Ruddon, R. W., Hartle, R. J., Peters, B. P., Anderson, C., Huot, R. I., & Stromberg, K. (1981b) *J. Biol. Chem. 256*, 11389–11392.
Suda, K., Sakamoto, S., Hida, K., Kano, Y., Takaku, F., & Miura, Y. (1987) *Cancer Res. 47*, 2782–2786.
Swaminathan, N., & Bahl, O. P. (1970) *Biochem. Biophys. Res. Commun. 40*, 422–427.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., & Maley, F. (1978) *J. Biol. Chem. 253*, 4508–4511.
Warren, L., Fuhrer, J. P., & Buck, C. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1838–1843.
Weintraub, B. D., Krauth, G., Rosen, S. W., & Rabson, A. S. (1975) *J. Clin. Invest. 56*, 1043–1052.
Weintraub, B. D., Stannard, B. S., & Rosen, S. W. (1977) *Endocrinology (Philadelphia) 101*, 225–235.
Whitaker, J. R. (1963) *Anal. Chem. 35*, 1950–1953.
Yamada, S., & Itano, H. A. (1966) *Biochim. Biophys. Acta 130*, 538–540.

Characterization of High Molecular Weight Transforming Growth Factor α Produced by Rat Hepatocellular Carcinoma Cells†

Noreen C. Luetteke,*,‡ George K. Michalopoulos,‡ Joaquin Teixidõ,§ Reid Gilmore,§ Joan Massagué,§ and David C. Lee‖

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710, Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and Department of Microbiology and Immunology and Lineberger Cancer Research Center, The University of North Carolina, Chapel Hill, North Carolina 27514

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ABSTRACT: In addition to the mature 50 amino acid transforming growth factor α (TGFα), some transformed cells appear to produce multiple higher molecular weight forms. The structure and derivation of most of these larger soluble TGFα species remain to be established. We previously reported that a chemically induced rat hepatocellular carcinoma cell line, JM1, secreted acid-stable proteins which bind to epidermal growth factor receptors and stimulate DNA synthesis in primary cultures of normal adult rat hepatocytes. Purification and characterization of these hepatoma-derived growth factors have indicated their relationship to TGFα. Two EGF-competing activities of apparent M_r 30K and 10K were separated by gel filtration of concentrated JM1-conditioned medium and further purified by ion-exchange chromatography and reverse-phase HPLC. Both growth factors were detected by a radioimmunoassay specific for TGFα. Western blotting with antibodies to the 50 amino acid TGFα revealed that the lower molecular weight factor comigrated with the synthetic 6-kDa rat TGFα. The higher molecular weight TGFα appeared on immunoblots as a diffuse band of 18–21 kDa, which converted to the mature 6-kDa form upon digestion with elastase, confirming a precursor–product relationship. However, the 18–21-kDa proteins did not react with antibodies directed against the carboxy-terminal cytoplasmic segment of the transmembrane TGFα precursor. Enzymatic deglycosylation of the 18–21-kDa TGFα species by sequential removal of sialic acids and O- and N-linked carbohydrate reduced the molecular weight to 11K. The size and soluble nature of this polypeptide suggest that it represents the extracellular domain of the transmembrane TGFα precursor. In vitro translation of JM1 mRNA, enriched for the TGFα mRNA by hybridization selection, yielded a protein of 17 kDa which was immunoprecipitated by antibodies directed against the carboxy-terminal regions of the mature TGFα and the TGFα precursor. In the presence of microsomes, the primary translation product converted to 20 kDa, which has previously been shown to be due to N-linked glycosylation. These data demonstrate that JM1 hepatoma cells release the mature TGFα plus larger processing intermediates which are proteolytically derived from a common transmembrane glycoprotein precursor.

Transforming growth factor (TGFα) is a single polypeptide of 50 amino acids which acts as a mitogen for mesenchymal and epithelial cells [for a review, see Derynck (1986)]. Rat TGFα shares 34% amino acid sequence identity, but no immunological cross-reactivity, with mouse EGF (Marquardt et al., 1984; De Larco & Todaro, 1978). Both EGF and TGFα bind with comparable affinity to a common 170-kDa receptor and activate the intrinsic tyrosine kinase, leading to receptor autophosphorylation, internalization, and down-regulation (Massagué, 1983). However, unlike EGF, TGFα synthesis is most prevalent and abundant in tumor cells and cells transformed by retroviruses, oncogenes, or chemicals. Recently, expression of TGFα mRNA and/or protein has also been detected in certain normal tissues and cultured cells including bovine anterior pituitary cells (Samsoondar et al., 1986), rat maternal decidua (Han et al., 1987), and human epidermis and primary keratinocytes (Coffey et al., 1987).

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* Address correspondence to this author at the Department of Pathology, Duke University Medical Center, Box 3432, Durham, NC 27710.
‡ Duke University Medical Center.
§ University of Massachusetts Medical School.
‖ The University of North Carolina.

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Analyses of cDNAs encoding human (Derynck et al., 1984) and rat (Lee et al., 1985) TGFα have revealed that they are synthesized as precursor proteins of 160 and 159 amino acids, respectively. These precursor sequences are more than 90% conserved, and each includes 2 regions of about 23 uncharged or apolar residues. The first hydrophobic region begins with the amino terminus and probably serves as a signal peptide. Immediately adjacent, at positions 25–27, lies a potential glycosylation site within the Asn-Ser-Thr triplet. The second hydrophobic region, which could span the membrane, is located beyond the carboxy terminus of the mature TGFα and is flanked by pairs of basic amino acids. This transmembrane domain is followed by a cytoplasmic domain that is rich in cysteines and extends to the carboxy terminus. The 50 amino acid TGFα is cleaved from the precursor between alanine and valine residues by a protease with elastase-like specificity.

These predictions of the precursor, based on DNA sequence analysis, have recently been confirmed by experimental evidence. First, cells transfected with expression vectors containing the partial TGFα cDNA produced membrane-associated proteins of 13–19 kDa which were recognized by antibodies directed against carboxy-terminal peptides of both the mature TGFα and the TGFα precursor (Bringman et al., 1987; Gentry et al., 1987). Some of these species were shown to contain Asn-linked carbohydrate and Cys-linked palmitate (Bringman et al., 1987). Second, immunoprecipitation of products from in vitro transcription and translation of rat TGFα cDNA in the presence of microsomes demonstrated the glycosylation and orientation of the transmembrane precursor (Teixidõ et al., 1987). Third, 17–19-kDa forms of TGFα secreted by retrovirally transformed fibroblasts were converted to the mature 6-kDa TGFα by treatment with elastase (Ignotz et al., 1986).

