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真島恵介

主論文

STUDY ON SEVERAL KINDS OF GROWTH INHIBITORS WITH DIFFERENT TARGET CELL SPECIFICITY

副論文

DIFFERENT SPECIES AND AMOUNTS OF mRNA IN VARIOUS NORMAL AND TUMOROUS CELLS OF RAT AS DETECTED BY IN VITRO TRANSLATION

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Keisuke MASHIMA

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ABBREVIATIONS

RSV, Rous sarcoma virus; DME/F12, 1 to 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 1.2 mg/ml $NaHCO_3$, 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate; FCS, fetal calf serum; PBS, Dulbecco's phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2); HBS, HEPES-buffered saline (10 mM HEPES-NaOH buffer (pH 7.2) containing 137 mM NaCl, 5 mM KCl, 0.01 mM $CaCl_2$ and 0.005 mM $MgCl_2$); BRL, an epithelial cell line established from liver cells of a Buffalo rat; RSV-BRL, BRL cells transformed by RSV; SDS, sodium dodecyl sulfate

SUMMARY

[I] Comparison of BRL and RSV-Transformed BRL Cells

The epithelial cell line BRL, which was established from liver cells of a normal Buffalo rat, was transformed by infection of Rous sarcoma virus (RSV). The RSV-transformed cells (RSV-BRL) showed a variety of characteristics compared to the parent BRL cells, such as tumorigenicity, anchorage-independent growth and altered morphorogy. The analysis of cellular proteins by two-dimensional polyacrylamide gel electrophoresis revealed the RSV-transformation had decreased the amounts of at least 13 proteins and increased those of at lease 16 proteins. The changes in proteins species were more marked with nuclear proteins than with total cellular proteins. When the expression of RSV proteins in RSV-BRL cells was immunologically analyzed, $Pr76^{gag}$ (precursor of internal structural proteins) was detectable, but other viral proteins including the oncogene product ($p60^{v\text{-}src}$) was not evident.

[II] Transformed Cell-Specific Growth Inhibitor (s-TGI) in Normal Rabbit Serum

Growth inhibitory factors were surveyed in sera of various kinds of animals using BRL and RSV-BRL cells as the target cells. Among sera of fetal calf, calf, horse, rat and rabbit, rat serum inhibited the growth of BRL cells more strongly than that of RSV-BRL cells, whereas rabbit serum inhibited the growth of RSV-BRL cells more strongly than that of BRL cells. The RSV-BRL-specific growth inhibitor (serum-derived transformed cell growth inhibitor, s-TGI) in rabbit serum was studied extensively. It existed as protein

complexes with 150-300k and pIs of about 5 and 6.5 in the serum. In the presence of 1 M NaCl and 6 M urea, it was dissociated into two active components with an apparent M_r of lower than 1.3k and with pI 7.5 and pI 9.5, respectively. The low M_r growth inhibitors did not pass through dialysis membrane with M_r cutoff of about 10k. s-TGI activity was precipitated by incubation with anti-rabbit immunoglobulins (IgA + IgG + IgM) but not with anti-rabbit IgG suggesting that s-TGI might be bound to immunoglobulin (IgA or IgM or both). s-TGI was purified to homogeneity from normal rabbit serum by anion-exchange chromatography on DEAE-Toyopearl 650M, adsorption to Amicon Diaflo YM5 membrane, molecular-sieve chromatography on Cellulofine GCL-300-m and reverse-phase high performance liquid chromatography (reverse-phase HPLC) on a C8 column (Ultron N-C8). The purified s-TGI showed a single band with M_r <10k on SDS-polyacrylamide gel electrophoresis. It showed 50% growth inhibition of RSV-BRL cells at about 40 ng/ml, but it hardly affected the growth of BRL cells. s-TGI was stable against treatment with 8 M urea or 1 M acetic acid (pH 2.5), but labile against treatment with 5 mM DL-dithiothreitol (DTT) or 1 mg/ml trypsin. The growth of B-32 (SV40-transformed cell line) and HSC-3 (cancer cell line) was effectively inhibited by s-TGI, whereas three non-transformed cell lines (BSC-1, LLC-RK1, YH-1) and HeLa-S3 (cancer cell line) were almost insensitive to the growth inhibitor.

[III] Two Types of Growth Inhibitors Secreted from BRL Cells

It was found that BRL cells secreted two types of growth inhibitors into culture medium (BRL-conditioned medium). One was

more inhibitory for BRL cells than RSV-BRL cells (conditioned medium-derived non-transformed cell growth inhibitor, c-NGI), and the other vice versa (conditioned medium-derived transformed cell growth inhibitor, c-TGI). Both activities of c-NGI and c-TGI were stable against treatment with 1 M acetic acid (pH 2.5) or 8 M urea, and labile against treatment with 5 mM DTT or 1 mg/ml trypsin.

Two c-NGIs (c-NGI-I and c-NGI-II) were purified to homogeneity from BRL-conditioned medium by molecular-sieve chromatography on Cellulofine GCL-300-m in the presence of 1 M acetic acid (pH 2.5), anion-exchange chromatography on DEAE-Toyopearl 650M in the presence of 6 M urea and reverse-phase HPLC on a C18 column (TSKgel LS-410K). c-NGI-I had a M_r of 53k, and c-NGI-II had a M_r of 20k as analyzed by SDS-polyacrylamide gel electrophoresis. c-NGIs effectively inhibited the growth of BRL cells at 10-20 ng/ml, but hardly that of RSV-BRL cells. Among six other cell line tested, HSC-3 cells were sensitive to c-NGI-I, and HSC-3, LLC-RK1 and B-32 cells were sensitive to c-NGI-II.

Two c-TGIs were purified from BRL-conditioned medium by molecular-sieve chromatography on Toyopearl HW55C in the presence of 1 M acetic acid and 6 M urea, molecular-sieve chromatography on the same column in the presence of 0.5 M acetic acid and 6 M urea and reverse-phase HPLC on Ultron N-C8. Both c-TGIs showed a single band with M_r <10k on SDS-polyacrylamide gel electrophoresis. The chromatographic and electrophoretic behaviors of c-TGIs suggested that they were identical or closely related to s-TGI from rabbit serum. Both c-TGIs inhibited the growth of RSV-BRL cells at 1-6

ng/ml, but hardly that of BRL cells. Among six cell lines tested, the growth of HSC-3 and LLC-RK1 cells were significantly inhibited by both c-TGIs.

INTRODUCTION

There is increasing evidence which supports the possibility that the proliferation of mammalian cells is at least in part controlled by the two groups of protein factors, growth-stimulating factors (growth factors) and growth-inhibiting factors (growth inhibitors). The abnormal growth of malignant cells *in vitro* or *in vivo* can be caused by disorder in the positive control mechanism by growth factors or in the negative control mechanism by growth inhibitors or both. Many studies have shown that in cell culture malignant cells have a tendency to grow without adding exogenous growth factors to the culture medium unlike non-malignant ones, and that they can produce growth factor (1-3). The production appears at least in part responsible for the autonomous growth of the cells. Recent molecular biology studies on carcinogenesis have shown that some oncogenes encode proteins which are structurally related to growth factors or their cell surface receptor proteins, such as platelet-derived growth factor (PDGF) (4, 5), epidermal growth factor (EGF) receptor (6), colony-stimulating factor (CEF-1) receptor (7) or tyrosine-specific protein kinases (8, 9). This suggests that the defective growth control in malignant cells is due to the abnormal expression of any of the elements involved in the normal mitogenic pathway.

The negative control mechanism by growth inhibitors was first proposed in the early 1950's by Weiss (10). The inhibitors were often called chalones. In the past, the isolation of the growth inhibitors was not successful; thus the mechanism has not been

accepted so widely as the positive control mechanism. However, during these few years, several types of growth inhibitors different in specificity for target cells have been highly purified from several sources, such as rat liver (11), platelets (12-14), the conditioned media of African green monkey kidney cell line (BSC-1) (15, 16), mouse fibroblast cell line (Swiss 3T3) (17) and human rhabdomyosarcoma cell line (A673) (18, 19) and sera of normal animals (20-22).

Recently, we have surveyed growth inhibitors present in normal animal sera. The two cell lines have been mainly used as the indicators; one is the non-malignant epithelial cell line (BRL), which was established in 1968 from normal Buffalo rat liver by Coon (23), and the other is the malignant (tumorous) BRL, which was obtained by infecting BRL with Rous sarcoma virus (RSV) in vitro (RSV-BRL) (24). In monolayer culture, the growth rate of BRL and RSV-BRL cells are practically the same and equally rapid in a culture medium containing an appropriate concentration of fetal calf serum. However, when subcutaneously injected into syngeneic rats (Buffalo rats), RSV-BRL cells grew forming tumor (malignant), but BRL cells did not (non-malignant). In addition, RSV-BRL cells showed the following characteristics; spindle-shaped morphology, loose intercellular junction, growth in semi-solid medium (anchorage-independent growth), loss of cell surface fibronectin, and elevated secretion of proteases. Such characteristics have been often observed in various kinds of virally transformed cells (25-28).

It was found that rat serum (host animal) inhibited the growth

of BRL cells to a significantly higher extent than that of RSV-BRL cells (24). Previously, BRL-specific growth inhibitors were highly purified from rat serum (29). In addition, similar BRL-specific growth inhibitors have been found in various tissues of normal rats and BRL and RSV-BRL cells themselves (14, 30).

On the other hand, rabbit serum, in contrast to rat serum, inhibited the growth of RSV-BRL cells more strongly than that of BRL cells, suggesting the existence of another type of growth inhibitor (RSV-BRL-specific growth inhibitor) in normal rabbit serum (24, 31). It was recently found that the culture medium conditioned by BRL cells showed both BRL-specific and RSV-BRL-specific growth inhibiting activity. In the present study, . we attempted to characterize growth inhibitors present in normal rabbit serum and the conditioned medium of BRL cells. The RSV-BRL-specific growth inhibitor was purified to homogeneity from normal rabbit serum. It was an acid-stable protein. Its apparent M_r was lower than 1.3k on molecular-sieve chromatography, but it did not pass through dialysis membrane (M_r cutoff: about 10,000). In addition, four kinds of growth inhibitors secreted from BRL cells were successfully purified to homogeneity, respectively. Two growth inhibitors with M_r 53k and M_r 20k exerted BRL-specific growth inhibition, whereas the others with apparent M_r lower than 1.3k did RSV-BRL-specific growth inhibition like the growth inhibitor purified from rabbit serum. The purification and properties of these growth inhibitors, together with their possible functions, are described in this paper.

MATERIALS AND METHODS

Cell Lines

The cell lines used were as follow: BRL (epithelial cell line from Buffalo rat liver), BSC-1 (epithelial cell line from African green monkey kidney), LLC-RK1 (epithelial cell line from rabbit kidney), YH-1 (diploid fibroblast cell line from human embryo skin), B-32 (SV-40 transformant of YH-1), HSC-3 (human tongue squamous carcinoma cell line) and HeLa-S3 (human uterine carcinoma cell line)

Cell Culture Conditions

Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The basal medium, DME/F12, consisted of a 1 to 1 mixture of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) and Ham's F12 medium (Gibco) supplemented with 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 1.2 mg/ml $NaHCO_3$, 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate. In most cases, cultures were supplemented with 10% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD). The standard culture medium was designated as 10% FCS + DME/F12. Falcon plastic culture dishes were purchased from Becton, Dickinson Labware (Oxnard, CA).

Transformation of Chicken Embryo Fibroblasts by RSV

Preparation and transformation of chick embryo fibroblasts (CEF) were performed basically by the method of Rubin (32). Fresh fertile eggs were purchased from Nisseiken (Tokyo) and incubated at 37°C for 10 days. Chicken embryos were asceptically removed from the incubated eggs and transferred into culture dishes. Head, limbs and viscera were removed from embryos with two forceps. The back of

embryos were washed with Dulbecco's phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) (PBS) and finely minced with curved scissors. The minced tissue was digested with 0.25% (w/v) trypsin (Difco) in PBS at 37°C for 15-30 min. The reaction was terminated by the addition of an equal volume of 10% FCS + DME/F12. Single cells were collected by passing the cell suspension through stainless steel filters with 60 mesh and 150 mesh and then centrifuging the filtrate at 10,000 rpm for 10 min. The CEF thus prepared were suspended in 10% FCS + DME/F12, plated in culture dishes (6 or 7 100-mm culture dishes per embryo) and cultured in a CO_2 incubator.

The Schmidt-Ruppin strain of RSV was kindly provided from Dr. M. Yutsudo (Research Institute for Microbial Diseases, Osaka University, Osaka). To prepare secondary culture of CEF, the primary cultures were harvested by washing once with PBS and then incubating with PBS containing 0.1% trypsin and 0.5% mM EDTA at room temperature. The harvested cells (10^6 cells) were seeded in 60-mm culture dishes containing 4 ml of 10% FCS + DME/F12, added with 1 ml of RSV stock solution (10^7 focus forming unit, FFU) and 50 μl of 0.2 mg/ml polybrene (Aldrich, Milwaukee, Wis.), and incubated overnight. Morphologically transformed cells appeared within one day after infection. After most cells had been transformed (2 or 3 days after infection), the culture fluid was harvested everyday and replaced with fresh medium. The pooled culture medium, which contained about 10^7 FFU/ml of RSV when the virus titter was assayed by the method of Rubin (32), was centrifuged at 800 x g for 2 hours, and the

precipitate of RSV was suspended in a small volume of fresh culture medium and stored at -80°C.

Transformation of Rat Liver Cells by RSV

The non-transformed epithelial cell line, BRL, which was originally cloned from liver cells of a normal Buffalo rat by Coon (23), was kindly provided by Prof. Nishikawa (Kanazawa Medical University, Ishikawa). Transformation of the rat liver cells was carried out according to the method of Hanafusa (33) for transformation of chicken embryo cells with some modifications. 2 x 10^6 BRL cells were seeded in 10% FCS + DME/F12 on a 60-mm culture dish. Two or three hours later, when a major part of cells had attached to plastic surface, the culture medium was sacked up, and 1 ml of fresh medium containing about 5 x 10^7 FFU of RSV and 2 μg polybrene was added. After incubation at 37°C for one hour, the culture was added with 3 ml of fresh medium containing 2 μg/ml polybrene and further incubated. Culture medium was refreshed twice a week. One week after infection, the cells were harvested by trypsinization and a fourth part of the harvested cells was transferred to a 100-mm culture dish. When the dish was incubated for 2 weeks, 3 to 5 small foci of morphologically transformed cells (RSV-BRL cells) appeared. The cultured cells were harvested, and a tenth part was further transferred to a new culture dish. During another incubation for one week, the number and size of foci of RSV-BRL cells increased appreciably. The transformed cells attached to substratum loosely and often piled up, whereas the non-transformed BRL cells attached firmly. Transformed cells were detached from

culture dishes by gentle pipetting, transferred to another dish and cultured. By repeating this procedure once again, almost pure culture of RSV-BRL cells was obtained.

For cloning of RSV-BRL cells, 50 cells were seeded in each of two 100-mm dishes. After 10 days in culture, 15 colonies were isolated from the dishes and subcultured. From them five clones which were different from each other in appearance, designated as RSV-BRL 1 to 5, were chosen and maintained in culture. In this study, RSV-BRL clone 1 was used as transformed cells (RSV-BRL cells).

Preparation of Samples for Electrophoretic Analysis

For analysis of cellular proteins by two-dimensional polyacrylamide gel electrophoresis, about 1 x 10^7 cells were harvested from surface of a culture dish, washed twice with PBS by centrifugation, and homogenized with a Potter-Elvehjem homogenizer in a mixture of 8 M urea, 2% 2-mercaptoethanol and 2% Nonidet P-40, and centrifuged at 100,000 x g for 1 hour. The resulting supernatant was added with Ampholine-carrier ampholytes (pH 3.5-10) (LKB Produkter AB, Bromma) to make a final concentration of 1% (w/v) and used as Nonidet P-40/2-mercaptoethanol/urea-extract (NP40/2-ME/UREA extract). 0.75 mg protein was analyzed by two-dimensional polyacrylamide gel electrophoresis.

For nuclear protein analysis, about 1 x 10^8 cells were harvested from 10 100-mm culture dishes and washed twice with PBS. The washed cells were suspended in 10 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 3 mM $CaCl_2$, 0.5% Triton X-100

and 1 mM phenylmethylsulfonyl fluoride (PMSF) and stirred at 4 °C overnight. This solution was centrifuged for 10 min at 1,200 x g. The resulting pellet was washed with the above buffer by centrifugation. The washed pellet was laid on 2 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.88 M sucrose, 1 mM PMSF, and centrifuged at 1,200 x g for 30 min. The pellet was washed twice with the same buffer as above. The washed pellet was dissolved in 1 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1% (w/v) sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 x g for 5 hours. 0.45 ml of the supernatant was mixed with 80 μl of Nonidet P-40, 20 μl of Ampholine-carrier ampholytes (pH 3.5-10) and 480 mg of solid urea. 0.4 mg protein was analyzed by two-dimensional polyacrylamide gel electrophoresis as "Nuclear protein fraction" (34).

For viral protein analysis with immuno-blotting, about 1 x 10^6 cells were harvested and washed twice with PBS by centrifugation. The washed cells were suspended in 1 ml of 62.5 mM Tris-HCl buffer (pH 6.8) containing 8 M urea, 1% SDS and 2% 2-mercaptoethanol, and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 x g for 3 hours. 0.2 mg protein of the resulting supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis on a polyacrylamide gel slab containing SDS was carried out by the method of Laemmli (35). In most cases, a KPI

electrophoresis apparatus (Model E-IE 17-30TR, Koike Precision Instruments, Kawasaki) was used. The separating gel slab (240 x 150 x 2 mm) was prepared from a mixture of 0.375 M Tris-HCl buffer (pH 8.8), 0.1% (w/v) SDS, acrylamide with a linear gradient from 10 to 20% (w/v), N,N'-methylene-bisacrylamide (BIS) with a linear gradient from 0.27 to 0.53% (w/v) and catalysts. The stacking gel slab (20 x 150 x 2 mm) was prepared from a mixture of 0.125 M Tris-HCl buffer (pH 6.8), 0.1% SDS, 2.5% acrylamide, 0.6% BIS and catalysts. Electrophoresis was carried out at 15 mA per gel slab at 10-15°C for 30 hours. The electrolyte consisted of 25 mM Tris, 192 mM glycine and 0.1% SDS .

In analysis of purified growth inhibitors, a small size of gel slabs were used. The size and acrylamide concentration of the gel slabs were 90 x 65 x 1 mm and 12.5% for the separating gel and 90 x 15 x 1 mm and 2.5% for the stacking gel.

After electrophoresis, the gel slab was stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 (CBB) containing 45% (v/v) ethanol and 10% (v/v) acetic acid. Destaining was carried out by washing the gel slab with a mixture of 25.5% (v/v) ethanol and 8.2% (v/v) acetic acid and then with a mixture of 5% (v/v) ethanol and 7.5% (v/v) acetic acid.

In some experiments, the gel slab was subjected to silver-staining. The gel slab was soaked in 50% (v/v) ethanol and 10% (v/v) acetic acid and washed twice with ultra-pure water. Silver-staining was carried out with the use of silver-staining reagents kit "Wako" (Wako Chemicals, Osaka) according to the technical manual. The gel

slab was dried under vacuum in a slab gel dryer (Koike Precision Inst. Kawasaki).

The molecular weight markers used were phosphorylase b (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,000).

Two-Dimensional Polyacrylamide Gel Electrophoresis

As the first dimensional electrophoresis, isoelectric electrophoresis was carried out on a cylindrical polyacrylamide gel formed in a glass tube (130 x 5 mm) in a KPI apparatus (model E-IE 12-10, Koike Precision Inst. Kanagawa). The gel was made from a mixture of 4% (w/v) acrylamide, 0.2% BIS, 2% Ampholine-carrier ampholytes (pH 3.5-10), 8 M urea and catalysts. A protein sample dissolved in a mixture of 2% Ampholine-carrier ampholytes (pH 3.5-10), 1% 2-mercaptoethanol and 8 M urea was applied on the top of the gel and overlaid with 50μl of 2% Ampholine-carrier ampholytes (pH 3.5-10) and 15% glycerol solution. In the apparatus, the upper reservoir was filled with 0.02 M H_3PO_4 (anode) and the lower reservoir with 1 N NaOH (cathode). Electrophoresis was carried out at 4°C at 200 V for initial 2 hours, at 300 V for next 16 hours and at 500 V until the colored pI makers used (cytochrome c of pI 10.6 and its acetylated derivatives of pI 4.1, 4.9, 6.3, 8.3 and 9.7) were focused at equilibrium. As the second dimensional electrophoresis, SDS polyacrylamide gel electrophoresis on an acrylamide-gradient gel slab was carried out as follows. Immediately after the first dimensional electrophoresis, the gel disc was taken

out of the glass tube, put into a 50 ml flask containing the equilibrating buffer (62.5 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol), and incubated at room temperature for 1 hour with renewals of the equilibration buffer every 20 min. The gel disc was then laid across the top of the stacking gel slab composed of 62.5 mM Tris-HCl (pH 6.8), 4% (w/v) acrylamide, 0.2% (w/v) BIS and 0.1% (w/v) SDS, and hot 1% (w/v) agarose solution containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol was poured around it. As soon as the agar had solidified, electrophoresis was started and carried out as described above.

Analysis of Intracellular RSV Proteins by Immuno-Blotting

Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose membrane was carried out according to the method of Towbin et al. (36), with Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Lab., Richmond, CA). After SDS-polyacrylamide gel electrophoresis, the gel slab used was placed onto a sheet of wet nitrocellulose membrane (Schleicher & Schüll, Dassel, W. Germany), which was cut to the size of gel slab and supported by a sheet of wet filter paper. A second filter paper sheet was placed on the gel surface. This sandwich was placed between two 0.5-cm thick scouring pads (Scotch-Brite), which were in turn supported by two rigid Plexiglas plates (gel holder) containing holes to allow free access of electrolyte to the gel sandwich and the flow of current through the transfer chamber filled with electrolyte, with the nitrocellulose sheet facing the anode. The electrolyte was consisted

of 25 mM Tris, 192 mM glycine and 15% methanol. After the electrophoresis, a part of the nitrocellulose sheet was stained for 10 min in 0.1% amidoblack solution containing 45% methanol and 10% acetic acid, to visualize the transferred polypeptide bands.

The nitrocellulose membrane with transferred polypeptides was soaked in PBS to remove electrolyte, followed by incubation in PBS supplemented with 3% (v/v) calf serum at room temperature to block protein-free site of the nitrocellulose membrane. The membrane was washed three times with TPBS (0.05% (w/v) Tween 20 in PBS) and incubated with 1:200 diluted anti-RSV antiserum (Transformation Research Inc.) for 4 hours at room temperature. After the antibody solution was removed, the membrane was washed with three times with TPBS, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Bio Rad Lab., Richmond, CA) diluted 1:1000 with TPBS for 2 hours at room temperature. The nonspecifically bound immunoglobulin was removed by washing the membrane three times with PBS, and the membrane was soaked in substrate solution at room temperature (about 5-20 min) until color development. The substrate solution was prepared as follows. 60 mg of 4-chloro-1-naphtol (Wako Chemicals Co. Ltd., Osaka) was dissolved in 20 ml methanol and mixed with 100 ml of PBS and 60μl of 30% H_2O_2 solution. The reaction was stopped by washing the membrane with water.

Assay of Growth Inhibitory Activity in monolayer Culture

During purification of growth inhibitors, their activities were routinely assayed by the dye reduction method of Green et al. (37) with a minor modification. 1 x 10^4 cells of BRL or RSV-BRL

cells were incubated in each well of 24-Multiwell plates (Falcon, #3047) containing 0.5 ml of 10% FCS + DME/F12 medium. Unless otherwise indicated, samples were dialyzed against 1 M acetic acid (pH 2.5), and their 25-μl portions were added to each well. Control culture were added with the same volume of 1 M acetic acid. The pH of the resulting medium in each well was neutralized with a corresponding volume of 1 M sodium carbonate. After 3 days in culture, each well was added with 20 μl of 5 mg/ml MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Louis, MO) in PBS, and incubated at 37°C for 4 hours to form insoluble MTT reductant (formazan). The culture medium containing unreacted MTT was removed, and 1 ml of isopropanol containing 0.04 N HCl was added to each well to sulubilize the MTT formazan. Absorbance at 565 nm was measured with the extract. The absorbance of the MTT reductant was proportional to the number of BRL or RSV-BRL cells in the wells.

In some cases, cell growth was directly determined by counting grown cells. In the cases, 2×10^4 cells of BRL or RSV-BRL cells were incubated per 35-mm plastic culture dish containing 2 ml of 10% FCS + DME/F12 supplemented with test samples (<0.2 ml). After 3 days in culture, the grown cells were counted with a hemocytometer.

When the growth of other cell lines was assayed, cells were incubated in 35-mm dishes for 4 days. The initial cell density was 4×10^4 cells per dish for HeLa S3, HSC-1, BSC-1 and LLC-RK1 cells and 8×10^4 cells per dish for YH-1 and B-32 cells.

Preparation of Lipoproteins

Lipoproteins were separated from chylomicron-free normal rabbit serum according to the method of Havel et al. (38). After centrifugation, the fractions with d < 1.210 mg/ml were collected. The collected fraction, which contained three types of lipoproteins (low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein (HDL)), was centrifugally washed twice with a salt solution of d = 1.210. The washed fraction was dialyzed against PBS and used as lipoprotein fraction. The bottom two-thirds of the tube content obtained by the centrifugation of the serum solution with d = 1.210 was dialyzed against PBS and used as "lipoprotein-free serum".

Analysis of s-TGI-binding Protein in Rabbit Serum by Immuno-Precipitation

Normal rabbit serum was diluted 2-fold with PBS and used for immuno-precipitation. The following antisera of sheep were obtained from Cappel (Malvern, PA): anti-rabbit IgG (Heavy and Light chains) (lot No. 22970), anti-rabbit immunoglobulins (IgA + IgG + IgM) (Heavy and Light chains) (lot No. 14100), anti-rabbit $alpha_2$-macroglobulin (Lot No. 21061) and anti-rabbit whole serum. Normal sheep serum (lot No. 26379) was used as control. The antisera and normal sheep serum (0.5 ml) were individually mixed with two-fold diluted rabbit serum (0.5 ml) and incubated at room temperature (25 °C) for 4 hours and stored at 4 °C for 2 days. The resulting immuno-precipitate was removed by centrifugation in Eppendorf Centrifuge Type 5414 for 5 min, and the growth inhibitory activity in the supernatant was determined.

Preparation of Conditioned Medium

BRL cells were grown to confluency in 150-mm culture dishes (Falcon, #3025) containing 25 ml of DME/F12 supplemented with 5% (v/v) FCS for 2-3 days. The confluent culture were washed twice with PBS and replaced with 25 ml of serum-free DME/F12. After 3-days culture, the medium was collected and centrifuged at 25,000 x g at 4°C for 30 min. The resulting supernatant was stored at -20°C and used as conditioned medium.

Concentration of Conditioned Medium and Adsorption to Amicon Diaflo YM5 Membrane

The conditioned medium of BRL cells was concentrated by ultrafiltration. For the analysis of growth inhibitors by molecular-sieve high performance liquid chromatography, the conditioned medium of BRL cells were added with solid NaCl and urea to make final concentrations of 1 M and 6 M, respectively, and the solution (about 500 ml) was concentrated to 5 ml by ultrafiltration through Diaflo YM2 membrane (M_r cutoff: 2,000; 25-mm diameter) in an Amicon Diaflo cell (Danvers, MA). For purification of growth inhibitors, the conditioned medium (8 liters) was concentrated to 100 ml by ultrafiltration through Diaflo YM5 membrane (M_r cutoff: 5,000; 76-mm diameter) at 50 rpm at 4°C. The concentrated conditioned medium was diluted with HBS (10 mM HEPES-NaOH buffer (pH 7.2) containing 137 mM NaCl, 5 mM KCl, 0.01 mM $CaCl_2$ and 0.005 mM $MgCl_2$) to 400 ml and reconcentrated by the same method as above. The dilution-concentration procedure was repeated twice. The finally concentrated solution was called "Concentrated fraction". After the

concentration, the YM5 membrane was rinsed with HBS, and the proteins adsorbed to the membrane was extracted by 50 ml of 1 M acetic acid containing 6 M urea. The extract was called "Extracted fraction".

Analysis of Growth Inhibitors by Molecular-Sieve High Performance Liquid Chromatography

The conditioned medium of BRL cells was concentrated in the presence of 1 M NaCl and 6 M urea and then dialyzed against 10 mM HEPES-NaOH buffer (pH 7.5) containing 1 M NaCl and 6 M urea. The dialyzed sample (2.5 ml) was applied to a TSKgel G3000SWG column (30 x 300 mm) (Toyo Soda, Tokyo) and developed with the same buffer at a flow rate of 2 ml/min. The eluate was collected in 5-ml fractions. Tyroglobulin (670K), ganmaglobulin (158K), ovalbumin (44K), myoglobulin (17K) and Vitamin B-12 (1.35K) were used as molecular weight markers.

Chromatofocusing

Growth inhibitor fraction obtained by molecular sieve chromatography in the absence of denaturant (non-dissociating condition) was dialyzed against 25 mM Imidazole-HCl buffer (pH 7.5). PBE 94 column (1 x 25 cm) (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was previously equilibrated with 25 mM Imidazole-HCl buffer (pH 7.5) degassed by under vacuum. After the dialyzed sample (20 ml) was applied to the column, the charged column was eluted with 400 ml of 8-fold diluted Polybuffer 74-HCl buffer (pH 4.0) (Pharmacia) at a flow rate of 30 ml/hours. Factions of 5 ml were collected, and the absorbance at 280 nm of each fraction was measured. Serial 5

fractions were combined and concentrated by ammonium sulfate precipitation at a concentration of 70% saturation. The concentrated factions were suspended in 5 ml of PBS, and 0.2-ml portions were dialyzed against PBS and used for the assay of growth inhibitory activity.

Polyacrylamide Gel Isoelectric Electrophoresis

Isoelectric electrophoresis was carried out on a gel formed in a cylindrical glass tube (130 x 5 mm ID) with a KPI apparatus (model E-IE 12-10, Koike Precision Inst. Kanagawa) by the same method as for two-dimensional gel electrophoresis. Electrophoresis was carried out at 4°C at 200 V for initial 2 hours, at 300 V for next 16 hours and at 500 V until the colored pI makers were focused at equilibrium. After electrophoresis, the gel was cut into 5-mm long pieces, put into glass tubes containing 0.5 ml of a mixture of 1 M acetic acid and 1 mg/ml of BSA. Each gel piece was homogenized with a glass lod and extracted by standing at 4°C overnight, and followed by centrifuging at 20,000 x g at 4°C for 30 min. The resultant supernatant was dialyzed against 1 M acetic acid and assayed for growth inhibitory activity. The control gel, which was run without protein sample, was fractionated in 5-mm length and extracted with 1 ml of water. The pHs of the gel extracts were determined at 0°C.

Molecular-Sieve Chromatography

Molecular-sieve chromatographies were carried out on a Cellulofine GCL-2000-m column (2.6 x 97 cm) (Chisso, Tokyo) equilibrated with PBS or 10 mM HEPES-NaOH buffer (pH 7.2) containing 1 M NaCl and 6 M urea, on a Cellulofine GCL-300-m column (2.6 x 96

cm) (Chisso, Tokyo) equilibrated with 1 M acetic acid or 0.5 M acetic acid, and on a Toyopearl HW55c column (2.6 x 95 cm) (Toyo Soda, Tokyo) equilibrated with 1 M acetic acid containing 6 M urea or with 0.5 M acetic acid containing 6 M urea. In most cases, about 50 ml of protein samples previously dialyzed against the respective buffer were applied to the columns and developed with the buffer at a flow rate of 30 ml/hour. The eluates were collected in 10-ml fractions.

