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Mitogenic activity from trout embryos

(serum-free cell culture/growth factors/fish cell culture/*Salmo gairdneri*)

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ABSTRACT **An extract of 21-day rainbow trout embryos stimulated growth of several piscine cell lines in the absence of added serum. Established lines from trout (RTG-2 and STE-137), salmon (CHSE-214), carp (EPC), and goldfish (CAR) and early-passage cells initiated from trout embryos grew in serum-free medium containing the embryo extract. In addition the extract was sufficient for maintaining long-term cultures of CHSE-214 cells for several months through a minimum of 20 passages (≈50 population doublings) in the absence of serum. Optimal response was achieved with 100 μg of extract protein per ml, but a significant growth-promoting effect was observed with as little as 2.5 μg/ml. The activity was nondialyzable, protease-sensitive, and stable in 200 mM acetic acid. The level of mitogenic response induced by the extract could not be duplicated with purified mammalian growth factors added individually or in combination, and the extract did not stimulate DNA synthesis in quiescent mouse fibroblasts. These results suggest that trout embryo extract may contain a novel growth-promoting activity for fish cells.**

Advantages of fish over mammalian models in some kinds of studies have led to increasing interest in the use of fish as an alternative experimental system for vertebrate developmental (1–4), neurological (5), and toxicological (6–8) studies. Methods for producing clonal strains of fish make available a virtually unlimited supply of genetically identical individuals (3, 9). Furthermore, unlike mammals, large amounts of synchronously developing fish embryos can be generated for biochemical and genetic studies. In addition fish embryos develop *ex utero*, making it convenient to experiment with embryos at early stages of development, and transfection of fish cells and production of transgenic fish has been accomplished (2, 10). For toxicology studies fish are sensitive indicators of toxicity to environmental carcinogens such as aflatoxin and aromatic hydrocarbons (8, 11, 12), and the relative inexpense of maintaining fish makes it feasible to carry out exposures using a large number of embryonic or juvenile individuals (8).

To coincide with this increased experimental utilization of fish there is a need to develop piscine cell culture models for use in *in vitro* studies complementing *in vivo* work (13). Traditionally, with few exceptions (14, 15), cultured fish cells have been used as a host for the propagation of pathogenic fish viruses, and the techniques involved have been borrowed almost entirely from analogous work with mammalian cells (16, 17). However, considerable potential remains in the broad application to fish cells of the *in vitro* experimental approaches commonly employed with mammalian cells (18, 19). A step toward this goal is an increased understanding of the extracellular factors influencing fish cell proliferation and differentiation in culture. Furthermore, with the increased dependence on farmed fish as a food source, a biochemical appreciation of the factors influencing fish growth would be of substantial practical importance.

Few studies have been directed toward defining the parameters of growth control for fish cells in culture (20, 21). The present study describes a mitogenic activity obtained from trout embryos that was identified by its ability to stimulate the serum-free growth of several established fish cell lines and early-passage cultures.

MATERIALS AND METHODS

Cell Culture. Fish cell lines were grown in a nutrient medium consisting of a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (GIBCO) supplemented with 10 nM sodium selenite, sodium bicarbonate (0.30 mg/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), penicillin (200 international units/ml), streptomycin sulfate (200 μg/ml), and ampicillin (25 μg/ml). Stock cultures were supplemented with 10% fetal bovine serum (FBS) (GIBCO).

For serum-free experiments cells were plated in 6-well (35-mm diameter) tissue culture plates (Falcon) precoated with human fibronectin (10 μg per well). For each experiment assays were conducted in duplicate. The medium was supplemented with bovine insulin (10 μg/ml; Sigma), human transferrin (25 μg/ml; Sigma), and mouse epidermal growth factor (EGF) (50 ng/ml; Upstate Biotechnical, Lake Placid, NY). All cell lines were grown at 20°C and the plates were wrapped in Parafilm to prevent evaporation. Medium was changed every 7 days. The cells were cultured in ambient air. Serum-free cells were passaged with trypsin and trypsin inhibitor (22) and suspensions of trypsinized cells in phosphate-buffered saline were counted with a Coulter particle counter.