There have been numerous other reports of bioactive higher molecular weight forms of TGFα (18–68K) found in supernatants of cultured human tumor cells (De Larco et al., 1985; Dickson et al., 1986; Stromberg, et al., 1986) and extracts of human platelets (Assoian et al., 1984) and urine (Kimball et al., 1984). In many cases, these proteins constitute the predominant TGFα activity, and their estimated size either equals or exceeds the molecular weight predicted for the transmembrane precursor. However, their precise structural relationship to the TGFα precursor remains undefined.

We have previously reported that two carcinogen-induced rat hepatocellular carcinoma cell lines produced similar heat- and acid-stable proteins that could bind to EGF receptors but not to EGF antibodies (Luetteke & Michalopoulos, 1985). The bioactivity of these hepatoma-derived growth factors was assessed by their stimulation of DNA synthesis and inhibition of EGF binding in primary cultures of normal adult rat hepatocytes. In the present study, gel filtration, ion-exchange chromatography, and reverse-phase HPLC were used to purify two molecular weight forms from media conditioned by JM1 hepatoma cells. The relationship of these proteins to TGFα was established by immunological criteria. Our results indicate that JM1 cells secrete, in addition to the mature rat TGFα, a higher molecular weight, heavily glycosylated intermediate form of TGFα. The latter heterogeneous species contain N- and O-linked carbohydrate and appear to correspond to the extracellular domain of the transmembrane precursor.

Experimental Procedures

Materials. Male Fischer F344 rats were obtained from the Frederick Cancer Research Facility. Tissue culture media and antibiotics were purchased from Gibco. Fetal bovine serum and rabbit anti-goat IgG were from HyClone Laboratories. Receptor-grade mouse EGF was obtained from Collaborative Research. Iodinated mouse EGF and protein A were purchased from Amersham. Goat antibodies to TGFα and TGFα RIA kit were obtained from Biotope, Inc. Rabbit antisera to TGF precursor-specific peptides were prepared as described (Gentry et al., 1987). Porcine pancreatic elastase was obtained from Elastin Products. *O*-Glycanase was purchased from Genzyme. Endoglycosidase F, grade II, was from Boehringer Mannheim Biochemicals. Neuraminidase, *N*-acetyltrialanyl methyl ester, bovine serum albumin, and other chemicals were obtained from Sigma.

Culture of Hepatocytes and Hepatoma Cells. Hepatocytes for primary culture were isolated from 150–250-g adult male Fischer 344 rats by a modified two-step collagenase liver perfusion method (Michalopoulos et al., 1982). Bioassays of hepatocyte DNA synthesis and radioreceptor assays of hepatocyte EGF binding were performed as previously described (Luetteke & Michalopoulos, 1985; Michalopoulos et al., 1984). JM1 hepatocellular carcinoma cells were clonally established from a liver tumor induced in a male Fischer 344 rat by initiation with a single dose of diethylnitrosamine 24-h post partial hepatectomy followed by promotion with phenobarbital in the drinking water (Novicki et al., 1983). JM1 cells were grown to confluency in Nunc cell factories in Eagle's MEM with Earle's salts supplemented with 50 μg/mL gentamicin and 10% Hyclone fetal bovine serum. Serum-free medium (1.5 L per cell factory) conditioned for 48 h was collected and centrifuged at 2500 rpm (1000*g*) for 5 min to remove cells. After addition of phenylmethanesulfonyl fluoride (PMSF) to 1 mM, medium was Millipore filter sterilized and stored at 4 °C.

Gel Filtration. Six liters of JM1-conditioned medium was concentrated by ultrafiltration through a hollow fiber cartridge apparatus (Amicon) and lyophilization. Proteins were reconstituted in and dialyzed against 0.15 M ammonium acetate (pH 7.0). The concentrate was filtered and applied to a 5 × 90 cm column of Sephadex G-100 (Pharmacia) and eluted at 4 °C with 0.15 M ammonium acetate (pH 7.0). Aliquots of 20-mL fractions were lyophilized and tested at 10% by volume in the hepatocyte DNA synthesis bioassay and 30% by volume in the EGF radioreceptor assay.

Ion-Exchange Chromatography. Gel filtration fractions comprising activity peaks were separately pooled, lyophilized, and reconstituted to 15–20 mL in 10 mM ammonium acetate (pH 5.5). Samples were filtered and loaded onto a 1.6 × 15 cm column of (carboxymethyl)-Sepharose (Pharmacia). Isocratic elution at 20 mL/h was followed by a 200-mL linear gradient of 10–500 mM ammonium acetate (pH 5.5). Aliquots of 8-mL fractions were lyophilized for testing at 30% in the radioreceptor assay. The higher molecular weight activity peak, which was not retained by CM-Sepharose, was subjected to anionic exchange chromatography on a 1.6 × 15 cm column of (diethylaminoethyl)-Sepharose under the same conditions.

Reverse-Phase HPLC. Active fractions from ion-exchange chromatography were pooled, lyophilized, and reconstituted in 2 mL of 10% acetonitrile and 0.05% trifluoroacetic acid in water. Samples (500 μL) were injected onto a 0.39 × 30 cm Waters μBondapak C18 column. After 5-min isocratic elution at 1 mL/min with 10% acetonitrile, a gradient of 10% to 60% acetonitrile in 0.05% TFA over 50 min was applied. Aliquots of 1-mL fractions were lyophilized and tested at 50% by volume in the EGF radioreceptor assay.

Northern Blot Analysis. RNA was isolated by homogenization of cultured cells or tissue in 4 M guanidine isothio-

cyanate followed by sedimentation through 5.7 M cesium chloride, as previously described (Lee et al., 1985). Poly(A^+) RNA was selected by chromatography on oligo(dT)–cellulose, and samples (10 μg) were electrophoresed through 1% agarose gels containing formaldehyde. RNA was transferred to nitrocellulose, probed with nick-translated cloned TGFα cDNA, and washed as previously described (Lee et al., 1985).

Western Immunoblot Analyses. Aliquots of the hepatoma-derived growth factors approximately equivalent to 200 ng of TGFα were subjected to reducing SDS–PAGE in 10–20% gradient minigels. Proteins were transferred (250 mA, 4 h) to 0.2-μm nitrocellulose and incubated at 4 °C overnight in Tris-buffered saline (TBS, pH 7.4) containing 5% bovine serum albumin (BSA), 0.05% Tween 20, and a 1:500 dilution of goat antibodies raised against synthetic 50 amino acid TGFα. Before and after subsequent incubations, immunoblots were washed 4× for 15 min each in TBS minus BSA. Secondary antibody incubation was for 2 h at room temperature in 1:500 dilution of rabbit anti-goat IgG in TBS plus BSA, followed by 2 h at room temperature with 0.5 μCi/mL ^{125}I protein A in TBS plus BSA.

