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Isolation of an Autocrine Growth Factor From Hepatoma HTC-SR Cells

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A growth factor has been isolated from HTC-SR rat hepatoma tissue culture cells which specifically stimulates DNA synthesis and cell proliferation of the HTC cells that produce it. The factor can be isolated from HTC cell conditioned medium or from an HTC cell extract. This autocrine factor has been purified 640-fold from a postmicrosomal supernatant by successive steps, involving ethanol precipitation, heating at 80°C for 10 min, chromatography on a DEAE Bio-Gel A column, and chromatography on a heparin-sepharose affinity column. The major peak of activity eluted from the heparin column migrates as a single band on SDS-PAGE with an apparent M_r of 60,000. The factor is resistant to acid, heat, and neuraminidase but sensitive to trypsin, papain, and protease.

The autocrine nature of the factor is indicated by the finding that several other types of cells do not respond with increased DNA synthesis. Mouse L-cells, BHK cells, Novikoff hepatoma cells, hepatocytes in primary culture, and an epithelial-like rat liver-derived cell line (Clone 9) were tested, and none of the cells could be stimulated. Small amounts of the factor could be extracted from the Clone 9 cells, however. This material had the same physical and purification properties as the factor extracted from HTC cells, but it did not stimulate DNA synthesis in Clone 9 cells, only in HTC cells.

Addition of the factor resulted in an almost immediate stimulation of DNA synthesis in a proliferating HTC cell population. When the factor was added together with [^{3}H]thymidine for 2 h, a significant stimulation of DNA synthesis was observed, provided the addition was made between 18 and 48 h after the cells had been plated. Autoradiographic studies indicated that the factor both accelerates DNA synthesis in cells already making DNA and increases the number of cells entering the S period. The stimulation of DNA synthesis was completely inhibited by 10 mM hydroxyurea, whether the factor was present for 2, 24, or 48 h in the culture. A significant increase in cell number due to addition of the factor was also observed. This accelerated proliferation was detectable only after the cells had been in culture for at least 48 h with the factor present.

Growth of mammalian cells seems to be regulated to a large extent by hormones and growth factors which are present in serum, as well as in other tissue fluids. In addition, there seem to be growth inhibitors in various tissue fluids contributing to the complexity of the regulatory process (O'Keefe and Pledger, 1983; Moses et al., 1985). Some of the growth factors are produced by specific tissues or cells in the organism and might affect a variety of cells throughout the organism. Others seem to be produced by the same cells that are also responsive to them (Kaplan et al., 1982; Sporn and Todaro, 1980; Sporn and Roberts, 1985). Several excellent review articles have discussed many of the various growth factors and their properties (O'Keefe and Pledger, 1983; Moses et al., 1985; Holley, 1974; Antoniades and Owen, 1982; Goustin et al., 1986; Ciba Symposium, 1985).

It has been known for some time that malignant cells seem to require fewer exogenous growth factors for optimal multiplication and growth than their cells of origin (Antoniades and Owen, 1982; Holley, 1975). Primarily by comparing 3T3 cells and virally transformed 3T3 cells in culture, it was shown that different serum concentrations and possibly different serum components were required to maintain and grow these cells (Holley, 1974; Antoniades and Owen, 1982; Holley, 1975). In general, much lower serum concentrations were needed to grow the virally transformed cells as well as other tumor cell lines (Antoniades and Owen, 1982). In

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recent years, an explanation for this self-sufficiency of malignant cells has emerged. It has been suggested that malignant cells can produce growth factors which act on their producer cells via external receptors (Kaplan et al., 1982; Sporn and Todaro, 1980; Burk, 1973; Todaro et al., 1976). This process is now referred to as "autocrine secretion" and provides an explanation for the ability of oncogenes to make cancer cells autonomous of exogenous growth factors (Sporn and Todaro, 1980; Sporn and Roberts, 1985).

Some of the first evidence for endogenous growth factors in tumor cells was provided with murine sarcoma virus transformed cells (Ozanne et al., 1980; DeLarco and Todaro, 1978). It was subsequently shown that these sarcoma growth factors were structurally related to, but distinct from, epidermal growth factor (EGF). The EGF-like substances competed with authentic EGF for EGF receptors (Ozanne et al., 1980; Roberts et al., 1983). Soon, similar growth factors, now collectively referred to as transforming growth factors (TGF), were found in the conditioned medium of various cultured transformed cells (Sporn and Todaro, 1980; Ozanne et al., 1980; Roberts et al., 1980; Todaro et al., 1980). Two molecular species have been identified, a 25 kDa TGF-β and a 5.6 kDa TGF-α. These factors have also been isolated from mouse embryos (Proper et al., 1982). TGF-α has been demonstrated in human placenta (Stromberg et al., 1982) and in mouse embryo (Twardzik et al., 1982), and a platelet-derived TGF-β has been purified (Childs et al., 1982). Human cancer cells produce and release TGF and have functional receptors for the peptides (Todaro et al., 1980; Marquardt and Todaro, 1982; Halper and Moses, 1983; Richmond et al., 1983).

We report the isolation and partial purification of a factor from rat hepatoma HTC-SR cells. This factor is produced by the HTC cells and stimulates DNA synthesis and growth specifically in these cells, thus meeting the criteria for an autocrine factor.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's medium (DME), Ham's F-12 medium, fetal bovine serum (FBS), antibiotic/antimycotic, and trypsin were obtained from GIBCO Laboratories, Grand Island, NY. DEAE Bio-Gel A was obtained from Bio-Rad Laboratories, Richmond, CA, and heparin-sepharose CL-6B from Pharmacia, Inc., Piscataway, NJ. [^{3}H]Thymidine (60–80 Ci/mmol) was purchased from New England Nuclear Research Products, Boston, MA. Other chemicals and enzymes were purchased from Sigma Chemical Company, St. Louis, MO.