Anion-Exchange Chromatography

Anion-exchange chromatographies were carried out under two different conditions. For the purification of s-TGI from rabbit serum, 500 ml of rabbit serum was added with glycerol to make a final concentration of 30% (w/v) and diluted with 10 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 30% (w/v) glycerol (HCMG buffer (pH 7.5)) to make a final volume of 2 liters. DEAE-Toyopearl 650M column (5 x 30 cm) was equilibrated with HCMG buffer (pH 7.5). The diluted serum was applied to the column, and the charged column was washed with the same buffer until the absorbance at 280 nm of the eluate decreased sufficiently. Proteins adsorbed to the column were eluted with a linear gradient of NaCl from 0 to 1 M in 4 liters of HCMG buffer (pH 7.5) at a flow rate of 60 ml/hour. The eluate was collected in 120-ml fractions.

For the purification of c-NGIs from the conditioned medium of BRL cells, the active fractions (total volume 150 ml) obtained by molecular-sieve chromatography were pooled, dialyzed against 10 mM HEPES-NaOH buffer (pH 8.0) containing 6 M urea (HU buffer (pH 8.0)),

and applied to a DEAE-Toyopearl 650M column (2.5 x 10 cm) equilibrated with HU buffer (pH 8.0). The charged column was washed with HU buffer (pH 8.0) and eluted with a linear gradient of NaCl from 0 to 0.75 M in 1 liter of HU buffer at a flow rate of 30 ml/hour. The eluate was collected in 10-ml fractions.

Reverse-Phase High Performance Liquid Chromatography (Reverse-Phase HPLC)

Reverse phase HPLC was carried out on two different columns, using a LKB HPLC system (Type 2150 HPLC pump, 2152 controller and 2151 variable wavelength monitor). For the purification of s-TGI from rabbit and c-TGIs from the conditioned medium of BRL cells, the samples were dialyzed against 1 M acetic acid and applied to a Ultron N-C8 (C-8) column (4.6 x 150 mm) (Shimwa Kagaku Inc. , Kyoto). Proteins adsorbed the column was eluted with 3-step linear gradients of acetonitrile in 1 M acetic acid from 0 to 20% for 20 min, from 20 to 60% for 120 min and from 60 to 90% for 20 min at a flow rate of 1.0 ml/min. The eluate was collected in 2-ml fractions.

c-NGIs from the conditioned medium were fractionated on a LS-410K (C-18) column (4.6 x 250 mm) (Toyo-Soda, Tokyo). Other experimental conditions were the same as above.

Treatments with Acetic acid, Dithiothreitol and Trypsin

s-TGI fraction prepared by molecular-sieve chromatography (see Fig. II-13), c-NGIs and c-TGIs fractions prepared by molecular-sieve HPLC were respectively concentrated. The concentrated solutions were supplemented with 0.1 mg/ml of BSA (Sigma), and dialyzed against HBS. Portions of the resultant solutions were mixed with 0.5 M

acetic acid, 5 mM DL-dithiothreitol (Sigma) or 1 mg/ml of trypsin from bovine pancreas (1280 U/mg, Type-I, Sigma). The mixtures with the acid or reducing reagent were incubated at 4°C for 1 hour, whereas those with the enzyme at 37°C for 1 hour. Only the mixtures with the acid were dialyzed against HBS. The solutions thus obtained were added at 4% volume to 10%FCS+DME/F12 for assay of growth inhibitory activity.

Determination of Protein Concentration

Protein concentrations of purified growth inhibitors (s-TGI, c-NGIs and c-TGIs) were determined by the dye method with Coomassie Brilliant Blue (CBB) G-250 (49), using bovine serum albumin as a standard protein.

RESULTS

[I] Comparison of BRL and RSV-Transformed BRL Cells

Transformation of Rat Liver Epithelial Cells by RSV

The epithelial cell line BRL was established by Coon (23) from liver of a normal Buffalo rat. When BRL cells were infected with Rous sarcoma virus (RSV) in culture and further incubated for a few weeks, foci of RSV-transformed cells appeared in the culture. The transformed cells, named RSV-BRL, were isolated and cloned. The morphology of BRL and RSV-BRL cells are shown in Fig. I-1. BRL and

Fig. I-1

RSV-BRL cells grew at almost the same rapid rate in monolayer culture with the fetal calf serum-containing medium. BRL cells displayed flat morphology and tight intercellular junction forming packed monolayer colonies, whereas RSV-BRL cells displayed irregular cell arrangement, loose intercellular junction and spindle-shaped morphology. BRL cells could not grow in soft agar medium, whereas RSV-BRL cells could grow forming colonies in soft agar medium (anchorage-independent growth). When subcutaneously injected into syngeneic rats (Buffalo rats), RSV-BRL cells, but not BRL cells, grew forming tumors (tumorigeneicity). These characteristics of RSV-BRL cells have been often observed in various kinds of virally transformed cells.

Analysis of Cellular Polypeptides

Total cellular proteins were extracted with a mixture of 2%

(w/v) Nonidet P-40, 2% (v/v) 2-mercaptoethanol and 8 M urea (NP40/2-ME/urea-extracted fraction) from BRL and RSV-BRL cells and analyzed by two-dimensional polyacrylamide gel electrophoresis with isoelectric electrophoresis in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension (Fig. I-2). When the two electrophoretic patterns were compared, the amount

Fig. I-2

of at least 9 polypeptides were lower and those of at least 7 polypeptides were higher in RSV-BRL cells than in BRL cells. Among these polypeptides, the polypeptide of M_r 45k and pI 6.3, which was increased in amount by the RSV-transformation, is likely to be actin. These results are summarized in Table I-1.

Table I-1

Nuclear proteins of BRL and RSV-BRL cells were analyzed by the same method (Fig. I-3). The amounts of at least 13 polypeptides were

Fig. I-3

changed by the RSV-transformation; the amounts of 9 polypeptides were higher in RSV-BRL cells than BRL cells, whereas those of 4 polypeptides were lower. These results are summarized in Table I-II.

Table I-II

As a whole, the change of cellular proteins was more marked with nuclear proteins than with total cellular proteins. Nuclear proteins had a tendency to increase in amount by the RSV-transformation of the rat liver epithelial cells.

Analysis of Viral Proteins Expressed in Transformed Cells

It is well known that RSV has a oncogene called *src*, which express a protein of M_r 60k ($p60^{v\text{-}src}$) having tyrosine-specific protein kinase activity. $p60^{v\text{-}src}$ is believed to be primarily responsible for the initiation and maintenance of neoplastic transformation of cells. When chick embryo fibroblasts (CEF) are transformed by RSV, the transformed cells produced RSV particles into culture medium. However, RSV-BRL cells produced no infectious RSV particles into culture medium. Therefore, the expression of $p60^{v\text{-}src}$ and other viral proteins of RSV was analyzed with non-transformed and RSV-transformed BRL and CEF cells by the immuno-blotting method with anti-RSV antiserum of rabbit. Total cellular proteins of the two pairs of cells were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The viral proteins were selectively colored by treating the membrane with the anti-RSV antiserum and then with horseradish peroxidase-conjugated anti-rabbit IgG (Fig. I-4). The

Fig. I-4

viral proteins, $Pr180^{gag\text{-}pol}$ (precursor of internal structural proteins (gag) and reverse transcriptase (pol)), $Pr90^{env}$ (precursor of envelope (env)), $Pr76^{gag}$ (precursor of the internal structural proteins (gag)) and $p60^{v\text{-}src}$ (oncogene product) were evident with RSV-transformed CEF cells, but no viral protein was detected with non-transformed CEF and BRL cells. On the other hand, only $Pr76^{gag}$ was visible with RSV-BRL cells. However, it can not be excluded that RSV-BRL cells express a very low level of $p60^{v\text{-}src}$.

[II] Transformed Cell-Specific Growth Inhibitor in Normal Rabbit Serum

Effect of Sera from Several Normal Animals on Growth of BRL and RSV-BRL Cells

As described above, although BRL and RSV-BRL cells equally grew under the standard culture condition, only RSV-BRL cells could grow when injected into syngeneic rats. Growth properties of BRL and RSV-BRL cells *in vitro* were investigated in more detail using culture media supplemented with different kinds of animal sera. Effect of sera from five kinds of normal animals on the growth of BRL and RSV-BRL cells are shown in Figs. II-1 - II-5. All kinds of sera tested

Fig. II-1

Fig. II-2

Fig. II-3

Fig. II-4

Fig. II-5

stimulated the cell growth at lower concentrations but suppressed it at higher concentrations, indicating that they contained both growth stimulatory substances (growth factors) and growth inhibitory ones (growth inhibitors). With fetal calf serum (FCS), which is most

widely used for animal cell cultures, calf serum or horse serum, BRL and RSV-BRL cells showed almost the same dose-response growth curves of cell growth. They maximally stimulated the cell growth at about 1%. However, the two kinds of cells differently responded to rat or rabbit serum. At final a concentration of 1% (v/v) rat serum maximally stimulated the growth of both BRL and RSV-BRL cells, but at increasing concentrations it was growth inhibitory. The growth of BRL cells was inhibited more strongly than that of RSV-BRL cells by higher concentrations of rat serum. Rabbit serum showed an optimal concentration at 1% for RSV-BRL cells and at 2% for BRL cells. In contrast to rat serum, rabbit serum was more growth inhibitory for RSV-BRL cells than for BRL cells at concentrations between 2% and 4%. These results suggested the existence of at least two types of growth inhibitors in sera of normal animals, different in specificity for BRL and RSV-BRL cells. One is specific for BRL cells and abundant in rat serum and the other is specific for RSV-BRL cells and abundant in rabbit serum. There are two possibilities that sera of fetal calf, calf and horse contain equal activities of BRL-specific growth inhibitor and RSV-BRL-specific one, and that they contain a non-specific growth inhibitor as the major growth inhibitor. BRL-specific growth inhibitor in rat serum has been highly purified in our laboratory (30). In the present study, we attempted to characterize RSV-BRL-specific growth inhibitor present in normal rabbit serum.

Since both BRL and RSV-BRL cells maximally grew in the presence of 10% (v/v) FCS (Fig. II-1), the growth inhibitory effect of normal

rabbit serum was determined in the presence of 10% FCS (Fig. II-6).

Fig. II-6

Under the condition, the growth of RSV-BRL cells was again inhibited more strongly than that of BRL cells, in proportion to the concentration of rabbit serum. Thereafter activities of various growth inhibitors were assayed in the presence of 10% FCS unless otherwise noted.

Growth Inhibitory Activities of Lipoprotein and Lipoprotein-Free Rabbit Serum

It has been reported that low-density lipoprotein (LDL) or very-low-density lipoprotein (VLDL) fraction inhibits the growth of several kinds of cells in culture (40-42). Rabbit serum was separated into lipoprotein fraction and lipoprotein-free serum fraction, and their effects on the growth of BRL and RSV-BRL cells were examined (Fig. II-7). It was found that the lipoprotein-free

Fig. II-7

serum fraction, similarly to whole serum, inhibited the growth of RSV-BRL cells more strongly than that of BRL cells. Growth inhibitory activity on BRL and RSV-BRL cells was hardly observed with the lipoprotein fraction. These results indicated that the growth inhibitory activity on RSV-BRL cells of normal rabbit serum was most likely due to a growth inhibitor other than LDL or VLDL.

The RSV-BRL-specific growth inhibitor was tentatively named as serum-derived transformed cell growth inhibitor (s-TGI).

Characterization of s-TGI

Normal rabbit serum was fractionated by ammonium sulfate precipitation. s-TGI activity was recovered between 30%- and 50%-saturated ammonium sulfate (30-50% sat. $AmSO_4$ fraction). A portion of the active fraction was dialyzed against PBS overnight and subjected to molecular-sieve chromatography on a Cellulofine GCL-2000-m column (2.6 x 97 cm) equilibrated with PBS (non-dissociating condition) (Fig. II-8). s-TGI activity was recovered forming a broad

Fig. II-8

peak of M_r 150-300k. The active fractions obtained by the molecular-sieve chromatography were pooled, dialyzed against 15 mM Imidazole-HCl buffer (pH 7.5) and subjected to chromatofocusing on a PEP 74 column (1 x 25 cm) equilibrated with the same buffer. The charged column was eluted with 7-fold diluted Polybuffer 94-HCl buffer (pH 4.0) (Fig. II-9). s-TGI activity was separated into two peaks at pH

Fig. II-9

5 and pH 6.5, respectively.

Another portion of 30-50% sat. $AmSO_4$ fraction was dialyzed against 10 mM HEPES-NaOH buffer (pH 7.2) containing 1 M NaCl and 6 M urea for 2-3 days. The dialyzed material was subjected to molecular-

sieve chromatography on a Cellulofine GCL-2000-m column (2.6 x 97 cm) equilibrated with 10 mM HEPES-NaOH buffer (pH 7.2) containing 1 M NaCl and 6 M urea (dissociating condition) (Fig. II-10). Under

Fig. II-10

this condition, s-TGI activity was separated into a large peak of M_r <1.3k and a small peak of M_r 40k. Little activity was recovered in a M_r 150-300k region. When the major active fractions of M_r <1.3k were dialyzed using a cellulose tube (M_r cutoff about 10k) or concentrated with Diaflo YM10 membrane (M_r cutoff 10k), the growth inhibitor did not pass through both membrane, suggesting its actual M_r might be around 10k or higher.

The major s-TGI fractions of M_r <1.3k were pooled, and dialyzed against 8.5 M urea and subjected to isoelectric electrophoresis in a cyrindrical polyacrylamide gel containing 2% Ampholine carrier ampholytes (pH 3.5-10) and 8.5 M urea (Fig. II-11). s-TGI activity

Fig. II-11

was again separated into two peaks of pI 7.5 and 9.5, respectively. These results indicated that s-TGI in rabbit serum can be separated into two major components (apparent M_r <1.3k, pI 7.5 & apparent M_r <1.3k, pI 9.5) and one minor component of M_r 40k, and that they form protein complexes of M_r 150-300k with a carrier protein(s) in the serum.

Immuno-Chemical Analysis

In order to identify s-TGI-binding protein(s), immunological analysis was carried out with the use of sheep antisera against several kinds of rabbit serum proteins. The antisera used were anti-rabbit IgG (Cappel), anti-rabbit (IgA + IgG + IgM) (Cappel), anti-rabbit alpha$_2$-macroglobulin (Cappel) and anti-whole rabbit serum (Cappel) antisera of sheep. As the control serum normal sheep serum (Cappel) was used. For the analysis, rabbit serum was mixed with each of the anti-sera or normal serum and incubated at 4°C for 2 days. After the incubation, immuno-precipitate was removed by centrifugation, and s-TGI activity in the resulting supernatant was determined (Table II-I). Both anti-rabbit whole serum and anti-

Table II-I

rabbit immunoglobulin (IgA + IgG + IgM) antisera significantly decreased s-TGI activity of rabbit serum, but not anti-rabbit IgG and anti-alpha$_2$-macroglobulin antisera as well as the normal sheep serum. These results suggested that s-TGI is bound with IgA or IgM in the serum.

Purification of s-TGI in Rabbit Serum

Normal rabbit serum (500 ml) was added with glycerol to make a final concentration of 30% (w/v) and diluted with 10 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 30% glycerol (HCMG buffer (pH 7.5)) to make a final volume of 2 liters. The diluted rabbit serum was applied to a DEAE-Toyopearl 650M column (5

x 50 cm) equilibrated with HCMG buffer (pH 7.5). The charged column was washed with an enough volume of HCMG buffer (pH 7.5) until the absorbance at 280 nm of the eluate became negligible, and then the proteins adsorbed to the column were eluted with a linear gradient of NaCl from 0 to 1 M in HCMG buffer (pH 7.5) (Figure II-12). Growth

Fig. II-12

inhibitory activity toward RSV-BRL cells (s-TGI activity) was eluted mostly at NaCl concentrations of 100-300 mM, and additionally at 500 mM, 700 mM and 850 mM. The major growth inhibitory fractions eluted at 100-300 mM NaCl and the minor ones at 700 mM and at 850 mM specifically inhibited the growth of RSV-BRL cells (s-TGI), whereas the active fraction eluted 500 mM NaCl appeared to equally inhibit the growth of BRL and RSV-BRL cells.

The major s-TGI fractions (fraction Nos. 8-11) were pooled, and the pooled material (480 ml) was concentrated to 50 ml by ultrafiltration through YM5 membrane (M_r cutoff: 5,000; 76 mm diameter) with stirring at 50 rpm and at 4°C in a Amicon Diaflo cell. The concentrated s-TGI was diluted to 400 ml with HBS, and reconcentrated to 50 ml under the same condition as above. The reconcentrated s-TGI fraction was decanted and saved. The YM5 membrane was rinsed with HBS, and the rinse was combined with the reconcentrated s-TGI fraction. s-TGI adsorbed to the membrane was extracted with 50 ml of 1 M acetic acid in the Diaflo cell at 300 rpm and 4°C for 30-60 min (acetic acid extract). The combined s-TGI

fraction of the concentrate and the rinse was again diluted to 400 ml with HBS and then concentrated to 50 ml as above. s-TGI was reextracted with 1 M acetic acid from the membrane. The procedures of dilution, concentration and acetic acid extraction were repeated once more. The three acetic acid extracts of s-TGI were combined. Most s-TGI activity was recovered in the combined extract. The extract showed 50% growth inhibition of RSV-BRL cells at a dose of 0.03 A_{280}/ml. s-TGI was purified about 6-fold by this acetic acid extraction.

The acetic-acid extracted s-TGI fraction (50 ml) was dialyzed against 0.5 M acetic acid and then subjected to molecular-sieve chromatography on a Cellulofine GCL-300-m column (2.6 x 96 cm) equilibrated with 0.5 M acetic acid (Fig. II-13). s-TGI activity was

Fig. II-13

separated into two peaks in fractions of M_r >90k (void fractions) and M_r <1.3k, respectively. The low M_r s-TGI (apparent M_r <1.3k) was effectively separated from contaminating proteins, but not the s-TGI at void fractions. The low M_r s-TGI was non-dialyzable against dialysis membrane.

The low M_r s-TGI fractions (fraction Nos. 48-52) were pooled, added with acetic acid to make a final concentration of 1 M, and subjected to reverse-phase high performance liquid chromatography (HPLC) on a Ultron N-C8 column (4.6 x 150 mm) equilibrated with 1 M acetic acid. The charged column was washed with 1 M acetic acid, and

then the proteins adsorbed to the column were eluted with three steps of acetonitrile gradient (0-20% for 20 min, 20-60% for 120 min and 60-90% for 20 min) in 1 M acetic acid monitoring absorbance at 280 nm (Fig. II-14). s-TGI was eluted forming two activity peaks at

Fig. II-14

53% (v/v) and 70% acetonitrile, respectively. These two peak fractions were respectively concentrated 5-fold with a Centricon-10 microconcentrator and analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and the following silver-staining. The 53% acetonitrile fraction still showed several stained bands and the active component could not be identified. On the other hand, the 70% acetonitrile fraction showed a single band (Fig. II-15). Since the protein band moved faster than Bromophenol

Fig. II-15

Blue (BPB) as the marker dye, its exact M_r could not be calculated.

Effects of the purified s-TGI (70% acetonitrile fraction) on the growth of BRL and RSV-BRL cells are shown in Fig. II-16. The

Fig. II-16

purified s-TGI inhibited the growth of RSV-BRL cells more strongly than that of BRL cells. About 40 ng/ml of s-TGI was required to

inhibit the growth of RSV-BRL cells by 50%.

The purification of s-TGI is summarized in Table II-II.

Table II-II

Stability of s-TGI

The stability of s-TGI was tested using the partially purified s-TGI preparation obtained by molecular-sieve chromatography in the presence of 0.5 M acetic acid (Fig. II-13, fraction Nos. 48-52). It was stable at 4°C in the presence of 8 M urea or 1 M acetic acid (pH 2.5) for at least 3 months, but labile against treatments with 5 mM dithiothreitol, 1 mg/ml trypsin and heat (Table II-III).

Table II-III

Cell Specificity

Effects of the partially purified s-TGI (Fig. II-13, fraction Nos. 48-52) on BRL, RSV-BRL and six other cell lines were examined (Table II-IV). Except for HeLa S3 (human carcinoma), all the cell

Table II-IV

lines were more or less sensitive to s-TGI. s-TGI inhibited the growth of RSV-BRL cells much more strongly than that of BRL cells, as described above. The growth of BSC-1 cells (non-transformed epithelial cells from African green monkey kidney), LLC-RK1 cells

(non-transformed epithelial cells from rabbit kidney) and YH-1 cells (non-transformed human skin fibroblasts) was inhibited to similar extents to that of BRL cells. On the other hand, HSC-3 cells (human tongue carcinoma cells) and B-32 cells (SV40-transformants of YH-1 cells) were inhibited to similar or higher extents compared with RSV-BRL cells (Table II-III). These results indicated that s-TGI is more effective for malignant cells, except for HeLa S3 cells, than non-malignant ones.

[III] Two Types of Growth Inhibitors Secreted by Rat Liver Epithelial Cells

Secretion of Growth Inhibitors by BRL Cells

It is well known that in culture most types of non-transformed cells stop growing or decrease the growth rate when the cultures reach confluency. This phenomenon is called "density-dependent growth inhibition" or "contact inhibition". There are reports indicating that several types of cells produce and secrete growth inhibitory factor (15-19, 43, 44). The secretion and accumulation of growth inhibitory factors may be one of the causes for the density-dependent growth inhibition.

BRL cells also showed the density-dependent growth inhibition, and it was more marked than that of RSV-BRL cells (Fig. III-1). In

Fig. III-1

the present study, autocrine growth regulatory factors were surveyed in the culture medium of BRL cells. BRL cells were grown to saturation in the standard medium, and the culture medium was replaced with fresh serum-free DME/F12 and further incubated for 3 days. The serum-free culture medium conditioned by BRL cells, called "conditioned medium", was pooled, supplemented with NaCl and urea to make final concentrations of 1 M and 6 M, respectively, and concentrated from 500 ml to 5 ml by ultrafiltration through Diaflo ultrafiltration membrane YM2 (M_r cutoff: 2,000) (Amicon Corp., Danvers, MA). To test the growth effect of the concentrated

conditioned medium, it was dialyzed against HBS and added to the culture media of BRL and RSV-BRL cells in the presence of 10% FCS (Fig. III-2). The growth of BRL and RSV-BRL cells was dose-

Fig. III-2

-dependently inhibited by the conditioned medium of BRL cells. Growth inhibition was higher with BRL cells than RSV-BRL cells. Both BRL and RSV-BRL cells could not grow when the serum-free conditioned medium was added to serum-free culture medium, suggesting that the conditioned medium contained more growth inhibitory activity than growth stimulatory activity.

Partial Characterization of Growth Inhibitors in Conditioned Medium

The concentrated conditioned medium of BRL cells was subjected to molecular-sieve high performance liquid chromatography (HPLC) on a TSKgel G3000SWG column in the presence of 1 M NaCl and 6 M urea (Fig. III-3). Growth inhibitory activity was separated into three

Fig. III-3

major peaks of M_r >670k (void fraction), M_r 40k and M_r 17k. The peak fractions of M_r > 670k and M_r 40k were more inhibitory for the growth of RSV-BRL cells than for that of BRL cells, but those of M_r 17k vice versa.

The concentrated conditioned medium of BRL cells was dialyzed against 0.5 M acetic acid containing 6 M urea, and then subjected to

polyacrylamide gel isoelectric electrophoresis in the presence of 8.5 M urea. The resulting fractions were assayed for the growth inhibitory activity toward BRL and RSV-BRL cells. Growth inhibitory activity toward BRL cells was recovered into two major peaks at pH 5 and pH 7, respectively (Fig. III-4). Growth inhibitory activity

Fig. III-4

toward RSV-BRL cells was recovered into two major peaks at pH 5 and pH 6, and one minor peak at pH 8 (Fig. III-5). These results

Fig. III-5

suggested that BRL cells secreted several types of growth inhibitors to the conditioned medium, and that these growth inhibitors were different in M_r, pI and cell specificity. With respect to the cell specificity, there appeared to be at least two different types of growth inhibitors in the conditioned medium of BRL cells; one is more growth inhibitory for BRL cells than RSV-BRL cells and the other vice versa. In this report the former growth inhibitor was tentatively called "conditioned medium-derived non-transformed cell growth inhibitor (c-NGI)" and the latter "conditioned medium-derived transformed cell growth inhibitor (c-TGI)".

Stability of c-NGI and c-TGI

The stability of c-NGIs and c-TGI was tested using partially purified c-NGI and c-TGI preparations obtained by molecular-sieve

HPLC (Fig. III-3). c-NGIs was stable at 4°C in the presence of 8 M urea or 1 M acetic acid (pH 2.5) for at least 3 months, but labile against treatments with 5 mM dithiothreitol and with 1 mg/ml trypsin (Table III-I). Like c-NGI, c-TGI was stable at 4°C in the presence

Table III-I

of 8 M urea or 1 M acetic acid (pH 2.5) for at least 3 months, but labile against treatments with 5 mM dithiothreitol and with 1 mg/ml trypsin (Table III-II).

Table III-II

Purification of c-NGI

The conditioned medium of BRL cells (8 liters) was concentrated to 150 ml by ultrafiltration through Diaflo YM5 membrane. During the ultrafiltration, considerable growth inhibitory activity was adsorbed to the membrane. The membrane-bound growth inhibitors were extracted with 50 ml of 1 M acetic acid containing 6 M urea and saved for another experiment. The concentrated conditioned medium was centrifuged at 10,000 x g for 15 min to remove cell debris. The resulting supernatant containing c-NGI was dialyzed against 1 M acetic acid, and subjected to a molecular-sieve chromatography on Cellulofine GCL-300-m column equilibrated with 1 M acetic acid (Fig. III-6). Growth inhibitory activity toward BRL cells (c-NGI) was

Fig. III-6

recovered forming a broad peak in a M_r 10-40k region. These fractions showed only a little growth inhibitory activity toward RSV-BRL cells (c-TGI activity).

The major c-NGI fractions (fraction Nos. 19-41) were pooled, concentrated and dialyzed against 10 mM HEPES-NaOH buffer (pH 8.0) containing 6 M urea. The dialyzed sample (100 ml) was applied to a DEAE-Toyopearl 650M column equilibrated with the same buffer as above. The charged column was washed with the 10 mM HEPES-NaOH buffer (pH 8.0) containing 6 M urea and eluted with a linear gradient of NaCl from 0 to 600 mM in the buffer (Fig. III-7). c-NGI

Fig. III-7

activity was eluted at NaCl concentrations from 50 to 200 mM. The fractions of Nos. 5-14 were collected and dialyzed against 1 M acetic acid. The dialyzed sample was subjected to reverse-phase HPLC on a LS-410K column equilibrated with 1 M acetic acid. The charged column was washed with 1 M acetic acid and eluted with linear gradients of acetonitrile from 0 to 20% (v/v) for 20 min, from 20 to 60% for 120 min and from 60 to 90% for 20 min in 1 M acetic acid (Fig. III-8). c-NGI activity was recovered into two major peaks at

Fig. III-8

acetonitrile concentrations of 35% and 65%, being separated from most of contaminating proteins.

35% (c-NGI-I) and 65% acetonitrile (c-NGI-II) fractions were respectively concentrated 5-fold with a Centricon-10 (Amicon), and analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and the following silver-staining (Figs. III-9 & III-10). Both c-NGI-I and c-NGI-II fractions showed a single protein

Fig. III-9

Fig. III-10

band. The M_rs of c-NGI-I and c-NGI-II were estimated to around 53k and 20k, respectively.

Effects of purified c-NGI-I and c-NGI-II on the growth of BRL and RSV-BRL cells were examined (Figs. III-11 & III-12). Purified

Fig. III-11

Fig. III-12

c-NGI-I and c-NGI-II inhibited the growth of BRL cells more strongly than that of RSV-BRL cells. The amount of c-NGI-I required for 50% growth inhibition of BRL cells was about 12 ng/ml, and that of c-NGI-II was about 15 ng/ml.

The purifications of c-NGI-I and c-NGI-II are summarized in

Table III-III.

Table III-III

Cell Specificity

Effects of the purified c-NGI-I and c-NGI-II on the growth of BRL, RSV-BRL and six other cell lines were examined (Table III-IV).

Table III-IV

c-NGI-I significantly inhibited the growth of HSC-3 cells, but hardly those of other cell lines. c-NGI-II inhibited the growth of LLC-RK1 and B-32 cells in addition to HSC-3 cells. The two growth inhibitors appeared to have different cell specificities.

Purification of c-TGI

The growth inhibitors adsorbed to the YM5 membrane during the ultrafiltration of the conditioned medium was extracted with 50 ml of 1 M acetic acid containing 6 M urea, as described above. This extract was subjected to molecular-sieve chromatography on Toyopearl HW55C column (2.6 x 95 cm) equilibrated with 1 M acetic acid containing 6 M urea (Fig. III-13). c-NGI activity was mostly eluted

Fig. III-13

in a M_r range of 10-40k forming a large peaks. These peak fractions also showed c-TGI activity. There was an additional small peak at M_r

1.3k, which showed both c-NGI and c-TGI activities at the same level.

The major active fractions (fraction Nos. 15-34) were pooled, concentrated by ultrafiltration through Diaflo YM5 membrane and dialyzed against 0.5 M acetic acid containing 6 M urea. The resultant dialyzed sample was subjected to molecular-sieve chromatography on a Toyopearl HW55C column (2.6 x 95 cm) equilibrated with 0.5 M acetic acid containing 6 M urea (Fig. III-14). The majority of c-NGI activity was eluted in the same M_r range

Fig. III-14

as in the preceding molecular-sieve chromatography (M_r 10-40k). c-TGI activity was observed in the fractions of apparent M_r <1.3k, where contaminating proteins and c-NGI activity were hardly detected.

The c-TGI fractions of apparent M_r <1.3k (fraction Nos. 49-57) were collected, concentrated by ultrafiltration and dialyzed against 1 M acetic acid. It should be noted that like s-TGI from rabbit serum, c-TGIs did not pass through both membranes for ultrafiltration and for dialysis in spite of the apparent M_r of <1.3k. The dialyzed sample was subjected to reverse-phase HPLC on a Ultron N-C8 column (4.6 x 150 mm) equilibrated with 1 M acetic acid. The charged column was washed with 1 M acetic acid and eluted by linear gradients of acetonitrile from 0 to 20% for 20 min, 20-60% for 120 min and 60-90% for 20 min in 1 M acetic acid (Fig. III-15).

Fig. III-15

c-TGI activity was eluted in the two peak fractions at acetonitrile concentrations of 36% (c-TGI-I) and 42% (c-TGI-II), respectively.

The 36% (c-TGI-I) and 42% acetonitrile (c-TGI-II) fractions were respectively concentrated 5-fold with a Centricon-10 concentrator (Amicon), and analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and the following silver-staining (Figs. III-16 & III-17). Both c-TGI-I and c-TGI-II

Fig. III-16

Fig. III-17

fractions showed a single band with the same mobility. The M_rs of c-TGI-I and c-TGI-II could not be calculated because they moved faster than BPB as the marker dye. Their electrophoretic mobilities were similar to that of s-TGI purified from rabbit serum.

Effects of these purified c-TGI-I and c-TGI-II on the growth of BRL and RSV-BRL cells were examined (Figs. III-18 & III-19). Both

Fig. III-18

Fig. III-19

c-TGIs inhibited the growth of RSV-BRL cells more strongly than that

of BRL cells. About 6 ng/ml of c-TGI-I and 1.7 ng/ml of c-TGI-II were required for 50% growth inhibition of RSV-BRL cells, respectively.

The purification of c-TGI-I and c-TGI-II is summarized in Table III-V.

Table III-V

Cell Specificity

Effects of the purified c-TGI-I and c-TGI-II on the growth of BRL, RSV-BRL and six other cell lines were examined (Table III-VI).

Table III-VI

Both c-TGI-I and c-TGI-II significantly inhibited the growth of HSC-3 and LLC-RK1 cells, but their activities on other cell lines were very low, if any.

Comparison of Properties of Growth Inhibitors Purified from Rabbit Serum and BRL-Conditioned Medium

Properties of the growth inhibitors purified in this study are summarized in Table III-VII. All the inhibitors were stable against

Table III-VII

acid and a denaturing reagent (urea), but unstable against a reducing reagent (DTT) and trypsin. These properties are common to

the growth inhibitors purified from rat serum (30). From the properties shown, it may be concluded that s-TGI from rabbit serum and c-TGIs from BRL-conditioned medium are identical or closely relative to each other. However, TGIs and NGIs are clearly different from each other in M_r and target cell specificity.

