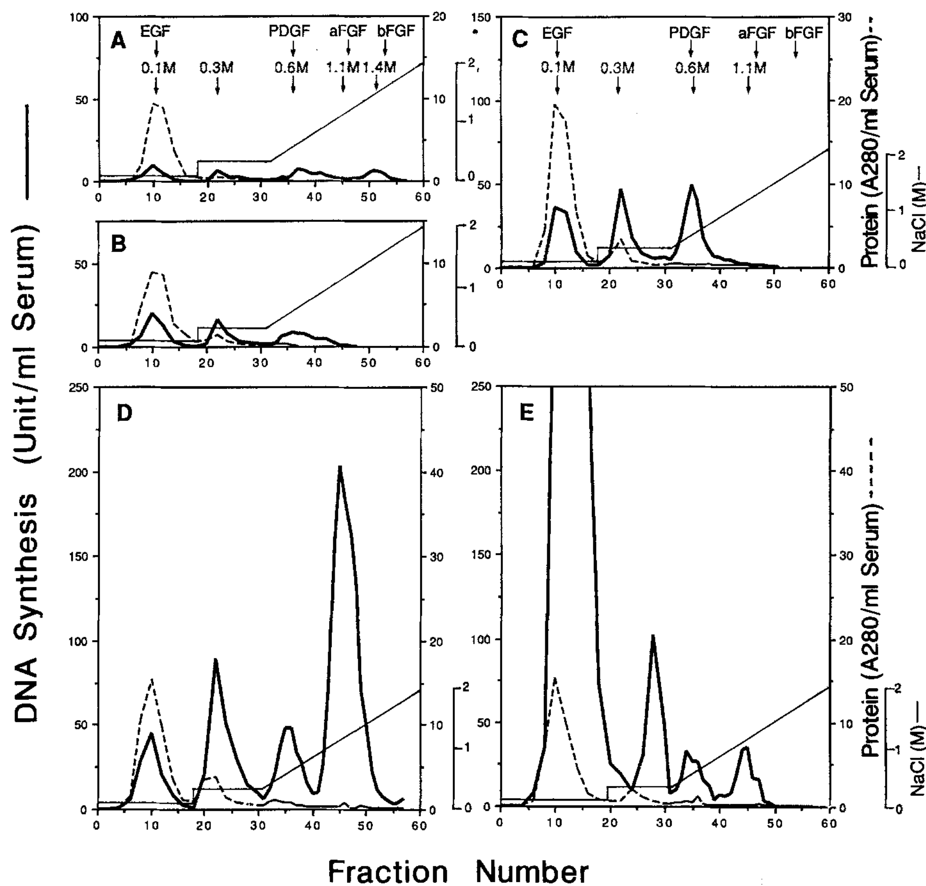

Figure 2. Chromatographic profiles of sera from various mammals determined using BALB/c 3T3 cells. FBS (*A*), CS (*B*), human serum (*C*), rat serum (*D*), and mouse serum (*E*), were applied to a heparin-sepharose CL-6B column and eluted as described in 'Materials and methods'. The thick solid lines represent the DNA synthesis-promoting activity of each fraction from 1 ml serum, and the thin solid line indicates NaCl molar concentration (M). The dotted line indicates absorbance at 280 nm, A_{280}, of each fraction from 1 ml serum. The arrows at the top point to the fraction numbers at which known growth factors were eluted and the arrows underneath point to those at which the peaks were eluted.

Mitogenic activity of each peak separated from the sera of different species in BALB/c 3T3 cells (A) and TIG-1 cells (B)

		0.1 M Peak	0.3 M Peak	0.6 M Peak	1.1 M Peak	1.4 M Peak	Total
Bovine (fetal)	A	27 ± 9	29 ± 10	45 ± 8	ND	18 ± 10	120 ± 30
	B	10	15	31	ND	ND	57
Bovine (calf)	A	85 ± 5	76 ± 11	99 ± 28	ND	ND	260 ± 41
	B	29	32	23	ND	ND	84
Human	A	166 ± 32	204 ± 74	205 ± 54	ND	ND	575 ± 156
	B	43 ± 2	60 ± 12	34 ± 12	ND	ND	137 ± 26
Rat	A	195 ± 21	356 ± 54	252 ± 12	1316 ± 387	ND	2119 ± 375
	B	29 ± 7	59 ± 19	47 ± 15	ND	ND	134 ± 37
Mouse	A	5064 ± 634	352 ± 191	164 ± 9	158 ± 52	ND	5738 ± 577
	B	51	78	32	ND	ND	161

