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Transforming Growth Factor α: Expression, Regulation, and Biological Activities*

DAVID C. LEE†, SUZANNE E. FENTON, EUGENE A. BERKOWITZ, AND M. ANDREW HISSONG

Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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† To whom correspondence should be addressed: Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599–7295.

I. Introduction

Since its discovery in the mid-1970s, TGFα‡ has been one of the most intensely studied polypeptide growth factors. This level of interest undoubtedly reflects not only evidence of EGF-R signaling in a wide array of tissues and cell types, but also the recognition that as a result of its deregulated expression, TGFα may be a frequent contributor to neoplastic progression. Moreover, TGFα is a prominent member of a class of polypeptide growth factors that is distinguished by the fact that soluble forms are proteolytically derived from integral membrane precursors. As a result of studies in several laboratories, proTGFα has emerged as an experimental paradigm for exploring the activities and roles of these precursor molecules. The aim of this review is to present a contemporary summary of investigations into these various areas of interest, with a goal of demonstrating the degree to which our understanding of this growth factor has matured over the past 15 years. Although every effort has been made to produce a comprehensive review, the final product almost certainly reflects the interests of its authors, and for this reason, valuable contributions by colleagues may not have always received the emphasis they deserve. Conversely, some topics may have been covered in excessive detail. There is no doubt that time will reveal any such errors in judgement. (For another recent review, see Derynck, 1992.)

‡ Abbreviations: TGFα, transforming growth factor α; EGF, epidermal growth factor; EGF-R, EGF-receptor; proTGFα, integral membrane form of TGFα; preproEGF, EGF precursor; VGF, vaccinia virus growth factor; HB-EGF, heparin-binding EFG; NRK, normal rat kidney; NDF, neu differentiation factor; CHO, Chinese hamster ovary; DNA, deoxyribonucleic acid; TNF, tumor necrosis factor; KL, c-*kit* ligand; TPA, 12-O-tetradecanoylphorbol-13-acetate; mRNA, messenger ribonucleic acid; LHRH, luteinizing hormone-releasing hormone; *wa*-1, waved-1; *wa*-2, waved-2; MT, metallothionein; UT, untranslated; kb, kilobase; A, adenine; C, cytosine; G, guanine; T, thymine.

II. Discovery and Initial Characterization

A. Sarcoma Growth Factor

The discovery of TGFα stemmed from observations that, relative to their normal counterparts, cells transformed by murine and feline sarcoma viruses showed reduced binding of exogenous EGF (Todaro et al., 1976; Todaro and De Larco, 1978). The effect was specific in that binding of insulin-like growth factor-II was not decreased (Todaro et al., 1977). These results suggested that sarcoma viruses encode a gene product that is functionally related to EGF and therefore able to compete for binding to the EGF-R. Trypsin- and dithiothreitol-sensitive, heat-stable activities of approximately 25,000, 12,000, and 7000 kDa were subsequently identified in media conditioned by sarcoma virus-transformed fibroblasts, and these were shown to compete with EGF for binding to its receptor (De Larco and Todaro, 1978). However, they were not recognized by anti-EGF antibodies, and their apparent ability to induce a reversible phenotypic transformation of normal cultured fibroblasts, as evidenced by anchorage-independent growth, also distinguished them from pure EGF. As a result, these activities were collectively named "sarcoma growth factor." Subsequent observations that similar activities were produced by various human tumor cell lines (Todaro et al., 1980) and by chemically transformed cells (Roberts et al., 1980) suggested that they were derived from cellular and not viral genes and prompted a change in nomenclature to "transforming growth factors." It is also led to enunciation of the autocrine hypothesis, namely that production of polypeptide

growth factors by cells capable of responding to those factors might promote the transformation of these same cells (Todaro and De Larco, 1978; Sporn and Todaro, 1980).

B. Distinct Classes of Transforming Growth Factors

In 1981, Roberts and colleagues described a new transforming growth factor activity, isolated from a variety of non-neoplastic tissues, whose ability to stimulate anchorage-independent growth was potentiated by exogenous EGF. This observation was rapidly followed by the demonstration that transforming growth factor activity from sarcoma virus-transformed cells could be separated into two, synergistically acting subsets by high-performance liquid chromatography (Anzano et al., 1982, 1983). The EGF-like component that bound (Massagué, 1983) and exerted its actions through (Carpenter et al., 1983) the EGF-R was named TGFα. The component whose colony-promoting activity was potentiated by EGF or TGFα was designated TGFβ. The realization that this latter component was present in early preparations of sarcoma growth factor provided an explanation not only for the apparent difference between the latter's activities and those of pure EGF, but also for the identification of an additional, nonEGF receptor that bound sarcoma growth factor but not EGF (Massagué et al., 1982). Subsequently, the NH_2-terminal sequences of purified human, rat, and mouse TGFα were reported (Marquardt et al., 1983), followed by the complete amino acid sequence of the mature rat protein (Marquardt et al., 1984) (fig. 1). A comparison of this sequence with those of mouse and human EGFs revealed clear relatedness, particularly with respect to preservation of the six cysteine residues. Later characterization of TGFβ confirmed it to be a structurally unrelated protein (Roberts and Sporn, 1990). As a result of studies in many laboratories, we now appreciate that TGFβ is a multifunctional protein whose various activities include regulation of immune function, extracellular matrix production, and cell growth and differentiation. Although it can stimulate the growth of fibroblasts, Its TGFβ is growth-inhibiting for many epithelial cells. Its co-identification with TGFα was an accident of the particular assay used in the early studies.

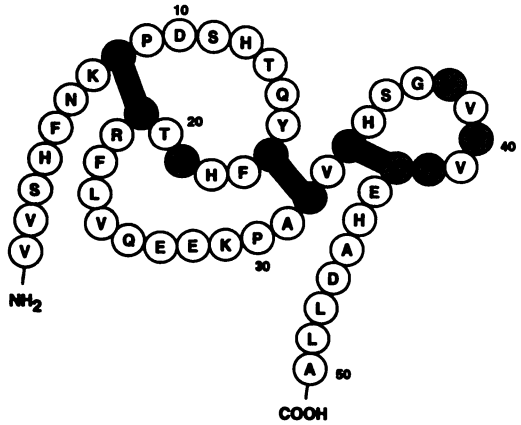

FIG. 1. The amino acid sequence of the mature, fully processed rat TGFα. Note that the sequence of the mature mouse protein is identical. Residues conserved by all EGF-R ligand family members are shaded, and disulfide bonds are indicated by heavy lines.

III. The EGF Superfamily

A. EGF Receptor Ligands

1. Family members. EGF and TGFα are now known to be members of a larger family of EGF-R ligands. Members of this family are recognized by a signature sequence of 36 to 37 amino acids having the following consensus: $CX_7CX_{2-3}GXCX_{10}CXCX_3YXGXRC$. In each case, this signature element (referred to hereafter as the EGF-like sequence) is contained within the external domain of an integral-membrane protein, and is released from the cell surface in the context of larger peptides as a result of proteolytic cleavage. For example, the integral-membrane, glycoprotein precursor to soluble TGFα (proTGFα) (fig. 2) is 159/160 amino acids in length (Derynck et al., 1984; Lee et al., 1985a), whereas the smallest, biologically active soluble form containing the EGF-like sequence is composed of 50 amino acids. Disulfide bonds formed between the first and third, second and fourth, and fifth and sixth cysteines of the EGF-like sequence result in the formation of a compact, three-loop structure that is essential for high-affinity binding to the EGF-R. In addition to EGF and TGFα, three related cellular EGF-R ligands have been identified. These are amphiregulin (Shoyab et al., 1988, 1989), heparin-binding EGF (Higashiyama et al., 1991), and betacellulin (Sasada et al., 1993; Shing et al., 1993). A potential sixth member, Schwannoma-derived growth factor (Kimura et al., 1990), is 70% homologous with amphiregulin and may, like human keratinocyte autocrine factor (Cook et al., 1991), correspond to the same gene product. An amino acid sequence comparison of the receptor-binding domains of these molecules (table 1) reveals that in addition to the six cysteines, only two glycines together with single tyrosine and arginine residues are conserved throughout the family. The comparison also indicates that of the five EGF-R ligands, betacellulin is most closely related to TGFα. Although the cellular protein cripto (Ciccodicola et al., 1989) has been referred to as a member of the EGF-R ligand family, variations in the positioning of its cysteine residues relative to the consensus sequence described above indicate that it is not an EGF-R ligand.

As previously mentioned, the cellular EGF-R ligand family is characterized by the fact that soluble forms are derived from integral membrane precursors (for a recent review of membrane-anchored growth factors, see Massagué and Pandiella, 1993); preproEGF is remarkable both with respect to its size (approximately 1200 amino acids), and the fact that its extracellular domain contains, besides the bona fide EGF sequence, eight other

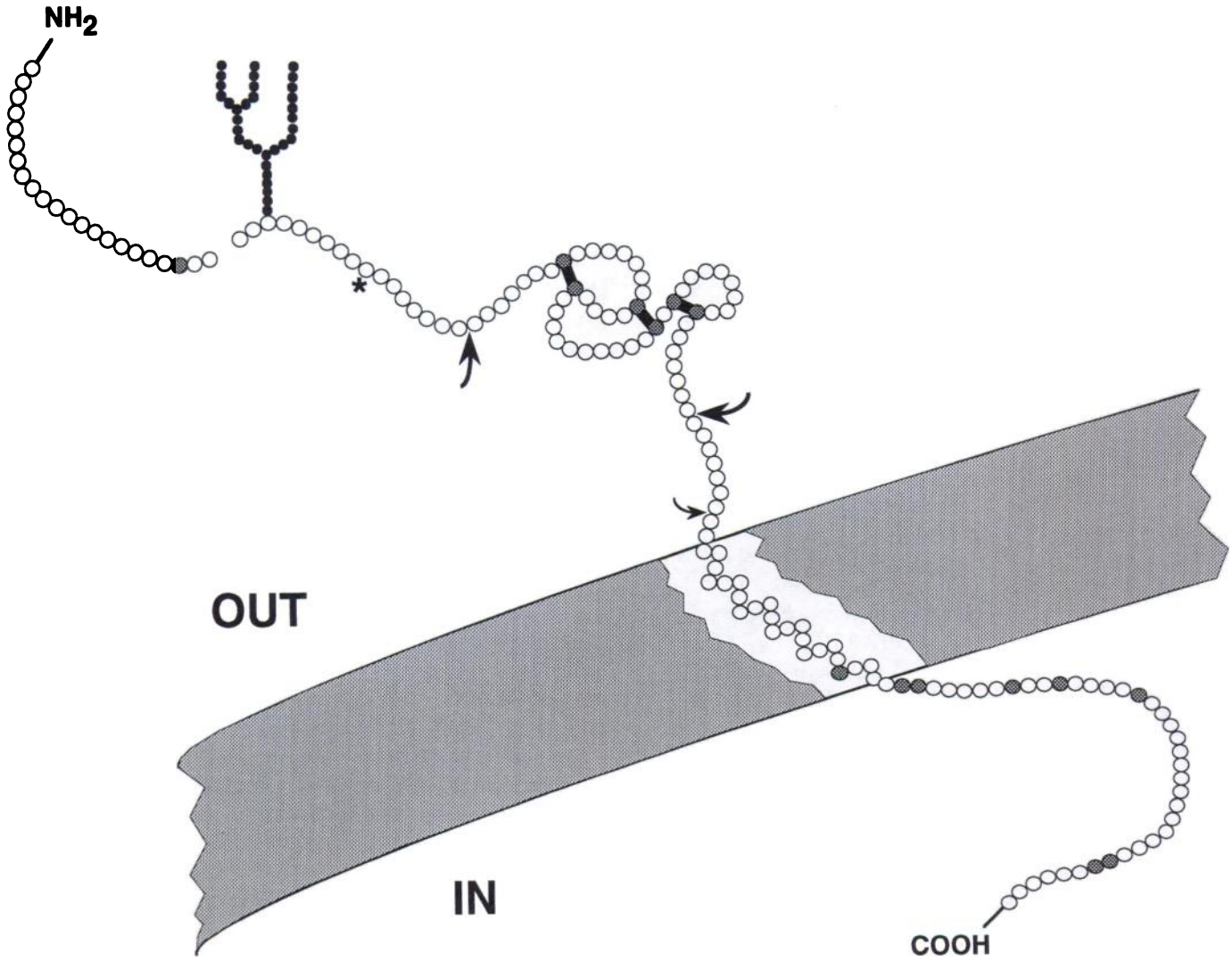

FIG. 2. A schematic representation of the 159/160-amino-acid rat TGFα precursor protein (proTGFα). Consistent with its biochemical properties, proTGFα is shown embedded in the plasma membrane as a glycoprotein. The putative signal peptide is shown detached from the remainder of the precursor. A site of N-glycosylation is marked by the branched structure of filled circles (note that the precursor is also modified by O-glycosylation). The location of a variably present alanine residue (see VII.A.2) is denoted by an asterisk, and cysteine residues are hatched. Disulfide bonds formed between cysteine residues in the mature protein are represented by heavy lines. Large arrows denote cleavage events that release the mature, 50-amino-acid TGFα; a light arrow denotes a lysine-lysine dipeptide immediately external to the membrane that could be cleaved to release larger, soluble forms of proTGFα.

EGF-like sequences that are roughly organized into two clusters (Scott et al. 1983; Gray et al. 1983). There is no evidence of any of these additional EGF-like sequences functioning as ligands, and variable deviations in the spacing of the cysteines likely preclude them from binding to the EGF-R. Precursors to the other EGF-R family members are similar to proTGFα in that they are considerably smaller proteins, and each contains only a single EGF-like sequence. With the exception of the actual EGF-like units, the various precursors display no significant degree of homology with one another. Interestingly, there is a commonality to the organization of the genes that encode the EGF-R ligands in that the three-loop structure is invariably encoded by two distinct exons (Bell et al., 1986; Plowman et al., 1990a). The 5′ exon encodes the first two disulfide loops, whereas the 3′ exon encodes the third loop together with the transmembrane domain of the precursor (fig. 3). Indeed, this organization may be diagnostic of cellular EGF-R ligands, inasmuch as exons encoding EGF-related sequences that do not function as ligands show no such consistency (e.g., as evidenced by preproEGF) (Bell et al., 1986).