Other purified growth factors were obtained commercially: porcine platelet-derived growth factor (PDGF) (R & D Systems, Minneapolis), rat transforming growth factor α (TGF-α) (Peninsula Laboratories), porcine transforming growth factor β (TGF-β) (R & D Systems), bovine acidic fibroblast growth factor (a-FGF) and basic fibroblast growth factor (b-FGF) (R & D Systems), recombinant human insulin-like growth factor I (IGF-I) (Amgen Biologicals), and rat insulin-like growth factor II (IGF-II) (multiplication-stimulating activity; Collaborative Research). Fish serum was prepared from the blood of Shasta strain rainbow trout as follows. After cells were removed by centrifugation, the plasma was allowed to clot at 4°C for 16 hr. The serum was then separated from the clotted protein by centrifugation and sterilized by filtration. Crude trypsin and insoluble trypsin (covalently linked to an agarose matrix) were obtained from Sigma.

Established fish cell lines (23) [carp epithelioma (EPC), goldfish fin (CAR), rainbow trout gonad (RTG-2) and hepa-

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Abbreviations: FBS, fetal bovine serum; EGF, epidermal growth factor; a-FGF, acidic fibroblast growth factor; b-FGF, basic fibroblast growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

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toma (RTH-149), steelhead trout embryo (STE-137), and chinook salmon embryo (CHSE-214)] were a gift from John Fryer (Department of Microbiology, Oregon State University). Early-passage trout embryo cell lines were derived from newly hatched (28-day) Shasta strain rainbow trout and passaged three times in medium supplemented with 10% FBS. Primary cultures were initiated as follows. After the yolk sac was removed, embryos were rinsed three times in culture medium and minced, and the cells were dissociated by incubation for 15 min at room temperature in crude trypsin (0.2% (wt/vol) trypsin/1 mM EDTA in phosphate-buffered saline). Large aggregates were dispersed by pipeting the mixture until a suspension of small cell aggregates was obtained. The aggregates were then collected by centrifugation and plated in 25-cm^2 tissue culture flasks in medium containing 10% FBS. [^{3}H]Thymidine incorporation assays with quiescent mouse 3T3 fibroblasts were performed as described (24).

Preparation of Embryo Extract. Shasta strain rainbow trout embryos (developed at 10°C) were obtained from the Oregon State University Marine Freshwater Biomedical Center and Environmental Health Sciences Center hatchery frozen in liquid nitrogen and were stored at −80°C. To prepare the extract the embryos were thawed and dissected from the egg and groups of ≈100 embryos were pooled and washed twice in culture medium to remove remaining yolk. The washed embryos were then suspended in 2–5 ml of culture medium (without supplementary growth factors) and homogenized three times (15 sec each time) on ice with a Tissuemizer cell homogenizer (Tekmar, Cincinnati). The resulting homogenate was centrifuged for 5 min in an Eppendorf microcentrifuge (15,600 × *g*) and the supernatant was collected. The supernatant was diluted 1:1 with culture medium and filter-sterilized through a 0.4-μm prefilter followed by a 0.2-μm filter. Protein concentrations were determined by the method of Bradford (25).

In experiments in which dialyzed extract was tested, the unconcentrated extract was filter-sterilized and assayed directly. The extract was stable for several weeks at 4°C and several months at −80°C. Extracts were prepared in the same way from newly hatched (28-day) embryos following removal of the yolk sac. Unfertilized eggs were disrupted and their contents were collected and processed as described above.

RESULTS

Growth-Promoting Activity of Trout Embryo Extract. In initial attempts to formulate a serum-free medium for salmon embryo cells analogous to previous work with rodent embryo cells (26, 27), we were unsuccessful in formulating a combination of medium supplements from known hormones, attachment proteins, binding proteins, and nutritional factors that would allow sustained growth of CHSE-214 chinook salmon embryo cells. These results led us to explore other potential sources of mitogenic activity for salmonid cells *in vitro*.

We found trout embryo extract to be strongly mitogenic (Fig. 1). The growth-promoting effect of trout embryo extract on CHSE-214 cells in serum-free medium supplemented with insulin, transferrin, and EGF could be detected with as little as 2.5 μg of extract protein per ml. Optimal growth response was achieved with 100 μg/ml, resulting in a 6-fold increase in cell number over the control after 14 days (Fig. 2). This is a conservative estimate of cell number because the CHSE-214 cells were difficult to dissociate to single cells after they had formed dense aggregates in the presence of the embryo extract. The effect of the extract is more apparent from the photomicrographs shown in Fig. 3.