Protease and Glycosidase Treatments. Aliquots of the higher molecular weight TGFα purified from JM1-conditioned medium were digested for 1 h at room temperature with 1 μg/mL pancreatic elastase in 20 μL of 50 mM glycylglycine (pH 8.8), with or without 50 mM *N*-acetyltrialanyl methyl ester, a competitive inhibitor of elastase. For glycosidase digestions, lyophilized samples were first resuspended and boiled in 10 μL of 0.2% SDS and 2% 2-mercaptoethanol. The reaction volume was increased to 20 μL by addition of 1% NP40, 100 mM sodium phosphate (pH 6.0), 1 mM PMSF, and 1–2 μL of enzymes. Samples were incubated at 37 °C for 2 h with 20 milliunits of neuraminidase, 6 h with 1.2 milliunits of *O*-glycanase (endo-α-*N*-acetylgalactosaminidase), and/or 12 h with 50 milliunits of endoglycosidase F (endo-β-*N*-acetylglucosaminidase F, grade II; also contains peptide:*N*-glycosidase F). All digestions were stopped by the addition of 20 μL 2× electrophoresis sample buffer. Samples were subjected to SDS–PAGE on 10–20% gradient gels and Western immunoblotting as described above.

In Vitro Translation of TGFα mRNA. Rat proTGFα was obtained by transcribing the pGEMα vector containing the TGFα cDNA as already described (Teixidõ et al., 1987). Poly(A^+) RNA from normal rat liver cells and JM1 rat hepatoma cells were subjected to hybridization selection as previously described (Cochet et al., 1979), using pEMBL containing the *Eco*RI insert of the rat TGFα cDNA. In vitro translation reactions were performed as described (Teixidõ et al., 1987). Dog pancreas rough microsomes were added where indicated at a concentration of 0.05 equiv/μL. Translation mixture proteins were immunoprecipitated with antisera to TGFα (Ignotz et al., 1986; Teixidõ et al., 1987), separated on 10–15% polyacrylamide gradient gels, and detected by fluorography using Enlightning (New England Nuclear).

Results

Untreated serum-free medium conditioned by confluent cultures of JM1 rat hepatoma cells stimulated DNA synthesis in primary cultures of adult rat hepatocytes in a dose-dependent and saturable manner (Luetteke & Michalopoulos, 1985). The maximal response of 75–80% labeling index is comparable to that induced by purified mouse EGF or synthetic rat TGFα (data not shown). Unconcentrated JM1-conditioned media also competed in the hepatocyte EGF radioreceptor assay, and typically contained greater than 10 ng/mL EGF equiv of activity. Previous results (Luetteke & Michalopoulos, 1985) had demonstrated that the JM1 activity shared physicochemical properties with other EGF-related growth factors, which are trypsin- and dithiothreitol-sensitive and heat- and acid-stable proteins. In addition, EGF antiserum did not block its biological activity. Therefore, we suspected that like many other transformed cells, the JM1 cells secreted TGFα proteins.

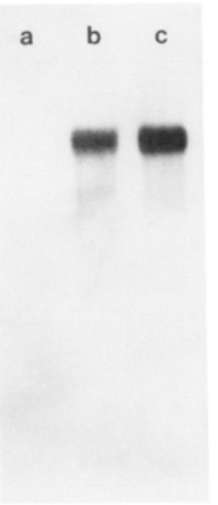

FIGURE 1: Expression of TGFα mRNA in JM1 hepatoma cells. The autoradiogram displays Northern analysis of polyadenylated RNA (10 μg) from normal rat liver (lane a), cultured JM1 cells (lane b), and JM1-induced tumor tissue (lane c) probed with the cloned rat TGFα cDNA. The position of bands corresponds to the previously described 4.5-kb mRNA (Lee et al., 1985).

Expression of TGFα was confirmed by Northern analysis of mRNA isolated from cultured JM1 cells and from tumors induced by inoculation of these cells. Figure 1 demonstrates that JM1 cells synthesize the 4.5-kb TGFα mRNA in vitro (lane b) and in vivo (lane c). In contrast, TGFα mRNA was not detectable in normal liver (lane a).

The high level of expression and secretion of TGFα-like activity by JM1 cells provided a suitable source for growth factor purification and characterization. Initial fractionation of 200 mL of JM1-conditioned media separated the activity into two molecular weight regions of approximately 25K and 10K (Luetteke & Michalopoulos, 1985). This gel filtration step was scaled up on a larger column of Sephadex G100 to accommodate the proteins concentrated over 100-fold from about 6 L of JM1-conditioned medium. The chromatogram (Figure 2) displays the activity profile of the fractions tested for stimulation of hepatocyte DNA synthesis and inhibition of hepatocyte EGF binding. The activities in both assays comigrated in two peaks with nearly equal amplitudes and apparent molecular weights of 30K and 10K. Pooled fractions from each peak were independently subjected to identical cationic exchange chromatography. At pH 5.5, the lower molecular weight activity was efficiently retained on (carboxymethyl)-Sepharose and eluted late in the linear salt gradient (Figure 3B). The higher molecular weight activity was not retained and eluted early in the flow-through fractions (Figure 3A). The higher molecular weight activity did absorb to (diethylaminoethyl)-Sepharose under the same column conditions and eluted in the gradient as expected for an anionic protein (data not shown). Thus, ion-exchange chromatography completely resolved the higher and lower molecular weight growth factors and eliminated cross-contamination due to peak overlap in the pools obtained from gel filtration. Since ion exchange can be adapted to batch adsorption and concentration of proteins, this high-capacity method might prove useful in

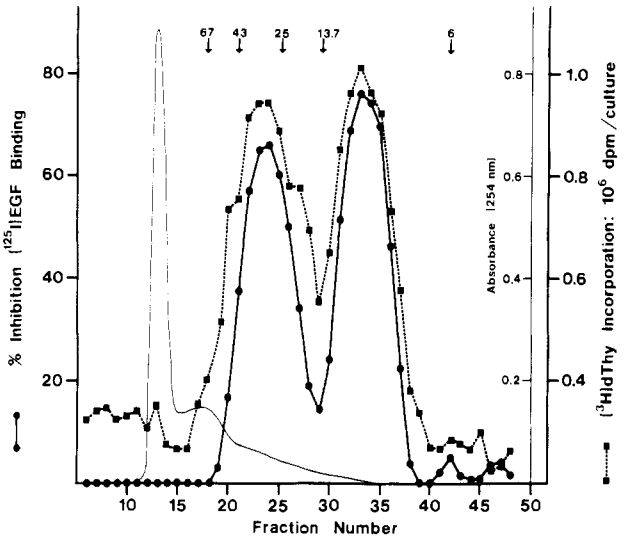

FIGURE 2: Gel filtration of JM1-conditioned medium. JM1-conditioned medium was collected, concentrated, and chromatographed on Sephadex G-100 as detailed under Experimental Procedures. Aliquots of fractions were tested at 10% by volume for stimulation of DNA synthesis (■) and at 30% for competitive inhibition of EGF binding (●) in primary cultures of rat hepatocytes. Data points represent the mean value from duplicate cultures. Molecular weight markers were bovine serum albumin (67K), ovalbumin, (43K), chymotrypsinogen (25K), ribonuclease (13.7K), and insulin (6K).

separating similar growth factors in large volumes of complex mixtures such as conditioned media, serum, or urine.