DISCUSSION

In 1911, Rous (45) found the presence of tumorigenic virus in an extract from a sarcoma which spontaneously occurred in chicken. Since then, this virus, later named Rous sarcoma virus (RSV), has been used as the representative oncogenic virus in numerous studies on cancer. Recent studies revealed that the oncogene of RSV, called v-src, encodes a protein of M_r 60,000 daltons ($p60^{v\text{-}src}$), which can phosphorylate tyrosine residures in its own and other kinds of proteins (8-9). However, the mechanism in which $p60^{v\text{-}src}$ or its autophosphorylated product $pp60^{v\text{-}src}$ transforms cells and maintains the transformed phenotype remains to be clarified.

BRL cells, which was originally obtained from Buffalo rat liver in 1968 by Coon (23), are one of well-known liver cell line. BRL cells express epithelial and parenchymal-like appearance in microscopic and electronmicroscopic observation.

We have previously transformed BRL cells with RSV. The RSV-transformed cells (RSV-BRL) were tumorigenic when injected into syngeneic rats. In addition, RSV-BRL cells showed the following properties: morphological changes, anchorage-independent growth and loss of contact inhibition. It is doubtless that these changes of cellular properties were caused from the changes of cellular proteins by the RSV-transformation. We have previously found that fibronectin, which is the major cell-cell binding protein on cell surface, disappeared by the RSV-transformation of BRL cells (to be published). In the present study, the analysis by two-dimensional polyacrylamide gel electrophoresis showed that the amounts of at

least 29 proteins increased or deceased as BRL cells were transformed by RSV. As a whole, RSV-BRL cells had higher amounts of nonhistone nuclear proteins than BRL cells. This result was in accordance with our previous finding that the content of nonhistone nuclear protein is higher in cancer tissues than normal ones (46). The protein of M_r 45k and pI 6.3, which increased in content by the RSV-transformation, is likely to be actin, the major component of cytoskeleton. It has been reported that neoplastic transformation is accompanied by disorganization and qualitative and quantitative changes of cytoskeletal proteins (47-50). More detailed studies area necessary to clarify the biological meanings of the changes of cellular proteins by the RSV-transformation.

When RSV is infected to host cells such as chicken embryo fibroblasts (CEF), its RNA genome is converted to DNA by RNA-dependent DNA polymerase (reverse transcriptase) and then inserted into chromosomal DNA of the hosts. Using the gene expression system of the host, the inserted viral genome express a precursor of the internal structural proteins and reverse transcriptase ($Pr180^{\underline{gag\text{-}pol}}$), a precursor of the envelope ($Pr90^{\underline{env}}$), a precursor of the internal structural proteins ($Pr76^{\underline{gag}}$), a oncogene product ($p60^{v\text{-}\underline{src}}$) and some mature proteins (51). These proteins, except for $p60^{v\text{-}\underline{src}}$, are used for the production of progeny viruses. However, RSV-BRL cells did not produce any progeny virus into culture medium. The immuno-blotting analysis confirmed that at least $Pr76^{\underline{gag}}$ was being appreciably expressed in the transformed cells. Although other viral proteins including $p60^{v\text{-}\underline{src}}$ were undetectable in RSV-BRL cells, this

result does not eliminate the possibility that a very low level of expression of $p60^{v\text{-}src}$ is sufficient for the maintenance of the transformed phenotype.

The growth and division of normal cells are well regulated *in vivo*. The control mechanism of cell growth is one of the most fundamental subjects in biology and closely related to the problem of cancer. It is now accepted that two groups of protein factors, growth factors and growth inhibitors, are involved in the control mechanism of cell growth. Recently much progress has been attained in the chemical characterization of growth factors and in understanding of their action mechanisms (52-55). Although the negative growth regulation mechanism by growth inhibitors was first proposed in the early 1950', it was not accepted for a long time because of difficulties in purifying growth inhibitors. For overall understanding of the control mechanism of cell growth, it seems essential to know molecular properties of growth inhibitors, the sites for their production and action and their growth inhibition mechanisms.

There are some reports indicating that sera of normal animals contain growth inhibitors (20-22, 56) as well as growth factors. Onda *et al*. (56) have reported that $alpha_1$-globulin fraction from normal rat plasma inhibited a regenerating proliferation of hepatocytes *in vivo* after partial hepatectomy and that this mitosis-suppressing activity was lost by heating at 65°C for 30 min. Harrington *et al*. (21) reported the presence of a growth inhibitor for normal but not neoplastic hepatocytes in $alpha_2$-globulin

fraction of normal calf serum. On the other hand, Pigott et al. (20) reported the presence of other types of growth inhibitors in normal human plasma-derived serum (PDS), which inhibited the growth of MCF-7 cells derived from a malignant breast pleural effusion but not that of HBL-100 cells established from human milk cells of a normal donor. These results suggest the existence of different types of growth inhibitors in sera of normal animals.

Recently we began to survey growth inhibitors in sera of various kinds of animals using BRL and RSV-BRL cells as non-transformed and transformed target cells of epithelial origins. Previous studies indicated that there are at least two types of growth inhibitors in sera, different in the specificity for BRL and RSV-BRL cells (24). Growth inhibitors having a specificity for BRL cells was highly purified from rat serum and human platelets (30). In the present study we attempted to characterize a growth inhibitor in rabbit serum, which had a specificity for RSV-BRL cells (transformed cell-growth inhibitor, s-TGI). In rabbit serum, s-TGI existed as high M_r proteins complexes, and they were dissociated into active components with apparent M_r <1.3k and with pI 7.5 and 9.5 in the presence of 1 M NaCl and 6 M urea. Immunological analysis suggested that s-TGI-binding protein(s) in rabbit serum might be IgA or IgM or both (Table II-I). We have reported that the growth inhibitor in rat serum existed forming a similar protein complex (apparent M_r 220k, pI 5.2) (24). In addition, epidermal growth factor (EGF) and insulin-like growth factors (IGFs or Somatomedins) have been reported to exist forming complexes with high-M_r-weight

carrier proteins in some situations (57, 58). However, the carrier proteins for these factors have not been identified.

s-TGI was purified to show a single band on SDS-polyacrylamide gel electrophoresis. the apparent M_r of s-TGI was lower than 1.3k (Vitamin B12) on molecular-sieve chromatography and lower than 0.67k (Bromophenol Blue, BPB) on the electrophoresis. However, s-TGI did not pass through dialysis membrane (M_r cutoff: about 10,000) and Diaflo YM10 membrane (M_r cutoff: 10,000). Therefore, its actual M_r seems to be around 10-15k. The purified s-TGI effectively inhibited the growth of malignant (transformed or cancerous) cell lines such as RSV-BRL, HSC-3 and B-32 except for HeLa-S3. All non-transformed cell lines tested (BRL, BSC-1, LLC-RK1 and YH-1) were insensitive or less sensitive to s-TGI (Table II-IV). This target cell specificity is in contrast to those of the growth inhibitors purified from rat serum, which was more growth inhibitory for non-transformed cells than transformed ones (30). Tumor necrosis factor (TNF) is a well-known antitumor factor (59, 60). It is produced by macrophages into serum when animals pretreated with Bacillus Calmette-Guérin (BCG) are injected with lipopolysaccharide (LPS). There are reports indicating that a TNF-like antitumor factor exists in sera of non-treated normal animals (61, 62). s-TGI purified from normal rabbit serum is similar to TNF in target cell specificity, but they are different from each other in the following properties: TNF is an acid-labile protein with M_r 40-50k (18k on SDS-polyacrylamide gel electrophoresis) and pI 5.1, whereas s-TGI is an acid-stable protein with M_r 10-15k and with pI 7.5 and 9.5. Few s-TGI-like growth

inhibitor has been reported so far. The amino acid sequence of s-TGI is currently under investigation.

During the last several years considerable data have been accumulated indicating the importance of the autocrine negative control mechanism of cell growth. Holley et al. (15, 16) purified a growth inhibitor of M_r 25,000 secreted from African green monkey kidney cells (BSC-1). This growth inhibitor effectively inhibited the growth of its producer (BSC-1) and some other non-transformed and transformed epithelial cell lines. Tucker et al. (63) demonstrated that the BSC-1 growth inhibitor is closely related to transforming growth factor-β (TGF-β). Hsu and Wnag (17) purified a growth inhibitor of M_r 13k from conditioned medium of 3T3 cells, which regulated the growth of its producer cells. On the other hand, Iwata et al. (18) and Fryling et al. (19) reported growth inhibitors secreted from human rhabdomyosarcoma cell line (A673). The growth inhibitors (tumor growth inhibitory factors; TIFs) were acid- and heat-stable low M_r polypeptides (M_rs 10-16k and 18-22k), and they inhibited the growth of malignant cells but stimulated that of non-malignant cells.

In the present study, we purified two types of growth inhibitors secreted from BRL cells: one was more growth inhibitory for BRL cells than for RSV-BRL cells (non-transformed cell growth inhibitor, c-NGI) and the other vice versa (transformed cell growth inhibitor, c-TGI). c-NGI was further separated into two components with M_r 53k (c-NGI-I) and M_r 20k (c-NGI-II). The growth of BRL cells was effectively inhibited by the purified c-NGI-I or c-NGI-II at 10-

20 ng/ml. Their target cell specificities and stabilities against acid are similar to those of BSC-1 growth inhibitor (15), the growth inhibitors purified from rat serum (29) and animals tissues (11-14), though there are differences in their M_r values. These growth inhibitors may belong to the same family.

c-TGI was also separated into two active components (c-TGI-I, II) by reverse-phase HPLC and individually purified to homogeneity. Both c-TGI-I and c-TGI-II effectively inhibited the growth of RSV-BRL cells at 1-6 ng/ml but hardly that of BRL cells. The chromatographic behaviors, electrophoretic mobilities and physical stabilities of c-TGIs were almost the same as those of s-TGI purified from rabbit serum, though minor differences were observed in the target cell specificity. These results suggest that s-TGI and c-TGIs may be identical or closely related to each other.

It is well known that malignant cells produce various types of growth factors capable of stimulating their own cells (self-stimulation). The growth factor production is believed to be responsible for the autonomous growth of cancer cells in vivo and their lesser requirement of exogenous growth factors for growth in culture. This type of growth control is currently called "autocrine control", distinguishing from "endocrine control" for the hormone-like action. Clearly the autocrine control model is applicable to the negative growth control mechanism by growth inhibitors. The present and previous studies indicated that different species of growth inhibitors are present in sera of various kinds of normal animals and produced by rat liver cells. Some of them seems to exist

commonly in sera and conditioned media of the cells. These results demonstrate that cell growth is regulated by both endocrine and autocrine actions of growth inhibitors in addition to growth factors.

It has been reported that BRL cells secrete a growth factor called Multiplication-Simulating Activity (MSA) or Insulin-like growth factor-II (IGF-II) (64). Our experimental model with BRL and RSV-BRL cells seems valuable to study the positive and negative growth regulatory mechanisms in the autocrine and endocrine fashions. The chemical characterization of the growth inhibitors found in this study and their growth inhibitory mechanisms are currently under investigation.

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TABLE I-I. comparison of contents of 16 kinds of cellular proteins between NP/2-ME/UREA-extracts from BRL and RSV-BRL cells

Protein	M_r(k)[1)]	pI[1)]	Change by transformation[2)]
1	120	7.0	decrease
2	100	6.1	increase
3	70	>10	increase
4	66	6.1	decrease
5	46	5.1	increase
6	46	< 5	increase
7	45	6.3	increase
8	42	8.8	decrease
9	39	8.6	decrease
10	37	>10	decrease
11	36	>10	decrease
12	34	>10	decrease
13	29	8.9	decrease
14	29	8.6	decrease
15	25	< 5	increase
16	9	5.6	increase

1) The M_r and pI of each protein were determined by two-dimensional polyacrylamide gel electrophoresis (Fig. I-3).

2) The relative content of each protein species was compared between the electrophoretic patterns of BRL and RSV-BRL cells after staining with CBB (Fig. I-3).

TABLE I-II. Comparison of contents of 14 kinds of proteins between nuclear fractions from BRL and of RSV-BRL cells

Protein	M_r(k)[1)]	pI[1)]	Change by transformation[2)]
1	>100	7.0	increase
2	72	7.5	increase
3	69	7.2	increase
4	64	6.9	increase
5	50	5.4	decrease
6	48	8.1	increase
7	46	5.8	decrease
8	45	7.5	increase
9	39	5.3	decrease
10	33	7.5	increase
11	33	7.1	increase
12	30	8.3	increase
13	26	5.3	decrease
14	25	5.5	decrease

1) The M_r and pI of each protein were determined by two-dimensional polyacrylamide gel electrophoresis (Fig. I-4)

2) The relative content of each protein species was compared between the electrophoretic patterns of BRL and RSV-BRL cells after staining with CBB (Fig. I-4).

Table II-I. Effect of antisera of sheep against rabbit serum proteins on growth inhibitory activity of rabbit serum

Addition	(μl/0.5 ml)	Relative cell number (%)	
		RSV-BRL	BRL
PBS	10	(100)	(100)
	50	(100)	(100)
Normal sheep serum	10	84	100
	50	47	84
Anti-IgG	10	70	100
	50	44	83
Anti-alpha$_2$-macroglobulin	10	84	100
	50	55	88
Anti-immunoglobulin (IgA + IgG + IgM)	10	95	100
	50	81	95
Anti-whole serum	10	98	100
	50	86	100

Experimental conditions are described in Materials and Methods.

Table II-II. Summary for purification of s-TGI from normal rabbit serum

Step	Total A_{280}	ED_{50}[1] (A_{280}/ml)	Purity	Yield (%)
Rabbit serum	25,000	1.5	(1)	(100)
DEAE-Toyopearl	3,400	0.45	3.3	45
Extract from YM5 membrane	300	0.048	21	37
Cellulofine GCL-300-m	0.5	0.0001	15,000	23
HPLC (Ultron N-C8)	<0.004	<0.00001	>150,000	5

1) Amount of protein (A_{280}) for 50% growth inhibition of RSV-BRL cells.

Table II-III. Effect of various treatments on growth-inhibitory activity of s-TGI

Addition	Relative cell number (%)	
	BRL	RSV-BRL
PBS	(100)	(100)
PBS + 5 mM dithiothreitol	86	81
PBS + 1 m/ml trypsin	97	101
s-TGI	72	40
s-TGI + 0.5 M acetic acid	69	32
s-TGI + 5 mM dithiothreitol	92	97
s-TGI + 1 mg/ml trypsin	101	104

BRL and RSV-BRL cells were cultured in 1.0 ml of 10%FCS+DME/F12, to which 80 μl of each sample was added as indicated. s-TGIs had been treated with acetic acid, dithiothreitol or trypsin as described in Materials and Methods. In the control, s-TGI was replaced with PBS. The cell number with PBS was regarded as 100.

Table II-IV. Effect of purified s-TGI on the growth of eight kinds of non-malignant or malignant cell lines

Cell line tested	Cell number (% of control)
Non-malignant	
BRL	75
BSC-1	66
LLC-RK1	57
YH-1	64
Malignant	
RSV-BRL	23
HeLa-S3	101
HSC-3	30
B-32	16

Table III-I. Effect of various treatments on growth-inhibitory activity of c-NGIs

Addition	Relative cell number (%)	
	BRL	RSV-BRL
PBS	(100)	(100)
PBS + 5 mM dithiothreitol	86	81
PBS + 1 m/ml trypsin	97	101
c-NGIs	32	72
c-NGIs + 0.5 M acetic acid	27	71
c-NGIs + 5 mM dithiothreitol	86	95
c-NGIs + 1 mg/ml trypsin	100	100

BRL and RSV-BRL cells were cultured in 1.0 ml of 10%FCS+DME/F12, to which 80 μl of each sample was added as indicated. c-NGIs had been treated with acetic acid, dithiothreitol or trypsin as described in Materials and Methods. In the control, c-NGI was replaced with PBS. The cell number with PBS was regarded as 100.

Table III-II. Effect of various treatments on growth-inhibitory activity of c-TGIs

Addition	Relative cell number (%)	
	BRL	RSV-BRL
PBS	(100)	(100)
PBS + 5 mM dithiothreitol	86	81
PBS + 1 m/ml trypsin	97	101
c-TGIs	68	35
c-TGIs + 0.5 M acetic acid	62	35
c-TGIs + 5 mM dithiothreitol	95	92
c-TGIs + 1 mg/ml trypsin	100	101

BRL and RSV-BRL cells were cultured in 1.0 ml of 10%FCS+DME/F12, to which 80 μl of each sample was added as indicated. c-TGIs had been treated with acetic acid, dithiothreitol or trypsin as described in Materials and Methods. In the control, c-TGI was replaced with PBS. The cell number with PBS was regarded as 100.

Table III-III. Summary for purification of c-NGI-I and c-NGI-II from conditioned medium of BRL cells

Step	Total A_{280}	ED_{50}[1] (A_{280}/ml)	Purity	Yield (%)
Concentrated conditioned medium	300	0.02	(1)	(100)
Cellulofine GCL-300-m	95	0.005	4	119
DEAE-Toyopearl	3.8	0.0005	40	48
c-NGI-I:				
HPLC (LS-410K)	<0.004	<0.00001	>2,000	3
c-NGI-II:				
HPLC (LS-410K)	<0.004	<0.00001	>2,000	3

1) Amount of protein (A_{280}) required for 50% growth inhibition of BRL cells.

Table III-IV. Effect of purified c-NGI-I and c-NGI-II on growth of eight kinds of non-malignant or malignant cell lines

Cell line	Cell number (% of control)	
	c-NGI-I	c-NGI-II
Non-malignant		
BRL	22	29
BSC-1	73	75
LLC-RK1	79	45
YH-1	80	77
Malignant		
RSV-BRL	71	65
HeLa-S3	111	107
HSC-3	53	33
B-32	92	50

Table III-V. Summary for purification of c-TGI-I and c-TGI-II from conditioned medium of BRL cells

Step	Total A_{280}	ED_{50}[1] (A_{280}/ml)	Purity	Yield (%)
Extracted fraction	57	0.05	(1)	(100)
1st Toyopearl HW54C	20	0.025	2	73
2nd Toyopearl HW55C	1.1	0.001	50	100
c-TGI-I:				
HPLC (Ultron N-C8)	<0.002	<0.00001	>5,000	18
c-TGI-II:				
HPLC (Ultron N-C8)	<0.002	<0.00001	>5,000	18

1) Amount of protein (A_{280}) required for 50% growth inhibition of RSV-BRL cells.

Table III-VI. Effect of purified c-TGI-I and c-TGI-II on growth of eight kinds of non-malignant or malignant cell lines

Cell line	Cell number (% of control)	
	c-TGI-I	c-TGI-II
Non-malignant		
BRL	55	57
BSC-1	77	74
LLC-RK1	61	54
YH-1	80	71
Malignant		
RSV-BRL	34	30
HeLa-S3	92	79
HSC-3	43	52
B-32	81	87

Table III-VII. Comparison of properties among s-TGI, c-TGI-I, c-TGI-II, c-TGI-II, c-NGI-I and c-NGI-II

	s-TGI	c-TGI-I	c-TGI-II	c-NGI-I	c-NGI-II
M_r	<1.3k[1)] <10k[2)]	<1.3k[1)] <10k[2)]	<1.3k[1)] <10k[2)]	17k[1)] 53k[2)]	17k[1)] 20k[2)]
Stability					
1 M acetic acid	stable	stable	stable	stable	stable
8 M urea	stable	stable	stable	stable	stable
5 mM DTT	unstable	unstable	unstable	unstable	unstable
1 mg/ml trypsin	unstable	unstable	unstable	unstable	unstable
Target cells	RSV-BRL HSC-3 B-32	RSV-BRL HSC-3	RSV-BRL HSC-3	BRL HSC-3	BRL HSC-3 LLC-RK1 B-32

1) Apparent M_r values determined by molecular-sieve chromatography. All the growth inhibitors were not passed through dialysis membrane and Diaflo YM10 (M_r 10,000).

2) M_r values determined by SDS-polyacrylamide gel electrophoresis.

Fig. I-1. Photomicrographs (x 100) of BRL (Normal) and RSV-BRL (Transformed) cells.

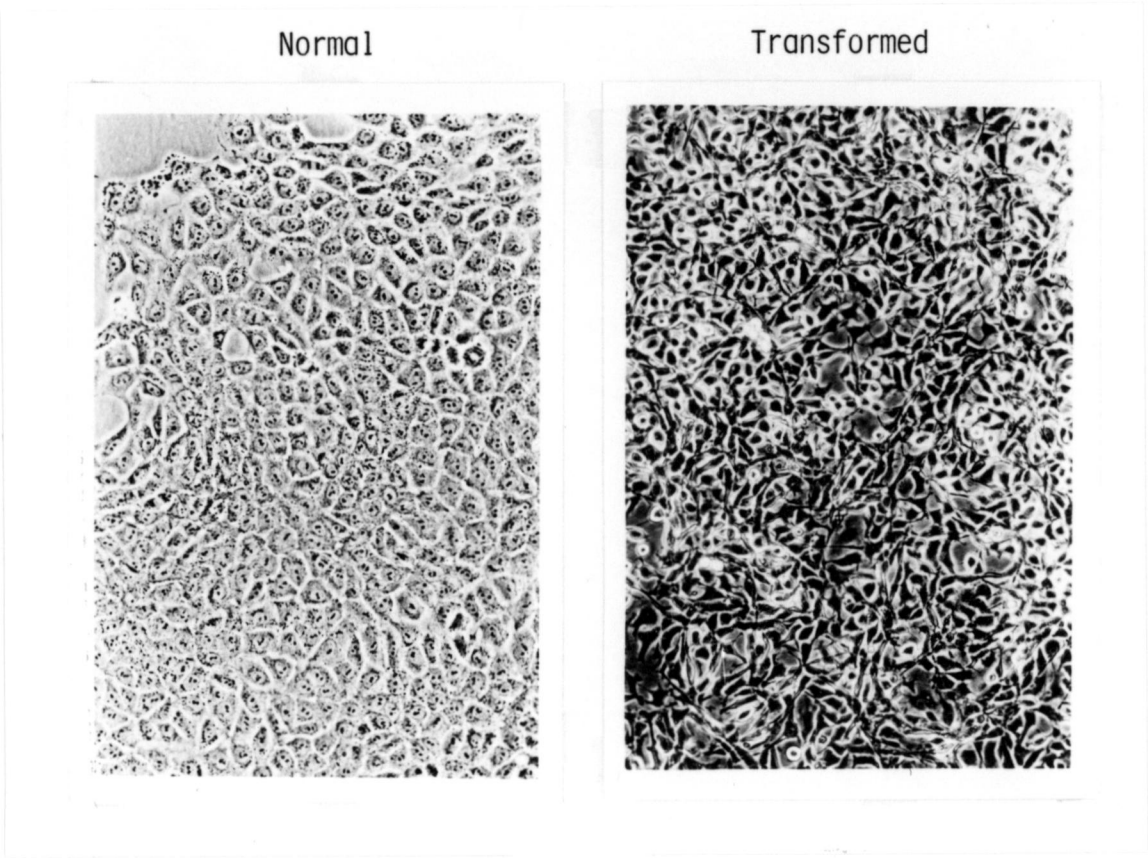


Fig. I-2. Two-dimensional polyacrylamide gel electrophoresis of total cellular proteins extracted from BRL (normal) and RSV-BRL cells (transformed).

Experimental conditions are described in Materials and Methods.

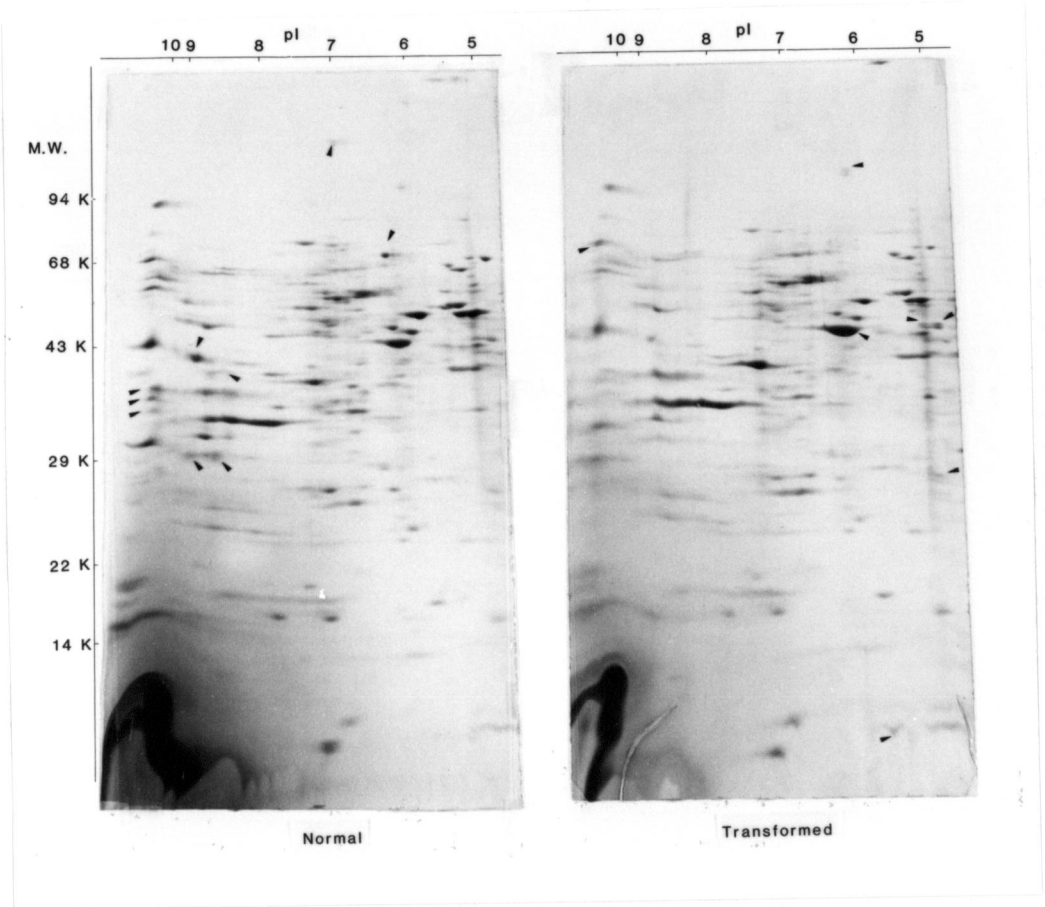


Fig. I-3. Two-dimensional polyacrylamide gel electrophoresis of nuclear proteins of BRL (normal) and RSV-BRL cells (transformed).

Experimental conditions are described in Materials and Methods.

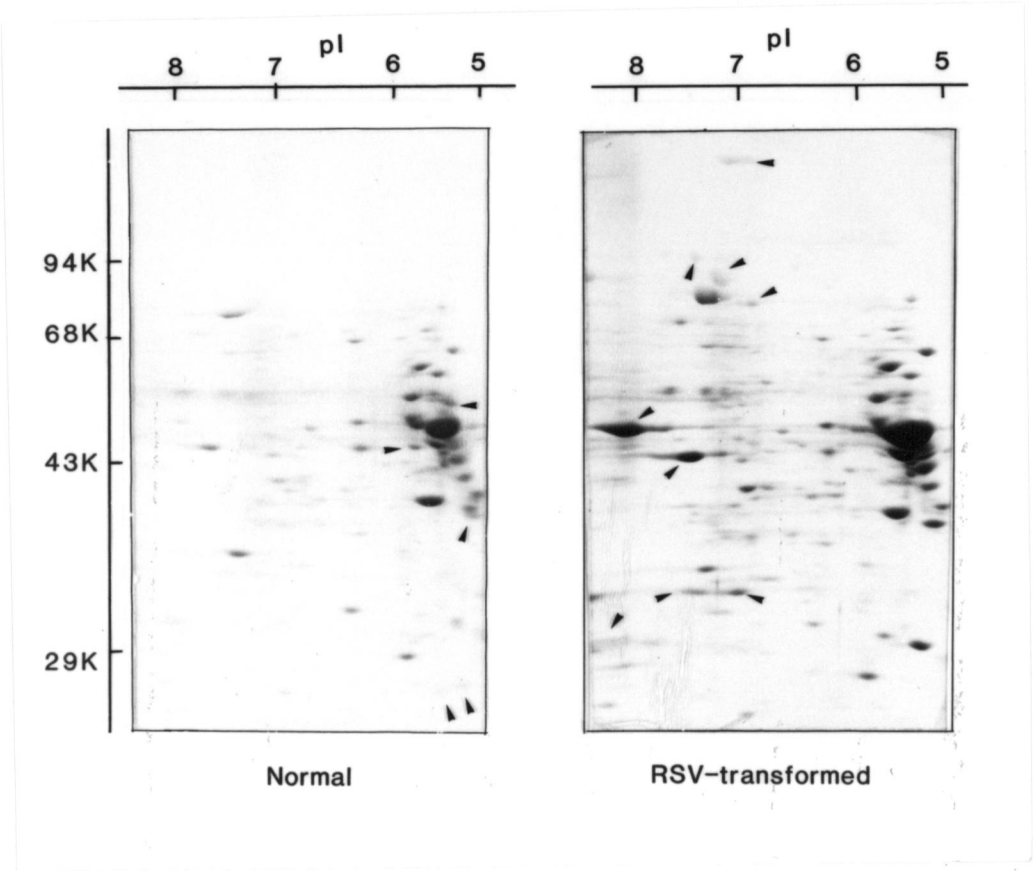


Fig. I-4. Analysis of intracellular RSV proteins by immuno-blotting.

Total cellular proteins of chicken fibroblasts (CEF) (Chicken, -), RSV-transformed CEF (Chicken, +), BRL (Rat liver, -) and RSV-BRL (Rat liver, +) were separated by SDS-polyacrylamide gel electrophoresis. Other experimental conditions are described in Materials and Methods.

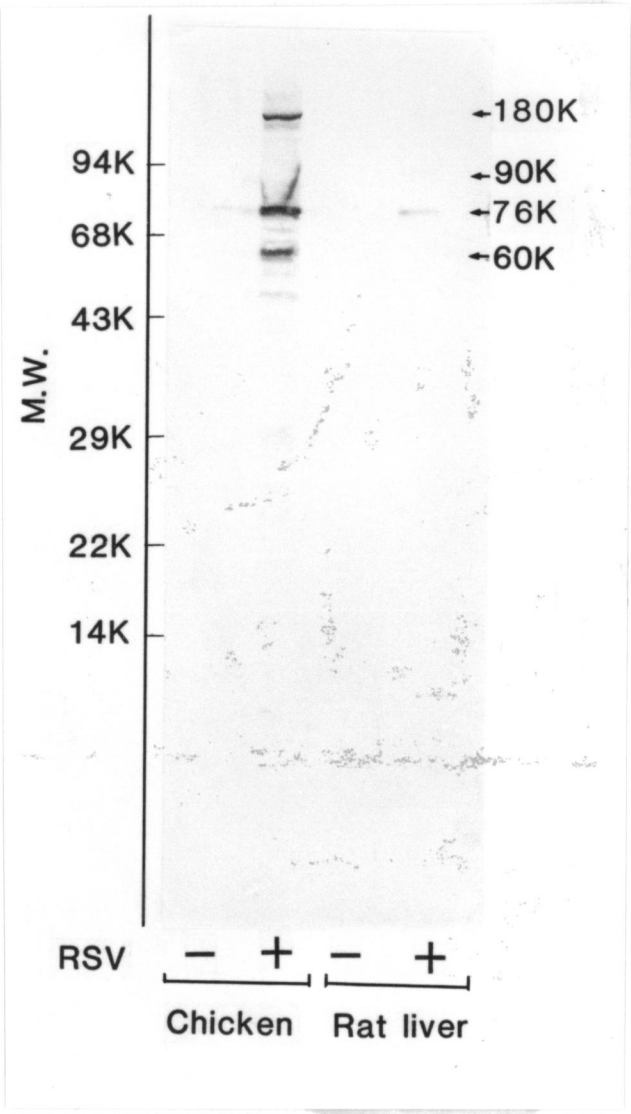


Fig. II-1. Growth response of BRL (○) and RSV-BRL (●) to various concentrations of fetal calf serum. DME/F12 was used as the basal medium.