Concentrations of extract protein >100 μg/ml did not stimulate cell growth to any greater degree, and in some preparations toxicity was apparent at this concentration. For most of the experiments described 20 μg/ml was used. This concentration produced a significant mitogenic effect but was well below the toxic level for all extract preparations tested. The embryo extract allowed long-term maintenance of serum-free cultures of CHSE-214 cells. These cells were grown continuously through 20 passages and ≈50 population doublings (10 months) in the presence of extract at 40 μg/ml.

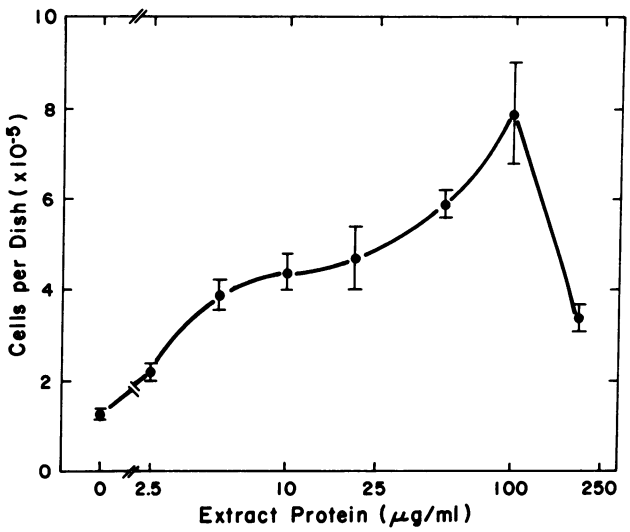

FIG. 1. Stimulation of CHSE-214 cell growth by trout embryo extract. Cells were plated at 10^5 per well in 6-well (35-mm diameter) tissue culture plates as described in *Materials and Methods*. A suspension of trypsinized cells in phosphate-buffered saline was counted on day 10. Bars denote standard deviations.

The embryo extract was mitogenic for established cell lines from carp (*Cyprinus carpio*), trout (*Salmo gairdneri*), and goldfish (*Carassius auratus*) but was not mitogenic when assayed on trout hepatoma cells (RTH-149). RTH-149 and EPC cells were capable of proliferation in serum-free medium supplemented with insulin, transferrin, and EGF, although growth of the cells under these conditions was less than that observed in medium supplemented with 10% FBS. The extract (20 μg/ml) also doubled the growth rate of early-passage cells initiated from rainbow trout embryos (Table 1).

Characteristics of the Growth-Promoting Activity. The mitogenic activity was present in extracts from both 21-day and 28-day embryos; the activity in unfertilized trout eggs was about 30% of that in extracts from 21-day embryos (Table 2).

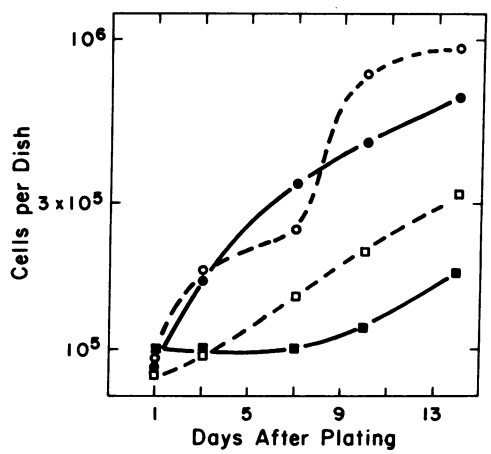

FIG. 2. Time course of CHSE-214 cell growth in the absence (■) or the presence of trout embryo extract protein at 2.5 μg/ml (□), 20 μg/ml (●), or 100 μg/ml (○). Cells were plated as described in Fig. 1 and were counted on the days indicated.