Reverse-phase HPLC on an analytical C18 column was performed separately on each pool of activity recovered from ion-exchange chromatography. Despite their significantly different size and net charge, the two hepatoma-derived growth factors display similar hydrophobicity by eluting near each other, but ahead of the bulk protein in a steep linear acetonitrile gradient (Figure 4). Both sets of fractions yielded a single peak of activity in the EGF radioreceptor assay. However, as in other purification steps, the peak from the higher molecular weight activity (Figure 4A) was broader with a shoulder suggesting heterogeneity. This peak spreading was more extensive when the higher molecular weight activity was eluted with a shallow acetonitrile gradient (0.1% mL^{-1}/min^{-1}; data not shown). The net recovery of the combined activities from JM1-conditioned media, as monitored and estimated by the EGF radioreceptor assay, was approximately 10%. The cumulative yields were 4.4 μg of EGF equiv of the higher molecular weight factor and 2.4 μg of EGF equiv of the lower molecular weight factor from 6 L of JM1-conditioned medium.

Following HPLC purification, samples of each growth factor preparation were reduced and denatured and tested in a TGFα-specific radioimmunoassay. This RIA utilizes rabbit antiserum raised against a synthetic peptide corresponding to the carboxy-terminal 17 amino acids of the mature rat TGFα. Figure 5 illustrates that both the higher and lower molecular weight hepatoma-derived growth factors were detected by this RIA, consistent with their identity to TGFα.

In order to more accurately determine the size of these proteins, they were subjected to SDS–PAGE and Western blotting with goat immunoglobulins directed against the synthetic 50 amino acid TGFα. These antibodies detected authentic rat TGFα and the growth factors purified from JM1-conditioned media but did not react with mouse EGF (Figure 6A). Since the protein band identified in the low molecular weight preparation (lane c) comigrated with the synthetic 6-kDa TGFα standard (lane b), it probably corresponds to the mature form of rat TGFα. The TGFα protein(s)

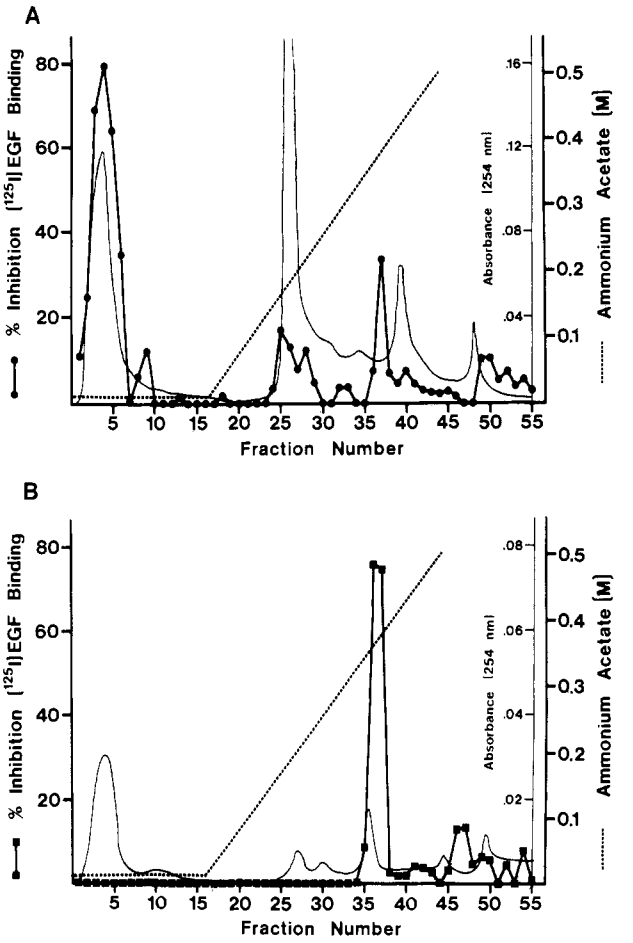

FIGURE 3: Cationic exchange chromatography of the hepatoma-derived growth factors. Pools of the high molecular weight and low molecular weight activities recovered from gel filtration of JM1-conditioned medium were subjected to chromatography on (carboxymethyl)-Sepharose at pH 5.5 as described under Experimental Procedures. A linear gradient of 10–500 mM ammonium acetate was begun at fraction 16 (···). Aliquots of fractions were tested at 30% by volume in the hepatocyte EGF radioreceptor assay (—). Data points represent the mean values from duplicate cultures. (A) Activity profile of the higher molecular weight growth factor (●). (B) Activity profile of the lower molecular weight growth factor (■).

in the higher molecular weight fraction (lane d) appeared as a broad band with mean molecular weight of 21K. In parallel control blots, no proteins were immunoreactive with normal goat IgG fraction (data not shown).

Processing of the mature 50 amino acid TGFα from its precursor requires cleavage at both termini between alanine and valine residues within alanine- and valine-rich sequences (Derynck et al., 1984; Lee et al., 1985). This specificity resembles that of elastase enzymes (Naughton & Sanger, 1961). Pancreatic elastase has been shown to convert 17–19-kDa TGFα proteins released by retrovirally transformed cells to the 6-kDa mature form (Ignotz et al., 1986). Similarly, elastase digestion of the higher molecular weight TGFα from JM1-conditioned media resulted in the loss of the 21-kDa species and the coincident appearance of a 6-kDa species which comigrated with synthetic 50 amino acid TGFα (Figure 6B, lanes a and b). The shift in immunoblot position was prevented by the addition of a competitive elastase inhibitor (lane c). These data suggest that the higher molecular weight activity in JM1-conditioned medium is related to or derived from the TGFα precursor.