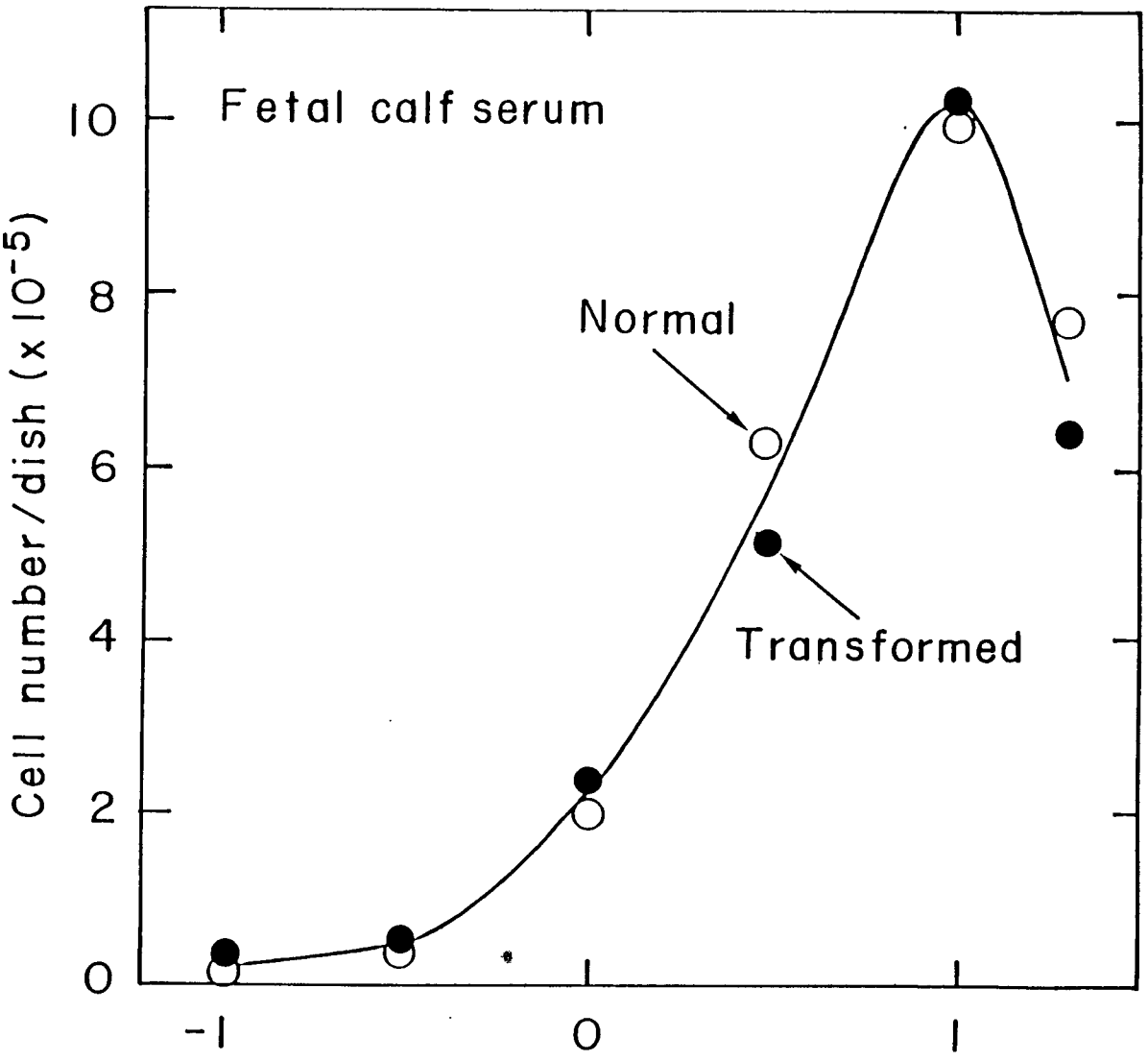


Fig. II-2. Growth response of BRL (○) and RSV-BRL (●) to various concentrations of calf serum. DME/F12 was used as the basal medium.

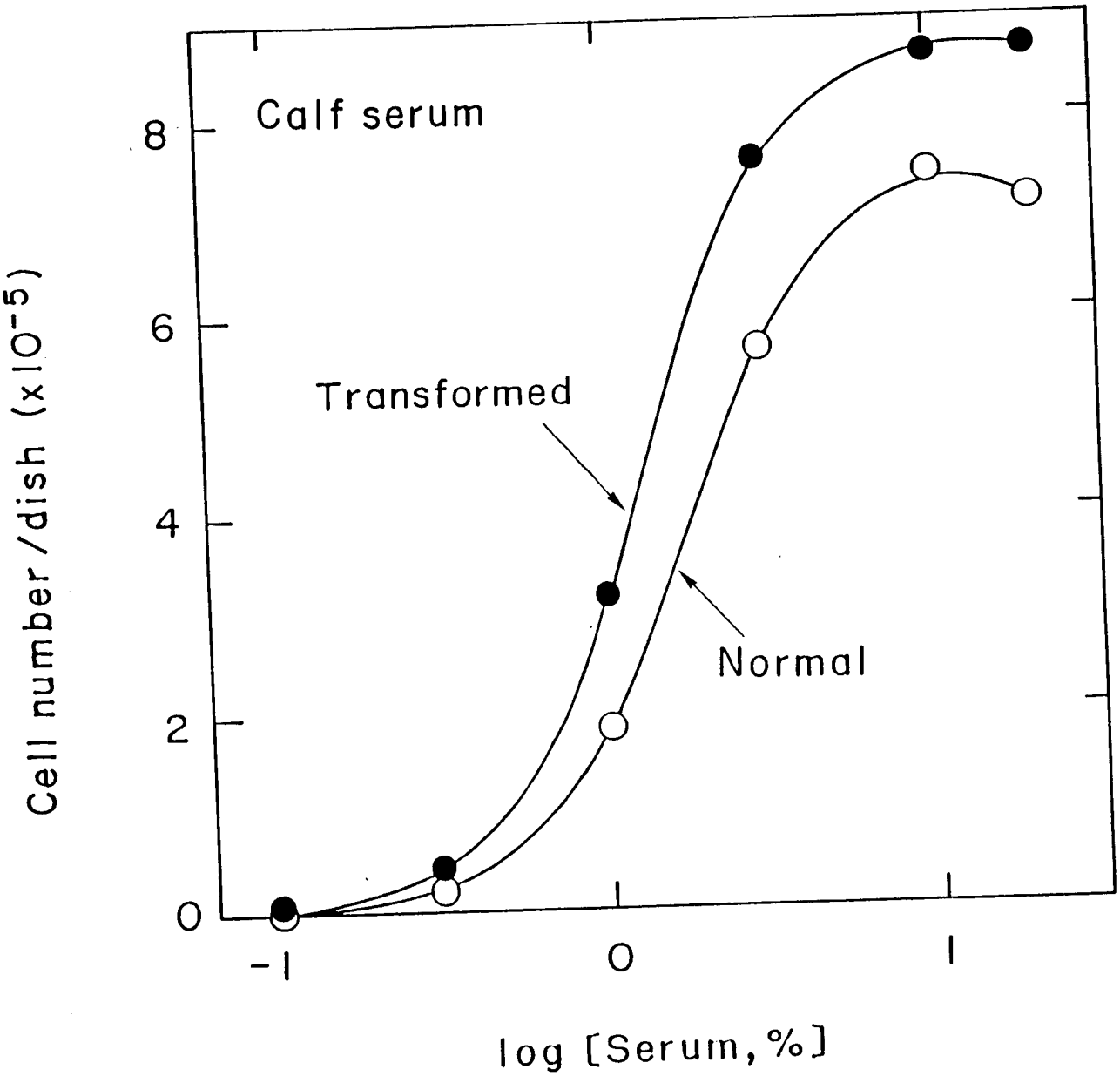


Fig. II-3. Growth response of BRL (○) and RSV-BRL (●) to various concentrations of horse serum. DME/F12 was used as the basal medium.

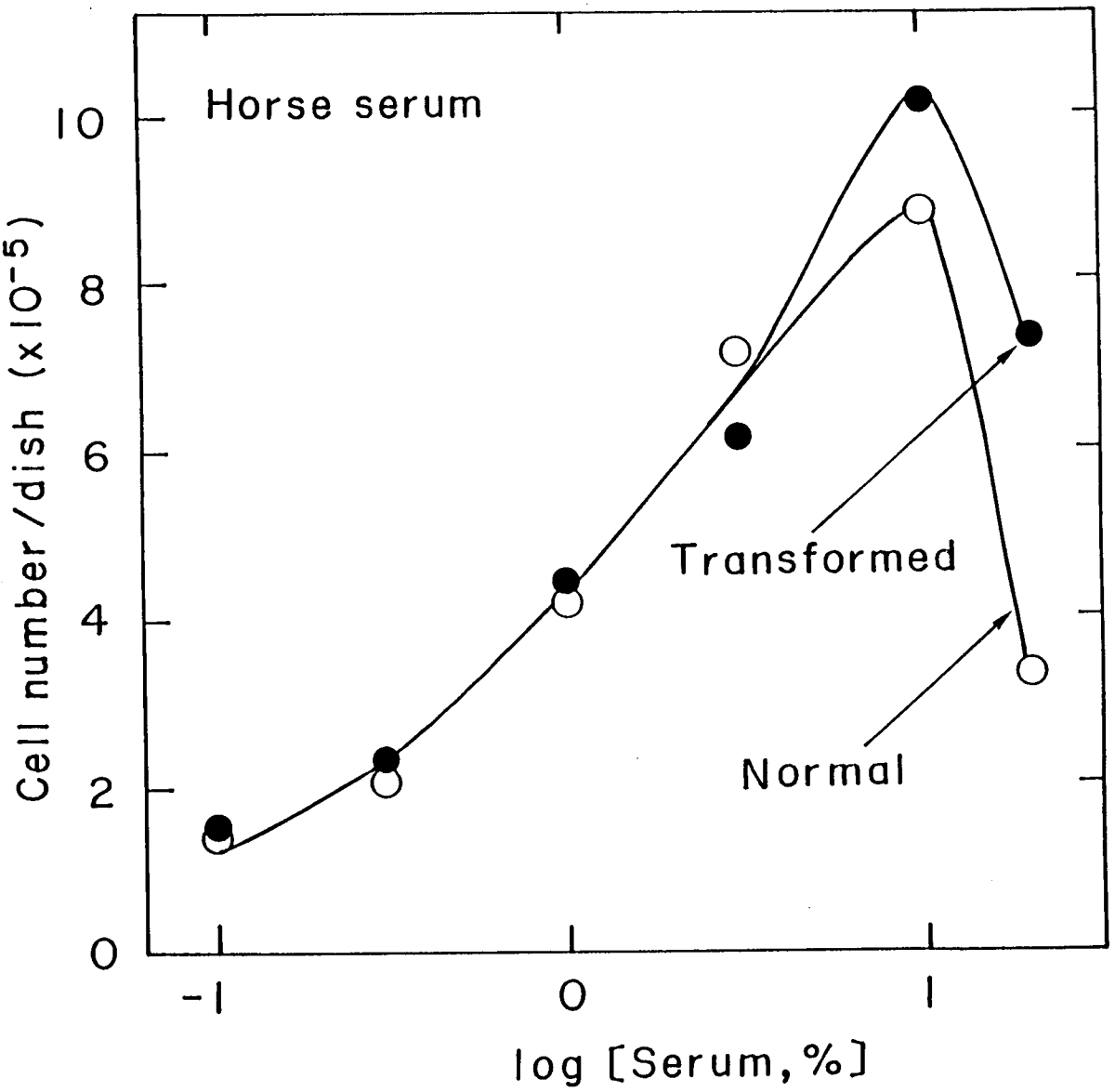


Fig. II-4. Growth response of BRL (○) and RSV-BRL (●) to various concentrations of rat serum. DME/F12 was used as the basal medium.

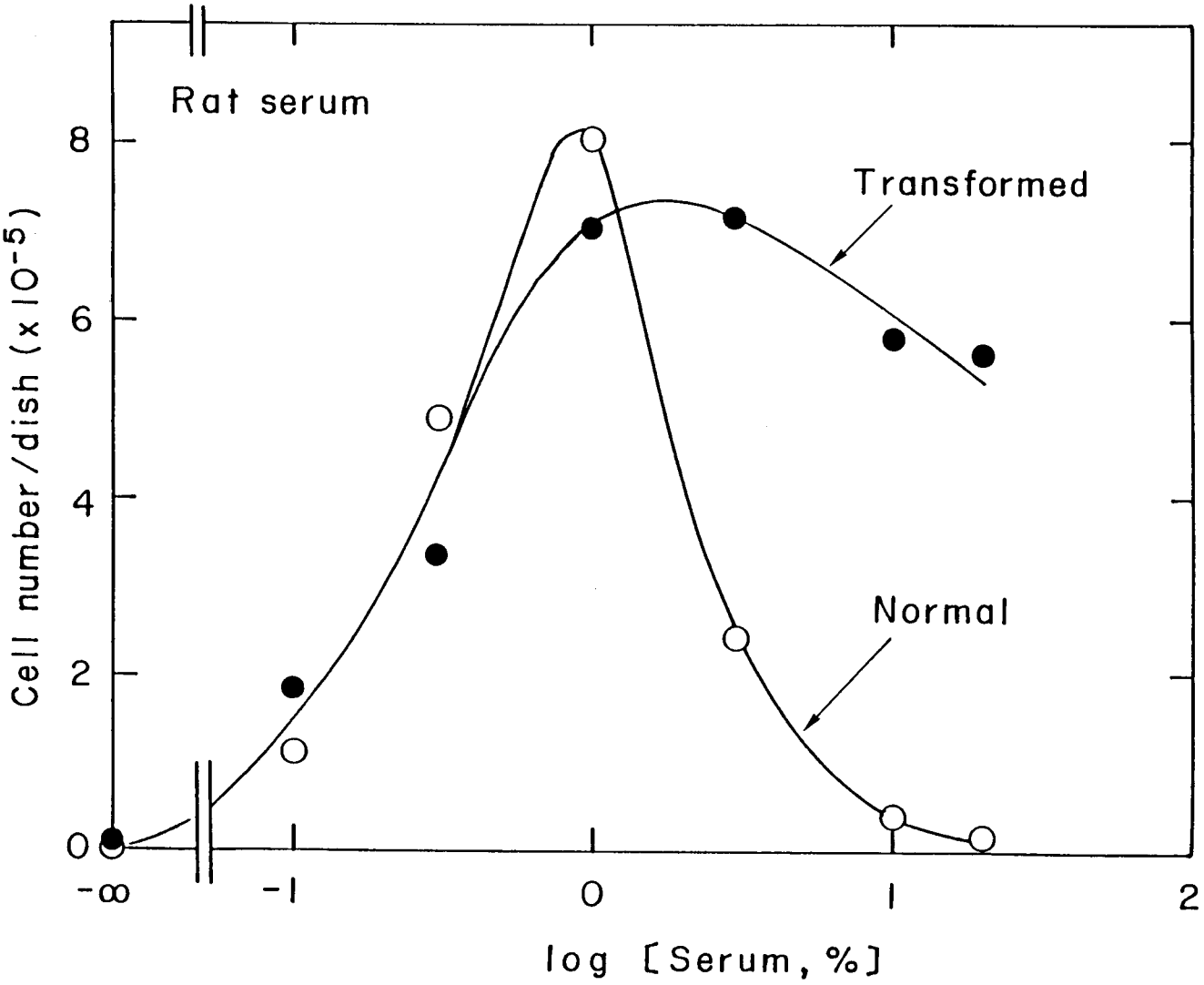


Fig. II-5. Growth response of BRL (○) and RSV-BRL (●) to various concentrations of rabbit serum. DME/F12 was used as the basal medium.

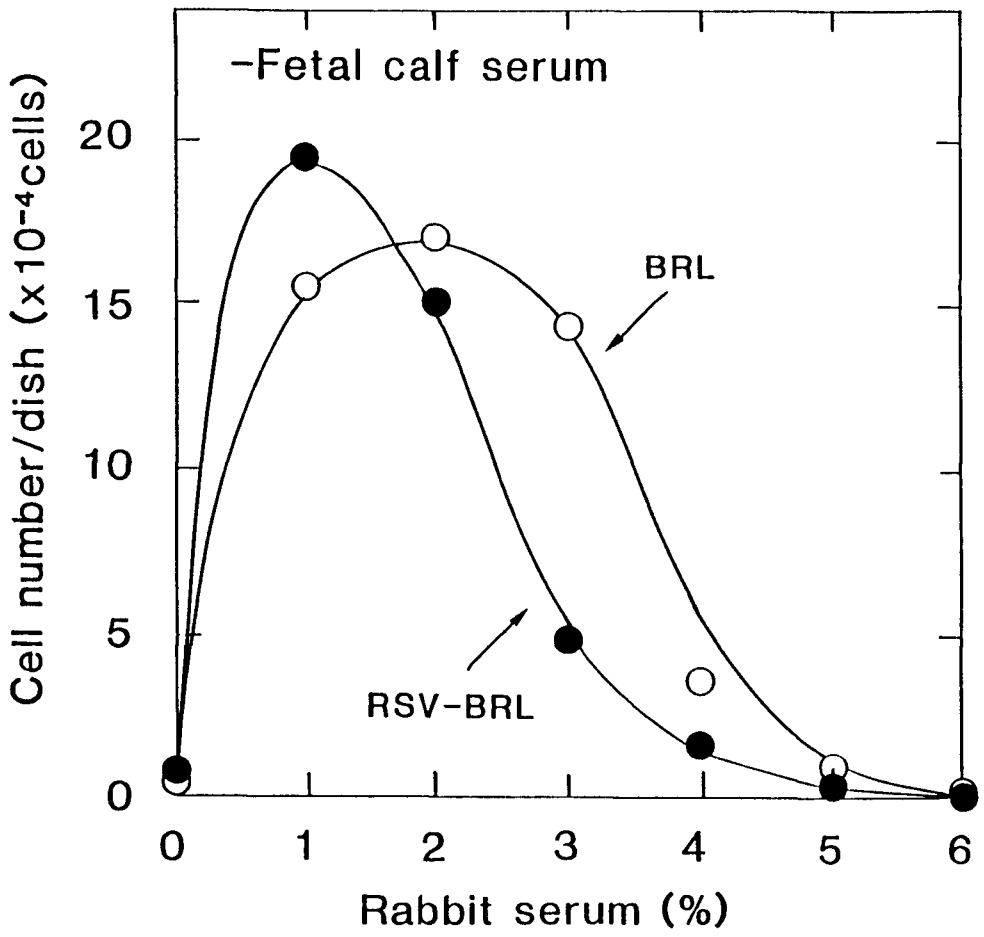


Fig. II-6. Dose-dependent growth inhibition of BRL and RSV-BRL cells by rabbit serum in presence of 10% FCS.

BRL (○) and RSV-BRL (●) cells were grown in the basal medium supplemented with the indicated concentrations of rabbit serum in the presence of 10% FCS.

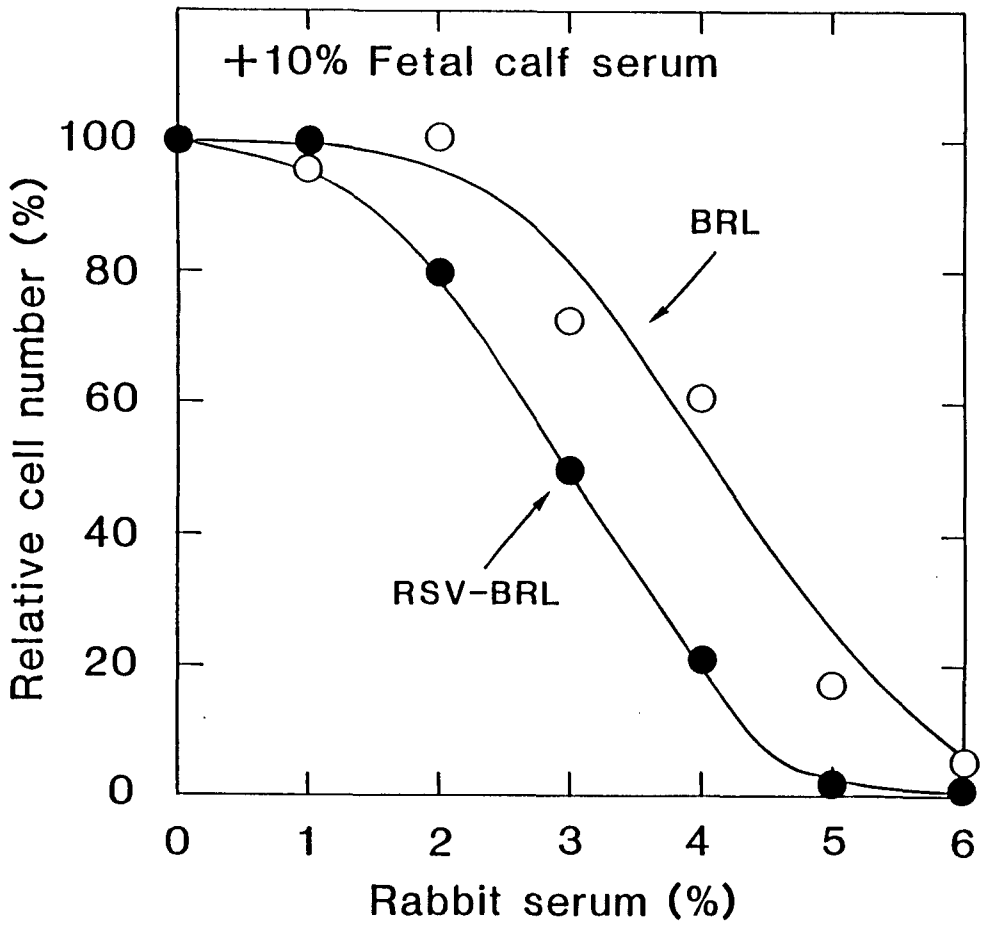


Fig. II-7. Growth inhibition of BRL (○) and RSV-BRL (●) cells by various concentrations of lipoprotein-free and lipoprotein fractions of rabbit serum in presence of 10% FCS.

Lipoprotein and lipoprotein-free fractions were prepared from rabbit serum as described in Materials and Methods.

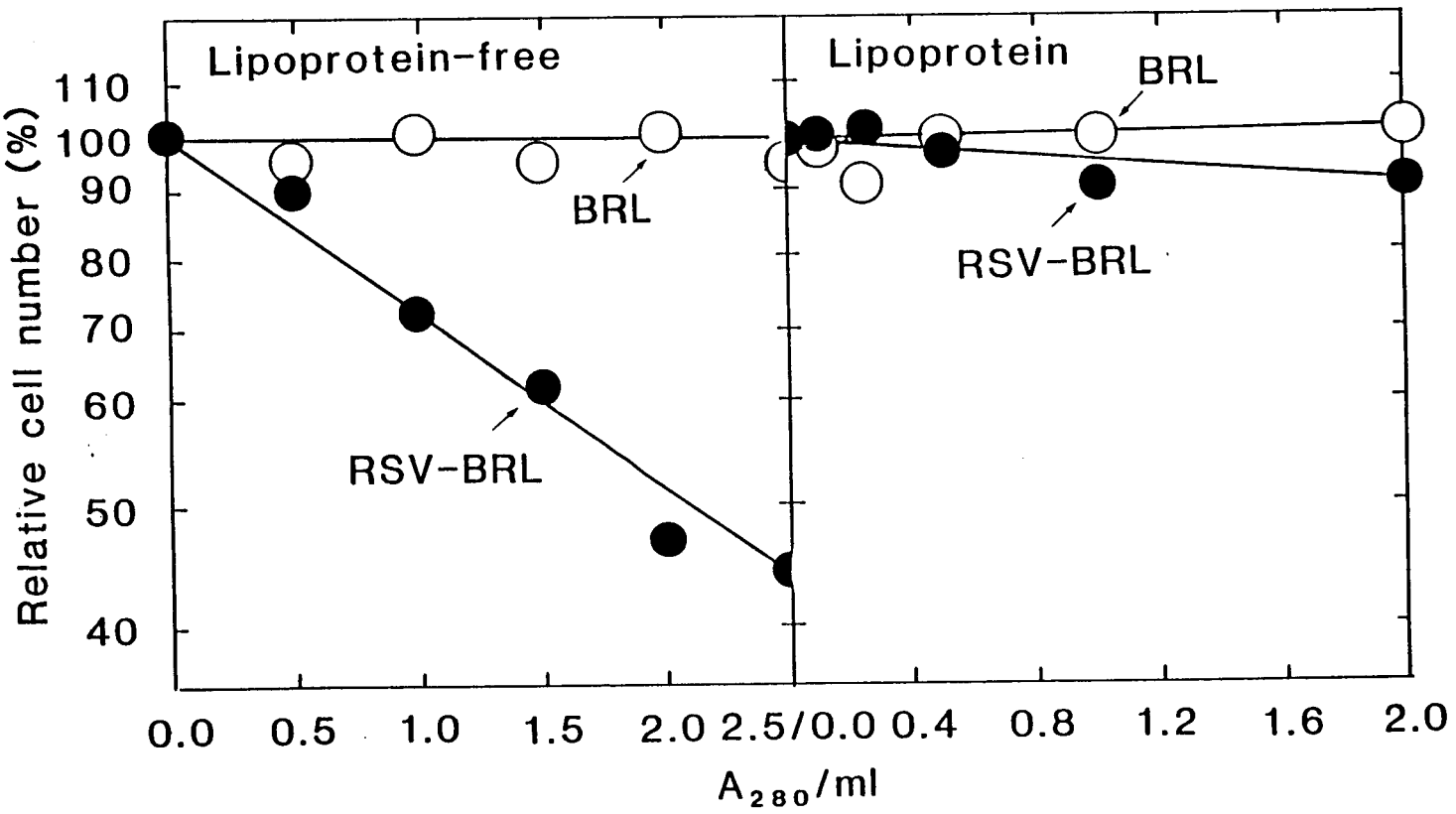


Fig. II-8. Molecular-sieve chromatography on Cellulofine GCL-2000-m column of 30-50% sat. $AmSO_4$ fraction of rabbit serum under the non-dissociating condition.

The 30-50% sat. $AmSO_4$ fraction of rabbit serum was dialyzed against PBS and applied to a Cellulofine GCL-2000-m column (2.6 x 97 cm) equilibrated with PBS and developed. The eluate was collected in 5.3-ml fractions. 0.2 ml each of every other fraction was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. o,A_{280}.

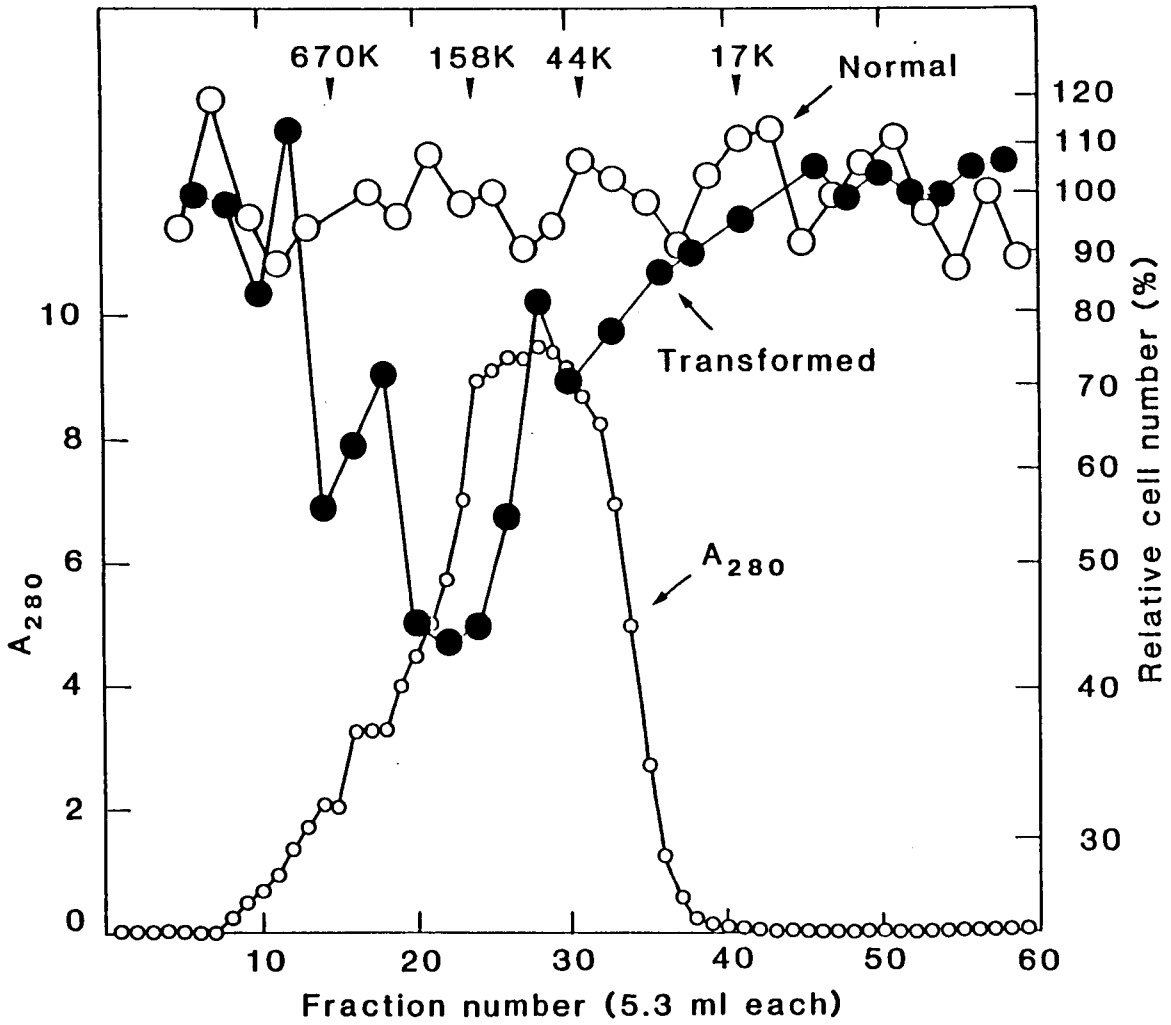


Fig. II-9. Chromatofocusing on PBE94 column of growth inhibitor fraction obtained by molecular-sieve chromatography under non-dissociating condition.

Experimental conditions are described in Materials and Methods. ○, relative number of BRL cells; ●, relative number of RSV-BRL cells; o, A_{280}; △, pH.