Values represent means ± SD of the activities determined from sera of three individuals, except that values for Bovine B and Mouse B represent the serum of one individual (Unit/ml serum). ND: not detected.

values for the 1.1 M peak of rat serum and the 0.1 M peak of mouse serum were extremely large in the case of 3T3 cells. The very high activities in rat serum and mouse serum shown in figure 1 A were obviously attributable to the 1.1 M peak in the rat and the 0.1 M peak in the mouse, because in all three sera, human, rat and mouse, the sum of the activities of the peaks other than the 0.1 M peak of mouse serum and the 1.1 M peak of rat serum was similar. This conclusion is also supported by the results shown in the table for TIG-1 cells, in which rat and mouse sera have no specific high-activity peaks, and all of the peaks of both sera have values similar to those of human serum. This explains the smallness of the difference in mitogenic activity in human, rat and mouse sera (fig. 1 B). Total activity, which was calculated by summing up the activities of all of the peaks contained in

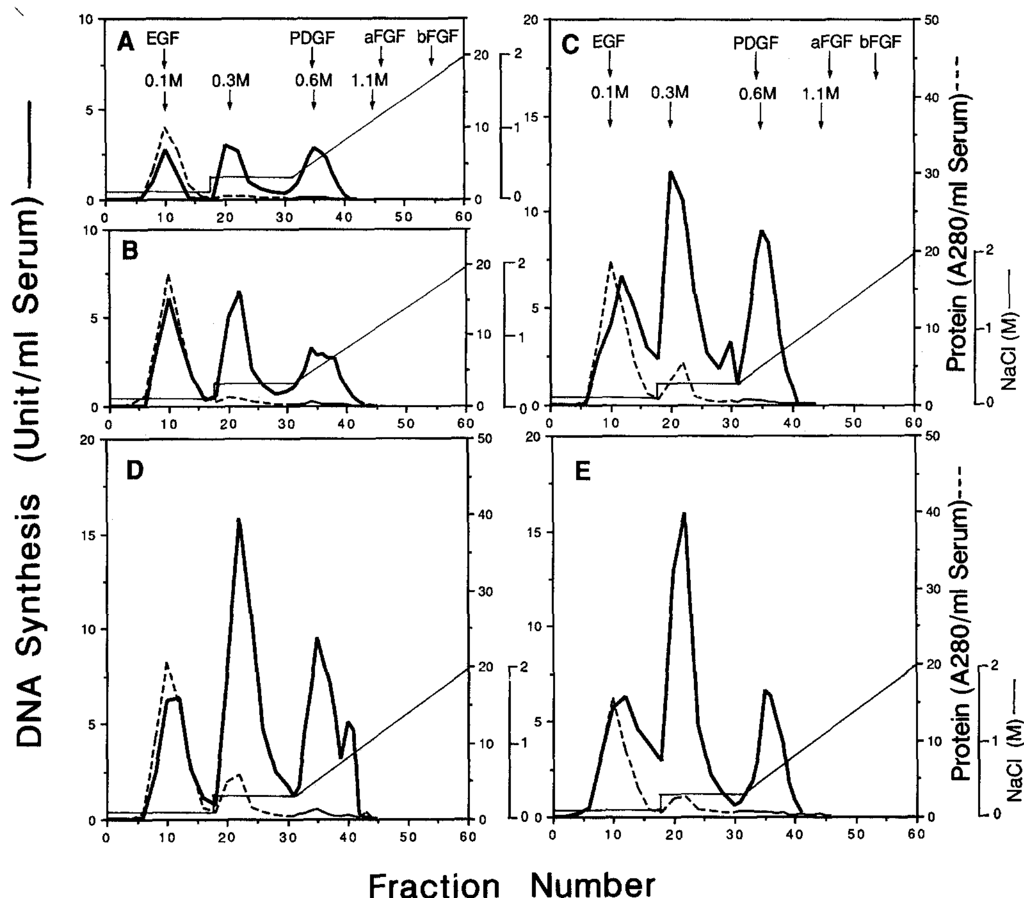

Figure 3. Chromatographic patterns of sera from various mammals obtained using TIG-1 cells. Heparin affinity chromatography was performed in the same way as in figure 2. Each fraction was tested for DNA synthesis-promoting activity as for 3T3 cells, except for the following changes in experimental conditions (see 'Materials and methods'): 1) cell suspension density 4×10^4 cells/ml, 2) incubation time before placement in serum-deficient medium (0.1 % FBS) 48 h, 3) incubation time for starvation 72 h, 4) incubation time for growth factor reaction 20 h, and 5) [^{3}H]thymidine pulse time 4 h.

each of the sera, showed differences similar to those for whole serum both with 3T3 cells and with TIG-1 cells (table and fig. 1).

