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(54) Titre : ADN ENCODANT UN FACTEUR DE CROISSANCE SPECIFIQUE POUR LES CELLULES EPITHELIALES

(54) Title: DNA ENCODING A GROWTH FACTOR SPECIFIC FOR EPITHELIAL CELLS

(57) **Abrégé/Abstract:**

Discoveries are disclosed that show particular aspects of recombinant DNA technology can be used successfully to produce hitherto unknown human keratinocyte growth factor (KGF) protein free of other polypeptides. These proteins can be produced in various functional forms from spontaneously secreting cells or from DNA segments introduced into cells. These forms variously enable biochemical and functional studies of this novel protein as well as production of antibodies. Means are described for determining the level of expression of genes for the KGF protein, for example, by measuring mRNA levels in cells or by measuring antigen secreted in extracellular or body fluids.



Discoveries are disclosed that show particular aspects of recombinant DNA technology can be used successfully to produce hitherto
5 unknown human keratinocyte growth factor (KGF) protein free of other polypeptides. These proteins can be produced in various functional forms from spontaneously secreting cells or from DNA segments introduced into cells. These forms
10 variously enable biochemical and functional studies of this novel protein as well as production of antibodies. Means are described for determining the level of expression of genes for the KGF protein, for example, by measuring
15 mRNA levels in cells or by measuring antigen secreted in extracellular or body fluids.

FIELD OF THE INVENTION

The present invention relates to growth factors, particularly to isolation of a polypeptide growth factor similar to a family of factors including known fibroblast growth factors (FGFs). This invention also relates to construction of complementary DNA (cDNA) segments from messenger RNA (mRNA) encoding the novel growth factor. Further, this invention pertains to synthesis of products of such DNA segments by recombinant cells, and to the manufacture and use of certain other novel products enabled by the identification and cloning of DNAs encoding this growth factor. In addition, a high affinity receptor is provided for the novel growth factor.

ABBREVIATIONS USED IN THIS APPLICATION

	aFGF	acidic fibroblast growth factor
	bFGF	basic fibroblast growth factor
	EGF	epidermal growth factor
5	HSAC	heparin-Sepharose affinity chromatography
	kb	kilobases
	K _d	dissociation constant
	kDa	kilodaltons
10	KGF	keratonicyte growth factor
	NaDodSo ₄ /PAGE	Sodium dodecylsulfate (SDS)/polyacrylamide gel electrophoresis
	RP-HPLC	reversed-phase high performance
	TGF α	transforming growth factor α

Growth factors are important mediators of intercellular communication. These potent molecules are generally released by one cell type and act to influence proliferation of other cell types (see reference I-1 in Experimental Section I, below). Interest in growth factors has been heightened by evidence of their potential involvement in neoplasia (reference II-2 in Experimental Section II, below). The v-sis transforming gene of simian sarcoma virus encodes a protein that is homologous to the B chain of platelet-derived growth factor (I-1, I-2). Moreover, a number of oncogenes are homologues of genes encoding growth factor receptors (I-1). Thus, increased understanding of growth factors and their receptor-mediated signal transduction pathways is likely to provide insights into mechanisms of both normal and malignant cell growth.

One known family of growth factors affecting connective tissue cells includes acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and the related products of the *hst* and *int-2* oncogenes.

Further, it is known that some growth factors, including the following, have heparin-

binding properties: aFGF (I-20, I-21); bFGF (I-19, I-20); granulocyte/macrophage colony stimulating factor (I-1); and interleukin 3 (I-1). Each of these polypeptide factors is
5 produced by stromal cells (I-1, I-2, I-25). Such factors appear to be deposited in the extracellular matrix, or on proteoglycans coating the stromal cell surface (I-1, I-25). It has been postulated that their storage, release and
10 contact with specific target cells are regulated by this interaction (I-25, I-28).

It is widely recognized, however, that the vast majority of human malignancies are derived from epithelial tissues (I-5). Effectors
15 of epithelial cell proliferation derived from mesenchymal tissues have been described (I-1, I-2, I-3), however, their molecular identities and structures have not been elucidated.

In light of this dearth of knowledge
20 about such mesenchymal growth factors affecting epithelial cells, it is apparent that there has been a need for methods and compositions and bioassays which would provide an improved knowledge and analysis of mechanisms of
25 regulation of epithelial cell proliferation, and, ultimately, a need for novel diagnostics and therapies based on the factors involved therein.

This invention contemplates the application of methods of protein isolation and recombinant DNA technologies to fulfill such needs and to develop means for producing protein factors of mesenchymal origin, which appear to be related to epithelial cell proliferation processes and which could not be produced otherwise. This invention also contemplates the application of the molecular mechanisms of these factors related to epithelial cell growth processes.

SUMMARY OF THE INVENTION

The present invention relates to developments of protein isolation and recombinant DNA technologies, which include production of novel growth factor proteins affecting epithelial cells, free of other peptide factors. Novel DNA segments and bioassay methods are also included.

The present invention in particular relates to a novel protein having structural and/or functional characteristics of a known family of growth factors which includes acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and the related products of the *hst* and *int-2* oncogenes. This new member of the FGF polypeptide family retains the heparin-binding properties of the FGFs but has evolved a unique target cell specificity. This growth factor appears to be specific for epithelial cells and is particularly active on keratinocytes. Therefore, this novel factor has been designated "keratinocyte growth factor" (KGF). Notwithstanding its lack of activity on fibroblasts, since it is the seventh known member of the FGF polypeptide family, KGF may also be referred to as FGF-7.

Accordingly, this invention relates, in part, to purified KGF or KGF-like proteins and

methods for preparing these proteins. Such purified factors may be made by cultivation of human cells which naturally secrete these proteins and application of isolation methods according to the practice of this invention. These proteins can be used for biochemical and biological studies leading, for example, to isolation of DNA segments encoding KGF or KGF-like polypeptides.

10 The present invention also relates to such DNA segments which encode KGF or KGF-like proteins. In a principal embodiment, the present invention relates to DNA segments, which encode KGF-related products, consisting of: human cDNA
15 clones 32 or 49, derived from polyadenylated RNA extracted from the human embryonic lung fibroblast cell line M426; recombinants and mutants of these clones; and related DNA segments which can be detected by hybridization to any of
20 the above human DNA segments, which related segments encode KGF-like proteins or portions thereof.

 In the practice of one embodiment of this invention, the DNA segments of the invention
25 are capable of being expressed in suitable host cells, thereby producing KGF or KGF-like proteins. The invention also relates to mRNAs produced as the result of transcription of the

sense strands of the DNA segments of this invention.

In another embodiment, the invention relates to a recombinant DNA molecule comprising a vector and a DNA of the present invention. These recombinant molecules are exemplified by molecules comprising a KGF cDNA and any of the following vector DNAs: a bacteriophage λ cloning vector (exemplified by λ pCEV9); a DNA sequencing plasmid vector (e.g., a pUC variant); a bacterial gene expression vector (e.g., pKK233-2); or a mammalian gene expression vector (such as pMMT).

In still another embodiment, the invention comprises a cell, preferably a mammalian cell, transformed with a DNA of the invention. Further, the invention comprises cells, including insect cells, yeast cells and bacterial cells such as those of *Escherichia coli* and *B. subtilis*, transformed with DNAs of the invention. According to another embodiment of this aspect of the invention, the transforming DNA is capable of being expressed in the cell, thereby increasing in the cell the amount of KGF or KGF-like protein encoded by this DNA.

The primary KGF translation product predicted from its cDNA sequence contains an N-terminal hydrophobic region which likely serves

as a signal sequence for secretion and which ^{is}
not present in the mature KGF molecule. In a
most preferred embodiment of the gene expression
aspect of the invention, the cell transformed by
5 the DNA of the invention secretes the protein
encoded by that DNA in the (truncated) form that
is secreted by human embryonic lung fibroblast
cells.

Still further, this invention
10 contemplates KGF or KGF-like proteins produced by
expression of a DNA of the invention, or by
translation of an RNA of the invention.
Preferably, these proteins will be of the
secreted form (i.e., lacking an apparent signal
15 sequence). These protein factors can be used for
functional studies, and can be purified for
additional structural and functional analyses,
such as qualitative and quantitative receptor
binding assays.

20 Moreover, the ability to produce large
quantities of this novel growth factor by
recombinant techniques will allow testing of its
clinical applicability in situations where
specific stimulation of growth of epithelial
25 cells is of particular importance. Accordingly,
this invention includes pharmaceutical
compositions comprising KGF or KGF-like
polypeptides for use in the treatment of such

conditions, including, for example, healing of 2038398
wounds due to burns or stimulation of
transplanted corneal tissue.

According to this embodiment of the
5 invention, the novel KGF-like proteins will be
protein products of "unmodified" DNAs and mRNAs
of the invention, or will be modified or
genetically engineered protein products. As a
result of engineered mutations in the DNA
10 sequences, modified KGF-like proteins will have
one or more differences in amino acid sequence
from the corresponding naturally occurring "wild-
type" proteins. According to one embodiment of
this aspect of this invention, the modified KGF-
15 like proteins will include "chimeric" molecules
comprising segments of amino acid sequences of
KGF and at least one other member of the FGF
peptide family.

Ultimately, given results of analogous
20 successful approaches with other peptide factors
having similar properties, development of such
chimeric KGF-like polypeptides should lead to
superior, "second generation" forms of KGF-like
peptides for clinical purposes. These modified
25 KGF-like products might be smaller, more stable,
more potent, and/or easier or less expensive to
produce, for example.

bioassay methods for determining expression in human cells of the mRNAs and proteins produced from the genes related to DNA segments of the invention. According to one such embodiment, DNAs of this invention may be used as probes to determine steady state levels or kinetics of induction of related mRNAs. The availability of the KGF-related cDNA clones makes it possible to determine whether abnormal expression of this growth factor is involved in clinical conditions characterized by excessive epithelial cell growth, including dysplasia and neoplasia (e.g., psoriasis, or malignant or benign epithelial tumors).

This invention also contemplates novel antibodies made against a peptide encoded by a DNA segment of the invention. In this embodiment of the invention, the antibodies are monoclonal or polyclonal in origin, and are generated using KGF-related polypeptides from natural, recombinant or synthetic chemistry sources.

The antibodies of this invention bind specifically to KGF or a KGF-like protein which includes the sequence of such peptide, preferably when that protein is in its native (biologically active) conformation. These antibodies can be used for detection or purification of the KGF or

KGF-like protein factors. In a most preferred embodiment of this aspect of the invention, the antibodies will neutralize the growth promoting activity of KGF, thereby enabling mechanistic studies, and ultimately, therapy for
5 clinical conditions involving excessive levels of KGF.

According to another aspect of the invention, a pharmaceutical composition is provided for treating conditions requiring specific stimulation of human keratinocytes or other epithelial cells while allowing
10 for normal differentiation as evidenced by appearance of differentiation markers, such as Keratin 1 (K1) and/or filaggrin. The composition comprises KGF purified from a culture of recombinant transformed cells and an acceptable pharmaceutical carrier.

15 According to yet another aspect of the present invention, a substantially pure human keratinocyte growth factor (KGF) protein or a portion of the KGF protein having at least 9 consecutive amino acids of the KGF protein, the KGF protein having an approximate molecular
20 weight between 25 and 30 kDa, as determined by SDS-PAGE, and having preferential mitogenic activity for an epithelial cell.

According to another aspect of the present invention, a pharmaceutical composition comprises a
25 protein or portion thereof as defined in the above aspects and a pharmaceutically acceptable carrier, for treating conditions requiring specific stimulation of epithelial cells.

According to another aspect of the present
30 invention, a pharmaceutical composition comprises a protein or portion thereof as defined in the above aspects and a pharmaceutically acceptable carrier, for acceleration or improvement of wound healing involving the epidermis.

35 According to another aspect of the present invention, a chimeric protein which comprises within a single polypeptide molecule a functional domain of human

KGF protein, wherein the functional domain is defined by amino acids responsible for the preferential mitogenic activity of KGF for an epithelial cell, and at least one other portion polypeptide of the fibroblast growth factor family.

According to yet another aspect of the present invention, an antibody directed against KGF protein or a portion of KGF protein as defined in the above aspects.

According to yet another aspect of the present invention, a pharmaceutical composition comprises an antibody as defined in the above aspects and a pharmaceutically acceptable carrier, for treating conditions requiring specific inhibition of stimulation of epithelial cells by KGF protein.

According to another aspect of the present invention, a DNA molecule encoding a human keratinocyte growth factor (KGF) protein or a portion of said KGF protein having at least 9 consecutive amino acids of said KGF protein, said KGF protein having an approximate molecular weight between 25 and 30 kDa, as determined by SDS-PAGE, and having preferential mitogenic activity for an epithelial cell.

According to another aspect of the present invention, a bioassay for mitogenic activity for a keratinocyte cell in a test sample which comprises the following steps: (i) growing keratinocytes in culture to confluence and maintaining the confluent culture in serum-free medium; (ii) adding a test sample to the confluent culture of keratinocytes; and (iii) determining the stimulation of DNA synthesis in the keratinocytes.

According to yet another aspect of the present invention, a method of producing KGF protein as defined in the above aspects comprises: (i) contacting a first solution comprising KGF protein with heparin under conditions such that the KGF protein binds to the heparin whereby a heparin-KGF complex is formed; (ii) separating the heparin-KGF complex from other components of the

first solution. And (iii) dissociating KGF protein from the heparin-KGF complex to form a second solution comprising KGF protein.

According to another aspect of the present invention, a keratinocyte growth factor defined by the amino acid sequence of Figure 11-1B, optionally lacking the N-terminal 31 amino acids, and proteins differing by the addition, deletion or substitution of one or more amino acids from the amino acid sequence of said factor, which retain the property of exhibiting at least about 500-fold greater stimulation of BALM/MK keratinocyte cells than NIH/3T3 fibroblast cells, and at least about 50-fold greater stimulation of BALB/MK keratinocyte cells than of BS/589 epithelial cells and CCL208 epithelial cells, as determined by H-thymidine incorporation.

According to an aspect of the invention, a chimeric protein which comprises within a single polypeptide molecule a segment of KGF protein, wherein the segment of KGF protein is a segment of amino acids of Figure 11-1B which comprises a sufficient number of consecutive amino acids 32 - 189 of Figure II-1B to confer on the polypeptide molecule preferential mitogenic activity for an epithelial cell, and at least one other polypeptide portion of the fibroblast growth factor family.

According to a further aspect of the invention, An antibody specifically directed against glycosylated or unglycosylated keratinocyte growth factor (KGF) polypeptide selected from the group consisting of (a) the following amino acid sequence, (b) a portion of the following amino acid sequence without the N-terminal 31

amino acids, and (c) an amino acid sequence that differs by the addition, deletion, or substitution of one or more amino acids from the following amino acid sequence:

	M	H	K	W	I	L	T	W	I	L	P	T	L	L	Y
5	R	S	C	F	H	I	I	C	L	V	G	T	I	S	L
	A	C	N	D	M	T	P	E	Q	M	A	T	N	V	N
	C	S	S	P	E	R	H	T	R	S	Y	D	Y	M	E
	G	G	D	I	R	V	R	R	L	F	C	R	T	Q	W
	Y	L	R	I	D	K	R	G	K	V	K	G	T	Q	E
10	M	K	N	N	Y	N	I	M	E	I	R	T	V	A	V
	G	I	V	A	I	K	G	V	E	S	E	F	Y	L	A
	M	N	K	E	G	K	L	Y	A	K	K	E	C	N	E
	D	C	N	F	K	E	L	I	L	E	N	H	Y	N	T
	Y	A	S	A	K	W	T	H	N	G	G	E	M	F	V
15	A	L	N	Q	K	G	I	P	V	R	G	K	K	T	K
	K	E	Q	K	T	A	H	F	L	P	M	A	I	T	

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. I-1 depicts results of heparin-Sephadex affinity chromatography of conditioned medium from M426 human embryonic fibroblasts showing that greater than 90% of the mitogenic activity for mouse keratinocytes (BALB/MK) eluted with 0.6 M NaCl.

Fig. I-2 illustrates results of further purification of the mitogen from human fibroblasts using HPLC with and adsorptive matrix. Panel (A) shows the profile on reversed-phase (C₄) HPLC of BALB/MK mitogenic activity. Panel (B) presents electrophoretic (NaDodSO₄/PAGE) analysis of selected fractions from the C₄ chromatography shown in panel A, demonstrating that the peak HPLC fractions contained a single band on the silver stained gel. Panel (C) is a bar graph of DNA synthesis in BALB/MK cells triggered by the fractions analyzed in Panel B, showing that the relative mitogenic activity correlated well with the intensity of the protein band across the activity profile.

Fig. I-3 presents an alternative purification step to RP-HPLC, using sieving chromatography with a (TSK G3000SW GlasPac)* column run in aqueous solution near physiologic

* trade mark

20 38 3 9 8

pH, which resulted in a major peak of mitogenic activity in the BALB/MK bioassay.

Fig. I-4 illustrates a comparison of BALB/MK DNA synthesis in response to TSK-purified mitogen and other growth factors.

Fig. I-5 shows comparisons of growth of BALB/MK cells in a chemically defined medium in response to different combinations of growth factors.

Table I-1 summarizes the results from various purification steps, documenting that sieving chromatography provided a far better recovery of activity than the adsorptive RP-HPLC approach.

Table I-2 recapitulates data on the target cell specificities of various growth factors, demonstrating that the newly isolated factor exhibited a strong mitogenic effect on keratinocytes (BALB/MK) and, in striking contrast, had no detectable effects on fibroblasts or human saphenous vein endothelial cells.

Fig. II-1 presents the nucleotide sequence and deduced amino acid sequence of KGF cDNA, as well as identification of RNAs transcribed from the KGF gene. Panel (A) outlines a schematic representation of human KGF cDNA clones. Panel (B) documents the KGF cDNA

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nucleotide and predicted amino acid sequences. (C)

Identification of RNA transcripts of KGF genes by

Northern blot analysis. (D) cDNA sequence and deduced amino acid sequence for aFGF.

5 Fig. II-2 illustrates the topological comparison of the FGF family of related molecules, including KGF, with emphasis on the two protein domains that share high homology, the putative signal peptide sequences, and the two conserved cysteine residues.

10 Fig. II-3 shows (Northern blot) analyses of expression of KGF-related mRNA in selected normal human cell lines and tissues, revealing that a single 2.4 kb transcript was present in RNA from human embryonic lung fibroblasts and from adult skin fibroblasts, while no
15 transcript was detected in the (B5/589) epithelial or (HA83) glial cell lines, or in primary cultures of human saphenous vein endothelial cells.

 Fig. II-4A represents the Mono-S-Chromatography* pattern of heparin-Sepharose purified
20 non-glycosylated KGF.

 Fig. II-4B shows the SDS-PAGE analysis of mitogenically active fractions from KGF preparation.

 Fig. II-4C shows the immunoblot analysis of selected fractions from the Mono-S-Chromatography.

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Fig. III-1 are phase contrast micrographs A, B and C of human epidermal keratinocytes grown in low Ca^{2+} .

5 Fig. III-2 is a dose response profile of KGF (solid dot) and EGF (open circle) on proliferation of cultured human epidermal keratinocytes.

Fig. III-3 is a time course of KGF and EGF-induced proliferation of human epidermal keratinocytes.

10 Fig. III-4 shows the effect of Ca^{2+} concentration on growth factor-induced proliferation of human epidermal keratinocytes.

Fig. III-5 are phase contrast micrographs A, B and C of human epidermal keratinocytes grown in high Ca^{2+} concentration.

15 Fig. III-6 is an immunoblot analysis of keratinocyte differentiation markers expressed in response to different Ca^{2+} concentrations and growth factors.

20 Fig. III-7 is a comparison of effects of $\text{TGF}\alpha$, EGF and KGF on keratin-1 expression by keratinocytes at low and high Ca^{2+} concentration.

25 Fig. IV-1 illustrates (A) ligand competition of ^{125}I -KGF specific binding to BALB/MK cells, (B) ligand competition of ^{125}I -KGF specific binding to NIH/3T3 cells, (C) ligand competition of ^{125}I -aKGF specific binding to BALB/MK cells and (D) ligand

competition of ^{125}I -FGF specific binding of ^{125}I -FGF to NIH/3T3 cells.

Fig. IV-2. (A) is a Scatchard analysis of ^{125}I -KGF and ^{125}I -aFGF specific binding to BALB/MK cells and (B) is a Scatchard analysis of ^{125}I -KGF binding on BALB/MK cells in presence or absence of heparin.

Fig. IV-3 shows the covalent affinity cross-linking of ^{125}I -KGF, ^{125}I -aFGF and ^{125}I -bFGF to intact BALB/MK and NIH/3T3 cells.

Fig. IV-4 is an autoradiogram of phosphotyrosyl proteins from intact Balb/MK and NIH/3T3 cells following treatment with KGF, aFGF or bFGF.

Fig. V-1 is (A) Southern blot analysis of SAL1-digested genomic DNA from transfectant and untransfectant NIH/3T3 cells and (B) Southern analysis of Eco RI-digested DNAs of different animal species and (C) Northern analysis of NIH/3T3 and BALB/MK RNA.

Fig. V-2 (A) is primary aminoacid structure of KGF receptor and (B) structural comparison of predicted KGF and bFGF receptors.

Fig. V-3 is competition of KGF, aFGF and bFGF for ^{125}I -labelled KGF binding on (A) BALB/MK cells and (B) NIH/ect1 cells ($\square$)-KGF, (O)-aFGF, (Δ)bFGF.

Fig. V-4 is an analysis of KGF receptor, expressed in NIH/3T3 cells. Panel A is covalent affinity cross-linking of ^{125}I -KGF to BALB/MK, NIH/3T3

and NIH/ect1 cultures and panel B is an autoradiogram of phosphotyrosyl-proteins from intact NIH/3T3 and NIH/ect1 cells.

5 Table II-1 summarizes a comparison of the
effect of heparin on KGF mitogenic activity with
effects on other growth factors, showing that thymidine
incorporation into DNA by BALB/MK cells in response to
KGF was inhibited by heprain, in contrast, to the
activities of both aFGF and bFGF which were increaed by
10 the same treatment.

This invention relates, in part, to purified KGF or KGF-like proteins and methods for preparing these proteins. A principal embodiment of this aspect of this invention relates to homogeneous KGF characterized by an apparent molecular weight of about 28 kDa based on migration in NaDodSO₄/PAGE, movement as a single peak on reversed-phase high performance liquid chromatography, and a specific activity of at least about 3.4×10^4 units per milligram, and preferably at least about 3.2×10^5 units per milligram, where one unit of activity is defined as that amount which causes half of the maximal possible stimulation of DNA synthesis in certain epithelial (keratinocyte) cells under standard assay conditions outlined below.

To identify novel growth factors specific for epithelial cell types, a clonal BALB/c mouse keratinocyte cell line, designated BALB/MK (I-6) was employed as an indicator cell to detect such factors. These cells are dependent for their growth upon an exogenous source of an epithelial cell mitogen even in medium containing serum (I-6). The development of chemically defined medium for these cells has made it possible to demonstrate that two major

proliferation. One involves insulin-like growth factor I (or insulin at high concentration) and the other is satisfied by epidermal growth factor (EGF), transforming growth factor α (TGF α), acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) (I-7).

By using BALB/MK as the prototypical epithelial cell line and NIH/3T3 as its fibroblast counterpart, conditioned media from various human cell lines were assayed for new epithelial cell-specific mitogens. These bioassays of this invention enabled the purification to homogeneity of one such novel growth factor, released by a human embryonic lung fibroblast line, and designated herein as keratinocyte growth factor (KGF).

In brief, the bioassay for KGF-like activity under standard conditions comprises the following steps:

- (i) Mouse keratinocytes (BALB/MK cells) are grown in culture to confluency and then maintained for 24-72 hr in serum-free medium;
- (ii) Following addition of test samples, stimulation of DNA synthesis is determined by incorporation of ^3H -thymidine into acid-precipitable DNA.

To determine the cell target specificity of a mitogenic growth factor, the DNA synthesis stimulation, expressed as ratio of stimulated synthesis over background incorporation of thymidine in the absence of added test sample, can be compared to analogous stimulation observed in cells other than keratinocytes under the same assay conditions. In such comparisons, KGF mitogenic activity will exhibit marked specificity for the keratinocytes as opposed to fibroblasts (at least about 500-fold greater stimulation) and lesser but significant (at least about 50-fold) greater activity on keratinocytes than on other exemplary epithelial cell types (see Table I-2 for further data, and Materials and Methods in Experimental Section I for details of the standard conditions of the bioassay).

By employing a method of KGF production involving culturing cells and isolating mitogenic activity, which method comprises ultrafiltration, heparin-Sepharose affinity chromatography (HSAC) and adsorptive reversed-phase high performance liquid chromatography (RP-HPLC) or, alternatively, molecular sieving HPLC (TSK-HPLC), according to the present invention, a quantity was isolated sufficient to permit detailed characterization of the physical and biological properties of this molecule.

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of KGF from producing cells such as M426 human embryonic fibroblasts (I-8), for example, comprises the following steps:

5 (i) Preparation of conditioned media (e.g., 10 liters) using monolayer cultures cycled from serum-containing to serum-free medium and storing the serum-free harvest at -70°C until further use;

10 (ii) Concentration by ultrafiltration using membranes having a 10 kDa molecular weight cutoff in several successive steps with intervening dilution in buffer (to facilitate removal of low molecular weight materials), followed by optional
15 storage at -70°C;

 (iii) Affinity chromatography on heparin attached to a polymeric support (e.g., Sepharose) with elution by a gradient of increasing NaCl concentration;

20 (iv) Concentration by a factor of at least ten- to twenty-fold with small scale ultrafiltration devices with a 10 kDa molecular weight cutoff (e.g., a Centricon-10 microconcentrator from Amicon) and storage at
25 -70°C.

The next step of the purification process comprises either step (v) or, alternatively, step (vi), as follows:

(v) Reversed-phase HPLC of active fractions
(0.6 M NaCl pool) from the previous HSAC step in 2038398
organic solvent systems;
or,

- 5 (vi) Molecular sieve HPLC (e.g, on a TSK-
G3000SW Glas-Pac Column from LKB) in aqueous
buffer at near physiological pH (e.g., Tris-HCl,
pH 6.8/0.5M NaCl) followed by storage at -70°C.

A preparation made by the TSK step (vi)
10 was almost as pure as one obtained from RP-HPLC,
as judged by silver-stained NaDodSO₄/PAGE (data
not shown); but the TSK approach provided a far
better recovery of activity (Table I-1).
Further, the TSK-purified material had a higher
15 specific activity than the RP-HPLC material. KGF
prepared by the TSK procedure above stimulated
DNA synthesis in epithelial cells at sub-
nanomolar concentrations, but failed to induce
any thymidine incorporation into DNA of
20 fibroblasts or endothelial cells at comparable or
higher concentrations (up to 5 nM). The activity
was sensitive to acid, heat and solvents used in
the RP-HPLC step. (See Experimental Section I for
data on sensitivities and further details of the
25 production method.)

Using standard methodology well known in
the art, an unambiguous amino acid sequence was
determined for positions 2-13 from the amino

terminus of the purified KGF, as follows: Asn-2038398
Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val (see
Experimental Section I).

5 The present invention also includes DNA
segments encoding KGF and KGF-like polypeptides.
The DNAs of this invention are exemplified by
DNAs referred to herein as: human cDNA clones 32
and 49 derived from polyadenylated RNA extracted
from the human embryonic lung fibroblast cell
10 line M426; recombinants and mutants of these
clones; and related DNA segments which can be
detected by hybridization to these DNA segments.

As described in Experimental Section II,
to search for cDNA clones corresponding to the
15 known portion of the KGF amino acid sequence, two
pools of oligonucleotide probes were generated
based upon all possible nucleotide sequences
encoding the nine-amino acid sequence,
Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala. A cDNA
20 library was constructed in a cDNA cloning vector,
 λ pCEV9, using polyadenylated RNA extracted from
the human embryonic lung fibroblast cell line
M426 which was the initial source of the growth
factor. Screening of the library (9×10^5
25 plaques) with the ^{32}P -labelled oligonucleotides
identified 88 plaques which hybridized to both
probes.

Of 10 plaque-purified clones that were analyzed, one, designated clone 49, had a cDNA insert of 3.5 kb, while the rest had inserts ranging from 1.8 kb to 2.1 kb. Analysis of the smaller clones revealed several common restriction sites, and sequencing of a representative smaller clone, designated clone 32, along with clone 49, demonstrated that they were overlapping cDNAs (Fig II-1A). Alignment of the two cDNAs established a continuous sequence of 3.85 kb containing the complete KGF coding sequence. The sense strand DNA nucleotide sequence, and the predicted primary protein sequence encoded, are shown for the full-length composite KGF cDNA sequence in Fig. II-1B.

These DNAs, cDNA clones 32 and 49, as well as recombinant forms of these segments comprising the complete KGF coding sequence, are most preferred DNAs of this invention.

From the cDNA sequence, it is apparent that the primary KGF and *hsk* translation products contain hydrophobic N-terminal regions which likely serve as signal sequences, based on similarity to such sequences in a variety of other proteins. Accordingly, this N-terminal domain is not present in the purified mature KGF molecule which is secreted by human embryonic fibroblasts.

Furthermore, KGF shares with all other members of the FGF family two major regions of homology, spanning amino acids 65-156 and 162 189 in the predicted KGF sequence, which are separated by short, non-homologous series of amino acids of various lengths in the different family members. The non-homologous sections may or may not play a role in determining the unique functional aspects of KGF. Furthermore, such non-homologous sections may also be unique in distinguishing from other known sequence portions of other growth factors.

The sequence of the purified form of KGF contains five cysteine residues, two of which are conserved throughout the family of FGF related proteins. Five pairs of basic residues occur throughout the KGF sequence. This same pattern has been observed in other FGF family members.

It should be obvious to one skilled in the art that, by using the DNAs and RNAs of this invention in hybridization methods (such as Southern blot analyses of genomic human DNAs), especially the most preferred DNAs listed herein above, without undue experimentation, it is possible to screen genomic or cDNA libraries to find other KGF-like DNAs or variants which fall within the scope of this invention. Furthermore, by so using DNAs of this invention,

genetic markers associated with the KGF gene, such as restriction fragment length polymorphisms (RFLPs), may be identified and associated with inherited clinical conditions involving this or other nearby genes. Such variants include, as is appreciated, any DNA sequence which hybridizes to the DNA sequence encoding KGF protein or polypeptide fragments thereof or any DNA sequence which has sufficient closeness in homology to have the same functional encoding capacity of DNA encoding KGF.

This invention also includes modified forms of KGF DNAs. According to a preferred embodiment of this aspect of the invention, such modified DNAs may encode KGF-like proteins comprising segments of amino acid sequences of KGF and at least one other member of the FGF peptide family. Thus, for example, since there is no significant N-terminal homology between the secreted form of KGF and analogous positions in other FGF-related proteins, polypeptides with novel structural and functional properties may be created by grafting DNA segments encoding the distinct N-terminal segments of another polypeptide in the FGF family onto a KGF DNA segment in place of its usual NH₂-terminal sequence.

The polypeptide chimeras produced by such modified DNAs are useful for determining whether the

KGF NH₂-terminal domain is sufficient to account for its unique target cell specificity. Studies on chimeras should also provide insights into which domains contribute the different effects of heparin on their biologic activities. As is understood, one or more regions of the KGF peptide act as function domain(s) for the protein to render it biologically useful. Such function domains are the critical sections of the protein in providing a growth function, receptor binding and/or medical treatment properties. Usually such functional domains comprise at least 10 amino acid residues and may be the same as or functionally the same as the amino acid sequence of the normal KGF polypeptide fragment. By functionally the same, it is understood to include embodiments which provide the same result by non-critical substitution of amino acid residues in the polypeptide or the cDNA sequence encoding the polypeptide.

Indeed, the utility of this approach has already been confirmed by the successful engineering and expression of a chimeric molecule in which about 40 amino acids from the NH₂-terminus of the secreted form of KGF (beginning with the amino terminal cys residue of the mature KGF form, numbered 32 in Fig. II-1, and ending at KGF residue 78, arg) is linked to about 140 amino acids of the CO₂-terminal core of aFGF (beginning

at residue 39, arg, and continuing to the C-terminal
end of the aFGF coding sequence. The sequence for aFGF
is set out in Fig. II-1D. This chimeric product has a
target cell preference for keratinocytes, like KGF, but
5 lacks susceptibility to heparin, a characteristic which
parallels that of aFGF rather than KGF. This novel
KGF-like growth factor may have advantages in clinical
applications where administration of an epithelial-
specific growth factor is desirable in the presence of
10 heparin, a commonly used anticoagulant. Further
details of the construction of this chimeric molecule
and the properties of the polypeptide are described in
Experimental Section II.

Other DNAs of this invention include the
15 following recombinant DNA molecules comprising a KGF
cDNA and any of the following exemplary vector DNAs: a
bacteriophage λ cloning vector (λ pCEV9); a DNA
sequencing plasmid vector (a pUC variant); a bacterial
expression vector (pKK233- 2); suitable yeast and plant
20 cell expression vectors; or a mammalian expression
vector (pMMT/neo). Such recombinant DNAs are
exemplified by_____

constructs described in detail in the
Experimental Sections.