Cell lines and culture conditions

HTC-SR rat hepatoma cells (a clonal line from Morris hepatoma 7288C) were a generous gift from Dr. H. Baumann, Department of Tumor Biology, Roswell Park Memorial Institute, Buffalo, NY. Stock cultures were maintained in DME supplemented with 10% FBS and antibiotic/antimycotic in 100-mm dishes at 37°C and a 5% CO_2 atmosphere.

Clone 9 normal rat liver cells were obtained from the American Type Culture Collection, Rockville, MD. Stock cultures were grown in Ham's F-12 medium, 10% FBS and antibiotic/antimycotic at 37°C and a 5% CO_2 atmosphere.

Cell bioassays

Determination of [^{3}H]thymidine incorporation. For the routine cell bioassay, stock cultures were subcultured when cells were not yet confluent and distributed at a cell density of 5×10^4 cells per 35-mm tissue culture dish in 2 ml complete medium required for that particular cell type. The medium was supplemented with 10% FBS unless indicated otherwise. Fractions, previously sterilized by UV irradiation, were added at the time of plating. The cells were left at 37°C and a 5% CO_2 atmosphere for 48 h with no additional medium change. The cells were exposed to 0.4 μCi/dish [^{3}H]thymidine for 2 h prior to harvest, unless otherwise indicated.

For harvesting, the cells were scraped into the medium and the dishes scraped again with 1 ml phosphate buffered saline (PBS). The rinse was combined with the cell suspension and centrifuged for 10 min at 5,000g. The cell pellet was solubilized in 1 ml 1 M NaOH by heating at 80°C for 10 min. DNA was precipitated by 5% trichloroacetic acid and collected on a Celite filter aid covered filter paper and radioactivity determined as previously described (Ove et al., 1971).

In those experiments designed to test the stimulatory activity of the conditioned medium, cells were subcultured as above, but were plated at a cell density of 5×10^4 cells per 35-mm dish in 0.5 ml DME:10% FBS. Conditioned medium was added at the concentrations indicated, and serum-free DME added to a final volume of 2.0 ml per dish. The final FBS concentration was 2.5%. Exposure to [^{3}H]thymidine and harvesting of cells was as described above.

Conditioned medium

To obtain conditioned medium, HTC cells were plated at a density of 10^4 cells/cm^2 in regular growth medium and incubated. After 48 h, the cells were washed twice with serum-free DME and then incubated in serum-free medium. After 24 h, the medium was collected, centrifuged to remove floating cells, and sterilized by filtration. Various concentrations of this medium were tested for stimulatory activity in the [^{3}H]thymidine incorporation assay.

Some conditioned medium was brought to 70% in ethanol, and the resulting precipitate was collected and subjected to the same purification procedures as the HTC cell extract.

Cell growth determination

Cells were subcultured as described above and plated at a cell density of 1.25×10^4 cells per 35-mm culture dish. Fractions (5 μg Bio-Gel F4) were added at the time of plating. On day 3, one-half of the experimental dishes received an additional aliquot of factor. At the times indicated, the medium was removed and the cells collected by mild trypsinization, and the cell number determined with a Coulter counter.

Extraction and purification of autocrine factor

HTC cells were grown in 100-mm dishes in complete medium and harvested for extraction when not confluent 48–72 h after subculturing. The dishes were washed three times with PBS and the cells scraped into extraction buffer, 250 mM sucrose: 10 mM Tris, pH 7.4: 10 mM EDTA according to the method of Hatase et al. (1979). Following homogenization, the cell suspension

was centrifuged at 10,000g for 10 min. The supernatant was subsequently centrifuged for 1 h at 100,000g, and the postmicrosomal supernatant brought to 70% in ethanol. After 2 h in ice, the precipitate was collected by centrifugation at 10,000g for 10 min and dissolved in H_2O. Following a 10-min heating at 80°C, any precipitate that formed was collected by centrifugation at 10,000g for 10 min and discarded. An aliquot of the supernatant was sterilized by UV irradiation for the cell bioassay and the remainder stored at −20°C for further purification.

DEAE Bio-Gel A chromatography

Four milligrams protein of 80°C supernatant were applied to a 10-cm × 1-cm column equilibrated with 5 mM Tris-HCl, pH 7.6. Four-milliliter fractions were collected. The column was washed with 20 ml 5 mM Tris-HCl, pH 7.6, and eluted stepwise with 20 ml each 100 mM NaCl, 250 mM NaCl, and 500 mM NaC1, each in 5 mM Tris, pH 7.6. The peak fractions, as determined by absorbance at 280 nm, were combined, lyophilized, dissolved in H_2O, and dialyzed for 6 h vs H_2O. Fractions were sterilized by UV irradiation before assay.

Heparin-sephrose CL-6B affinity chromatography

Three hundred micrograms DEAE Bio-Gel A fraction 4 was applied to a heparin-sepharose CL-6B column prepared as follows: One gram heparin-sepharose was allowed to swell in 50 ml distilled H_2O and the slurry poured to form a 5-cm × 2-cm column. The column was washed extensively with 200 ml distilled H_2O and equilibrated with 20 ml 5 mM Tris-HCl, pH 7.6 before application of the sample. Following adsorption of the fraction, the column was washed with 20 ml 5 mM Tris-HCl, pH 7.6, and the activity eluted with 100 mM NaCl:5 mM Tris-HCl, pH 7.6. Two-milliliter fractions were collected. The peak fractions as determined by absorbance at 280 nm were combined, lyophilized, dissolved in H_2O, and dialyzed for 18 h vs H_2O. The fractions were sterilized by UV irradiation before assay.