2. Related viral proteins. Pox family viruses encode related EGF-R ligands, the best characterized of which is VGF (Blomquist et al., 1984; Brown et al., 1985; Stroobant et al., 1985). VGF is a 140-amino-acid integral membrane protein that contains a single EGF-like sequence in its extracellular domain. Soluble VGF seems to be derived by a cleavage event that releases the entire

TABLE 1

Sequence comparison of EGF-R ligands*

	1 10 20 30 40 50	Homology with TGFα (%)
hTGFα:	D F D A	
r/mTGFα:	VVSHFNK **C** PDSHTQY **C** FH- **G** T **C** RFLVQEEKPA **C** V **C** HSG **Y** V **G** V **RC** EHADLLA	
mβC:	VKTHFSR **C** PKQYKHY **C** IH- **G** R **C** RFVVDEQTPS **C** I **C** EKG **Y** F **G** A **RC** ERVDLFY	50
mEGF:	NSYPG **C** PSSYDGY **C** LNG **G** V **C** MHIESLDSYT **C** N **C** VIG **Y** S **G** D **RC** QTRDLRWWELR	32
hHB-EGF:	LGKKRDP **C** LRKYKDF **C** IH- **G** E **C** KYVKELRAPS **C** I **C** HPG **Y** H **G** E **RC** HGLSLPV	32
hAR:	NRKKKNP **C** NAEFQNF **C** IH- **G** E **C** KYIEHLEAVT **C** K **C** QQE **Y** F **G** E **RC** GEKSMKT	26
hHRGα:	GTSHLVK **C** AEKEKTF **C** VNG **G** E **C** FUVKDLSNPSRYL **C** K **C** QPG F T **G** A **RC** TENVPMK	

* Amino acid sequence comparison of the EGF-like sequence of the known EGF-R ligands. A partial sequence of human heregulin (hHRGα) is also shown for comparison—note the differential cysteine spacing. Ligands are abbreviated as follows: βC, betacellulin; HB-EGF, heparin-binding EGF; AR, amphiregulin. Note that, in each case, the amino acid sequence shown is contained within a larger, integral-membrane protein. Numbering is relative to the mature TGFα protein. Gaps are inserted in the sequences of the EGF-R ligands for optimal alignment. Amino acids that show overall conservation are bolded. Those in the EGF-R ligands that are conserved relative to TGFα are underlined. The degree of homology with TGFα from the relevant species is indicated. Note that of the various family members, betacellulin is most closely related, whereas amphiregulin shows the lowest degree of homology. Amino acid abbreviations are as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Other abbreviations: h, human; r, rat; m, mouse.

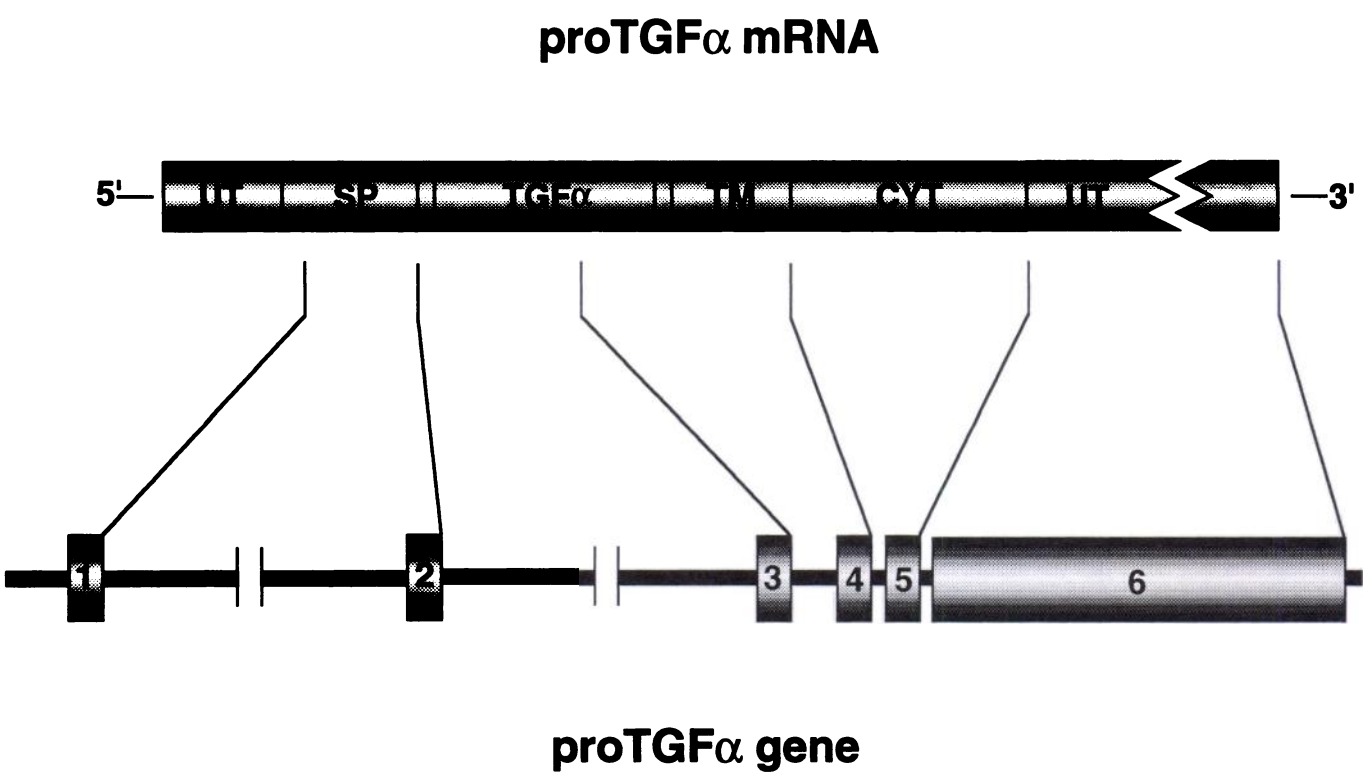

FIG. 3. A diagrammatic representation of rat TGFα mRNA and coding sequences. Above, the organizational domains of the 4.5 to 4.8 kb mRNA. UT, untranslated sequence; SP, signal peptide; TGFα, the mature, 50-amino-acid protein; TM, transmembrane domain; CYT, cytoplasmic domain. Below, the exon/intron organization of the 85 to 90 kb rat TGFα gene. Exons are indicated by large, shaded bars and numbered 1 to 6.

77-amino-acid extracellular domain. A distinction from other EGF-R ligands is the presence within the EGF-like sequence of two canonical N-glycosylation sites, at least one of which seems to be utilized. Despite this modification, VGF binds to the EGF-R with high affinity and is mitogenic for fibroblasts and keratinocytes. Expressed in infected cells during the early phase of viral replication, VGF may facilitate viral infection by inducing hyperproliferation of adjacent uninfected cells (Buller et al., 1988). A truncated form of VGF was reported to act as an EGF-R antagonist (i.e., bind the receptor without inducing mitogenesis) for some cell lines (Lin et al., 1990). Related proteins that are encoded by the Shope fibroma (Chang et al., 1987) and myxoma (Upton et al., 1987) viruses contain putative signal peptides but seem to lack transmembrane domains, suggesting that they may be directly secreted. A 16 kDa intracellular form of the Shope fibroma growth factor was converted to 6 kDa (approximately the size of the mature EGF-like sequence) in the presence of tunicamycin, indicating that (like VGF) it is glycosylated (Chang et al., 1990).

3. Differences in biological response. The five cellular ligands are presumed to activate EGF-R signaling pathways in a similar manner (fig. 4). Nevertheless, quantitative differences in biological response have been reported. For example, TGFα was found to be a more potent stimulator of keratinocyte migration (Barrandon and Green, 1987) and angiogenesis (Schreiber et al., 1986), and some distinctions in biological response have persisted, even when higher doses of EGF are used. Thus, in certain contexts, the effects of TGFα have been described as being stronger and/or more prolonged than those of EGF. Other indications of differences in biological activity include the findings that (*a*) HB-EGF was 40-fold more potent than EGF in stimulating smooth aortic muscle cell proliferation (Higashiyama et al., 1991), and (*b*) amphiregulin, which promoted the proliferation of BALB/MK cells in a manner similar to that of EGF, was (in contrast to EGF or TGFα) unable to induce anchorage-independent growth of NRK cells in the presence of TGFβ (Shoyab et al., 1989). Some of these ligand-specific differences could reflect both qualitative and quantitative distinctions in the interactions between the various ligands and the EGF-R. For instance, amphiregulin does not fully displace ^{125}I-EGF bound to EGF-R on the surface of human A431 cells (Shoyab et al., 1989). HB-EGF, amphiregulin, and betacellulin are distinguished from EGF and TGFα by an affinity for heparin, a property that is apparently conferred by hydrophilic sequences located NH_2-terminal to the EGF-like sequence in each case. Thus, binding to heparin could influence presentation of these ligands to the EGF-R in a manner analogous to that described for fibroblast growth factors (Yayon et al., 1991). It could also be at least partially responsible for observed differences in biological response, including the fact that HB-EGF has

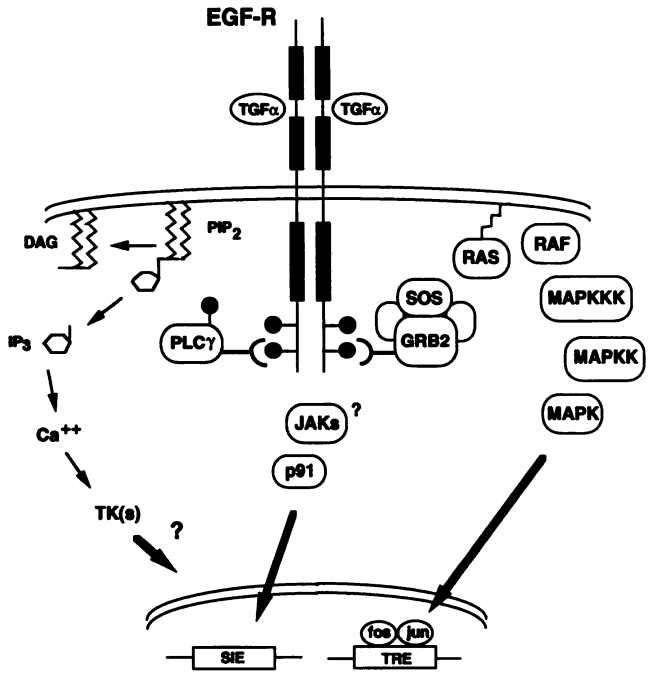

FIG. 4. EGF-R signaling pathways. Binding of ligand induces dimerization of the EGF-R, with concomitant activation of the receptor's intrinsic tyrosine kinase. The resultant autophosphorylation of tyrosine residues in the receptor's COOH-terminal portion produces docking sites to which signaling intermediates (e.g., phospholipase Cγ and GRB2) bind via SH2 domains. Currently, EGF-R is believed to signal through at least three distinct pathways: (*a*) a Ras/Raf/mitogen-activated protein kinase-dependent pathway that is associated with the activation of Fos/Jun; (*b*) a phospholipase C-γ-mediated activation of one or more Ca^{2+}-dependent tyrosine kinase(s); and (*c*) a newly elucidated pathway involving the activation of latent transcription factors (e.g., p91) which then translocate from the cytoplasm to the nucleus. By analogy with cytokine receptors, this latter pathway may involve Janus family kinases. Cysteine-rich sequences in the extracellular domain of the EGF-R are shown as solid boxes, the tyrosine kinase portion of the cytoplasmic sequence as a hatched box, and autophosphorylation sites as solid circles.

a greater affinity for EGF-R on smooth muscle cells than does EGF (Higashiyama et al., 1991). With regard to TGFα and EGF, it is unfortunate that comparisons of their relative EGF-R binding affinities have generally been confounded by the frequent use of components derived from different species. That species-specific differences in ligand binding could be significant is suggested by the finding that chicken EGF-R binds human TGFα with 100-fold greater affinity than murine EGF (Lax et al., 1988). Based on comparisons with homologous components derived from the same species, Ebner and Derynck (1991) recently reported that EGF and TGFα bind EGF-R with similar affinities. However, they made the intriguing observation for mouse L cells and human keratinocytes that, whereas both receptor and ligand were preferentially recycled to the cell surface from an internalized TGFα/EGF-R complex, they were efficiently degraded from an EGF/EGF-R complex. This difference in routing, which may result from the fact that TGFα dissociates from the EGF-R at a much higher pH than EGF, could account for some of the observed differences in the biological potencies of these two ligands. Suggesting that such differences in processing can be cell-type-specific, Korc and Finman (1989) described differential degradation of TGFα versus EGF in several cell lines, and Garcia and coworkers (1989) identified growth factor degrading activities that showed a preference for TGFα. Regardless of the underlying mechanism(s), mounting evidence of differences in biological responsiveness points to the desirability of using physiologically relevant ligands in in vitro studies. Given its more restricted pattern of expression, it is unfortunate that EGF is most commonly used as an exogenous ligand.

