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# Separation and microanalysis of growth factors by Phast system gel electrophoresis and by DNA synthesis in cell culture

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ABSTRACT

A simple, micro-scale method was established for the characterization of growth factors at picogram levels using Phast system gel electrophoresis followed by monitoring the mitogenic activity by DNA synthesis in cell culture instead of staining methods. The separations and bioassays were carried out with a procedure involving Phast polyacrylamide gel electrophoresis or isoelectric focusing, gel slicing along the template, elution of growth factors through Transwell membranes and measurement of [$^3$H]thymidine incorporation into DNA of normal rat kidney (NRK) fibroblasts. Transwell cell culture chamber inserts separated sliced gel pieces from culture cells and also permitted the direct elution of growth factors into the culture medium. The lower limit of sensitivity for human epidermal growth factor (hEGF) and transforming growth factor type alpha (TGF-α) were about 50 and 200 pg, respectively. At these concentrations, they were not detectable by the current most sensitive silver staining technique. Iodinated hEGF and TGF-a were also used to demonstrate the feasibility of determining the isoelectric point and molecular weight of peptides at picogram levels. This method is reliable, reproducible and can improve current methods for the characterization of growth factors.

## INTRODUCTION

**Polypeptide growth factors and growth inhibitors are regulatory agents that act to control cell proliferation and differentiation *in vitro* and *in vivo* [1–5]. The isolation of growth factors in homogeneous form, such as epidermal growth factor (EGF), transforming growth factor type alpha (TGF-α), fibroblast growth factor (FGF) and**

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platelet-derived growth factor (PDGF), made structural and functional characterization possible and allowed studies of the molecular mechanisms of their actions and genetic analysis [6–12]. However, many biologically important polypeptide growth factors or inhibitors remain to be isolated and characterized.

During the isolation process for peptide growth regulators, the identification of any specific factor in a complex mixture of growth factors is difficult. Moreover, only a small amount of the purified peptide factors will be available at the final stage of purification for identification and characterization. In addition, other complicated problems are that at very low concentrations, in the nanogram range, peptide factors are easily washed away from the gel matrix by the standard staining procedure after polyacrylamide gel electrophoresis (PAGE), and some growth regulators cannot be detected with Coomassie blue or silver nitrate, even if retained in the gel matrix in sufficient amounts.

To circumvent these problems, a simple microanalytical method is desirable. In this paper, we describe an effective method combining Phast gel electrophoresis and the Transwell cell culture system to measure growth factor mitogenic activity. This technique does not require staining procedures and provides a significant improvement over current identification methods.

## EXPERIMENTAL

### *Materials*

Phast gel, silver staining kit and standard proteins for measuring p*I* and molecular weight were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Transwell cell culture chamber inserts were obtained from Costar (Cambridge, MA, U.S.A.) and recombinant human EGF (hEGF) was purchased from Amgen Biologicals (Thousand Oaks, CA, U.S.A.). Recombinant human TGF-a was obtained from Triton Biosciences (Alameda, CA, U.S.A.). Carrier-free $^{125}$I and [$^{3}$H]thymidine were purchased from ICN Biomedicals (Irvine, CA, U.S.A.). Other chemicals were of analytical reagent grade.

### *Cell line and cell culture*

Normal rat kidney (NRK) fibroblast cells, clone 49F, were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured at 37°C in a humidified atmosphere of carbon dioxide-air (5:95). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) newborn calf serum (Hazelton Product, Denver, PA, U.S.A.), 50 μg/ml gentamicin and 30 m*M* tricine. The cells ($1 \times 10^4$ cells/ml) were seeded in each l-ml well of 24-well multi-dishes with DMEM-10% calf serum for 24 h and then the medium was replaced with DMEM-O. 1% calf serum for at least 24 h prior to use.