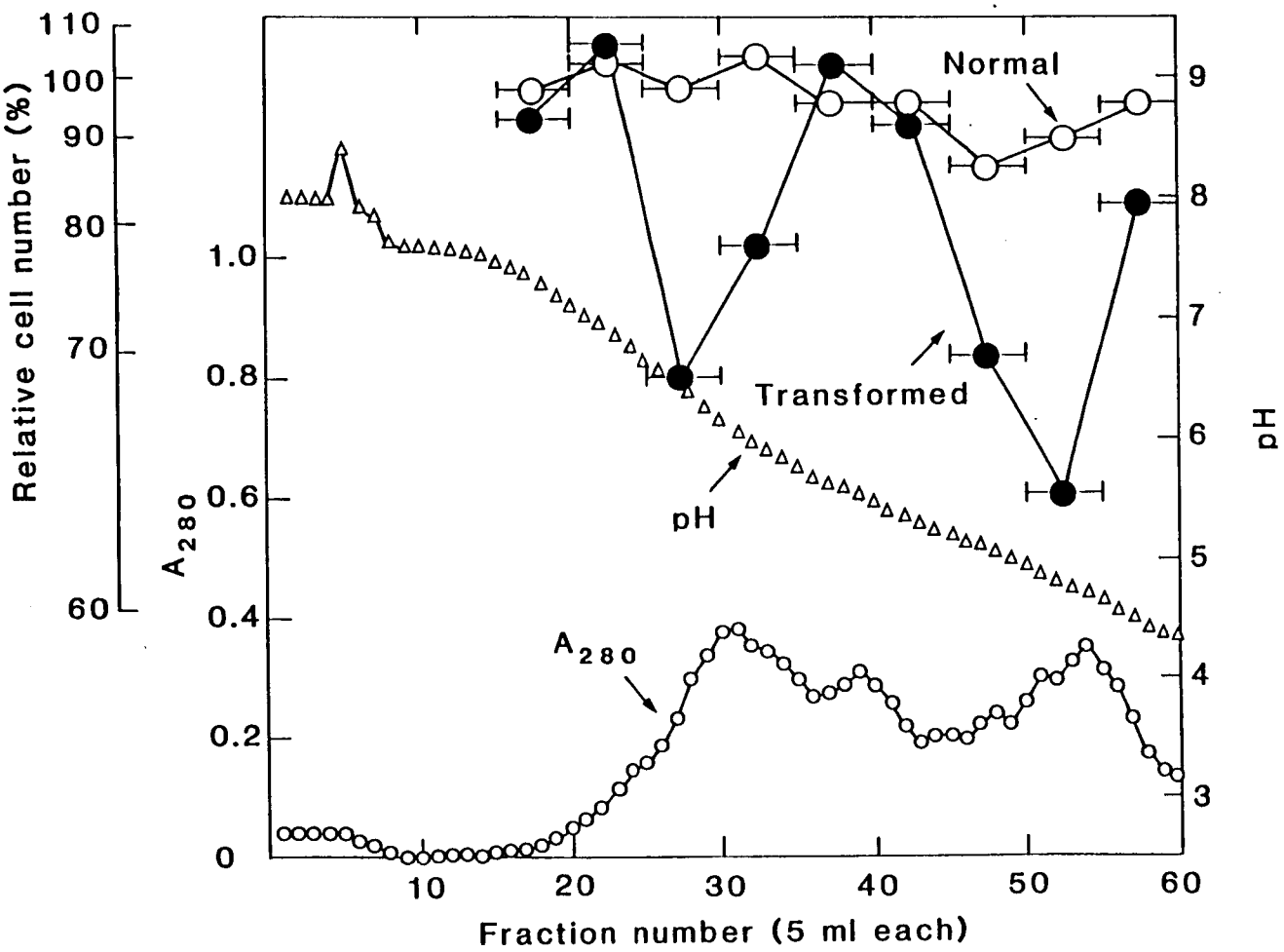


Fig. II-10. Molecular-sieve chromatography on Cellulofine GCL-2000-m column of 30-50% sat. $AmSO_4$ fraction of rabbit serum under the dissociating condition.

The 30-50% sat. $AmSO_4$ fraction of rabbit serum was dialyzed against 10 mM HEPES-NaOH buffer (pH 7.2) containing 1 M NaCl and 6 M urea, and applied to a Cellulofine GCL-2000-m column (2.6 x 97 cm) equilibrated with the same buffer and developed. Eluate was collected in 10-ml fractions. One ml each of every other fraction was dialyzed against PBS and its 0.2-ml portion of was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. o, A_{280}

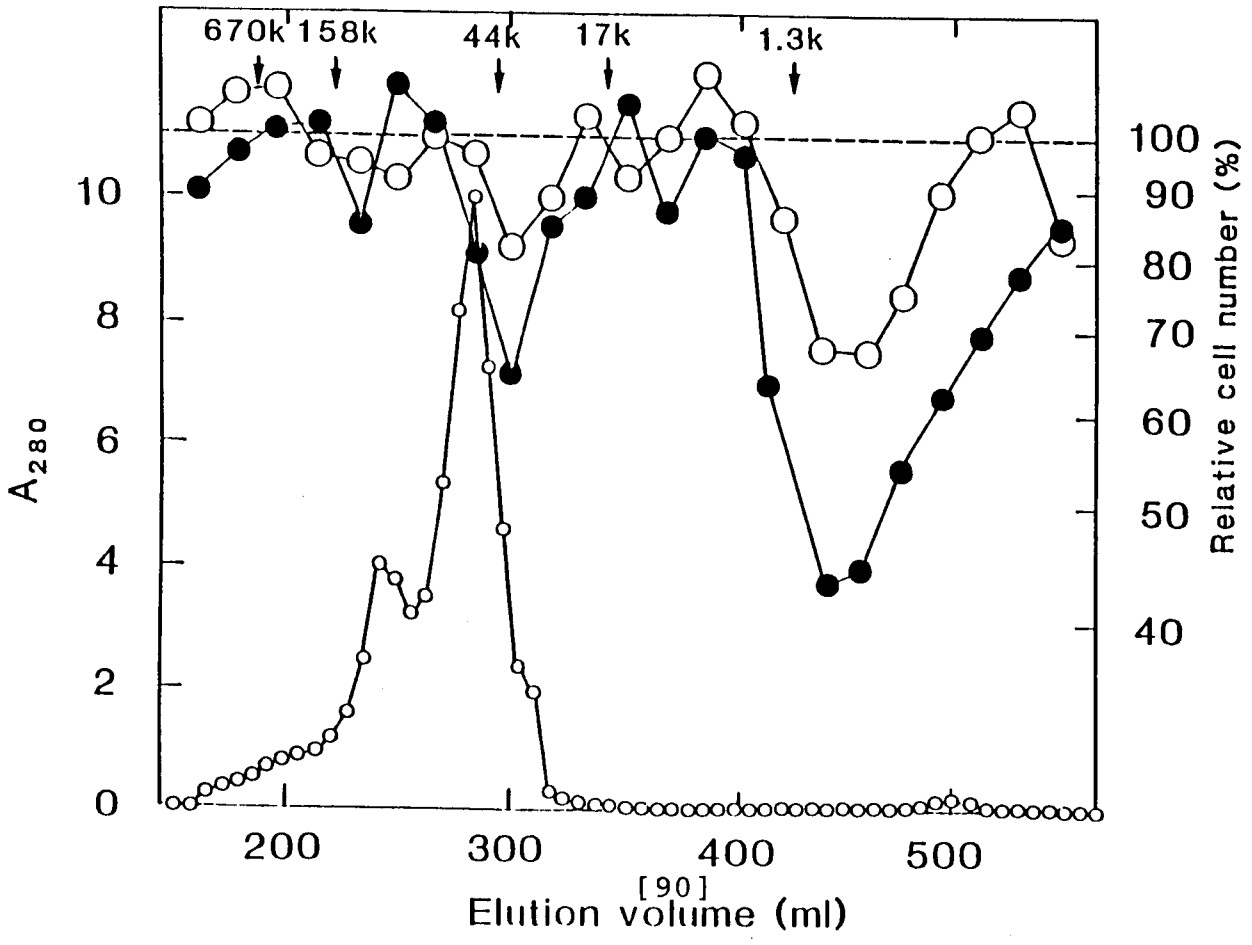


Fig. II-11. Polyacrylamide gel electrophoresis of growth inhibitor fraction obtained by molecular-sieve chromatography under dissociating condition.

25 μl each of extracts from sliced gel pieces was assay for growth inhibitory activity on RSV-BRL (●) cells. Other experimental conditions are described in Materials and Methods. ○, pH

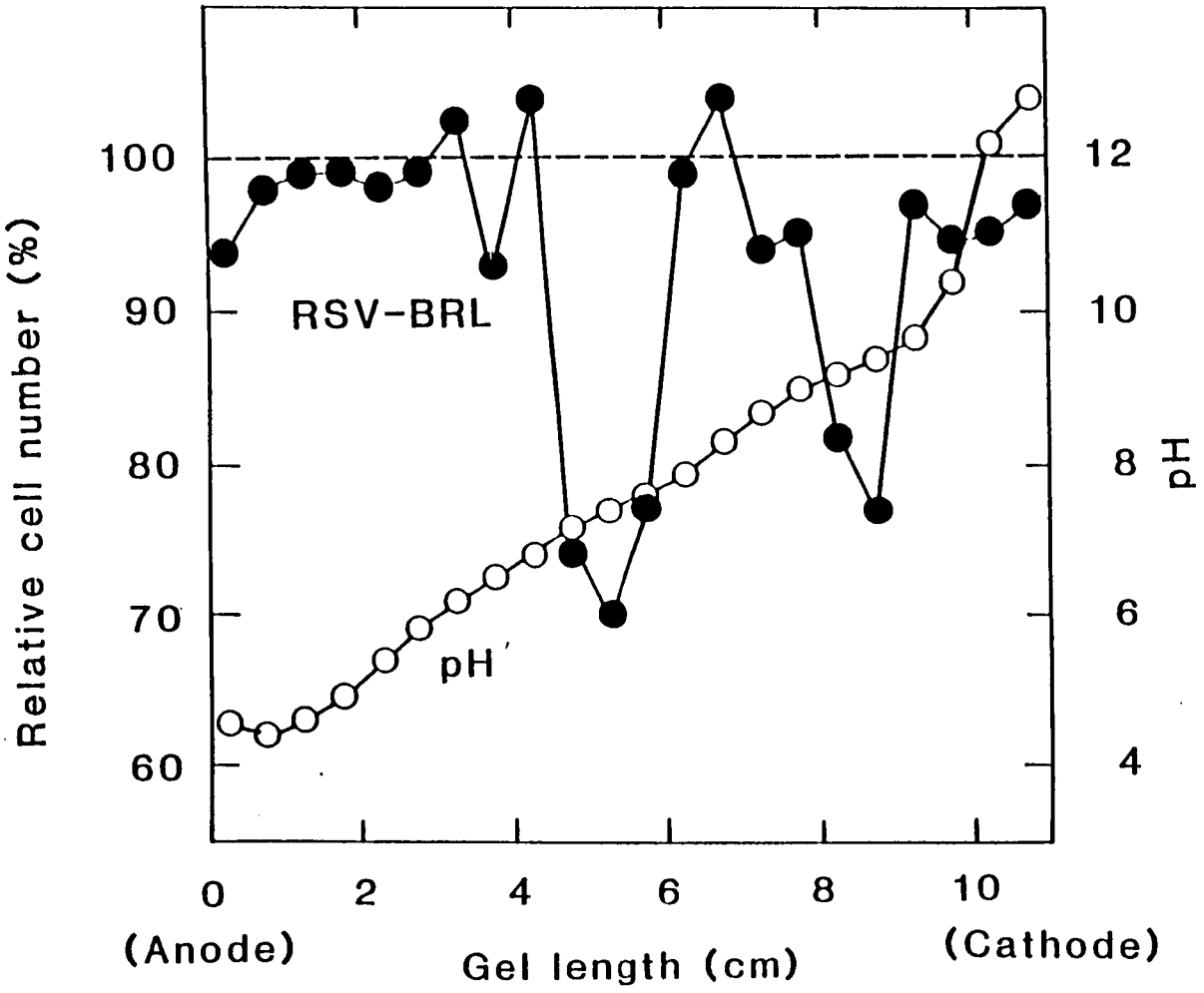


Fig. II-12. Anion-exchange chromatography on DEAE-Toyopearl 650M column of rabbit serum.

0.5-ml each of every other fraction was dialyzed against 0.5 M acetic acid and its 25 μl was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. Other experimental conditions are described in Materials and Methods. o, A_{280};——, NaCl.

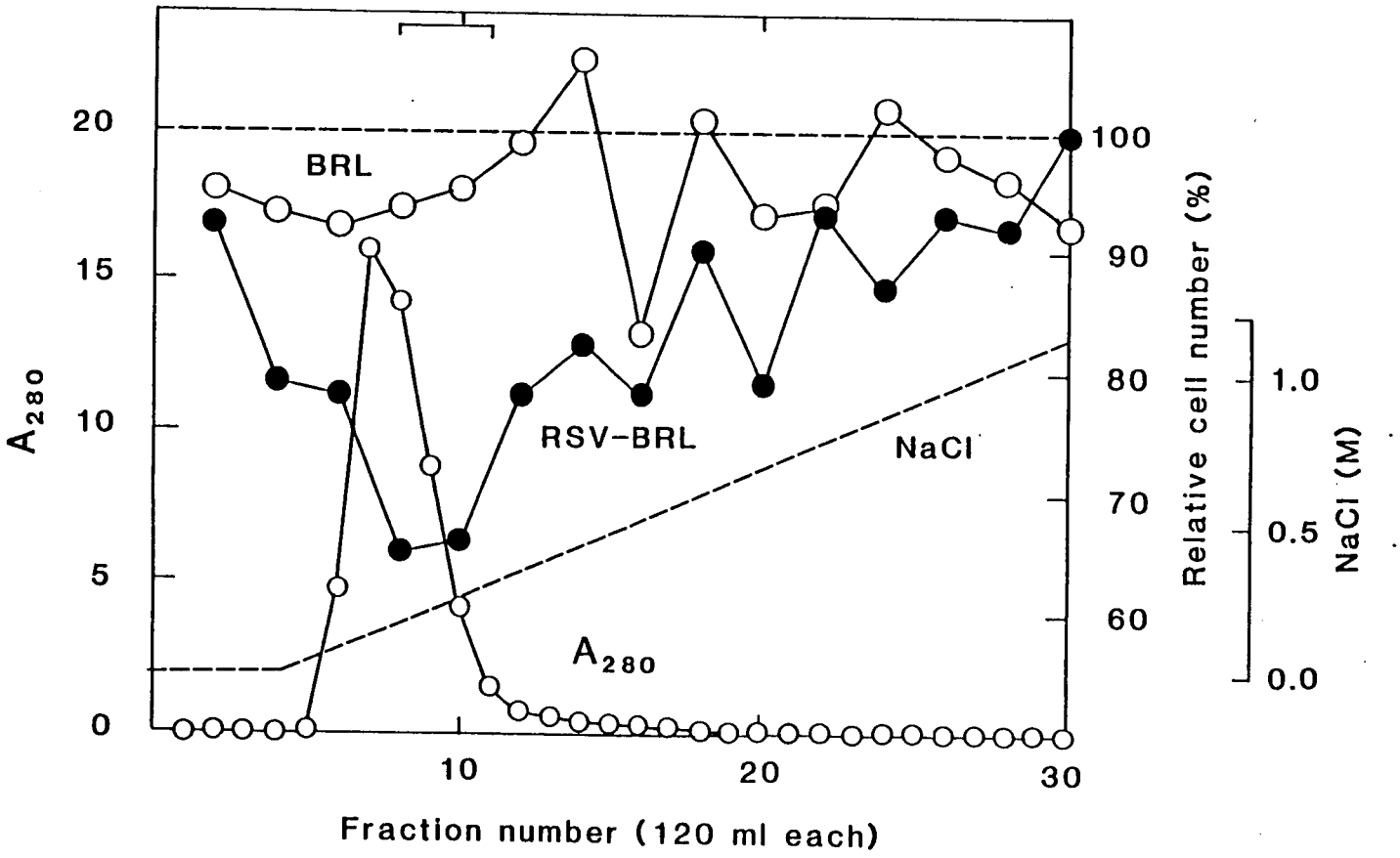


Fig. II-13. Molecular-sieve chromatography on Cellulofine GCL-300-m column of acetic acid-extracts.

The growth inhibitor extract with 1 M acetic acid from Diaflo YM5 membrane was dialyzed against 0.5 M acetic acid, applied to a Cellulofine GCL-300-m column (2.6 x 96 cm) equilibrated with 0.5 M acetic acid and developed. The eluate was collected in 10-ml fractions. 25 μl each of every other fraction was assayed for growth inhibitory activity on RSV-BRL cells (●). o, A_{280}.

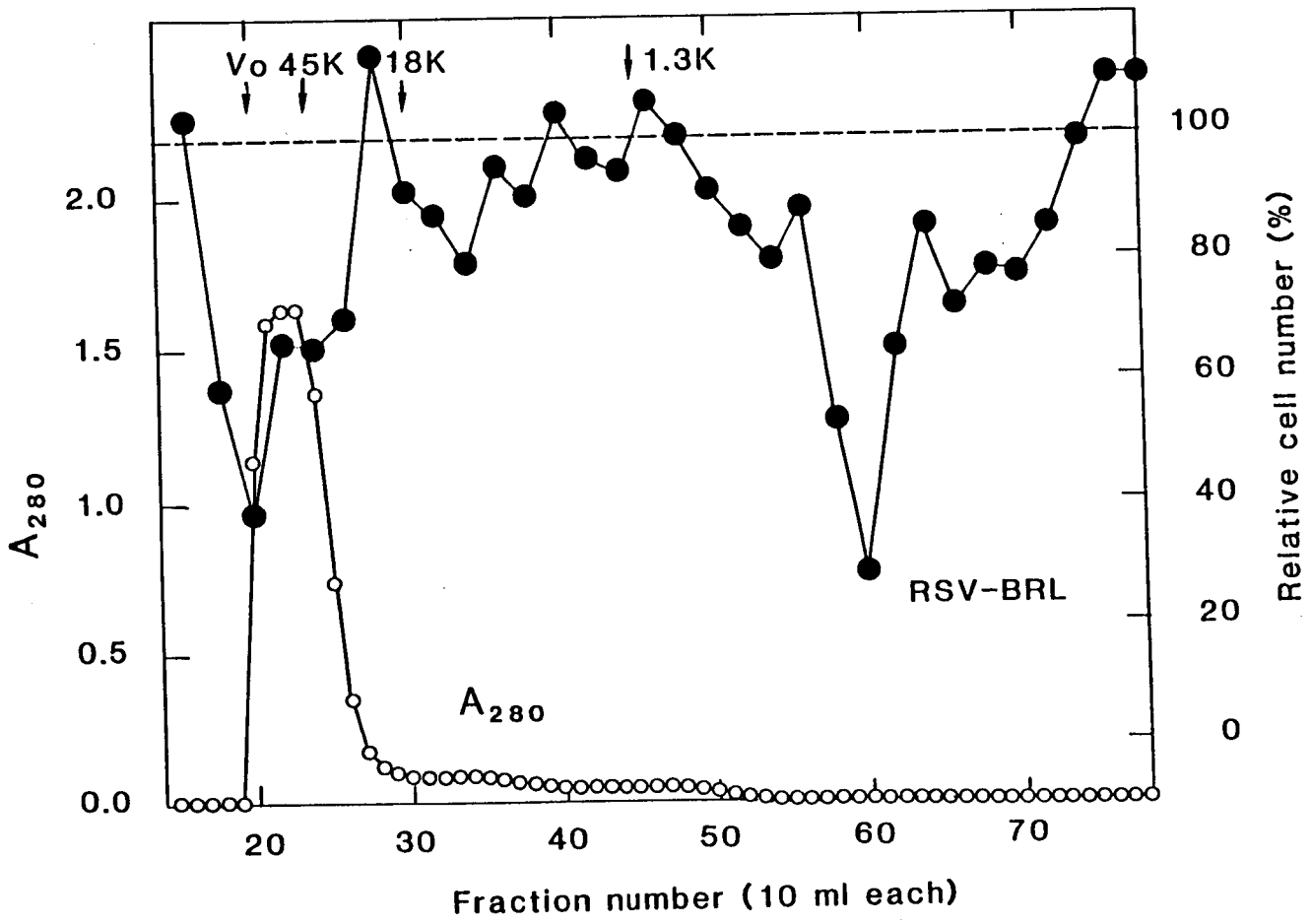


Fig. II-14. Reverse-phase high performance liquid chromatography (HPLC) on Ultron N-C8 column (C-8) of growth inhibitor fraction obtained by molecular-sieve chromatography.

25 μl each of every other fraction was assayed for growth inhibitory activity on RSV-BRL (●) cells. Other experimental conditions are described in Materials and Methods. ——, A_{280}; – –, acetonitrile.

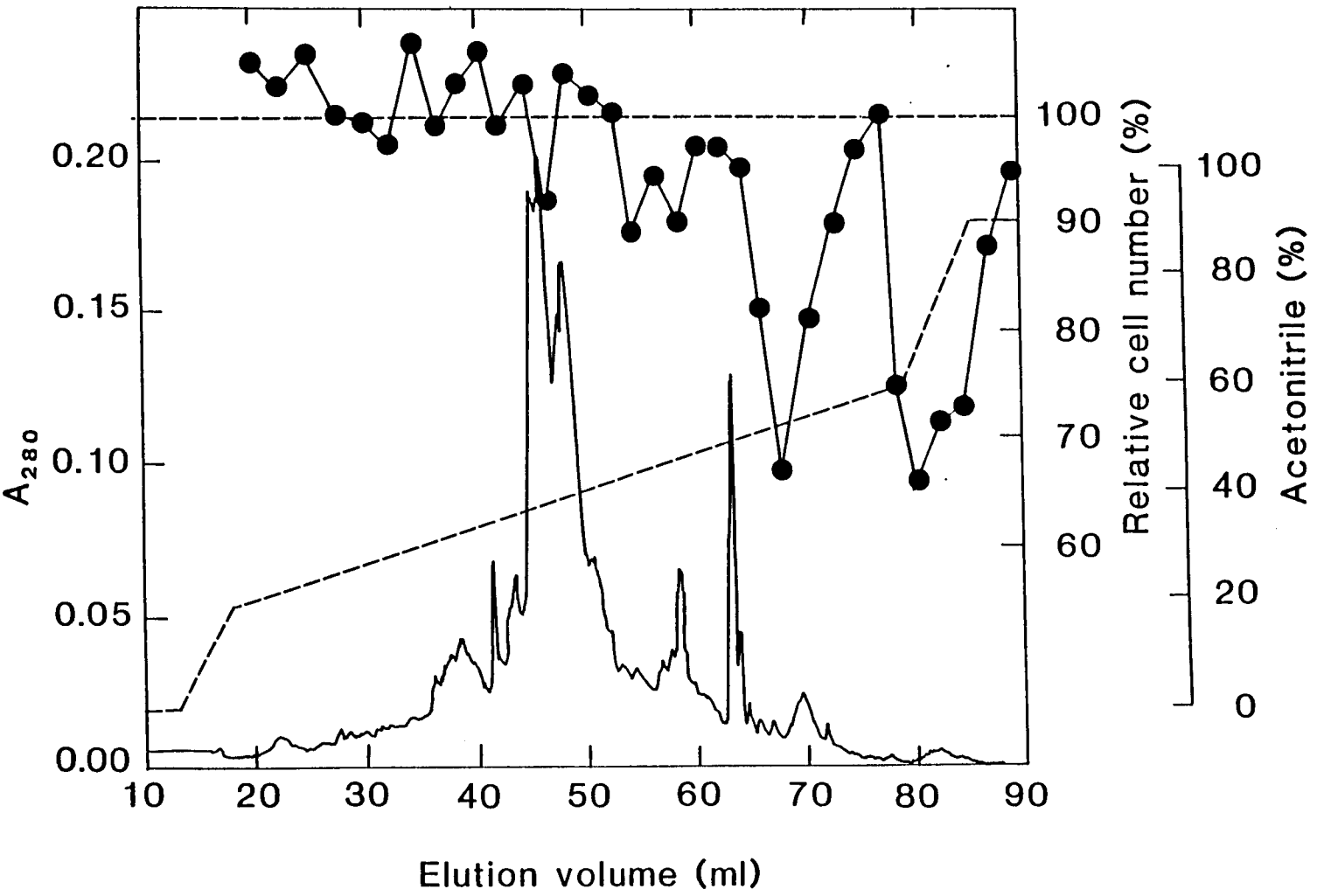


Fig. II-15. SDS-polyacrylamide gel electrophoresis of purified s-TGI.

Experimental conditions are described in Materials and Methods.

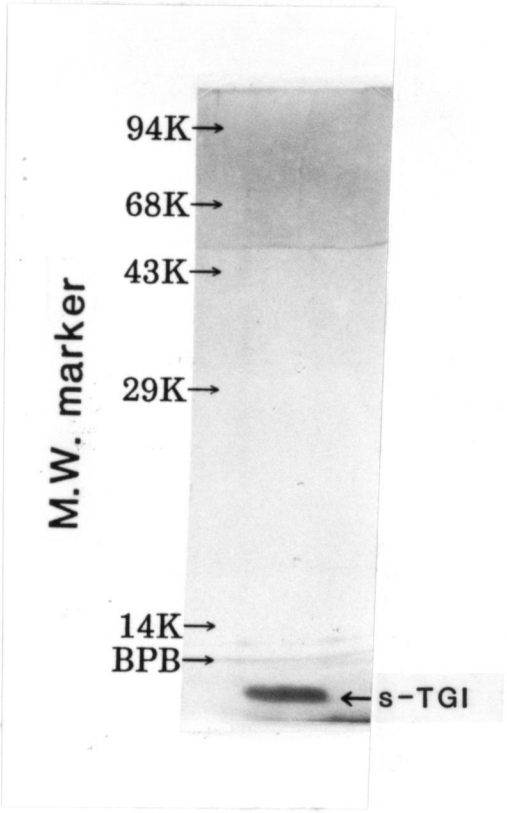


Fig. II-16. Dose-response of purified s-TGI to the growth of BRL and RSV-BRL cells in presence of 10% FCS.

Purified s-TGI was added to test medium at the indicated concentrations, and its growth inhibitory activity on BRL (○) and RSV-BRL (●) cells was determined.

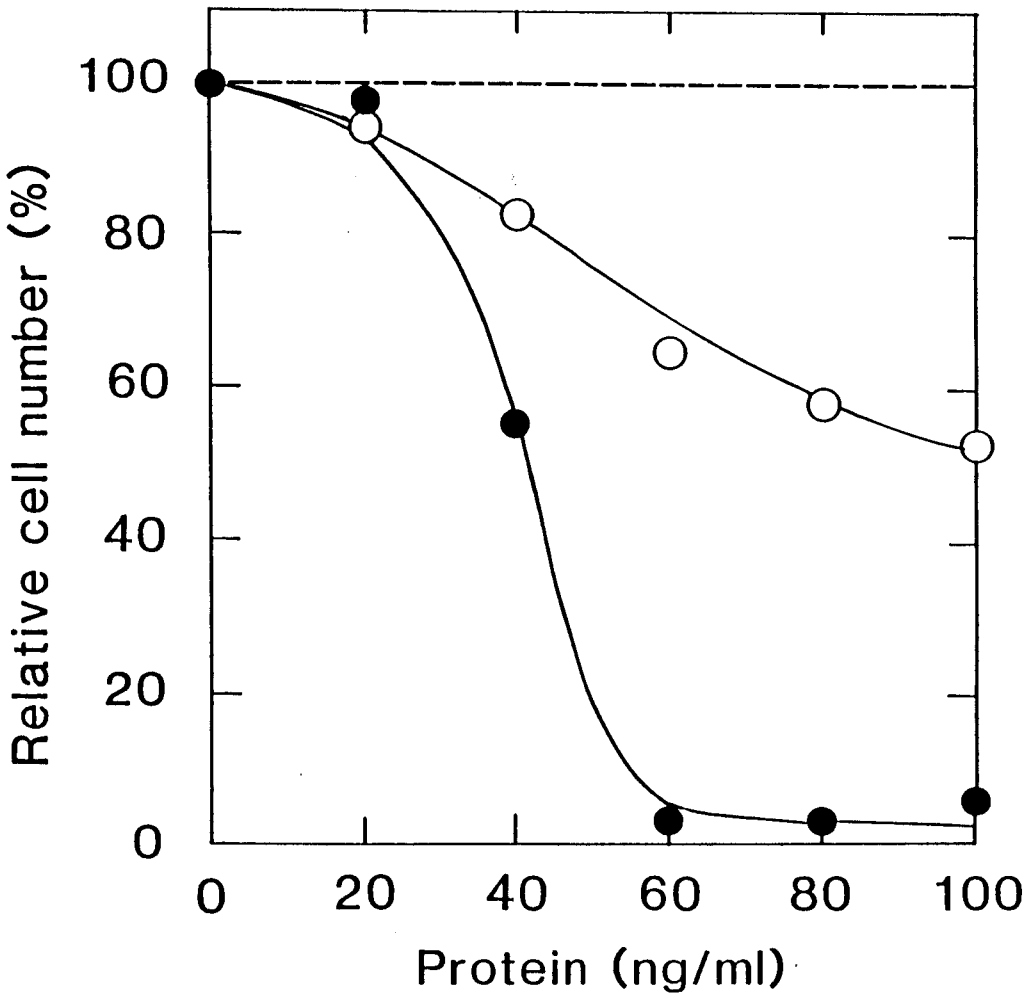


Fig. III-I. Growth curves of BRL (○) and RSV-BRL (●) cells in presence of 10% FCS.

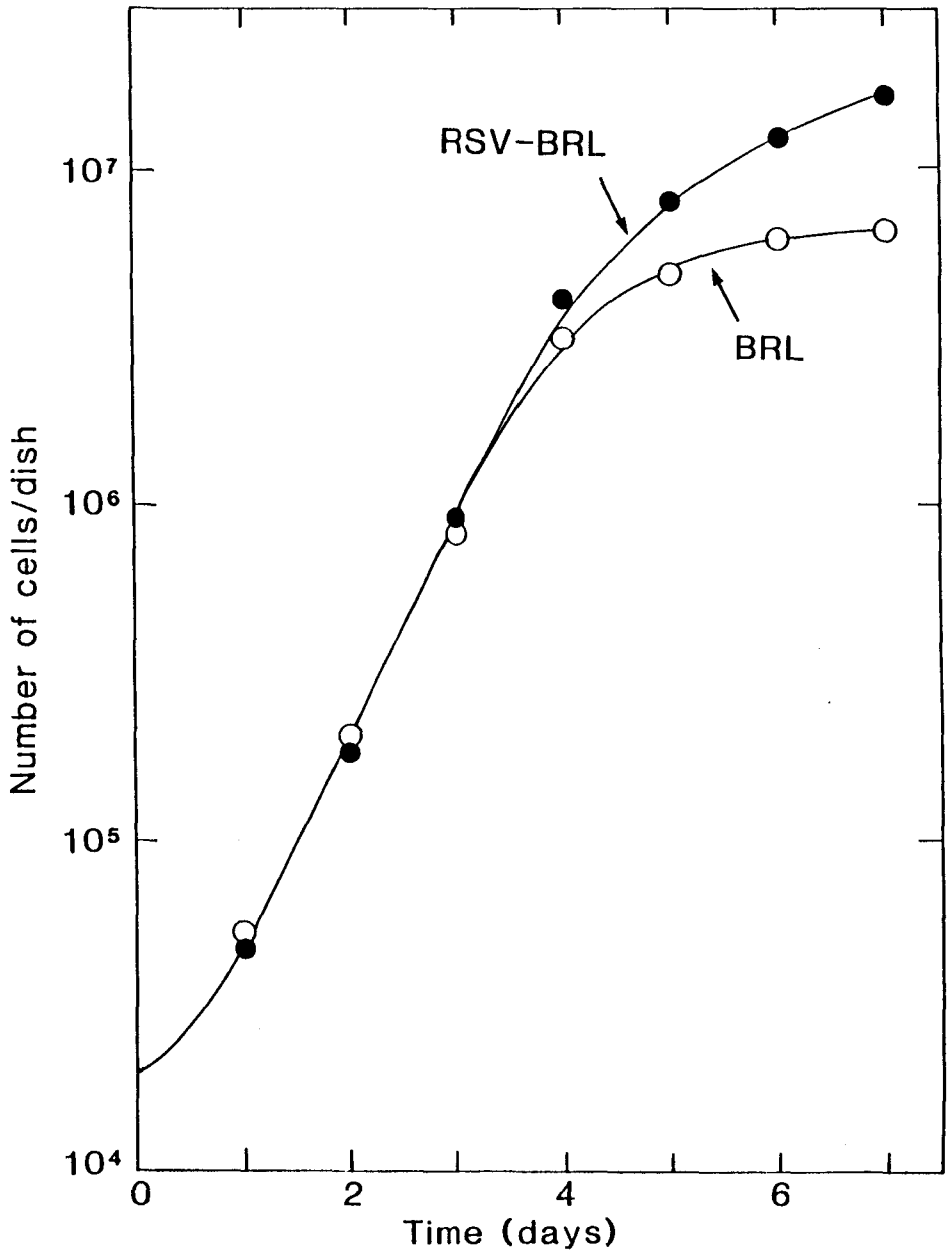


Fig. III-2. Effect of concentrated conditioned medium of BRL cells on growth of BRL (○) and RSV-BRL (●) cells in presence of 10% FCS

Experimental conditions are described in Materials and Methods.