B. Related Ligands

1. Mammalian ligands. Three EGF-R-related proteins have been identified: the HER2/erbB2/neu proto-oncogene (Bargmann et al., 1986), HER3/erbB3 (Kraus et al., 1989; Plowman et al., 1990b), and HER4/erbB4 (Plowman et al., 1993a). The identification of these orphan receptors has initiated an intense search for their respective ligands. Human and rat proteins that were originally thought to serve as ligands for HER2/neu have been cloned. Heregulin was isolated from media conditioned by a human breast cancer cell line (Holmes et al., 1992), and NDF was isolated from ras-transformed rat fibroblasts (Peles et al., 1992; Wen et al., 1992). Although these factors were initially identified on the basis of their ability to induce tyrosine phosphorylation of HER2/neu, very recent studies have shown that heregulin is unable to induce phosphorylation of HER2/neu in the absence of HER4 (Plowman et al., 1993b; Peles et al., 1993). Hence, HER4 may transphosphorylate HER2/neu upon binding of heregulin/NDF. In addition, HER3 has been shown to function as a receptor for heregulin/NDF and to form heterodimers with HER2 (Carraway III et al., 1994). In contrast, HER2 does not directly bind hergulin/NDF; its ligand has not yet been identified.

At least four distinct heregulins of 640, 645, 637 and 241 amino acids, and multiple NDF species (Wen et al., 1994), are generated from single genes by alternate splicing. Although they lack a canonical signal peptide, NDF and the larger forms of heregulin are predicted to be integral membrane glycoproteins that, like the precursors to the EGF-R ligands, contain a single EGF-like sequence in their ectodomains in close proximity to the transmembrane domain. Soluble ligands may be generated from these transmembrane precursors by proteolytic release of the entire ectodomain. Interestingly, the smaller form of heregulin lacks a membrane anchor, suggesting that it might be directly secreted. The EGF-like sequence of heregulin/NDF is most closely related to HB-EGF, with which it also shares the property of heparin binding. However, the spacing between the third and fourth cysteines (and, hence, the size of the second

disulfide loop) is increased by three amino acids relative to EGF-R ligand family members, and tyrosine[38], which is conserved throughout the EGF-R ligand family, is replaced. In light of these differences, it is not surprising that heregulin/NDF do not bind to the EGF-R. Curiously, NDF was reported to inhibit the growth of cultured human breast cancer cells and induce their differentiation (hence, the name) (Peles et al., 1992). This is a surprising observation, given the fact that overexpression of HER2/neu is associated with cell transformation.

Sheng et al. (1992) recently cloned a transmembrane protein component of a mammary adenocarcinoma sialomucin complex. The predicted 744-amino-acid protein contains a relatively large, heavily glycosylated extracellular domain with two EGF-like sequences flanking the transmembrane domain. Variations in the spacing of the cysteines relative to those in EGF-R ligands suggest that neither sequence directly interacts with the EGF-R. However, they could be ligands for a related receptor.

2. Nonmammalian proteins. The *spitz* (*spi*) gene of *Drosophila* is required for normal dorsal-ventral axis formation and neurogenesis. The predicted *spi* product is a 230-amino-acid transmembrane protein that contains an EGF-like sequence in its ectodomain (Rutledge et al., 1992). Although the actual EGF-like sequence is most closely related to EGF, the *spi* product resembles the integral membrane precursors of the other EGF family members in terms of its overall size and domain structure. The observation that, in addition to the six cysteine residues, the *spi* protein also preserves other residues that are conserved throughout the various EGF family members (including the equivalent of arginine[42] of TGFα) suggests that it might be functionally related. It is tempting to speculate that it could act as a ligand for the ***Drosophila*** **EGF-R**-related (Der) protein, and indeed weak alleles of the Der-encoding *faint little ball* locus yield a phenotype similar to that of *spi* mutants. Overall, however, the phenotype of *spi* mutants most closely resembles those of the *rhomboid* locus. The latter encodes a putative membrane receptor with several membrane-spanning domains but no discernible signaling elements. Another EGF/TGFα-like *Drosophila* protein, Gurken (Grk), was recently identified (Neuman-Silberberg and Schupback, 1993).

Lin-3 is an EGF-related gene product that was discovered as a result of its developmental roles in the nematode *Caenorhabditis elegans* (Hill and Sternberg, 1992). One of these roles is to promote the development of the vulva, which forms from six vulval precursor cells. The vulval precursor cells are stimulated to differentiate in response to a signal, mediated by *lin*-3, from an anchor cell that is located in the adjacent gonadal tissue. The *lin*-3 gene has recently been cloned and shown to encode alternate transmembrane proteins of 438 and 423 amino acids that contain an EGF-like sequence in their extracellular domain. Although differences in the spacing of the cysteines suggest that Lin-3 is less related to the EGF-R ligands than Spi or Grk, it may nevertheless function as a ligand, inasmuch as the EGF-R homologue Let-23 is also required for vulval fate determination. Given that the inductive signal can apparently act at a distance, Lin-3 could be proteolytically processed to release the EGF-related sequence.

C. Other Peptides Containing EGF-Like Sequences

As defined by the characteristic arrangement of six cysteine residues within a unit length of roughly 40 amino acids, EGF-like motifs have been identified in a large group of secreted and integral membrane proteins that are devoid of mitogenic activity (Carpenter and Wahl, 1990). These include but are not limited to (*a*) proteases (e.g., factors IX, X and XII, and both urokinase- and tissue-type plasminogen activators) and their cofactors (e.g., protein S and thrombomodulin), (*b*) proteins that mediate cell-cell (e.g., the *Drosophila* Notch and Delta gene products, and *Caenorhabditis elegans* product Lin-12) and cell-extracellular matrix (e.g., cytotactin and entactin) interactions, and (*c*) receptors (e.g., low-density lipoprotein receptor). Often, the EGF-like units in these proteins are present as repeats. For example, the *Drosophila* protein Notch includes 36 EGF-like elements in its extracellular domain. The spacing of the cysteines in these elements shows greater variation than that of the known EGF-R ligands, and one or more of the cysteine residues is often missing (Bell et al., 1986). Additionally, glycine and arginine residues that are preserved by members of the EGF-R and HER2/erbB2/neu ligand families are not conserved in EGF-like units lacking growth factor activity. A unifying concept regarding their function(s) is suggested by the fact that many of the proteins containing these EGF-like elements are dependent on Ca^{2+} for activity, and some of the EGF-like elements contain a consensus signal for aspartate-β-hydroxylation, a modification that contributes to the formation of high-affinity Ca^{2+}-binding sites (Handford et al., 1991; Huang et al., 1991; Selander-Sunnerhagen et al., 1992).

IV. Structure/Function Relationships

A. Peptide Requirements

An early report that a peptide corresponding to the third loop of mature human TGFα had significant biological activity (Nestor et al., 1985) has not been reproduced, and a consensus has emerged that all three disulfide loops are required for significant receptor binding/activation. Thus, multiple synthetic peptides corresponding to the NH_2 terminus, the COOH terminus, or the individual disulfide-constrained rings of TGFα did not exhibit appreciable receptor binding or mitogenic activity (Darlak et al., 1988; Defeo-Jones et al., 1988). Moreover, destruction of any of the disulfide bonds through the substitution of alanines for cysteines at positions 8 and 21, 16 and 32, and 34 and 43, or

simultaneously at positions 8, 21, 34 and 43, yielded inactive mutant proteins (Defeo-Jones et al., 1988). These results are consistent with predictions based on hydrophilicity plots that three discrete sequences (one in each loop) are exposed on the growth factor's surface and hence are potentially able to interact with receptor (Darlak et al., 1988). On the other hand, deletion or mutation of the NH_2-terminal leader sequence that precedes the first cysteine and includes amino acids 1 through 7 had relatively little (< three- to four-fold) effect on biological activity (Defeo-Jones et al., 1988).

B. Site-directed Mutagenesis

Predictably, it is more difficult to draw a clear consensus from attempts to assess the importance of individual (noncysteine) amino acids by site-directed mutagenesis. The consequence of a substitution mutation can depend on the nature of the substitution. Given the effort required to replace a particular residue with a series of conservative and nonconservative substitutions, it is not surprising that only a few amino acid positions in mature TGFα have been analyzed in such detail. Moreover, inasmuch as all studies to date have utilized recombinant growth factor expressed in bacteria, the difficulty of ensuring that the same amount of correctly folded TGFα protein is compared in each case cannot be underestimated. Finally, the effect of a given mutation can also vary depending on the nature of the assay (i.e., receptor binding versus mitogenesis).

With these caveats in mind, the collective impression from a number of studies is consistent with the limited overall homology between the various EGF-R ligand family members—namely that, with the exception of the cysteine residues, there are relatively few critical amino acids. Perhaps not surprisingly, three of the four noncysteine residues that are conserved throughout the EGF-R ligand family seem to be critical for activity as assessed both by receptor binding and by stimulation of mitogenesis. Thus, individual mutations of glycine[19] (Feild et al., 1992), glycine[40] (Feild et al., 1992), and arginine[42] (Defeo-Jones et al., 1989; Lazar et al., 1989; Feild et al., 1992) all yielded proteins with greatly reduced or no activity (amino acid numbers refer to positions in the mature, 50-amino-acid protein as shown in table 1). Mutation of tyrosine[38], on the other hand, produced variable effects, depending on the nature of the substitution. Although substitution with phenyalanine only inhibited activity by a few-fold, replacement with alanine produced a much larger diminution (Lazar et al., 1989; Defeo-Jones et al., 1989). However, contradictory results were obtained by replacing tyrosine[38] with tryptophan. Whereas Lazar et al. (1989) observed a relatively small decrease in activity, leading them to suggest a requirement for an aromatic side chain at this position, Feild et al. (1992) reported a greater loss of activity with the same substitution.

Several amino acid positions are partially conserved throughout the family. For example, all EGF-R ligands contain either a tyrosine or phenylalanine at position 15, and mutation of phenylalanine[15] of human TGFα to alanine markedly reduced activity (Defeo-Jones et al., 1988). Mutation of histidine[18], which is conserved by all members with the exception of EGF, produced small to moderate decreases in activity, depending on the nature of the substitution (Defeo-Jones et al., 1989; Feild et al., 1992). Asparagine[47] is conserved between TGFα, EGF, and betacellulin. Substitution of various amino acids at this position generally produced significant inhibition of activity (Defeo-Jones et al., 1988; Lazar et al., 1988), although in contrast, Feild et al. (1992) reported that replacement with alanine had relatively little effect. Finally, leucine[48] is conserved between all family members but amphiregulin, and even the conservative substitution of an alanine at this position greatly diminished activity (Lazar et al., 1988; Feild et al., 1992).

Of the relatively nonconserved amino acids, substitution of serine[11], histidine[12], phenylalanine[17], arginine[22], leucine[24], glutamic acid[27], lysine[29], valine[33], histidine[35], glutamic acid[44], and alanine[46] all decreased activity by small to moderate extent (Defeo-Jones et al., 1988, 1989; Feild et al., 1992). Deletion of the COOH-terminal leucine[49]alanine[50] dipeptide also moderately reduced activity, although the absolute effect depended on the nature of the assay (Yang et al., 1990). Deletion of alanine[41], on the other hand, abolished activity (Lazar et al., 1989), whereas its substitution by serine had only a modest effect (Feild et al., 1992). Finally, replacement of histidine[45] with lysine markedly reduced activity (Defeo-Jones et al., 1989), but substitution with alanine had only a small effect (Feild et al., 1992).

C. Polypeptide Folding

Sequence-specific ^{1}H-nuclear magnetic resonance assignments have been deduced for human recombinant TGFα (Kohda et al., 1989; Montelione et al., 1989; Kline et al., 1990). These data predict that, as in the case of EGF, the mature TGFα protein is composed of two independently folded domains. The first of these includes the NH_2-terminal peptide and the first two loops (through approximately residue 33). The second corresponds to the third loop and the short COOH-terminal peptide. These two domains are predicted to form a mitten-like structure similar to that previously proposed for EGF. The first and second disulfide loops form the palm of the mitten, the third loop forms the thumb, and the NH_2-terminal polypeptide segment contributes to the backbone. If these predictions are correct, TGFα could bind to the EGF-R as a mitten would grasp an object. The prediction regarding the independent folding of two domains is consistent with the finding that a hybrid protein corresponding to the NH_2-terminal segment (residues 2 to 32) of TGFα and the COOH-terminal

segment (residues 33 to 52) of VGF was as biologically active as either parent molecule (Purchio et al., 1987).

V. Characterization of the TGFα Precursor

A. proTGFα as an Integral-Membrane Glycoprotein

1. Synthesis and processing of proTGFα. The cloning of cDNAs encoding human (Derynck et al., 1984) and rat (Lee et al., 1985a) TGFα led to predictions that the mature 50-amino-acid polypeptide is derived from a larger, integral-membrane glycoprotein (proTGFα) (fig. 4). Human proTGFα was predicted to be 160 amino acids in length, whereas the rat protein, which lacked an alanine residue in the short region between the signal peptide and EGF-like sequence, was predicted to be 159 amino acids in length. However, it is now apparent that microheterogeneity in splicing leads to production of both the 159- and 160-amino-acid polypeptides in the same cell (see VII.A.2.). ProTGFα includes a putative signal peptide of 22 amino acids, followed by a short 16- or 17-amino-acid sequence containing an N-linked glycosylation consensus site. This latter sequence, which is retained by larger secreted TGFα molecules, is followed by the 50-amino-acid (EGF-like) sequence, a juxta-membrane region of about 9 amino acids, and a hydrophobic transmembrane domain of roughly 23 residues. The cytoplasmic sequence of proTGFα contains approximately 39 amino acids, 7 of which are cysteines. Interestingly, a comparison of the human and rat precursors reveals that the 62 amino acids comprising the transmembrane and cytoplasmic domains differ only by a single conservative substitution, whereas the 50-amino-acid, EGF-like sequence contains four alterations (Todaro et al., 1985).