### *Iodination of hEGF and TGF-a*

Growth factors were labeled with carrier-free $^{125}$I according to the chloramine method using published procedures [13,14]. Carrier-free $^{125}$I (0.2 mCi) was added to 1 μg of growth factor in 12.5 μl of 1 *M* phosphate buffer (pH 7.4). Iodination was initiated by the addition of 2.5 μl of chloramine-T (2 mg/ml in 0.05 *M* phosphate

buffer, pH 7.4). The reaction was carried out for 1 min at room temperature with gentle shaking, then 10 μl of sodium metabisulfite (5 mg/ml) and 30 μl of sodium iodide (10 mg/ml) were added to stop the reaction. Bovine serum albumin (30 μl, 5%) was added and the labeled growth factor was separated from unreacted iodine by passage through a Sephadex G-25 column (20 cm × 0.5 cm I.D.). The labeled growth factors were stored at − 20°C.

*Bioanalysis of growth factors*

*Phast system PAGE.* Samples were separated on Phast gel media (50 × 43 × 0.45 mm) or Phast gel isoelectric focusing (IEF) media (50 × 43 × 0.35 mm) with a Phast system gel electrophoresis apparatus (Pharmacia, Uppsala, Sweden). For sodium dodecyl sulfate (SDS) PAGE, the growth factors were dissolved in 10 m*M* Tris HCl buffer (pH 6.8) containing 2.5% (v/v) of SDS and heated at 100°C for 5 min. It was carried out for 20–50 min depending on the kind of electrophoresis.

***Elution** of growth factors.* After completion of electrophoresis, the slab gel was sliced along the template (Fig. 1A) with a razor blade into equal pieces of 4 × 3 mm, scraped and each piece transferred into an indivual Transwell (Fig. 1 B), that had been prewashed with sterilized water and phosphate-buffered saline solution. The wells were then placed in a 24-well plate containing quiescent NRK cells and incubated for 18 h at 37°C. The gels in the Transwells were rinsed with the original medium in the wells and then the gels and the Transwells were removed before adding [$^3$H]thymidine to measure the rate of DNA synthesis.

*DNA synthesis.* A 20-μl volume of [$^3$H]thymidine (0.5 μCi) was added to each well and incubated at 37°C for 4 h. The incorporation was terminated by diluting with 1 ml of cold thymidine (100 μg/ml). DNA was precipitated with cold 10% trichloroacetic acid (TCA) and washed once with 5% TCA, then 95% ethanol. [$^3$H]DNA was dissolved in 0.5 ml of 0.2 A4 sodium hydroxide solution and transferred to vials containing 2.5 ml of Ecolume scintillation fluid (ICN Biomedicals). [$^3$H]DNA was measured using a Beckman Model LS 6800 scintillation counter.

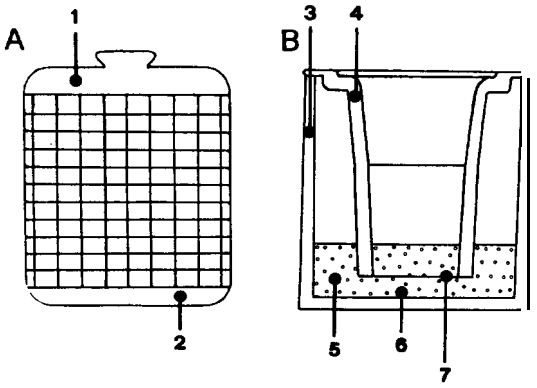


**Fig. 1. Illustration of microanalysis of growth factors. After electrophoresis, the gel was sliced along the designed template (A) and transferred into a Transwell (B). The measurement of [$^3$H]thymidine incorporation is described under Experimental. 1 = Cathode; 2 = anode; 3 = 24-well cell culture plate; 4 = Transwell; 5 = culture medium; 6 = monolayer of cell; 7 = porous membrane.**

## RESULTS

### *Determination of biological activity of growth factor by direct elution from gels through Transwell membranes*

We have successfully determined the biological activity of hEGF at the picogram level by eluting the growth factor from small, ultra-thin pieces of acrylamide gels (4 × 3 × 0.45 mm). After placing the gels on Transwell membranes and inserting the Transwell chamber into the cell culture medium, we were able to eliminate inhibition to the cells caused by direct contact of the gels. To determined the sensitivity, hEGF and TGF-a in amounts ranging from 2 to 400 pg were separated by 20% Phast gel native PAGE. The sliced gels were then transferred into the Transwells and the mitogenic activities were measured (Fig. 2). The sensitivity for hEGF and TGF-a were 50 and 200 pg, respectively.