Cell-dependent differences in the mitogenic activity of growth factors present in the 0.1 M peak and the 1.1 M peak. BALB/c 3T3 cells responded intensely to the growth factor(s) in the 1.1 M peak of rat serum (1.1 M factor) and in the 0.1 M peak of mouse serum (0.1 M factor) (fig. 2), while TIG-1 cells did not respond at all to the 1.1 M factor and responded less to 0.1 M factor than did 3T3 cells (fig. 3). This finding raised the question of whether these factors were consistently more active on cells from the same family of animals, the rodent family, that is on 3T3 cells, which were derived from the mouse. To answer this question fractions from rat and mouse sera were tested for their effects on DNA synthesis using normal rat lung fibroblasts (RL cells) at an early passage (5 population doublings). As shown in figure 4, the profiles were completely different from those for 3T3 cells in figure 2 and for TIG-1 cells in figure 3. No activity of a 1.1 M peak from either serum could be detected at all, and the 0.1 M peak exhibited high activity. This suggested that the 1.1 M factor is not rodent family-specific, but the 0.1 M factor probably is. Since 3T3 cells are non-malignant immortalized cells derived from mouse, an immortalized cell line derived from normal rat kidney, NRK cells, then tested. Figure 5 shows the chromatography profiles of the same sera as in figure 4, when activity was tested on NRK cells. The activity of the 0.1 M peak increased and the 1.1 M peak again exhibited activity in both rat and mouse sera. These results show that these growth factors tend to have a greater effect in immortalized cells than in normal cells, and act in a different manner.

Discussion

Serum is an important factor in initiating and promoting cell growth in cell cultures. The mitogenic activity of sera from several species of mammals was examined by measuring their DNA synthesis-promoting activity in BALB/c 3T3 cells and in TIG-1 cells (fig. 1). In both cases, the order of activity among the sera tested was similar. However, the quantitative differences in activity varied, depending on the cells used for the DNA assay. When 3T3 cells were used, a large difference was found especially between human, rat and mouse sera (fig. 1 A), whereas