The predicted characteristics of proTGFα have been confirmed in several laboratories. Gentry and coworkers (1987) used polyclonal antisera specific for peptides in the cytoplasmic domain to demonstrate the preferential immunoprecipitation of precursor molecules from the membrane fraction of cells transfected with a proTGFα expression vector. Bringman et al. (1987), who used a similar approach, additionally reported that the cytoplasmic domain of the precursor remained membrane-associated after the cleavage of extracellular sequences. Teixido and coworkers (1987), on the other hand, examined for possible membrane association of proTGFα produced by in vitro translation reactions. They observed that a 17 kDa species (the predicted size of proTGFα) was derived when translation reactions were carried out in the absence of membranes, whereas an additional product of approximately 20 kDa was obtained in the presence of microsomes. The fact that this larger product was converted to approximately 17 kDa by exposure to endoglycosidase H suggested that it corresponded to a glycosylated form of the core 17 kDa protein. Importantly, a portion of the 17 kDa molecule, and all of the detectable 20 kDa species, was pelleted with microsomes, confirming their association. To demonstrate that proTGFα was inserted into vesicle membranes as opposed to being secreted into the vesicle lumen, they challenged post-translational membrane products for sensitivity to exogenous proteases. Secretion into the vesicle lumen was expected to render the entire proTGFα molecule resistant to digestion, whereas membrane insertion was predicted to leave the exposed cytoplasmic domain (which would be on the outside of the membrane vesicles) sensitive to digestion. Consistent with insertion, exposure to proteinase K converted the 20 and 17 kDa proteins to new species of 18 and 15 kDa that were recognized by antibodies directed toward the mature EGF-like sequence, but not by antibodies raised against the cytoplasmic domain of proTGFα. These results were further corroborated by showing that post-translational vesicles treated with alkali, which promotes release of lumen contents, retained a significant proportion of the 17 kDa and most of the 20 kDa proteins. Finally, the fact that the core 17 kDa species was observed both in the absence and presence of membranes, with no smaller products detected in their presence, suggested that the signal peptide was not cleaved from proTGFα in these in vitro translation reactions. It is not known whether the signal peptide is also retained in vivo.

Pulse-chase analyses of proTGFα species in transfected cells (Bringman et al., 1987; Gentry et al., 1987; Teixido et al., 1990) revealed a sequence of events that is consistent with the results of the in vitro translation studies. An initial product of 17 to 19 kDa was rapidly converted to a diffuse set of species of approximately 23 to 25 kDa as a result of both N- and O-linked glycosylation (Ignotz et al., 1986; Luetteke et al., 1988). Within 1 to 2 h, the 23 to 25 kDa species were reduced to a 14 to 15 kDa product that, on the basis of its reactivity toward different antisera, was predicted to include the transmembrane and cytoplasmic domains, but not the EGF-like sequence. In some instances, an intermediate 17 kDa form that still retained mature growth factor sequence was also detected (Teixido et al., 1990). The fact that the 14 to 15 kDa species was larger than expected solely on the basis of its amino acid content may be explained by the finding that the cytoplasmic domain of proTGFα is modified by addition of palmitate, presumably to cysteine residues (Bringman et al., 1987). Finally, Bringman et al. (1987) noted the conversion of the 14 to 15 kDa protein to a product of about 12 kDa. This decrease in size could reflect the loss of the short juxtamembrane sequence owing to cleavage of a lysine-lysine bond immediately external to the transmembrane sequence (fig. 4). As predicted by this type of analysis, the half-life of proTGFα in transfected cells was on the order of 1 to 3 h.

2. Proteolytic cleavage of proTGFα. Release of the 50-amino-acid, mature TGFα from proTGFα requires the cleavage of alanine-valine bonds at both termini, and the

cleaved alanine-valine dipeptides are flanked in each case by additional small apolar amino acids. Thus, the sequence surrounding the NH_2 terminus of the mature EGF-like peptide growth factor is

valine-alanine-alanine-alanine-/-valine-valine,

whereas the COOH-terminal sequence is,

leucine-leucine-alanine-/-valine-valine-alanine-alanine

(/indicates the cleaved bond). This cleavage specificity is reminiscent of elastase enzymes that preferentially cleave alanine-, leucine- and valine-rich sequences. In fact, larger soluble forms of TGFα retaining the NH_2-terminal leader sequence together with EGF-like sequence are cleaved by pancreatic elastase to a 6 kDa species that is indistinguishable in size from the fully processed, 50-amino-acid polypeptide (Ignotz et al., 1986; Luetteke et al., 1988). These results establish that elastase can act on the NH_2-terminal cleavage site, but its ability to cleave the COOH-terminal alanine-valine bond is less clear. Although Teixido et al. (1990) found that pancreatic elastase would not release soluble TGFα from the cell surface, treatment of a proTGFα-expressing NRK cell line with polymorphonuclear neutrophil leukocytic elastase resulted in the accumulation of a 6 kDa TGFα polypeptide in the medium (Mueller et al., 1990). It is not known whether this apparent discrepancy results from differences in the specificity of the enzymes used, the extent of proTGFα glycosylation, or yet another explanation.

A pulse-chase analysis of proTGFα processing in transfected CHO cells indicated that cleavage of the NH_2-terminal site in the cell type was relatively rapid (occurred within 1 h), whereas the COOH-terminal cleavage that releases soluble TGFα species was slow (required up to 4 h) (Teixido et al., 1990). This observation raised the possibility that different proteases cleave the two sites, but the similarity in flanking sequences and the fact that cleavage at both sites was repressed by the same protease inhibitors (Pandiella et al., 1992) argue against this. In fact, slow cleavage of the COOH-terminal site may be peculiar to CHO cells, inasmuch as it is difficult to resolve with findings that many cell types secrete larger TGFα species owing to cleavage of the COOH-terminal site alone (see next paragraph.). Finally, the observation that COOH-terminal cleavage occurred over a period of several hours, with precursor molecules accumulating on the cell surface, suggests that processing of proTGFα occurs at the plasma membrane.

The results of numerous studies indicate that proteolysis of the alanine-valine bonds is limiting not only in cells expressing transfected vectors, but also in those that are expressing the endogenous gene. Thus, in addition to the fully processed mature growth factor, many cell types secrete larger species that often appear heterogeneous in size. Bringman and colleagues (1987) identified a diffuse 18 kDa species secreted by transfected CHO cells that was reduced to products of 15 and 13 kDa by treatment with N-glycanase. The apparent size difference between these two species could reflect variable retention of the signal peptide or differential cleavage at either the COOH-terminal alanine-valine bond or the previously described lysine-lysine dipeptide (fig. 4). In a similar manner, TGFα species of 17 to 19 and 18 to 21 kDa secreted by transformed rat embryo fibroblasts and hepatocellular carcinoma cells, respectively, were reduced to 11 kDa core proteins by sequential removal of both N- and O-carbohydrate (Teixido and Massagué, 1988; Luetteke et al., 1988). The smaller size of these core proteins relative to the 13 to 15 kDa species described above could reflect a more complete removal of carbohydrate. Significantly, both the 17 to 19 kDa and 18 to 21 kDa species were shown to comigrate with fully processed, mature TGFα after exposure to pancreatic elastase (Ignotz et al., 1986; Luetteke et al., 1988). Inasmuch as the single N-glycosylation consensus site is located between the signal peptide and the mature EGF-like sequence, these results imply that the larger soluble forms are derived from the extracellular domain of proTGFα owing to selective cleavage of the COOH-terminal alanine-valine (or possibly the lysine-lysine described in fig. 2) bond. Their inherent size is increased as a result of both N- and O-linked glycosylation, with N-linked glycosylation accounting for most of the size heterogeneity. Larger, soluble TGFα species of approximately 30 to 40 kDa have also been reported (Sherwin et al., 1983; Linsley et al., 1985). Whether these include larger portions of the precursor, or simply contain more carbohydrate, is unknown. Also, the impact of the glycosylation on expression and processing of proTGFα is uncertain. Although treatment of certain cells with the N-glycosylation inhibitor, tunicamycin, blocked secretion of mature TGFα and led to the reduced accumulation of larger forms (Bringman et al., 1987; Teixido and Massagué, 1988), processing and release of TGFα species was not significantly altered in an O-glycosylation-defective CHO clone (Teixido et al., 1990). In any event, an important point is that all soluble forms of TGFα are biologically active (Ignotz et al., 1986; Bringman et al., 1987; Luetteke et al., 1988).

Recent work suggests that release of soluble TGFα species could be a regulated event. Pandiella and Massagué (1991a, b) found that, whereas processing of proTGFα was normally slow in transfected CHO cells, it was rapidly accelerated by phorbol esters (e.g., TPA), crude serum factors, and calcium ionophores. Stimulation of processing, which seemed to be mediated through both protein kinase C-dependent and protein kinase C-independent pathways, was not specific to proTGFα as evidenced by the fact that release of the soluble c-*kit* ligand, KL, from its precursor (preproKL) was similarly induced. However, the differential effects of a panel of

inhibitors suggested that distinct proteases were nevertheless activated to cleave the KL and TGFα precursors (Pandiella et al., 1992). ProTGFα retained in Golgi compartments in response to brefeldin A treatment was not susceptible to cleavage. Conversely, stimulated cleavage of surface precursor was insensitive to drugs or conditions that interfere with membrane maturation in the Golgi, vesicular trafficking, or protein degradation in the lysomes (Bosenberg et al., 1992). These observations suggested that induced processing affects proTGFα molecules at the cell surface rather than those in intracellular compartments. This conclusion was corroborated by the observation that TPA-induced processing was observed with cytosol-deficient cells that had been deliberately permeabilized with streptolysin O (Bosenberg et al., 1993). Surprisingly, mutagenesis of proTGFα revealed that the phorbol-dependent stimulation of processing was most strongly dependent on the presence of the terminal valine residue of the precursor's cytoplasmic domain (Bosenberg et al., 1992). The significance of this latter observation is underscored by the fact that the KL precursor also contains a COOH-terminal valine residue and that activation of processing was similarly observed with a chimeric TGFα/KL protein that featured the ectodomain of proTGFα attached to the cytoplasmic region of the KL precursor. Finally, in a related observation, Brown et al. (1993) reported that low doses of ultraviolet irradiation also activated release of soluble TGFα. They suggested that this occurs as part of a global activation of ectopeptidase activity—perhaps the mammalian equivalent of a genetic stress response.

Efforts are underway in many laboratories to identify the proTGFα processing enzyme(s). Sensitive peptidase assays have been developed (Brown et al., 1992), and a candidate, membrane-associated serine protease with elastase-like specificity, has been described (Cappelluti et al., 1993).

3. Biological activities of proTGFα. The existence of integral membrane forms of EGF-R ligands, together with observations that both the TGFα and EGF (Rall et al., 1985) precursors accumulate on the cell surface, raised the intriguing possibility that the activities of these growth factors could be spatially regulated by controlling the level of processing. In the absence of processing, membrane-tethered forms could bind and activate only receptors on adjacent cell surfaces (or in membrane compartments of the same cell); with processing activated, soluble growth factors would be released to act on distant cell types. This notion of spatial regulation assumes that membrane-anchored growth factors are indeed biologically active. Relevant to this concern, it should be noted that processing of hormone precursors is generally required for activation. Additionally, in the case of the EGF family precursors, close proximity to the membrane could potentially inhibit binding of EGF-like sequences to the EGF-R.

To examine the biological activity of the precursor, two laboratories used site-directed mutagenesis to generate noncleavable forms of proTGFα. Inasmuch as both of the alanine-valine cleavage sites are flanked by additional alanines and valines and the specificity of the protease has not been established, our laboratory created a series of mutants in which the sequences flanking the two cleavage sites were progressively altered through the substitution of nonconservative amino acids (Wong et al., 1989). Cassettes containing either wild-type or mutant sequences were then cloned into expression vectors that were transfected into baby hamster kidney cells. Comparisons of media conditioned by baby hamster kidney clones that expressed either wild-type and mutant forms of proTGF in which the levels of soluble TGFα were assessed by EGF-R binding competition, NRK cell colony formation, and Western blot analysis, confirmed that processing was completely inhibited only when the flanking sequences were also mutated. Brachmann and coworkers (1989), on the other hand, conservatively replaced the alanine-/-valine-valine tripeptide at the COOH-terminal cleavage site and simultaneously mutated the lysine-lysine bond in the juxtamembrane domain, on the assumption that it could serve as an alternate cleavage site (see fig. 2.). Media conditioned by CHO cell clones expressing this mutant proTGFα molecule also did not contain detectable levels of TGFα. To test for biological activity, both laboratories then utilized a coculture approach in which baby hamster kidney or CHO cells expressing the mutant forms of proTGFα were co-incubated with an unrelated target population of EGF-R-expressing cells. In response to co-incubation of the two cell populations, both groups reported a rapid and sustained activation of the target cell receptor as shown by its autophosphorylation. Moreover, as is true of the response to soluble ligand, receptor activation by the noncleavable precursor was accompanied by a rapid and transient rise in the level of free intracellular Ca^{2+} (Wong et al., 1989) as well as induction of *c-fos* mRNA (Brachmann et al., 1989). Brachmann and coworkers (1989) further showed that solubilized precursor promoted NRK colony growth in the presence of TGFβ, and Blasband et al. (1990a) found that NRK cells formed TGFβ-dependent colonies in agar and tumors in nude mice in response to expression of noncleavable forms of proTGFα on their cell surface. Collectively, these various observations confirmed that integral membrane forms of proTGFα molecules are biologically active, although whether they are as active on a per molecule basis as the secreted growth factor is less clear.