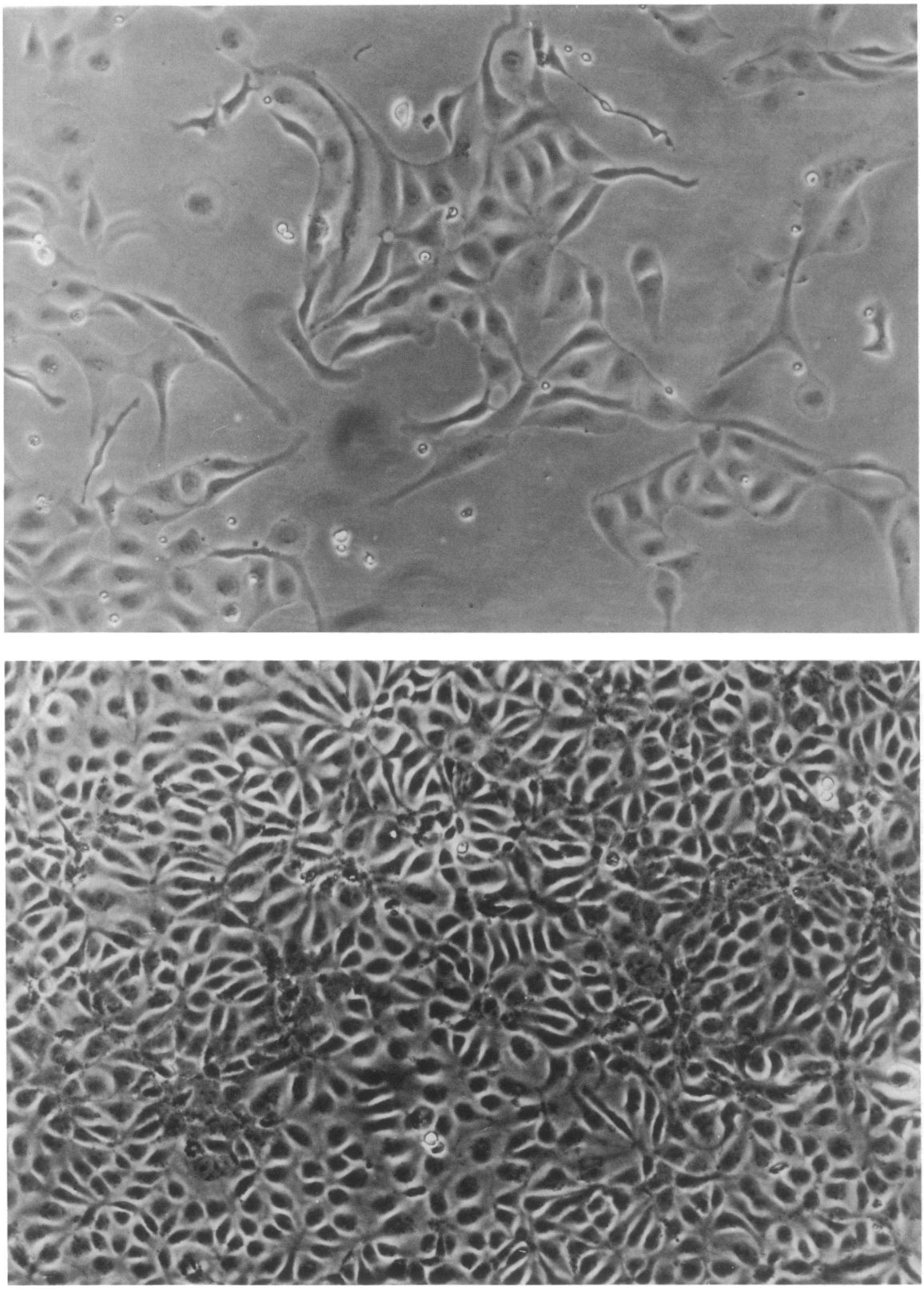

FIG. 3. Photomicrographs of CHSE-214 cells grown 14 days in the absence (*Upper*) or presence (*Lower*) of trout embryo extract at 20 μg/ml. Cells were plated as described in Fig. 1. (× 90.)

The activity was nondialyzable (M_r 3500 cutoff membrane) against either 10 mM Hepes, pH 7.4/130 mM NaCl or 200 mM acetic acid. Proteolysis (24 hr at 37°C in the presence of 20 units of insoluble trypsin) or boiling (100°C for 5 min) reduced the activity by 48% and 35%, respectively, whereas treatment with 1 mM dithiothreitol caused no decrease in activity.