### *Recovery of growth factors from acrylamide gels through Transwell membranes.*

The kinetics of the elution profile of a growth factor after PAGE was examined. A 1-ng amount of hEGF was separated by 20% Phast gel native PAGE and then transferred into Transwells. The gel pieces were removed from the culture medium at different time intervals between 15 min and 20 h. Mitogenic activity was tested by [$^3$H]thymidine incorporation. As shown in Fig. 3, more than 95% of hEGF was eluted into the medium within 1 h of incubation. There was no need for shaking or a special extraction procedure for eluting the growth factor from gel slices under these conditions.

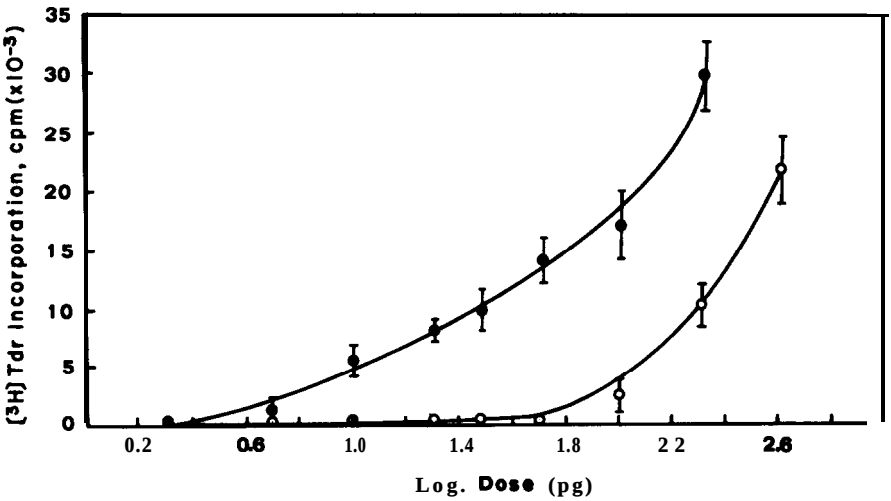


**Fig. 2. Concentrations of hEGF and TGF-a that affect DNA synthesis of NRK cells. Different concentrations of (●) hEGF and (0) TGF-a, as indicated, were separated by 20% Phast gel native PAGE. [$^3$H]Thymidine incorporation into DNA was determined after incubation of the sliced gels for 18 h. Data points represent means ± standard errors of the difference between the presence and absence of the growth factors from triplicate experiments.**