Subsequent to the above-described studies, Anklesaria and colleagues (1990) reported that episomal expression of wild-type proTGFα in a bone marrow stromal cell line prompted these cells to adhere to an EGF-R-expressing, EGF/interleukin-3-dependent hematopoietic cell line in mixed cell culture. Confirmation that adhe-

sion was promoted by interactions between the TGFα precursor and EGF-R was provided by showing that it was disrupted in the presence of an excess of soluble EGF-R ligand. The additional finding that a proportion of the adhered progenitor cells were stimulated to undergo DNA replication provided indirect confirmation of the precursor's biological activity and suggested that proTGFα might have dual functions in this context. The term "juxtacrine action" was suggested to distinguish precursor-mediated interactions between adjacent cells from autocrine and paracrine actions involving soluble growth factor. It should be noted, however, that adhesion mediated by proTGFα/EGF-R interactions is relatively weak compared with that of classical cell-cell adhesion molecules, and hence it remains to be established whether this is a physiologically relevant activity.

B. Integral Membrane Forms of Related Ligands

As previously noted, the derivation of soluble forms from integral membrane precursors is a conserved characteristic of the cellular EGF-R ligand family that also extends to the related growth factor, heregulin/NDF, as well as to several unrelated growth factors including TNF-α, colony stimulating factor-I, and c-*kit* ligands (Massagué and Pandiella, 1993). The fact that the cellular EGF-R ligands are all expressed in the context of larger, integral membrane proteins could indicate that membrane insertion facilitates the correct folding of the three-loop structure. However, findings that a directly secreted, recombinant form of TGFα is biologically active (Watanabe et al., 1987), and that related ligands encoded by Shope family viruses may be directly secreted (Chang et al., 1987), argue against this. A comparison of the amino acid sequences flanking the NH_2- and COOH- termini of the various mature growth factors does not reveal a common proteolytic recognition sequence and in fact suggests that the proTGFα cleaving enzyme(s) is distinct from enzymes that process the other precursors.

The obvious similarities between proTGFα and integral membrane forms of the other EGF-R ligands suggest that the latter are also likely to be biologically active, and this prediction has been confirmed for solubilized preparations of preproEGF (Mroczkowski et al., 1989). It is important to stress, however, that whereas the potential for biological activity has been established, no proof has yet been provided that transmembrane precursors to EGF family ligands are physiologically important signaling entities. Indications of the concept's validity instead come from molecular and genetic analyses of (*a*) the c-*kit* ligand and (*b*) photoreceptor cell differentiation in the *Drosophila* compound eye (Massagué and Pandiella, 1993). Two forms of the c-*kit* ligand (KL1 and KL2) are derived from the mouse *Steel* gene by alternate splicing. Both are integral membrane proteins, but one is preferentially processed to release soluble ligand because it contains an insert sequence that includes the proteolytic cleavage site. Defects in melanogenesis, gametogenesis, and hematopoiesis that are associated with loss of *Steel* gene function are also observed with the *Steel-Dickie* allele. Significantly, the *Steel-Dickie* mutation has been shown to result in the expression of a single truncated KL ligand that lacks the membrane anchor and is, therefore, directly secreted (Flanagan et al., 1991; Brannan et al., 1991; Huang et al., 1992). The fact that phenotypes are associated with the loss of the transmembrane protein indicates that it is physiologically important and not simply a precursor to soluble growth factor.

Sevenless/bride-of-sevenless interactions in the developing *Drosophila* compound eye provide another elegant demonstration of cell-cell signaling. The compound eye is composed of hundreds of ommatidia, each of which contains eight photoreceptor ("R") cells. During development of the eye, the eight photoreceptor cells differentiate in a stereotypical stepwise fashion in response to inductive stimuli. The R7 cell is the last photoreceptor cell to differentiate, and this event is triggered by an inductive signal from the adjacent, previously differentiated R8 cell. Significantly, combined genetic and molecular analyses have established that the R7 inducing signal is mediated by interactions between a membrane-anchored ligand (bride-of-sevenless or boss) expressed on the R8 cell surface, and an EGF-R-like tyrosine-kinase receptor (sevenless) expressed on the surface of the R7 cell (Reinke and Zipursky, 1988; Hart et al., 1990). Analyses of boss/sevenless interactions have further allowed investigators to address an interesting question that arises concerning interactions between transmembrane ligands and receptors on adjacent membranes, and that is how signaling is terminated. In the case of soluble ligands, signaling is thought to be controlled by rapid clustering and internalization of ligand/receptor complexes ("down-regulation"). Recent studies suggest that boss/sevenless complexes are internalized by the sevenless-expressing cell (Cagan et al., 1992), indicating that either boss is pulled out of its membrane, or perhaps more likely, there is exchange of membranes between ligand- and receptor-expressing cells.

The overall resemblance between preproEGF and growth factor receptors, which are characterized by the presence of cysteine-rich sequences in their extracellular domains, led Pfeffer and Ullrich (1985) to suggest that the EGF precursor could function as a receptor in some contexts. Although this notion, which could be extended to the other EGF family precursors, has not yet been validated in a physiological sense, it is interesting to note that preproHB-EGF serves as an internalizing receptor for diptheria toxin (Naglich et al., 1992). A distinct but related possibility is that the integral membrane precursors signal through their cytoplasmic domains as a result of binding to the EGF-R. This possibility is perhaps consistent with the fact that the transmembrane and cytoplasmic domains are the most

highly conserved regions of proTGFα (Todaro et al., 1985). It may also be consistent with the recent finding by Shum and colleagues (1994) that proTGFα co-immunoprecipitated with proteins of 86 and 106 kDa in transfected CHO cells. Whereas association of p86 was dependent on the presence of proTGFα's cytoplasmic domain, the binding of p106, which was tyrosine phosphorylated and exposed on the cell surface, was not. Significantly, the proTGFα/p86/p106 complex displayed kinase activity towards tyrosine, serine, and threonine residues. Hence, the possibility exists that this complex participates in reverse signaling in response to ligand-receptor interactions.

VI. Expression of proTGFα

A. Physiological Roles

1. Biological activities. A large body of literature describes the effects of exogenous EGF on various tissues and cell types. Originally purified as an activity that (*a*) accelerated eyelid opening and incisor eruption (Cohen, 1962) and (*b*) inhibited gastric acid secretion (Gregory, 1975), EGF has been shown to regulate fundamental cellular behaviors including proliferation, migration, and differentiation (Carpenter and Cohen, 1979; Carpenter and Wahl, 1990; Lee and Han, 1990). Additionally, it influences membrane function, cellular morphology, and gene expression. Given its overall similarities, TGFα would be expected to demonstrate these same activities (though perhaps with greater potency); indeed, to the extent that any of the activities are physiological, these activities are more likely to be mediated by TGFα, inasmuch as TGFα is more broadly expressed (see VI. A.3.). In fact, TGFα has been shown to mimic EGF's actions in newborn mice (Smith et al., 1985; Tam, 1985), to be a potent mitogen for epithelial and mesenchymal cells in vivo (Sandgren, et al., 1990; Jhappan et al., 1990), to induce migration of certain cell types in culture (Barrandon and Green, 1987; Bade and Feindler, 1988), and to regulate gene expression and differentiation (Serrero, 1987; Luetteke et al., 1993a; Davidson et al., 1993). It has also been shown to be active in a variety of biological processes including angiogenesis (Schreiber et al., 1986), wound healing (Schultz et al., 1987, 1992), and bone resorption (Ibbotson et al., 1985). Given these numerous activities and its widespread pattern of expression, it is perhaps surprising that targeted inactivation of the TGFα gene in mice has revealed roles for TGFα only in skin and eyes (see VI.A.4), and the more conspicuous skin phenotype is not obviously related to cellular proliferation. Whether these targeting results are influenced by a redundancy of both related and unrelated growth factor/receptor signaling systems or instead underscore our general ignorance regarding the roles of growth factors in vivo remains to be determined.

2. Developmental expression. The first indications of developmental expression were reports that TGFα-like activity could be extracted from midgestation rodent fetuses (Twardzik et al., 1982a, 1985; Matrisian et al., 1982), human placenta (Stromberg et al., 1982), and the urine of pregnant women (Twardzik et al., 1982b). These were followed by the reported detection of TGFα mRNA in whole rat fetuses from days 8 to 10 of gestation (Lee et al., 1985b). Expression was highest at day 9, diminished by day 11, and nondetectable at days 13 and 18. Given our present state of knowledge, the possibility exists that these early findings were compromised by the contribution of TGFα mRNA and/or protein from contaminating maternal decidua which, as described below, expresses the growth factor at high levels around days 9 and 10 of gestation. Hence, a more convincing demonstration of fetal expression was provided by Wilcox and Derynck (1988a), who detected TGFα transcripts in midgestation mouse fetuses by in situ hybridization. TGFα transcripts were found in cells of the placenta, otic vesicle, oral cavity, pharyngeal pouch, first and second branchial arches, and developing kidneys of 9- and 10-day fetuses. Surprisingly, specific hybridization was not observed with sections of older embryos. Subsequently, Rappolee and coworkers (1988a) used a highly sensitive reverse transcriptase-polymerase chain reaction assay to demonstrate the presence of TGFα transcripts during the earliest stages of mouse embryogenesis. Transcripts derived from maternal sources were present in the unfertilized ovulated oocyte but were replaced by embryo-derived transcripts in pre-implantation blastocysts. Consistent with the active translation of these transcripts, anti-TGFα antibodies specifically localized to punctate structures in the perinuclear region of all cells of the blastocyst. Finally, TGFα protein was also detected by specific radioimmunoassay in fetal rats immediately before birth (Brown et al., 1990), and was extracted from ovine fetal kidneys (Freemark and Comer, 1987).

As noted above, relatively high levels of TGFα transcripts were detected in the maternal rat decidua by both Northern blot analysis and by in situ hybridization (Han et al., 1987). Lower levels of TGFα mRNA were also present in the uterus and placenta. Expression in the decidua was observed at day 7 of gestation, peaked between days 8 and 10, and then slowly declined through the period of decidual resorption. Interestingly, a gradient of hybridization was observed, with the highest levels occurring adjacent to the embryo. Although the existence of this gradient could indicate that expression of the growth factor is triggered by implantation of the conceptus, TGFα mRNA and protein were induced in a pseudopregnant model in the absence of the conceptus (Bonvissuto et al., 1992). Using the latter model, immunoreactivity was localized to the majority of stromal cells in the antimesometrial area, with no immunoreactivity detected in mesometrial cells. These various results suggest that TGFα could play an important role in the growth and development of the decidua.

TGFα expression was localized to mouse uterine epithelial (glandular and tubular) and stromal cells during the peri-implantation period (days 1 to 4) (Tamada et al., 1991). Their additional finding that the presence of the growth factor improved the inferior development of singly cultured mouse embryos in vitro led Paria and Dey (1990) to suggest that TGFα has beneficial effects on peri-implantation embryo development and blastocyst functions in vivo. Finally, TGFα mRNA and protein were markedly induced in estrogen-treated uterine epithelia (Nelson et al., 1992).

3. Expression in adult tissues. Numerous studies indicate that expression of TGFα is widespread in adult mammals. TGFα transcripts have been detected at low to moderate levels in a large number of tissues, with relatively high levels found in (rodent) brain, lung, and decidua. The presence of TGFα antigen in various tissues has also been demonstrated (though the specificities of the antisera have not always been clearly established). As a result, the emerging view is that TGFα is a more common EGF-R ligand than is EGF. Its widespread expression in adult tissues further suggests that references to TGFα as an "oncofetal" growth factor are misleading and inappropriate.

THE NERVOUS SYSTEM. Detection of its mRNA in whole rat brain was the first indication that TGFα is expressed in a normal adult tissue (Lee et al., 1985a). This observation was followed by reports of specific TGFα immunostaining in brain (Code et al., 1987; Loughlin et al., 1989). However, some of the latter results may have been confounded by possible cross-reactivity of the polyclonal antisera to the neuropeptide, synenkephalin. In the first detailed characterization of brain expression, Wilcox and Derynck (1988b) localized transcripts to cell bodies of the caudate nucleus, dentate gyrus, anterior olfactory nuclei, and a laminar distribution of mitral cells in the olfactory bulb. In situ hybridization seemed to be predominantly neuronal, although glial cell expression was not excluded. Subsequently, Kudlow and colleagues (1989) demonstrated specific TGFα immunoreactivity in neurons distributed throughout human and rat brains, and they confirmed the presence of TGFα mRNA in bovine brainstem, cerebellum, hypothalamus, and cerebral cortex by Northern blot analysis. They further observed that fetal rat brain contained two-fold higher levels of TGFα transcripts than adult brain. Corroborating and extending these previous studies, Seroogy et al. (1991) found significant and equivalent levels of expression of TGFα mRNA in samples of rat olfactory bulb, anterior olfactory nucleus, corpus striatum, hippocampus, ventral mesencephalon and caudal brainstem. Of several adult peripheral tissues examined, only adrenal gland expressed similar levels of transcript. Interestingly, expression in the cervical spinal cord was highly variable, but in some samples was five- to six-fold higher than in other tissues. Finally, lower but detectable expression of TGFα mRNA was observed in the prefrontal and cerebral cortexes, as well as in the cerebellum. The latter levels were similar to those observed in the peripheral tissues, lung, liver and kidney. In a follow-up study, Seroogy and colleagues (1993) localized TGFα transcripts by in situ hybridization to neuronal perikarya in numerous regions of the forebrain, as well as to several thalamic and hypothalamic nuclei. TGFα-expressing cells were also evident in regions of white matter, confirming that glial cells express the growth factor. Collectively, these several studies indicate widespread but differential distribution of TGFα throughout the mammalian central nervous system.