Physicochemical treatments of DEAE Bio-Gel fraction 4

To test for sensitivity to trypsin, an aliquot was incubated at 37°C for 30 min at a concentration of 4 μg enzyme per 1 μg factor. The reaction was stopped by the addition of 2 μg soybean trypsin inhibitor/μg trypsin. Sensitivity to papain and protease was determined by treating the factor at 37°C for 30 min at the same concentration of 4 μg enzyme/μg factor. For neuraminidase treatment, 50 μg factor was exposed to 0.5 units of enzyme. The enzymes were inactivated by heating at 90°C for 30 min. An aliquot was also heated at 90°C for 30 min to determine heat lability. In order to test for acid sensitivity, an aliquot was brought to 1 M HCl and left at room temperature for 1 h, followed by neutralization of the fraction. All fractions were sterilized by UV irradiation before bioassay.

SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed essentially according to Laemmli (1970), using a 1.5 mm thick slab gel. The separating gel was 10% acrylamide and the stacking gel 5%. Electrophoresis was performed at 6 mA for 18 h, using the buffer system of Laemmli (1970). Gels were stained for 2 h in 0.25% Coomassie blue R250: 50% methanol: 10% acetic acid and destained in 50% methanol: 10% acetic acid for 1 h and 5% methanol: 5% acetic acid for 8 h.

TABLE 1. The effect of conditioned medium from HTC cells on [^{3}H]thymidine incorporation in HTC and Clone 9 cells[1]

Conditioned medium (%)	[^{3}H]thymidine incorporation (cpm/10^5 cells)	
	HTC cells	Clone 9 cells
0	1,426 ± 78[2]	964 ± 102
25	1,740 ± 196	853 ± 36
50	2,367 ± 141	951 ± 136
75	3,622 ± 310	817 ± 75

[1]The final serum concentration was 2.5%.
[2]Values are the averages of six determinations ± SD.

RESULTS

It appears that HTC cells release an autocrine factor into the medium. HTC cell conditioned medium stimulated [^{3}H]thymidine incorporation when added to newly plated cells, as shown in Table 1. The stimulation was dose-dependent and was specific for HTC cells. Clone 9 cells, a liver-derived epithelial-like cell line, did not respond with increased [^{3}H]thymidine incorporation to HTC cell conditioned medium. The activity could be extracted from the conditioned medium by ethanol precipitation and has been further purified. The factor could also be extracted from HTC cells by homogenization and ethanol precipitation of a postmicrosomal supernatant, as described in Materials and Methods, and most of our work has been done with factor extracted from the cells directly.

By heating the dissolved ethanol precipitate at 80°C for 10 min, a modest purification could be obtained with the activity in the 80°C supernatant. Addition of this fraction to HTC cells resulted in a several-fold stimulation of [^{3}H]thymidine incorporation. As can be seen in Figure 1, slight stimulation was apparent at 12 h of exposure to the factor with maximal stimulation occurring between 18 and 48 h of exposure. The cells were grown in regular growth medium (DME supplemented with 10% FBS) and the cells were exposed to [^{3}H]thymidine for 2 h prior to harvest, as indicated on the abscissa. It can also be seen from the data in Figure 1 that most of the newly plated cells seem to be in a lag phase for the first 12 to 15 h after plating. At 18 h after plating, many more cells seem to enter the S phase, and it is only at this time, or following 18 h in culture, that the cells respond to the factor, even though the factor was present from the time of plating. We confirmed the [^{3}H]thymidine incorporation studies by autoradiography. Dishes were processed for autoradiography after a 2-h pulse with [^{3}H]thymidine. Between 2 and 12 h after plating, around 17% of the cells were labeled, regardless of whether the factor was present or absent. By 18 h in culture, many more cells had entered the S period, and the effect of the factor became apparent. Without factor, 49% of the cells were labeled at 18 h, and this had increased to 76% when the factor had been present from the time of plating. At 24 and 48 h in culture, about 30% of the cells were labeled with no exogenous factor added, and around 60% were labeled in the presence of the factor. In addition, the presence of the factor resulted in a significant increase in the number of grains per nu-

cleus over the number found when no factor was present. The number of grains per nucleus was not affected by the presence of the factor during the first 12 h after plating.

A dose response curve is shown in Figure 2. Two different fractions were used in these experiments. There is a good dose response between 10 and 100 μg/dish of

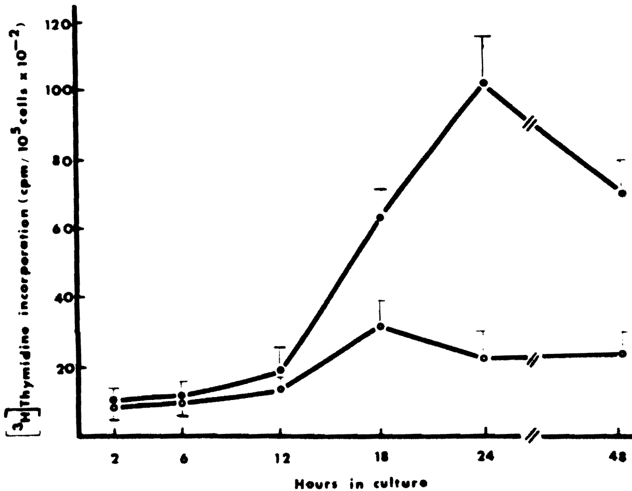

Fig. 1. Incorporation of [^{3}H]thymidine at various times after factor addition. HTC cells were plated at a density of 5 × 10^4 cells per 35-mm dish ± 50 μg/ml of 80°C supernatant, in complete medium supplemented with 10% FBS. For each time point, control dishes and dishes with factor were exposed for 2 h to 0.4 μCi [^{3}H]thymidine per dish. Cells were harvested and [^{3}H]thymidine incorporation was determined at indicated times. ○, Control dishes; ●, addition of 80°C supernatant. Each point represents the average of three determinations, with the SD indicated by the vertical bars.