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Most preferred recombinant molecules
include the following: molecules comprising the
5 coding sequence for the secreted form of KGF and
a bacterial expression vector (e.g., pKK233-2) or
a cDNA encoding the entire primary translation
product (including the NH₂-terminal signal
peptide) and a mammalian expression vector
10 (exemplified by pMMT) capable of expressing
inserted DNAs in mammalian (e.g., NIH/3T3) cells.

Construction of recombinant DNAs
containing KGF DNA and a bacterial expression
vector is described in Experimental Section II.
15 In brief, KGF cDNA was expressed to produce
polypeptide in *E. coli* by placing its coding
sequence under control of the hybrid trk promoter
in the plasmid expression vector pKK233-2 (II-
31).

20 Construction of recombinant DNAs
comprising KGF DNA and a mammalian vector capable
of expressing inserted DNAs in cultured human or
animal cells, can be carried out by standard gene
expression technology using methods well known in
25 the art for expression of such a relatively
simple polypeptide. One specific embodiment of a
recombinant DNA of this aspect of the present

invention, involving the mammalian vector pMMT, is described further below in this section under recombinant cells of this invention.

5 DNAs and sense strand RNAs of this invention can be employed, in conjunction with protein production methods of this invention, to make large quantities of substantially pure KGF or KGF-like proteins.

Substantially pure KGF protein thus produced can be employed, using well-known techniques, in diagnostic
10 assays to determine the presence of receptors for this protein in various body fluids and tissue samples.

Accordingly, this invention also comprises a cell, preferably a bacterial, yeast, plant, insect or mammalian cell, transformed with a DNA of the
15 invention, wherein the transforming DNA is capable of being expressed. In a preferred embodiment of this aspect of the invention, the cell transformed by the DNA of the invention produces KGF protein in a fully mitogenic form. Most preferably, these proteins will
20 be of a secreted form (i.e., lacking an apparent signal sequence). These protein factors can be used for functional studies, and can be purified for additional biochemical and functional analyses, such as
25 qualitative and quantitative receptor binding assays.

constructed in a bacterial expression vector,
pKK233-2, for production of KGF, as detailed in
Experimental Section II. In summary, several
5 recombinant bacterial clones were tested for
protein production by the usual small scale
methods. All recombinants tested synthesized a
protein that was recognized by antibodies raised
against an amino-terminal KGF peptide (see
10 below). One recombinant was grown up in a one
liter culture which produced recombinant KGF that
efficiently stimulated thymidine incorporation
into DNA of BALB/MK keratinocyte cells, but was
only marginally active on NIH/3T3 fibroblasts.
15 Half-maximal stimulation of the BALB/MK cells in
the standard keratinocyte bioassay was achieved
with a concentration of between 2 to 5 ng/ml,
compared to a concentration of 10 to 15 ng/ml for
KGF purified from M426 cells.

20 One liter of bacterial cells yielded
approximately 50 µg of Mono-S purified
recombinant KGF. It will be apparent to those
skilled in the art of gene expression that this
initial yield can be improved substantially
25 without undue experimentation by application of a
variety known recombinant DNA technologies.

Recombinant mammalian (NIH/3T3 mouse)
cells have also been constructed using the entire

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KGF cDNA coding sequence (including the NH₂-
terminal signal peptide) and the vector pMMT/neo,
which carries mouse metallothionine (MMT)
promoter and the selective marker gene for
neomycin resistance. The cells are being
evaluated for KGF production, particularly for
secretion of the mature form (lacking signal
peptide) produced by human fibroblasts, using
bioassays of the present invention. This same
vector and host cell combination has been used
successfully to express several other similar
recombinant polypeptides, including high levels
of Platelet-Derived Growth Factor (PDGF) A and B
chains (II-32). Accordingly, it will be
recognized by those skilled in the art that high
yields of recombinant KGF can
be achieved in this manner, using the
aforementioned recombinant DNAs and transformed
cells of this invention.

Ultimately, large-scale production can
be used to enable clinical testing in conditions
requiring specific stimulation of epithelial cell
growth. Materials and methods for preparing
pharmaceutical compositions for administration of
polypeptides topically (to skin or to the cornea
of the eye, for example) or systemically are well
known in the art and can be adapted readily for

administration of KGF and KGF-like peptides without undue experimentation.

This invention also comprises novel antibodies made against a peptide encoded by a DNA segment of the invention. This embodiment of the invention is exemplified by several kinds of antibodies which recognize KGF. These have been prepared using standard methodologies well known in the art of experimental immunology, as outlined in Experimental Section II. These antibodies include: monoclonal antibodies raised in mice against intact, purified protein from human fibroblasts; polyclonal antibodies raised in rabbits against synthetic peptides with sequences based on amino acid sequences predicted from the KGF cDNA sequence [preferably sequences comprising 10 to 20 amino acids, such as exemplified by a peptide with the sequence of KGF residues 32-45, namely, NDMTPEQMATNVR (using standard one-letter code for amino acid sequences; see Fig. II-1)]; polyclonal antibodies raised in rabbits against both naturally secreted KGF from human fibroblasts and recombinant KGF produced in *E. coli* (see above).

All tested antibodies recognize the recombinant as well as the naturally occurring KGF, either in a solid-phase (ELISA) assay and/or in a Western blot. Some exemplary antibodies, which are

preferred antibodies of this invention, appear to neutralize or inhibit mitogenic activity of KGF in the BALB/MK bioassay.

5 Fragments of antibodies of this invention, such as Fab or F(ab)' fragments, which retain antigen binding activity and can be prepared by methods well known in the art, also fall within the scope of the present invention. Further, this invention comprises pharmaceutical compositions of the antibodies of this
10 invention, or active fragments thereof, which can be prepared using materials and methods for preparing pharmaceutical compositions for administration of polypeptides that are well known in the art and can be adapted readily for administration of KGF and KGF-like
15 peptides without undue experimentation.

 These antibodies, and active fragments thereof, can be used, for example, for detection of KGF in bioassays or for purification of the protein factors. They may also be used in approaches well
20 known in the art, for isolation of the receptor for KGF, which, as described in Experimental Section II, appears to be distinct from those of all other known growth factors.

 Those preferred antibodies, and fragments and
25 pharmaceutical compositions thereof, which neutralize or inhibit mitogenic activity of KGF for epithelial

cells, as indicated by the BALB/MK assay, for instance, may be used in the treatment of clinical conditions characterized by excessive epithelial cell growth, including dysplasia and neoplasia (e.g., psoriasis, or malignant or benign epithelial tumors or benign mixed stromal/epithelial tumors).

This invention further comprises novel bioassay methods for detecting the expression of genes related to DNAs of the invention. In some exemplary embodiments, DNAs of this invention were used as probes to determine steady state levels of related mRNAs. Methods for these bioassays of the invention, using KGF DNAs, and standard Northern blotting techniques, are described in detail in Experimental Section II.

One skilled in the art will recognize that, without undue experimentation, such methods may be readily applied to analysis of gene expression for KGF-like proteins, either in isolated cells or various tissues. Such bioassays may be useful, for example, for identification of various classes of tumor cells or genetic defects in the epithelial growth processes.

In accordance with the invention, KGF is also as potent as EGF in stimulating proliferation of primary or secondary human keratinocytes in tissue culture. Exposure of KGF or EGF stimulated keratinocytes to 1.0 mM calcium, an inducer of

differentiation, leads to cessation of cell growth. However, immunological analysis of early and late markers of terminal differentiation, in the form of the proteins K1 and filaggrin, reveal striking differences in the keratinocytes growth in the presence of these two growth factors. With KGF the normal differentiation response is evident as demonstrated by association with the expression of both markers whereas their appearance was retarded or blocked by EGF. Furthermore, TGF α which also interacts with the EGF receptor gave similar response to that observed with EGF. Such significant differences in treated human keratinocytes distinguishes KGF from the EGF family of growth factors. This information confirms efficacy of KGF in stimulating the proliferation of human epithelial cells and simultaneously permit the normal differentiation of the cells.

KGF has specific high affinity binding to surface receptors on intact BALB/MK mouse epidermal keratinocytes, but not on NIH/3T3 fibroblasts. KGF binding on BALB/MK cells competed efficiently with aFGF and with 20-fold lower efficiency with bFGF. In contrast, aFGF and bFGF bind competitively on both BALB/MK keratinocytes and NIH/3T3 fibroblasts. Covalent affinity cross-linking of ^{125}I -KGF to its receptor on BALB/MK cells reveals two species of 115

and 140 kDa. KGF stimulates the rapid tyrosine
phosphorlation of a 90 kDa protein in BALB/MK cells but
not in the NIH/3T3 fibroblasts. Hence, the BALB/MK
keratinocytes possess high affinity KGF receptors to
5 which FGF may also bind, however, these receptors are
distinct from the receptors for aFGF and bFGF on
NIH/3T3 fibroblasts which fail to interact with KGF.

A cDNA encoding a KGF receptor from the
BALB/MK cells was isolated and sequenced. The amino
10 acid sequence deduced from the coding region of the KGF
receptor is set out in Fig. V-2A. The cDNA of the
receptor has a variety of additional uses. For
example, the receptor cDNA and KGF binding analysis, as
described above, could have a variety of uses, for
15 example, in diagnostic studies wherein knowledge of KGF
receptor levels could be of prognostic or therapeutic
benefit. Furthermore, a functional fragment of the
receptor protein can be useful for treating cell
proliferative disorders where excessive activation of
20 receptor molecules is associated with the ailment to be
treated. The receptor protein fragment would be
biologically functional to bind and thereby inactivate
excess KGF in the mammal circulatory system.

Without further elaboration, it is believed
25 that one of ordinary skill in the art, using the
preceding description, and following the methods of the

Experimental Sections below, can utilize the present invention to its fullest extent. The material disclosed in the Experimental Sections, unless otherwise indicated, is disclosed for illustrative purposes and therefore should not be construed as being limitive in any way of the appended claims.

**IDENTIFICATION AND CHARACTERIZATION OF A NOVEL
GROWTH FACTOR SPECIFIC FOR EPITHELIAL CELLS**

This section describes experimental work
5 leading to identification of a growth factor
specific for epithelial cells in conditioned
medium of a human embryonic lung fibroblast cell
line. The factor, provisionally termed
keratinocyte growth factor (KGF) because of its
10 predominant activity on this cell type, was
purified to homogeneity by a combination of
ultrafiltration, heparin-Sepharose affinity
chromatography and hydrophobic chromatography on
a C₄ reversed-phase HPLC column, according to
15 methods of this invention. KGF was found to be
both acid and heat labile, and consisted of a
single polypeptide chain with an apparent
molecular weight of approximately 28,000 daltons.
Purified KGF was a potent mitogen for epithelial
20 cells, capable of stimulating DNA synthesis in
quiescent BALB/MK epidermal keratinocytes by more
than 500-fold with activity detectable at 0.1 nM
and maximal at 1.0 nM. Lack of mitogenic
activity on either fibroblasts or endothelial
25 cells indicated that KGF possessed a target cell
specificity distinct from any previously
characterized growth factor. Microsequencing

revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this novel growth factor by human embryonic fibroblasts indicates that KGF plays a
5 role in mesenchymal stimulation of normal epithelial cell proliferation.

METHODS AND MATERIALS

Preparation of Conditioned Media. An early passage of M426 human embryonic
10 fibroblasts (I-8) was plated onto 175 cm² T-flasks and grown to confluence over 10-14 days in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% calf serum (GIBCO). Once
15 confluent, the monolayers were cycled weekly from serum-containing to serum-free medium, the latter consisting of DMEM alone. The cells were washed twice with 5 ml of phosphate buffered saline
prior to addition of 20 ml of DMEM. After 72 hrs, culture fluids were collected and replaced
20 with 35 ml of serum-containing medium. The conditioned medium was stored at -70°C until further use.

Ultrafiltration. Approximately ten liters of conditioned medium were thawed,
25 prefiltered through a 0.50 micron filter (Millipore HAWP 142 50*) and concentrated to 200

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ml using the Pellicon cassette system (Millipore XX42
00K 60) and a cassette having a 10 kDa molecular weight
cutoff (Millipore PTGC 000 05^{*}). After concentration,
the sample was subjected to two successive rounds of
5 dilution with one liter of 20 mM Tris-HCl, pH 7.5/0.3 M
NaCl, each followed by another step of ultrafiltration
with the Pellicon system. Activity recovered in the
retentate was either immediately applied to heparin-
Sephadex resin or stored at -70°C.

10 Heparin-Sephadex Affinity

Chromatography (HSAC) The retentate from
ultrafiltration was loaded onto heparin-Sephadex resin
(Pharmacia) which had been equilibrated in 20 mM Tris-
HCl, pH 7.5/0.3 M NaCl. The resin was washed
15 extensively until the optical density had returned to
baseline and then subjected to a linear-step gradient
of increasing NaCl concentration. More particularly,
approximately 150 ml of ultrafiltration retentate
derived from 5 liters of M426 conditioned medium were
20 loaded onto a heparin-Sephadex column (6 ml bed
volume) in 1 hr. After washing the column with 150 ml
of the equilibration buffer, 20 mM Tris-HCl, pH 7.5/0.3
M NaCl, the retained protein (<5% of the total protein
in the retentate) was eluted with a modified linear
25 gradient of increasing NaCl concentration. Fraction

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size was 3.8 ml and flow rate during gradient elution was 108 ml/hr. Two μ l of the indicated fractions were transferred to microtiter wells containing a final volume of 0.2 ml for assay of ^3H -thymidine

5 incorporation of BALB/MK cells as described in the Methods. After removing aliquots from the fractions for the thymidine incorporation bioassay, selected fractions were concentrated ten- to twenty-fold with a Centricon-10 microconcentrator (Amicon) and stored at
10 -70°C .

Reverse-Phase HPLC (RP-HPLC) Active fractions (0.6 M NaCl pool) from the HSAC were thawed, pooled and further concentrated with the Centricon-10 to a final volume of $\leq 200 \mu\text{L}$. The sample was loaded onto a Vydac
15 HPLC column (The Separations Group, Hesperia, CA) which had been equilibrated in 0.1% trifluoroacetic acid (TFA, Fluka)/20% acetonitrile (Baker, HPLC grade) and eluted with a linear gradient of increasing acetonitrile. Aliquots for the bioassay were
20 immediately diluted in a 10-fold excess of 50 $\mu\text{g/ml}$ BSA (Fraction V, Sigma)/20 mM Tris-HCl, pH 7.5. The remainder of the sample was dried in a Speed-Vac (Savant) in preparation for structural analysis.

A preferred technique for the above was to
25 elute active fractions from heparin-Sepharose with 0.6M NaCl process with the Centricon-10 and load directly

onto a C₄ Vydac column (4.6 x 250 mm) which had been equilibrated in 0.1% trifluoroacetic acid/20% acetonitrile (ACN). After washing the column with 4 ml of equilibration buffer, the sample was eluted with a modified linear gradient of increasing percentage of ACN. Fraction size was 0.2 ml and flow rate was 0.5 ml/min. Aliquots for the assay of ³H-thymidine incorporation in BALB/MK cells were promptly diluted 10-fold with 50 µg/ml bovine serum albumin/20 mM Tris-HCl, pH 7.5, and tested at a final dilution of 200-fold. (B) NaDodSo₄/PAGE analysis of selected fractions from the C₄ chromatography shown in panel A. Half of each fraction was dried, redissolved in NaDodSo₄/2-mercaptoethanol, heat denatured and electrophoresed in a 14% polyacrylamide gel which was subsequently stained with silver. The position of each molecular weight marker (mass in kDa) is indicated by an arrow. (C) DNA synthesis in BALB/MK cells triggered by the fractions analyzed in Panel B. Activity is expressed as the fold stimulation over background which was 100 cpm.

Molecular Sieve HPLC

Approximately 50 µl of the twice concentrated heparin-Sepharose fractions were loaded onto a TSK-G3000SW Glas-Pac Column (LKB, 8 x 300 mm) which had been equilibrated in 20 mM Tris-HCl, pH 6.8/0.5M NaCl. The sample was eluted in this buffer at a flow rate of

0.4 ml/min and 0.2 ml fractions were collected.

Aliquots of 2 μ l were transferred to microtiter wells containing a final volume of 0.2 ml for assay ^{3} H-thyminidine incorporation in BALB/MK cells. The

5 elution positions of molecular weight markers (mass in kDa) were as indicated by the arrows. After removing aliquots for the bioassay, the fractions were stored at -70°C .

A preferred technique for the above is to
10 load approximately 50 μ l of a Centricon-processed, 0.6M NaCl pool from HSAC onto a LKB Glas-Pac TSK G3000SW column (8 x 300 mm), previously equilibrated in 20 mM Tris-HCl, pH 6.8/0.5M NaCl, and eluted as 0.2 ml fractions at a flow rate of 0.4 ml/min.

15 NaDodSO₄-Polyacrylamide Gel

Electrophoresis (NaDodSO₄/Page) Polyacrylamide gels were prepared with NaDodSO₄ according to the procedure of Laemmli (I-9). Samples were boiled for 3 min in the presence of 2.5% 2-mercaptoethanol (vol/vol). The gels
20 were fixed and stained with silver (I-10) using the reagents and protocol from BioRad. Molecular weight markers were from Pharmacia.

DNA Synthesis Stimulation. Ninety-six well microtiter plates (Falcon No. 3596) were precoated with human fibronectin (Collaborative Research) at $1 \mu\text{g}/\text{cm}^2$ prior to seeding with
5 BALB/MK cells. Once confluent, the cells were maintained for 24-72 hr in serum-free medium containing $5 \mu\text{g}/\text{ml}$ transferrin (Collaborative Research) and 30 nM Na_2SeO_3 (Baker).
10 Incorporation of ^3H -thymidine ($5 \mu\text{Ci}/\text{ml}$ final concentration, NEN) into DNA was measured during a 6 hr period beginning at 16 hrs following addition of samples. The assay was terminated by washing the cells once with ice cold phosphate-buffered saline and twice with 5% trichloroacetic
15 acid. The precipitate was redissolved in 0.25 M NaOH , transferred into liquid scintillation fluid (Biofluor, NEN) and counted.

Stimulation of DNA synthesis was monitored as described above for BALB/MK cells on
20 a variety of other cell lines. NIH/3T3 fibroblasts (I-11) were available from the National Institutes of Health, while CCL208 Rhesus monkey bronchial epithelial cells (I-12) were obtained from the American Type Culture
25 Collection. The B5/589 human mammary epithelial cell line, prepared as described in (I-13), was obtained from Martha Stampfer (University of California, Berkeley). The mammary cells were

grown in RPMI 1640 supplemented with 10% fetal calf serum and 4 ng/ml EGF. When maintained in serum-free conditions, the basal medium was DMEM. Primary cultures of human saphenous vein endothelial cells were prepared and maintained as described elsewhere (I-14). Epidermal growth factor and insulin were from Collaborative Research. Acidic FGF and bFGF were obtained from California Biotechnology, Inc. Recombinant TGF α was obtained from Genentech, Inc. Media and serum were either from GIBCO, Biofluids, Inc. or the NIH media unit.

Proliferation Assay. Thirty-five mm culture dishes were precoated sequentially with poly-D-lysine (20 μ g/cm²) (Sigma) and human fibronectin, and then seeded with approximately 2.5×10^4 BALB/MK cells. The basic medium was a 1:1 mixture of Eagle's low Ca²⁺ minimal essential medium and Ham's F-12 medium, supplemented with 5 μ g/ml transferrin, 30 nM Na₂SeO₃ and 0.2 mM ethanolamine (Sigma). Medium was changed every 2 or 3 days. After 10 days, the cells were fixed in formalin (Fisher Scientific Co.) and stained with Giemsa (Fisher Scientific Co.).

Protein microsequencing. Approximately 4 μ g (-150 pmol) of protein from the active fractions of the C₄ column were redissolved in 50% TFA and loaded onto an Applied Biosystems gas-

phase protein sequenator. Twenty rounds of Edman degradation were carried out and identifications of amino acid derivatives were made with an automated on-line HPLC (Model 120A, Applied Biosystems).

RESULTS

Growth Factor Detection and Isolation.

Preliminary screening of conditioned media from various cell lines indicated that media from some fibroblast lines contained mitogenic activities detectable on both BALB/MK and NIH/3T3 cells. Whereas boiling destroyed the activity on BALB/MK, mitogenic activity on NIH/3T3 remained intact. Based on the known heat stability of EGF (I-15) and TGF α (I-16), it was reasoned that the BALB/MK mitogenic activity might be due to an agent different from these known epithelial growth factors.

M426, a human embryonic lung fibroblast line, was selected as the most productive source of this activity for purification of the putative growth factor(s). Ultrafiltration with the Pellicon system provided a convenient way of reducing the sample volume to a suitable level for subsequent chromatography. Various combinations of sieving, ion exchange and

isoelectric focusing chromatography were tried during the development of a purification scheme, but all resulted in unacceptably low yields.

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On the other hand, heparin-Sepharose affinity chromatography (HSAC), which has been employed in the purification of other growth factors (I-17--I-22), proved to be useful as an early purification step in the present invention. While estimates of recovered specific activity were uncertain at this stage because of the likely presence of other factors, the apparent yield of activity was 50-70% with a corresponding enrichment of approximately 1000 fold.

As shown in Fig. I-1, greater than 90% of the BALB/MK mitogenic activity eluted from the HSAC column with 0.6M NaCl. This peak of activity was not associated with any activity on NIH/3T3 cells (data not shown). A much smaller peak of BALB/MK mitogenic activity consistently emerged with 0.8 - 1.2M NaCl.

Due to the reproducibility of the HSAC pattern, active fractions could be identified presumptively on the basis of the gradient and optical density profile. Prompt concentration of 10-20 fold with the Centricon-10 was found to be essential for stability, which could be maintained subsequently at -70°C for several months.

Final purification was achieved by RP-HPLC
with a C₄ Vydac column, a preparative method 2038398
suitable for amino acid sequence analysis. While
the yield of activity from the C₄ step was usually
5 only a few percent, this loss could be attributed
to the solvents employed. In other experiments,
exposure to 0.1% TFA/50% acetonitrile for 1 hr at
room temperature reduced the mitogenic activity
of the preparation by 98%. Nonetheless, as shown
10 in Fig. I-2, a single peak of BALB/MK stimulatory
activity was obtained, coinciding with a distinct
peak in the optical density profile. The peak
fractions produced a single band upon
NaDodSO₄/PAGE and silver staining of the gel (Fig.
15 I-2B), and the relative mitogenic activity of
each tested fraction (Fig. I-2C) correlated well
with the intensity of the bands across the
activity profile.

An alternative purification step to the
20 HPLC technique described above, using sieving
chromatography with a TSK G3000SW GlasPac column
run in aqueous solution near physiologic pH,
resulted in a major peak of activity in the
BALB/MK bioassay (Fig. I-3). This preparation
25 was almost as pure as the one obtained from RP-
HPLC as judged by silver-stained NaDodSO₄/PAGE
(data not shown) but provided a far better
recovery of activity (Table I-1). The TSK-

purified material was used routinely for biological studies as it had a higher specific activity.

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5 In both types of purified preparations (i.e., purified by HPLC or molecular sieving), the profile of mitogenic activity was associated with a distinct band on NaDodSO₄/PAGE which appeared to be indistinguishable in the two preparations.

10 Physical and Biological Characterization
of the Growth Factor. The purified factor had an estimated molecular weight of about 28 kDa based on NaDodSO₄/PAGE under reducing (Fig. I-2) and non-reducing conditions (data not shown). This
15 value was in good agreement with its elution position on two different sizing columns run in solvents expected to maintain native conformation (TSK-G3000-SW, Fig. I-3, and superose-12, data not shown). From these data, the mitogen appears
20 to consist of a single polypeptide chain with a molecular weight of 25-30 kDa.

The heat and acid lability of the mitogenic activity were demonstrated using the BALB/MK mitogenesis bioassay. While activity was
25 unaffected by a 10 min incubation at 50°C, it was reduced by 68% after 10 min at 60°C and was undetectable after 3 min at 100°C. Exposure to 0.5M acetic acid for 60 min at room temperature

resulted in a decline in activity of 15% of the control. In comparison, the mitogenic activity of the known growth factor, EGF, was not diminished by any of these treatments.

5 The dose response curve for the purified growth factor depicted in Fig I-4 illustrates that as little as 0.1 nM led to a detectable stimulation of DNA synthesis. Thus, the activity range was comparable to that of the other growth factors analyzed to date. A
10 linear relationship was observed in the concentration range 0.1 - 1.0 nM with maximal stimulation of 600-fold observed at 1.0 nM. The novel factor consistently induced a higher level of maximal thymidine incorporation than EGF, aFGF or bFGF in the BALB/MK
15 keratinocytes (Fig. I-4). Incorporation of ³H-thymidine into trichloroacetic acid-insoluble DNA, expressed as fold stimulation over background, was measured as a function of the concentration of the indicated growth factors. Background values with no
20 sample added were 150 cpm. The results represent means values of two independent experiments. Replicates in each experiment were within 10% of mean values. TSK-purified mitogen, •—• EGF, ▲—▲, aFGF, •—• bFGF, ○—○.

25 The distinctive target cell specificity of this factor was demonstrated by comparing its

activities on a variety of cell types with those of other growth factors known to possess epithelial cell mitogenic activity. As shown in Table I-2, the new isolated factor exhibited a strong mitogenic effect on BALB/MK but also induced demonstrable incorporation of thymidine into DNA of the other epithelial cells tested. In striking contrast, the factor had no detectable mitogenic effects on mouse (or human data not shown) fibroblasts or human saphenous vein endothelial cells.

By comparison, none of the other known growth factors appeared to preferentially stimulate keratinocytes. TGF α and EGF showed potent activity on fibroblasts, while the FGFs were mitogenic for endothelial cells as well as fibroblasts (Table I-2). Because of its specificity of epithelial cells and the sensitivity of keratinocytes in particular, the novel mitogen was provisionally designated as keratinocyte growth factor (KGF).

To establish that KGF not only would stimulate DNA synthesis but would also support sustained cell growth, the ability of BALB/MK cells to grow in a fully-defined, serum-free medium supplemented with this growth factor was assessed. With reference to Figure I-5, cultures were plated at a density of 2.5×10^4 cells per dish on 35 mm Petri dishes precoated

with poly-D-lysine/fibronectin in a 1:1 mixture of Eagle's minimal essential medium and Ham's F12 medium supplemented with transferrin, Na_2SeO_3 , ethanolamine and the growth factors indicated below. After 10 days, the plates were fixed and stained with Giemsa. Key: a) no growth factor; b) EGF alone; c) insulin alone; d) KGF alone; e) EGF + insulin. Final concentrations of the growth factors were as follows: EGF, 20ng/ml; insulin, 10 $\mu\text{g/ml}$; and KGF, 40 ng/ml. As shown in Fig. I-5, KGF served as an excellent substitute for EGF but not insulin (or insulin-like growth factor I) in this chemically defined medium. Thus, KGF appears to act through the major signalling pathway shared by EGF, aFGF and bFGF for proliferation of BALB/MK cells. Further aspects of the impact of KGF on cell proliferation and in particular on human keratinocyte growth is discussed in Experimental Section III.

Microsequencing Reveals a Unique N-Terminal Amino Acid Sequence of KGF. To further characterize the growth factor, approximately 150 pmol of C_4 -purified material were subjected to amino acid sequence analysis. A single sequence _____

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was detected with unambiguous assignments made
for cycles 2-13, as follows: X-Asn-Asp-Met-Thr-
Pro-Glu-Gln-Met-Ala-Thr-Asn-Val. High background
noise precluded an assignment for the first
5 position which is, therefore, indicated by an X.

A computer search using the FASTP
program (I-24) revealed that the N-terminal amino
acid sequence of KGF showed no significant
homology to any protein in the National
10 Biomedical Research Foundation data bank, thus
supporting the novelty of this epithelial growth
factor.

DISCUSSION

The studies described in this
15 Experimental Section identified a human growth
factor which has a unique specificity for
epithelial cells. By employing ultrafiltration,
HSAC and RP-HPLC or TSK sieving chromatography
according to the present invention, a quantity
20 sufficient to permit detailed characterization of
the physical and biological properties of this
molecule was isolated.

A single silver-stained band
corresponding to a molecular weight of about
25 28,000 daltons was detected in the active
fractions from RP-HPLC, and the intensity of the

band was proportional to the level of mitogenic activity in these fractions. A band indistinguishable from that obtained by RP-HPLC was seen in the active fractions from TSK chromatography. The purified protein stimulated DNA synthesis in epithelial cells at sub-nanomolar concentrations, but failed to induce any thymidine incorporation in fibroblasts or endothelial cells at comparable or higher concentrations (up to 5 nM). This distinctive target cell specificity combined with the single novel N-terminal amino acid sequence determined from the purified molecule lead to the conclusion that KGF represents a new growth factor.

15 In a chemically defined medium the purified factor was able to complement the insulin-like growth factor I/insulin growth requirement of BALB/MK cells and therefore must act through a signal transduction pathway shared with EGF, TGF α and the FGFs. Moreover, the new factor was more potent than any of the known epithelial cell mitogens in stimulating thymidine incorporation in BALB/MK cells. Preliminary evidence indicates that this factor is also capable of supporting proliferation of secondary cultures of human keratinocytes (data not shown).

25 Handling and storage of KGF were problematical during its purification. Besides

its inherent lability to acid and heat, it was
unstable to lyophilization or dialysis. After
HSAC, complete loss of activity occurred within
24 hr despite the use of carrier proteins,
5 heparin, protease inhibitors, siliconized tubes
or storage at either 4° or -20°C. Only
concentrating the sample at this stage could
preserve its activity.

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Furthermore, in order to transfer the
10 dried, purified factor it was necessary to
utilize either strong acid or detergent,
consistent with an adsorptive tendency or
insolubility. Thus, for preservation of
activity, the purified factor was maintained in
15 solution at high concentrations at -70°C where it
remained stable for several months.

The ability of KGF to bind heparin may
signify a fundamental property of this factor
that has a bearing on its function *in vivo*. Growth
20 factors with heparin-binding properties include
aFGF (I-20--I-22) bFGF (I-19, I-22)
granulocyte/macrophage colony stimulating factor
and interleukin 3. (I-25) Each of these is
produced by stromal cells (I-25--I-27). Such
25 factors appear to be deposited in the
extracellular matrix, or on proteoglycans coating
the stromal cell surface (I-25, I-28). It has
been postulated that their storage, release and

contact with specific target cells are regulated
 by this interaction (I-25, I-28). While
 mesenchymal-derived effectors of epithelial cell
 proliferation have also been described (I-29--I-
 5 31), their identities have not been elucidated.
 Its heparin-binding properties, release by human
 embryonic fibroblast stromal cells, and
 epithelial cell tropism provide KGF with all of
 the properties expected of such a paracrine
 10 mediator of normal epithelial cell growth.

The partial amino acid sequence
 determined for this new growth factor has enabled
 molecular cloning of its coding sequence and
 determination of its structural relationship to
 15 known families of growth factors, as described in
 Experimental Section II, below.

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**CDNA SEQUENCE OF
A NOVEL EPITHELIAL CELL SPECIFIC GROWTH FACTOR
DEFINES A NEW MEMBER OF THE FGF FAMILY**

5 Work in the previous Experimental
Section I identified and purified a novel
heparin-binding growth factor, designated
keratinocyte growth factor (KGF), which is
particularly active on keratinocytes and appears
10 to be specific for epithelial cells. This second
Experimental Section describes the isolation and
characterization of cDNA clones encoding KGF,
using synthetic oligonucleotides, based upon the
experimentally determined NH₂-terminal amino acid
15 sequence, as hybridization probes. Nucleotide
sequence analysis identified a 582-bp open
reading frame which would code for a 194-amino
acid polypeptide that is between 41% and 33%
identical to the heparin-binding acidic and basic
20 fibroblast growth factors (FGFs), and the related
products of the *hst* and *int-2* oncogenes. The KGF
gene RNA transcript is expressed in normal
fibroblasts of both embryonic and adult origin,
but not in epithelial, endothelial or glial
25 cells. Thus, KGF appears to be normally
expressed by the mesenchyme, indicating a role in
the regulation of epithelial cell proliferation.

Isolation of cDNA clones. The

purification and N-terminal sequencing of KGF has been previously described (see Experimental
5 Section I, above and II-3). Pools (50 pmole) of deoxyoligonucleotides described under Results were 5' end-labelled using 83 pmole of γ - ^{32}P -ATP (3000 Ci/mmole, Amersham) and 10 units of T4 polynucleotide kinase. The recombinant phage
10 carrying cDNA clones were replica plated onto nitrocellulose filters and hybridized with ^{32}P -labelled deoxyoligonucleotides in 20% formamide, 10% dextran sulphate, 10 mM Tris-HCl (pH 7.5), 8 x SSC, 5x Denhardt's and 50 $\mu\text{g/ml}$
15 denatured salmon sperm DNA, overnight at 42°C. Filters were washed in 0.5 x SSC, 0.1% SDS at 50°C and exposed to Kodak X-omat AR film.