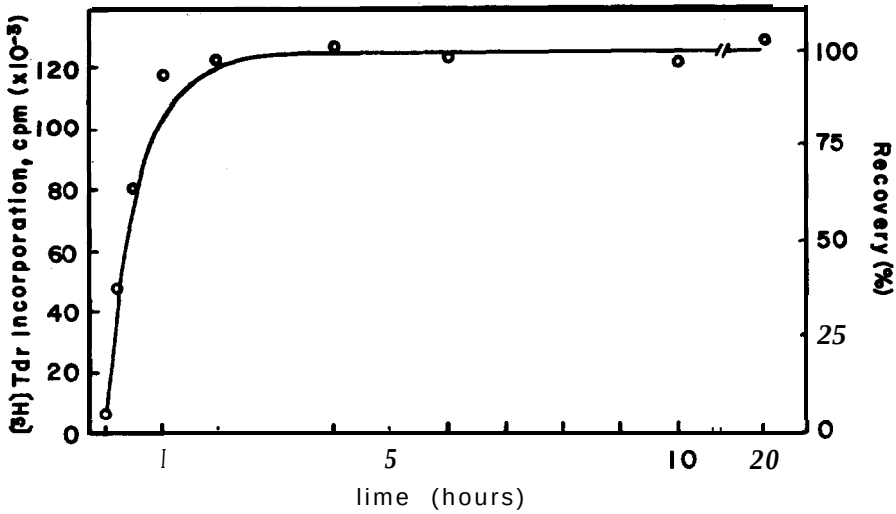


Fig. 3. Recovery of hEGF activities from Phast polyacrylamide gels. An equal concentration of hEGF (1 ng) was separated by 20% Phast gel native PAGE. At various time intervals, the gels were removed from the Transwells. [$^3$H]Thymidine incorporation was initiated after 20 h. The percentage recoveries were calculated based on the maximum activity of hEGF eluted from the gels. The efficiency was more than 75% of that obtained from hEGF (1 ng) added directly to culture medium for 20 h under the same conditions.

***Determination of isoelectric points by monitoring mitogenic activity after isoelectric focusing***

A 2-ng amount of hEGF or TGF-α was used to determine its isoelectric point. Neither hEGF nor TGF-a could be detected by silver staining at this low concentration. A 1-μl volume of the samples (2 ng/μl) was separated on Phast gel IEF 3-9 by IEF, followed by slicing of the gel into 4 × 3 mm pieces. The sliced gels were added to Transwell without washing. As shown in Fig. 4, the profiles of mitogenic activity after isoelectric focusing revealed that hEGF and TGF-a were well separated and their p*I* values were calculated to be 4.4 and 6.8, which were consistent with published data [ 15,161. The above results illustrate that one can determine isoelectric points of mitogens at nanogram levels or less by IEF. This is much lower than the range detectable by the most sensitive silver staining technique [17].

***Determination of isoelectric points, molecular weight and purity of iodinated growth factors.***

[$^{125}$I]hEGF and [$^{125}$I]TGF-α were used to determine isoelectric points and molecular weights by the proposed microanalytical methods. As shown in Fig. 5, [$^{125}$I]hEGF and [$^{125}$I]TGF-α were separated with isoelectric points of 4.4 and 6.8. respectively, confirming the observation in Fig. 4 using mitogenic activity as a marker. The iodinated growth factors were also used as examples to determine molecular weights by SDS-PAGE. [$^{125}$I]hEGF and [$^{125}$I]TGF-α were separated and their molecular weights were calculated to be 6000 and 5000 dalton, respectively (Fig. 6), compared with standard molecular weight markers. These results demonstrate that