80°C supernatant and between 1 and 10 μg/dish of fraction 4 from a DEAE Bio-Gel A column, which represents the next step in the purification scheme. The results shown in Figure 2 also indicate that the factor does not stimulate [^{3}H]thymidine incorporation in Clone 9 cells, a rat liver-derived epithelial-like cell line, thus indicating the specificity of this factor.

As already mentioned, the 80°C supernatant was applied to a DEAE Bio-Gel A column, and the activity was recovered in fraction 4. The elution profile is shown in Figure 3. Fractions 1 and 2 had no activity, but fraction 3 had some activity. The specific activity in fraction 3 was between 10 and 20% of the specific activity found in fraction 4.

Purification to apparent homogeneity could be achieved by applying the DEAE Bio-Gel fraction 4 to a heparin-sepharose CL-6B affinity column. As can be seen in the elution profile in Figure 4, most of the material applied passed through the column, but most of the activity associated with a small amount of protein eluted with 100 mM NaCl in absorbance peak II. The material in the tubes under the absorbance peaks was pooled, concentrated, and assayed. Despite a large difference in the absorbance profile between fractions I and II, there was only three times more protein in fraction I than in fraction II, but the specific activity of fraction II was ten times higher than that of fraction I. Application of this material to an SDS polyacrylamide gel and staining with Coomassie blue resulted in a single band migrating to a M_r of about 60,000, as shown in Figure 5. That the biological activity is indeed associated with a molecule of this size was confirmed by passing the heparin-sepharose fraction II through a Sephadex G-200 column. The activity eluted at the same position as a bovine serum albumin standard, and no activity eluted in later

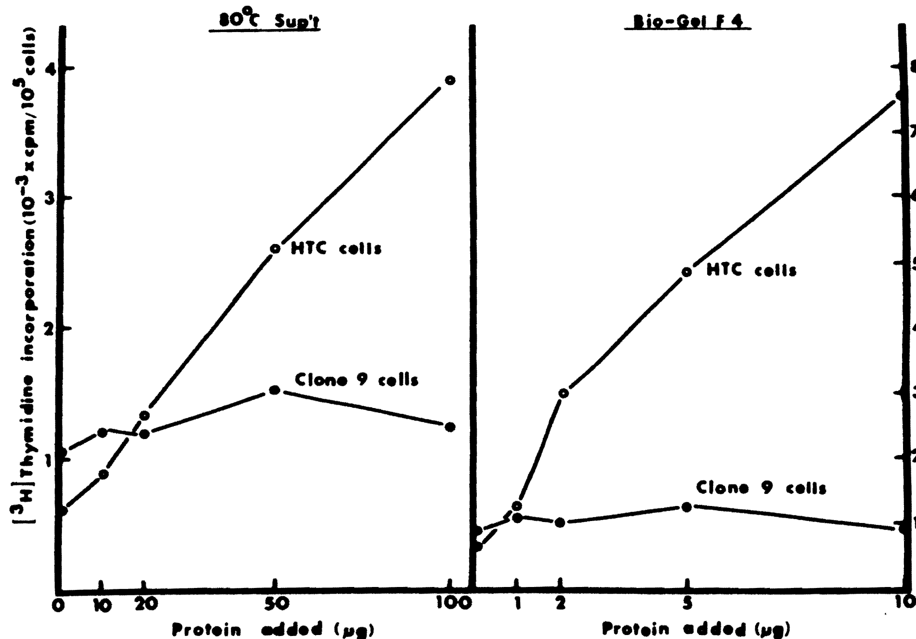

Fig. 2. Dose response of HTC and Clone 9 cells. Assay conditions were as described for Figure 1 but all dishes were exposed to 0.4 μCi [^{3}H]thymidine from 46 to 48 h after plating and addition of growth factor. Both cell types were seeded at the same density of 5 × 10^4 cells per dish. The numbers on the abscissa indicate the protein added per dish. Each point represents the average of at least three determinations, with individual values within 5% of the mean.

fractions, including fractions containing EGF as a standard. Furthermore, commercially obtained growth factors, including EGF, multiplication stimulatory activity (MSA), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), did not stimulate [^{3}H]thymidine incorporation under our assay conditions.

The activities in the various fractions and the increase in specific activity is shown in Table 2. We have so far achieved a purification of 640-fold with the postmicrosomal supernatant as the starting material. A similar purification of the activity found in the conditioned medium can also be achieved. The activity can be precipitated from the medium by the addition of ethanol and elutes from the columns in the same positions as the cell extract activity on further purification. A comparison of the specific activities in the DEAE Bio-Gel fraction 4 and the heparin-sepharose fraction II obtained from cell extracts or from conditioned medium is shown in Table 3.