DNA sequencing. The nucleotide sequence of the KGF cDNA was determined by the dideoxy
20 chain termination method (II-26), of overlapping restriction fragments, subcloned into pUC vectors (II-27)

Construction of a bacterial expression vector for KGF cDNA. KGF cDNA encoding the
25 mature, secreted form of the polypeptide was placed under control of the hybrid trk promoter

in the plasmid expression vector pKK233-2 (II- 2038398
31), as follows. To accomplish this, a specific
length of KGF cDNA that contained the information
to code for the mature KGF molecule (i.e.,

5 without its signal peptide) was amplified using
the polymerase chain reaction (PCR) technique
(II-32). The fragment was directionally inserted
between two sites in the vector, namely the *Nco*I
site, made blunt ended by *S*I nuclease digestion,
10 and the *Hind*III site, using standard recombinant
DNA methodology. The ends of the KGF cDNA
produced by the PCR method were as follows: the
5' end was blunt and began with an ATG codon,
followed by the codon TGC for cys residue, number
15 33, which is the amino terminal residue of the
mature form of KGF (see Fig. II-1), and then the
entire KGF coding sequence. The stop codon, TAA,
and the four bases immediately following, TTGC,
were also included on the 3' end of the cDNA.
20 The primer used in the PCR method to direct DNA
synthesis to the desired position on the 3' end
of the cDNA included a *Hind*III site for insertion
of the amplified cDNA into the vector DNA.

Production of antibodies against KGF and
25 KGF-related peptides. Monoclonal antibodies were
raised in mice against intact, purified protein
from human fibroblasts using 5 or more

subcutaneous injections. Test bleeds were

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screened with a solid-phase (ELISA) assay using highly purified KGF from human epithelial cells as antigen. Hybridomas were prepared by routine

5 methods and supernatants were screened with the ELISA assay to detect KGF-reactive antibodies. Positive clones were serially subcloned by the usual methods, and selected subclones were grown as ascites tumors in mice for production of large
10 amounts of antibodies. Antibodies were purified from ascites fluids employing standard techniques (e.g., hydroxyapatite or immunoaffinity resins).

Polyclonal antibodies against a synthetic peptide were raised in rabbits by
15 standard methods, as follows. The peptides were made by solid phase technology and coupled to thyroglobulin by reaction with glutaraldehyde. Serial subcutaneous injections were made and test bleed were screened by ELISA as well as other
20 techniques, including Western blot analysis and mitogenesis bioassay. IgG immunoglobulins were isolated by affinity chromatography using immobilized protein G.

Polyclonal antibodies were raised in
25 rabbits against both naturally secreted KGF from human fibroblasts and recombinant KGF produced in *E. coli* (see next section), using the following protocol:

i) Initial injection and first boost were administered in the inguinal lymph nodes;

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ii) subsequent boosts were made intramuscularly.

5

Screening of test bleeds included ELISA as well as Western blot analysis and mitogenesis bioassay, and IgG was purified as for antibodies against synthetic peptides, above.

Isolation of cDNA clones encoding the novel growth factor. To search for cDNA clones corresponding to the KGF coding sequence, two
5 pools of oligonucleotides with lengths of 26 bases were generated based upon a nine-amino acid sequence, Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala, as determined by microsequencing of purified KGF
(see Experimental Section I, above and reference
10 II-3). One oligonucleotide pool contained a mixture of all 256 possible coding sequences for the nine amino acids, while the other contained inosine residues at the degenerate third position of the codons for Thr and Pro.

15 This latter design reduced the number of possible coding sequences in the pool to 16. Inosine in a tRNA anticodon can form hydrogen bonds with A, C or U (II-4), and oligonucleotides that contain deoxyinosine have been shown to
20 hybridize efficiently with the corresponding cDNA (II-5).

A cDNA library was constructed in a cDNA cloning vector, pCEV9 (II-6) using polyadenylated RNA extracted from the human
25 embryonic lung fibroblast cell line M426 (II-7), the initial source of the growth factor. Screening of the library (9×10^5 plaques) with

the ^{32}K -labelled 26-mer oligonucleotides identified 88
plaques which hybridized to both pools of
oligonucleotide probes.

Characterization and sequencing of selected

5 cDNA clones. Of 10 plaque-purified clones that were
analyzed, one, designated clone 49, had a cDNA insert
of 3.5 kb, while the rest had inserts ranging from 1.8
kb to 2.1 kb.

Analysis of the smaller clones revealed
10 several common restriction sites. Sequence of a
representative small clone, designated clone 32, along
with clone 49, demonstrated that they were overlapping
cDNAs (Fig II-1A). Overlapping pCEV9 clones 32 and 49,
used in sequence determination, are shown above a
15 diagram of the complete structure in which untranslated
regions are depicted by a line and the coding sequence
is boxed. The hatched region denotes the sequence of
the signal peptide and the open region denotes the
sequence of the mature protein. Selected restriction
20 sites are indicated. Whereas clone 49 was primed from
the poly(A) tail of the message, clone 32 arose during
the construction of the library by hybridization of the
oligo (dT) primer to an A-rich sequence in the 3'
noncoding region of the KGF mRNA.

25 Description of the sequence encoding the KGF
polypeptide. Alignment of the two cDNAs (clones 32 and

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49) established a continuous sequence of 3.85 kb containing the complete KGF coding sequence (Fig. II-1B). (B) KGF cDNA nucleotide and predicted amino acid sequences. Nucleotides are numbered on the left; amino acids are numbered throughout. The N-terminal peptide sequence derived from purified KGF is underlined. The hydrophobic N-terminal domain is italicized. The potential asparagine-linked glycosylation site is overlined. The variant polydenylation signals, AATTAA and AATACA, close to the 3' end of the RNA, are boxed. An ATG, likely to be an initiation codon was located at nucleotide position 446, establishing a 582-base paid open reading frame that ended at a TAA termination codon at position 1030. This open reading frame

would encode a 194-amino acid polypeptide with a calculated molecular weight of 22,512 daltons.

The sequence flanking the ATG codon did not conform to the proposed GCC(G/A)CCATGG
5 consensus for optimal initiation by eukaryotic ribosomes (II-8), however, there was an A three nucleotides upstream of the ATG codon. An A at this position is the most highly conserved nucleotide in the consensus. This ATG codon was
10 preceded 85 nucleotides upstream by a TGA stop codon in the same reading frame.

A 19-amino acid sequence that was homologous to the experimentally determined NH₂-terminus of purified KGF began 32 amino acids
15 downstream of the proposed initiation codon. There was complete agreement between the predicted and experimentally determined amino acid sequences, where unambiguous assignments could be made.

20 To search for homology between KGF and any known protein, a computer search of the National Biomedical Research Foundation data base using the FASTP program of Lipman and Pearson was conducted (II-9). By this approach, a striking
25 degree of relatedness between the predicted primary structure of KGF and those of acidic and

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basic FGF, as well as the related *nst* and *int-2*-encoded proteins was revealed.

Expression of mRNA transcripts of the KGF

gene in human cells. In preliminary attempts to

5 examine expression of KGF mRNA in human cells, a probe spanning the majority of the KGF coding sequence (Probe A, Figure II-1A) detected a single 2.4 kb transcript by Northern blot analysis of total M426 RNA (Fig. II-1C, lane b). Lanes a and c, poly(A)-selected M426 RNA; 10 lanes b and d, total cellular M426RNA. The filter was hybridized with a ³²P-labeled 695 bk *Bam*/HI/*Bcl*I fragment from clone 32 (Probe A, Fig. II-1A). This was considerably shorter than the length of the composite cDNA sequence, 3.86 kb.

15 However, on screening poly(A)-selected M426 RNA, an additional transcript of approximately 5 kb was detected (Fig. II-1C, lane a). Furthermore, a probe derived from the untranslated region of clone 49, 3' to the end of clone 32 (Probe B, Figure II-1A), hybridized 20 only to the larger message (Fig. II-1C, lane c). Thus, it appears that the KGF gene is transcribed as to alternate RNAs. Two other members of the FGF gene family, bFGF (II-29) and *int-2* (II-30), also expresses multiple RNAs, the significance of which remains to be 25 determined.

To investigate the normal functional role of KGF, the expression of its transcript in a variety of human cell~~s~~ lines and tissues was examined (Fig. II-3). Northern blot analysis of KGF mRNA in normal human cell
5 lines and tissues, and comparison with mRNA expression of other growth factors with known activity of epithelia cells was conducted. Total cellular RNAs were isolated by cesium trifluoro-acetate gradient centrifugation. 10 μ g of RNA were denatured and
10 electrophoresed in 1% formaldehyde gels. Following mild alkali denaturation (50 mM NaOH for 30"), RNA was transferred to nitrocellulose filters using 1 M ammonium acetate as a convectant. Filters were hybridized to a ^{32} P-labeled cDNA probe containing the
15 647 bk *Eco*RI fragment from the 5' end of the KGF coding sequence (A) or similar probes from the other growth factor DNAs. The following human cell types were used: squamous cell carcinomas (A253, A388 and A431); mammary epithelial cells (B5/589); immortalized bronchial
20 epithelial cells (S6 and R1); keratinocytes immortalized with Ad12-SV40; primary human keratinocytes; neonatal foreskin fibroblasts (AG1523); adult skin fibroblasts (501T); and embryonic lung fibroblasts (WI-39 and M426). As shown in Fig. II-3,
25 the _____

predominant 2.4 kb KGF transcript was detected in²
each of several stromal fibroblast lines derived
from epithelial tissues of embryonic, neonatal
and adult sources, but not from epithelial cell
5 lines of normal origin. The transcript was also
detected in RNA extracted from normal adult
kidneys and organs of the gastrointestinal tract,
but not from lung or brain. The striking
specificity of KGF RNA expression in stromal
10 cells from epithelial tissues indicated that this
factor plays a normal role in mesenchymal
stimulation of epithelial cell growth.

For comparison, the mRNAs of other
growth factors with known activity on epithelial
15 cells were also analyzed in the same tissues as
listed above. Among the epithelial and stromal
cell lines analyzed, there was no consistent
pattern of expression of aFGF or bFGF transcripts
(Fig. II-3). The EGF transcript was not
20 expressed in any of the same cell lines, and was
only observed in kidney, among the various
tissues. Finally, the TGF α message was not
detected in any of the stromal fibroblast lines
and was expressed at varying levels in each of
25 the epithelial cell lines. It was also detected
at low levels in kidney among the tissues
examined (Fig. II-3).

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heparin. Heparin has been shown to substantially increase the mitogenic activity of aFGF for a variety of target cells in culture, and to stabilize it from heat inactivation (II-21, II-22). Despite binding tightly to bFGF, heparin had minimal effects on its mitogenic activity (II-22). In view of the relatedness of KGF to the FGFs, the effect of heparin on KGF mitogenic activity was examined. As shown in Table II-1, thymidine incorporation by BALB/MK cells in response to KGF was inhibited 16 fold when heparin was included in the culture medium. In contrast, the activities of both aFGF and bFGF were increased by the same treatment.

Production of anti-KGF antibodies.

Several kinds of antibodies which recognize KGF or KGF-like polypeptides have been prepared using standard methodologies well known in the art of experimental immunology and summarized in the Methods section, above. These include: monoclonal antibodies raised in mice against intact, purified protein from human fibroblasts; polyclonal antibodies raised in rabbits against synthetic peptides with sequences based on amino acid sequences predicted from the KGF cDNA sequence; polyclonal antibodies raised in rabbits against both naturally secreted KGF from

human fibroblasts and recombinant KGF produced in
E. coli (see next section).

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Monoclonal antibodies from three
different hybridomas have been purified. All
5 three recognize the recombinant as well as the
naturally occurring KGF in a solid-phase (ELISA)
assay. None cross-reacts with KGF under
denaturing conditions (in a Western blot), and
none neutralizes mitogenic activity of KGF in the
10 BALB/MK bioassay.

Polyclonal antibodies were generated
with a synthetic peptide with the amino acid
sequence NDMTPEQMATNVR, corresponding to residues
numbered 32 through 44 in KGF (see Fig. II-1),
15 plus an R (arg) residue instead of the actual asp
residue encoded by the cDNA at position 45. The
asp residue is probably glycosylated in the
natural KGF polypeptide and, therefore, appeared
to be an arg in the amino acid sequencing data
20 obtained directly from that polypeptide (see
Discussion, below). Polyclonal antibodies
generated with this synthetic peptide recognize
both naturally occurring and recombinant KGF in
ELISA and Western blot analyses at a level of
25 sensitivity of at least as low as 10 ng protein.
These antibodies, however, do not neutralize
mitogenic activity of KGF in the BALB/MK
bioassay.

Polyclonal antisera against intact
natural KGF protein recognizes KGF in both ELISA
and Western blot assays. Such antibodies also
appear to inhibit mitogenic activity of KGF in
the BALB/MK bioassay.

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Expression of KGF cDNA in *E. coli*. KGF

cDNA was expressed to produce polypeptide in *E. coli*
by placing its coding sequence under control of
the hybrid trk promoter (comprising elements of
trp and lac promoters), in the plasmid pKK233-2
(II-31). To accomplish this, a specific length
of KGF cDNA that contained the information to
code for the mature KGF molecule (i.e., without
its signal peptide) was amplified using the
polymerase chain reaction technique (II-32). The
fragment was directionally inserted between two
sites in the vector, namely the *Nco*I site, made
blunt ended by S1 nuclease digestion, and the
*Hind*III site, using standard recombinant DNA
methodology. Selected recombinants were
sequenced at their cDNA 5' ends to ensure correct
alignment of the ATG initiation codon with the
regulatory elements of the trk promoter.

Several recombinants were tested for
protein production by the usual small scale
methods. In brief, the clones were grown to mid-
exponential phase ($OD_{595} \sim 0.5$), treated with 1 mM

isopropyl β -D-thiogalactopyranoside (IPTG) for 90 minutes, and cell extracts were run on SDS-polyacrylamide gels for Western blot analysis. All recombinants tested synthesized a protein that was
5 recognized by antibodies raised against an amino-terminal KGF peptide. One recombinant was selected which showed the greatest induction from IPTG, for further protein analyses.

One liter of bacteria was grown up in NZY
10 broth containing 50 μ g/ml ampicillin and 12.5 μ g/ml tetracycline, to OD₅₉₅ 0.5, and treated for 90 min with IPTG. The cells were collected by centrifugation, resuspended in 50 mM sodium phosphate (pH 7.3), 0.2 M NaCl, and lysed by sonication. Cell debris was removed
15 by centrifugation, and lysate applied directly to a heparin-Sepharose affinity column.

As determined by Western blot analysis and mitogenic activity in keratinocytes, recombinant KGF was eluted in 0.5 - 0.6 M NaCl. Subsequent
20 purification of the HSAC material with a Mono-S (FPLC) column (Pharmacia) yielded a preparation of KGF estimated to be $\geq 90\%$ pure, as judged by electrophoretic analysis using SDS-polyacrylamide gels and silver-staining.

25 The following specific test was carried out to determine the estimated molecular weight size of the

non-glycosylated bacterially expressed recombinant KGF protein that is represented in the Fig. II-4.

Fig. II-4A represents the Mono-S chromatography pattern of heparin-Sepharose purified, non-glycosylated KGF. The mitogenic activity (· - · - ·) coincides with elution of protein peaks, as indicated by optical absorbance read at 280 nm (_____)

Fig. II-4B shows the SDS-PAGE analysis of mitogenically active fractions from KGF preparation. Silver-stain of 14% polyacrylamide gel demonstrates purification of major active species at or slightly retarded relative to the 21.5 kD molecular weight marker, as well as minor species (here appearing as a doublet) with apparent molecular weight of approximately 16 kD. Lane 1: crude lysate; lanes 2 and 3: peak fractions from heparin-Sepharose chromatography; lanes 4-9: fractions 26-31 from Mono-S chromatography shown in Figure II-4A.

Fig. II-4C shows the immunoblot analysis of selected fractions from the Mono-S-chromatography. The purified proteins in mitogenically active Mono-S fractions cross-react with a rabbit neutralizing polyclonal antiserum raised against highly purified human KGF. Lanes 1-6 correspond to fractions 26-31 from Mono-S-chromatography shown in Fig. II-4A and silver-stained lanes 4-9 shown in Fig. II-4B.

Recombinant KGF efficiently stimulated thymidine incorporation into BALB/MK keratinocyte cells, but was only marginally active on NIH/3T3

fibroblasts. Half-maximal stimulation of the 2038398
BALB/MK cells in the standard keratinocyte
bioassay was achieved with a concentration of
between 2 to 5 ng/ml, compared to a concentration
5 of 10 to 15 ng/ml for KGF purified from M426
cells. One liter of bacterial cells yielded
approximately 50 µg of Mono-S purified
recombinant KGF.

Construction of a chimera containing KGF
10 and aFGF sequences. The studies above indicated
that KGF possessed two distinctive
characteristics which might be encoded by
distinct portions or domains of the polypeptide
sequence, as is well known to occur in coding
15 sequences of other multifunctional polypeptides.
To test this possibility, a chimeric DNA segment
encoding the NH₂-terminal sequence of KGF grafted
onto the C-terminal core of aFGF was constructed,
as follows. A *Sau3AI* restriction enzyme site
20 (GATC) in the 5' end of the KGF cDNA, within
codons for residues 78 and 79 (arg and ile,
respectively; see Fig. II-1) was cut and joined
to an homologous site in the aFGF cDNA within
codons for amino acids 39 (arg) and 40. The 3'
25 and 5' ends of this chimeric DNA were joined to
the vector DNA of the plasmid pKK233-2 by the
same method used for insertion of the KGF cDNA

encoding the secreted form of polypeptide (see
Methods, above).

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When recombinant *E. coli* cells were
constructed using the vector carrying the
5 chimera, and expressions tests were conducted as
described for mature KGF, above, a novel product
with properties of both KGF and aFGF was
produced. The peptide was enriched by heparin-
Sephacrose chromatography and found to have a
10 target cell preference for keratinocytes, like
KGF, with minimal activity on fibroblasts
(NIH/3T3). The mitogenic activity of this
chimeric polypeptide lacks, however,
susceptibility to inhibition by heparin, a
15 characteristic which parallels that of aFGF
rather than KGF. In fact, the mitogenic activity
on keratinocytes is actually enhanced by heparin,
as is the case for aFGF. Thus the peptide
domains responsible for cell target specificity
20 and heparin sensitivity are clearly distinct and
readily separable in KGF, according to the
practice of the present invention.

DISCUSSION

The experiments described in this
25 section illustrate the practice of several
principal embodiments of the present invention.
These include isolation of cDNAs encoding KGF,

expression of such cDNAs in recombinant cells,
production of various antibodies reactive with
KGF, and construction and expression of a
chimeric cDNA encoding a novel growth factor with
5 amino acid sequences and related functionalities
of both KGF and aFGF. The following points
related to these embodiments may also be noted to
enhance the understanding of the present
invention.

10 The sequence predicted from the KGF cDNA
agreed with the amino acid sequence determined
from the purified KGF form secreted by human
fibroblasts. Moreover, the sequence offered
potential explanations for positions where
15 definitive amino acid assignments could not be
made by direct amino acid sequencing. Residues
32 and 46 are predicted from the cDNA sequence to
be cysteines, and hydrolyzed derivatives of
unmodified cysteine residues are not detectable
20 following Edman degradation. The predicted KGF
amino acid sequence also contained one potential
N-linked glycosylation site (Asn-X-Ser/Thr) from
residues 45 through 47. If Asn 45 were
glycosylated, it would not be detected by the
25 amino acid sequencing methods employed here. In
fact, KGF migrates as a broad band on
NaDodSO₄/PAGE at a higher molecular weight than

predicted for the purified protein. This may be accounted for by glycosylation.

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The FGFs are heparin-binding mitogens with broad target cell specificities (II-10).

5 The *hst* gene was identified as a transforming gene from a human stomach tumor (II-11), adjacent normal stomach tissue (II-12), and from Kaposi's sarcoma (II-13), by standard NIH/3T3 transfection assays. The product of the *int-2* gene is expressed
10 normally during mouse embryogenesis (II-14) and aberrantly after proviral integration of mouse mammary tumor virus (II-15).

KGF is the sixth member of the fibroblast growth factor family to be identified
15 (II-28). While the name FGF-6 does not seem suitable because KGF is devoid of activity on fibroblasts, this nomenclature may also be used for this growth factor, to denote its structural relationship to the FGF family. As all
20 previously characterized growth factors either exclude epithelial cells as targets or include them among a number of sensitive target cells, the highly specific nature of KGF mitogenic activity for epithelial cells, and the
25 sensitivity of keratinocytes in particular, make it unique.

In studies to date, expression of the KGF transcript appears to be specific for stromal cells derived from epithelial tissues, suggesting its function in normal epithelial cell

5 proliferation. The availability of the KGF cDNA clone will make it possible to determine whether abnormal expression of this growth factor can be implicated in clinical conditions characterized by epithelial cell dysplasia and/or neoplasia.

10 Moreover, the ability to produce large quantities of this novel growth factor by recombinant techniques should allow testing of its clinical applicability in situations where specific growth of epithelial cells is of particular importance.

15 Alignment of the KGF sequence with the five other proteins of the FGF family revealed two major regions of homology, spanning amino acids 65-156 and 162-189 in the predicted KGF sequence, which were separated by a short,

20 nonhomologous series of amino acids with varying lengths in different members of the family (Fig. II-2). In the case of *int-2*, the length of this sequence was 17 residues, while in *hst*, the two homologous regions were contiguous. In KGF the

25 intervening sequence consisted of five amino acids.

In the aligned regions, the KGF amino acid sequence was about 44% identical to *int-2* (mouse), 39% identical to bFGF (human), 37% identical to aFGF (human) and 33% identical to *hst* (human). In this same region, all six proteins were identical at 19% of the residues, and allowing for conservative substitutions, they showed 28% homology. 2038398

As shown in Fig. II-2, the amino termini of these related proteins are nonhomologous and of variable length. The primary KGF and *hst* translation products contain hydrophobic N-terminal regions which likely serve as signal sequences (II-16). The fact that this N-terminal domain is not present in the mature KGF molecule (Fig. II-1B) further supports this conclusion. In contrast, the FGFs are synthesized apparently without signal peptides (II-10). The *int-2* protein contains an atypically short region of N-terminal hydrophobic residues (II-17), but it is not known if the protein is secreted. Moreover, the *int-2* protein contains a long C-terminal extension compared to the other family members.

Purified KGF contains five cysteine residues, two of which are conserved throughout the family of FGF related proteins (Fig. II-2). Also of note are the five pairs of basic residues

throughout the KGF sequence. This same pattern
has been observed in other FGF family members and
may be involved in their interaction with heparin
(II-18). Dibasic sites are also common targets
5 for proteolytic processing and such processing
might account for the microheterogeneity observed
in some KGF preparations (unpublished data).

The KGF cDNA sequence was AT rich
throughout its length, but particularly so in the
10 3' untranslated region where the AT content was
70% as compared to 60% in the putative coding
sequence and 63% in the 5' untranslated region.
The 3' untranslated region contained a large
number of ATTTA sequences, which have been
15 proposed to be involved in the selected
degradation of transiently expressed, unstable
RNAs (II-19). There was no classical AATAAA
polyadenylation signal but two variant sequences,
AATTAA and AATACA (II-20), were detected 24 and
20 19 nucleotides, respectively, upstream of the
poly(A) sequence at the 3' end of the cDNA.

It has been suggested that the heparin
effect on acidic FGF is either due to
stabilization of the active conformation of the
25 growth factor or to formation of a tertiary
complex with acidic FGF and its receptor (II-21,
II-22). If so, heparin may stabilize a
conformation of KGF that is not as active as the

free molecule, or form a tight complex that is
unable to efficiently interact with its receptor.

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While its ability to bind heparin
reflects the structural similarities of KGF with
5 the FGF's, the differences in target cell
specificities between these related mitogens is
remarkable. The FGF's induce division of most
nonterminally differentiated cells of both
embryonic mesodermal and neuroectodermal origin.
10 In addition to fibroblasts and vascular
endothelial tissues, mesodermally derived targets
in culture include myoblasts, chondrocytes and
osteoblasts (II-23). FGF's are also mitogenic
for glial astrocytes and neuroblasts (II-24).
15 The product of the oncogene isolated from
Kaposi's sarcoma, which is identical to *hst*, also
stimulates proliferation of NIH/3T3 and capillary
endothelial cells (II-25). To date, KGF induced
mitogenesis has only been observed in epithelial
20 cells, and the absence of any detectable activity
in fibroblasts or endothelial cells has also been
demonstrated (see Experimental Section I, above
and II-3). It seems likely, therefore, that KGF
acts through a different cell surface receptor
25 than the FGFs.

There is no significant N-terminal
homology between KGF and other FGF-related
proteins. Thus, the construction of chimeric

molecules between KGF and a prototype FGF was undertaken to determine whether the KGF

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N-terminal domain is sufficient to account for its unique target cell specificity. The results
5 on the first such recombinant polypeptide sequence indicate that the N-terminal domain of KGF essentially encodes the cell preference for keratinocytes, while the susceptibility of KGF to heparin is encoded somewhere in the C-terminal
10 core region which was replaced by sequences of aFGF. This novel KGF-like growth factor may have advantages in clinical applications where administration of an epithelial-specific growth factor is desirable in the presence of heparin, a
15 commonly used anticoagulant. Additional studies on chimeras should also provide insights into which specific domains in the C-terminal core contribute the different effects of heparin on their biologic activities.

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EXPERIMENTAL SECTION III**MATERIALS AND METHODS****Human keratinocyte culture**

5

Cultures of human epidermal cells were derived from full-thickness biopsies of neonatal foreskin as previously described (III-26). For proliferation assays in serum-free medium, secondary cultures were transferred to 60 mm petri dishes or 24 well cluster plates (Falcon) in standard medium consisting of MCDB 153, supplemented with 0.03 mM Ca^{2+} , 0.4 $\mu\text{g/ml}$ hydrocortisone, 5 $\mu\text{g/ml}$ insulin, 0.1 mM ethanolamine, and 70 $\mu\text{g/ml}$ whole bovine pituitary extract (WBPE) (Clonetics Corp. San Diego, CA). Cell adhesion was achieved by precoating petri dishes sequentially with poly-L-D-lysine (10 $\mu\text{g/cm}^2$), and human fibronectin (1 $\mu\text{g/cm}^2$) for 30 min at 37°C (III-16). Medium was changed every 2 days.

20

EGF and TGF α were obtained from Collaborative Research and Bacham, Inc. (Torrance, CA), respectively. Preliminary experiments were performed with KGF purified from culture fluids of human embryonic fibroblasts as previously described (III-29). Most of the studies described were performed with recombinant KGF expressed in *Escherichia coli* as described in

25

Experimental Section II. Recombinant KGF was purified by heparin Sepharose chromatography and was at least 90% pure as assessed by SDS-PAGE analysis. With subsequent purification using Mono-S ion exchange chromatography, it was possible to show that all activity on keratinocytes was due to KGF.

To measure cell number, cultures were harvested by incubation in 0.5% trypsin-EDTA (0.2%) solution for 15 min at 37°C, and cell counts were performed by haemocytometer.

Antisera

Rabbit anti-human keratin 1 (anti K1) (III-8, 11 and 12) was provided by S. Yuspa, Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI. This affinity purified antibody was used at a 1:1,000 dilution. Mouse anti-human filaggrin was purchased from BTI, Biomedical Technologies, Inc., (Stoughton, MA), and used at a 1:500 dilution.

20

Immunoblotting

Cell lysates were prepared by scraping cells from 100 mm petri dishes into OFLB lysis buffer (10.M urea, 2% NP-40, 100 mM dithiothreitol, 1 mM sodium vanadate, 1.6% LKB ampholine pH 5-7, and 0.4% LKB ampholine pH 3-10). Protein measurements were made by the Bradford

procedure (Bio-Rad Laboratories, Richmond, CA) using thyroglobulin as a standard (III-3). Approximately 100 μ g protein was loaded in each lane and resolved in 8% polyacrylamide-SDS gels (III-20).

5 Protein bands were electrophoretically transferred to Immobilon-PVDF membranes (Millipore) in 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol for 90 min at 1 Amp. PVDF membrane blots were processed with the relevant antisera and ^{125} I-labeled protein A as
10 described in (III-10).

RESULTS

Morphology of human keratinocytes cultured in KGF

15 In order to examine the effects of KGF on human keratinocytes, serum-free medium (Clonetics) was supplemented with 70 μ g/ml whole bovine pituitary gland extract (WBPE). In addition, petri dishes were
20 precoated with polylysine and human fibronectin as previously described (III-6,34). Under these conditions, secondary human keratinocytes attached, spread well, but grew very slowly. Figure III-1 A, B and C show a typical culture 4 days after plating in
25 this medium. Panel A was a control. In Panel B, cultures exposed to 10 ng/ml of recombinant human KGF demonstrate an obvious increase in cell proliferation.

For comparison, the effects of 10 ng/ml of EGF under the same conditions were examined. As shown in Figure III-1C EGF-exposed cultures also showed increased keratinocyte proliferation. With EGF, there was also an obvious outgrowth both of fibroblasts and melanocytes that was not observed in KGF-stimulated cultures with repeated cell passage.

Comparison of proliferation of human keratinocytes in response to KGF and EGF

In order to quantitatively compare the effects of KGF and EGF on human keratinocyte proliferation, the dose response to each growth factor was investigated. Cells were seeded at a density of 10^4 cells per well in a 24 well cluster plate and grown for 4 days in standard low Ca^{2+} medium supplemented with varying KGF or EGF concentration. Experiments were performed in triplicate, and results represent mean values $\pm$ standard deviations. In a 4-day assay, both growth factors induced significant increases in cell proliferation, ranging from approximately 4.5-fold under optimal conditions with EGF (O) to 6-fold in response to KGF (●) (Fig. III-2). Moreover, both growth factors significantly increased proliferation at low concentration. For example, there was a greater

than two-fold increase in cell number with as little as 0.1 ng/ml of KGF.

No significant additive effects on proliferation were observed using 10 ng/ml of EGF in combination with 10 ng/ml KGF. The kinetics of keratinocyte proliferation in response to the two growth factors are shown in Figure III-3. Following a short lag phase, KGF (10 ng/ml, ●) and EGF (10 ng/ml, ○) induced proliferation with similar kinetics. In contrast, untreated cultures (Δ) grew more slowly and plateaued in their proliferation after 10 days, while the growth factor-stimulated cultures continued to increase in cell number. Thus, under these experimental conditions, KGF was at least as effective as EGF for inducing proliferation of human keratinocytes.

The effects of KGF on colony formation by keratinocytes plated at low cell density were determined. Colony formation was significantly increased both in number and size over that observed in control cultures. Moreover, colonies in KGF-treated cultures showed a relative abundance of small cells that have been previously shown to represent less well differentiated keratinocytes (III-26). A similar pattern was observed for colonies that formed in EGF-treated cultures.

**Effects of the calcium-induced differentiation signal
on KGF- and EGF-induced keratinocyte proliferation**

It has been reported that keratinocytes in culture differentiate in response to calcium concentrations greater than 0.15 mM (III-2,16,18). The effects of increasing calcium concentration on proliferation and differentiation in response to KGF were determined. Cells were plated at a density of 10^4 cells per well in a 24 well cluster plate and grown for 4 days in standard medium alone (Δ) or supplemented with 10 ng/ml of either KGF ($\bullet$) or EGF ($\circ$). Experiments were performed in triplicate, and results represent mean values $\pm$ standard deviations. As shown in Figure III-4, the peak of cell proliferation in response to KGF (10 ng/ml) was observed at 0.05 mM, and Ca^{2+} concentrations of 0.1 and 0.5 mM were associated with increased cell proliferation as well. At 1.0 mM Ca^{2+} , there was no significant net increase in cell number in a 4-day assay. The results with EGF (10 ng/ml) were essentially the same (Fig. III-4). By use of colony formation as a measure of keratinocyte proliferation, it was reported (III-38) that the peak of cell growth was at 0.3 mM Ca^{+} with 10 ng/ml EGF. They also observed the above noted decreased growth at higher Ca^{+} concentrations. Thus, neither KGF nor EGF

was able to block the inhibitory effects of high Ca^{2+} on keratinocyte proliferation.

The morphologic effects of high Ca^{2+} were readily detectable as well. In control as well as growth factor-treated cultures, cells appeared more flattened, less refractile, and cell borders became less distinct. However, the effects were less dramatic in either of the growth factor-supplemented cultures (Fig. III-5). Cells were maintained for 4 days either in standard medium plus 1.0 mM Ca^{2+} (control) or supplemented with KGF (10 ng/ml) or EGF (10 ng/ml).

Calcium-dependent expression of differentiation markers

Epidermal differentiation is characterized by a number of morphological and biochemical changes that result in the development of stratified squamous epithelium (III-9,14,28). In the course of differentiation, there is a sequential expression of specific markers. Among such markers, keratins including K1 (67 kd) and K10 (59 kd), are expressed early in the differentiation program as cells begin their maturation in the basal or first suprabasal layer (III-12,14,23,28,31). Filaggrin (III-17,21) is expressed later as cells enter the granular cell layer (III-9). Expression of keratins and filaggrin genes is presumably regulated as a function of the stage of

keratinocyte differentiation by various external agents such as calcium and growth factors (III-27,32,37,39).