The relatively high expression of TGFα in the central nervous system has prompted considerable interest in its function. The ability of EGF to promote neuronal survival (Morrison et al., 1987; Kornblum et al., 1990) and stimulate astrocyte proliferation (Leutz and Schachner, 1981) suggests that TGFα may have both neurotrophic and mitogenic activity in brain. The additional possibility that these activities may be important in early central nervous system development is raised by the finding that TGFα induced proliferation of neuroepithelial progenitor cells (Anchan et al., 1991). Yet another possible function for TGFα in brain is suggested by observations that both EGF and TGFα stimulate LHRH release from the median eminence of the hypothalamus (Ojeda et al., 1990). Inasmuch as hypothalamic lesions causing sexual precocity resulted in dramatic activation of TGFα gene expression in reactive astrocytes surrounding the site of injury (Junier et al., 1991), a hypothesis was put forth that TGFα contributes to the acceleration of puberty induced by anterior hypothalamic injury. The latter finding, however, also raised the possibility that TGFα has a role in regulating the function of LHRH neurons, a specialized set of hypothalamic cells that are essential for sexual maturation and the maintenance of reproductive function. This notion received support from findings that: (*a*) TGFα mRNA and protein were localized to hypothalamic regions concerned with LHRH control, (*b*) increases in hypothalamic TGFα mRNA levels correlated with LHRH-dependent events, (*c*) gonadal steroids that regulate LHRH secretion induced TGFα mRNA, and (*d*) inhibition of EGF-R signaling by tyrphostin resulted in delayed puberty (Ma et al., 1992). These findings have been interpreted to suggest that TGFα may be a component in the process by which the brain controls mammalian sexual maturation.

THE INTEGUMENT. Another early confirmation of TGFα expression in normal adult tissues was provided by Coffey and coworkers (1987a), who demonstrated expression of the growth factor by primary cultures of human keratinocytes. In situ hybridization and immunohistochemical analyses of normal skin biopsies revealed the presence of TGFα mRNA and protein in the stratified epidermis, with increased expression of both in psoriatic lesions (Gottlieb et al., 1988; Elder et al., 1989; Turbitt

et al., 1990). In addition, TGFα transcripts were recently localized to the inner root sheath of active mouse hair follicles (Luetteke et al., 1993b). By comparison, the level of expression in active hair follicles of neonatal mice was significantly higher than in the overlying epidermis, and physiological roles for both TGFα and EGF-R in hair follicle development and function have recently been demonstrated (Luetteke et al., 1993b; Mann et al., 1993; Luetteke et al., 1994). Conversely, skin is sensitive to deregulated expression of the growth factor as shown by the fact that mice harboring a TGFα transgene under the control of the human keratin K14 promoter displayed a thicker epidermis and stunted hair growth (Vassar and Fuchs, 1991). These various results, together with observations that TGFα (*a*) stimulated migration and proliferation of keratinocytes (Barrandon and Green, 1987; Coffey et al., 1988; Turksen et al., 1991) and (*b*) regulated expression of specific keratins (Cheng et al., 1993) as well as extracellular matrix-degrading enzymes (Turksen et al., 1991), are consistent with important roles for this EGF-R ligand in skin.

Transcripts encoding TGFα and EGF-R were detected in mouse adipose tissue by Northern blot analysis (Luetteke et al., 1993a), and TGFα peptide was detected in fractionated adipocytes, with higher levels in epididymal versus perirenal depots (Crandall et al., 1992, 1993). Inasmuch as both TGFα and EGF inhibited the differentiation of pre-adipocytes in vitro (Serrero, 1987; Luetteke et al., 1993a) and caused a decrease in fat pad size and cellularity in vivo (Serrero and Mills, 1991; Luetteke et al., 1993a), one or both may have a role in the regulation of adipose tissue development. Related to these observations, transgenic expression of TGFα also inhibited the growth of mesodermally-derived muscle and bone (Luetteke et al., 1993a), suggesting that it might inhibit development and/or proliferation of a shared stem cell compartment.

Finally, TGFα transcripts were detected by in situ hybridization in ductal and alveolar epithelial cells of the virgin rat mammary gland, as well as in cells of nulliparous and parous human mammary glands (Liscia et al., 1990). Expression was judged to be two- to three-fold higher in pregnant and lactating glands, and in contrast to results with virgin glands, transcripts were also detected in a fraction of stromal cells. Corroborating these findings, Snedeker and colleagues (1991) used polymerase chain reaction methodology to confirm the presence of TGFα transcripts in virgin and pregnant mouse mammary glands. However, in contrast to the preceding study, they did not detect expression in lactating glands. Immunohistochemical analysis localized TGFα antigen to the epithelial cap-cell layer of the advancing terminal end bud, as well as to fibroblasts at the base of the terminal end bud. Interestingly, EGF was differentially localized to the inner layers of the terminal end bud and to ductal cells of the mammary epithelium. The significance of these findings is underscored by observations that mammary gland growth (as shown by the development of alveolar structures and branched ducts) was induced in nonpregnant and postlactational female mice by implantation of TGFα-releasing pellets (Vonderhaar, 1987; Snedeker et al., 1991). Moreover, ectopic expression of a TGFα transgene not only induced precocious development of mammary glands but also inhibited their postlactational regression (E. P. Sandgren, J. Schroeder, and D. C. Lee, unpublished observation). These findings suggest that TGFα (and perhaps other EGF-R ligands) could play varied roles in the regulation of mammary processes.

THE DIGESTIVE TRACT. TGFα mRNA and protein were detected at low levels in adult rodent liver, with higher levels present in fetal and neonatal hepatocytes (Mead and Fausto, 1989; Brown et al., 1990; Seroogy et al., 1991). Additionally, TGFα mRNA and protein are induced during liver regeneration. Mead and Fausto (1989) observed a nine-fold increase in TGFα mRNA 24 h after hepatectomy and localized both the transcript and protein to hepatocytes. More recently, Evarts et al. (1993) demonstrated that expression of TGFα, which they predominantly localized to basophilic foci of hepatocytes but also to oval and Ito cells (Evarts et al., 1992), was induced as early as 4 h after partial hepatectomy. As a result, they suggested that TGFα, which is a potent inducer of hepatocyte proliferation, may be the primary mitogen for activation of the hepatic stem cell compartment.

Expression of TGFα by gastric mucosal cells has been described (Beauchamp et al., 1989), and as previously reported for EGF, TGFα was shown to inhibit gastric acid secretion (Rhodes et al., 1986). Surprisingly, the latter activity was reportedly retained by a small synthetic peptide corresponding to the third disulfide loop (Goldenring et al., 1993). TGFα mRNA and antigen have also been detected in other regions of the gut. Barnard and colleagues (1991) reported expression in the rat jejunum, with epithelial cells from the villus tip expressing higher levels of mRNA and protein than cells from the crypt. This observation was unexpected, given that the proliferative compartment is contained in the crypt, but was corroborated by Thomas et al. (1992), who found that TGFα immunostaining was restricted to the differentiated, nonproliferative compartments of the human stomach, small intestine, and colon. These observations are suggestive of roles for TGFα in cellular differentiation rather than proliferation, although transgenic mouse studies confirm the fact that TGFα is a potent mitogen for cells of the gastrointestinal tract (Sandgren et al., 1990). Finally, TGFα transcripts were found throughout the gut in human fetuses of 15 to 20 weeks gestation, with particularly high levels in the duodenum (Miettinen, 1993).

ENDOCRINE ORGANS. The purification of TGFα from untransformed, cultured bovine pituitary cells was another early demonstration of its expression by a normal

adult cell type (Samsoondar et al., 1986; Kobrin et al., 1986). Subsequently, a monoclonal antibody directed against the COOH-terminus of mature TGFα stained adenohypophysial cells of the bovine anterior pituitary (Kobrin et al., 1987). Other endocrine tissues that have stained positive for TGFα include the hypothalamus, thyroid, parathyroid, adrenal cortex, adrenal medulla, and pancreatic islets (Driman et al., 1992).

REPRODUCTIVE ORGANS. TGFα immunostaining was observed in thecal but not granulosa cells of the bovine ovary (Kudlow et al., 1987; Lobb et al., 1989). Specific staining was detected after thecal cell formation, and increased in intensity during the progression from the preantral stage to the early antral follicle. The finding that the levels of TGFα immunoreactivity followed the peak and decline in granulosa cell mitosis, together with the observation that EGF is a mitogen for porcine granulosa cells (May and Schomberg, 1981), suggests that TGFα could be responsible for driving granulosa cell proliferation through a paracrine mechanism. Perhaps related to this observation, TGFα was found to inhibit the spontaneous onset of apoptosis in cultured rat granulosa cells and follicles in culture (Tilly et al., 1992). Finally, a number of reports also suggest that TGFα could have a role in regulating ovarian cell function. Thus, EGF and/or TGFα were shown to inhibit the follicle-stimulating hormone-dependent inductions of luteinizing hormone receptor (Mondschein and Schomberg, 1981) and granulosa cell aromatase activity (Adashi and Resnick, 1986), as well as both basal and human chorionic gonadotropin-stimulated estradiol secretion by cultured porcine theca cells (Caubo et al., 1989). Although these results point to a largely inhibitory role, TGFα was found to induce basal estrogen production in cultured prepubertal porcine ovarian granulosa and theca cells. Whether this apparent discrepancy has an underlying developmental or species-specific basis is presently unclear.

Skinner and colleagues (1989) reported that cultured peritubular and Sertoli cells of the testis express and secrete TGFα protein. The growth factor induced peritubular cell migration, proliferation, and colony formation in vitro but had no affect on the growth of Sertoli cells. Interestingly, although TGFα did not influence transferrin expression in pure Sertoli cell cultures, it did promote the production of transferrin by Sertoli cells that were cocultured with peritubular cells. These results were interpreted to suggest possible paracrine roles for the growth factor in the maintenance of testicular function. However, in contrast to these results, Teerds et al. (1990) failed to detect TGFα-specific immunostaining in Sertoli cells in vitro or in vivo. Additionally, although peritubular-myoid cells in culture stained positive for the growth factor, those in tissue sections were negative. Although the basis for these apparent discrepancies is unclear, both laboratories reported germ cells to be negative for TGFα expression.

THE URINARY AND RESPIRATORY SYSTEMS. Low levels of TGFα protein and/or mRNA were detected in normal human (Gomella et al., 1989) and rat (Brown et al., 1990; Seroogy et al., 1991) kidney. Additionally, TGFα mRNA and protein were present in rat (Brown et al., 1990; Seroogy et al., 1991) and human (Liu et al., 1990) lung.

HEMATOPOIETIC CELLS AND THE VASCULATURE. Two observations suggest that TGFα might be an important product of hematopoietic cells. First, activation of cultured human alveolar macrophages with lipopolysaccharide was accompanied by the induction of TGFα mRNA and the secretion of TGFα protein (Madtes et al., 1988), and noncultured macrophages from wound sites also expressed TGFα mRNA (Rappolee et al., 1988b). Although these results imply that macrophage-produced TGFα could have an important role in epithelial proliferation and repair, it should be noted that skin and corneal wound healing was not impaired in TGFα-deficient mice (Luetteke et al., 1993b). Second, the long-term self-renewal of an avian erythroid progenitor population was promoted by a TGFα/EGF-R loop (Hayman et al., 1993). Finally, in a related finding, TGFα was immunohistochemically localized to vascular smooth muscle cells in arterioles (Mueller et al., 1990).

4. Targeted inactivation of the TGFα gene. As a first step to identifying physiological roles for TGFα, two laboratories recently derived "knockout" mice in which both alleles of the TGFα gene were rendered nonfunctional. Our laboratory constructed a targeting vector in which exon 4 (encoding the third loop of the mature growth factor) was disrupted by insertion of a thymidine kinase promoter-driven neomycin cassette (Luetteke et al., 1993b) (fig. 5). Mann and colleagues (1993), on the other hand, designed a targeting construct in which exon 3 (encoding the first two loops of the mature growth factor) was disrupted by the insertion of a phosphoglycerate kinase-promoter-neomycin gene expression cassette. Additionally, multiple stop codons were introduced in all reading frames between the sequences encoding the signal peptide and the mature growth factor. Both groups electroporated their targeting constructs into the E14 clone of embryonic stem cells and then used positive-negative selection to enrich for correctly targeted embryonic stem clones. Embryonic stem clones that harbored disrupted TGFα alleles as shown by Southern blot and polymerase chain reaction analyses, were subsequently microinjected into blastocysts. The latter were implanted into pseudopregnant foster mothers, and offspring displaying germ line chimerism were bred to establish lines of mice heterozygous for the disrupted TGFα gene. Matings of heterozygous mice produced homozygous offspring that were shown to lack detectable TGFα mRNA and protein by application of various sensitive assays.