The autocrine factor also stimulated an increase in cell number, as can be seen from the results in Figure 6. The first significant difference in cell number, when control cultures were compared with cultures in the presence of the factor, was apparent after 72 h in culture. The difference in cell number increased until day 5, at which time the dishes were confluent. In control

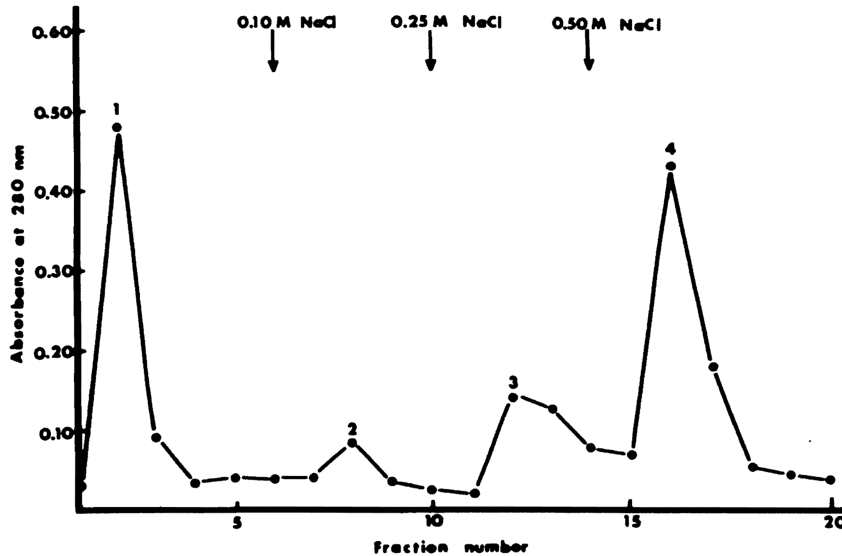

Fig. 3. Elution profile of DEAE Bio-Gel A chromatography. The activity was recovered in fraction 4. Four milligrams protein of 80°C supernatant were applied to a 10 cm × 1 cm column equilibrated with 5 mM Tris - HCl, pH 7.6. Four milliliter fractions were collected. Following adsorption of the sample the column was washed with 20 ml 5 mM Tris - HCl, pH 7.6 and eluted stepwise with 20 ml each: 100 mM NaCl, 250 mM NaCl, and 500 mM NaCl each in 5 mM Tris, pH 7.6.

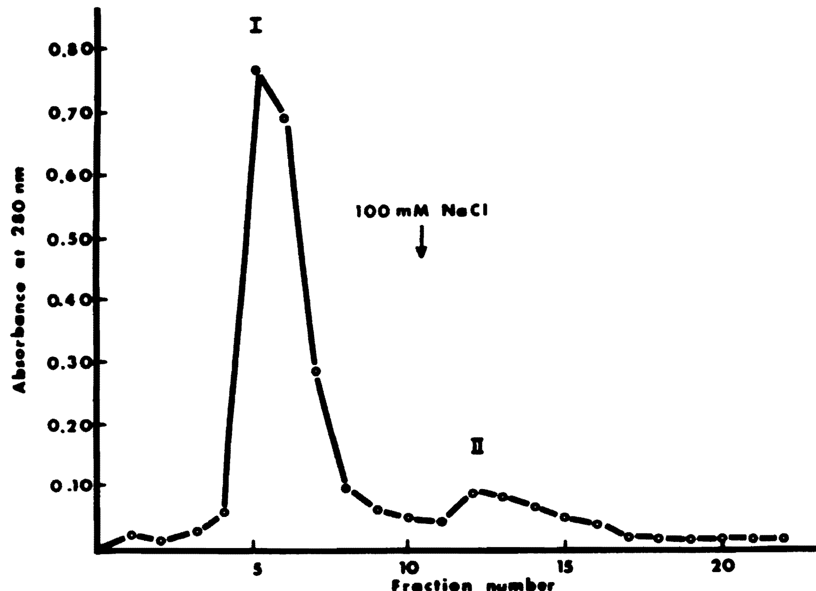

Fig. 4. Elution profile of heparin-sepharose affinity chromatography. The activity eluted in fraction II. Three hundred micrograms DEAE Bio-Gel A fraction 4 were applied to a heparin-sepharose CL-6B column prepared as described in Materials and Methods. Following adsorption of the fraction the column was washed with 20 ml 5 mM Tris - HCl, pH 7.6 and the activity eluted with 100 mM NaCl: 5 mM res-HCl, pH 7.6. Two millimeter fractions were collected.

dishes, there was no increase in cell number during the first 24 h. At 48 h, the cell number per dish had increased from 25,000 to 42,000, and they then doubled every 24 h. The values shown in Figure 6 indicate the difference between control dishes and dishes that contained 5 μg/dish of the Bio-Gel F4. An even greater difference in cell number resulted when dishes were refed after 72 h in culture with the autocrine factor. Some physical properties of the factor are shown in Table 4. The activity was destroyed by treatment with trypsin, papain, or protease, but was not affected by acid treatment, heat treatment, or by neuraminidase. In fact, acid treatment enhanced the activity.

Further evidence for the autocrine nature of the growth factor is provided by the results shown in Table 5. Despite our finding that the factor can also be extracted from Clone 9 cells, these cells did not respond to the factor, whether Clone 9 cells or HTC cells were the source of the extract. HTC cells, on the other hand, responded to factor obtained from Clone 9 cells. Several

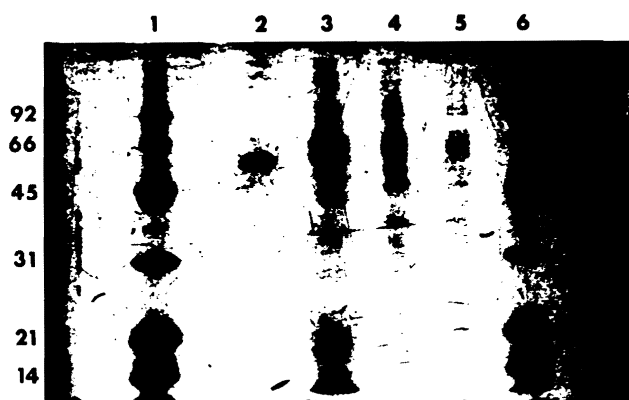

Fig. 5. SDS polyacrylamide gel electrophoresis of the autocrine factor. Lanes 1 and 6, low M_r standards; lane 2, heparin-sepharose FII, 20 μg; lane 3, Bio-Gel F4, 50 μg; lane 4, 80°C supernatant, 30 μg; lane 5, 80°C supernatant, 10 μg.