To examine the effects of the growth factor on these markers of differentiation, KGF- or EGF- stimulated cultures were exposed to increasing calcium concentration for 6 days. Human keratinocyte cultures were maintained for 6 days in the presence of varying Ca^{2+} concentrations as indicated, except for the last group which was exposed to 1.0 mM Ca^{2+} for 18 hr. Cells were maintained either in standard medium (control) or supplemented with KGF (10 ng/ml) or EGF (10 ng/ml). Cells were lysed, processed and immunoblotting was performed with anti-keratin 1 or anti-filaggrin sera, as described in the "Materials and Methods". As shown in Figure III-6, immunoblots prepared from cell lysates under different treatment conditions were probed with the human anti-K1 antibody. At 0.03 mM Ca^{2+} , a K1 reactive protein species migrating at 67 kd was observed in untreated keratinocytes, while neither KGF- nor EGF- exposed cultures showed this protein. At 0.15 mM Ca^{2+} , the 67 kd species was increased in control cultures and could be seen at lower level in KGF but not in EGF-supplemented cultures. This same pattern was observed at 1 mM Ca^{2+} . Because the appearance of K1 is known to be time-dependent (III-39), cultures treated with 1.0

mM Ca^{2+} for 18 hr were examined. The K1-reactive protein was readily detectable in control cultures and was observed at lower levels with KGF but not EGF exposure (Fig. III-6). This indicates that human
5 keratinocytes were rendered significantly more resistant by EGF than KGF to the appearance of this early differentiation marker in response to the calcium signal.

Filaggrin is synthesized as a 400 kd precursor, which is sequentially processed to a final
10 product of around 39 kd (III-17,21). Figure III-6 shows that in human skin, multiple intensely staining filaggrin immunoreactive proteins were detectable. In contrast, these proteins were not observed in secondary
15 human keratinocyte cultures at low Ca^{2+} in the presence or absence of either growth factor. At increasing calcium concentration, control cultures demonstrated multiple bands similar to the pattern observed in skin. As was the case with K1, the appearance of filaggrin
20 was specifically inhibited in cultures exposed to EGF but not KGF (Fig. III-6). At 18 hr, there also appeared to be relatively less induction of filaggrin relative to K1 (Fig. III-6), consistent with the known kinetics of appearance of these markers.

25 To further explore the differential effects of KGF and EGF on the appearance of biochemical markers

of terminal differentiation, we examined the Ca^{2+} response of keratinocytes propagated in $\text{TGF}\alpha$, which like EGF, interacts with the EGF receptor (III-5,33). Human keratinocyte cultures were maintained for 6 days with 0.03 or 1.0 mM Ca^{2+} in standard medium (control) or supplemented with 10 ng/ml of KGF or $\text{TGF}\alpha$. Cells were lysed, processed and immunoblotting analysis was performed with anti-keratin 1 serum as described in the "Materials and Methods". The appearance of K1 was blocked similarly in response to $\text{TGF}\alpha$ and EGF, while the marker was induced despite the presence of KGF (Fig. III-7). This indicates very different patterns of biochemical markers induced in response to the Ca^{2+} differentiation signal in keratinocytes stimulated by KGF as opposed to members of the EGF family.

DISCUSSION

Keratinocyte growth factor (KGF) is identified hereinabove as a human epithelial-specific growth factor. The growth factor is released in culture by stromal cells derived from major epithelial organs including skin and gastrointestinal tract. "In vivo", the KGF transcript is in stromal cells of these same normal tissues. This demonstrates that KGF plays an important role in normal epithelial cell proliferation (III-13). The results of this

Experimental Section further demonstrate that KGF acts as a potent mitogen for human keratinocytes in culture, equivalent to or more active on a molar basis than EGF.

5 In the sequential program of keratinocyte differentiation, the basal cell at the innermost layer of the epidermis is the stem cell. Progeny cells migrate into the upper epidermal layers, ultimately terminally differentiating to form the stratum corneum (III-37). During this process, the cells change
10 dramatically both morphologically and biochemically. Thus, one could expect that growth factors of physiologic importance for epidermal cells would be able to sustain proliferation of undifferentiated basal cells and yet not interfere with proper signals for
15 terminal differentiation. According to this Experimental Section, the results indicate that in response to the differentiation signal induced by a high calcium concentration, KGF-treated keratinocytes ceased to proliferate. The cells developed morphologic
20 features of terminally differentiated keratinocytes as well. Finally, under conditions of high calcium exposure, KGF-stimulated human keratinocytes expressed both K1 and filaggrin, early and late markers, respectively, of keratinocyte differentiation.

25 In contrast to these results with KGF, the well-characterized EGF molecule was able to block or at

least significantly retard expression of biochemical markers of keratinocyte differentiation under identical conditions of high calcium exposure. Thus, both K1 and filaggrin were either not detectable or were much
5 reduced in expression in EGF-treated keratinocytes. EGF, itself, is not thought to be normally expressed in epidermal or dermal cells. In contrast, there is evidence that TGF α , which also binds and activates the EGF receptor, is expressed in certain epithelial cells
10 including keratinocytes (III-6,15). This section demonstrates that TGF α , like EGF, blocked the appearance of K1 in response to the calcium differentiation signal. The results of this section demonstrate potentially significant differences in the
15 abilities of specific epithelial growth factors to modulate differentiation responses to normal physiologic stimuli "in vivo".

There is most likely a molecular basis for the differing responses to the calcium signal in
20 keratinocytes exposed to KGF as opposed to either EGF or TGF α . EGF may interfere with the normal calcium-induced response by blocking some critical intracellular signalling pathway. There is accumulating evidence that the ability of keratinocytes
25 to respond to the calcium signal is dependent at least in part upon the substratum that they produce (III-1).

Production of substances such as fibronectin, laminin, or collagen in response to KGF or EGF could significantly differ which may explain the patterns observed.

5 Considerable attention has been focused on the potential clinical application of growth factors to wound healing (III-4,22) and tissue repair. In particular, epithelium derived from keratinocytes cultured on feeder layers has been successfully applied
10 to such clinical conditions as burns (III-7,19) and chronic ulcers (III-25). The ability to speed wound repair by direct application of growth factors to the wound site has been tested experimentally with a variety of such factors. EGF and TGF α have been
15 reported to stimulate regeneration of epithelium (III-4,24,30), but in one study differentiation abnormalities were reported as well (III-22). This section in determining that KGF is associated with a normal differentiation response in vitro demonstrates
20 its clinical application for epithelial cell regeneration and repair.

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MATERIALS AND METHODS

Recombinant KGF was purified as described in
5 Experimental Section II. Acidic and basic FGF purified
from bovine brain and their ^{125}I -labelled derivatives
(150-200 $\mu\text{Ci}/\mu\text{g}$) were obtained from R & D Systems.
Affinity-purified antiphosphotyrosine ($\alpha\text{P-Tyr}$) was
prepared as described by White and Kahn (IV-11).
10 Heparin-Sepharose CL-6B was purchased from Pharmacia
LKB Biotechnology Inc. GammaBind-G agarose* was
obtained from Genex, ^{125}I -Labelled sodium ($>5000 \text{ Ci/mM}$)
was purchased from Amersham Corp. Recrystallized bovine
serum albumin (BSA) was obtained from ICN.
15 Disuccinimidyl suberate, Triton X-100*, BCA protein
assay reagent, and dithiothreitol were purchased from
Pierce Chemical Co. Heparin, aprotinin, and
phenylmethylsulfonyl fluoride were obtained from Sigma.
Tissue culture plasticware was obtained from Costar.
20 Reagents for SDS-PAGE were purchased from Bio-Rad.

Iodination of KGF. Recombinant KGF was
radiolabelled with ^{125}I -labelled sodium by the
chloramine-T method (IV-12). KGF (3 $\mu\text{g}/50 \mu\text{l}$ in 20 mM
phosphate buffer + 1.0 M NaCl, pH 7.4) was reacted with
25 chloramine-T (1.2 $\mu\text{g}/4 \mu\text{l}$ of phosphate buffer) and ^{125}I -
labelled sodium (1 mCi/10 μl) at 24°C for 1 min. The

* trade mark

reaction was terminated by the addition of sodium metabisulfite (10 μ g/8 μ l), and the mixture was then diluted with phosphate buffer + 0.1% BSA (200 μ l) and applied to a column (300 μ l packed volume) of heparin-
5 Sepharose CL-6B preequilibrated in phosphate-buffered saline + 0.1% BSA (PBS/BSA). The sample was recycled several times, and the column was washed with 30ml of PBS/BSA. Elution with aliquots (200 μ l) of PBS/BSA + 0.85M NaCl removed >98% of trichloroacetic acid-
10 precipitable radioactivity from the column. Peak fractions (specific activity, 150-250 μ Ci/ μ g) were >99% trichloroacetic acid precipitable, migrated as a single band on SDS-PAGE, and retained full mitogenic activity on Balb/MK cells.

15 Receptor Binding Assays. Confluent Balb/MK (IV-13) or NIH/3T3 (IV-14) cultures in 24-well plates were serum-starved for 24 h, then incubated with HEPES binding buffer (100 mM HEPES, 150 mM NaCl, 5 mM KCl, 31.2 mM MgSO₄, 8.8 mM dextrose, and 0.1% BSA pH 7.4)
20 containing ¹²⁵I-KGF for 3 h at 15°C. The cells were washed (3 x 1 ml) with cold PBS or, alternatively, washed with PBS (2 x 1 ml) followed by an extraction with PBS + 0.5 M NaCl (1 x 1 ml). The cells were lysed with 0.5% SDS (2 x 250 μ l), and radioactivity in the
25 NaCl and SDS extracts of triplicate samples was measured in a γ counter. ¹²⁵I-aFGF and -bFGF binding

assays were performed similarly, except the salt extraction with 1.0 M NaCl (in aFGF binding assays) or 1.5 M NaCl (in bFGF binding assays) was substituted appropriately.

5 Bound counts/min were normalized according to protein content of SDS extracts as measured by BCA protein assay (Pierce Chemical Co.). Specific binding was determined by subtracting normalized counts/min of samples incubated with 100-fold excess unlabelled
10 ligand from the normalized counts/min bound in the absence of unlabelled ligand. In some experiments, heparin (3 μ g/ml) was added during the binding incubation. In competition assays, samples contained tracer levels of radiolabelled ligand (1 ng/ml) and
15 several concentrations of competitor (0-300 ng/ml) for processing as outlined above. For Scatchard analysis, samples contained several concentrations of radiolabelled ligand (1-100 ng/ml) in the presence or absence of 100-fold excess unlabelled ligand and were
20 processed similarly. Estimates of receptor affinity and total binding capacity were made using LIGAND software (IV-15).

Cross-linking of ^{125} I-KGF, -aFGF, and -bFGF to
25 Receptors. Samples for covalent cross-linking were prepared from confluent serum-starved Balb/MK or

NIH/3T3 cultures in 10-cm dishes using 10-30 ng/ml ^{125}I -KGF, -aFGF, or -bFGF in the presence or absence of 100-fold excess unlabelled ligand. After binding (as described above), cross-linking with disuccinimidyl suberate was performed as described (IV-16). The cells were then scraped into cold HEPES binding buffer containing protease inhibitors (0.1 mM aprotinin and 1.0 mM phenylmethylsulfonyl fluoride), and a crude membrane fraction was generated by brief sonication (50 watts, 10s), low speed centrifugation (600 x g, 10 min), and high speed centrifugation (10,000 x g, 30 min) of the low speed supernatant. The membrane pellet was solubilized in sample buffer (IV-17) containing 100 mM dithiothreitol and boiled for 3 min. ^{125}I -Labelled proteins were resolved by 7.5% SDS-PAGE (IV-17) and autoradiography at -70°C .

Western Blot Detection of Phosphotyrosyl

Proteins. Confluent cell cultures in 10-cm dishes were serum-starved for 24 h and then treated with KGF, aFGF, or bFGF (30-100 ng/ml) for 10 min at 37°C . The medium was aspirated, and the cells were solubilized in cold HEPES buffer containing 1% Triton X-100, and protease and phosphatase inhibitors (IV-7). The lysate was cleared by centrifugation (14,000 x g, 3 min), and phosphotyrosyl proteins were immunoprecipitated with

α P-Tyr absorbed to GammaBind G-agarose. Phosphotyrosyl proteins were specifically eluted using 50 mM phenyl phosphate, diluted in sample buffer, and resolved by 7.5% SDS-PAGE. Proteins were then transferred to
 5 nitrocellulose and detected with α P-Tyr and ^{125} I-protein A as described (IV-7).

DISCUSSION OF RESULTS

10 Specific Binding of ^{125} I-KGF to Receptors on
Balb/MK. Saturable specific binding of ^{125} I-KGF to intact Balb/MK cells could be detected in the presence of low concentrations of heparin (1-3 μ g/ml) or after briefly extracting the cell surface with 0.5 M NaCl.
 15 In the absence of heparin or salt extraction, specific binding to Balb/MK was obscured by an excess of low affinity binding. Heparin appeared to block the binding of ^{125} I-KGF to the salt-extractable cell surface or extracellular matrix component, as salt extractable
 20 counts/min were dramatically lower in its presence. At these low heparin concentrations (1-3 μ g/ml), KGF retained a potent mitogenic effect of Balb/MK cells.

In Figure IV-1, specific binding of ^{125} I-KGF (1 ng/ml) to Balb/MK cells is depicted, expressed as
 25 femtomoles bound per 10^5 cells, competed by increasing concentrations (nM) of unlabeled KGF ($\square$), aFGF (O), or

bFGF (Δ). Values shown are the mean of triplicate samples $\pm$ standard deviation (SD). Where no error bars are shown, the error is less than the symbol size. Similar results were obtained using either low

5 concentrations of heparin (1-3 μ g/ml) or brief salt extraction to block low affinity ligand binding in all competition studies shown. In panel B, specific ^{125} I-KGF binding on NIH/3T3 cells is displayed, competed by unlabeled KGF, aFGF, or bFGF. In panel C, specific

10 binding of ^{125} I-aFGF (1 ng/ml) to Balb/MK cells is shown, competed by unlabeled KGF, aFGF, or bFGF. In panel D, specific ^{125} I-aFGF binding on NIH/3T3 cells is displayed, competed by unlabeled KGF, aFGF or bFGF. Using either heparin or salt extraction to block low

15 affinity binding ^{125} I-KGF binding was reduced 50% by 0.05 nM KGF (Fig. IV-1A).

High affinity ^{125} I-KGF binding to Balb/Mk was competed by aFGF with 4-fold lower efficiency than by KGF (50% displacement at 0.2 nM aFGF, Fig. IV-1A).

20 bFGF also competed for ^{125} I-KGF binding but with 20-fold lower efficiency than KGF (50% displacement at 1 nM, Fig. IV-1A). In the presence of heparin (1-3 μ g/ml) or after brief salt extraction, specific high affinity binding of ^{125} I-KGF to NIH/3T3 cells was not detected

25 (Fig. IV-1B), consistent with its lack of mitogenic activity for this cell type (IV-1). However, without

added heparin or prior salt extraction, low affinity binding of ^{125}I -KGF to NIH/3T3 was observed. ^{125}I -aFGF also bound specifically and with high affinity to Balb/MK cells and was competed with similar efficiency by aFGF and KGF (50% displacement at 0.2 and 0.5 nM, respectively; (Fig. IV-1C). In contrast, bFGF competed 20-fold less efficiently than the other two ligands for ^{125}I -aFGF binding (50% displacement at 4 nM; Fig. IV-1C). Finally, high affinity ^{125}I -aFGF binding to NIH/3T3 fibroblasts was competed with similar efficiency by aFGF or bFGF (50% displacement at 0.2 and 0.3 nM, respectively) but was not competed by KGF at any concentration tested (Fig. IV-1D). Thus the pattern of ^{125}I -KGF and -FGF high affinity binding and competition exhibited by Balb/MK cells was fundamentally different from that of NIH/3T3 fibroblasts.

These results indicate that:

- 1) KGF and aFGF competed for the same site on Balb/MK,
- 2) NIH/3T3 cells lack high affinity KGF binding and,
- 3) bFGF competed poorly for ^{125}I -aFGF binding on Balb/MK, yet efficiently for ^{125}I -aFGF binding on NIH/3T3 cells,

which distinguishes at least two receptors to which a FGF can bind, one with KGF cross-reactivity and one with bFGF cross-reactivity.

Scatchard Analysis of ^{125}I -KGF and ^{125}I -aFGF

5 Binding. Scatchard analysis of ^{125}I -KGF binding on Balb/MK yielded a curvilinear pattern, most simply interpreted as representing two receptor subpopulations of different affinities (Fig. IV-2A). Values (expressed as femtomoles bound per μg of cell protein) 10 are the mean of triplicate samples $\pm$ SD. In the inset of Fig. IV-2A. Saturation binding of ^{125}I -KGF ($\square$) and ^{125}I -aFGF (O) on Balb/MK cells is expressed as ligand bound (femtomoles) per μg of cell protein. Values are the mean triplicate samples $\pm$ SD. B, Scatchard analysis 15 of ^{125}I -KGF specific binding on Balb/MK cells in the presence of ($\square$) or absence (O) of added heparin.

The higher affinity component predicted fewer than 5,000 sites/cell with dissociation constant (K_D) of 25 pM, while the lower affinity component predicted 20 approximately 65,000 sites/cell with a K_D of 400 pM. The 50% effective dose (ED) of the recombinant KGF used in these studies was approximately 50 pM for Balb/Mk cells of Experimental Section II, suggesting that both receptor subpopulations described here may mediate KGF 25 mitogenic activity. Scatchard analysis of ^{125}I -KGF binding performed in the absence of salt extraction or

heparin reversed an additional low affinity component that was not saturable under the conditions tested (Fig. IV-2B). We attribute this low affinity binding to cell-associated heparin-like moieties similar to those demonstrated previously for bFGF (IV-19).

Scatchard analysis of ^{125}I -aFGF binding on Balb/MK revealed a single receptor population consisting of approximately 80,000 sites/cell with a K_D of 350 pM (Fig. IV-2A). Similar analysis of ^{125}I -aFGF binding on NIH/3T3 fibroblasts revealed a single receptor population of approximately 100,000 sites/cell with a K_D of 250 pM. These affinity and capacity values are within the range of values previously published for the high affinity aFGF receptor ($K_D = 50$ -500 pM, 0.5 - 5×10^4 sites/cell (IV-3)) and bFGF receptor ($K_D = 10$ -200 pM, 0.2 - 10×10^4 sites/cell (IV-3, 19)). This work also confirmed the added presence of low affinity receptors for the FGFs on both Balb/MK and NIH/3T3, similar to the low affinity bFGF receptors on BHK cells previously characterized by Moscatelli (IV-19).

Cross-linking of ^{125}I -KGF, -aFGF, and -bFGF to Their Receptors. Covalent affinity cross-linking of ^{125}I -KGF to its receptor on Balb/MK cells revealed two labelled species of 162 and 137 kDa that were specifically competed by 100-fold excess KGF (Fig. IV-

3). Assuming a 1:1 receptor:ligand interaction, the corrected molecular masses of the putative KGF receptors were 140 and 115 kDa, respectively.

Predictably, no proteins were specifically labelled when ^{125}I -KGF was cross-linked to NIH/3T3 fibroblasts (Fig. IV-3). Similar results were obtained when heparin was added or after brief salt extraction suggesting that only high affinity KGF receptors were cross-linked by this method. On Balb/MK cells, ^{125}I -aFGF was specifically cross-linked to two proteins, one similar in size to the larger species labelled by ^{125}I -KGF and one with a corrected molecular mass of 120 kDa (Fig. IV-3). On NIH/3T3 cells, however, ^{125}I -aFGF bound specifically to two species with corrected molecular masses of 155 and 135 kDa (Fig. IV-3). Attempts to cross-link ^{125}I -bFGF to receptors on Balb/MK indicated weak cross-reactivity with the KGF/aFGF-associated species, but on NIH/3T3 fibroblasts, ^{125}I -bFGF and ^{125}I -aFGF appeared to label similar protein species (Fig. IV-3).

Both protein species cross-linked to ^{125}I -KGF on Balb/Mk cells were efficiently competed with excess aFGF, and conversely, excess KGF competed for the ^{125}I -aFGF-labelled species. These results are consistent with the binding studies described above and, together with the size similarity of species labelled by KGF and

aFGF indicates that both ligands may act through the same receptors on Balb/MK. Furthermore, the data indicate that an analogous situation may exist for FGFs acting on NIH/3T3 fibroblasts. bFGF competed efficiently for both ^{125}I -aFGF-labelled species, and conversely, aFGF competed for both ^{125}I -bFGF-labelled species; KGF did not compete for any cross-linked species on NIH/3T3 cells. Together with the size and similarity of proteins labelled by ^{125}I -aFGF and ^{125}I -bFGF on fibroblasts, the results support previous reports that aFGF and bFGF cross-react with two receptors of approximately 150 and 130 kDa (IV-18).

Phosphotyrosyl Proteins Induced by KGF, aFGF, and bFGF. To enrich for phosphotyrosyl proteins, cell lysates were sequentially immunoprecipitated and immunoblotted with affinity-purified antiphosphotyrosine antibodies. By this method several phosphotyrosyl proteins were observed in quiescent Balb/MK and NIH/3T3 cells (Fig. IV-4). KGF (30 ng/ml) specifically stimulated the rapid tyrosine phosphorylation of a 90-kDa protein (pp90) in Balb/MK cells (Fig. IV-4). KGF-induced phosphorylation of pp90 in Balb/Mk cells reached maximum within 10 min at 37°C and decreased thereafter. The phosphorylation of pp90 was dose-dependent and was detectable using KGF concentrations from 10 to 100 ng/ml. aFGF also induced

the tyrosine phosphorylation of a 90-kDa protein in Balb/MK cells, although less effectively than KGF (Fig. IV-4). pp90 was not observed in bFGF-treated Balb/MK cells over a concentration range of 10-100 ng/ml (Fig. IV-4). pp90 migrated similarly under reducing and non-reducing SDS-PAGE conditions, suggesting that it was not disulfide-linked to either of the higher molecular weight KGF-binding entities observed by covalent affinity cross-linking.

A decrease in the electrophoretic mobility of a diffuse 70-kDa phosphotyrosyl protein (pp70) was also reproducibly observed in KGF- or aFGF-treated Balb/MK cells and in FGF-treated NIH/3T3 cells (Fig. IV-4). Such a shift indicates changes in phosphorylation of pp70 triggered by these growth factors.

KGF failed to induce tyrosine phosphorylation of any cellular proteins in NIH/3T3 fibroblasts (Fig. IV-4), consistent with its lack of mitogenic effect as established in Experimental Section I or high affinity binding. In contrast, both aFGF and bFGF stimulated the phosphorylation of a 90-kDa protein in NIH/3T3 fibroblasts (Fig. IV-4), as reported previously in (IV-6). The FGFs also stimulated the phosphorylation of a 150-kDa protein in NIH/3T3 cells (Fig. IV-4) similar to that demonstrated by Friesel et al. (IV-7), and later reported by Ruta et al (IV-10) to be

phospholipase C γ . The phosphorylation of phospholipase C γ on tyrosine by the epidermal growth factor and platelet-derived growth factor receptors was described previously (IV-21, 22).

5 Although both Balb/MK cells and NIH/3T3 fibroblasts displayed low affinity heparin-like receptors for KGF, the binding and cross-linking data of this Section IV show that only the Balb/MK keratinocytes express high affinity KGF receptors.
10 Such high affinity receptors are believed to be required for transduction of the KGF mitogenic signal.

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EXPERIMENTAL SECTION V

As established in Section IV, KGF exhibits specific binding to keratinocytes but not fibroblasts. A cDNA library (4.5×10^6 independent clones) was prepared from BALB/MK epidermal keratinocytes (V-10) in an improved vector, λ pCEV27 (V-11), and transfected into NIH/3T3 mouse embryo fibroblasts (V-12) which synthesize KGF (V-13). Fifteen transformed foci were detected among a total of 100 individual cultures. Each was shown to be resistant to G418, indicating that it contained integrated vector sequences. Three representative transformants were chosen for more detailed characterization based on differences in their morphologies. Several plasmids were isolated from each transformant after plasmid rescue. This was accomplished by cloning genomic DNA from each transformant by one of the infrequent cutters that can release the plasmids containing cDNA inserts. Digested DNA was ligated under diluted conditions and used to transform bacterial-competent cells. Plasmids were isolated from ampicillin- and kanamycin-resistant transformants and used to transfect NIH/3T3 cells to examine for focus formation. The ect1 plasmid was rescued by Xho I, while the ect2 and ect3 plasmids were rescued by Not I digestion. A single cDNA clone rescued from each transformant was found to possess

high-titered transforming activity ranging from 10^3 to 10^4 focus-forming units per nanomole of DNA.

Transfectants induced by the individual plasmids containing these epithelial cell-forming cDNAs (designated *ect1*, *ect2*, and *ect3*) were used in subsequent analyses.

To determine if any of the three genes encodes for the KGF receptor, binding studies with recombinant ^{125}I -labeled KGF were performed. BALB/MK cells showed specific high affinity binding of ^{125}I -labeled KGF, which was not observed when NIH/3T3 cells were used. Expression of the *ect1* gene by NIH/3T3 cells resulted in the acquisition of 3.5-fold more ^{125}I -labeled KGF binding sites than BALB/MK cells. Under the same conditions, control NIH/3T3 as well as transfectants containing either *ect2* or *ect3* did not bind the labeled growth factor. These results determined that *ect1* encoded the KGF receptor (KGFR), whose introduction into NIH/3T3 cells had completed an autocrine transforming loop.

To characterize *ect1*, 4.2-kb cDNA released by Sal I digestion was transformed as a molecular probe to hybridize Sal I-digested genomic DNAs. Since Sal I is an infrequent cutter, the large genomic DNA fragments migrated near the origin of the gel. The expected 4.2-kb DNA fragment was detected in the *ect1* transformant

(Fig. V-1A), but neither NIH/3T3 nor the other transfectants showed evidence of Sal I fragment hybridized by the cDNA insert. These results indicate that the *ect2* and *ect3* represented independent transforming genes. When Eco RI was used to cleave normal mouse DNA, several distinct *ect1*-hybridizing DNA fragments were observed, which reflected endogenous *ect1* sequences or closely related genes (Fig. V-1B). These *ect1*-related sequences were also observed in the DNAs of other species analyzed, including human, indicating its high degree of conservation in vertebrate evolution. A single *ect1* transcript of around 4.2 kb was observed in BALB/MK cells (Figure V-1C). Thus, the cDNA clone represented essentially the complete *ect1* transcript. In NIH/3T3 cells, a transcript of comparable size was only faintly detectable under stringent hybridization conditions. Thus, if this transcript were to represent *ect1* rather than a related gene, its expression was markedly lower in fibroblasts as compared to epithelial cells.

Method for Genomic Analysis of *ect1* DNA and Comparative RNA Expression.

Figure V-1A is a Southern analysis of the Sal I-digested DNAs from NIH/3T3 and its transformants. The blot was probed with the entire *ect1* cDNA insert.

Lane 1, NIH/ect1; lane 2, NIH/ect2; lane 3, NIH/ect3;
lane 4 NIH/3T3.

Figure V-1B is a Southern analysis of Eco RI-digested DNAs of different animal species (Clontech Labs, Inc.). The blot was probed with the 5'-half of the ect1 cDNA insert (Fig. V-2B) and washed under reduced stringency conditions. Lane 1, human; lane 2, rhesus monkey; lane 3, mink; lane 4, cat; lane 5, mouse; lane 6, cow; lane 7, chicken; lane 8, dog, lane 9, guinea pig; lane 10, pig.

Figure V-1C is a Northern analysis of NIH/3T3 and BALB/MK RNA. The blot was probed with the 5'-half of the ect1 cDNA (lanes 1 and 2) or a β -actin cDNA (lanes 3 and 4) and washed under stringent conditions. Lanes 1 and 3, NIH/3T3; lanes 2 and 4, BALB/MK.

In the above analysis of V-1A and V-1B, the blot was probed by digesting DNA (10 μ g) by Sal I (Fig. V-1A) or Eco RI (Fig. V-1B), fractionated by agarose gel electrophoresis, and transferred to a nylon-supported nitrocellulose paper (Nitrocellulose-GTG, FMC Corp.). The blot in Fig. V-1A was hybridized with the ^{32} P-labeled entire ect1 insert at 42°C and washed at 65°C in 0.1 x saline sodium citrate (SSC), while the blot in Fig. V-1B was hybridized with the ^{32} P-labeled 5'-ect1 probe (Fig. V-2B) at 37°C and washed at 55°C in 0.1 x SSC. Hybridization experiments were performed at

the indicated temperature in a solution containing 50% formamide, 5 x SSC, 2.5 x Denhardt solution, 7 mM tri-HCl (pH 7.5), denatured calf thymus DNA (0.1 mg/ml), and tRNA (0.1 mg/ml). Location of DNA markers (BRL, Gaithersburg, MD) is indicated in kilobases.

Polyadenylated RNA preparations (5 μ g each) were fractionated by formaldehyde gel, transferred to Nitrocellulose-GTG, and hybridized with the 5'-ect1 probe for the Northern analysis of V-1C. After autoradiography, the filter was boiled to remove the probe and then hybridized with a β -actin probe (lanes 3 and 4). Location of markers (BRL, Gaithersburg, MD) is indicated in kilobases.

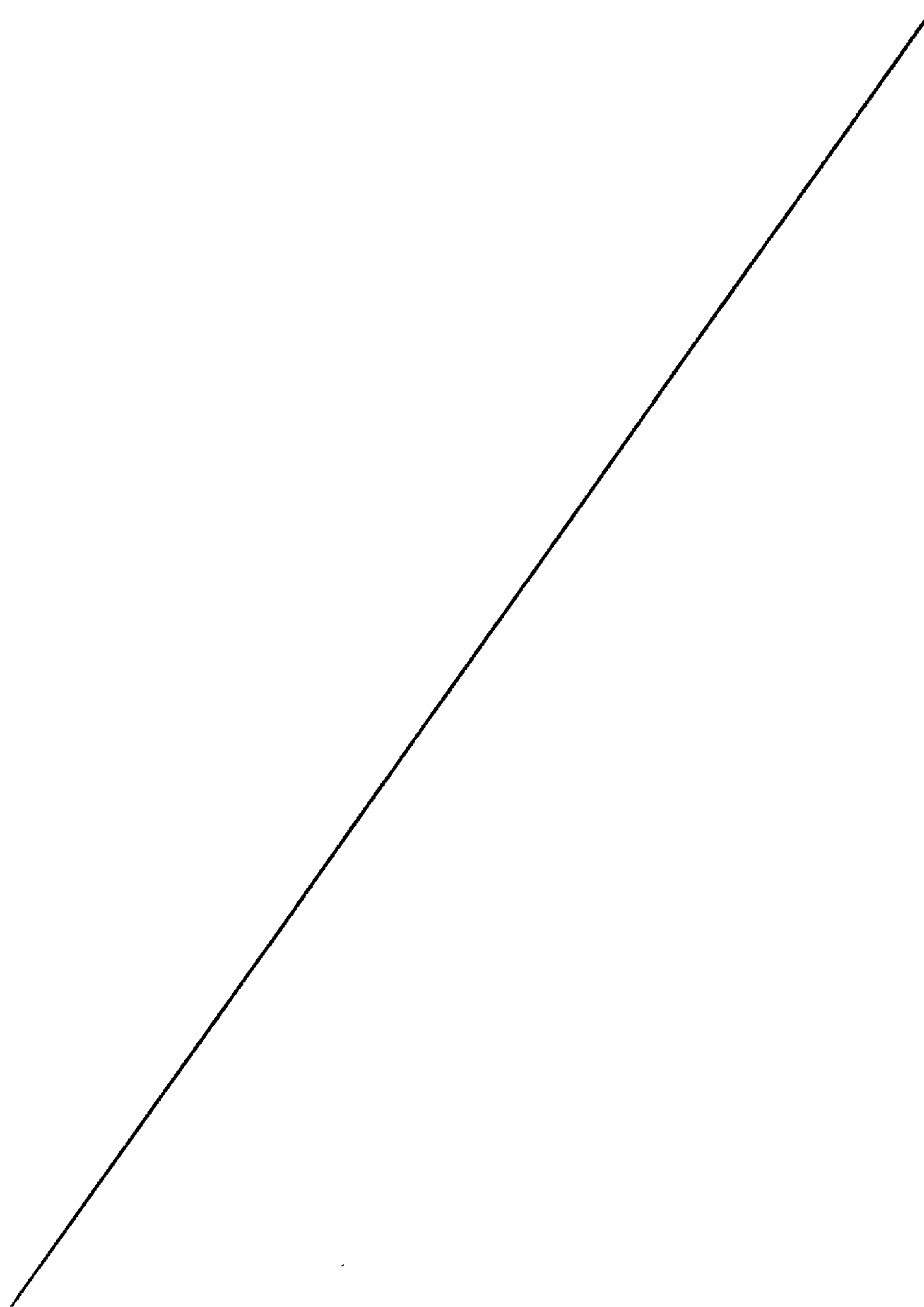
Receptor Nucleotide Sequence. Nucleotide sequence analysis of the 4.2-kb ect1 cDNA revealed a long open reading frame of 2235 nucleotides (nucleotide position 562 to 2796). Two methionine codons were found at nucleotide positions 619 and 676 respectively. The second methionine codon matched the Kozak's consensus for a translational initiator sequence (A/GC-CATGG) (V-15). Moreover, it was followed by a characteristic signal sequence of 21 residues, 10 of which were identical to those of the putative signal peptide of the mouse basic FGF (bFGF) receptor (V-16,17). Thus, the second AGT is the initiation codon. The receptor polypeptide is believed therefore to

comprise 707 amino acids with a predicted size of 82.5-kD (Fig. V-2A).