Given evidence of TGFα expression in a large array of developing and adult tissues, it is perhaps surprising

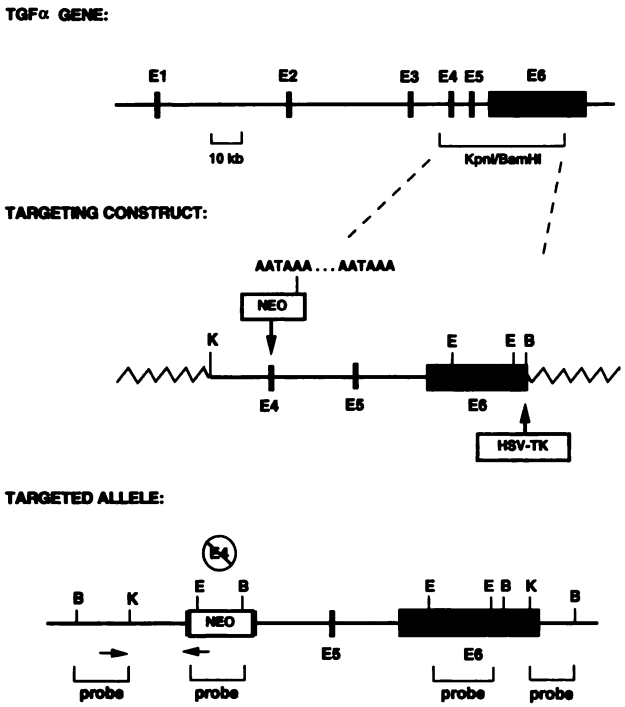

FIG. 5. TGFα gene targeting strategy. The organization (see also fig. 3) of the six exons (E1–E6) of the TGFα gene is indicated together with the relative position of a Kpn1/BamH1 fragment used to prepare the targeting vector (*upper*). This vector was modified by insertion into exon 4 of a neomycin (neo)-resistance gene cassette that is driven by the herpes simplex virus (HSV)-thymidine kinase (tk) (HSV-tk) promoter and polyoma virus enhancer (*middle*). Note that the neo cassette also contains two polyadenylation signals to discourage read-through transcription. The (HSV-tk) gene was inserted in reverse orientation at the 3′ end of the mouse sequence to allow for a positive-negative selection strategy that discriminates against random integrants (the tk gene should not be retained in a homologous recombination event). The *lower* schematic shows the predicted structure of the targeted allele together with the location of various probes used in Southern analysis to confirm the correct targeting event. Arrows represent the positions of polymerase chain reaction primers used to confirm the generation of a unique 2.2 kb product in homologous recombinants. B, BamH1; E, EcoR1; K, Kpn1.

that both laboratories reported homozygous mutant mice to be viable, generally healthy, and fertile. In fact, only two phenotypes were observed. The most conspicuous of these was that homozygous mutant mice displayed striking waviness of the fur and whiskers, which was most prominent during the first hair growth cycle. Individual hairs of all types were curly and kinky, with frequent irregularities in the caliber of the hair shaft and in the deposition of pigmented medullary cells. Histologically, neonatal skin from targeted homozygous mice showed dramatic disorganization of active hair follicles. In contrast to hair follicles in wild-type skin, which were precisely aligned at a particular depth in the dermis, follicles in the targeted mice were located throughout the dermis and were variably oriented with respect to one another. In targeted skin, follicles with erupting hair shafts were observed next to, and parallel with, the subcutaneous muscle layer, and some sections contained adjacent follicles in both cross-sections and longitudinal sections. Other follicles appeared distinctly serpentine. These dramatic effects on hair growth and follicle organization established physiological roles for TGFα in skin. The localization of TGFα (Finzi et al., 1991; Luetteke et al., 1993b) and EGF-R (Green et al., 1984; Luetteke et al., 1994) expression to the inner and outer root sheaths, respectively, suggested that interactions between these two cell layers, which are believed to be important for normal hair growth, could be mediated by the growth factor and its receptor. Interestingly, hair growth was accelerated in the TGFα-minus mice (Mann et al., 1993) but was stunted in transgenic mice that overexpressed TGFα in skin under the control of a keratin K14 promoter (Vassar and Fuchs, 1991).

In addition to the skin phenotype, which similarly affected all homozygous mutant mice, both lines of targeted mice displayed eye problems of variable incidence and severity. Observations common to both lines were that a proportion of the homozygous mutant mice were born with open eyes, and some older animals showed evidence of corneal scarring and inflammation. Luetteke and colleagues (1993b) reported that approximately 40% of adult homozygous mice displayed some form of ocular anomaly that ranged from mild corneal opacity to shrunken eyes that sometimes lacked a lens. Histological examination revealed frequent evidence of corneal inflammation and ulceration, retinal dysplasia, and anterior segment dysgenesis. Additionally, the eyelids of some TGFα-deficient mice were poorly developed and lacked organized muscle and glandular structures. None of these alterations were consistently observed, and in fact, the extent of pathology often differed between eyes of the same animal. Given the open-eye-at-birth phenotype, the possibility exists that these various eye abnormalities result from a primary defect in eyelid closure during development. In fact, a recent study suggests that eyelid closure could be variably delayed in a high proportion of TGFα-deficient mice (N. C. Luetteke and D. C. Lee, unpublished observations).

Both groups of investigators noted phenotypic similarities between the TGFα targeted mice and the mutant mouse line, (*wa*-1) (Crew, 1933). *wa*-1 mice also display pronounced waviness of the hair and whiskers, and some are born with open eyes at birth (Crew, 1933; Bennett and Gresham, 1956). Moreover, TGFα and *wa*-1 map to the same vicinity of mouse chromosome 6 (Fowler et al., 1993; Barrow et al., 1994). To determine whether *wa*-1 and TGFα are allelic, both laboratories mated homozygous TGFα-deficient and *wa*-1 mice. Of a total of 41 TGFα × *wa*-1 offspring born in the two studies, all had curly whiskers and wavy hair, confirming that TGFα and *wa*-1 behave as alleles. Consistent with this conclusion, the levels of TGFα mRNA (Luetteke et al., 1993b) and protein (Mann et al., 1993) were reduced approximately ten-fold in tissues of *wa*-1 mice. Inasmuch as the *wa*-1 proTGFα coding region is identi-

cal to that of wild-type mice (C. A. Harrison and D. C. Lee, unpublished observations), the *wa*-1 mutation is presumably regulatory in nature.

The mutant mouse line, *wa*-2, also displays hair and eye phenotypes that are similar to those of TGFα-deficient and *wa*-1 mice (Keeler, 1935). Because *wa*-2 maps to the vicinity of the EGF-R (c-*erbB*) gene on mouse chromosome 11, our laboratory recently investigated the possibility that the *wa*-2 mutation affects either the expression or activity of EGF-R (Luetteke et al., 1994). We found that the levels of EGF-R mRNA and protein were comparable in tissues of *wa*-2 and wild-type mice and that the ligand-binding ability of the *wa*-2 EGF-R was not detectably altered. However, the level of ligand-dependent receptor autophosphorylation was markedly reduced in liver membranes isolated from *wa*-2 mice when compared with those from controls. Western blot analysis using antiphosphotyrosine antibodies also revealed a significant reduction in the EGF-dependent phosphotyrosine content of EGF-R and other phosphotyrosine substrates in tissues from *wa*-2 mice compared with their normal counterparts. These results suggested that the *wa*-2 mutation might affect the receptor's tyrosine kinase activity, and in fact, a nucleotide sequence comparison of full-length wild-type and *wa*-2 EGF-R cDNAs revealed a single nucleotide discrepancy that was localized to the tyrosine kinase domain. A G-to-T transversion in the NH_2-terminal portion of the *wa*-2 tyrosine kinase sequence was predicted to result in the substitution of a glycine for a wild-type valine residue. Significantly, this valine is either conserved or conservatively replaced in a large group of receptor and nonreceptor tyrosine kinases, as well as in protein kinases with serine/threonine specificity. Based on studies of *ras* codon 12 mutations that result in the converse substitution, the *wa*-2 valine-to-glycine mutation could significantly perturb local structure in the vicinity of the receptor's ATP binding site. To assess the importance of the G-to-T alteration, the identical mutation was introduced into an otherwise normal EGF-R coding sequence. Transfection into receptorless CHO cells of an expression vector encoding the mutated EGF-R yielded expression of a receptor protein that, compared with a wild-type control, showed markedly reduced autophosphorylation in response to exogenous EGF (Luetteke et al., 1994). Hence, these results, together with the characterization of the TGFα-deficient and *wa*-1 mice, confirmed the importance of TGFα/EGF-R interactions in skin. The fact that EGF-R is not completely inactivated in *wa*-2 mice provides a likely explanation for the fact that the phenotype is limited to skin and eyes.

Given the widespread expression of TGFα in developing and adult tissues, as well as its ability to influence a variety of biological processes, it is perhaps surprising that loss of TGFα was not associated with a wider spectrum of phenotypes. Although it is possible that patterns of expression are poor indicators of physiological function, a more likely explanation is that the redundancy of EGF-R ligands compensates for loss of TGFα in other tissues. Confirmation of the latter hypothesis awaits the targeting of additional EGF family members.

B. Role in Transformation

1. Expression in neoplastic cells and tissues. Deregulated expression of growth controlling molecules has proved to be the hallmark of neoplastic transformation, and TGFα is a conspicuous example of this phenomenon. Early indications that tumor-derived and transformed cell lines produced much higher levels of TGFα than did their normal counterparts have been reinforced by reports from many laboratories. In an initial broad survey of small sample size, Derynck and colleagues (1987) found the levels of TGFα mRNA to be markedly elevated in a variety of primary and metastatic tumors compared with those in their normal tissue counterparts. Tumors with high levels of expression included (*a*) squamous carcinomas of the head, neck, and lung, (*b*) renal and mammary carcinomas, (*c*) adenocarcinomas of the stomach and endometrium, (*c*) large cell carcinomas of the lung, and (*d*) melanomas. Additionally, many of those same tumors expressed EGF-R mRNA at high levels. Induced expression of TGFα was observed with carcinoma-derived, and to a lesser extent, sarcoma-derived cell lines, but not with cell lines derived from hematopoietic tumors. The results of this initial survey have been corroborated by numerous studies that have focused on particular tumor types. Examples of these are cited in the following paragraphs.

Overexpression of TGFα is characteristic of gastrointestinal tract tumors and tumor-derived cell lines. For example, diffuse TGFα immunostaining was detected in the epithelium of 80 of 84 human pancreatic adenocarcinomas (Barton et al., 1991), and cultured pancreatic cancer cells produced and utilized TGFα as an autocrine growth factor (Smith et al., 1987; Ohmura et al., 1990; Hofer et al., 1991). Conversely, declines in TGFα mRNA levels correlated with the differentiation of a human pancreatic cell line that occurred either spontaneously or in response to sodium butyrate treatment (Estival et al., 1992). Additionally, inductions of TGFα have characterized liver carcinogenesis. Compared with the levels in their normal counterparts, TGFα mRNA and protein were markedly induced (at least 25- to 50-fold) in chemically-transformed rat liver epithelial clones (Liu et al., 1988; Grisham et al., 1990; L. W. Lee et al., 1991), as well as in chemically induced rat hepatocellular carcinoma lines (Luetteke et al., 1988). Interestingly, analysis of growth factor and proto-oncogene expression in 16 transformed rat liver epithelial lines suggested a possible correlation between the degree of tumorigenicity, and combined overexpression of TGFα and c-*myc*. Consistent with these in vitro studies, the levels of TGFα were on average several-fold higher in urine from patients with hepatocellular carcinoma as compared with

controls (Yeh et al., 1987). Emphasizing the potential importance of these observations, overexpression of TGFα in transgenic mice produced hepatic tumors with a frequency that depended on the genetic background of the mouse. Finally, immunoreactive TGFα has been frequently detected in colorectal neoplasias (Tanaka et al., 1991), and Liu et al. (1990) found the average TGFα content of 29 primary colonic tumors to be four-fold higher than that in normal mucosa. In comparing tumor and normal specimens from the same individual, the levels of TGFα protein were increased four- to 260-fold in 12 of 22 tumors. In light of these increases, it is surprising that expression of TGFα mRNA was generally unaffected. Increased production of TGFα by many human colon carcinoma cell lines (Coffey et al., 1987b; Hanauske et al., 1987; Watkins et al., 1988; Anzano et al., 1989; Ciardiello et al., 1991; Baldwin and Zhang, 1992), some of which also harbored an amplified EGF-R gene (Untawale et al., 1993), was associated with their autonomous proliferation (Karnes et al., 1992) and tumorigenicity (Modjtahedi et al., 1992).

Overexpression of TGFα has also been associated with tumors of the skin and mammary glands. In an early study, the growth factor was purified from the urine of melanoma patients (Kim et al., 1985), and TGFα immunostaining was subsequently detected in 16 of 17 human appendageal tumor types that were distinguished on the basis of their differentiation toward hair follicles, eccrine, apocrine, and sebaceous glands (Finzi et al., 1992). The level of TGFα immunostaining correlated with the differentiation status of the various tumor cell types, and decreased in the order hyperplasias > adenomas > benign epitheliomas > primordial epitheliomas. In addition, TGFα acted as an autocrine growth factor for several human squamous carcinoma cell lines that coincidentally overexpressed EGF-R (Reiss et al., 1991), and it may have a role in the development of cutaneous paraneoplastic syndromes (Ellis et al., 1987). Finally, Finzi et al. (1988) observed that TGFα stimulated the growth of skin papillomas but did not induce their neoplastic progression.

Using a radioimmunoassay, Stromberg and colleagues (1987) detected TGFα protein in urine samples from patients with disseminated breast cancer but not in those from normal subjects. Numerous studies of human breast cancer cell lines have described the expression of TGFα, together with its induction by estrogen (Dickson et al., 1987; Liu et al., 1987; Bates et al., 1988) and repression by the anti-estrogen, tamoxifen (Arteaga et al., 1988a). Use of EGF-R blocking antibodies to determine whether TGFα might mediate estrogen's growth-promoting activity have produced conflicting results. Arteaga and coworkers (1988a) reported that EGF-R blocking antibodies inhibited the TGFα-induced growth of hormone-dependent breast cancer cells but did not interfere with estrogen-mediated growth. Ahmed et al. (1991), on the other hand, reported that EGF-R blocking antibodies inhibited both TGFα- and estrogen-mediated growth (though not growth induced by prolactin or progesterone). In related studies, introduction of *ras* oncogenes was shown to elevate expression of TGFα by normal mouse (Salomon et al., 1987; Ciardello et al., 1988) and human (Ciardiello et al., 1990a) mammary epithelial cell lines, as well as by human MCF-7 breast cancer cells (Dickson et al., 1987). Although these various observations are consistent with the hypothesis that induced expression of the growth factor contributes to mammary tumorigenesis, Valverius and colleagues (1989) described a high level of TGFα secretion by normal human mammary epithelial cells derived from reduction mammoplasty. As a result, they suggested that malignant mammary transformation might be more related to differential growth responses to TGFα than to changes in the levels of its production. Nevertheless, it should be noted that transgenic expression of TGFα consistently produced tumors of the mammary gland in postlactational mice (Sandgren et al., 1990; Matsui et al., 1990).