TABLE 2. Specific activity at various steps during the purification

Fraction	Total protein per fraction (mg)	Protein added (μg/dish)	[^{3}H]Thymidine incorporation (cpm/10^5 cells)	Specific activity (units/mg protein)	Total units[1]
No addition	0	0	1,328 ± 47[2]	—	—
PMS[3]	80	100	3,864 ± 129	39	3,120
70% ethanol precipitate	25	100	5,178 ± 112	52	1,300
80°C supernatant	15	50	5,776 ± 202	116	1,740
DEAE Bio-Gel F4	0.15	5	9,812 ± 248	2,962	294
Heparin-sepharose FII	0.010	0.5	12,480 ± 212	24,960	250

[1]One unit is defined as the incorporation of 1,000 cpm [^{3}H]thymidine per 10^5 cells.
[2]Values are the average of duplicate dishes from at least three separate experiments ± SD when applicable.
[3]Postmicrosomal supernatant.

TABLE 3. Stimulation of [^{3}H]thymidine incorporation by purified fractions from cell extracts and from conditioned medium in HTC cells

Fraction	Amount added (μg)	[^{3}H]Thymidine incorporation (cpm/10^5 cells): Cell extract	Conditioned medium
None	0	1,289 ± 96[1]	
DEAE Bio-Gel F4	5	9,586 ± 720	7,795 ± 325
Heparin-sepharose FII	0.5	12,480 ± 1,490	11,345 ± 860

[1]Values are the average of duplicate dishes for three separate experiments ± SD.

TABLE 4. Physicochemical properties of the factor

Treatment of Bio-Gel F4	Amount added (μg)	[^{3}H]Thymidine incorporation (cpm/10^5 cells)	Activity after treatment (%)
None	5	7,275[1]	100
Trypsin	5	2,309	32
Papain	5	3,771	52
Protease	5	100	1
Neuraminidase	5	7,816	107
1 M HCl	5	12,560	173
90°C 30 min	5	7,451	102

[1]Values are the averages of duplicate dishes for three separate experiments. A control value of 1,250 cpm/10^5 cells with no additions has been subtracted from the experimental values to give these values.

TABLE 5. Growth factor activity with different cell populations[1]

Cell bioassay	Source of growth factor			
	HTC cells (units/mg protein)[2]		Clone 9 cells (units/mg protein)	
	80°C Supernatant	Bio-Gel F4	80°C Supernatant	Bio-Gel F4
HTC	96	1,342	39	520
Clone 9	14	6	13	5
Hepatocytes in primary culture	2	0	1	3
BHK (baby hamster kidney cells)	16	5	11	13
L-cells	6	7	0	8
Novikoff hepatoma	2	8	1	4

[1]The cell bioassay is similar for all cell lines, with each cell line requiring its own medium. BHK cells, DME: 10% tryptose phosphate broth: 5% newborn calf serum; L-cells, DME: 4% newborn calf serum; and Novikoff hepatoma cells, DME: 5% calf serum. The isolation and incubation conditions for hepatocytes in primary culture were as described by Francavilla et al., 1986. The values are the averages of duplicate dishes from at least three separate experiments.
[2]One unit is defined as the incorporation of 1,000 cpm [^{3}H]thymidine per 10^5 cells.

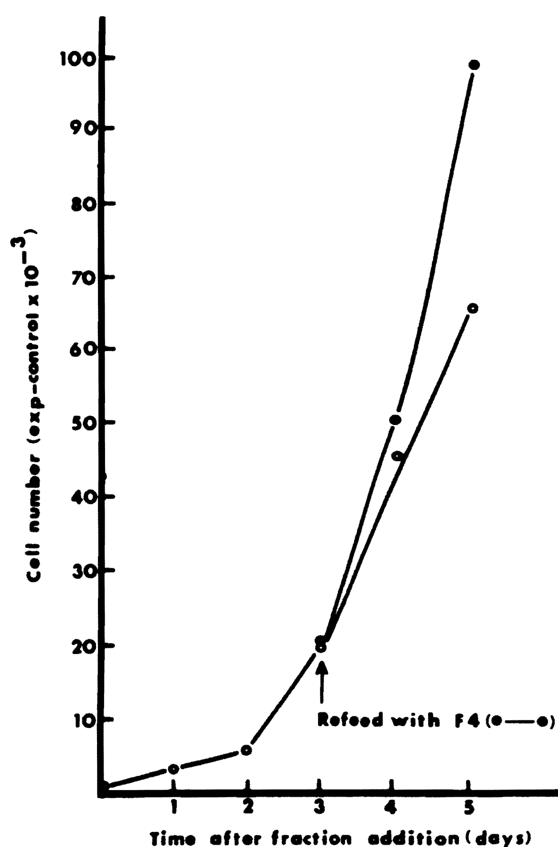

Fig. 6. Stimulation of cell proliferation. HTC cells were subcultured and distributed at a cell density of 1.25 × 10^4 cells per 35-mm dishes in complete medium ± 5 μg Bio-Gel fraction 4. No medium changes were made, and cells were dispersed by trypsin treatment at indicated times and counted with a Coulter counter. On day 3, some of the experimental dishes received an additional 5 μg factor. The values indicated were expressed as cell number in experimental dishes minus cell number in control dishes and were from duplicate determinations for each point.