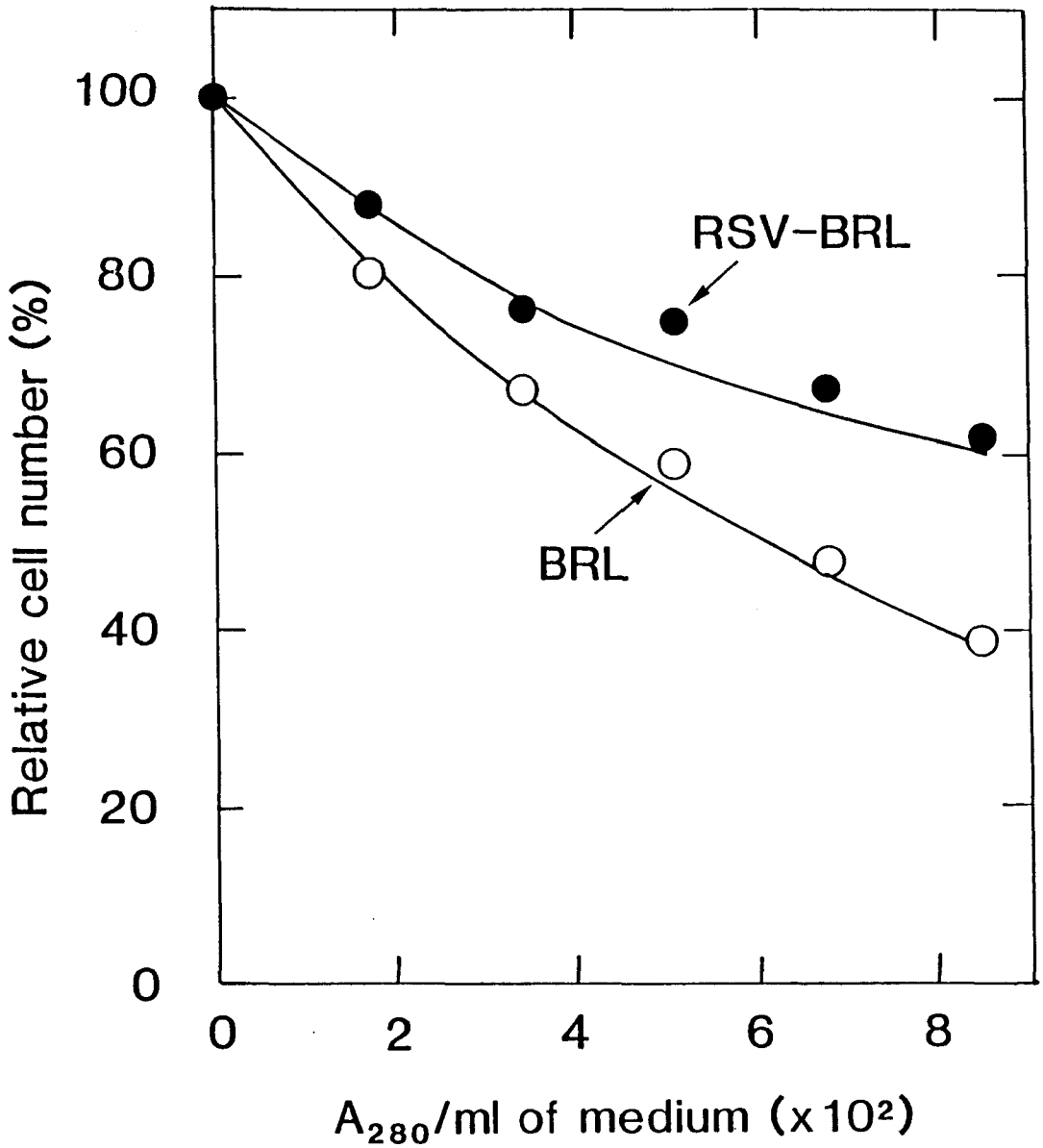


Fig. III-3. Molecular-sieve HPLC on TSKgel G3000SWG column of conditioned medium of BRL cells in presence of 1 M NaCl and 6 M urea.

0.5 ml each of every other fraction was dialyzed against PBS, and its 0.2 ml portion was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. Other experimental conditions are described in Materials and Methods. o, A_{280}.

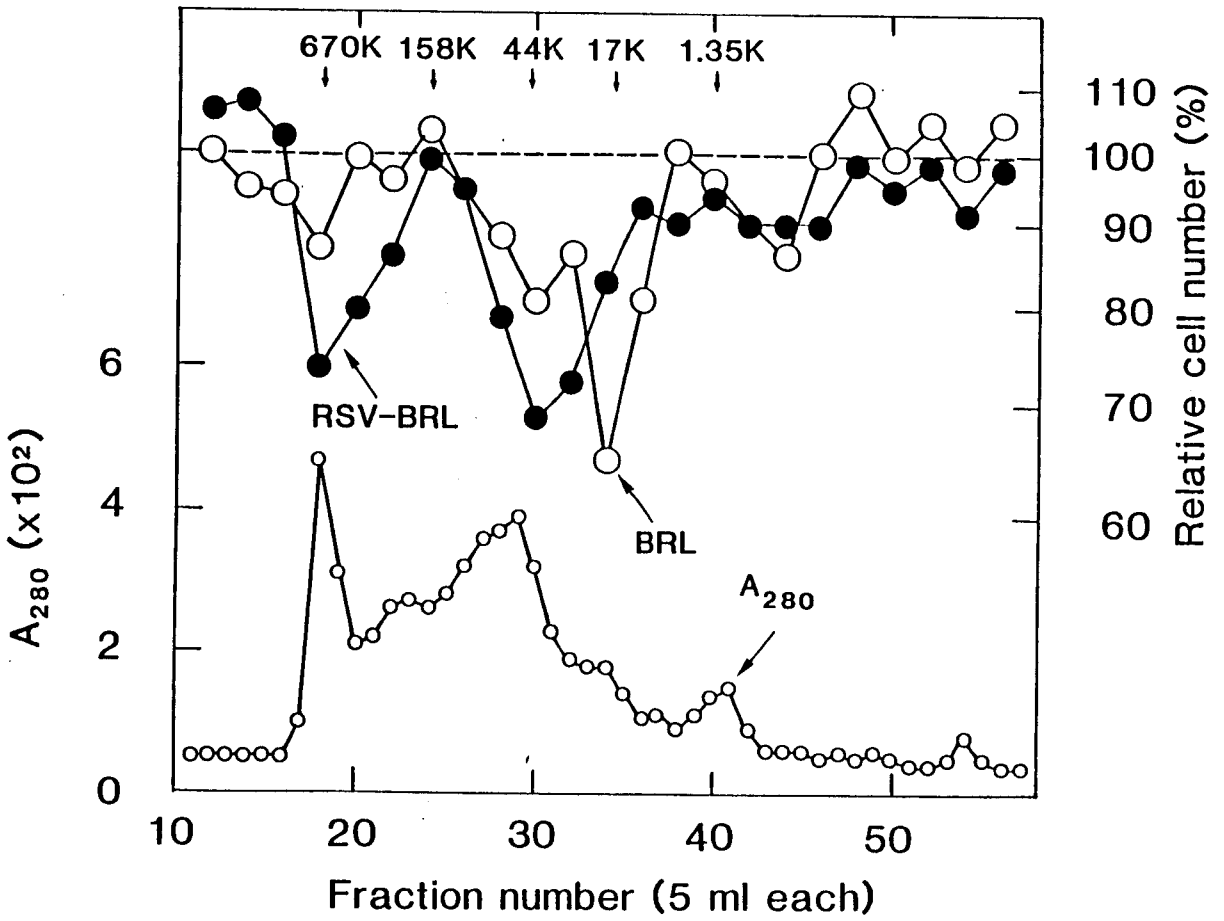


Fig. III-4. Polyacrylamide gel isoelectric electrophoresis of conditioned medium of BRL cells in presence of 8.5 M urea.

Growth inhibitory activity on BRL (◯) cells was assayed. Other experimental conditions are described in Materials and Methods. ○, pH.

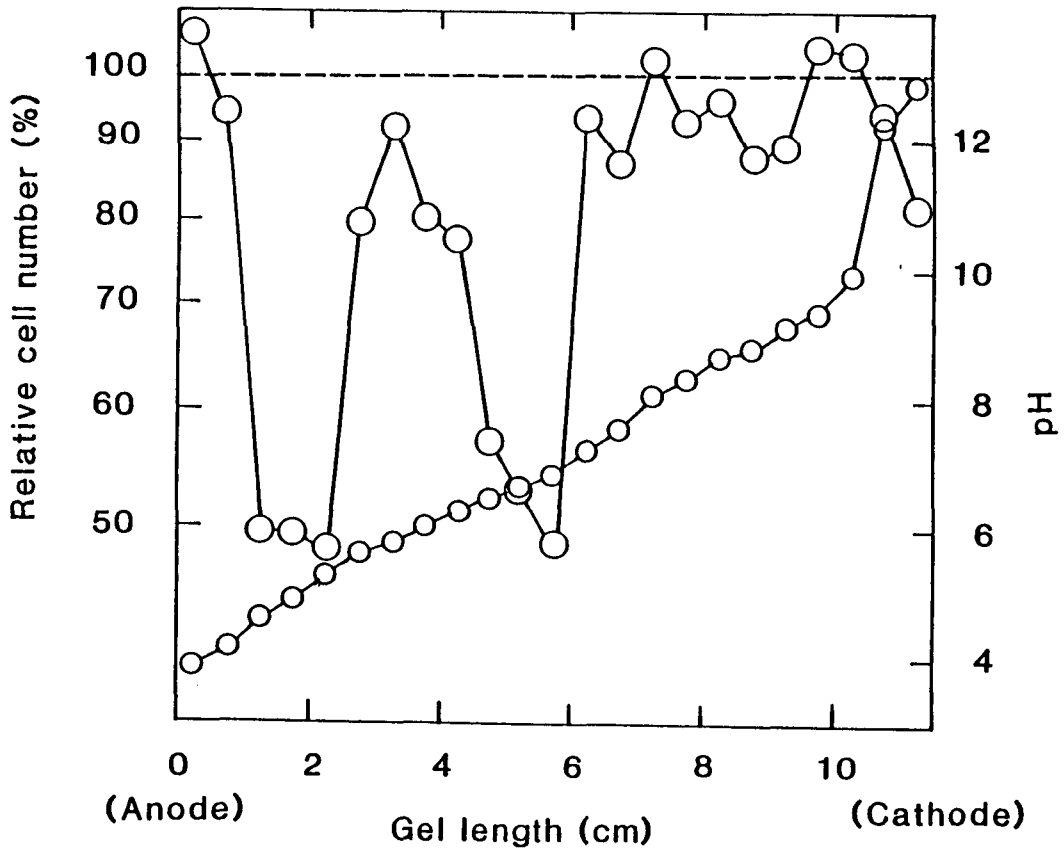


Fig. III-5. Polyacrylamide gel isoelectric electrophoresis of conditioned medium of BRL cells in presence of 8.5 M urea.

Growth inhibitory activity on RSV-BRL (●) cells was assayed. Other experimental conditions are described in Materials and Methods. ○, pH.

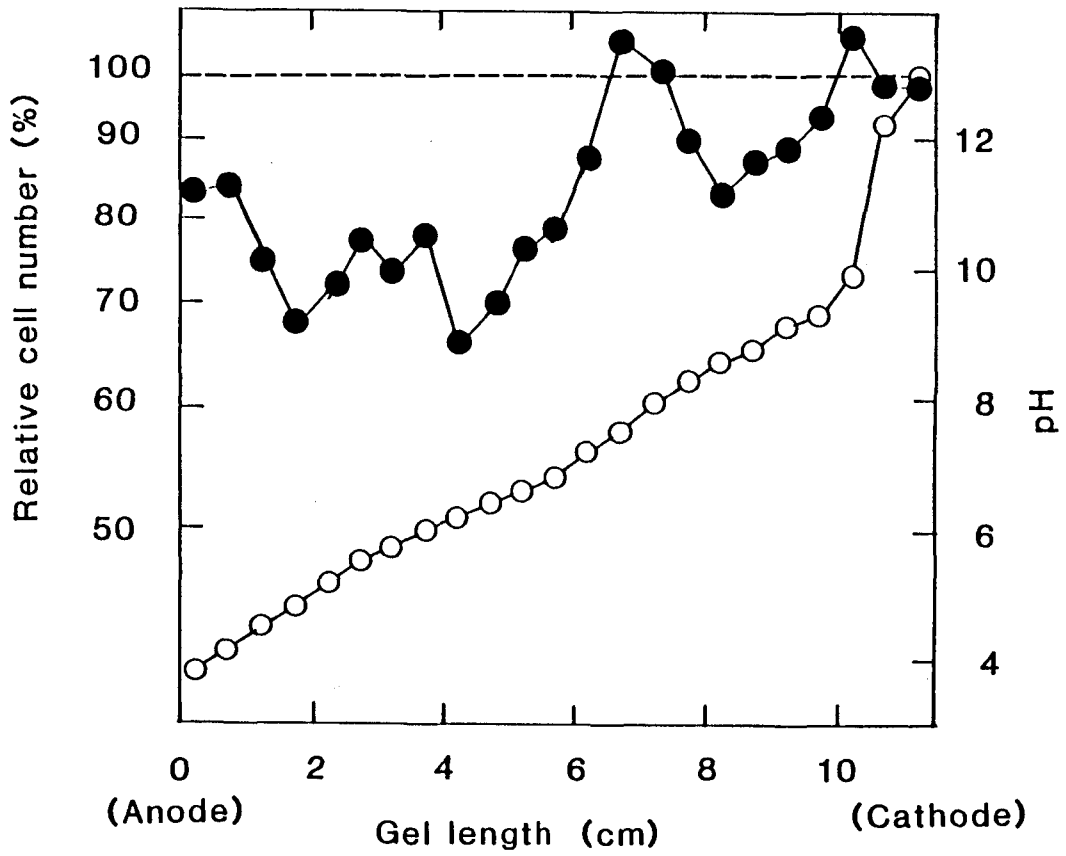


Fig. III-6. Molecular-sieve chromatography on Cellulofine GCL-300-m column of concentrated conditioned medium of BRL cells.

The conditioned medium of BRL cells was concentrated as described in Materials and Methods. The concentrated conditioned medium was dialyzed against 1 M acetic acid, and its 50 ml portion was applied to a Cellulofine GCL-300-m column (2.6 x 96 cm) equilibrated with 1 M acetic acid and developed. 25 μl each of every other fraction was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. o, A_{280}.

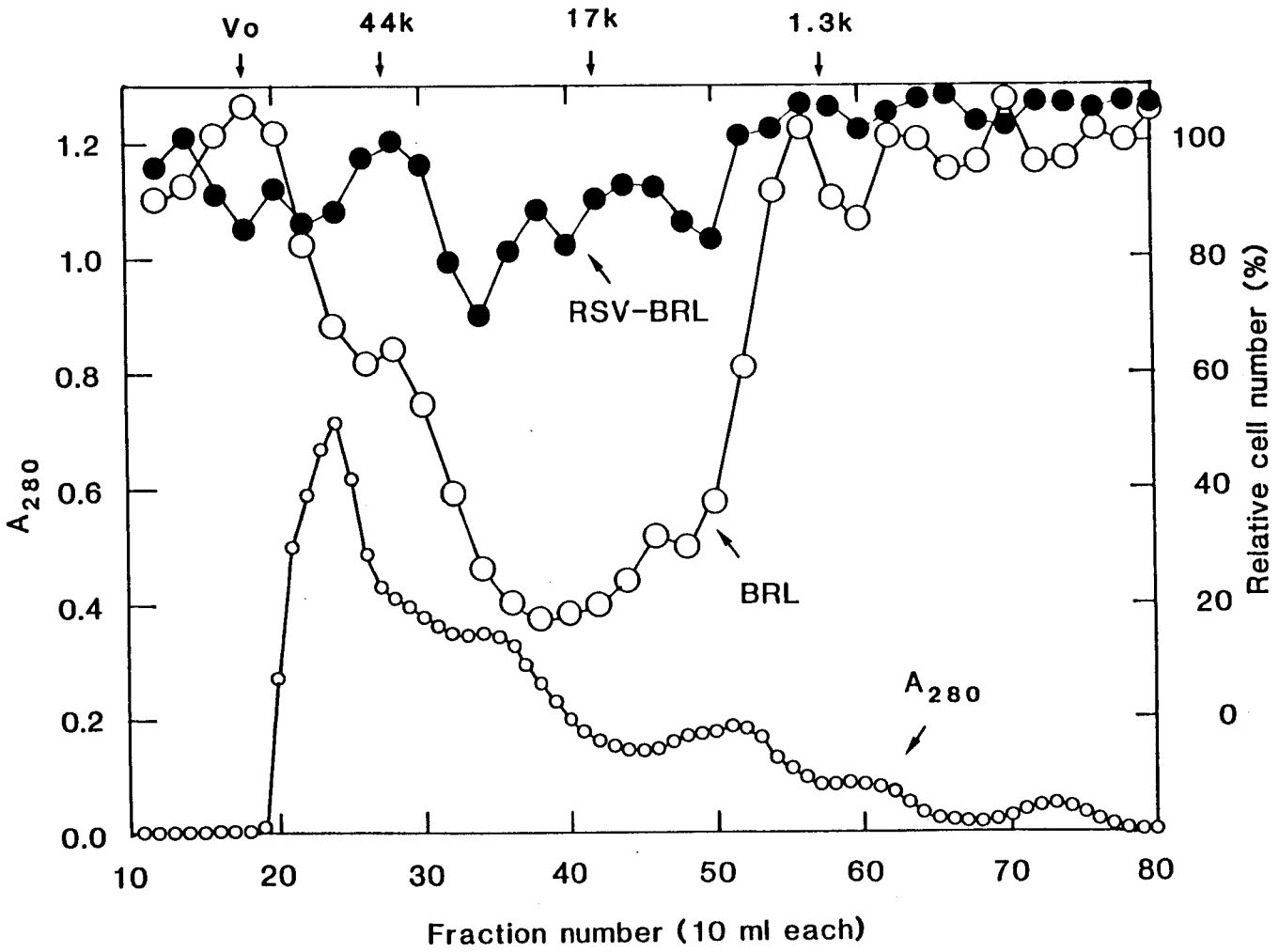


Fig. III-7. Anion-exchange chromatography on DEAE-Toyopearl 650M column of growth inhibitor fraction obtained by molecular-sieve chromatography.

0.5 ml each of every other fraction was dialyzed against 1 M acetic acid, and its 25 l was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●). Other experimental conditions are described in Materials and Methods. o, A_{280}; ——, NaCl.

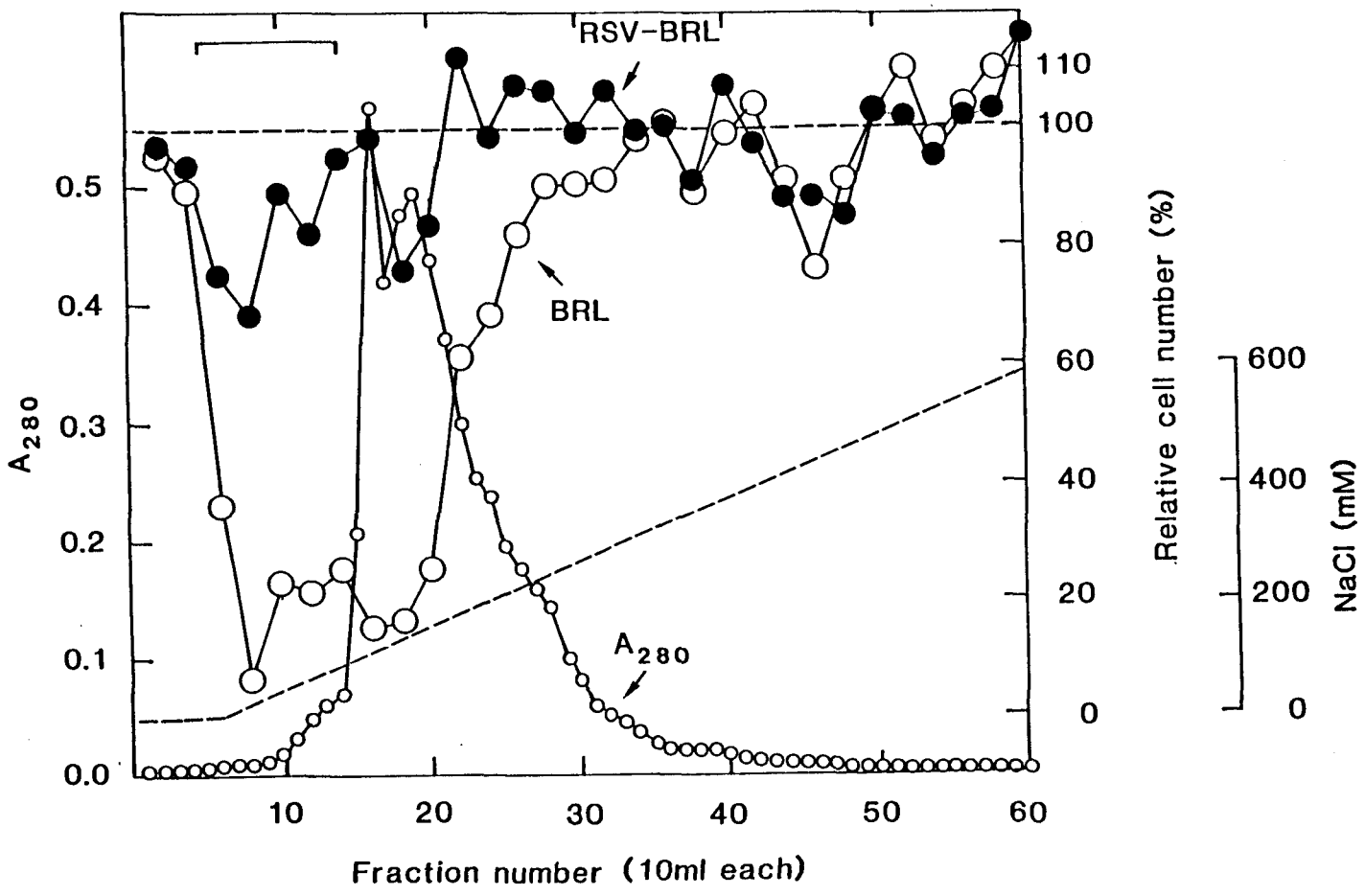


Fig. III-8. Reverse-phase HPLC on LS-410K (C-18) column of growth inhibitor fraction obtained by anion-exchange chromatography.

Growth inhibitor fraction obtained by anion-exchange chromatography was dialyzed against 1 M acetic acid and applied to a LS-410K column (4.6 x 250 mm). 25 μl of each fraction was assayed for growth inhibitory activity on BRL (◯) cells. Other experimental conditions are described in Materials and Methods. —, A_{280}; ——, acetonitrile.

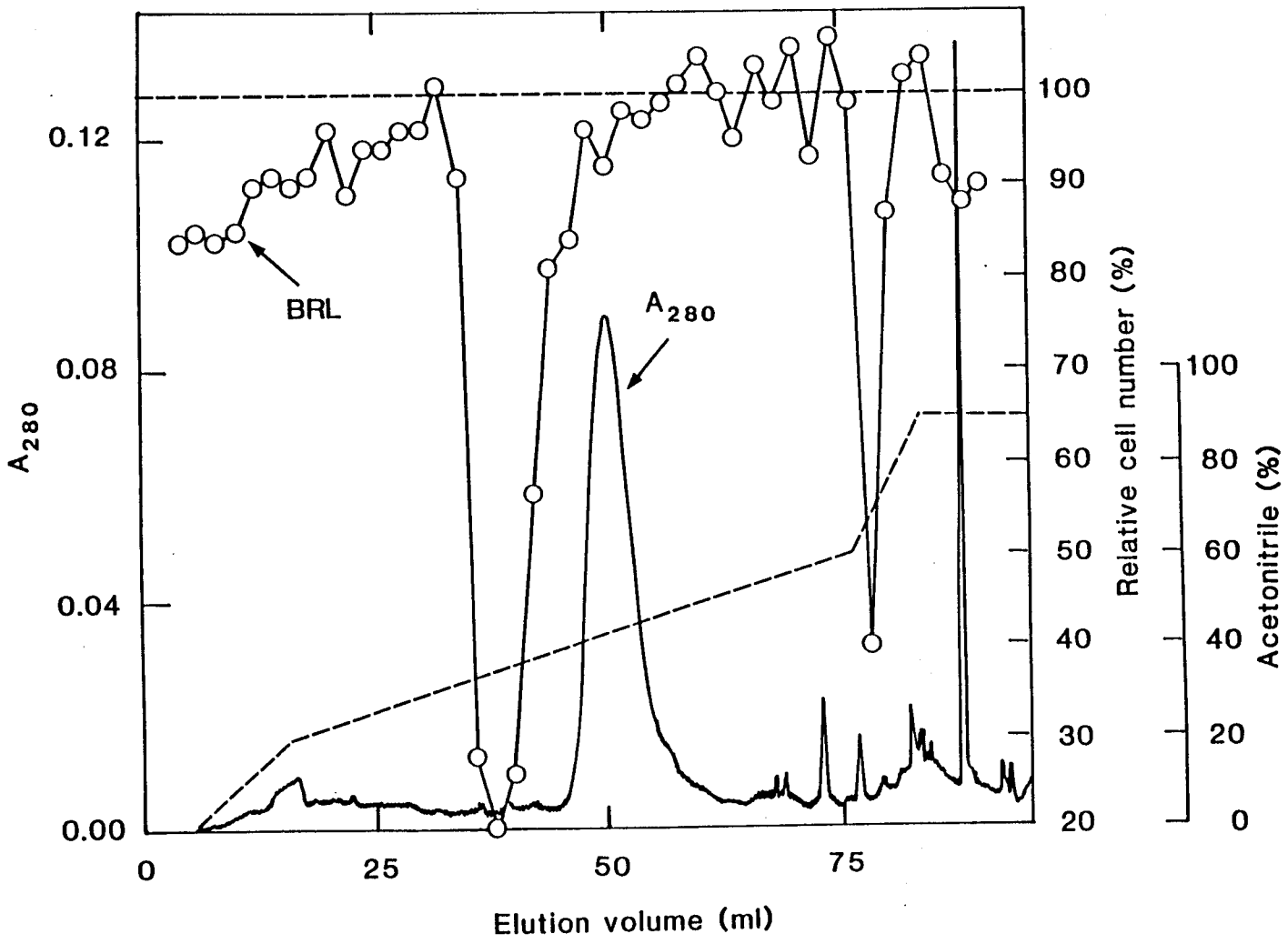


Fig. III-9. SDS-polyacrylamide gel electrophoresis of purified c-NGI-I under non-reducing conditions.

Experimental conditions are described in Materials and Methods.

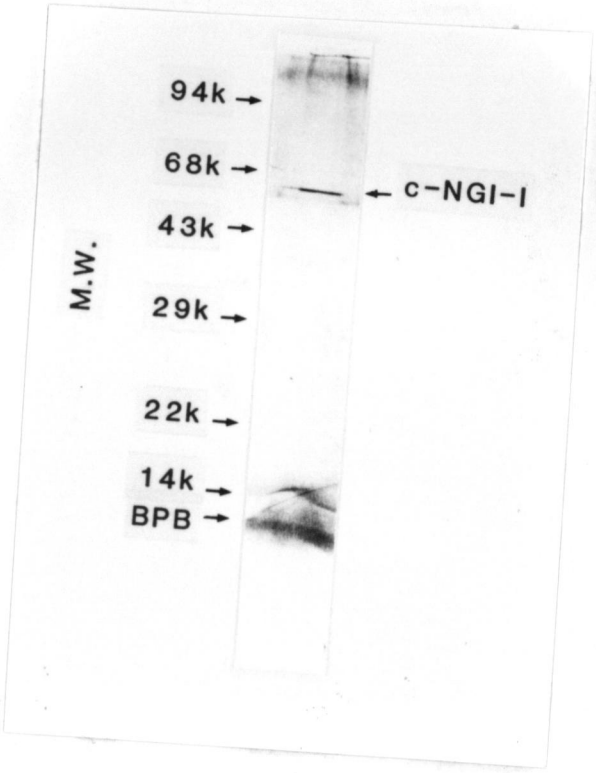


Fig. III-10. SDS-polyacrylamide gel electrophoresis of purified c-NGI-II under non-reducing conditions.

Experimental conditions are described in Materials and Methods.

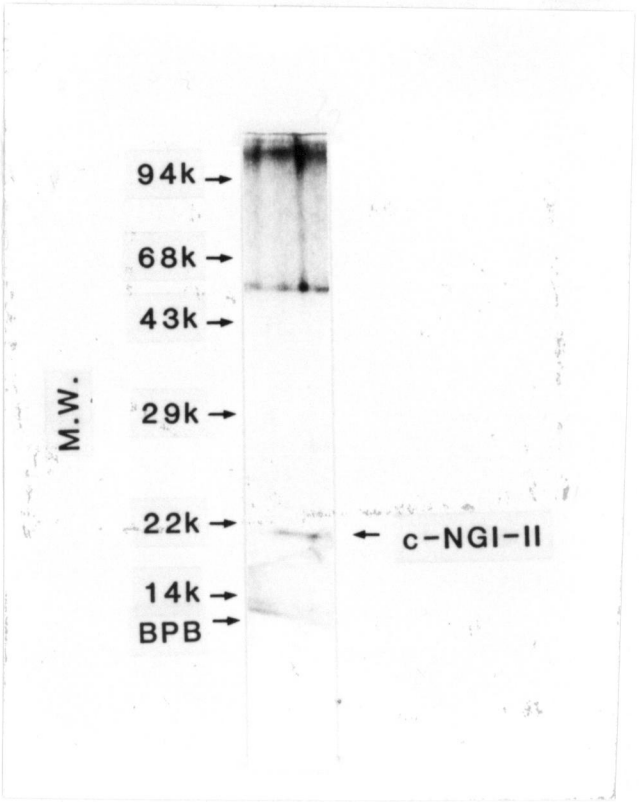


Fig. III-11. Dose-response curves of purified c-NGI-I for the growth of BRL and RSV-BRL cells in presence of 10% FCS.

Purified c-NGI-I was added to test medium at the indicated concentrations and assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells.

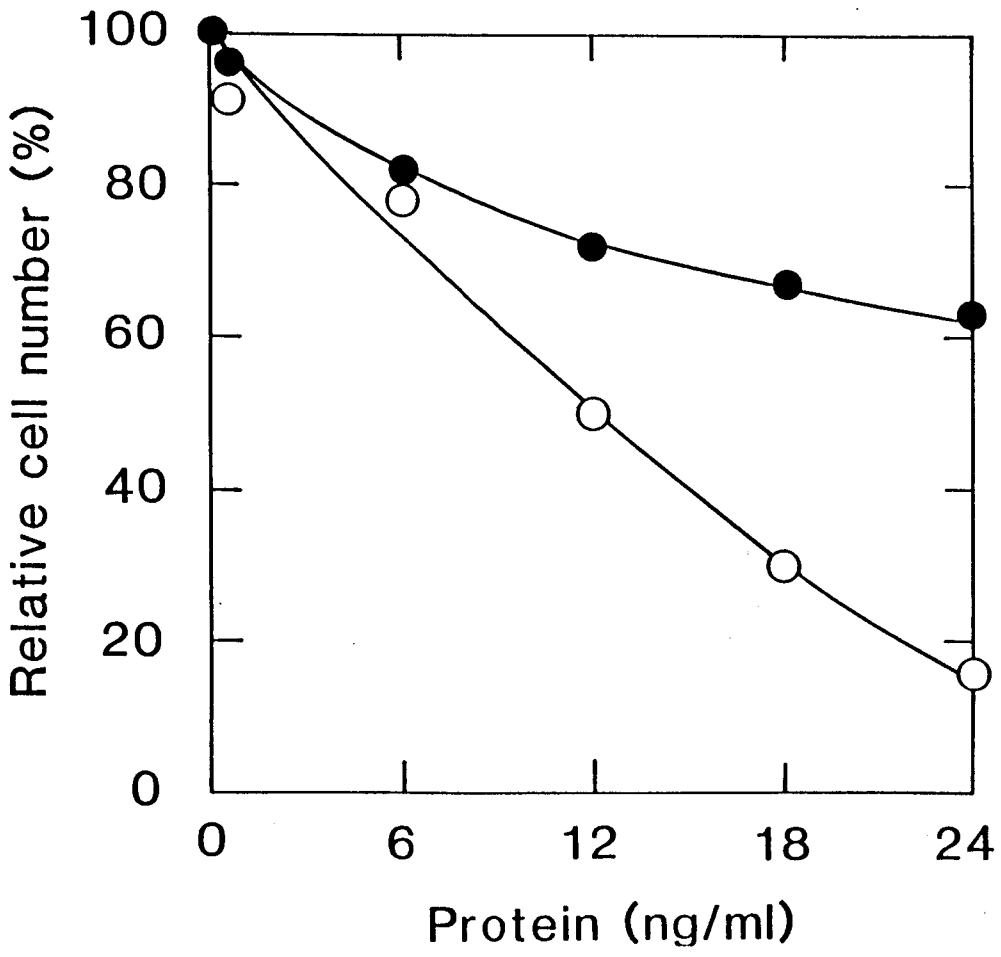


Fig. III-12. Dose-response curves of purified c-NGI-II to the growth of BRL and RSV-BRL cells in presence of 10% FCS.