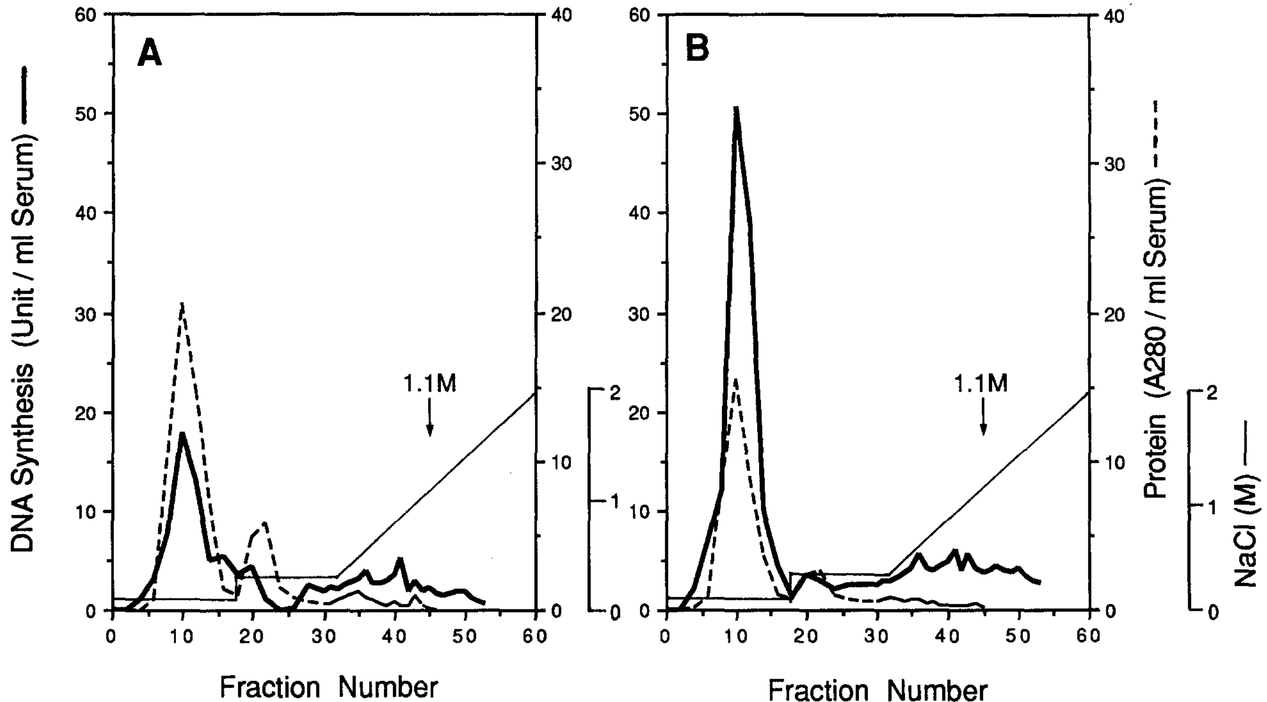

Figure 4. Chromatographic patterns of sera from the rat (*A*) and mouse (*B*) obtained using rat lung fibroblasts. Each chromatographic fraction was tested for DNA synthesis-promoting activity in the same way as in the case of 3T3 cells, except for the following changes in experimental conditions (see 'Materials and methods'): 1) cell suspension density 4×10^4 cells/ml, 2) incubation time before placement in serum-deficient medium (0.2% FBS) 24 h, 3) incubation time for starvation 24 h, 4) incubation time for growth factor reaction 19 h, and 5) [^{3}H]thymidine pulse time 4 h.