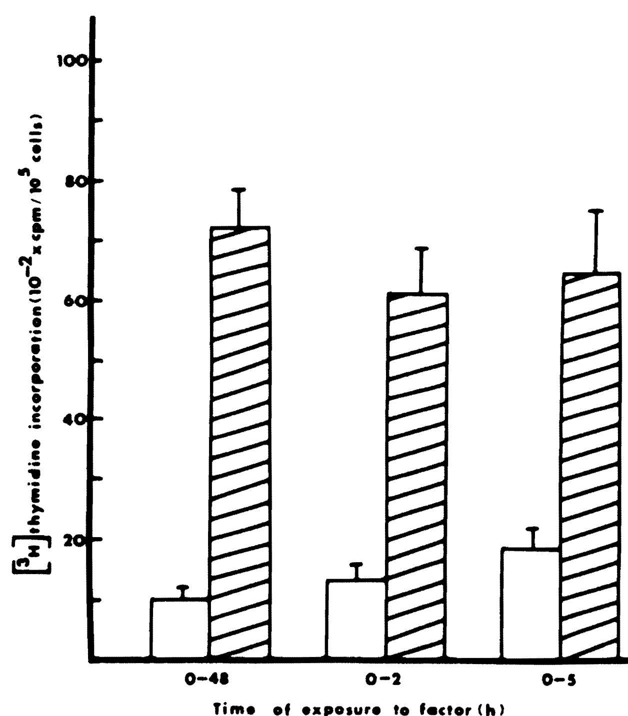

Fig. 7. DNA synthesis after short-term exposure to the factor. Assay conditions were as described in Figure 1. After 2 and 5 h, medium was removed from control and experimental dishes, the dishes were washed once, and complete medium without factor was added to both control and experimental dishes. Cells were exposed to [^{3}H]thymidine from 46–48 h. The values indicated are the averages of five determinations with the SD. Open bar, control dishes; shaded bar, + 50 μg/ml 80°C supernatant.

other cell types, including hepatocytes in primary culture and Novikoff hepatoma cells, did not respond to the factor with increased DNA synthesis.

To gain some insight into the mechanism and the time course of the stimulation, we removed the factor at 2 and 5 h after the cells had been plated and the factor added. As can be seen from the results in Figure 7, this short exposure was sufficient for stimulation of DNA synthesis, which was determined 48 h after plating. We also added the factor at various times after plating and determined DNA synthesis after 48 h in culture. These results are shown in Figure 8. Stimulation of DNA synthesis was similar, whether the factor was added at 0 time or as late as 36 h after plating. Also shown in Figure 8 is the finding that the stimulation was com-

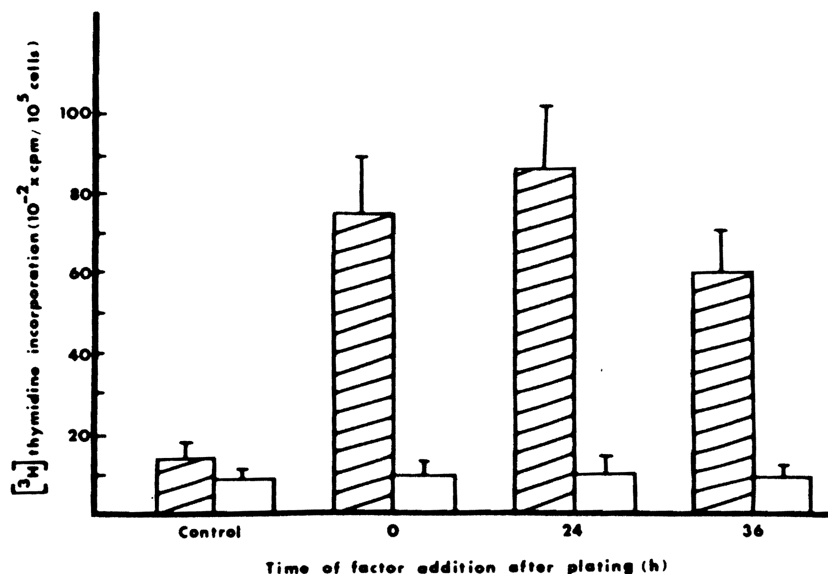

Fig. 8. Inhibition of DNA synthesis by hydroxyurea. Cells were plated as described in Figure 1. Five micrograms of Bio-Gel fraction 4 were added at indicated times. Hydroxyurea (10 mM final concentration) was added at 0 time to controls and at times indicated on the abscissa to the experimental dishes. All dishes received [^{3}H]thymidine between 46–48 h. The values are the averages of five determinations with the SD. Shaded bar, − hydroxyurea; open bar, + hydroxyurea.

TABLE 6. Stimulation of DNA synthesis after brief exposure to factor and its inhibition by hydroxyurea[1]

Time of [^{3}H]thymidine exposure (h)	Hydroxyurea (10 mM)	[^{3}H]Thymidine incorporation (cpm/10^5 cells)	
		No addition	Bio-Gel F4 (5 μg/dish)
0–2	−	1,410 ± 42	1,385 ± 35
10–12	−	1,326 ± 39	1,789 ± 57
22–24	−	1,286 ± 31	8,679 ± 250
22–24	+	637 ± 7	890 ± 9
46–48	−	1,486 ± 47	9,970 ± 231
46–48	+	912 ± 10	967 ± 12

[1]The growth factor and hydroxyurea, where indicated, were present only during the 2 h of exposure of the HTC cells to [^{3}H]thymidine. The values are the averages of five determinations ± SD.

pletely abolished in the presence of 10 mM hydroxyurea, which was added at the time factor additions were made.