Purified c-NGI-II was added to test medium at the indicated concentrations and assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells.

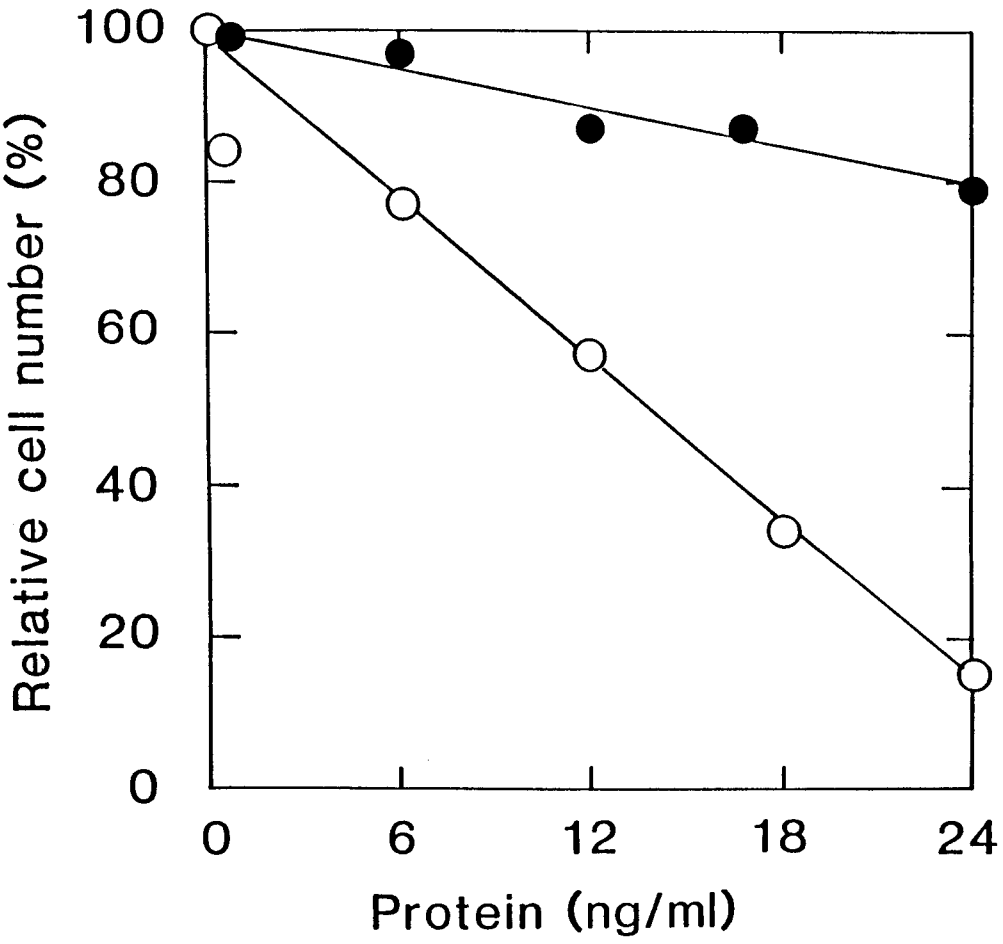


Fig. III-13. Molecular-sieve chromatography on Toyopearl HW55C column in presence of 1 M acetic acid and 6 M urea of extract from YM5 membrane in presence of 1 M acetic acid.

c-TGI adsorbed to YM5 membrane was extracted as described in Materials and Methods. After dialysis against a mixture of 1 M acetic acid and 6 M urea, extract (55 ml) was applied to a Toyopearl HW55C column (2.6 x 95 cm) equilibrated with the same mixture and developed. Eluate was collected in 10-ml fractions. 0.5 ml each of every other fraction was dialyzed against 1 M acetic acid, and its 25 μl was assayed growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. o, A_{280}.

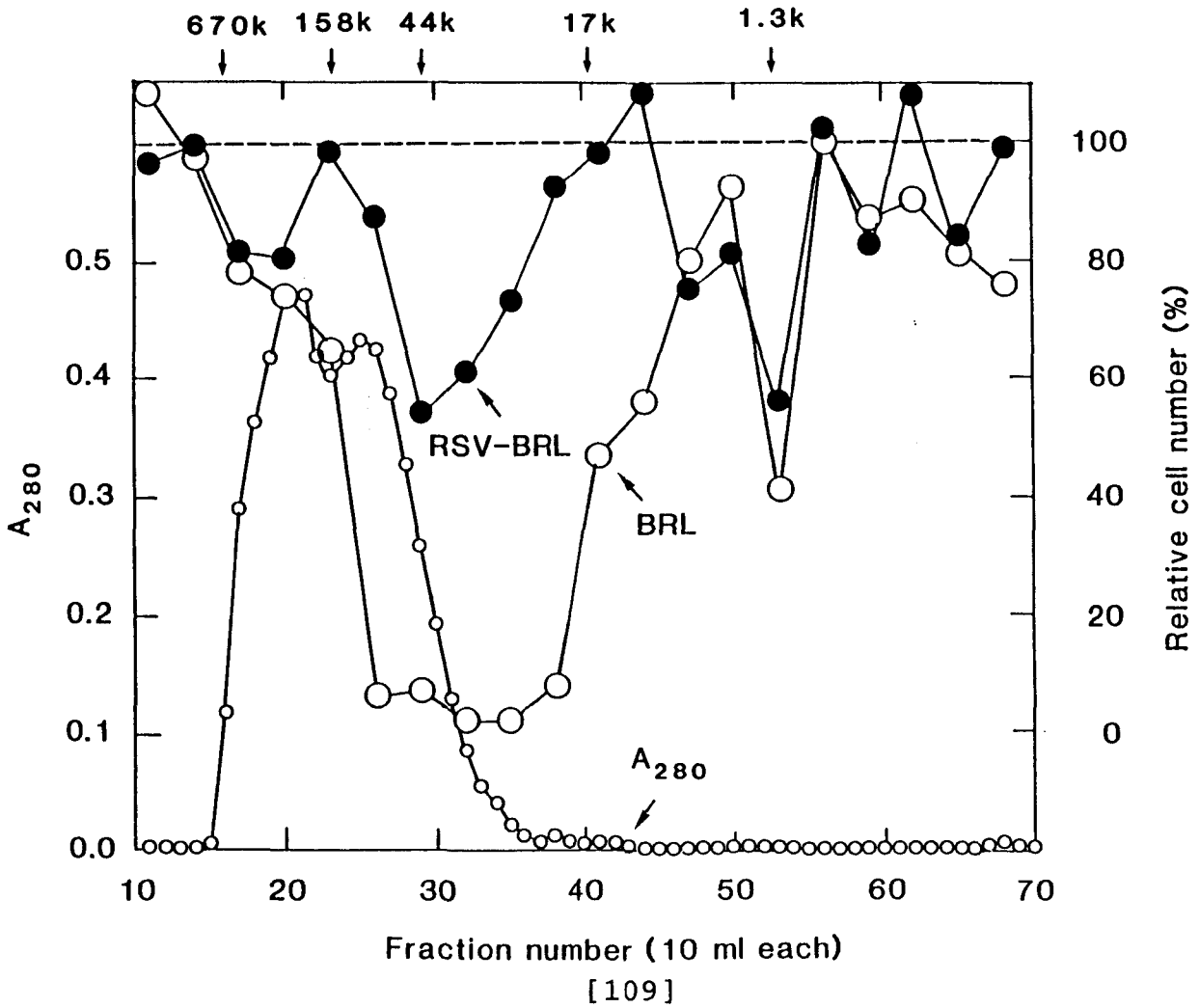


Fig. III-14. Molecular-sieve chromatography on Toyopearl HW55C column in presence of 0.5 M acetic acid and 6 M urea of growth inhibitor fraction obtained by molecular-sieve chromatography.

Growth inhibitor fraction obtained by molecular-sieve chromatography (Fig. II-13, fraction Nos. 15-34) was dialyzed against a mixture of 0.5 M acetic acid and 6 M urea, applied to a Toyopearl HW55C column (2.6 x 95 cm) equilibrated a mixture of 0.5 M acetic acid and 6 M urea and developed. Eluate was collected in 10-ml fractions. 0.5 ml each of every other fraction was dialyzed against 1 M acetic acid, and its 25 μl was assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells. o, A_{280}.

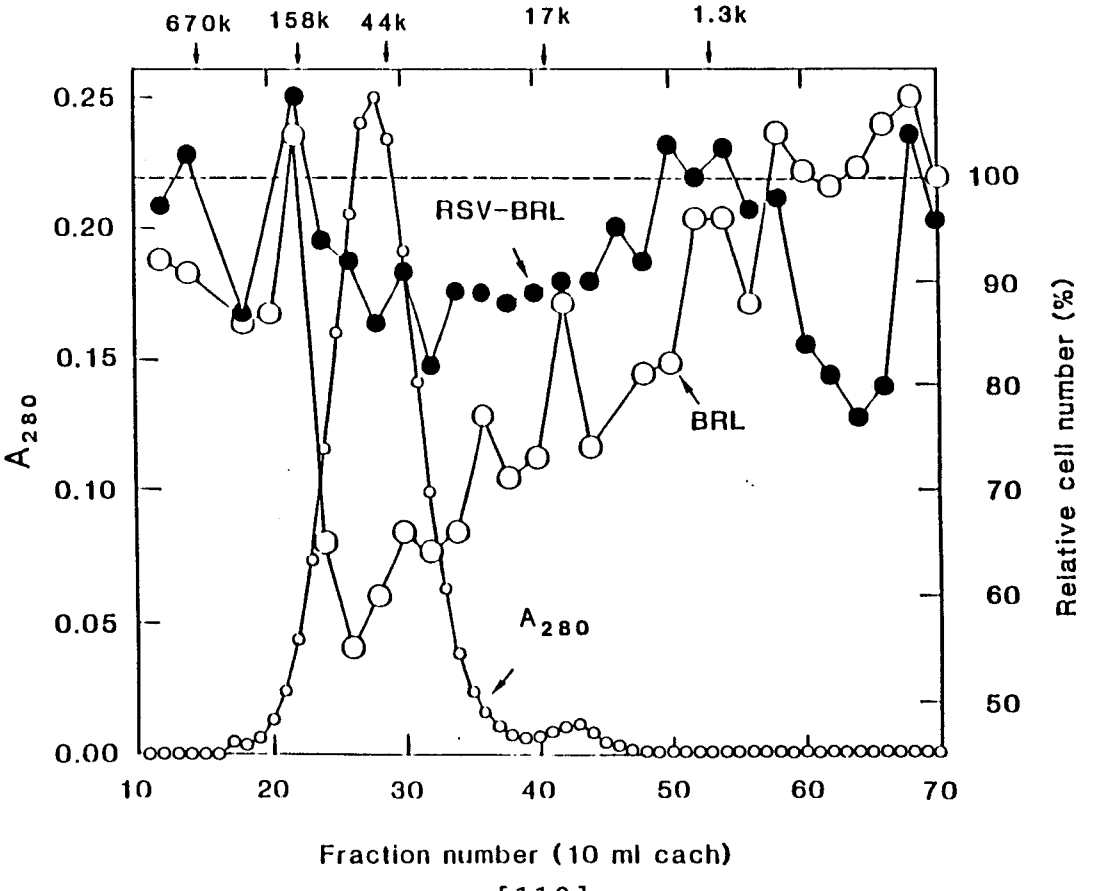


Fig. III-15. Reverse-phase HPLC on Ultron N-C8 (C-8) column of growth inhibitor (c-TGI) fraction obtained by molecular-sieve chromatography.

c-TGI fraction obtained by molecular-sieve chromatography (Fig. III-14, Nos. 49-57) was dialyzed against 1 M acetic acid and applied to a Ultron N-C8 column (4.6 x 150 mm). 25 μl portion of each fraction was assayed for growth inhibitory activity on RSV-BRL (●) cells. Other experimental conditions are described in Materials and Methods. —, A_{280}; ––, acetonitrile.

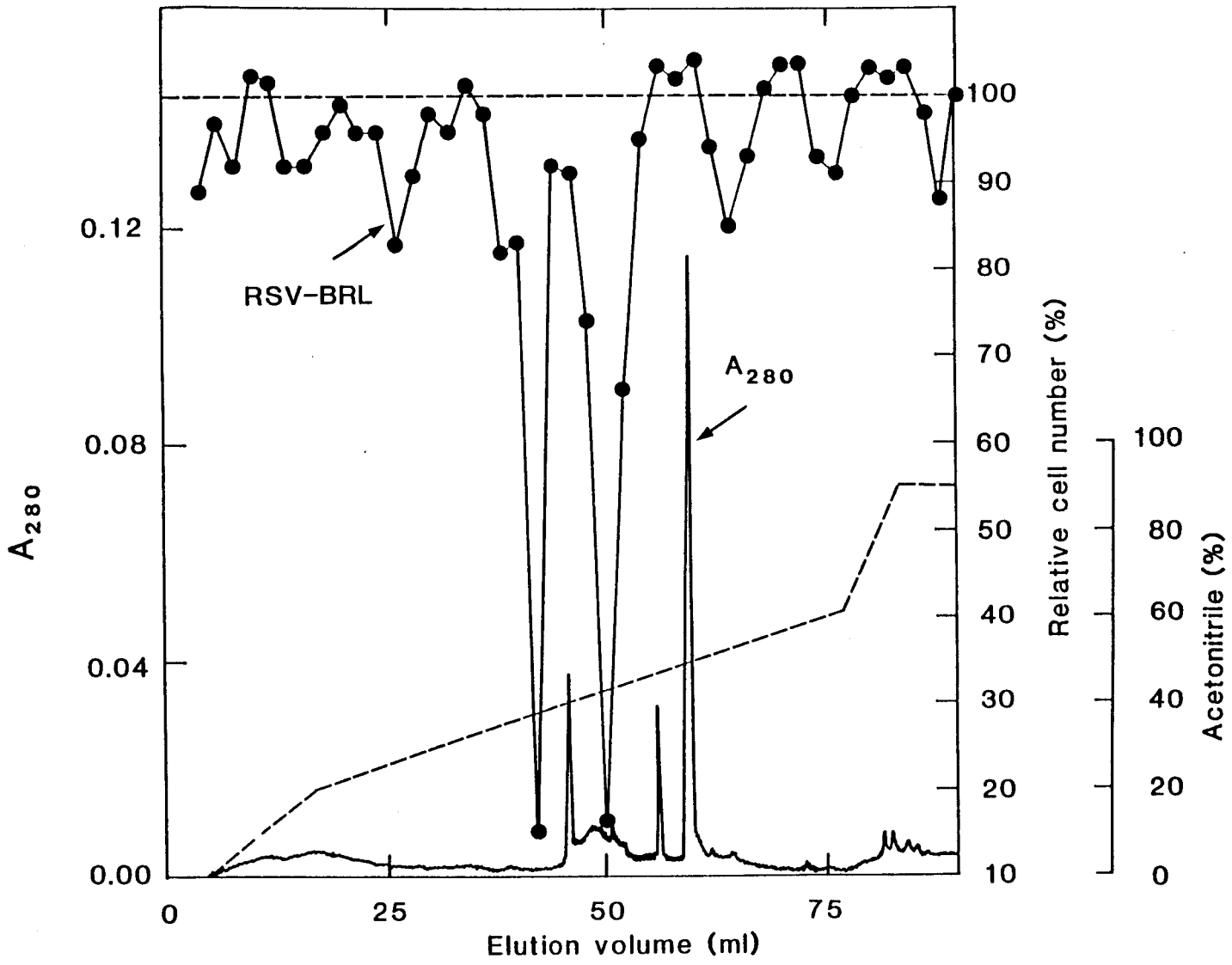


Fig. III-16. SDS-polyacrylamide gel electrophoresis of purified c-TGI-I.

Experimental conditions are described in Materials and Methods.

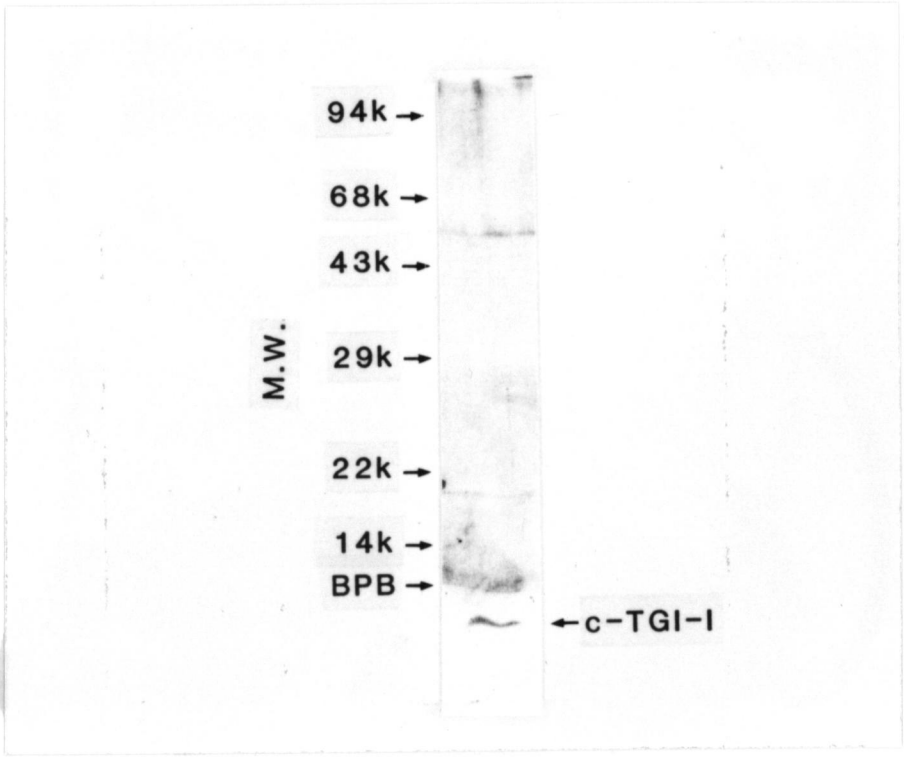


Fig. III-17. SDS-polyacrylamide gel electrophoresis of purified c-TGI-II.

Experimental conditions are described in Materials and Methods.

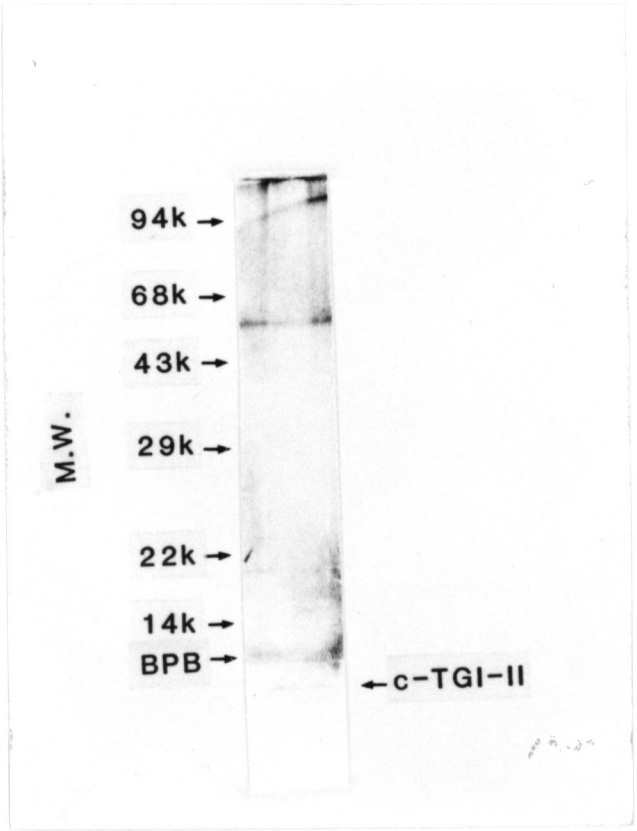


Fig. III-18. Dose-response curves of purified c-TGI-I for growth of BRL and RSV-BRL cells in presence of 10% FCS.

Purified c-TGI-I was added to test medium at the indicated concentrations and assayed for growth inhibitory activity on BRL (○) and RSV-BRL (●) cells.

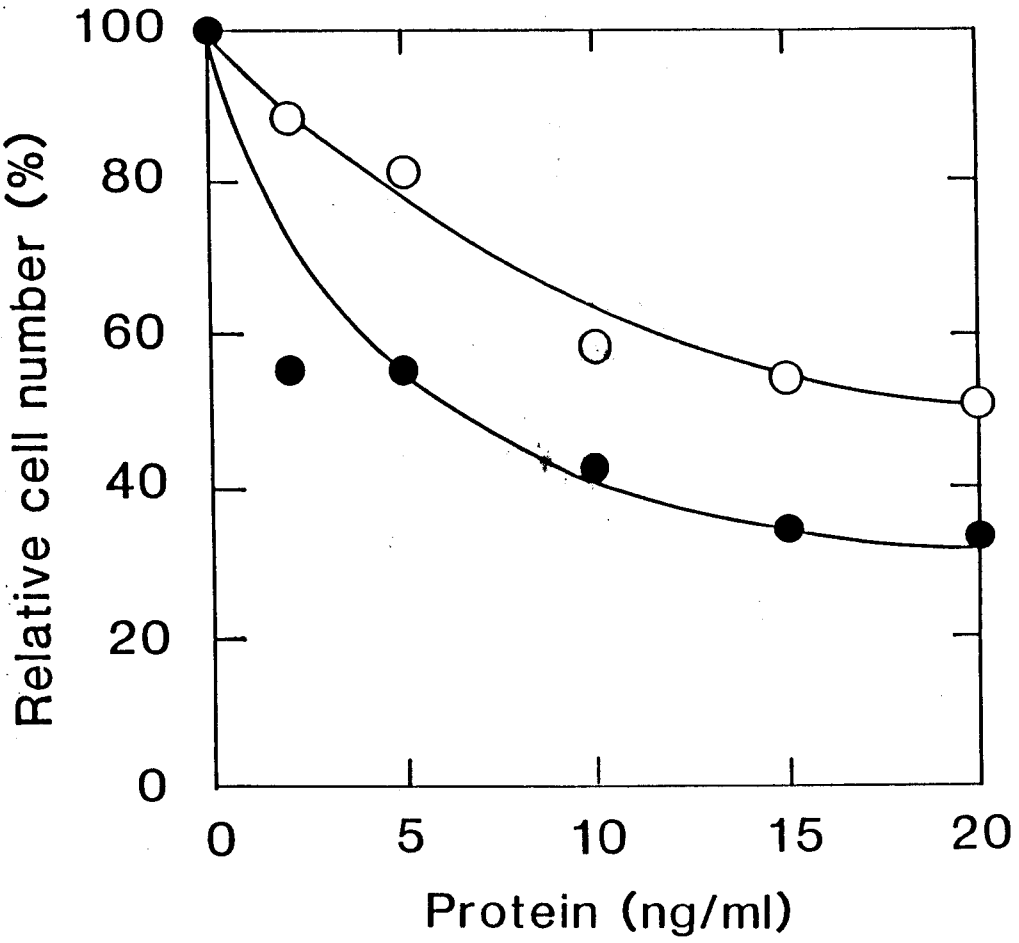
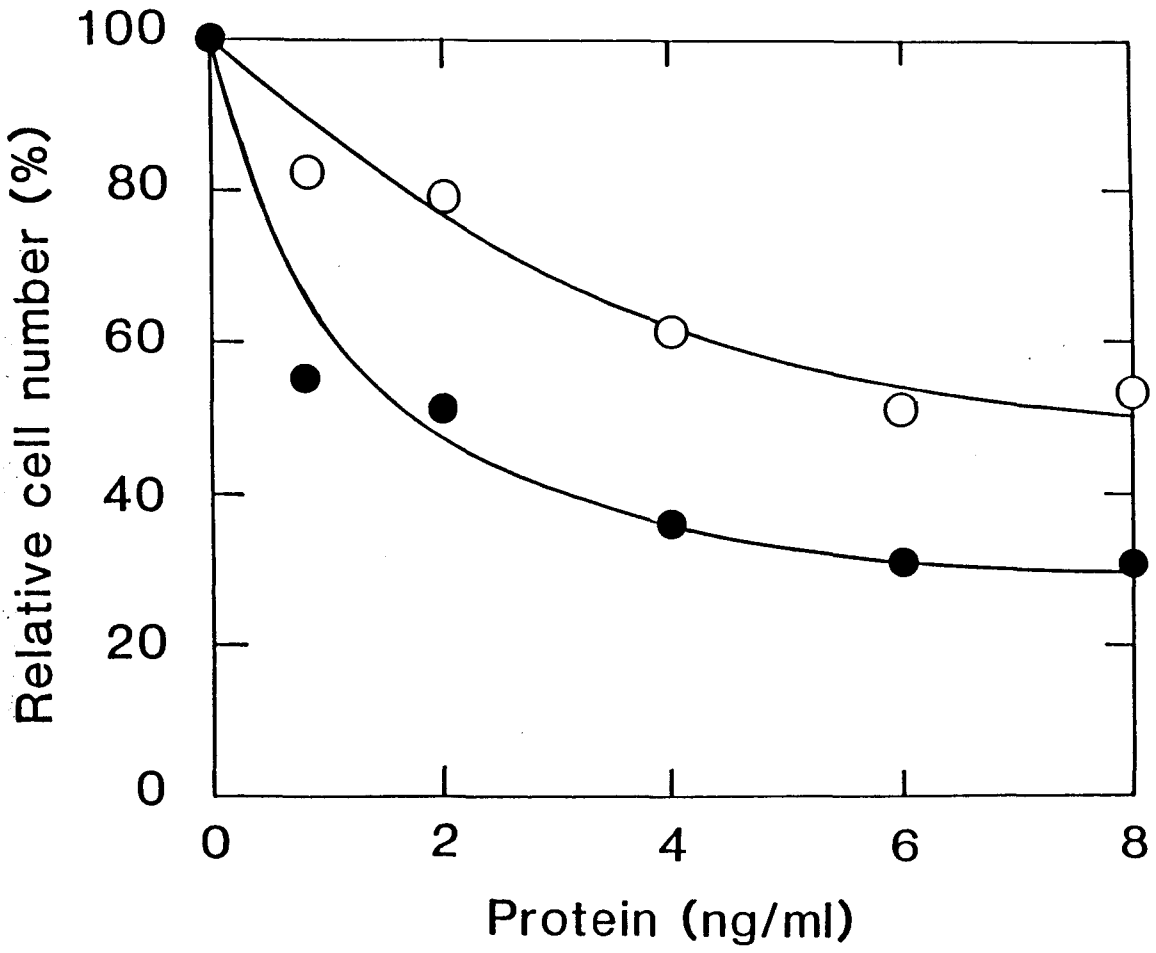


Fig. III-19. Dose-response curves of purified c-TGI-II for growth of BRL and RSV-BRL cells in presence of 10% FCS.

Purified c-TGI-II was added to test medium at the indicated concentrations and assayed growth inhibitory activity on BRL (○) and RSV-BRL (●) cells was determined.



DIFFERENT SPECIES AND AMOUNTS OF mRNA IN VARIOUS NORMAL AND TUMOROUS CELLS OF RATS AS DETECTED BY *IN VITRO* TRANSLATION

Keisuke MASHIMA

CONTENTS

SUMMARY

The species and amounts of mRNA in various normal and tumorous cells of rats were *in vitro* translated into proteins in the presence of [^{35}S]methionine, and the radioactive proteins thus synthesized were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorography. The kind of cells used were the normals: livers with and without regeneration, kidney, spleen and muscle, and tumors: Rhodamine fibrosarcoma, Yoshida ascites sarcoma and AH130 hepatoma. Of the tumors examined, the proteins were similar in species and amounts for all the kinds. A protein with M_r of 37k and pI of 7.5 (p37) was detected with the tumors, but not with the normals. In addition, the tumors had eight proteins (p34, p32, p27, p20, p18, p17.5, p17, p16 and p15), the amounts of which were three to ten times higher than with the normals. With the normals, however, p34 (pI 10) was detected in regenerating liver and spleen, p32 (pI 6.1) in regenerating liver, p20 in kidney, and p18 in muscle and p16 (pI 5.0) in spleen.

Cultured cells of Rhodamine fibrosarcoma were pulse-labeled with [^{14}C]leucine, and the radioactive proteins thus formed were analyzed. The results were similar to those by *in vitro* translation. p37 was evenly located between nuclei and extra-nuclei.

INTRODUCTION

Different kinds of cells carry out metabolisms, which are more or less different from one kind to another. On acquisition of tumorigenicity, cells change some of the properties, so as to become similar to the fetal cells (1, 2). Obviously, these differences are caused by changes in the gene expression. It is reasonable to consider that the rapid growth of normal cells such as regenerating liver are achieved without alteration of the genes. There are, however, many findings indicating that tumoregenicity is acquired by alteration of one or more genes; the altered genes are called onc genes (3-5). In any cases, changes of the gene expression should be reflected on differences of the species and amounts of mRNA and then those of proteins present in the cells.

Differences of the proteins present in normal (non-tumor) and tumorous cells have been studied for these several years, and the results became more precise as the analytical methods were improved. SDS-polyacrylamide gel electrophoresis was applied to study the tissue-specific distribution of nonhistone proteins in nuclei of various tissues of rats including a tumor (6). Two-dimensional polyacrylamide gel electrophoresis (7) was also applied to study differences in protein species between normal and tumorous cells (8, 9). The hybridization of DNA and the cloning of complementary DNA were used to study the differences (10-12) and to analyze onc genes (3-5). Methods for isolation of mRNA and for its translation into the corresponding protein were also improved significantly (13, 14).

In the present paper, the species and amounts of mRNA dominant in various normal and tumorous cells of rats were compared through

analysis of the proteins translated in vitro with the total RNA from the cells with attempts for surveying the proteins associated with the rapid growth of normal and tumorous cells.

MATERIALS AND METHODS

Animals and tumors ——— Adult male albino rats of Donryu strain were used. Rhodamine sarcoma was subcutaneously implanted on the back of animals by the method of Matuo et al. (15). On the other hand, cells of AH130 and Yoshida sarcoma were implanted intraperitoneally. Regenerating liver was obtained by a partial hepatectomy, in which about 70% of the total liver was removed by the method of Higgins et al. (16).

Cells and cell culture ——— A cell line of Rhodamine sarcoma (RdF4) was a gift from Prof. K. Nishikawa (Kanazawa Medical University, Ishikawa). Cells were grown routinely in plastic tissue-culture dishes (#3002, Falcon Plastic Co., Oxnard, California) at 37 °C in a humidified atmosphere of 5% CO_2 in the air. The culture medium used was a mixture of equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco, Grand Island, New York), which was supplemented with 10% (v/v) fetal calf serum (M. A. Bioproducts, Walkersville, Maryland), 100 units/ml of penicillin G (Meiji Seika Co., Ltd., Tokyo) and 100 μg/ml of streptomycin sulfate (Meiji Seika Co., Ltd., Tokyo).