The size of the higher molecular weight TGFα estimated from immunoblots was greater than the molecular weight predicted for the entire transmembrane precursor. Therefore,

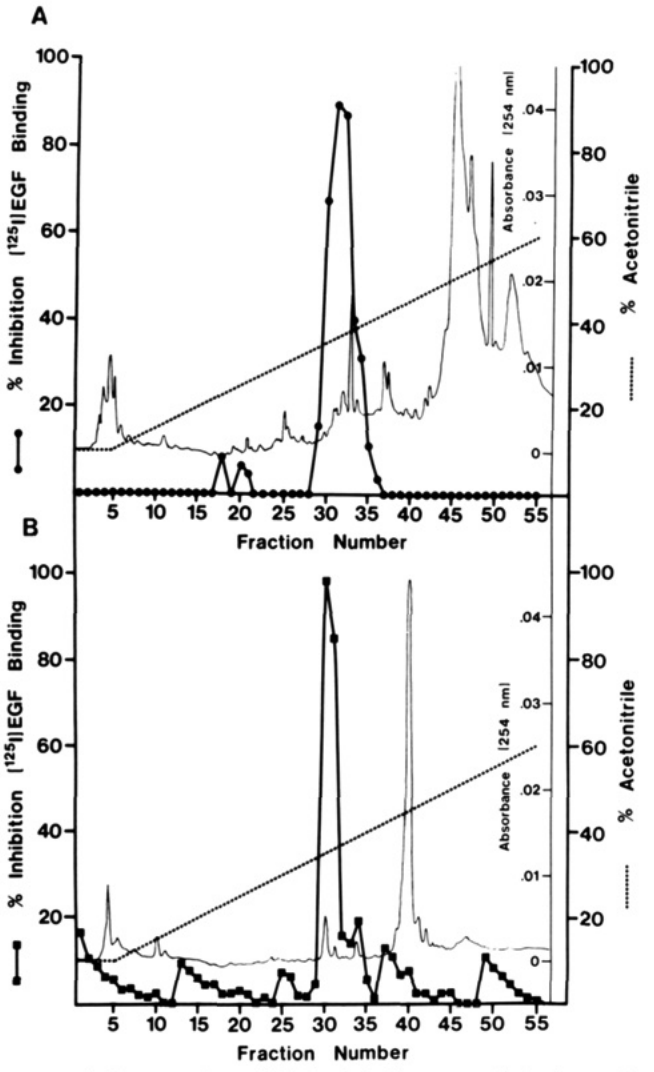

FIGURE 4: Reverse-phase HPLC of the hepatoma-derived growth factors. Pools of the high molecular weight and low molecular weight activities recovered from cationic exchange chromatography were applied to an analytical C18 column and eluted at 1 mL/min within a 50-min linear gradient of 10–60% acetonitrile in 0.05% trifluoroacetic acid (···). Aliquots of fractions were tested at 50% by volume in the hepatocyte EGF radioreceptor assay (—). Data points represent the mean value of duplicate cultures. (A) Activity profile of the higher molecular weight growth factor (●). (B) Activity profile of the lower molecular weight growth factor (■). Both factors elute between 35 and 37% acetonitrile.

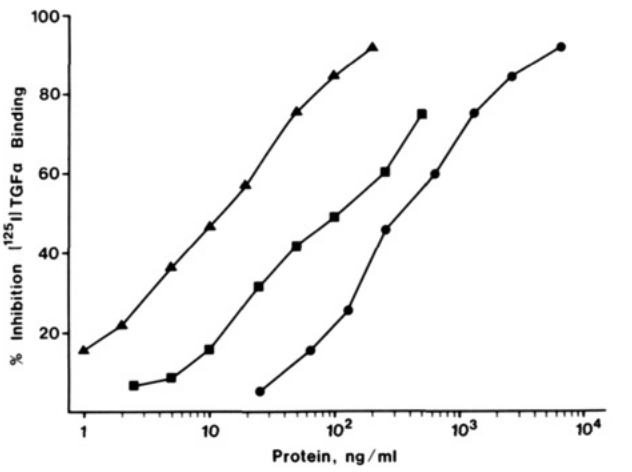

FIGURE 5: TGFα radioimmunoassay. Dilutions of synthetic rat TGFα (▲) and the low molecular weight (■) and high molecular weight (●) activities secreted by JM1 cells were reduced and denatured and tested in a TGFα-specific RIA which utilizes rabbit antibodies raised against a 17 amino acid peptide located at the carboxy terminus of the mature TGFα. Protein content in the JM1 samples was determined by amino acid analysis. Data points represent the mean value from duplicate samples.

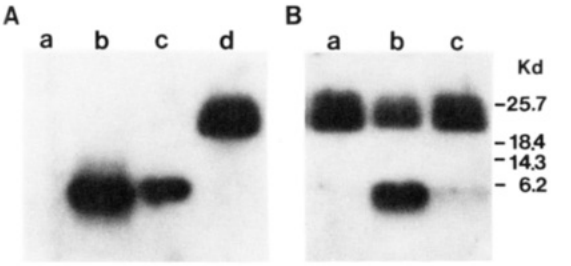

FIGURE 6: Western analysis of the TGFα proteins secreted by JM1 hepatoma cells. Aliquots of HPLC-purified high molecular weight and low molecular weight activities approximately equivalent by RIA to 200 ng of TGFα were subjected to reducing SDS–PAGE in 10–20% gradient minigels and blotted with goat antibodies to the 50 amino acid TGFα as described under Experimental Procedures. Autoradiogram shows the immunoreactivity of 500 ng of mouse EGF (lane a), 200 ng of synthetic 50 amino acid rat TGFα (lane b), and the lower molecular weight (lane c) and higher molecular weight (lane d) TGFα proteins from JM1 cells. (B) Conversion of the 21-kDa TGFα to the 6-kDa mature form by elastase. Aliquots of the higher molecular weight TGFα were incubated for 1 h at room temperature with buffer alone (lane a), 1 μg/mL pancreatic elastase (lane b), or 1 μg/mL elastase plus 50 mM *N*-acetyltrialanyl methyl ester (lane c) and analyzed by immunoblotting. Migration positions of molecular weight markers are indicated.

similar blots were incubated separately with three rabbit antisera raised against three synthetic peptides corresponding to amino acid sequences at the carboxy terminus of the rat TGFα precursor (residues 137–151, 137–159, and 153–159). These precursor–peptide-specific antibodies have been used to immunoprecipitate higher molecular weight TGFα proteins from cell lysates and membrane fractions (Gentry et al., 1987). In Western blots, each of these antisera recognized a 3-kDa peptide representing residues 137–159, which encompasses the sequences of the three synthetic antigens. However, all three antisera failed to react with either the higher or the lower molecular weight TGFα species secreted by JM1 cells (data not shown). This finding suggests that despite its apparent size, the higher molecular weight TGFα does not contain the carboxy-terminal region of the transmembrane precursor.