The activity was stable to incubation at 60°C and was not removed by passage through a heparin-agarose column. When embryo extract was chromatographed on a phenyl-Sepharose column (loaded in 3 M NaCl, eluted in 20 mM sodium phosphate, pH 7.0) followed by chromatography on a Sephadex G-50 column in 0.2 M acetic acid, activity was

Table 1. Mitogenic activity of trout embryo extract assayed on fish cell lines

Cell line	Cells per plate, no. × 10^{-5}	
	No extract	Extract at 20 μg/ml
CAR	0.57 ± 0.01	1.85 ± 0.12
EPC	6.29 ± 0.50	19.80 ± 0.01
RTG-2	0.69 ± 0.02	1.50 ± 0.04
RTH-149	2.32 ± 0.23	2.63 ± 0.12
STE-137	0.78 ± 0.00	2.16 ± 0.21
Early-passage trout	1.30 ± 0.15	2.95 ± 0.50

Cells were plated at 10^5 per well and grown for 14 days with or without trout embryo extract (20 μg/ml). Data shown are the average cell count of duplicate plates and the standard deviation. The cell lines tested are from goldfish fin (CAR), carp epithelioma (EPC), rainbow trout hepatoma (RTH-149), rainbow trout gonad (RTG-2), steelhead trout embryo (STE-137), and early-passage trout embryo cells initiated from rainbow trout embryos.

divided equally between unretarded material and a peak of M_r ≈10,000.

The embryo extract was ≈3 times more effective at stimulating CHSE-214 cell growth than FBS at 20 μg/ml (Table 2). However, extract at 20 μg/ml was less than half as effective as 10% FBS (≈5 mg of protein per ml), the concentration of serum routinely used to grow these cells. Trout serum was also mitogenic at 20 μg/ml; however, at a concentration of 10% the fish serum was toxic. The toxic effect of 10% fish serum has also been reported by others (16).

Purified peptide growth factors at optimal concentrations for growth effects did not mimic the effect of trout embryo extract on CHSE-214 cell growth (Fig. 4). Optimal concentration of each growth factor was determined by dose–response experiments. TGF-β, IGF-I, and IGF-II were moderately mitogenic in this system but did not produce effects comparable to trout embryo extract, even when added at optimal concentrations in combinations of two or more (data not shown). Conversely, the embryo extract (50 μg/ml) did not stimulate DNA synthesis in quiescent mouse 3T3 fibroblasts (Fig. 5). Results identical to those of Fig. 5 were obtained using embryo extract concentrations from 1 to 100 μg/ml.

Table 2. Stability of embryo extract activity and comparison to serum activity

Addition	Cells per plate, no. × 10^{-5}
Experiment 1	
No extract	1.49 ± 0.08
21-day embryo extract	6.70 ± 0.15
28-day embryo extract	5.54 ± 0.67
Unfertilized egg extract	3.02 ± 0.00
21-day embryo extract dialyzed against 10 mM Hepes, pH 7.4/130 mM NaCl	5.78 ± 1.49
21-day embryo extract dialyzed against 200 mM acetic acid	5.27 ± 0.59
Experiment 2	
No extract or serum	2.70 ± 0.25
21-day embryo extract	10.90 ± 0.09
FBS, 20 μg/ml	3.75 ± 0.50
FBS, 10%	24.30 ± 0.50
Trout serum, 20 μg/ml	14.50 ± 1.08
Trout serum, 10%	No live cells

CHSE-214 cells were plated at 10^5 per well and counts were taken after 14 days. Data shown are the average cell count of duplicate plates and the standard deviation. Embryo extract protein was assayed at 20 μg/ml.