The amino acid sequence predicted a transmembrane tyrosine kinase closely related to the mouse bFGF receptor (bFGFR). The percent similarity between both proteins is shown in Fig. V-2B. The putative KGFR extracellular portion contained two immunoglobulin (Ig)-like domains, exhibiting 77% and 60% similarity with the Ig-like domains 2 and 3, respectively, of the mouse bFGFR. The sequence NH₂-terminal to the first Ig-like domain of the KGFR is 63 residues long in comparison to 88 residues found in the shorter form of the mouse bFGFR. The mouse bFGFRs contain a stretch of eight consecutive acidic residues between the first and second Ig-like domains (V-16-18). The KGFR lacked such an acidic amino acid domain (Fig. V-2B).

The kinase domain of the KGFR was 90% related to the bFGFR tyrosine kinase (Fig. V-2B). The central core of the catalytic domain was flanked by a relatively long juxtamembrane sequence, and the tyrosine kinase domain was split by a short insert of 14 residues, similar to that observed in mouse, chicken, and human bFGF receptors (V-16-19). Hanafusa and co-workers isolated a partial cDNA for a tyrosine kinase gene, designated *bek*, by bacterial expression

cloning with phosphotyrosine antibodies (V-20). The reported sequence of *bek* was identical to the KGFR in the tyrosine kinase domain (Fig. V-2B).



Method for Determining Primary Structure of
the KGF Receptor.

Figure V-2A is the amino acid sequence deduced for the coding region of the KGF receptor cDNA. Amino acids are numbered from the putative initiation site of translation. Potential sites of N-linked glycosylation are underlined. The potential signal peptide and transmembrane domains are boxed. The interkinase domain is shown by underlined italic letters. Glycine residues considered to be involved in ATP (adenosine triphosphate) binding are indicated by asterisks. Cysteine residues delimit two Ig-like domains in the extracellular portion of the molecule are shown by bold face. Nucleotide sequence was determined by the chain termination method (V-30). Figure V-2B is a structural comparison of the predicted KGF and bFGF receptors. The region used as a probe for Southern and Northern analysis (Fig. V-1B and C) is indicated. The region homologous to the published *bek* sequence (V-20) is also shown. The schematic structure of the KGF receptor is shown below the restriction map of the cDNA clone. Amino acid sequence similarities with the smaller and larger bFGF receptor variants are indicated. S, signal peptide; A, acidic region; IG1, IG2 and IG3, Ig-like domains; TM, transmembrane domain, JM, juxtamembrane domain; TK1 and TK2, tyrosine kinase

domains; IK, interkinase domain; C, COOH-terminus domain.

Competitive Binding Scatchard analysis of ^{125}I -labeled KGF binding to the NIH/ect1 transfectant revealed expression of two similar high-affinity receptor populations. Out of a total of $\sim 3.8 \times 10^5$ sites per cell, 40% displayed a dissociation constant (K_d) of 180 pM and the remaining 60% showed a k_d of 480 pM as demonstrated in Section IV. These values are comparable to the high-affinity KGF binding sites displayed by BALB/MK cells (V-9). The pattern of KGF and FGF competition for ^{125}I -labeled KGF binding to NIH/ect1 cells was also very similar to that observed with BALB/MK cells (Fig. V-3). Although maximum ^{125}I -labeled KGF binding to NIH/ect1 cells was 3.5 times higher than to BALB/MK, there was 50% displacement by 2 ng/ml of either KGF or acidic FGF (aFGF) with each cell type. Similarly, both cells showed 15 times less efficient competition by bFGF for bound ^{125}I -labeled KGF. Thus, the cloned KGFR exhibited the characteristic pattern of KGF and FGF competition displayed by BALB/MK cells, which indicates that the KGFR is a high-affinity receptor for aFGF as well as KGF.

When ^{125}I -labeled KGF is cross-linked to its receptors on BALB/MK cells, two protein species of 162-

and 137-kD have been observed as established in Section IV. Taking into account the size of KGF itself, we have estimated the cross-linked receptors to be around 140- and 115-kD, respectively. When ¹²⁵I-labeled KGF cross-linking was performed with NIH/ect1 cells, a single species corresponding in size to the smaller, 137-kD complex in BALB/MK cells (Fig. V-4A) is observed. Moreover, detection of this band was specifically and efficiently blocked by unlabeled KGF. When glycosylation is considered, the size of the KGFR predicted by sequence analysis corresponds reasonably well with the corrected size (115 kD) of the cross-linked KGFR in the ect1 transfectant.

To examine the functional nature of the KGFR expressed in NIH/ect1 cells, its capacity to induce tyrosine phosphorylation of cellular proteins was investigated. NIH/3T3 or NIH/ect1 cells were exposed to KGF for 10 min and cell lysates were subjected to immunoprecipitation and immunoblotting analysis with antibody to phosphotyrosine (anti-Ptyr). NIH/ect1 cells contained several tyrosine-phosphorylated proteins that were not detectable in control or KGF-stimulated NIH/3T3 cells (Fig. V-4B). Addition of KGF to NIH/ect1 cells resulted in the detection or increased tyrosine phosphorylation of several putative substrates. These included p55, p65, p90, p115, p150

and p190. These findings established that the KGFR was enzymatically activated in response to KGF. In Section IV, it was noted that similar-size proteins are phosphorylated in response to KGF triggering of BALB/MK cells. Moreover, the 115-kD phosphoprotein matched the corrected size of the KGFR cross-linked by ^{125}I -labeled KGF.

Method for Analysis of the KGF Receptor
Expressed in NIH/3T3 Cells.

Figure V-4A shows covalent affinity cross-linking of ^{125}I -labeled KGF to BALB/MK, NIH/3T3, and NIH/ect1 cultures. The left and center panels of this autoradiogram were exposed to Kodak XAR^{*} film for 72 hours at -70°C ; the right lane is an 18-hour exposure of the same autoradiogram. The second lane (labeled +) for each cell type shows cross-linking performed in the presence of excess unlabeled KGF. Molecular weight markers ($\times 10^3$) are indicated on the left; the positions of ^{125}I -labelled KGF-cross-linked complexes are indicated by arrows. Cross-linking was carried out as described previously in Section IV.

Figure V-4B shows autoradiogram of phosphotyrosyl-proteins from intact NIH/3T3 and NIH/ect1 cells before and after treatment with KGF. Molecular weight markers are indicated on the left; the estimated molecular weights of proteins displaying KGF-
* trade mark

stimulated phosphorylation on tyrosine are shown at right. Analysis of phosphoproteins was performed as described previously in Section IV.

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V-30

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For the purposes of completion, the background description and present disclosure, each of the published articles, patents and patent applications heretofore identified in this specification are hereby
5 incorporated by reference into the specification.

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various combinations in form and detail can be made without departing from the
10 scope of the invention.

Table I-1. Growth Factor Purification

Purification step	Protein (mg)	Total activity (units) ^a	Specific activity (units/mg)
5 Conditioned medium (10 liters)	1.4×10^{3a}	2.5×10^4	1.8×10^1
Ultrafiltration (retentate)	1.3×10^{3a}	3.2×10^4	2.5×10^1
NSAC 0.6 M NaCl pool	0.73^b	1.6×10^4	2.2×10^4
TSK-G3000 SW	8.4×10^{-3b}	2.7×10^3	3.2×10^5
10 C ₄ -HPLC	6.1×10^{-3b}	2.1×10^2	3.4×10^4

Recoveries were calculated by assuming that all of the mitogenic activity in the starting material was due to the isolated factor.

15 ^a One unit of activity is defined as half of the maximal stimulation of thymidine incorporation induced by TSK-purified factor in the Balb/MK bioassay, in which approximately 3 ng of the TSK-purified factor stimulated 1 unit of activity.

20

^a Protein was estimated by using the Bradford reagent from BioRad (I-23).

^b Protein was estimated by using $A_{214}^{1\%} = 140$.

Table I-2. Target Cell Specificity of Growth Factors

Growth Factor	<u>Epithelial</u>			<u>Fibroblast</u>	<u>Endothelial</u>
	BALB/MK	BS/589	CCL208	NIH/3T3S	Human saphenous vein
KGF	500-1000	2-3	5-10	<1	<1
EGF	100-200	20-40	10-30	10-20	n.d.
TGF α	150-300	n.d.	n.d.	10-20	n.d.
aFGF*	300-500	2-3	5-10	50-70	5
bFGF	100-200	2-3	2-5	50-70	5

Comparison of maximal thymidine incorporation stimulated by KGF and other growth factors in a variety of cell lines, expressed as fold stimulation over background.

This data represents a summary of four different experiments.

*Maximal stimulation by aFGF required the presence of heparin (Sigma), 20 μ g/ml.

n.d. = not determined.

TABLE II-1. Effect of Heparin on KGF Mitogenic Activity.

Growth Factor	<u>BALB/MK</u>		<u>NIH/3T3</u>	
	+	-	+	-
KGF	150	9.5	<1	<1
aFGF	106	259	10.4	68
bFGF	30	124	45.7	70

Cells were plated in microtiter plates, grown to confluence in serum containing media and then placed in a serum-free medium for 24-72 hr prior to sample addition. Mitogenesis assays were performed as described (see Experimental Section I, above and II-3). Where indicated, heparin was included in the culture media at a final concentration of 20 μ g/ml. The concentration of all growth factors was 50 ng/ml. The results represent fold stimulation of ^{3} H-thymidine incorporation in the indicated assay cell in the presence (+) or absence (-) of heparin. Each value represents the mean result from two independent experiments in which each point, in turn, represents the mean value of duplicate analyses.

CLAIMS:

1. A chimeric protein which comprises within a single polypeptide molecule a segment of KGF protein, wherein
5 the segment of KGF protein is a segment of amino acids of Figure 11-1B which comprises a sufficient number of consecutive amino acids 32 - 189 of Figure II-1B to confer on the polypeptide molecule preferential mitogenic activity for an epithelial cell, and at least one other
10 polypeptide portion of the fibroblast growth factor family.
2. The chimeric protein of claim 1, wherein the fibroblast growth factor is acidic fibroblast growth
15 factor (aFGF).
3. The chimeric protein of claim 1, wherein the polypeptide portion of aFGF is defined by amino acid residues in that portion of aFGF that is homologous to
20 the KGF sequence responsible for heparin sensitivity of KGF.
4. The chimeric protein according to claim 2, wherein said segment of KGF protein is defined by amino acid
25 residues numbered 32 through 78 in Figure II-1B and said polypeptide portion of aFGF is defined by the carboxyl terminus of aFGF, beginning at residue number 39.
5. A pharmaceutical composition comprising a chimeric
30 protein according to any one of claims 1 to 4, and a pharmaceutically acceptable carrier.

6. Use of a chimeric protein according to any one of claims 1 to 4 in a composition for the treatment of conditions requiring specific stimulation of epithelial cells in the presence of heparin.

5

7. Use of a chimeric protein according to any one of claims 1 to 4 for preparing a medicament for acceleration or improvement of wound healing involving the epidermis.

10 8. Use of a chimeric protein according to any one of claims 1 to 4 for preparing a medicament that is to be topically applied to the skin or eye.

9. An antibody specifically directed against
 15 glycosylated or unglycosylated keratinocyte growth factor (KGF) polypeptide selected from the group consisting of (a) the following amino acid sequence, (b) a portion of the following amino acid sequence without the N-terminal 31 amino acids, and (c) an amino acid sequence
 20 that differs by the addition, deletion, or substitution of one or more amino acids from the following amino acid sequence:

	M	H	K	W	I	L	T	W	I	L	P	T	L	L	Y
	R	S	C	F	H	I	I	C	L	V	G	T	I	S	L
25	A	C	N	D	M	T	P	E	Q	M	A	T	N	V	N
	C	S	S	P	E	R	H	T	R	S	Y	D	Y	M	E
	G	G	D	I	R	V	R	R	L	F	C	R	T	Q	W
	Y	L	R	I	D	K	R	G	K	V	K	G	T	Q	E
	M	K	N	N	Y	N	I	M	E	I	R	T	V	A	V
30	G	I	V	A	I	K	G	V	E	S	E	F	Y	L	A
	M	N	K	E	G	K	L	Y	A	K	K	E	C	N	E
	D	C	N	F	K	E	L	I	L	E	N	H	Y	N	T

Y A S A K W T H N G G E M F V
A L N Q K G I P V R G K K T K
K E Q K T A H F L P M A I T

5 10. The antibody of claim 9 which neutralizes the
mitogenic activity of human KGF.

11. The antibody of claim 9 which is a polyclonal
antibody.

10

12. The antibody of claim 9 which is a monoclonal
antibody.

13. The antibody of claim 9 which reacts against the
15 peptide NDMTPEQMATNVR.

14. A pharmaceutical composition comprising at least one
antibody of claim 9 and a pharmaceutically acceptable
carrier.

20

15. Use of the antibody of any one of claims 9 to 13 for
preparing a medicament for treating conditions requiring
specific inhibition of stimulation of epithelial cells by
KGF.

25

16. A recombinant DNS molecule which encodes a chimeric
protein according to claim 1.

17. A recombinant DNA molecule which encodes a chimeric
30 protein according to claim 2.

18. A recombinant DNA molecule which encodes a chimeric protein according to claim 3.

19. A recombinant DNA molecule which encodes a chimeric
5 protein according to claim 4.

20. A method for detecting a KGF protein in a sample comprising:

- i) reacting polypeptides from body fluids or
10 tissue samples with an antibody of claim 9 to form an antibody-polypeptide complex; and
- ii) detecting said complex.

FIG. I-1

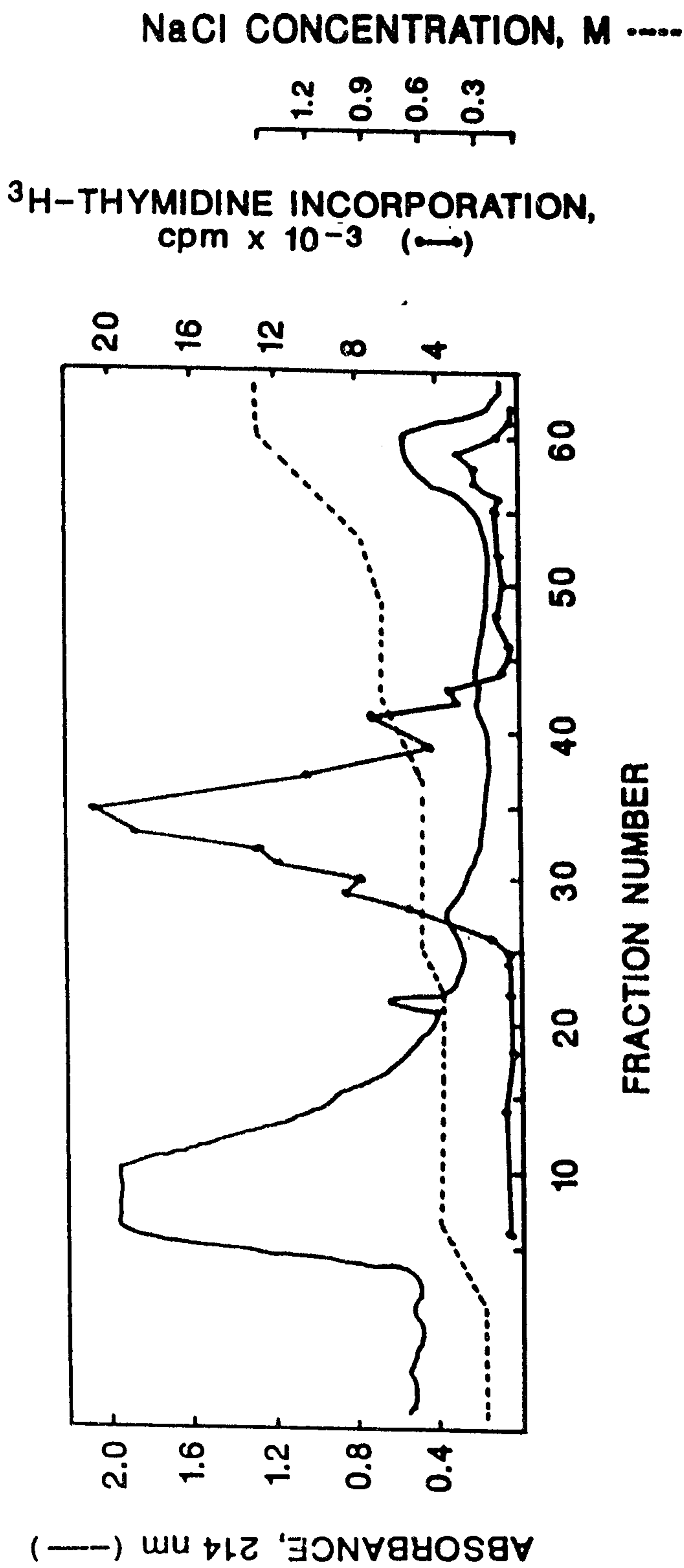


FIG. 1-2A

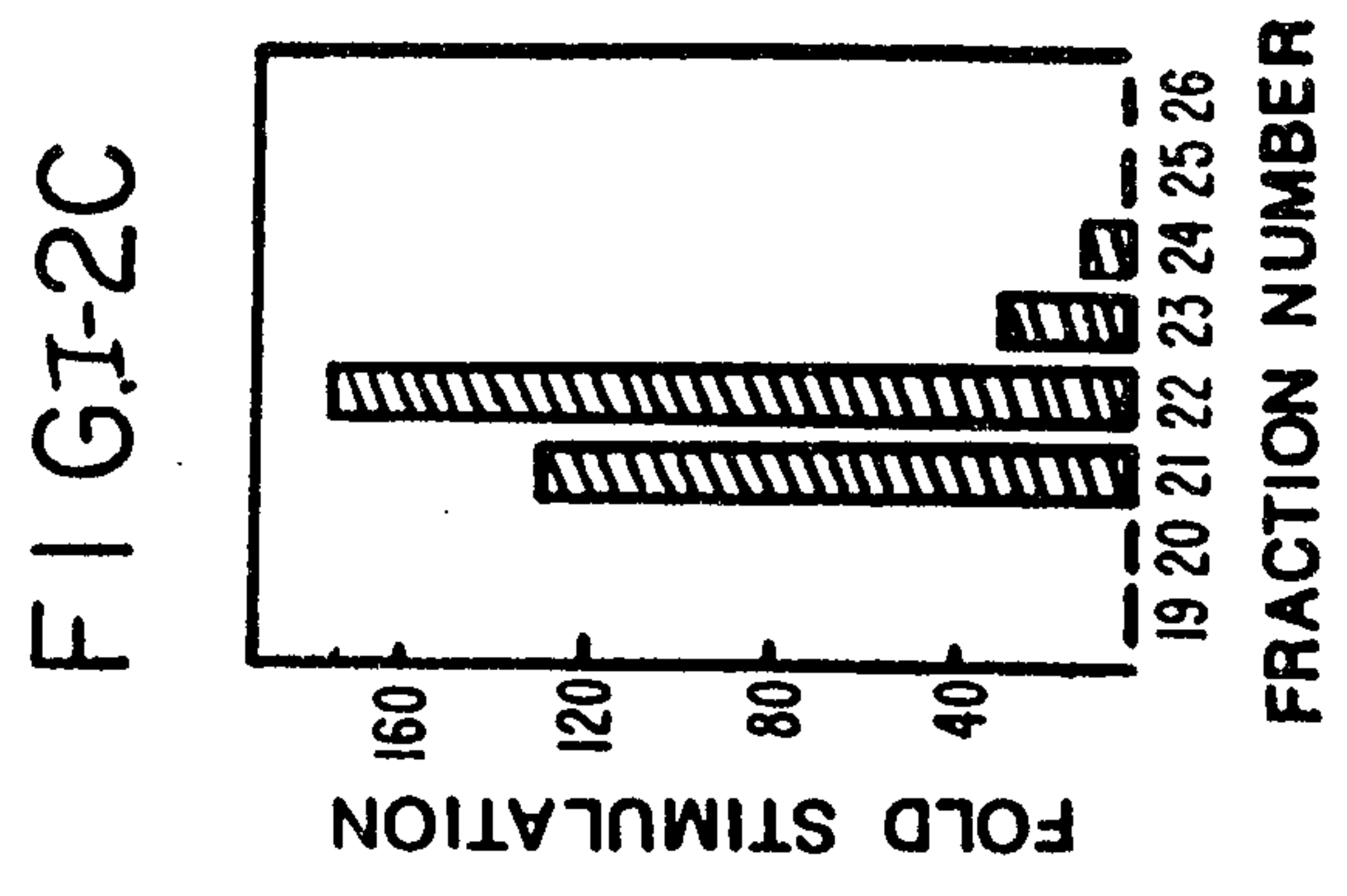
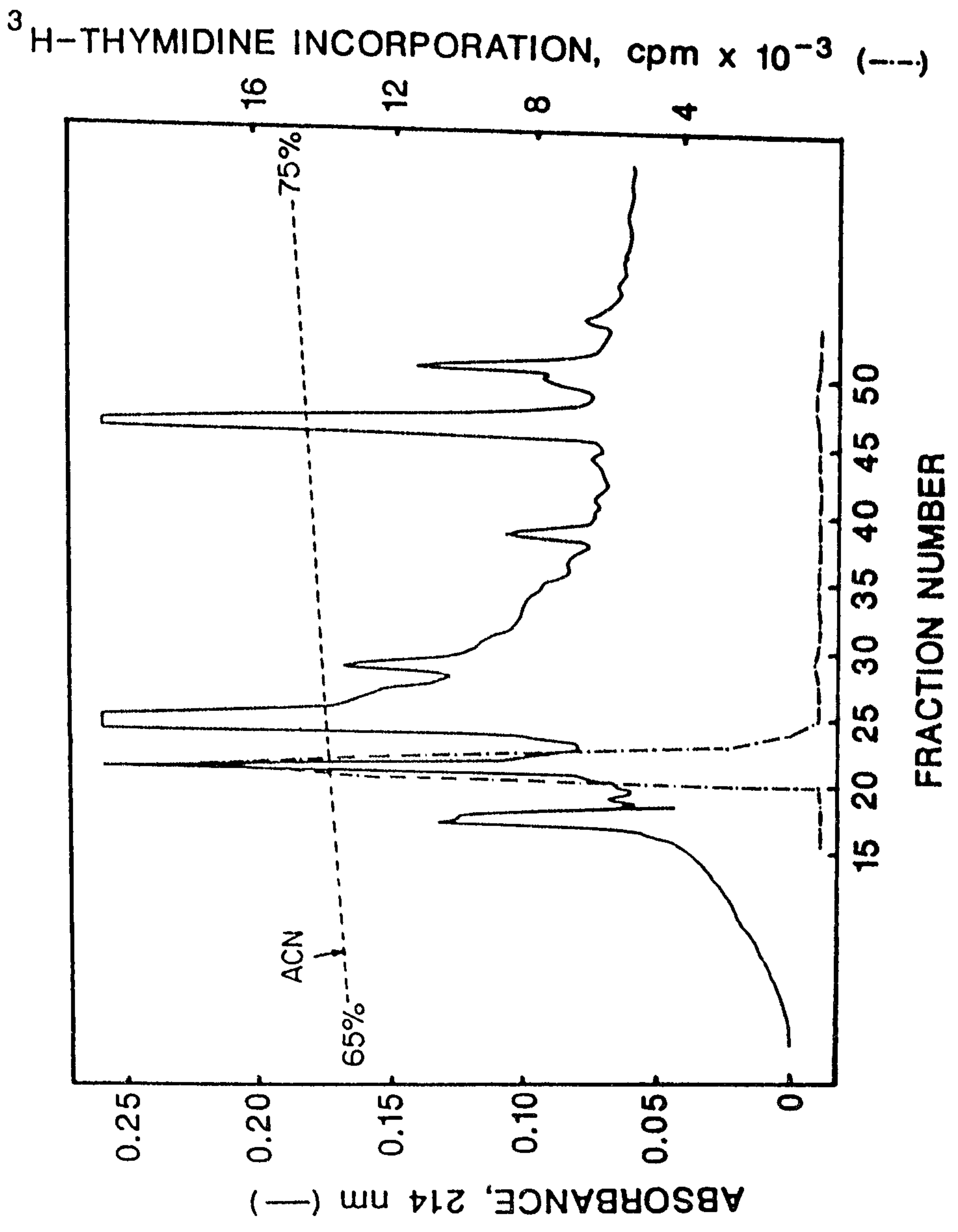
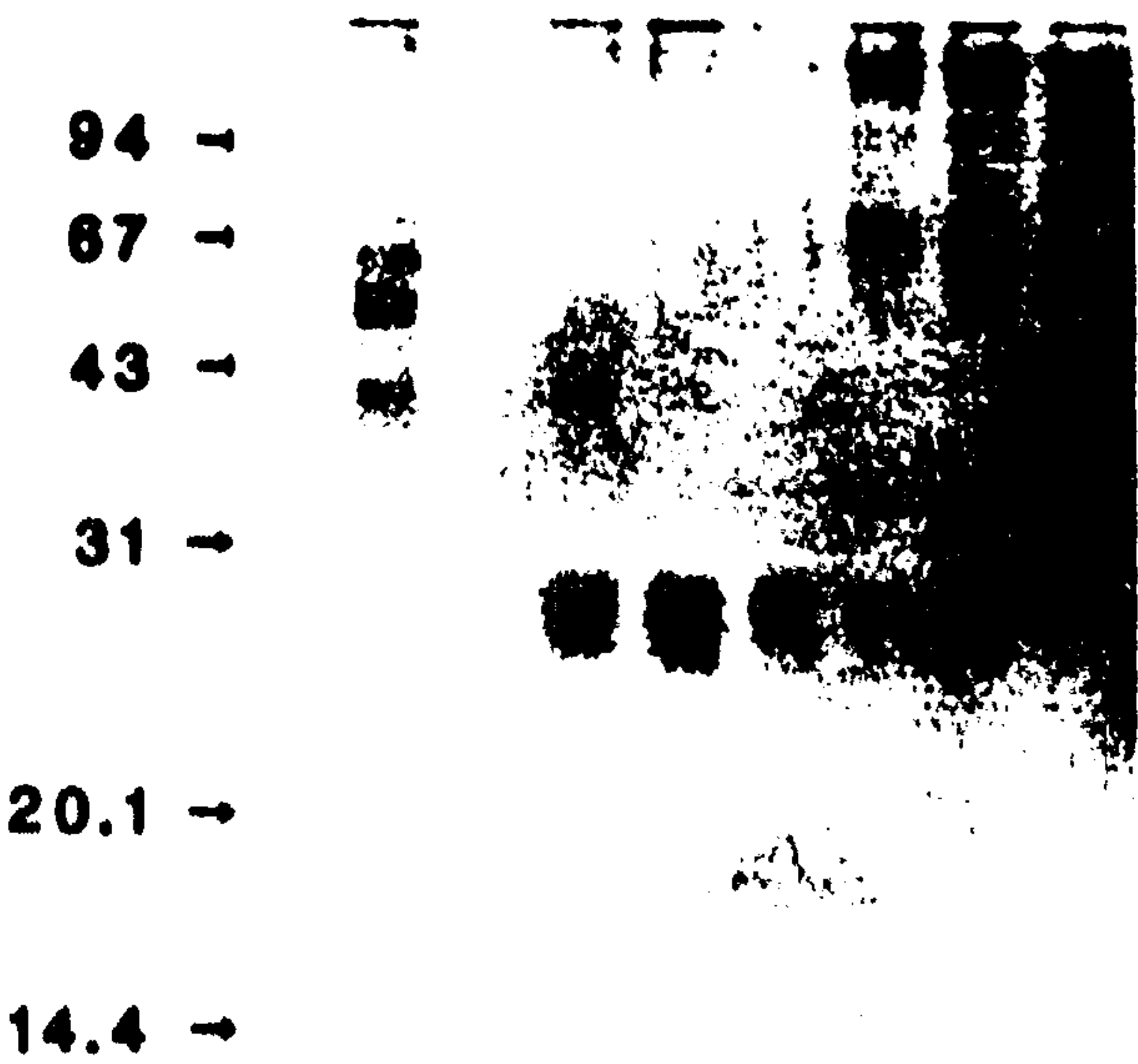
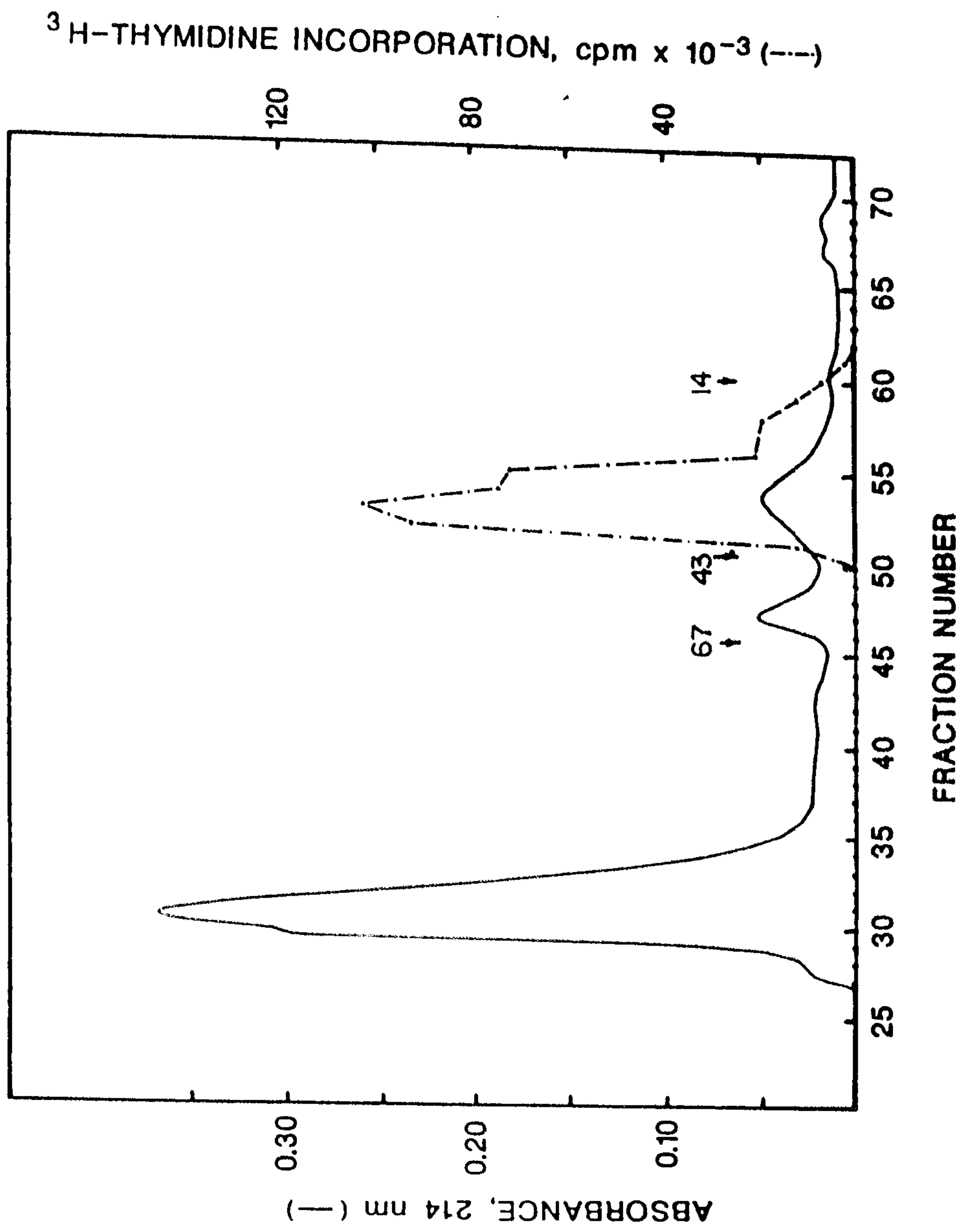