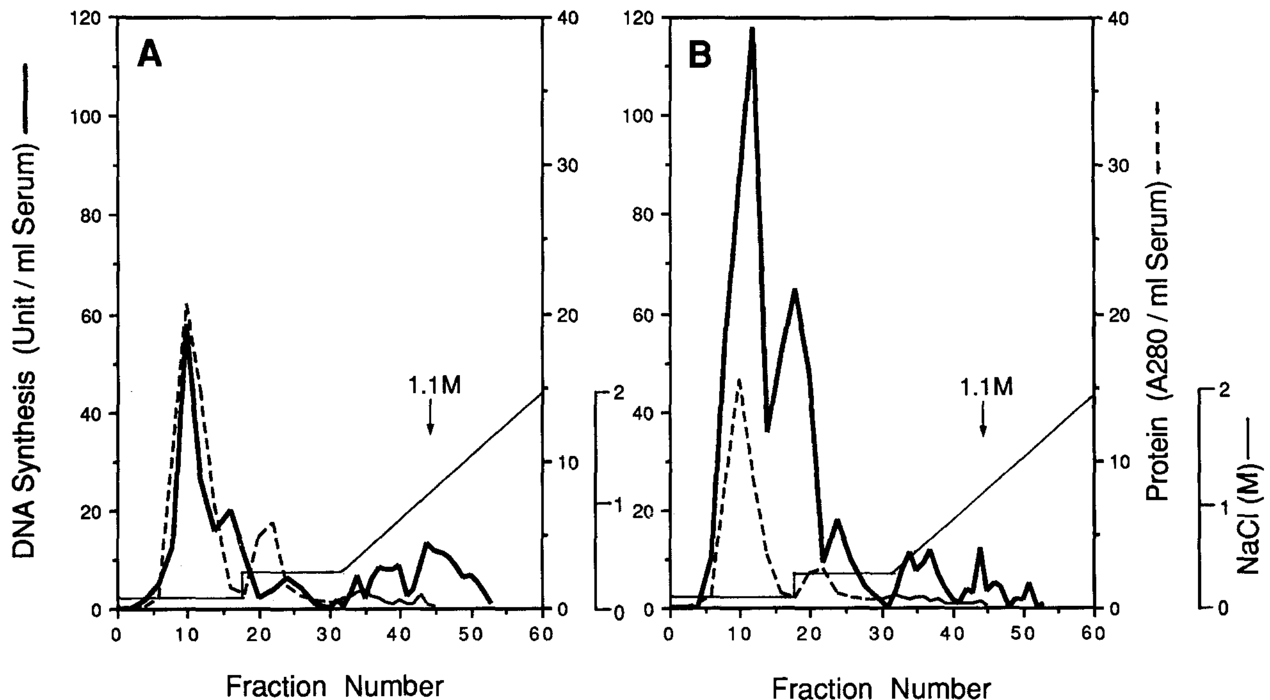

Figure 5. Chromatographic patterns of sera from the rat (*A*) and mouse (*B*) obtained using NRK cells. Each chromatographic fraction was tested for DNA synthesis-promoting activity as for 3T3 cells, except for the following: 1) cell suspension density 4×10^4 cells/ml, 2) incubation time before placement in serum-deficient medium (0.2% FBS) 16 h, 3) incubation time for starvation 27 h, 4) incubation time for growth factor reaction 19 h, and 5) [^{3}H]thymidine pulse time 4 h.

when TIG-1 cells were used only small differences were found between the three (fig. 1 B). FBS showed the least activity on both types of cells, and although this fell short of our expectations, it was not inconsistent with certain earlier reports [7-9]. A similar order of serum mitogenic activity was found using RL cells and NRK cells, and the

quantitative differences among sera were greater in the case of the NRK cells than the RL cells (unpublished data). The fact that this similarity in the order of serum mitogenic activity is found for these four kinds of cells implies that this order is of biological relevance.

To separate the serum growth factors which might be responsible for the variations in serum activity, we used heparin-affinity chromatography. We devised a new elution system, and obtained clear profiles of various growth factors which could be simultaneously separated from serum with high reproducibility (fig. 2). When 3T3 cells were used as responsive cells to assay stimulation of DNA-synthesis, profiles explained the diversity of mitogenic ability among the sera (fig. 1), showing that the factor from rat serum eluted with 1.1 M NaCl, and the 0.1 M factor of mouse serum, were the principal factors responsible for the differences. We are now studying age-related changes in the growth factors in rat serum using the same method.

The 1.1 M peak in rat serum and the 0.1 M peak in mouse serum exhibited extremely high activity in 3T3 cells. Using Swiss 3T3 cells, Bowen-Pope et al. found that rat and mouse sera have relatively high mitogenic activity despite their low PDGF content, suggesting that PDGF is not the major mitogen in these sera [7]. Our findings may be evidence in support of his conclusion.

The factor(s) present in the 1.1 M peak exhibited no activity on DNA-synthesis in human TIG-1 cells, but also had no effect on RL cells (fig. 4), so we can conclude that this factor is not rodent-specific. When immortalized cells derived from the rat, NRK cells, were used, activity of the 1.1 M peak was again observed (fig. 5), which indicated that the 1.1 M factor functions more effectively in immortalized cells than in normal cells. Since it has been reported that cells spontaneously transformed from primary liver cells (non-malignant transformed cells) react to autocrine growth factor(s) from the cells, whereas the primary cells do not [20], we are planning to investigate whether the 1.1 M factor functions differently in primary cells and cells spontaneously transformed in the course of subcultivation.

In order to determine whether the 1.1 M factor is related to already-known heparin-binding growth factors such as PDGF and aFGF, which are eluted from our heparin column at 0.6 M NaCl and 1.2 M NaCl, respectively, we carried out Western blotting analysis of the 1.1 M peak fractions using rabbit polyclonal antibody to human PDGF or to bovine aFGF. PDGF and aFGF were not detected in our fractions (data not shown). Several researchers have recently reported finding new growth factors eluted from heparin columns at around 1.1 M NaCl [21–23]. We are now engaged in purifying the 1.1 M factor, to find out whether our factor and theirs are the same.

The factor(s) present in the 0.1 M peak of mouse serum also have not been finally unidentified. Since EGF is known to be eluted in the 0.1 M peak (fig. 2), and mouse serum has been reported to have relatively high amounts of EGF [24], the peak probably contained EGF. However, there is a possibility that some unknown factor(s) which functions more effectively in immortalized cells than in normal cells is also present, since the ratio of the 0.1 M peak to other peaks in terms of mitogenic activity was much larger for the two immortalized cell-lines than for normal cells.

The total activity of all of the peaks from each serum was about 30–60% of the entire activity of each serum (depending on the species), on both 3T3 cells and TIG-1 cells (fig. 1 and table). The reason for this relatively low recovery is unclear. We suppose that synergism among the factors present in the serum caused much greater activity in the serum as a whole.

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