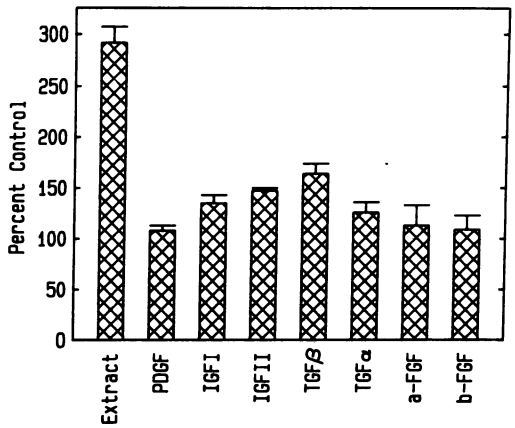

FIG. 4. Mitogenic activity of mammalian growth factors on CHSE-214 cells. Cells were plated as in Fig. 1 and cultured in medium containing insulin, transferrin, EGF, and each growth factor as indicated. Data shown are for the following concentrations: embryo extract, 20 μg/ml; PDGF, 10 ng/ml; IGF-I, 100 ng/ml; IGF-II, 1.0 μg/ml; TGF-β, 10 ng/ml; TGF-α, 50 ng/ml; a-FGF, 10 ng/ml; b-FGF, 10 ng/ml. Cells were counted on day 14 and the results are presented as percentages of the control (cells grown in medium with insulin, transferrin, and EGF only).

DISCUSSION

This study demonstrates mitogenic activity of rainbow trout embryo extract for piscine cell lines from several species. The activity associated with trout embryo extract was nondialyzable and stable in acetic acid and dithiothreitol. Preliminary evidence indicates that a major portion of the extract-associated activity behaves as a molecule of $M_r \leq$ 10,000 upon Sephadex G-50 gel filtration chromatography in 0.2 M acetic acid and the remaining activity is not retarded by the column. Mitogenicity was partially destroyed by protease treatment or boiling; multiple factors with different sensitivities to those treatments may be present.

The level of activity found in the embryo extract could not be duplicated by adding purified mammalian growth factors individually or in combination, and the embryo extract could

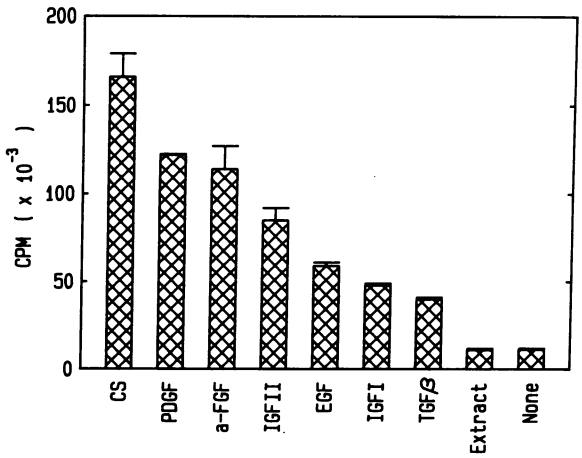

FIG. 5. Stimulation of DNA synthesis in quiescent mouse 3T3 cells. BALB/c 3T3 cells were plated in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 10% calf serum (CS) at 2 × 10^5 cells per 35-mm-diameter tissue culture dish and allowed to form a confluent monolayer. [^{3}H]Thymidine incorporation (4-hr pulse; 4 μCi/ml; 1 μCi = 37 kBq), as described (19), was measured 20 hr after the addition of individual growth factors or trout embryo extract. The following concentrations of growth factors were assayed: PDGF, 10 ng/ml; a-FGF, 10 ng/ml; IGF-II, 1 μg/ml; EGF, 20 ng/ml; IGF-I, 100 ng/ml; TGF-β, 10 ng/ml; dialyzed trout embryo extract, 50 μg/ml.

not mimic the stimulatory effect of these growth factors on DNA synthesis in quiescent mouse fibroblasts. If the extract-associated activity is a peptide growth factor (particularly the activity with $M_r \leq 10,000$), it may be a fish-specific version of a known mammalian growth factor or a factor not previously described. In this context it should be noted that mammalian growth factors frequently exhibit activity on nonmammalian species (28–32), suggesting that the trout embryo activity may be novel.

The results reported here represent a first step in understanding the extracellular conditions regulating growth of piscine cells. Defining these biochemical parameters is a prerequisite for *in vitro* work utilizing fish cells for toxicological and developmental studies as well as for *in vivo* work directed toward maximizing fish growth. Further work is required to determine the biochemical nature of the embryo extract activity and its role in growth control in the developing and adult organism.

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