FIG. 2B



19 20 21 22 23 24 25 26
FRACTION NUMBER



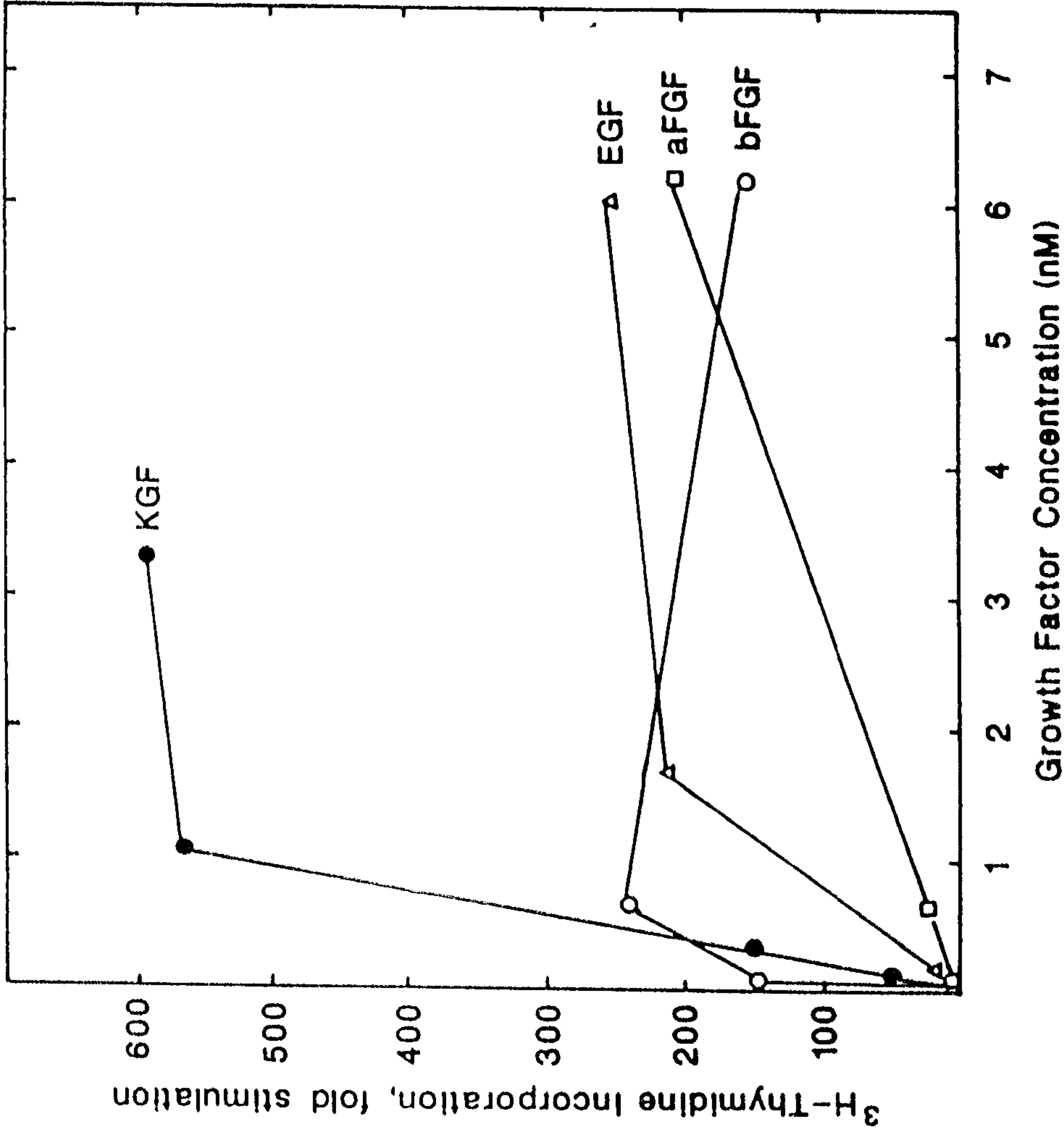


FIG 14

Sim & McBurney

Sim: H. Burroughs

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FIG 5

FIG 5(a) FIG 5(b) FIG 5(c) FIG 5(d)

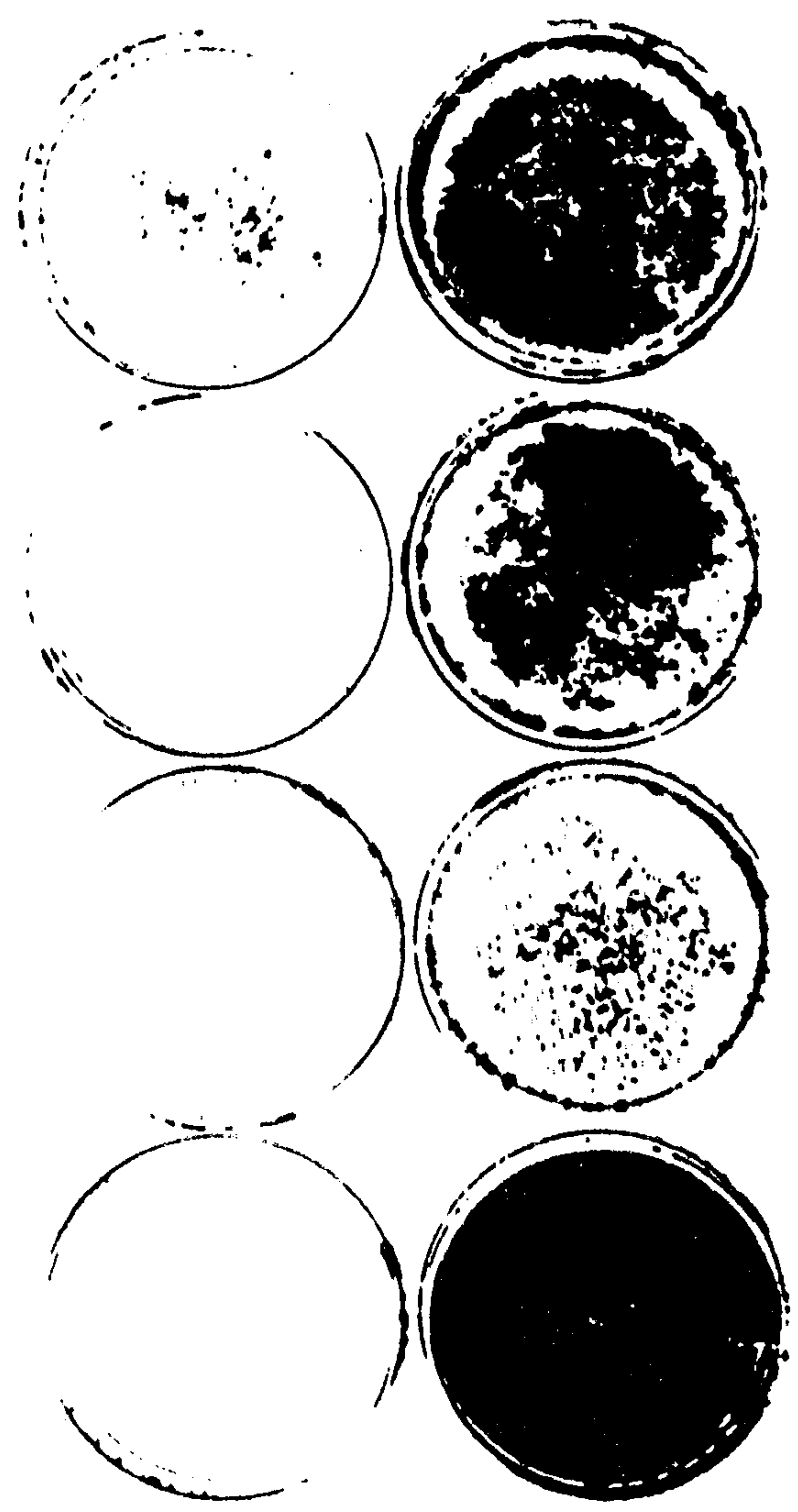


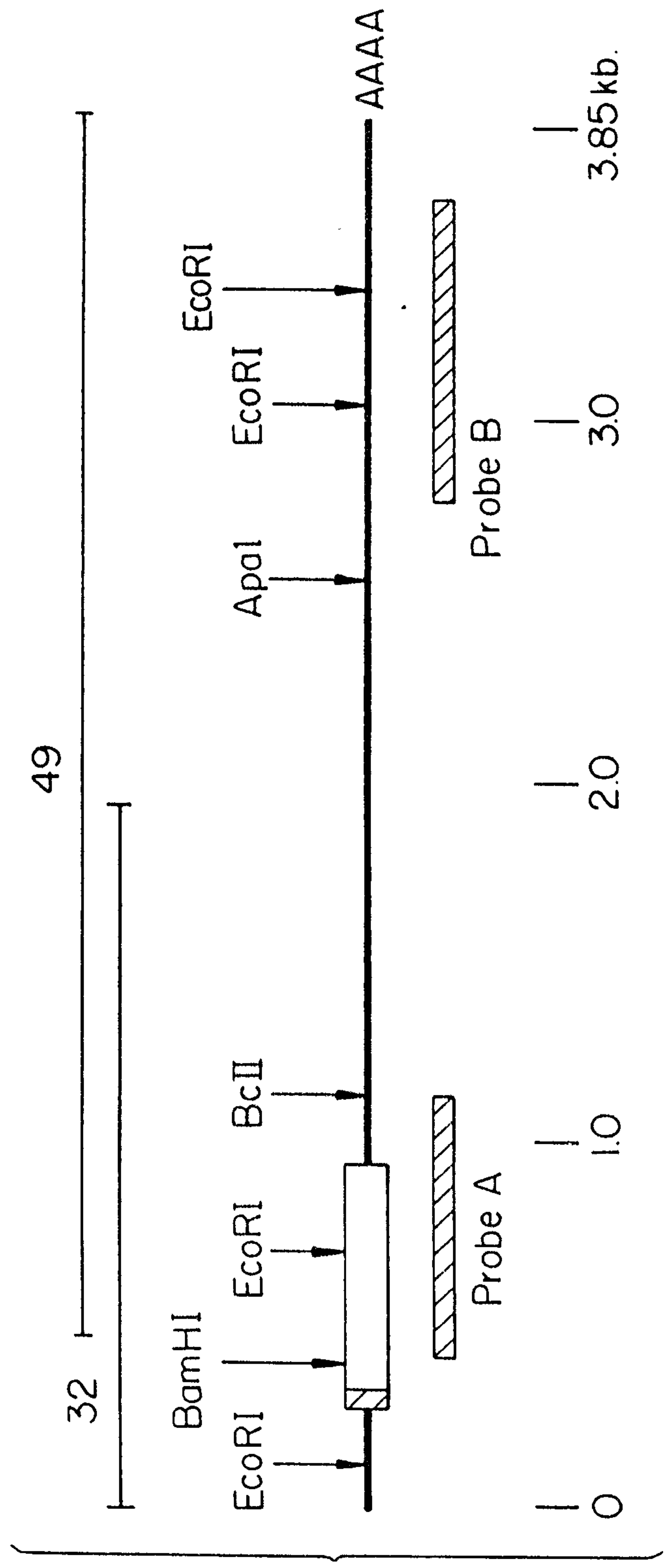
FIG 5(e) FIG 5(f) FIG 5(g) FIG 5(h)

Sim: H. Burroughs

Gen. & M. Bureau

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FIG. IV-14



Gen. & M. Bureau

II-13(1)

FIG.

FIG. 1B(2)

1 ACGCGCTCACACACAGAGAGAAAATCCTTCTGCCTGTTG
121 GCACCAGGCAGACAAACAGACATGGAATTCTTATATATCC
241 TTATCAACAGAGTTATTTAAGGAGGAATCCTGTGTTGTT

361 AAGAGGTCAATGACCTAGGAGTAACAATCAACTCAAGAT

481 ^{L L Y R S C F H I I C L V}
TTTGCTCTACAGATCATGCTTTCACATTATCTGTCTAGT

601 ^{T R S Y D Y M E G G D I R}
CACAAGAAGTTATGATTACATGGAAGGAGGGGATATAAG

721 ^{N N Y N I M E I R T V A V}
GAATAATTACAATATCATGGAAATCAGGACAGTGGCAGT

841 ^{C N E D C N F K E L I L E}
ATGCAATGAAGATTGTAACCTTCAAAGAACTAATTCTGGA

961 ^{P V R G K K T K K E Q K T}
TCCTGTAAGAGGAAAAAAACGAAGAAAGAACAAAAAAC

1081 TGGACTGTTTTTCTTTCTTCTCAAAATTTTTCTTTTCCTTTTT
1201 ACACCTGCATTAAAGAAAGATTTGAAAAGTATACACAAAAA
1321 TAAATTAAATTTACCCCTTAAGAGTATGTTAGATTTTGATTA
1441 GGTATATCAGACCTACAGGCTTCTGGCAGGATTTTGTCAG
1561 AATCAGAAAAAAATTTCTCAAAAAAACTATTATGAAAGT
1681 TCAAGTGGAAAGGGTATTGCTAAAAGGATGTTTTCCAAAA
1801 CCTCAAAGTAAAATTGAGAAAATCTTTTAAGTTTTTTTCAA
1921 TTCCTATGGTTACAGCATTAACCTCTATTTTAAGTTGTT
2041 TTTTAATTTTAAAGGAATAACAAAACCTGTCTGGCTCAAC
2161 ATAAGAGCCTGAAGCAATGCTTACAATAGATGTCTCACA
2281 ATATAAGTATTTACAGGATTTTAAAGTTAGAATATATTT
2401 TGTTCAAAAGGTGGCAGCACTGAAAGTTGTTTTCTGTT
2521 CCTACAGATAACAGGATTATTACAAGGATGAATTTCCAC
2641 GTATGCTAACCCTGTGGTTTTTAATTTCAAAATATTTGT
2761 CAATAGATTCAATTTAATTTTCTGTGGTTGACCTATACG
2881 CACCTGATTCAAGGACTTTGCTAGCTAGGTTTTTGAGGTC
3001 GCAGACTATCTGTTTCATAATCAGTTTTTCAGTGTGAATTC
3121 TTAAATAGAAATAGTGTATATACATATAAAATACAAGCT
3241 ATTTAGTGGTAAATCCATTCTGTTAGTATAAGTCACCT
3361 AAATTTGCTCTAGTTACACACCTTTAGAATTCTAGAATA
3481 GCTGGGTAGATATACAGCTGTACACAAGAGTCTAGATCAG
3601 AGATATAGCCTTTTACATTTGTACACAAATGTGACTATGT
3721 TCAATTCTGATTCTTATTACCTTTTTGTTTATGAATGGA
3841 TCTAACAATTAGAAAAAAACAAAAAAACAAAAAAAC

FIG. 1B(2)

II- 1B(2)

FIG.-

FIG. 1B(1)

FIG. 1B(3)

ATTTATGGAAACAATTATGATTCTGCTGGAGAACTTTT
AGCTGTTAGCAACAAAACAAAAGTCAAATAGCAAACAG
ATCAGGAACTAAAAGGATAAGGCTAACAAATTTGGAAAG

TCATTTTTCATTATGTTATTTCATGAACACCCGGAGCACT

30
G T I S L A C N D M T P
GGGTACTATATCTTTAGCTTGCAATGACATGACTCCAG

70
V R R L F C R T Q W Y L
AGTGAGAAGACTCTTCTGTCTGAACACAGTGGTACCTGA

110
G I V A I K G V E S E F
TGGAATTGTGGCAATCAAAGGGGTGGAAAGTGAATTCT

150
N H Y N T Y A S A K W T
AAACCATTACAACACATATGCATCAGCTAAATGGACAC

190
A H F L P M A I T
AGCCCACTTTCTTCCTATGGCAATAACTTAAATGCATA

ATTTTTTTAGTAATCAAGAAAGGCTGGAAAAAACTACTGA
ATCAGATTTTAGTAACATAAAGGTTGTAAAAAATTGTAAA
TCTGATAATGATTATTTTAAATATTTCTCTATCTGCTTATA
ATAATCAAGCCACACTAACTATGGAAAAATGAGCAGCAT
CAATAAAAATAGATAATTTTAAACAAAAGTACAGGATTAGA
ATCTTGTATATAAGATAGCAACAGTGATTGATGATAAT
GTAACATAATCTATCTTTTGTATAATTCATATTTTGGGAA
TTTGAACCTTTATTGTTTGTATTATTAAAGTTTATGTTAT
GGCAAGTTTCCCTCCCTTTTCTGACTGACACTAAGTCT
CAGAACAAATACAAATATGTAAAAAACTCTTTTCACCACAT
GAATGCATGGGTAGAAAAATATCATATTTTAAAACTATG
AGATGGCAAGAGCACAATGCCCAAAATAGAAGATGCAG
TTCAAAAAGTCTTTTCATTGGCAGATCTTGGTAGCACTTT
CATTCAAGTCCCTTTACATAAAATAGTATTTTGGTAATAC
ACCAGGATGTAGAAAACTAGAAAGAAGTGCCTTTCCTC
AGGCTTTCAGTAACCTGTAGTCTTGTGAGCATATTGAGGG
ACTGAATGTTTATAGACAAAAGAAAAATACACACTAAAA
ATGTTAGGACCAAAATGCTCTTTGTCTATGGAGTTATAC
AAAAAAGACTTCTAGAAATATGTACTTTAATTATTTGT
TTAAAACTGTAAGGGGCCCTCCATCCCTCTTACTCATTT
TTAGCACATGCTTTTCTACTCTTCGATTATTAGTATTAT
CTTGGCAATGCACTTCATACACAATGACTAATCTATAC
AAGCTTTGTGCAAAATATACATATAAGCAGAGTAAGCC

FIG. 1B(1)

FIG. 1B(3)

II-1B(3)

FIG.

FIG. 1B(2)

CAGCTGAGAAATAGTTTGTAGCTACAGTAGAAAGGCTCAAGTT
 CGTCACAGCAACTGAACTTACTACGAACTGTTTTTTATGAGGAT
 AGCAAGTACTCTTTCTTAAATCAATCTACAATTACAGATAGG

ACACTATAATGCACAAATGGATACTGACATGGATCCTGCCAAC¹⁰

E Q M A T N V N C S S P E R H^{40 50}
 AGCAAATGGCTACAAATGTGAACTGTTCCAGCCCTGAGCGACA

R I D K R G K V K G T Q E M K^{80 90}
 GGATCGATAAAAGAGGGCAAAGTAAAAGGGACCCAAGAGATGAA

Y L A M N K E G K L Y A K K E^{120 130}
 ATCTTGCAATGAACAAGGAAGGAAAACCTCTATGCAAAGAAAGA

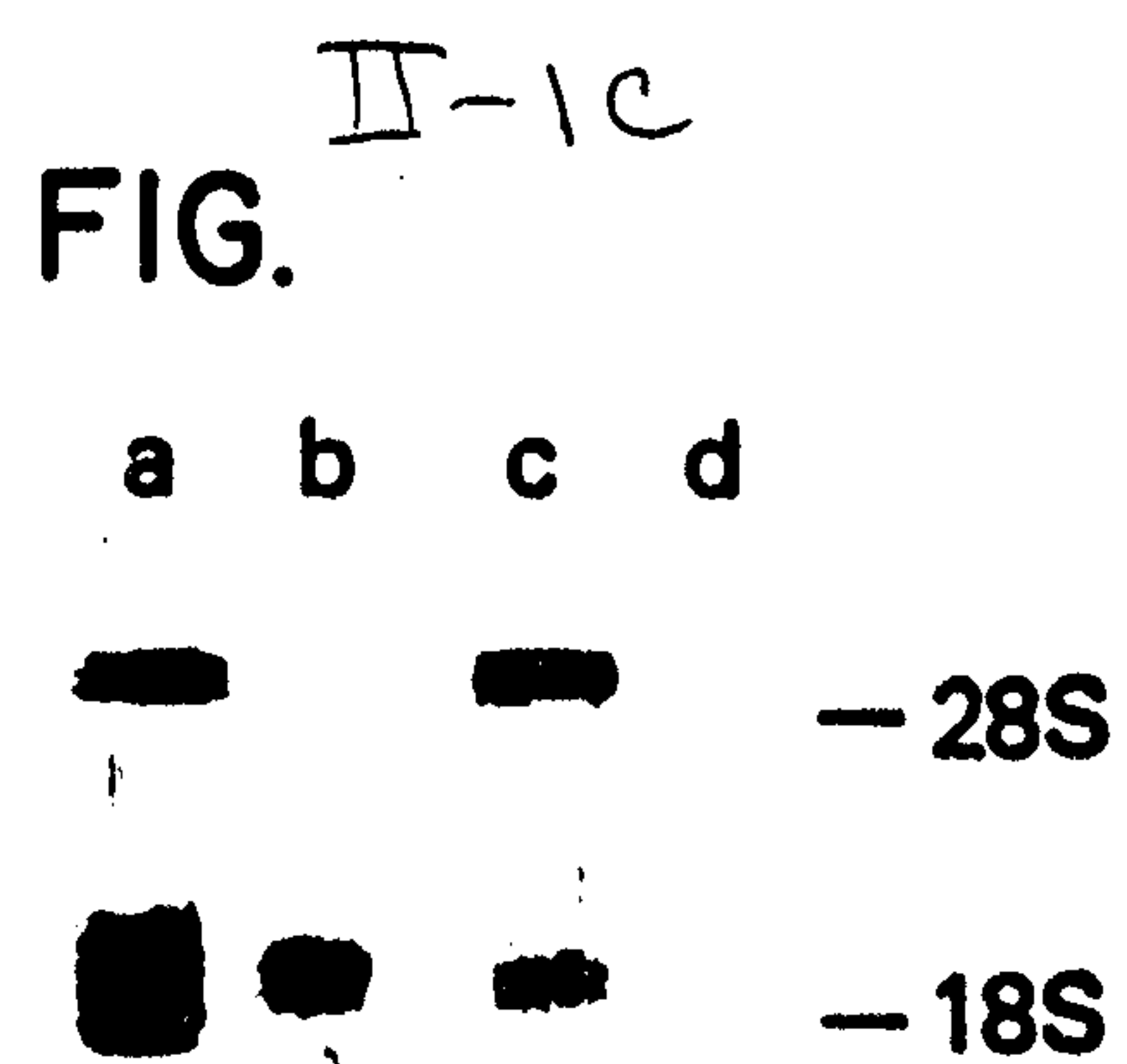
H N G G E M F V A L N Q K G I^{160 170}
 ACAACGGAGGGGAAATGTTTGTGCTTAAATCAAAGGGGGAT

TGGTATATAAAGAACCCAGTTCCAGCAGGGAGATTTCTTTAAG

AAAACCTGATCAAGCTGGACTTTGTGCATTTTATGTTTTGTTTTTAAG
 ACTGGTTGTACAATCATGATGTTAGTAACAGTAATTTTTTTTCT
 AAATGGCTGCTATAATAATAATAATACAGATGTTGTTATATAA
 TTTAAATGCTTTTCTAGTGAAAAATTATAATCTACTTAAACTCT
 ACATGCTTATACCTATAAATAAGAACAATAATTTCTAATGCTGC
 ACTGTACTTCATCTTACTTGGCCACAAAAATAACATTTTATAAAT
 TATGGCTTTTATAATAATGTTCTTCCCACAAAAATAATCATGCTTTT
 TTATAAAAAAATAACCTTAATAAGCTGTATCTGTTTTCATATGC
 AGCACACAGCACTTGGGCCCAGCAAATCCTGGAAGCAGACAAAAA
 ATTCTTGCCCAATTAAATTGGATCATATAAGTAAAATCATTACAA
 TATATTTAAATTTAGTAATTTTCTAATCTCTAGAAATCTCTGC
 TTAAGAATAAGGGGCCCTGAATGTCATGAAGGCTTGAGGTCAG
 ATATGTTTACCAATGGGAGGTCAATATTTATCTAATTTAAAAG
 ATTTATAGATGAGAGTTATATGAAAAGGCTAGGTCAACAAAAA
 AGATATACTCTTGGGAGAGAGCATGAATGGTATTCTGAACCTAT
 CAGAGGAGGACTTAGTTTTTTCATATGTGTTTTCTTAGTGCCCTA
 CTAATCTTCAATTTTAAAAGGGTAAAACATGACTATACAGAAAT
 TTCCATCAAATTACATAGCAATGCTGAATTAGGCAAAACCAAC
 TTTTCTCCTATTTTTTAAATTTATTATGCAAATTTTAGAAAAA
 GTAGTCTAGGAATTGAGATTTTGTATACACCTAAGGTCACGCA
 TAGCTAATGGTCTTTGGCATGTTTTTTGTTTTTTTATTTCTGTTG
 TGTGATGATTTGACTCAAAAGGAGAAAAGAAATTATGTAGTTT
 TTTTAAAAATGTTCTTTGAAAGATAAATTAATAACATGAGTT

FIG. 1B(2)

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GAATTGGGAAGCGGCCACAGCAGCAGCTGCTGAGCC
-1
120
ATGGCTGAAGGGGAATCACCACCTTCACAGCCCTGACCGAGAGGTTTAACTCCCTCCAGGGATACAGAGGCCCAACTCCCTCTAGCTAGCAACGGGGCCACTTCTGAGGATC
M A E G E I T T F T A L T E K F N L P P G H Y K K P K L L Y C S N G G H F L R I
240
CTTCGGATGGCACAGTGGATGGGACAGGGACAGGAGCGACAGCACAATTCAGCTGCAGCTCAGTCCGGAAGCGTGGGGAGGTGTATATAAGAGTACCGAGACTGGCCAGTACTTG
L P D G T Y D G T R D R S D Q H I Q L S A E S Y G E Y Y I K S T E T G Q Y L
360
GCCATGGACACCGAGGGCTTTTATACGGCTCACAGACACCAATGAGGAATGTTTCTGGGAAGGCYGGAGGAGAACCATTAACACCTATATATCCAGAGCATGCCAGAGAG
A M D T D G L L Y G S Q T P W E E C L F L E R L E E N H Y N T Y I S K H A E K
480
AATYGGTTTGTGGCTCAAGAGAAATGGGAGCTGGCAACGCGGCTCGGACTCAGTAAGCCAGAGCAATCTTGTTCCTCCCTCCAGTCTCTCTGATTAAAGAGATCTGTTT
M W F Y G L K K M G S C K R G P R T M Y G Q K A I L F L P L P V S S D
600
YGGTGTGACCACATCCAGAGAAGTTTCAGGGGGTCTTCACCTGGTTGACCCCAAAATGTCCCTTGACCATYGGCTGCCYACCCGAGCCACAGAGCCTGAATTTGTAAAGCAACTT

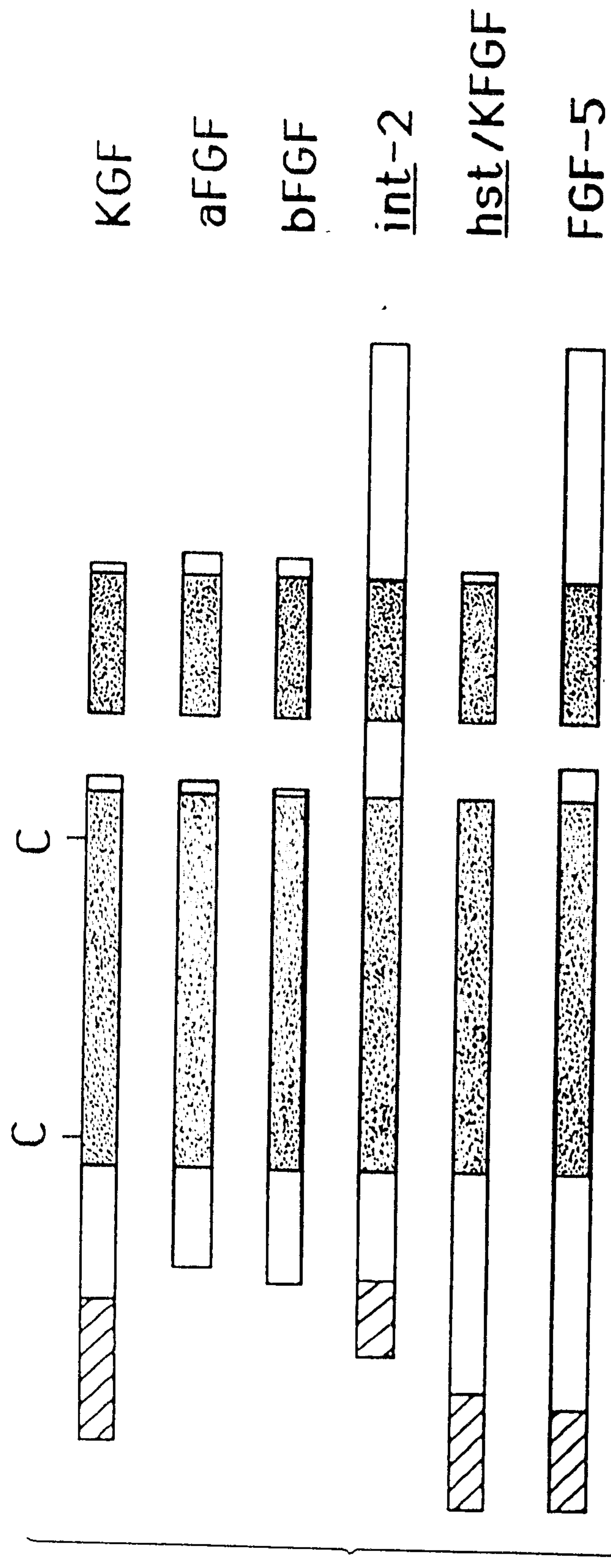
FIG. II-1D

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Scott H. Gunning

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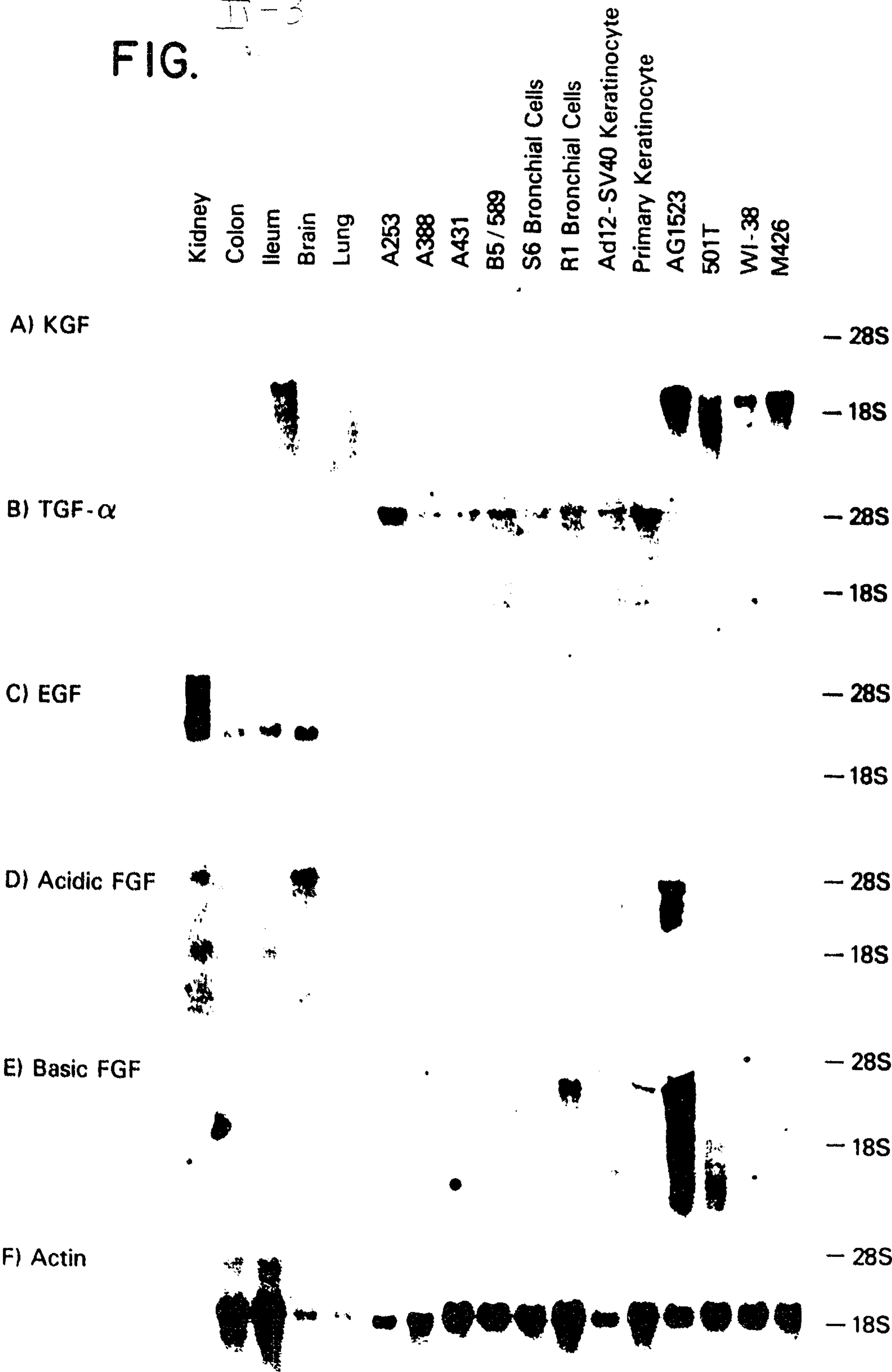
II-2
FIG.



— 20 aa.