Preparation of total RNA from various cells ——— The total RNA was prepared from various tissues and cultured cells by the method of Chirgwin et al. (13). With a Potter-Elvehjem homogenizer, fresh tissue or cultured cells were homogenized in a guanidinium thiocyanate solution, the volume of which was sixteen times as much as that of the original material. The guanidinium thiocyanate solution consisted of 4 M guanidinium thiocyanate, 0.5% (w/v) sodium N-lauroylsarcoside, 25 mM sodium citrate and 0.1 M 2-mercaptoethanol

(pH 7.0) and was sterilized with an autoclave. The homogenate described above was centrifuged at 10 °C for 10 min at 10,000 x g. The resultant supernatant was mixed with 0.025 volume of 1 M acetic acid and 0.75 volume of ethanol. One volume is the same as the total volume of the guanidinium thiocyanate solution used for homogenization. The mixture was allowed to stand overnight at -20 °C, followed by centrifugation at -10 °C at 6,000 x g for 10 min. The resultant precipitate was suspended in 0.5 volume of a guanidine hydrochloride solution, and mixed with 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The guanidine hydrochloride solution consisted of 7.5 M guanidine hydrochloride, 25 mM sodium citrate, 5 mM dithiothreitol (pH 7.0) and was sterilized with an autoclave. The mixture was allowed to stand at -20 °C for 3 h and centrifuged at -10 °C at 6,000 x g for 10 min. The resultant precipitate was suspended in 0.25 volume of the guanidine hydrochloride solution, and mixed with 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The mixture was allowed to stand at -20 °C for 3 h and centrifuged at -10 °C at 6,000 x g for 5 min. The resultant precipitate was dispersed in ethanol at a room temperature (20-25 °C), and centrifuged at 10 °C at 6,000 x g for 5 min. The precipitate thus obtained was placed under a stream of nitrogen gas in order to remove the remaining ethanol, and then dissolved in 1.0 ml of sterile water per g of the original tissue, followed by centrifugation at 10 °C at 28,000 x g for 10 min. The resultant supernatant was collected by decantation. For re-extraction, the resultant precipitate was suspended in 0.5 ml of sterile water per g of the original tissue, followed by centrifugation at 10 °C at 28,000

x g for 10 min. The re-extraction was repeated once more. The three supernatants thus obtained were mixed. The mixture was supplemented with 0.1 volume of 2 M potassium acetate (pH 5.0) and 2 volumes of ethanol, and allowed to stand overnight at -20 °C, followed by centrifugation at -10°C at 16,000 x g for 20 min. The resultant precipitate was washed with 95% (v/v) ethanol, dried under a stream of nitrogen gas and dissolved in sterile water. The resultant solution was used as total RNA. The concentration of RNA was determined, regarding that an 1% (w/v) RNA would exhibit an absorbance at 260 nm of 200.

In vitro translation of total RNA ——— The total RNA was subjected to in vitro translation by the method of Pelham et al. (17) with some modifications. The reaction mixture consisted of 16μl of rabbit reticulocyte lysate (code N. 90, Amersham-Japan, Tokyo), 4 μl of [^{35}S]methionine (0.02 μCi) (specific activity of 1,280 Ci/mmol, code SJ. 204, Amersham-Japan, Tokyo), 2 μl of the total RNA (1-2 μg as RNA) and water to make the total volume 22 μl. The reaction was carried out at 30°C for 1 h, and stopped by lowering to an ice-cold temperature. For determining the extent of the translation, 1 μl of the resultant reaction mixture was mixed with 0.5 ml of 1 N sodium hydroxide containing 5% (w/v) hydrogen peroxide, and incubated at 37 °C for 10 min. The incubated solution was cooled in an ice bath, mixed with 1 ml of ice-cold 25% (w/v) TCA, and allowed to stand in an ice bath for 30 min. The resultant solution was filtered through on Whatman glass filter (GF/A, Whatman Ltd., Springfield Mill, England). The TCA-insoluble material thus trapped on the filter was washed twice with 5 ml in each time of

ice-cold 8% (w/v) TCA and once with 5 ml of ethanol. The together with the filter, it was dried, put into a scintillation cocktail, which consisted of toluene containing 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]-benzene, and measured of the radioactivity with a liquid scintillation spectrometer (model LSC-900, Aloka, Co., Ltd., Mitaka). About 5,000 cpm of [^{35}S]methionine was incorporated into the TCA-insoluble fraction (translated proteins) per μl of the reaction mixture. About 5 and 10 μl of the reaction mixture were used for analyzing the translated proteins by SDS-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis, respectively.

In some experiments, the total RNA was subjected to chromatography on a poly(U)-Sepharose 4B column. The mRNA having polyadenine tail was collected and used for *in vitro* translation. The results thus obtained were essentially the same as those with the total RNA.

Pulse-labeling of proteins in vitro ——— In a 35-mm plastic tissue-culture dish (#3002, Falcon, Oxnard, California) were incubated at 37°C for 1 h in leucine-free Eagle's minimum essential medium (leucine-free MEM, Gibco, Grand Island, New York) supplemented with 10% (v/v) of fetal calf serum previously dialyzed against PBS(-), and L-[U-^{14}C]leucine (10 Ci/ml) (specific activity of 330 mCi/mmol, code CFB. 183, Amersham-Japan, Tokyo). The incubated cells were harvested by exposing to 1 ml of PBS(-) containing 0.5 mM EGTA and 0.5 mM EDTA. They were washed twice with PBS(-) by centrifugation. The washed cells were subjected to SDS-

polyacrylamide gel electrophoresis. In some cases, the washed cells were subjected to cellular fractionation (18). They were suspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 3 mM $CaCl_2$ and 0.5% (w/v) Triton X-100, and stirred at 4°C for 30 min, followed by centrifugation at 4°C at 1,200 x g for 10 min. The resultant supernatant is called "extranuclei fraction". The resultant precipitate was suspended in 0.5 ml of the same buffer containing sucrose, $CaCl_2$ and Triton X-100, and layered onto 0.5 ml of 0.88 M sucrose in a eppendorf 1.5 ml tube (Eppendorf, Homburg), followed by centrifugation at 4°C at 1,200 x g for 10 min. The resultant precipitate is called "nuclei fraction". The nuclei and extranuclei fractions were measured of radioactivity as described above.

SDS-polyacrylamide gel electrophoresis ——— Electrophoresis in a polyacrylamide gel containing SDS was carried out by the method of Laemmli (19) using a KPI electrophoresis apparatus (Model E-IE 17-30TR, Koike Precision Inst., Kanagawa), as reported previously (6). The separating gel slab (240 x 150 x 2 mm) was prepared from a mixture of 0.375 M Tris-HCl buffer (pH 8.8), 0.1% (w/v) SDS, acrylamide with a linear gradient from 10 to 20% (w/v), N,N'-methylene-bisacrylamide (Bis) with a gradient from 0.27 to 0.53% (w/v) and 0.0175% (w/v) ammonium persulfate and 0.05% (w/v) N,N,N',N'-tetraethylmethylenediamine (TEMED). The stacking gel slab (20 x 150 x 2 mm) was prepared from a mixture of 0.125 M Tris-HCl buffer (pH 6.8), 0.1% SDS, 2.5% acrylamide, 0.6% Bis, 0.0375% ammonium persulfate and 0.1% TEMED. Samples were dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% (v/v) 2-

mercaptoethanol and 7% (v/v) glycerol, and the solutions were heated at 100°C for 2 min. Electrophoresis was carried out at 15 mA per gel slab at 10°C for 30 h, using a mixture of 25 mM Tris, 192 mM glycine and 0.1% SDS as the electrode buffer. The M_r makers used were phosphorylase b (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 29,000), sobean trypsin inhibitor (M_r 21,500) and lysozyme (M_r 14,000).

Two-dimensinal polyacrylamide gel electrophoresis ——— Two-dimensinal polyacrylamide gel electrophoresis was performed by the method of O'Farrel as modified by Tsuji et al. (20). The first-dimensional isoelectric electrophoresis was carried out in cylindrical gel (130 x 5 mm) containing 4% (v/v) acrylamide, 2% (v/v) Ampholine (pH 3.5-10), 8 M urea and 2% (v/v) Nonidet P-40. The second-dimensional SDS-gel electrophoresis was carried out in a separating gel slab (240 x 150 x 2 mm) with a linear gradient of acrylamide concentration from 10 to 20% (w/v), as described above.

Fluorography ——— After electrophoresis, the gel slab was subjected to fluorography by the method of Bonner et al. (21), using dimethysulfoxide and 2,5-diphenyloxazyole. It was exposed to Fuji X-ray film (RX) (Fuji Photo Film Co., Ltd., Minami-Ashigara) at -80°C for about 1 week, unless otherwise stated. Densitogram of the exposed film was measured with a Shimadzu Dual-Wavelength TLC Scanner (Model CS-910, Shimadzu Seisakusho Ltd., Kyoto) at the single wavelength mode with a sample wavelength of 550 nm.

RESULTS

Comparison of species and amounts of proteins in vitro translated with total RNA from various cells ——— Total RNA preparations were made from the five kinds of normal cells (livers with and without regeneration, spleen, kidney and muscle), and the three kinds of tumorous cells (Rhodamine sarcoma, Yoshida sarcoma and AH130 hepatoma). The mRNAs present in the preparations were translated in vitro into the corresponding proteins in presence of [^{35}S]methionine. The radioactive proteins thus synthesized were separated by SDS-polyacrylamide gel electrophoresis, and then analyzed by fluorography (Figs. 1 & 2). With the kinds of normal

Fig. 1

Fig. 2

cells examined, the proteins detected were significantly different in species and amount from one kind to another; some of them were specific to the respective tissues. The nomenclature of proteins refers to the M_r values (x 10^{-3}) affixed to "p" (protein). Relative to the other tissues, spleen was most abundant in p34, p32 and p16, kidney in p27 and p20, and muscle in p18. On the other hand, with the kinds of tumorous cells examined, the proteins detected were similar to one kind to another both in species and amount, but significantly different from those of all the normal cells. Ubiquitous in all tumors, p37 was detected in a significant amount, but not in the normals. The other nine proteins, p34, p32, p27, p20,

p18, p17.5, p17, p16 and p15 were significantly higher in amount with the tumors than with the normals.

In order to examine which of the ten proteins were associated with the rapid growth, total RNA preparations were also made from regenerating liver, and subjected to *in vitro* translation (Fig. 3).

Fig. 3

The p34, p32, p27, p17 and p15, which had not been detected before hepatectomy, became detectable, although the amounts of these proteins were significantly lower than those in the tumors. p37 was not detected with regenerating liver, either.

In some experiments, mRNAs were collected from the total RNA, and subjected to *in vitro* translation. The results thus obtained were essentially the same as those with the total RNA.

Two-dimensional polyacrylamide gel electrophoresis of proteins translated in vitro with total RNA from Rhodamine sarcoma ———

The proteins translated *in vitro* with the total RNA form Rhodamine sarcoma were analyzed by two-dimensional polyacrylamide gel electrophoresis and fluorography. The pI values determined were 7.5 for p37, 10 for p34, 6.1 for p32 and 5.0 for p16. The other six proteins, p27, p20, p18, p17.5, p17 and p15 were not detectable in the first-dimensinal gel for isoelectric electrophoresis, perhaps, due to their too high or low pI values.

Table I summarizes the results described above.

Table I

Pulse-labeling of proteins in vivo ——— The cell line of Rhodamine sarcoma (RdF4) was cultured. The cultured cells were pulse-labeled for 1 h with [^{14}C]leucine, and then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 4). The

Fig. 4

pulse-labeled proteins were similar in species and amount to the translated proteins, indicating that the analysis by in vitro translation reflected the rates of protein synthesis rather than the amounts of the proteins present in the cells.

The cultured tumorous cells, previously pulse-labeled with [^{14}C]leucine, were divided into nuclei and extranuclei fractions, and then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 5). It was found that p37 and p18 were evenly

Fig. 5

located in both fractions, p34, p32, p27, p20 and p16 in extranuclei fraction, and p17.5, p17 and p15 in the nuclei fraction. Table II summarizes the results described above.

Table II

DISCUSSION

By isoelectric electrophoresis, polyacrylamide gel electrophoresis in the presence and absence of SDS and two-dimensional polyacrylamide gel electrophoresis, we studied differences of pyruvate kinase isozymes (22, 23), catalase (24) and nuclear proteases (25), nuclear nonhistone proteins (6), and serum proteins (26) among various normal and tumorous tissues of rats with and without bearing tumor. By two-dimensional gel electrophoresis, Leavitt and Kakunaga (8) reported that at least 24 protein species are different between human fibroblasts before and after transformation *in vitro* by a single chemical treatment, and that a variant from of actin with M_r 44k and pI 5.2 is formed to the highest extent after transformation. By molecular hybridization with complementary DNA, Hirsh *et al.* (11) found quantitative differences of mRNA species among livers before and after hepatectomy and Novikoff hepatoma. A similar study was carried out with L-cells and various tissues of mice by Ryffel and McCarthy (10). There have been, however, few reports on the comparison of mRNA species among normal and tumorous cells.

Conceivably, the proteins associated with the rapid growth of cells are synthesized at rates faster than the other proteins. In the present study, the species and amounts of mRNA in various normal and tumorous cells of rats were measured by the method, in which the mRNAs present in total RNA preparations from cells were translated *in vitro* into the corresponding proteins and then the proteins thus synthesized were analyzed by SDS-gel electrophoresis and fluorography. It was found that this method gave differences among

normal and tumorous cells to an extent significantly more pronounced than the analysis of the proteins present in cells.

The proteins synthesized by *in vitro* translation were similar in species and amount for all the kinds of tumorous cells examined, although they were significantly different from those of the normal cells examined. In the tumors examined, the ten species of mRNA coding p37, p34, p32, p27, p20, p18, p17.5, p17, p16 and p15 existed at amounts significantly higher than those in the normals examined. Of the ten species of mRNA, that coding p37 was detectable only in the tumors, almost evenly distributing in both of the outside and inside of the nuclei. The species of mRNAs coding p34, p32 and p27 were not detectable in normal liver, but they were in regenerating liver. Probably, p17.5, p17 and p15 were histones, because their migrations in SDS-gel electrophoresis corresponded to those of histone H3, H2A and H2B, respectively, and they were localized in the nuclei fraction. The amounts of the histones per weight of DNA are essentially the same for all the cells, normal and tumorous (6). This indicates that the mRNA species coding these histones were present in the tumors to a significantly higher extent than in the normals; thus, their amounts increased in parallel to the increasing amount of DNA in the tumor.

Miyazaki *et al.* (6) reported that nuclear proteins with M_r of 54k and 34k are inherent to Rhodamine sarcoma. Possibly, their 34k-protein corresponded to the nuclear p37 in the present study, with some difference in M_r. By *in vitro* translation with the total mRNA of normal and regenerating liver, and Novikoff hepatoma, Wu *et al.* (27) reported that the amounts of the six proteins of the 40S

ribosomal subunit (M_r: 35k, 34k, 33k, 32k, 30k and 25k) were 3 to 5-fold higher with the hepatoma and regenerating liver than with the normal liver. It seems likely that some of the ribosomal proteins corresponded to p34, p32 and p27.

Recently, various onc genes and their products have been characterized (8-10). It is not known, however, how the proteins coded on onc genes can control the phenotype of tumorous cells, in particular their ability to grow in the host animals. It is important, therefore, to find the functions of the proteins associated with rapid growth and tumorigenicity.

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Table I. Relative amounts and pI values of proteins abundant in tumors. than The relative amounts of the proteins were measured the areas from the densitograms in Figs. 1, 2 and 3, taking the amounts of the respective poteins in Rhodamine sarcoma as 100. nd, not detectable; regenerating liver, 20 h after hepatectomy.

Protein		Relative amount							
	(pI)	Rhodamine sarcoma	Yoshida sarcoma	AH130	Liver	Spleen	Kidney	Muscle	Regenerating liver
p37	7.5	(100)	70	80	nd	nd	nd	nd	nd
p34	10	(100)	120	80	nd	50	10	10	40
p32	6.1	(100)	110	90	nd	20	nd	nd	40
p27	---	(100)	140	60	nd	20	50	30	30
p20	---	(100)	120	80	10	nd	20	nd	10
p18	---	(100)	60	30	10	10	10	20	10
p17.5	---	(100)	120	90	10	10	20	nd	10
p17	---	(100)	110	70	nd	30	20	10	20
p16	5.0	(100)	140	80	20	50	20	10	20
p15	---	(100)	130	70	nd	30	30	30	10

Table II. Intracellular distribution of proteins abundant in cultured cells of Rhodamine sarcoma. The relative amounts of the proteins were measured from the densitograms in Fig. 4 in the same method as that for Table I, taking the amount of p37 in the nuclei fraction as 100. nd, not detectable.

Fraction	Relative amount									
	p37	p34	p32	p27	p20	p18	p17.5	p17	p16	p15
Nuclei	(100)	20	nd	5	nd	20	60	60	nd	30
Extranuclei	100	100	80	40	20	30	nd	nd	50	nd

Fig. 1. SDS-polyacrylamide gel electrophoresis of *in vitro* translated ^{35}S-proteins with total RNA from liver, spleen, kidney and muscle. Experimental conditions are described in the text. L, with liver; S, with spleen; K, with kidney; M, with muscle.

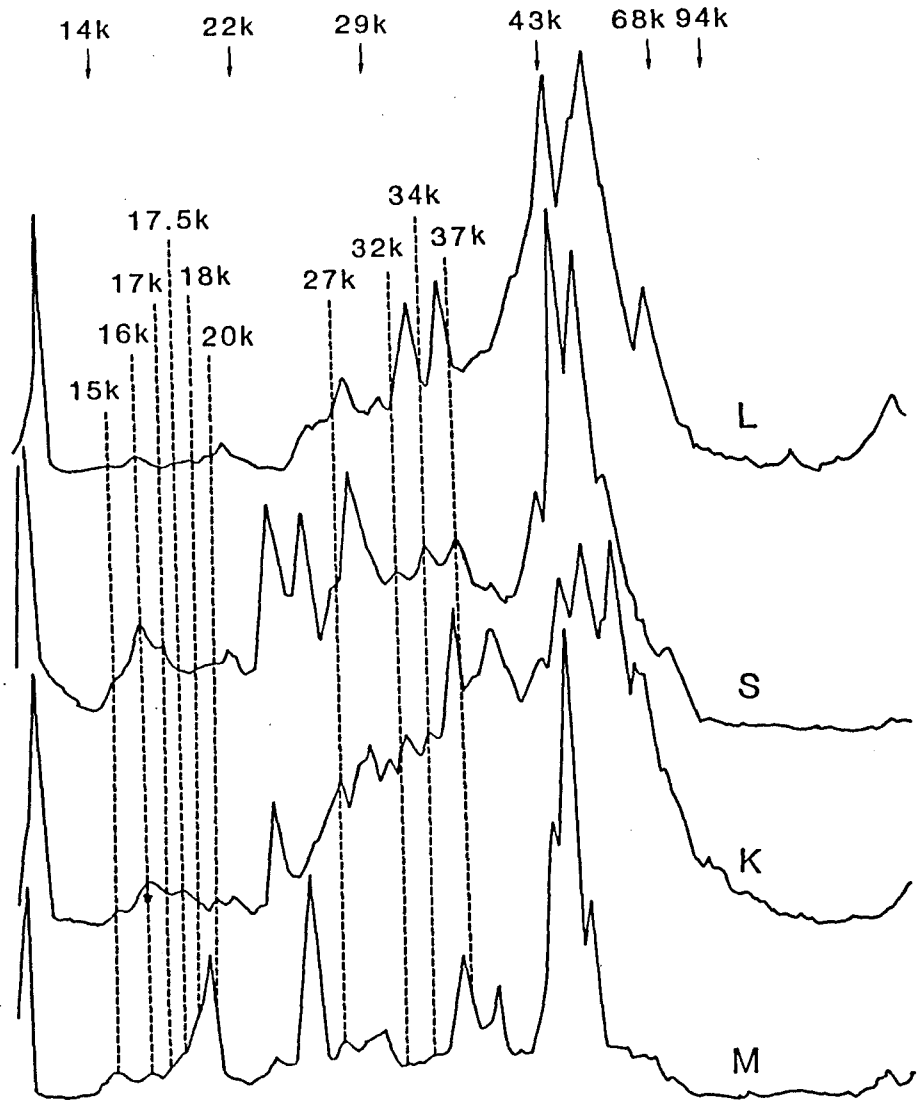

Fig. 2. SDS-polyacrylamide gel electrophoresis of <u>in vitro</u> translated ^{35}S-proteins with Rhodamine sarcoma, Yoshida sarcoma and AH130. Experimental conditions are described in the text. R, with Rhodamine sarcoma; Y, with Yoshida sarcoma; AH130, with AH130 hepatoma.

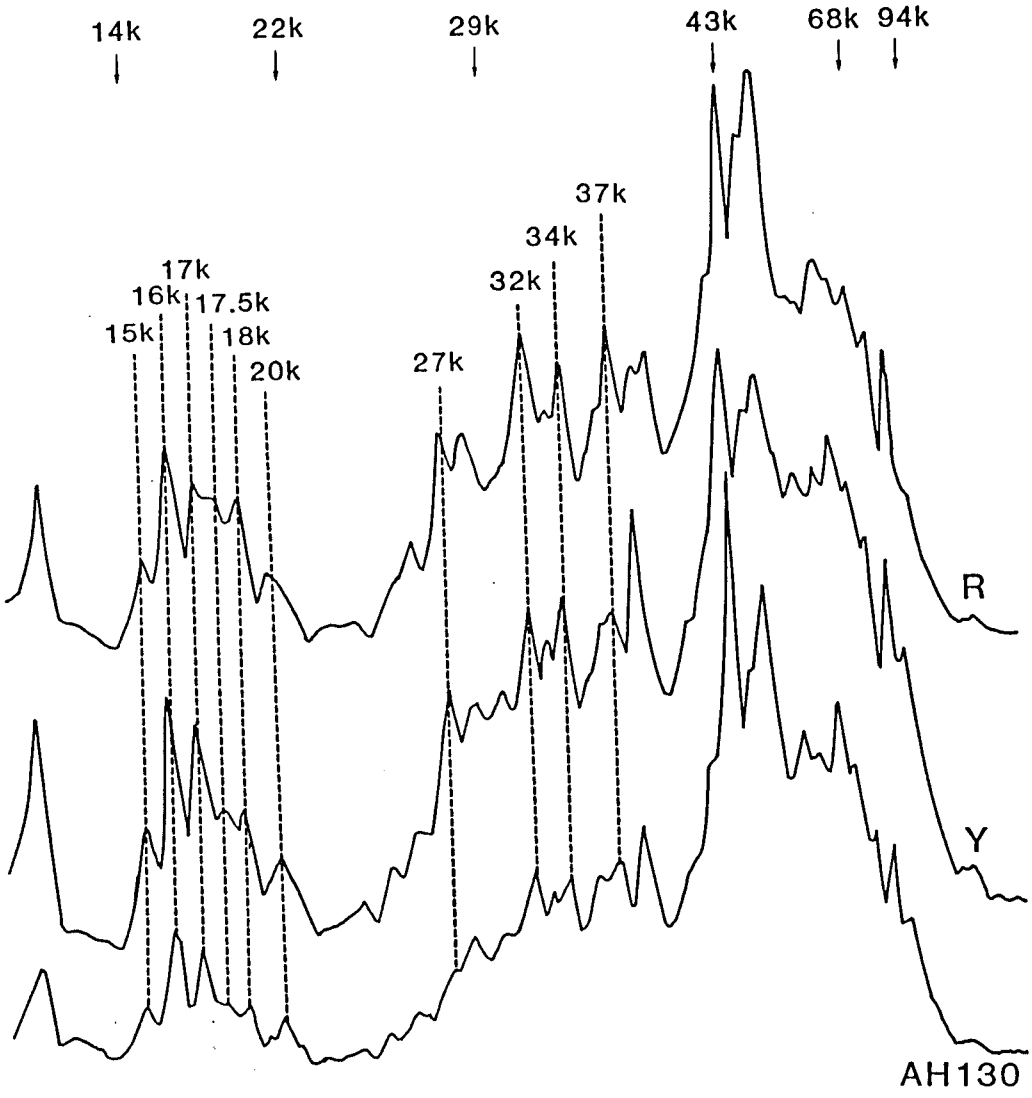

Fig. 3. SDS-polyacrylamide gel electrophoresis of <u>in vitro</u> translated ^{35}S-proteins with control and regenerating livers. Regenerating livers were obtained 20 h (20-R) and 26 h (26-R) after hepatectomy. Control livers (C) were obtained 20 h after Sham-operation. Other experimental conditions are the same as for Fig. 1.

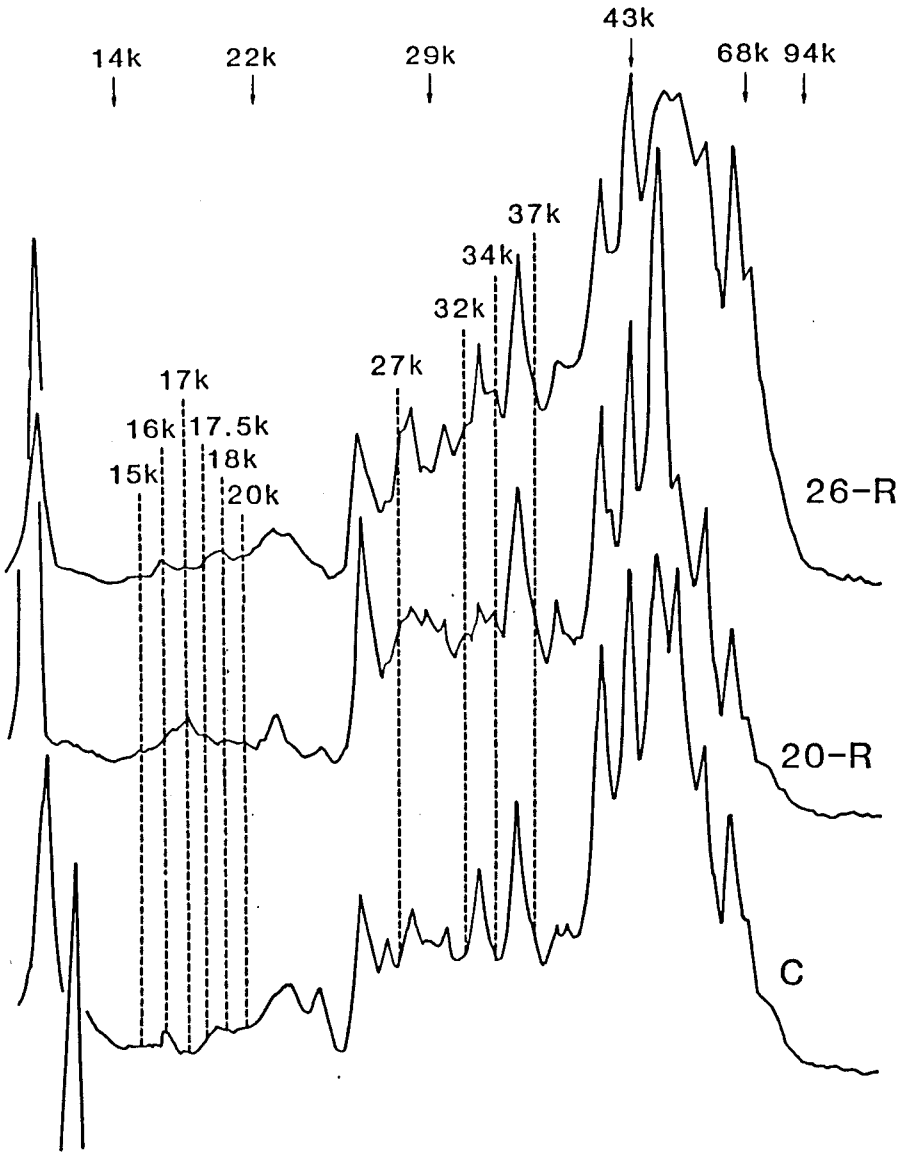

Fig. 4. SDS-polyacrylamide gel electrophoresis of in vitro translated ^{35}S-proteins and in vivo ^{14}C-pulse-labeled proteins with cultured cells of Rhodamine sarcoma RdF4. Total RNA was prepared from cultured cells of Rhodamine sarcoma RdF4 and translated in vitro. In parallel, the cultured cells were incubated for 1 h in the culture medium containing [^{14}C]leucine. Both the in vitro translated ^{35}S-proteins (TR) and the in vivo ^{14}C-pulse-labeled proteins (PL) were analyzed. Other experimental conditions are described in the text.

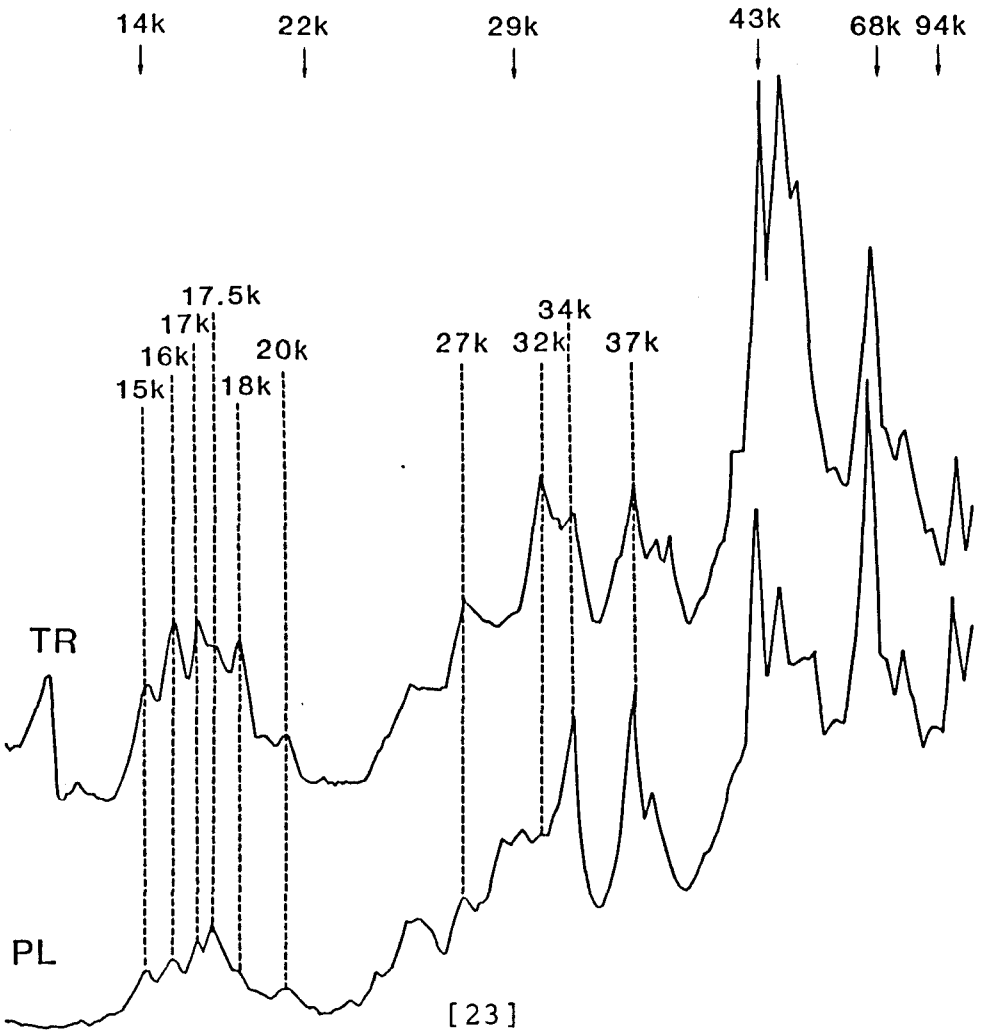

Fig. 5. SDS-polyacrylamide gel electrophoresis of <u>in vivo</u> ^{14}C-pulse-labeled proteins in nuclei and extranuclei fractions with cultured cells of Rhodamine sarcoma. Pulse-labeling was carried out in the same manner as for Fig. 4. The resultant cells were divided into nuclei and extranuclei fractions. These two fractions were respectively analyzed. Other experimental conditions are described in the text. N, nuclei fraction; EX, extranuclei fraction.