Even more striking were the results shown in Table 6. These results indicate that stimulation of DNA synthesis by the factor was very rapid. The presence of the factor, exclusively during the 2-h exposure to [^{3}H]thymidine, was sufficient to elicit a full response, provided this 2-h exposure was done after the cells had established themselves in culture for 18 h. No, or very little, stimulation occurred when cells were exposed for 2 h in the presence of factor during the first 12 h after plating. It seems that the cells in the culture must be actively growing before this rapid stimulation can occur. We have demonstrated that a factor can be extracted from HTC-SR cells, which meets the criteria of an "autocrine factor," since it stimulated DNA synthesis and growth of the same HTC cells that produced it but had no effect on several other cell lines. Our results suggest that proliferation in at least this tumor-derived cell line might be regulated by an autocrine growth factor.

DISCUSSION

The growth factor isolated from HTC-SR cells has in common with other growth and transforming factors its stability to heat and acid treatment, but differs on the basis of its estimated molecular weight from the well-characterized TGF (Todaro et al., 1980; Marquardt and Todaro, 1982; Halper and Moses, 1983; Richmond et al., 1983) and PDGF-like molecules (DeVare et al., 1983; Johnsson et al., 1984; Bowen-Pope et al. 1984; Owen et al., 1984). Our factor seems to be a larger molecule than many of the growth factors characterized. It is unlikely that the activity in our heparin-sepharose FII is due to a small molecule present in nanogram amounts, since upon gel filtration on Sephadex G-200, the activity eluted at the same position as a bovine serum albumin standard, with no activity evident in later fractions. In addition, the activity could not be dissociated into a small molecule by treatment with acid. In the last few years, evidence has been provided for the autocrine hypothesis, first formulated by Sporn and Todaro (1980). Using SSV-transformed cells, Huang et al. (1984) provided evidence in support of a mechanism in SSV-NIH 3T3 and SSV-NRK cells whereby autocrine stimulation of DNA synthesis and cell growth resulted from secretion of the transforming protein p $28^{v\text{-}sis}$, acting through external PDGF/p $28^{v\text{-}sis}$ cell surface receptors. A similar relationship exists between type TGF-α and the EGF receptors (DeLarco and Todaro, 1978; Todaro et al., 1980). We have no direct evidence that our autocrine factor binds to receptors and might be internalized, but our results suggest that a ligand-receptor interaction is important for the action of our growth factor. Our finding that Clone 9 cells produce small amounts of the factor, but do not respond with increased DNA synthesis to their own factor nor to the factor extracted from the

HTC cells, whereas HTC cells respond to both factors, suggests that the HTC cells bind and/or internalize the factor and that Clone 9 cells do not.

Our factor also differs from TGF and PDGF-like molecules on the basis of its specificity. Most growth factors described are active on a variety of different cell lines, but as far as we have determined, our factor seems to be specific for HTC-SR cells. Although we have not investigated a wide variety of different neoplastic cell lines, Novikoff hepatoma cells, which might be expected to have characteristics similar to HTC cells, did not respond to the factor. As yet, we have not attempted to extract a similar factor from Novikoff hepatoma cells. The amount of heparin-sepharose FII needed to show significant stimulation of DNA synthesis is 100 ng/ml. Many of the well-characterized growth factors are active at a lower concentration. It is quite possible that the factor is active at lower concentrations, but this is difficult to detect in our assay system. The HTC cells are proliferating at a fast rate, and they also produce the factor endogenously. It would, therefore, be difficult to detect stimulation by exogenous factor at nanogram amounts.

For most of our assays, we have added the factor when the cells were plated. DNA synthesis was determined 48 h later. However, the factor needs to be present for only a short time to elicit a comparative response, as indicated by the results in Figure 7. Similarly, the factor could be added at any time, up to the time of the 2-h exposure to [^{3}H]thymidine at 46 h after plating and DNA synthesis was stimulated. The finding that between 18 and 48 h in culture, when the cells were actively growing, the factor needed to be present only during the duration of a 2-h exposure, as shown in Table 4, indicates that the factor stimulates DNA synthesis directly. This was also confirmed by our finding that the stimulation due to the factor, whether present for 48 h or for only 2 h, was completely inhibited by 10 mM hydroxyurea. The results with autoradiography suggest that the factor enhances the number of cells entering the S period and also seems to initiate the activation of additional replication sites as the number of grains per nucleus was increased in its presence.

There was, however, no instantaneous response during the first 12 h in culture when cells were exposed to the factor during a 2-h exposure to [^{3}H]thymidine, as shown in Table 4. Before the cells were plated, they were detached from stock culture dishes by trypsin treatment, and it appears that this treatment induces a lag period in the newly seeded cultures. This also seems to account for our finding that the cell population does not double during the first 24 h in culture as was observed at any time after 24 h in culture. On the other hand, the trypsin treatment does not seem to alter the ability of the cells to bind the factor or to internalize it. A 2-h exposure to the factor at the time of plating did not result in an immediate stimulation of DNA synthesis, but the cells were apparently programmed for increased DNA synthesis which could be detected when DNA synthesis was measured after 18 h or later in culture.

The possibility must be considered that the factor might not be a true "growth factor" but a permissive factor required for optimal growth, such as an attachment or spreading factor. This seems unlikely, however, since the HTC cells grow rapidly in the absence of the factors, and the presence of the factor does not seem to shorten the lag period following plating. In addition, the finding that the factor can stimulate DNA synthesis rapidly is inconsistent with the action of spreading or attachment factors. Observation with the light microscope did not indicate any effect of the factor on the morphological appearance of the cells.

At present, we do not know by what mechanism our factor induces DNA synthesis. We assume, that at the cellular level the factor binds to receptors and might be internalized by receptor mediated endocytosis. To provide evidence for such an occurrence will be our next goal. The fact that the factor's action is inhibited by hydroxyurea and the rapidity of the response when the factor is added to actively growing cells suggests that DNA synthesis might be affected directly.

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