Scott H. Gunning

FIG. ^{IV-3}



A FIG II-4A

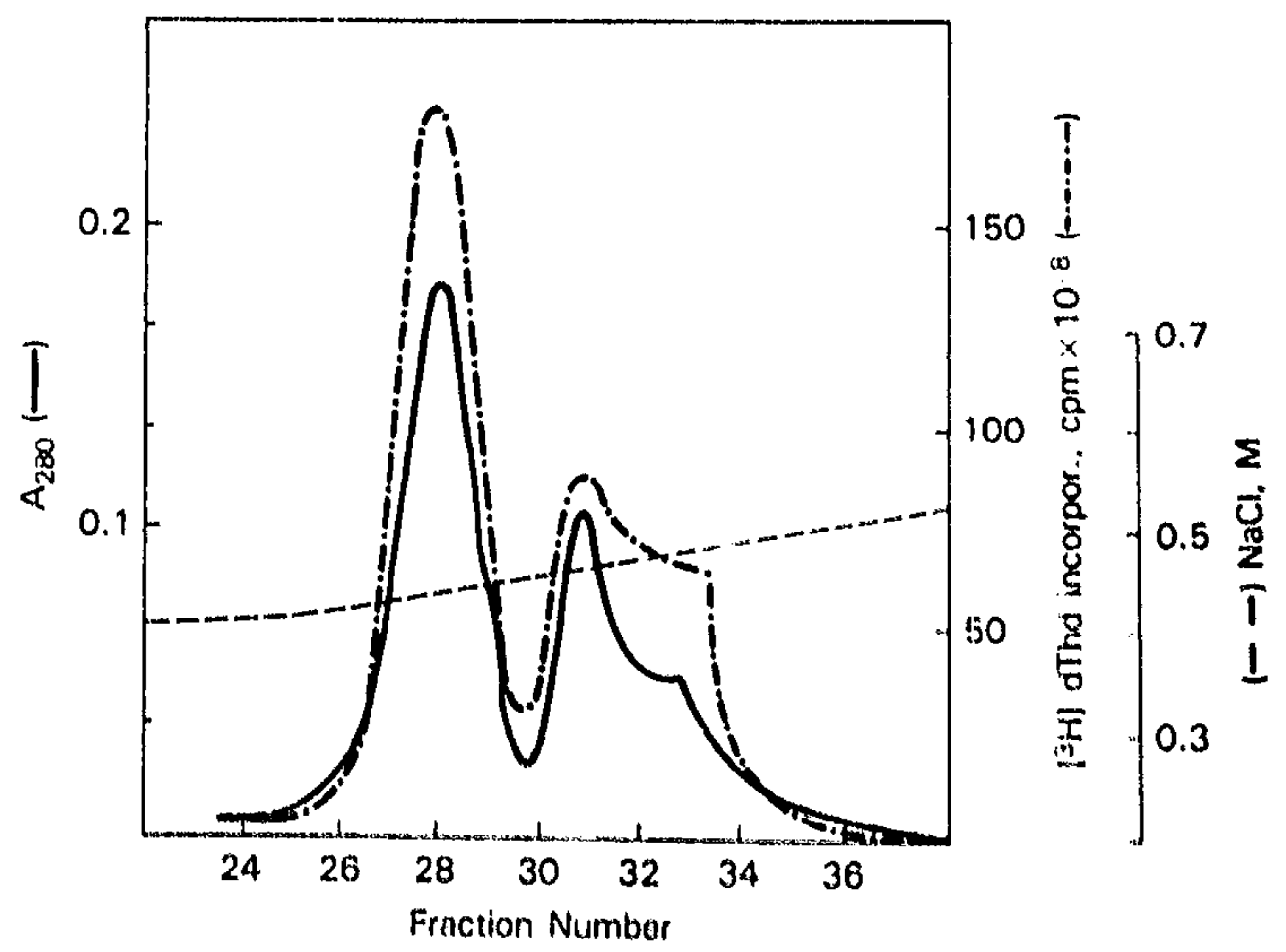


FIG. II-4B



FIG II-4C

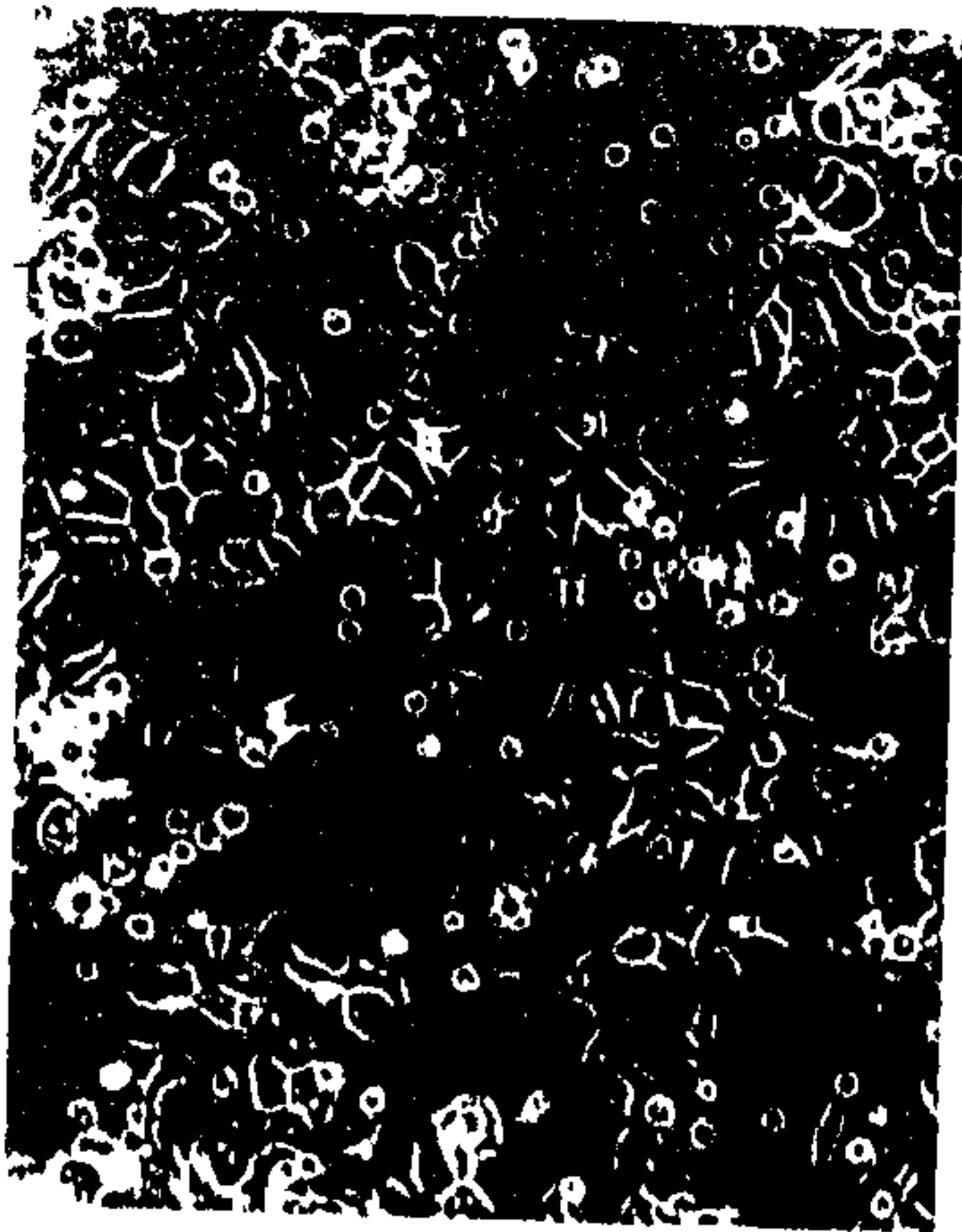
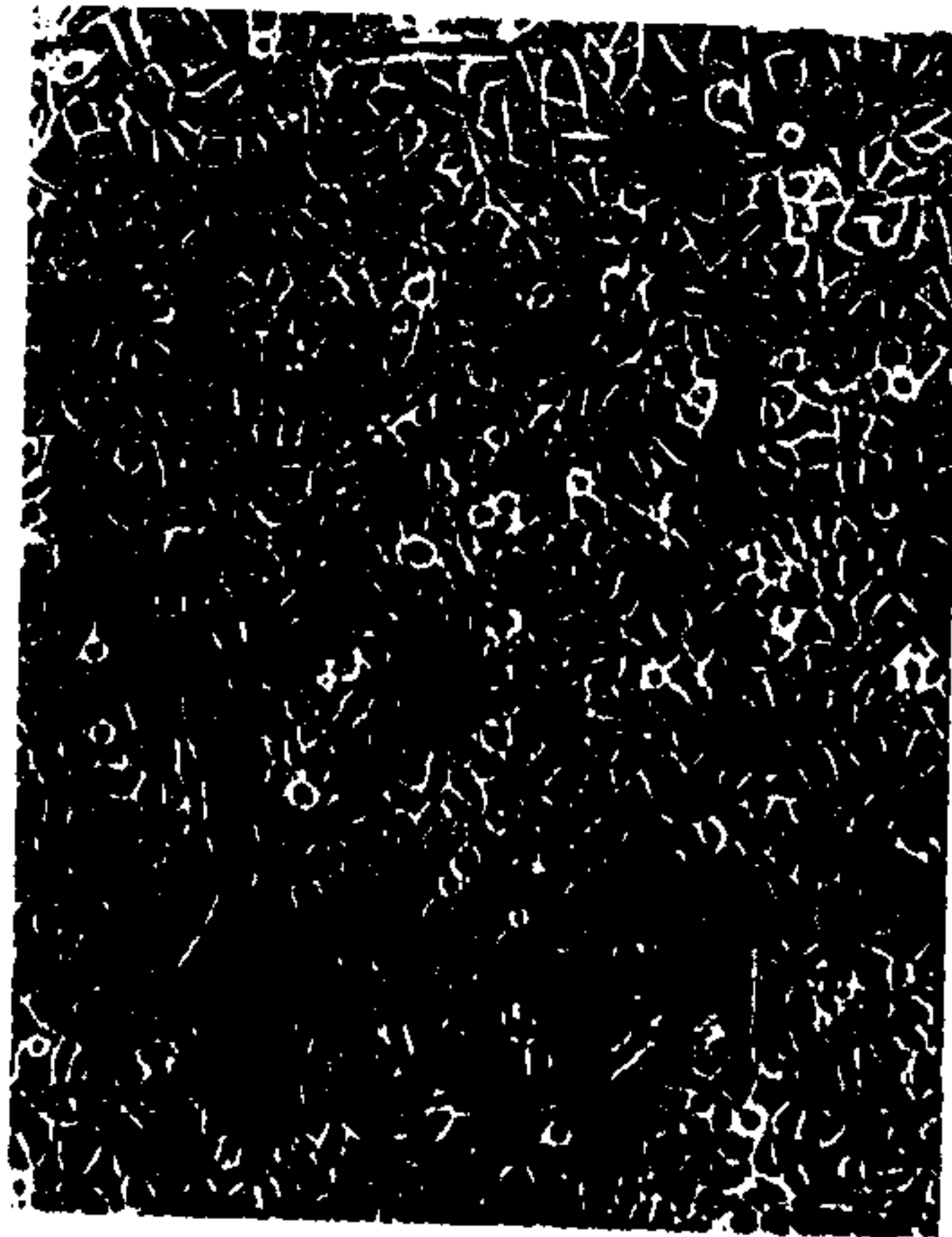
low Ca^{2+} Control *A*+ KGF *B*+ EGF *C*

FIG. III -1

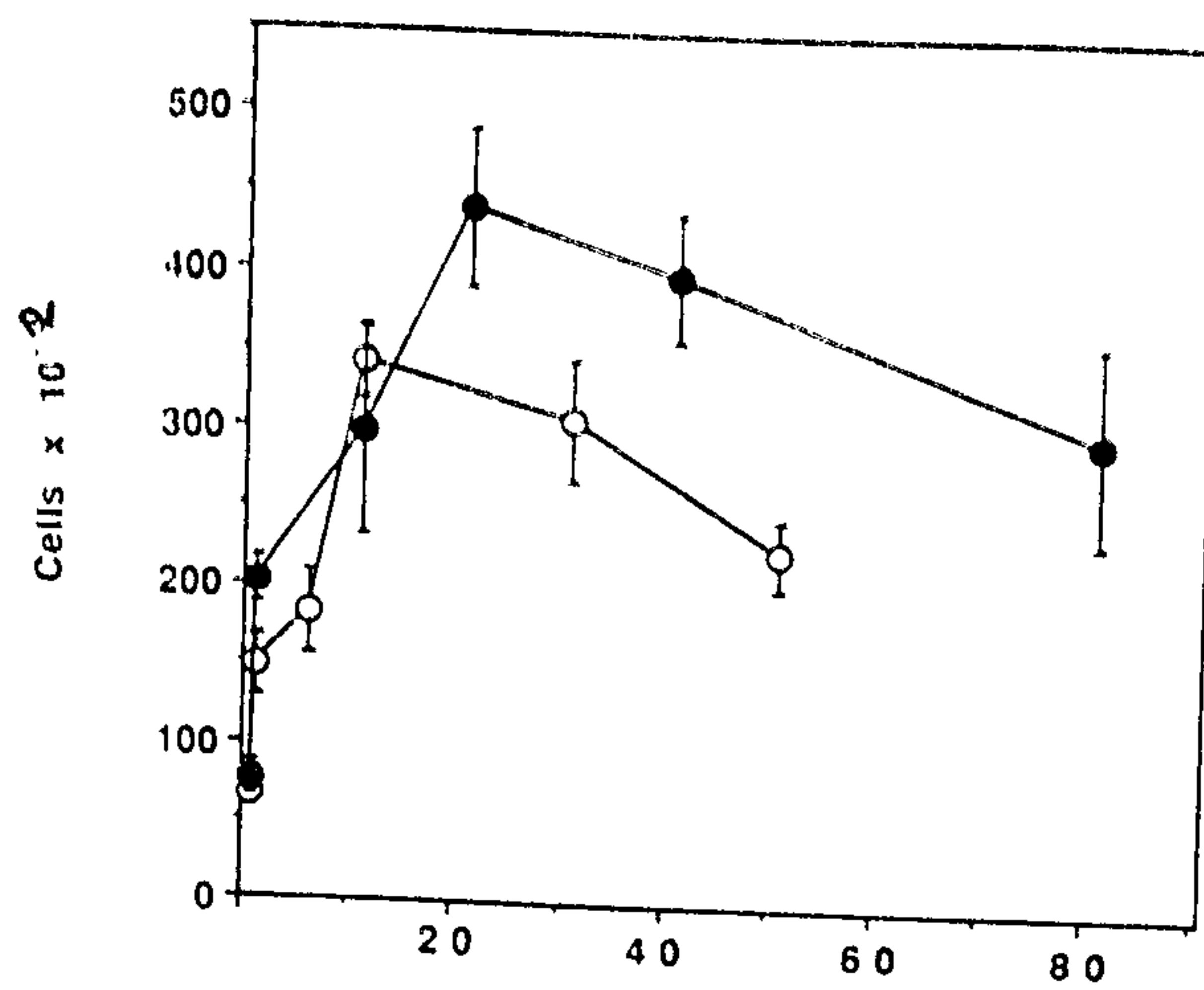


FIG. III-2

DIFFERENTIATION RESPONSE TO KGF AND EGF FAMILY

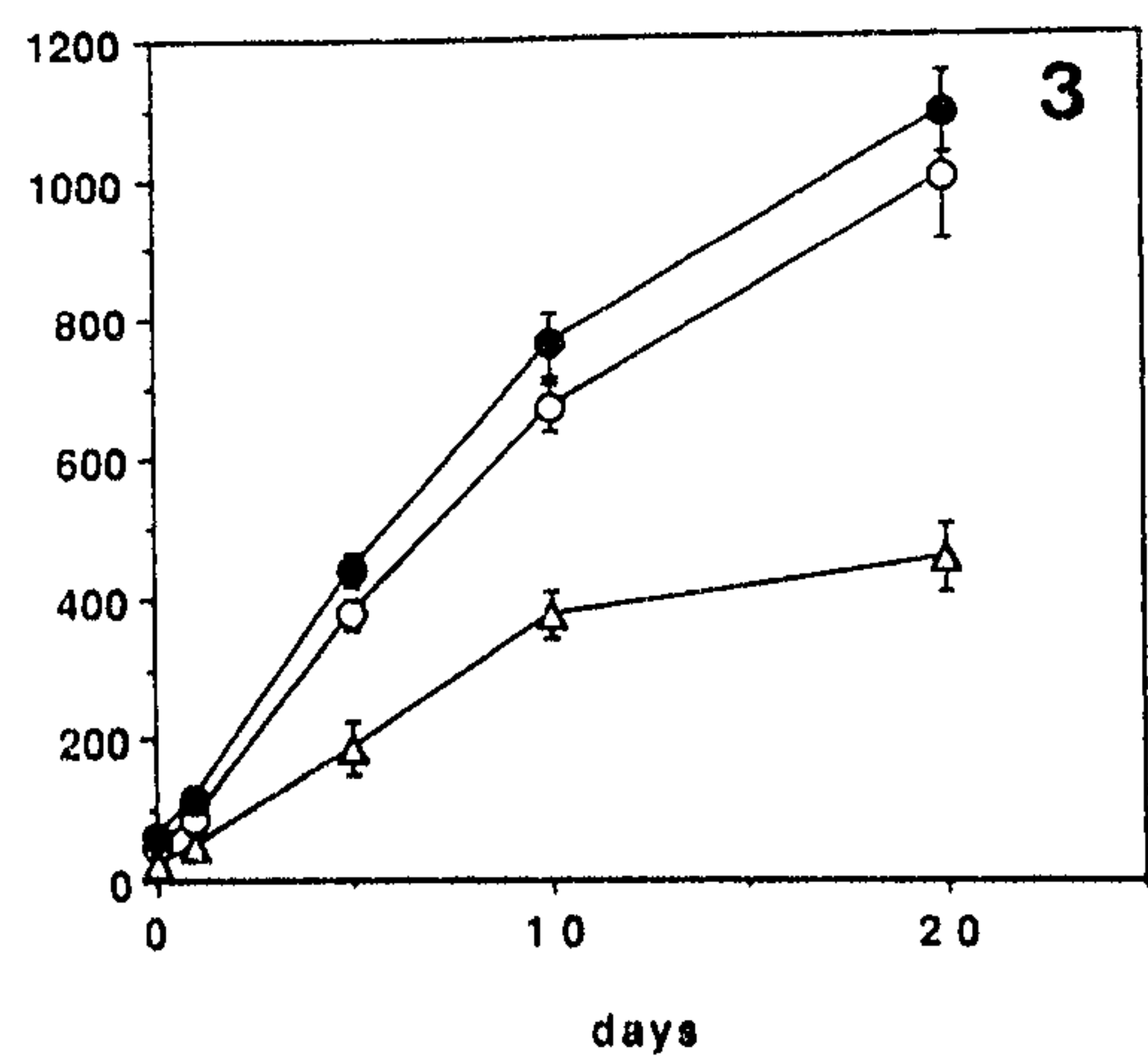


FIG. III-3

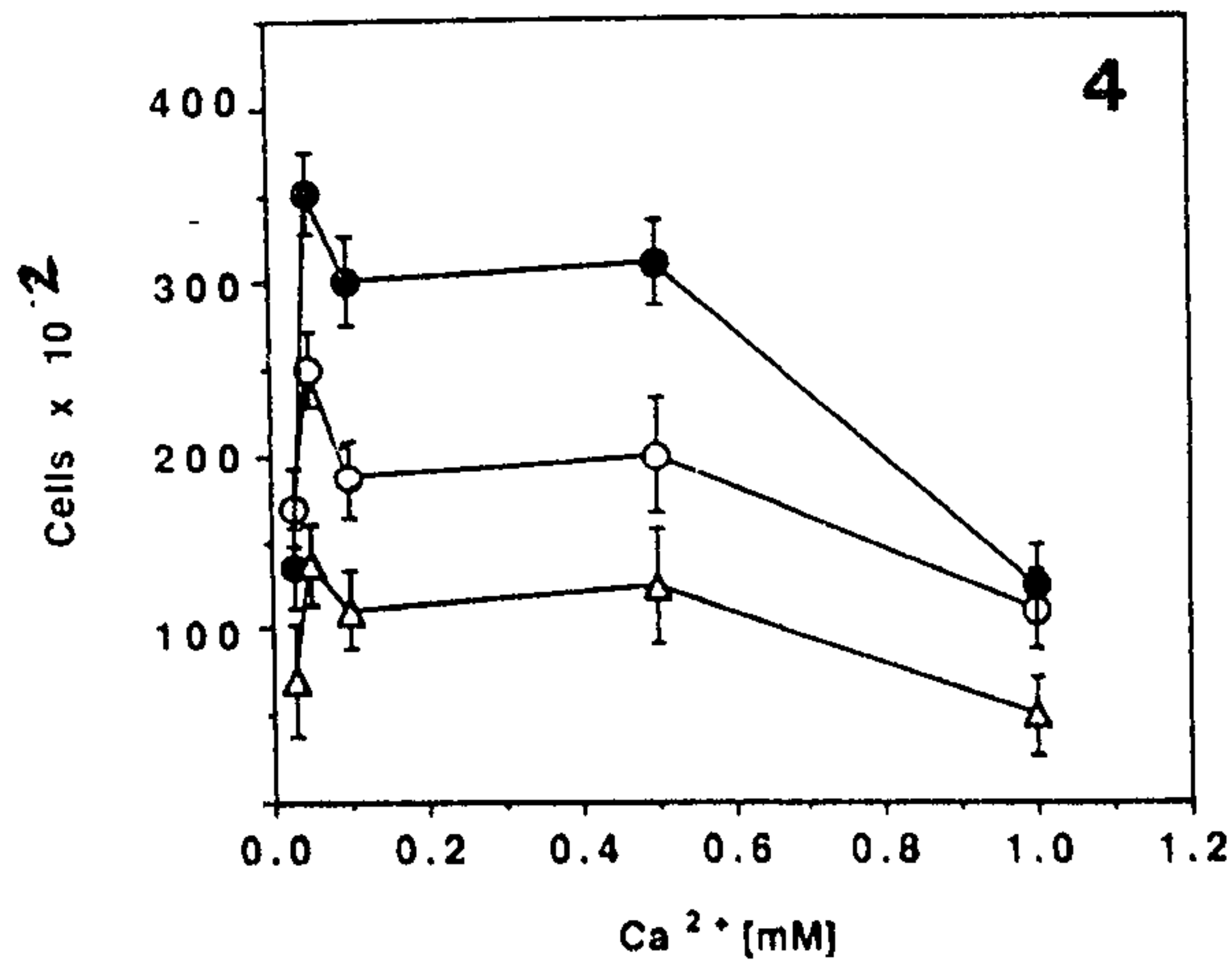


FIG. III-4

high Ca^{2++}

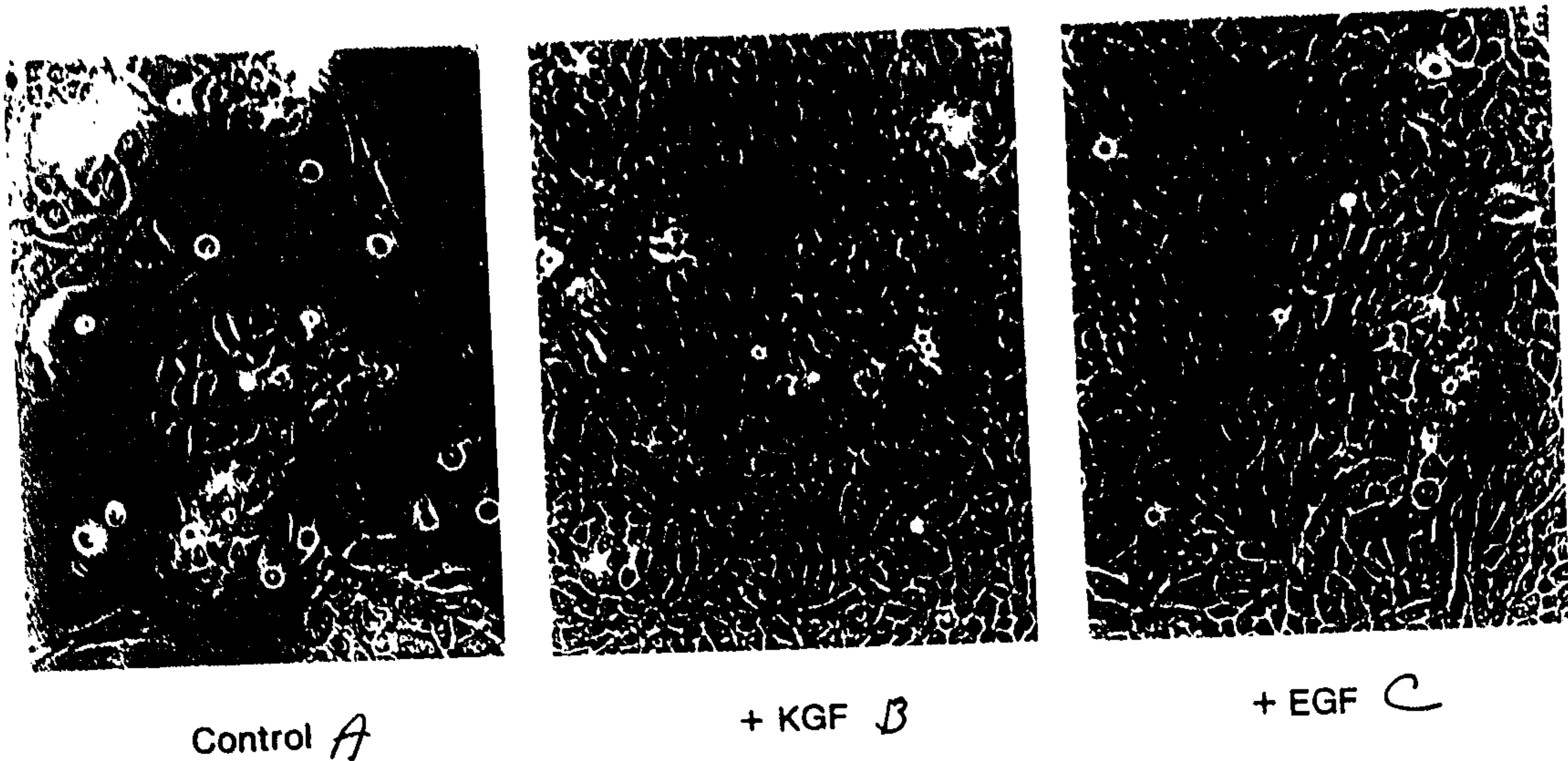


FIG. III-5

Sim. of ...