Analyses of the rat and human TGFα cDNA sequences revealed an Asn-Ser-Thr triplet that could serve as a potential glycosylation site (Derynck et al., 1984; Lee et al., 1985). Immunoprecipitation of labeled proteins from cells transfected with a human TGFα expression vector indicated that the precursor contained N-linked carbohydrate (Bringman et al., 1987). In vitro translation of rat TGFα mRNA in the presence of rough microsomes yielded a glycosylated protein (Teixidó et al., 1987). Therefore, we suspected that the higher molecular weight and heterogeneity of the TGFα secreted by hepatoma cells might also be due, at least in part, to glycosylation. Accordingly, aliquots of the higher molecular weight TGFα were treated with neuraminidase, *O*-glycanase, and endoglycosidase F alone, and in combination, and examined by Western blotting. The untreated protein, which migrated as a broad 21-kDa band in minigels (see Figure 6), was partially resolved in larger gradient gels as a diffuse doublet of 18–21 kDa (Figure 7, lane a). Each of the enzymes tested decreased the apparent molecular weight of these TGFα proteins. Neuraminidase caused a reduction of about 2 kDa to 16–19 kDa (lane b) but did not alter the appearance of the bands. Further digestion with *O*-glycanase caused a slight diminution in the size and intensity of the signal, particularly

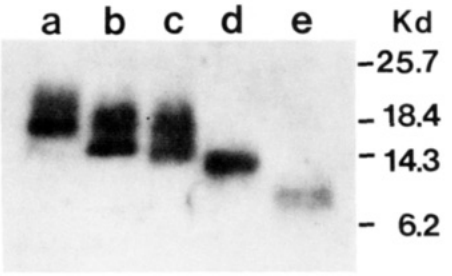

FIGURE 7: Enzymatic deglycosylation of the higher molecular weight TGFα from JM1 cells. Samples equivalent to 200 ng of TGFα activity were incubated as described under Experimental Procedures with buffer alone (lane a), neuraminidase (lane b), neuraminidase and *O*-glycanase (lane c), endoglycosidase F/glycopeptidase F (lane d), or all three enzyme preparations (lane e) and examined by Western blotting with antibodies against the 50 amino acid TGFα. Migration positions of molecular weight markers are indicated.

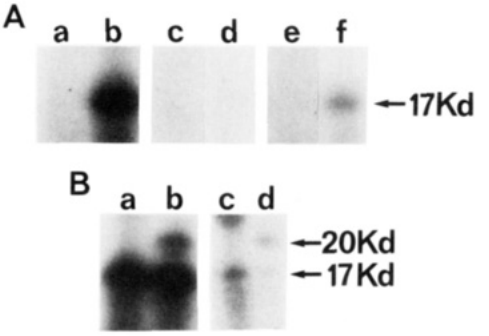

FIGURE 8: In vitro translation of TGFα mRNA from JM1 cells. (A) No products were obtained from reaction mixtures without RNA (lane a). Poly(A^+) RNA samples isolated from normal rat liver cells (lanes c and d) and JM1 rat hepatoma cells (lanes e and f) were subjected to hybridization selection with pEMBL DNA alone (lanes c and e) or with pEMBL containing the rat TGFα cDNA (lanes d and f). These RNA samples and transcripts derived from the cloned rat TGFα cDNA (lane b) were translated in vitro in a wheat germ system as described (Teixidó et al., 1987). (B) The TGFα mRNA samples derived from the partial cDNA clone (lanes a and b) and selected from JM1 cells (lanes c and d) were translated in vitro in the absence (lanes a and c) and presence (lanes b and d) of rough microsomes from dog pancreas. The higher molecular weight band (>20K) at the top of lane c was nonspecific and nonreproducible (compare with panel A, lane f).

in the lower band (lane c). Treatment with the endoglycosidase F/glycopeptidase F mixture, which hydrolyzes N-linked high mannose and complex carbohydrate (Plummer et al., 1984; Tarentino et al., 1985), sharpened and shifted the signal to a single band of 14 kDa (lane d). Sequential digestion with all three enzyme preparations, which should remove virtually all carbohydrate, produced an 11–12-kDa TGFα species (lane e). On immunoblots, this deglycosylated protein was occasionally discernible as a doublet. These results indicate that the higher molecular weight TGFα secreted by JM1 hepatoma cells contains sialic acid and N- and O-linked carbohydrate. The N-linked glycosylation appears to primarily account for the heterogeneous nature of these species.

The molecular weight of the deglycosylated TGFα was substantially lower than that predicted for the unmodified transmembrane precursor. Repeated efforts to identify the TGFα transmembrane precursor by immunoprecipitation of JM1 cell extracts failed, even though the same reagents and procedures were used to immunoprecipitate TGFα precursors from lysates of TGFα-transfected cells (Gentry et al., 1987; unpublished data). We speculate that the JM1 cells, compared to the TGFα-transfected cells, produce less of the precursor and process a greater proportion of it to soluble extracellular forms. Alternatively, we sought to confirm the size of the TGFα precursor by immunoprecipitating proteins translated from JM1 mRNA. Coupled in vitro transcription and translation of the cloned rat TGFα cDNA was previously utilized to demonstrate the integral membrane glycoprotein properties of the TGFα precursor (Teixidó et al., 1987). Although it encodes the putative precursor, this partial cDNA corresponds to the 3′ half of the natural 4.5-kb TGFα mRNA and does not include an in-frame termination codon upstream from the presumed initiation site (Lee et al., 1985). Therefore, we cannot exclude the possibility that the full-length mRNA contains other initiation sites upstream which could generate larger or additional TGFα proteins. This question is of particular relevance with regard to reports of TGFα activities of much higher molecular weights [40K and 42K (Linsley et al., 1985); 30K and 68K (Dickson et al., 1986)]. Accordingly, we immunoprecipitated the products derived from in vitro translation of full-length TGFα transcripts isolated by hybridization selection to the cloned TGFα cDNA. TGFα proteins were not detected when the translation reactions were performed in the absence of RNA (Figure 8A, lane a) or in the presence of RNA selected from normal rat liver cells (lanes c and d). However, a 17-kDa TGFα protein was immunoprecipitated from translation reactions containing hybridization-selected JM1 poly(A^+) RNA (lane f). This primary translation product comigrated with the TGFα protein obtained by in vitro transcription and translation of the cloned rat TGFα cDNA (lane b) and was not detected when JM1 RNA was selected by hybridization to vector DNA alone (lane e). Furthermore, when in vitro translation was carried out in the presence of rough microsomes, a 20-kDa product was obtained from both the JM1 TGFα transcripts and the cloned TGFα cDNA transcripts (Figure 8B, lanes b and d). The 20-kDa product has previously been shown to arise by cotranslational addition of N-linked carbohydrate to the 17-kDa protein (Teixidó et al., 1987). The 17- and 20-kDa proteins were recognized by antibodies against both the mature and precursor TGFα peptides. These data demonstrate that transcripts of the partial TGFα cDNA and the full-length TGFα mRNA from JM1 cells direct the in vitro synthesis of the same immunoreactive proteins. By comparison with the previous findings (Teixidó et al., 1987), we infer that the TGFα precursor in JM1 cells is also glycosylated and integrated into the membrane, consistent with the structure predicted by cDNA analysis (Lee et al., 1985). Finally, the observation that the completely deglycosylated higher molecular weight TGFα protein secreted by JM1 cells is only 11K suggests that it represents a partially processed, soluble form of the original 17-kDa precursor.