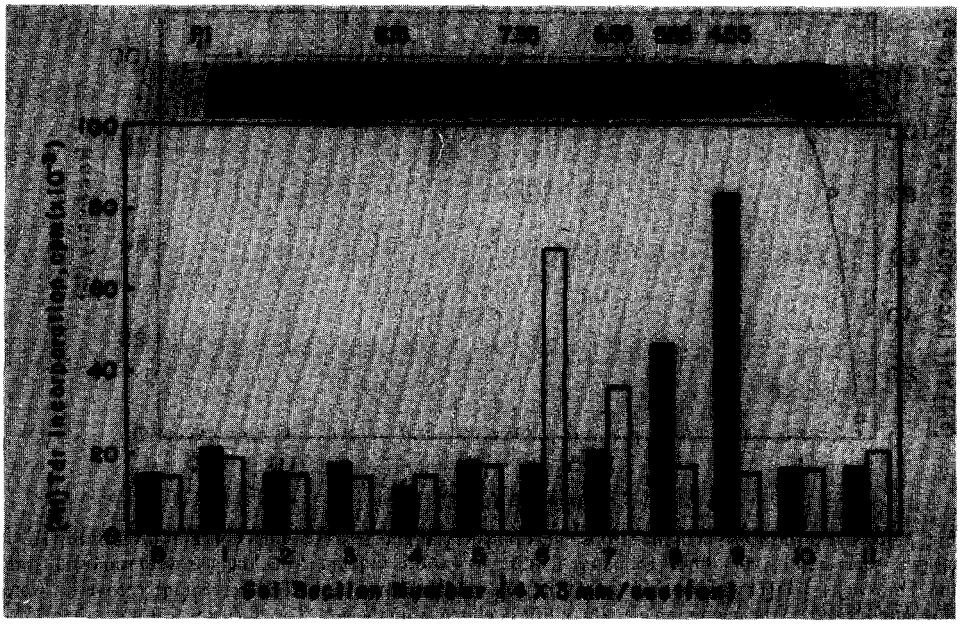

Fig. 4. Microanalysis of hEGF and TGF-a by isoelectric focusing. ( ■ ) hEGF (2 ng) and (0) TGF-a (2 ng) were separated on Phast gel IEF 3-9 by IEF and determined by monitoring DNA synthesis, as described under Experimental. Standard proteins stained by silver staining were used as markers. B = background, Nos. 1–11 = gel section numbers from cathode to anode.

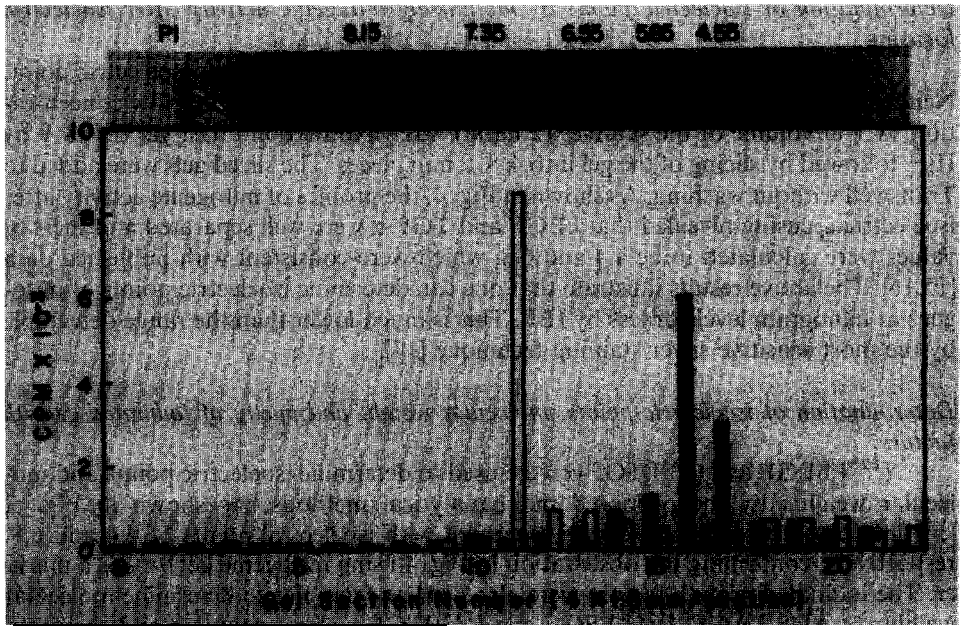

Fig. 5. Separation of [$^{125}$I]hEGF and [$^{125}$I]TGF-$\alpha$ by isoelectric focusing. A 1-$\mu$l volume (■) [$^{125}$I]hEGF ($1.4 \cdot 10^4$ cpm/$\mu$l) and (0) [$^{125}$I]TGF-$\alpha$ ($1.7 \cdot 10^4$ cpm/$\mu$l) were used for separation on Phast gel IEF 3-9 by IEF and determined by measuring radioactivity in a $\gamma$ counter. Standard proteins stained by silver staining were used as markers. B = background; Nos. 1-22 = gel section numbers from cathode to anode.