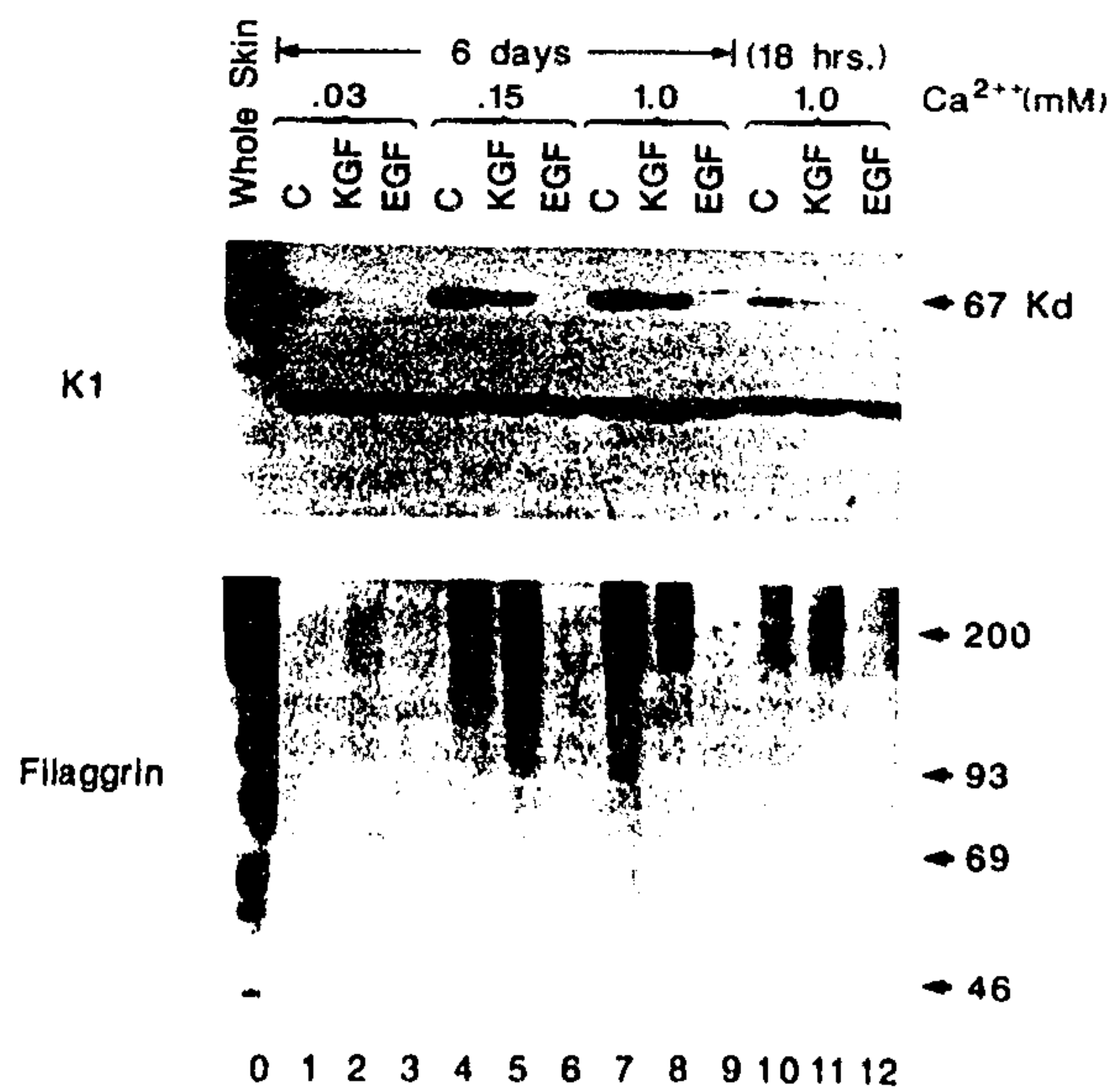


FIG III-6

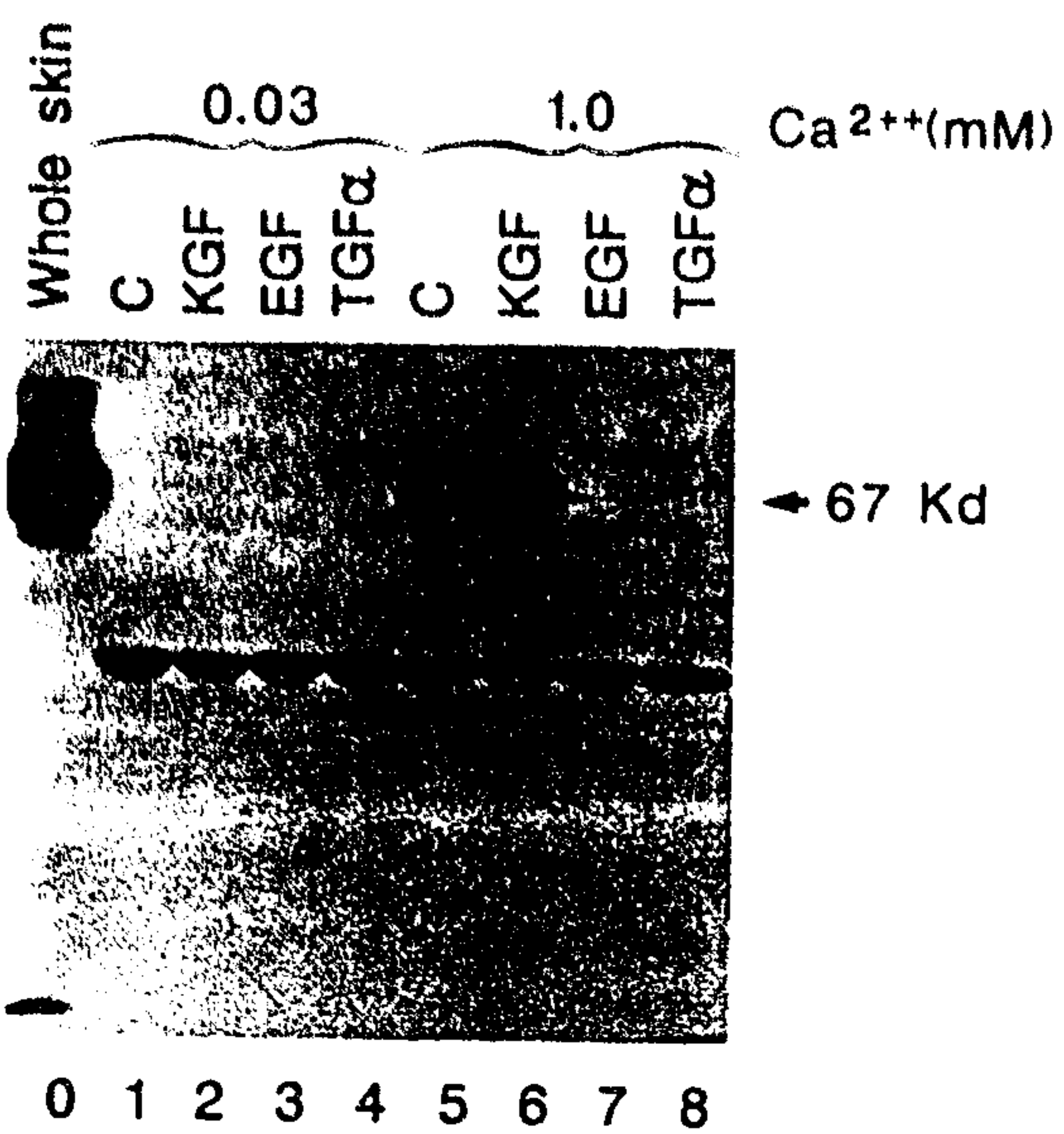


FIG III-7

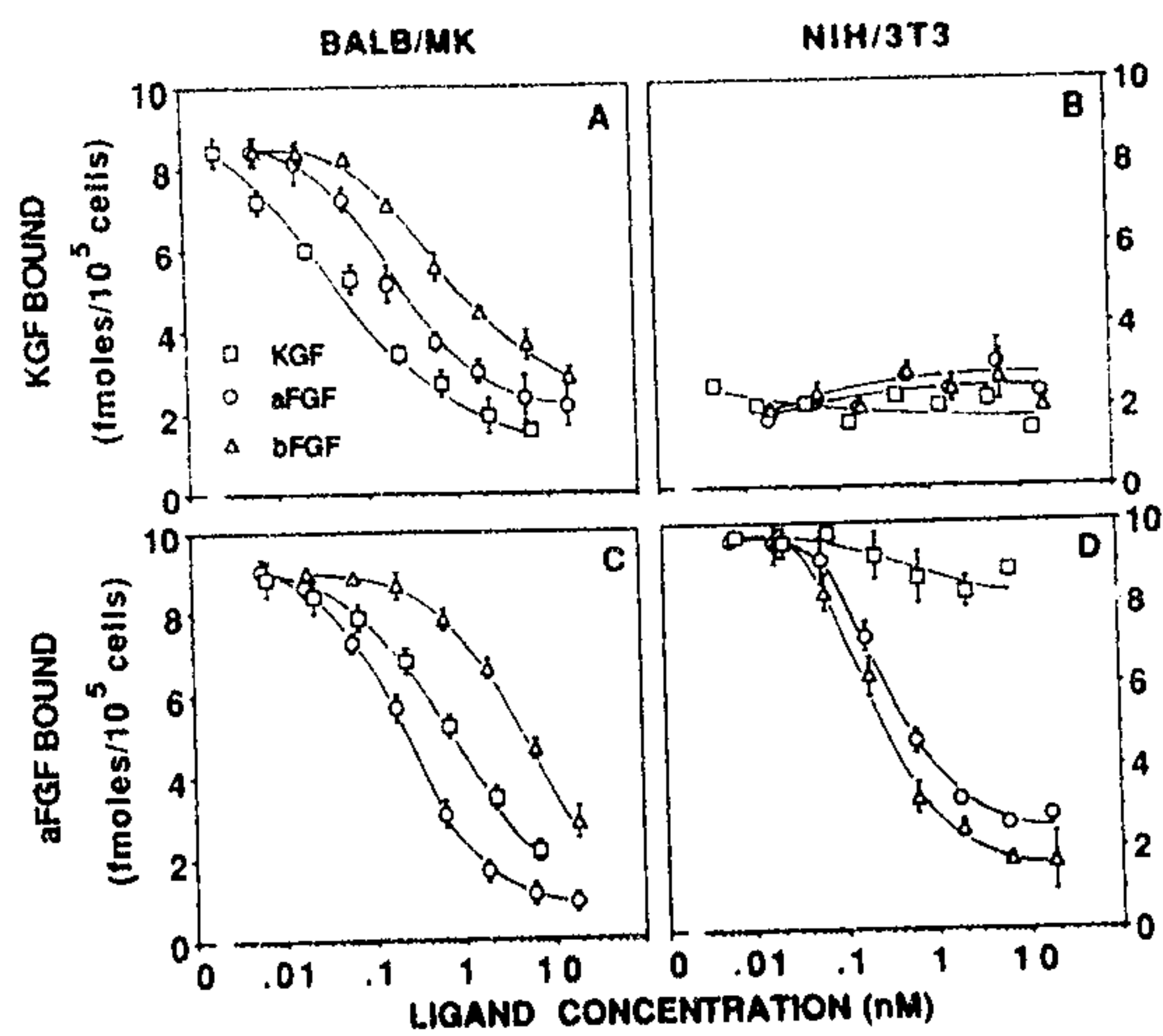


FIG. IV-1

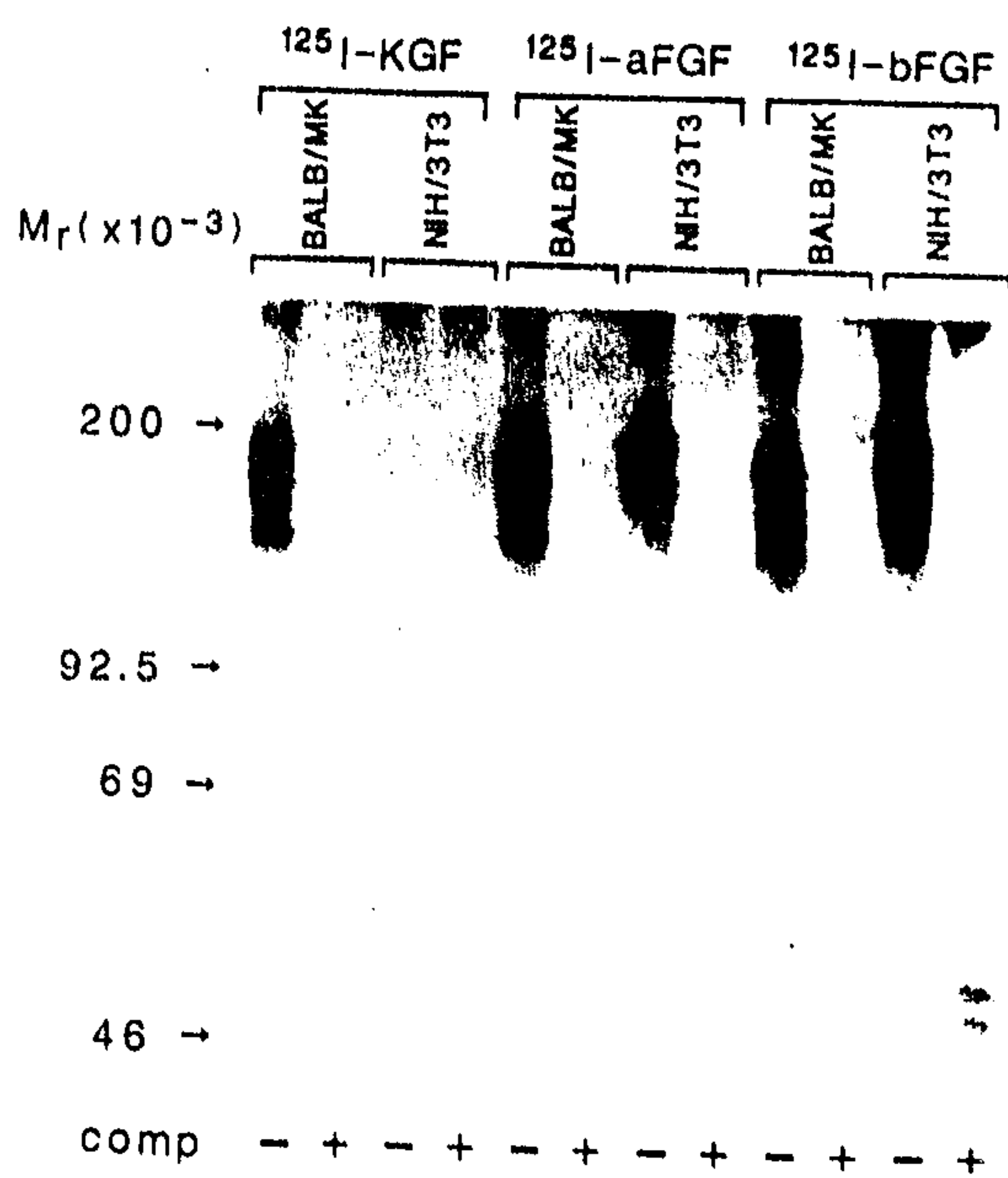


FIG. IV-3

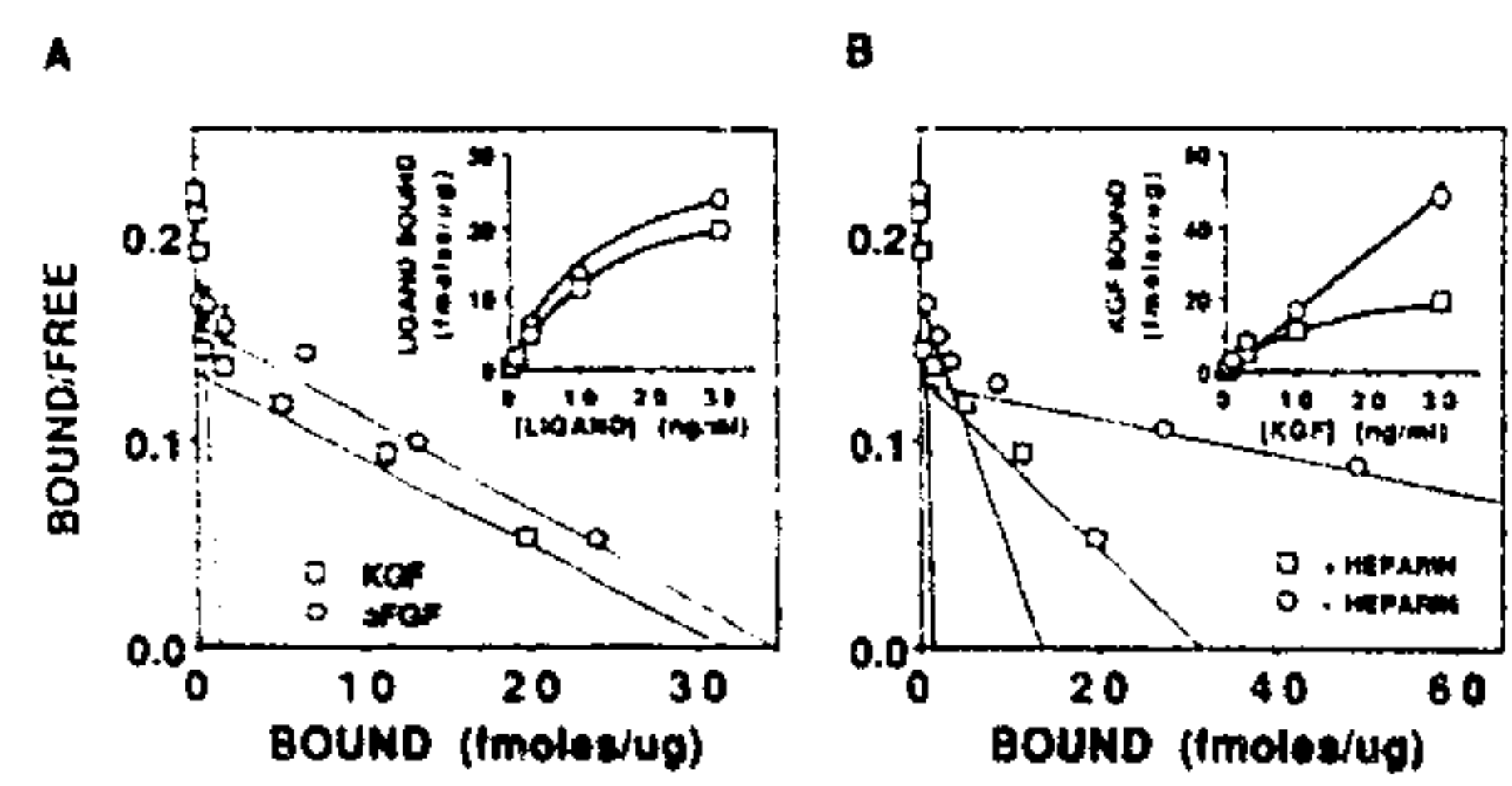


FIG. IV-2A

FIG. IV-2B

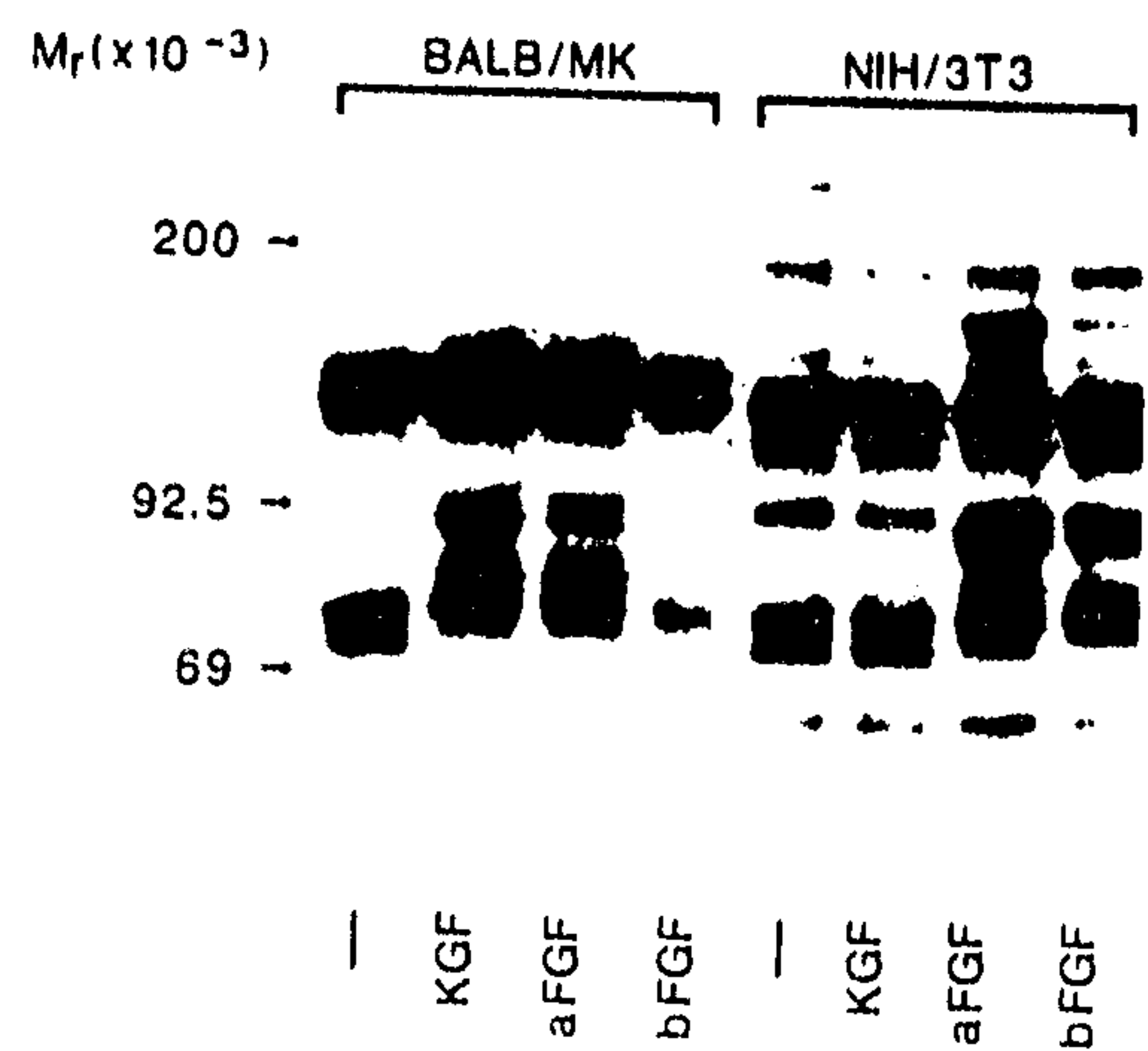
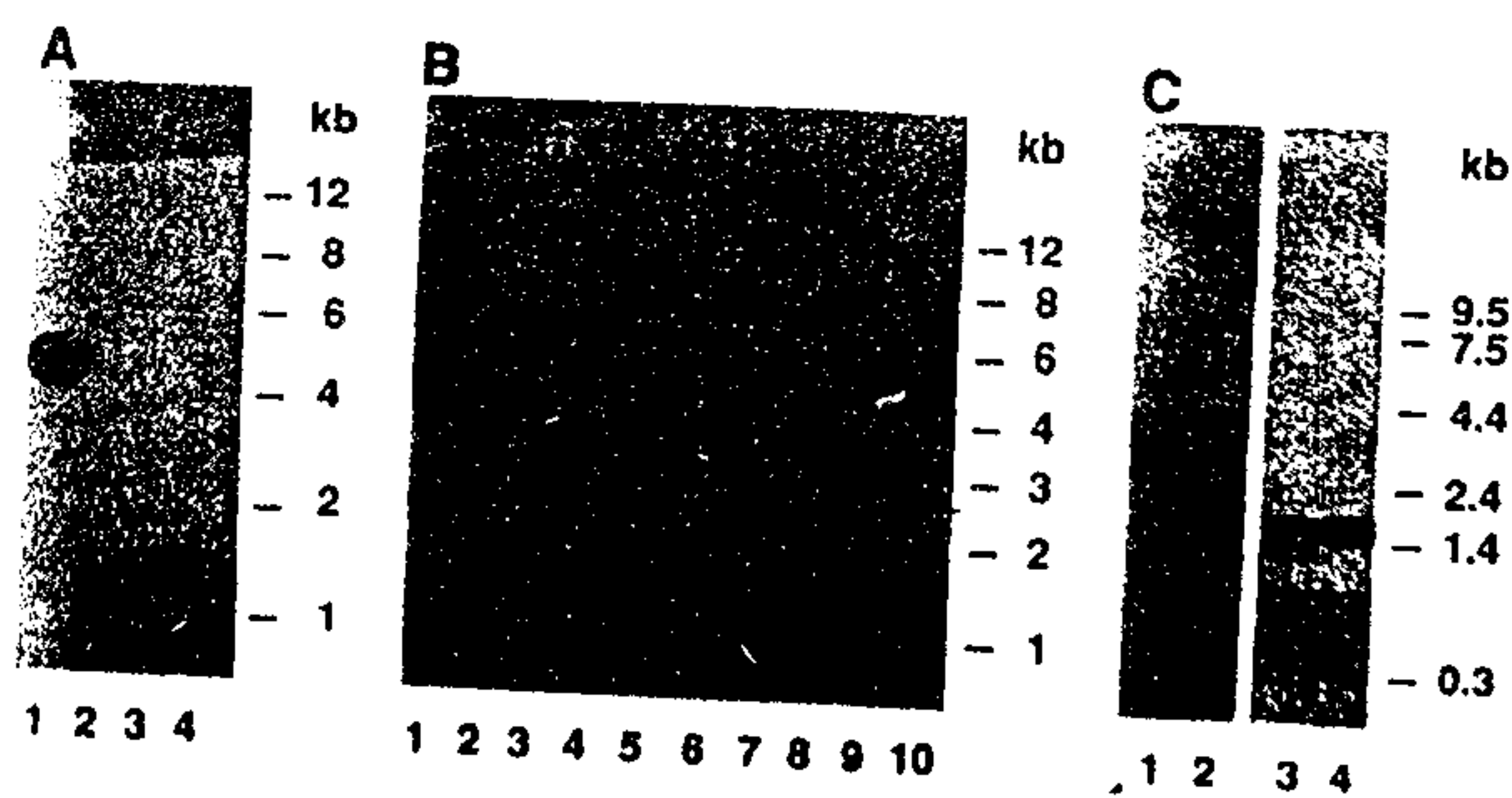


FIG. IV-4



IFG. V-1

A 1 NVSWQRFICLVIVTMTLSLRPSFSLVEDTTLEPEGAPYNTNTEKMEKRLHAVPAANTVKFRCPAGGNETPTMRNLKNG
81 KEFKQEHRIIGGYKVRNQHWSLIMESVVP SDKGNYTCLVENEYGS INHTYHLDVVERSPHRP ILQAGLPANASTVVGGDVE
161 FVCKVYSDAQPHIQWIKHVEKNQSKYGPDLPLKVLKHSQINSNAEVLALFNVTENDAGEYICKVSNYICQANOSAWL
241 TVLPKQQAPVREKEITASPDYLE TAIYCIGVFLIACHVVTVIFQRMKTTTKKPDFSSQPAVHKLTKRIP LRRQVTVSAES
321 SSSMNSNTPLVRITRLSSTADTPMLAGVSEYELPEDPKNEFPDKLT LGKPLGEGCFGQVMAEAVGIDKDKPKEAVTV
401 AVKMLKDDATEKDLSDLVSEMEMMKHIGKHKNI INLLGACTQDGP LYVIVEYASKGNLREYL RARRPPGMEYSYDINRVP
481 EEQMTTFKDLVSCTYQLARGMEYLASQKCIHRDLAARNVLVTENNVMKIADFGLARDINNIDYKKTTNGRLPVKWMAPFA
561 LFDVRYTHQSDVWSFGVLMWEIFTLGGSPPYFGITVEELFKLLKEGHRMDKPTNCTNELYMMHRDCNHAVPSQRPTFKQLV
641 EDLDRIITLTNEEYLDLTQPLEQYSPSPOTRSCSSGDDSVFSPDPMPYEPCLPQYPHINQGVKT

FIG. V-2A

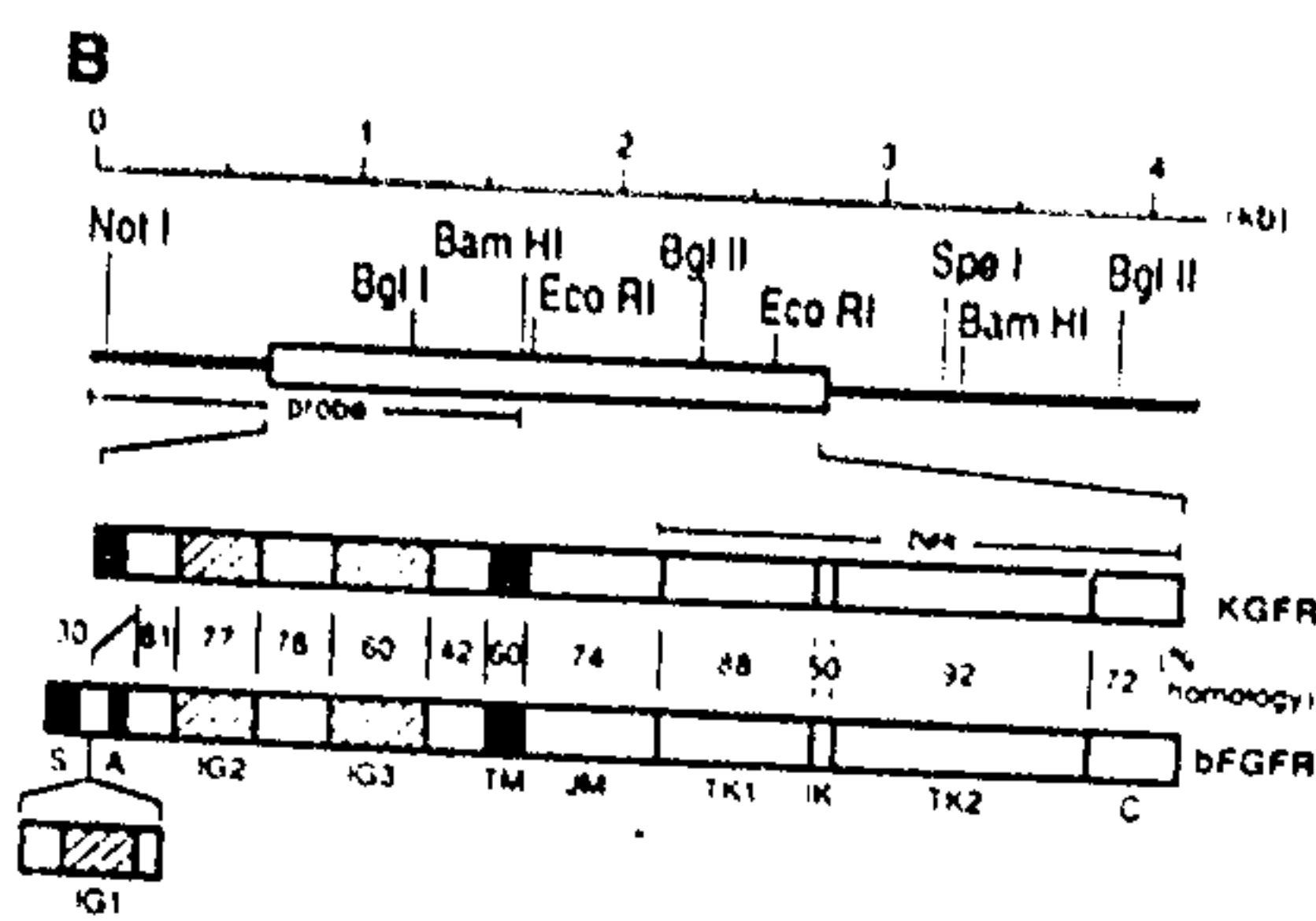


FIG. V-2B

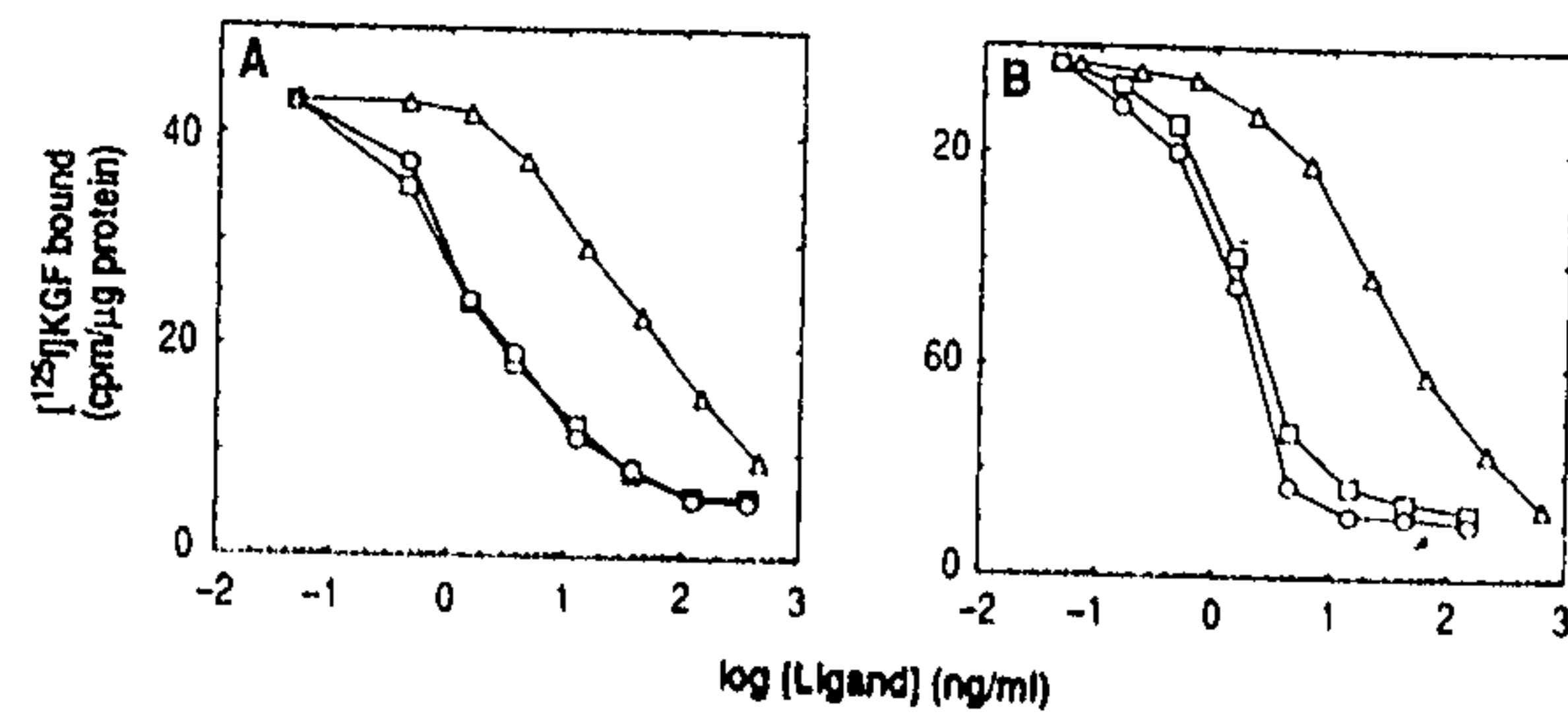


FIG. V-3A

FIG. V-3B

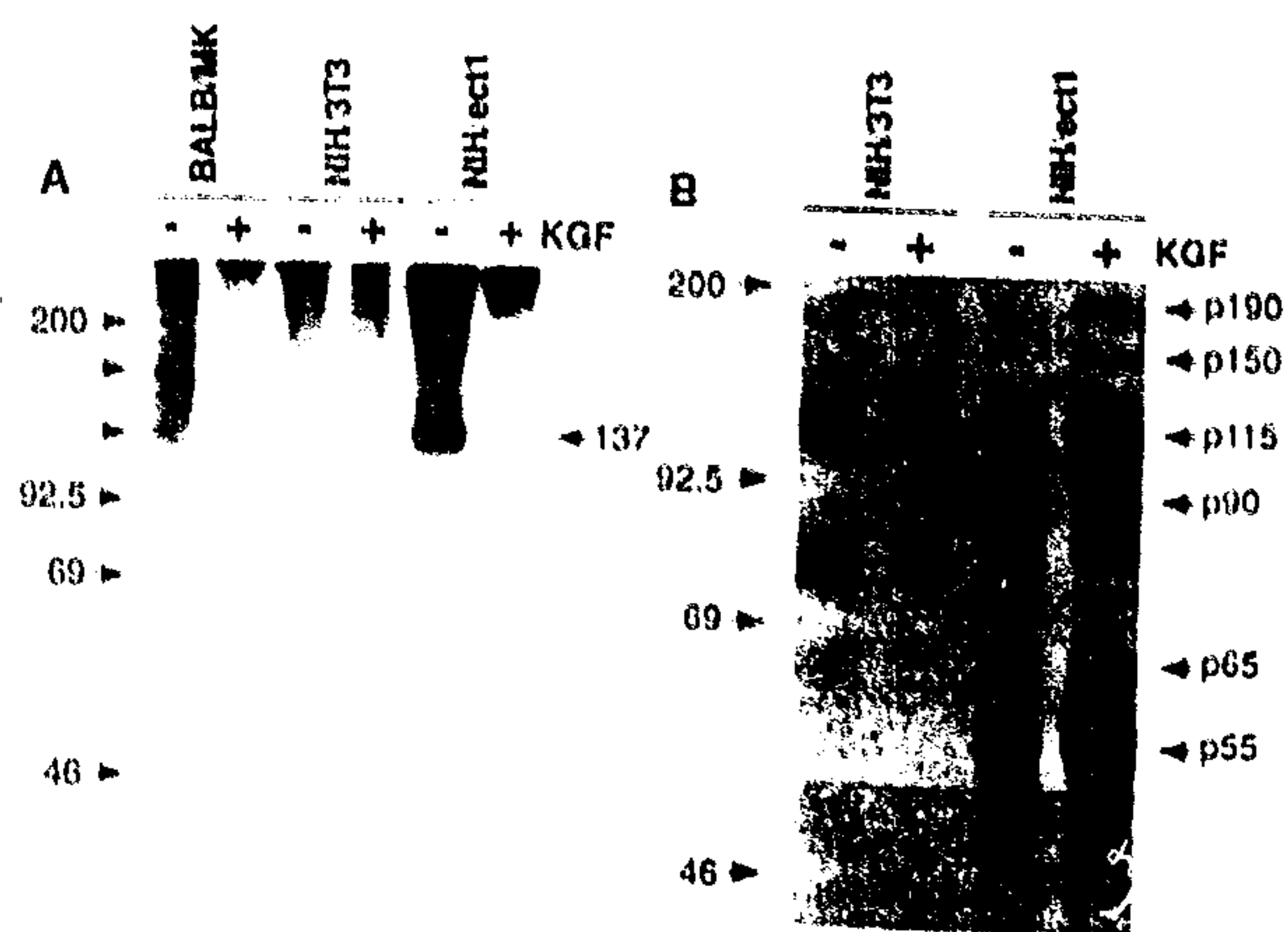


FIG. V-4