Discussion

The present report describes the purification and characterization of type α transforming growth factors secreted by rat hepatocellular carcinoma cells. The JM1 hepatoma cells released into their medium nearly equivalent, abundant amounts of the mature 6-kDa rat TGFα and a 18–21-kDa glycosylated intermediate form of the TGFα precursor, which was converted to the mature form by elastase treatment. In addition, in vitro translation of the 4.5-kb TGFα mRNA from JM1 cells yielded a 17 kDa protein which was converted to 20 kDa in the presence of microsomes. The 17- and 20-kDa TGFα species correspond respectively to the unmodified and glycosylated transmembrane precursor encoded within the rat TGFα cDNA (Teixidó et al., 1987). The finding that the same molecular weight proteins were synthesized from both the full-length TGFα mRNA and the transcript from the partial

TGFα cDNA supports the previously assigned initiation site (Lee et al., 1985).

The higher molecular weight and heterogeneity of the 18–21-kDa TGFα species secreted by JM1 cells were found to be due to glycosylation at Asn and Ser/Thr residues. Removal of sialic acid and O- and N-linked carbohydrate by sequential enzymatic deglycosylation yielded an 11-kDa immunoreactive protein. This molecular weight is significantly lower than the 17K molecular weight predicted and confirmed here for the unmodified primary translation product of the TGFα mRNA. However, 11K closely approximates the molecular weight calculated for that portion of the TGFα precursor which extends from the amino terminus up to, but not into, the transmembrane domain. In vitro translation experiments have established that this segment of the TGFα precursor retains the signal sequence and protrudes outside the membrane, whereas the carboxy-terminal region lies inside the cell (Teixidõ et al., 1987). The TGFα proteins in medium conditioned by JM1 cells failed to react in Western blots with antibodies directed against various sequences within the carboxy-terminal region of the precursor, suggesting that they lack the cytoplasmic domain. Therefore, these findings support the conclusion that the soluble, bioactive higher molecular weight TGFα species correspond to the glycosylated extracellular domains released from the transmembrane precursor.

The TGFα species produced by JM1 hepatoma cells resemble those secreted by Chinese hamster ovary cells transfected with a partial human TGFα cDNA (Bringman et al., 1987) and Fischer rat embryo cells transformed by feline sarcoma virus (Ignotz et al., 1986; Teixidõ & Massaguê, 1988). Immunoprecipitation of medium conditioned by the transfected cells with antibodies against human recombinant TGFα identified a single 5-kDa and diffuse multiple 18-kDa protein bands. Treatment with *N*-glycanase alone (peptide:*N*-glycosidase F) converted the 18-kDa species to 13.5- and 15-kDa species. In another study, Western blotting with antibodies to the carboxy-terminal region of mature rat TGFα detected a doublet of 17- and 19-kDa proteins purified from the medium of feline sarcoma virus transformed cells (Ignotz et al., 1986). These TGFα species were not recognized by antibodies directed against carboxy-terminal regions of the transmembrane precursor. The 17- and 19-kDa proteins also contained sialic acid and O-linked carbohydrate in addition to complex N-linked carbohydrate, and chemical deglycosylation (with trifluoromethanesulfonic acid) decreased their molecular weight to 11K (Teixidõ & Massaguê, 1988). Thus, the soluble higher molecular weight TGFα proteins secreted by the transfected, virally transformed, and chemically transformed cells share common structural properties expected for the extracellular domain of the transmembrane precursor.

Several other reports have described higher molecular weight TGFα species. For example, higher molecular weight forms comprised the predominant TGFα activity extracted from lysates or supernatants of human rhabdomyosarcoma [18K (Dart et al., 1985); 15–48K (Stromberg et al., 1986)], melanoma [22.5K (De Larco et al., 1985)], and mammary carcinoma cells [30K and 68K (Dickson et al., 1986)] and from human platelets [24K (Assoian et al., 1984)] and the urine of cancer patients [30K (Kimball et al., 1984)]. Multiple larger TGFα proteins (24K, 40K, and 42K) have been found in conditioned medium from retrovirus transformed cells (Linsley et al., 1985). However, it remains unclear whether these proteins correspond to all or part of the TGFα precursor. Their higher molecular weight could be due to glycosylation or association with other proteins. While some of these various species, particularly the extracellular proteins, might be similar in structure to the higher molecular weight TGFα secreted by JM1 cells, direct comparisons are limited or complicated by the use of different assays to detect the TGFα species, as well as different methods to estimate their size.

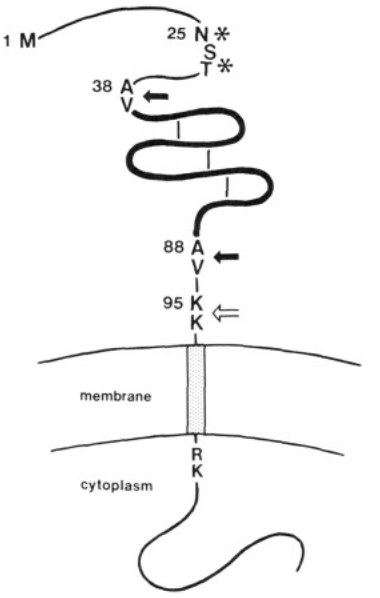

FIGURE 9: Structural model of the TGFα precursor. The transmembrane domain (shaded box) is flanked by pairs of basic amino acids. The Met at the amino terminus is the previously assigned initiation site. The N- and O-linked glycosylation presumably occurs within the Asn-Ser-Thr sequence beginning at residue 25 (asterisks). The mature, 50 amino acid TGFα (bold line) is released by cleavage at both ends between Ala and Val residues (bold arrows) by an elastase-like enzyme. The glycosylated extracellular domain could be released by preferential cleavage at the Ala^{88}-Val^{89} site or by cleavage at the Lys^{95}-Lys^{96} site (open arrow) by a protease with trypsin-like specificity.

The characterization of soluble, bioactive higher molecular weight TGFα species raises questions regarding the specificity and significance of precursor processing. The terms "meso TGFα" and "pro TGFα" have been proposed to denote, respectively, the soluble intermediate and membrane-bound precursors (Teixidõ & Massaguê, 1988). Meso TGFα could be generated by preferential cleavage of pro TGFα at the Ala^{88}-Val^{89} site by an elastase-like enzyme (see Figure 9). Cleavage at the Ala^{38}-Val^{39} site, as well as cleavage of the signal peptide, might be hindered by heavy glycosylation at proximal residues. In those instances where the elastase-type activity is limiting, mature TGFα may be deficient or absent. However, meso TGFα could still be released by cleavage at the Lys^{95}–Lys^{96} site by an alternate protease with trysin-like specificity. Processing at either or both sites may depend upon a particular cell type or environment. Variable cleavage at both sites would release meso TGFα species differing in molecular weight by less than 1K, which could explain the observance of doublet protein bands in some deglycosylated samples. Since both mature TGFα and meso TGFα are bioactive, precursor processing need not be complete or even precise.