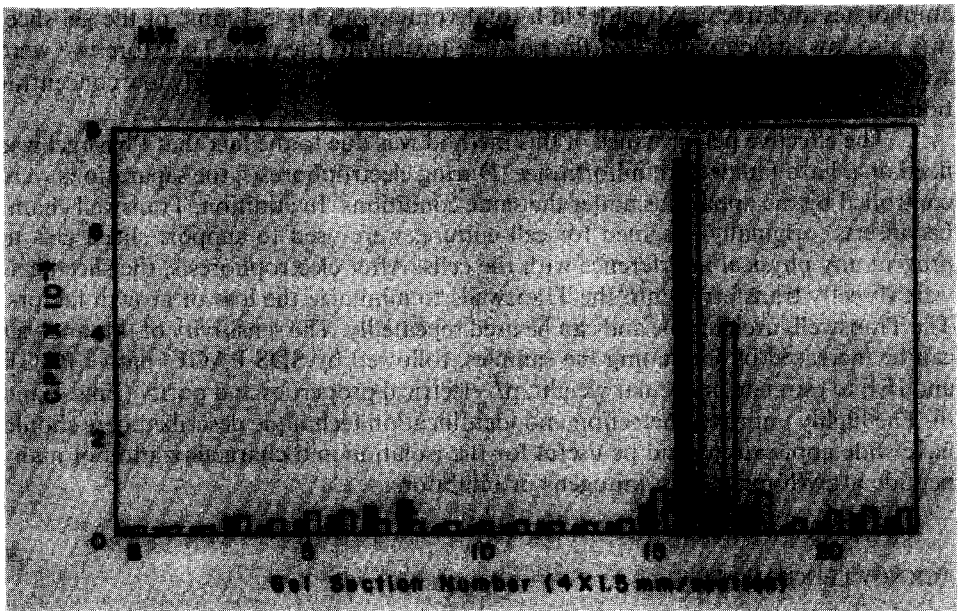

Fig. 6. Determination of molecular weight of [$^{125}$I]hEGF and [$^{125}$I]TGF-$\alpha$ by 20% Phast gel SDS-PAGE. (■) [$^{125}$I]hEGF (1.8 $10^4$ cpm/$\mu$l) and (□) [$^{125}$I]TGF-$\alpha$ (2.0 $10^4$ cpm/$\mu$l) were used for separation by Phast gel SDS-PAGE (20% polyacrylamide gel) and determined by measuring radioactivity in a $\gamma$ counter. Other details as in Fig. 5.

isoelectric points and molecular weights of growth factors and purity of preparation can be determined by this technique with much higher sensitivity than using standard staining procedures.

## DISCUSSION

Electrophoresis is widely employed for determining the properties of purified proteins. In general, the sensitivity of protein detection in the gel has been improved significantly by utilizing the silver staining method [17]. However, some proteins do not stain well with silver nitrate. Low concentrations of growth factors, e.g., IGF-I (50 ng), hEGF (30 ng) and TGF-a (100 ng), cannot be detected in gels by existing staining methods. In this paper, we have shown that as little as 50 pg of hEGF can be separated by electrophoresis and shown an increase in DNA synthesis in NRK cells. Hence picogram amounts of growth factors, which cannot be detected by conventional staining techniques, can now be detected and characterized. We have also shown that the isoelectric point of growth factors can be determined by their biological activity with better sensitivity than using the silver nitrate staining method.

Picogram amounts of growth factors were successfully transferred into a cell culture system by cutting ultra-thin layer of gel into slices (4 × 3 × 0.45 mm) and placing them in Transwells. It was shown that 95% of the growth factor was eluted within 1 h by examining mitogenic activity for NRK cells. Further. the content of

ampholytes and free acrylamide, in a total volume of only 5.4 $mm^3$ of the gel slice, was also low, which minimized the possible toxicity to the cells. Therefore, we were able to determine the biological activity of growth factors at extremely low concentrations.

The effective performance of this method was due to the fact that Phast gel was fixed on a hard film with a uniform size. During electrophoresis, the separations were controlled by the apparatus under the same conditions. In addition, Transwell chamber inserts, originally desgined for cell culture, were used to support sliced gels to prevent any physical interference with the cells. After electrophoresis, the sliced gels were directly transferred into the Transwells 'to minimize the loss of growth factors. The Transwells are durable and can be used repeatedly. The sensitivity of this analysis can be increased by iodinating the samples, followed by SDS-PAGE, native PAGE and IEF to estimate molecular weight, p*I*, electrical properties and purity (Figs. 5 and 6). The highly improved detection and identification technique described here should have wide applications and be useful for the isolation and characterization of many peptide growth regulators, mitogens or inhibitors.

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