Most studies of TGFα production have been performed with transfected cells, virally transformed fibroblasts, and human tumor cells. The present study demonstrates that the model of TGFα secretion and processing also applies to chemically transformed epithelial cells. Relatively high levels of expression of both TGFα and EGF receptor mRNA have been found in human tissue or cell lines from squamous carcinomas and renal carcinomas (Derynck et al., 1987). TGFα and TGFβ mRNA

was expressed in the HepG2 human hepatoma cell line. Furthermore, TGFα protein levels measured by RIA were significantly elevated in the urine of patients with hepatocellular carcinoma (Yeh et al., 1987). Thus, TGFα may play a role in the development or progression of tumors of the liver and other epithelia. The rat liver tumor from which the JM1 cells were derived was induced with an initiation and promotion protocol (Novicki et al., 1983). Administration of hepatocarcinogens and liver tumor promoters has been shown to decrease EGF receptor binding or phosphorylation in hepatic membranes by as yet undetermined mechanisms (Carr et al., 1986; Lev-Ran et al., 1986; Madhukar et al., 1984). JM1 cells (or their preneoplastic progenitor cells) could have acquired the ability to produce TGFα in vivo, during early or late stages in chemical carcinogenesis, or in vitro, during clonal selection. The effects of hormones, growth factors, and oncogenes in vivo can often be simulated in vitro. Primary cultures of rat hepatocytes respond to TGFα and thereby provide a useful system for investigating its function in growth regulation.

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REFERENCES

Assoian, R. K., Grotendorst, G. R., Miller, D. M., & Sporn, M. B. (1984) *Nature (London) 309*, 804–806.

Bringman, T. S., Lindquist, P. B., & Derynck, R. (1987) *Cell 48*, (*Cambridge, Mass.*) 429–440.

Carr, B. I., Roitman, A., Hwang, D. L., Barseghian, G., & Lev-Ran, A. (1986) *JNCI, J. Natl. Cancer Inst. 77*, 219–225.

Cochet, M., Perrin, F., Gannon, F., Krust, A., Chambon, P., McKnight, G. S., Lee, D. C., Mayo, K. E., & Palmiter, R. (1979) *Nucleic Acids Res. 6*, 2435–2452.

Coffey, R. J., Derynck, R., Wilcox, J. N., Bringman, T. S., Goustin, A. S., Moses, H. L., & Pittelkow, M. R. (1987) *Nature (London) 328*, 817–820.

Dart, L. L., Smith, D. M., Meyers, C. A., Sporn, M. B., & Frolik, C. A. (1985) *Biochemistry 24*, 5925–5931.

De Larco, J. E., & Todaro, G. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4001–4005.

De Larco, J. E., Pigott, D. A., & Lazurus, J. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5015–5019.

Derynck, R. (1986) *J. Cell. Biochem. 32*, 293–304.

Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., & Goeddel, D. V. (1984) *Cell (Cambridge, Mass.) 38*, 287–297.

Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., & Berger, W. H. (1987) *Cancer Res. 47*, 707–712.

Dickson, R. B., Bates, S. E., McManaway, M. E., & Lippman, M. E. (1986) *Cancer Res. 46*, 1707–1713.

Gentry, L. E., Twardzik, D. R., Lim, J., Ranchalis, J. E., & Lee, D. C. (1987) *Mol. Cell. Biol. 7*, 1585–1591.

Han, V. K. M., Hunter, S., Pratt, R. M., Zendegui, J. G., & Lee, D. C. (1987) *Mol. Cell. Biol. 7*, 2335–2343.

Hazarika, P., Pardue, R. L., Earls, R., & Dedman, J. R. (1987) *Biochemistry 26*, 2067–2070.

Ignotz, R. A., Kelly, B., Davis, R., & Massagué, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6307–6311.

Kimball, E. S., Bohn, W. H., Cockley, K. D., Warren, T. C., & Sherwin, S. A. (1984) *Cancer Res. 44*, 3613–3619.

Lee, D. C., Rose, T. M., Webb, N. R., & Todaro, G. J. (1985) *Nature (London) 313*, 489–491.

Lev-Ran, A., Carr, B. I., Hwang, D. L., & Roitman, A. (1986) *Cancer Res. 46*, 4656–4659.

Linsley, P. S., Hargreaves, W. R., Twardzik, D. R., & Todaro, G. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 356–360.

Luetteke, N. C., & Michalopoulos, G. K. (1985) *Cancer Res. 45*, 6331–6337.

Madhukar, B. V., Brewster, D. W., & Matsumura, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7407–7411.

Marquardt, H., Hunkapiller, M. W., Hood, L. E., & Todaro, G. J. (1984) *Science (Washington D.C.) 223*, 1079–1082.

Massagué, J. (1983) *J. Biol. Chem. 258*, 13614–13620.

Michalopoulos, G. K., Cianculli, H. D., Novotny, A. R., Kligerman, A. D., Strom, S. C., & Jirtle, R. L. (1982) *Cancer Res. 42*, 4673–4682.

Michalopoulos, G. K., Houck, K. A., Dolan, M. L., & Luetteke, N. C. (1984) *Cancer Res. 44*, 4414–4419.

Naughton, M. A., & Sanger, F. (1961) *Biochem. J. 78*, 156–163.

Novicki, D. L., Jirtle, R. L., & Michalopoulos, G. (1963) *In Vitro 19*, 191–202.

Plummer, T. H., Elder, J. H., Alexander, S., Phelan, A. W., & Tarentino, A. L. (1984) *J. Biol. Chem. 259*, 10700–10704.

Samsoondar, J., Kobrin, M. S., & Kudlow, J. E. (1986) *J. Biol. Chem. 261*, 14408–14413.

St. Hilaire, R. J., & Jones, A. L. (1982) *Hepatology 2*, (*N.Y.*) 601–613.

Stromberg, K., Hudgins, W. R., Fryling, C. M., Hazarika, P., Dedman, J. R., Pardue, R. L., Hargreaves, W. R., & Orth, D. N. (1986) *J. Cell. Biochem. 32*, 247–259.

Tarentino, A. L., Gomez, C. M., & Plummer, T. H. (1985) *Biochemistry 24*, 4665–4671.

Teixidó, J., & Massagué, J. (1988) *J. Biol. Chem. 263*, 3924–3929.

Teixidó, J., Gilmore, R., Lee, D. C., & Massagué, J. (1987) *Nature (London) 326*, 883–885.

Umemoto, J., Bhavanandna, V. P., & Davidson, E. A. (1977) *J. Biol. Chem. 252*, 8609–8614.

Yeh, Y., Tsai, J., Chuang, L., Yeh, H., Tsai, J., Florine, D. L., & Tam, J. P. (1987) *Cancer Res. 47*, 896–901.