Deregulated expression of TGFα has also been observed with tumors of reproductive and endocrine tissues. TGFα protein and/or activity were detected in the urine (Sherwin et al., 1983) and ascites (Arteaga et al., 1988b) of patients with ovarian cancer, and 15 EGF-R-positive primary ovarian cancers expressed mRNA for TGFα but not EGF (Morishige et al., 1991a). Pointing to the importance of this expression, the addition of TGFα to cell cultures derived from these tumors stimulated ^{3}H-thymidine incorporation, and anti-TGFα and anti-EGF-R antibodies were growth-inhibitory (Morishige et al., 1991b). In addition, TGFα was implicated in the autonomous growth of androgen-independent human prostatic carcinoma cells (Hofer et al., 1991), and a variety of endocrine tumors, including hypothalamic gangliocytomas, pheochromocytomas, and adrenal cortical carcinomas, displayed a higher level of TGFα immunoreactivity than their normal counterparts. However, no correlation existed between the level of staining and tumor grade or stage (Driman et al., 1992). Interestingly, TGFα was growth-inhibitory for the GH4 pituitary tumor cell line (Ramsdell, 1991).

Increased expression of TGFα has also been observed in kidney, lung, and brain tumors. Compared with levels in adjacent normal tissue, TGFα mRNA was increased two- to eight-fold in renal cell carcinomas from six of ten patients (Gomella et al., 1989). In a separate study, five of five kidney tumors expressed higher levels of TGFα mRNA than were present in normal autologous kidney tissue (Mydlo et al., 1989), and some kidney tumor-derived cell lines expressed and utilized TGFα as an autocrine growth promoter (Atlas et al., 1992). Production of TGFα-like activity by lung carcinoma cell lines has been described (Siegfried et al., 1987), and 16 primary lung carcinomas contained an average two-fold higher level of TGFα protein than a comparable number

of normal parenchyma samples (Liu et al., 1990). Additionally, high levels of TGFα protein were detected in 87 of 131 primary human lung adenocarcinomas by immunohistochemistry (Tateishi et al., 1990). For patients whose EGF-R-positive tumors displayed high and low TGFα staining, the 5-year survival rates were 36% and 85%, respectively. More recently, increased expression of TGFα was found to correlate with that of the EGF-R in lung carcinomas, as well as in cell lines derived from them (Liu and Tsao, 1993). Of 20 patients examined, 55% expressed at least two-fold higher levels of TGFα and EGF-R than those in the corresponding normal tissue. Detection of TGFα in lung tumor effusions is described below. Finally, TGFα mRNA was overexpressed in several human glioblastoma cell lines compared with normal human brain tissue (Saxena and Ali, 1992).

In conclusion, the numerous studies cited above create the collective impression that induction of TGFα is a frequent event in neoplastic transformation. This impression now needs to be critically evaluated through large-scale studies of primary tumors.

2. TGFα as a prognosticator. Efforts to determine whether measurement of TGFα protein could have diagnostic and/or prognostic value were encouraged by the detection of transforming growth factor-like activity in the urine of patients with disseminated cancer (Twardzik et al., 1982b; Sherwin et al., 1983; Kimball et al., 1984; Stromberg et al., 1987). Using a specific radioimmunoassay, Yeh and colleagues (1987) found the average urinary TGFα concentration to be four- to five-fold higher in a group of 31 hepatocellular carcinoma patients when compared with 33 age- and sex-matched controls. Twenty of 31 hepatocellular carcinoma patients, and 13 of 15 hepatocellular carcinoma-probable patients, displayed markedly elevated levels of TGFα protein, whereas the level of EGF was not significantly different. Based on these results, it was suggested that TGFα might be a useful complementary tumor marker for management of hepatocellular carcinoma patients who present with low levels of α-fetoprotein (a commonly used marker). Measurement of TGFα in urine of patients with advanced squamous cell carcinoma of the head and neck, on the other hand, produced ambiguous results (Fazekas-May et al., 1990). Although the absolute level of growth factor was not significantly elevated in a group of patients compared with controls, comparison of the same individual's preoperative and postoperative levels showed a correlation with the course of the disease in 43% of the cases. Finally, radioimmunoassay detected TGFα at levels from 1.6 to 50 ng/ml in tumor effusions from 13 of 34 breast cancers, 12 of 24 lung cancers, and 13 of 31 ovarian cancers (Arteaga et al., 1988b). Sarcomas and cancers of the pancreas, stomach, and colon also contained elevated levels of the growth factor. In contrast, TGFα was detected at concentrations less than 2 ng/ml in only three of 17 control effusions. Among the cancer patients, increased levels of TGFα correlated with several known prognosticators of poor survival rate, including the number of metastatic sites. Thus, 48 patients with a detectable level of TGFα had a median survival of 3 months, whereas the median survival of 72 patients with no detectable activity was 6 months. In sum, these studies are consistent with the possibility that measurement of TGFα protein could provide useful diagnostic and/or prognostic information. Whether its measurement would have advantages relative to currently used markers, however, has not yet been established.

3. TGFα-induced transformation of cultured cells. The fact that TGFα was discovered and purified on the basis of its ability to promote anchorage-independent growth of normal cultured fibroblasts prompted speculation that overproduction of the growth factor could contribute to neoplastic progression. The cloning of TGFα complementary DNAs in the mid-1980s allowed the testing of this notion both in cultured cells and in animals. Thus, Rosenthal et al. (1986) found that overexpression of TGFα under the control of the simian virus 40 early promoter induced stably transfected Rat-1 cells to form foci in monolayer, colonies in soft agar, and small tumors in nude mice. Consistent with the growth factor being responsible for these effects, anchorage-independent growth was inhibited in the presence of an anti-TGFα monoclonal antibody, and the size of individual soft agar colonies correlated with their levels of TGFα expression. Although these results confirmed TGFα's transforming activity, this activity is apparently not be as robust as that of certain oncogene products, inasmuch as comparable expression of a Ha-*ras* oncogene resulted in a higher rate of colony formation, and cells transformed with *ras* produced larger nude mouse tumors with higher frequency and shorter latency. Subsequently, NRK cell transformation was demonstrated in response to either infection with a retrovirus encoding a synthetic, secreted form of TGFα (Watanabe et al., 1987) or transfection with an expression plasmid encoding a noncleavable form of proTGFα (Blasband et al., 1990a). In addition, transformation of mouse mammary epithelial cell lines by expression of TGFα complementary DNAs has been described (Shankar et al., 1989; McGeady et al., 1989). Transformation of these cells was inhibited by the cyclic adenosine monophosphate analog, 8-Cl-cyclic adenosine monophosphate (Ciardiello et al., 1990b), but the significance of this observation is presently unclear.

In apparent contrast to the preceding studies, Finzi et al. (1987, 1990) reported that overexpression of TGFα under the control of either the cellular MT or a retroviral promoter failed to induce morphological transformation of various rodent cell lines. These included mouse (NIH-3T3 and BALB-3T3) and rat (NRK and Fisher) fibroblasts, as well as BALB/MK mouse keratinocytes. Surprisingly, retroviral expression of TGFα also failed to relieve the BALB-MK cell growth requirement for exogenous EGF (or TGFα). Although isolated NIH-3T3

clones secreted TGFα protein at relatively high levels, showed evidence of EGF-R down-regulation, and were growth-inhibited by an anti-TGFα antibody, they did not form foci in monolayer and were not tumorigenic in nude mice. In contrast, coinfection with retroviruses encoding both TGFα and EGF-R produced NIH-3T3 cell clones that efficiently formed colonies in soft agar and tumors in nude mice (Di Marco et al., 1989). These findings confirmed that TGFα-mediated transformation is dependent on the level of EGF-R expression, and suggested a possible quantitative discrimination between the levels of EGF-R required for normal growth stimulation and transformation. They also pointed to the importance of findings that the growth factor and its receptor are often co-overexpressed in human tumors and tumor-derived cell lines (Derynck et al., 1987; Di Marco et al., 1989).

4. Studies in transgenic mice. To explore the pathological consequences of its overexpression in vivo, three laboratories independently derived TGFα transgenic mouse lines. Sandgren and colleagues (1990) and Jhappan and coworkers (1990) both placed proTGFα coding sequences under the control of the zinc-inducible MT promoter. As a consequence, multiple tissues displayed high levels of TGFα transgene RNA and protein. Responses to this overexpression, which were tissue-specific and partly dependent on the background genetics of the host mouse strain, confirmed TGFα's potency as an epithelial and mesenchymal cell mitogen but also revealed unexpected activities. Most importantly, they showed that, although TGFα is not directly oncogenic in most tissues, it can initiate neoplastic growth in certain tissues. Specifically, Sandgren and colleagues (1990) found that TGFα promoted a uniform epithelial hyperplasia of several organs of the gastrointestinal tract without causing major changes in tissue architecture. For example, the wet weights of liver, stomach, small intestine, and colon were all increased two- to three-fold relative to controls. Despite these sizable increases in prominent organs, the body weights of MT-TGFα mice were actually reduced as a result of unexpected reductions in the weights of mesodermally-derived tissues including fat, muscle, and bone (Luetteke et al., 1993a). In the case of muscle and fat, declines in wet weight were accompanied by similar decreases in the cellularity of the tissues and occurred in the absence of significant changes in the metabolic rates of TGFα transgenic mice compared with nontransgenic littermates.

In marked contrast to the orderly growth of the gastrointestinal tract, TGFα produced unique effects in pancreas that included hyperproliferation of connective tissue and acinoductular metaplasia (Sandgren et al., 1990; Jhappan et al., 1990). The latter was characterized by the redifferentiation or dedifferentiation of acinar cells to ductal-like cells that stained negative for pancreatic amylase and occasionally positive for mucin (Sandgren et al., 1990; Bockman and Merlino, 1992). Although analogous changes have been previously reported to accompany pancreatic carcinogenesis in rodents (Longnecker, 1984), TGFα transgenic mice did not display an increased incidence of pancreatic tumors. TGFα overexpression also produced preneoplastic-like alterations in the stomachs of transgenic mice (Dempsey et al., 1992; Takagi et al., 1992a). Severe adenomatous hyperplasia resulted in hypertrophy of the gastric mucosa, and gastric acid secretion was repressed. Menetrier's disease, which is associated with carcinoma of the stomach, is characterized by similar aberrations, and four patients with Menetrier's disease showed abnormally high TGFα immunoreactivity in cells of the gastric mucosa (Dempsey et al., 1992). However, TGFα transgenic mice did not display an increased incidence of stomach cancer.

In contrast to its affects in other tissues, TGFα overexpression induced frank neoplastic growth in liver and mammary gland. Jhappan et al. (1990) described the frequent appearance in livers of CD1 transgenic mice of multifocal tumors. These included adenomas and well-differentiated, α-fetoprotein-positive hepatocellular carcinomas that developed from dysplastic parenchyma or occasionally adenomas (Lee et al., 1992). Their occurrence was significantly repressed in castrated transgenic males, but correspondingly increased in ovariectomized females (Takagi et al., 1992b). Tumors often expressed the transgene at markedly higher levels than adjacent normal tissue, whereas EGF-R expression was either unchanged or reduced relative to surrounding tissue. Tumors also frequently overexpressed the endogenous c-*myc* and insulin-like growth factor II genes but did not harbor mutated Ha-*ras* or Ki-*ras* genes. Sandgren et al. (1990, 1993), who utilized a mouse strain (C57BL/6) with a reduced susceptibility to liver cancer, observed a low incidence of benign liver tumors in aged mice. The implication from these contrasting findings that background genetics could influence the TGFα-mediated liver tumorigenesis was supported by the finding that the incidence of liver tumors in CD1 × FVB/N offspring was substantially reduced relative to the parental CD1 transgenic mice (19% versus 74%, respectively, at 13 months of age) (Takagi et al., 1992b).

In addition to occasional liver tumors, Sandgren et al. (1990) described the appearance in MT-TGFα mice of postlactational mammary adenocarcinomas. Simultaneously, Matsui and colleagues (1990) reported that directing expression of a TGFα transgene to mammary gland under the control of the mouse mammary tumor virus enhancer/promoter also produced tumors in that tissue. The latter investigators observed a range of growth abnormalities in transgenic females (but not males) that included solid and cystic hyperplasia, dysplasia, adenoma, and adenocarcinoma (Halter et al., 1992). Although abnormalities were more prevalent in multiparous mice, older virgins showed a similar spectrum of aberrations. Interestingly, some tumors dis-

played increased expression of the endogenous EGF-R. In recent unpublished work, Sandgren and colleagues specifically targeted TGFα overexpression to lactating mammary glands under the control of the whey acidic protein promoter. As a result, they observed a high incidence (> 90%) of breast tumors in postlactational females. In both MT- and whey acidic protein-TGFα mice, focal tumors coincided with large numbers of hyperplastic alveolar nodules. These nodules, which generally resembled normal lactating glands and may have corresponded to preneoplastic lesions, increased in number with consecutive pregnancies. Their origin may be explained by the preliminary observation that overexpression of TGFα blocked the postlactational regression of mammary glands in TGFα transgenic mice (E. P. Sandgren, J. Schroeder, and D. C. Lee, unpublished observations).

Subsequent to the aforementioned studies, two laboratories targeted expression of TGFα transgenes to skin under the control of keratin promoters. Vassar and Fuchs (1991) used the *K14* promoter to overexpress TGFα in stratified squamous epithelia, and as a result, observed a thickening of the epidermis in areas containing relatively few hair follicles. They also noted a stunting of hair growth, the frequent appearance of benign papillomas in areas of irritation or wounding, and changes associated with psoriasis. Papillomas in the TGFα transgenic mice were similarly induced by topical application of phorbol esters, which synergized with TGFα to enhance epidermal hyperproliferation (Vassar et al., 1992). Significantly, whereas the development of chemically-induced skin papillomas has been frequently associated with c-Ha-*ras* mutations, papillomas from the TGFα mice did not harbor c-Ha-*ras* mutations. TGFα overexpression under the control of the keratin *K1* promoter produced smaller than normal neonatal mice that had wrinkled, scaly skin and generalized hair loss (Dominey et al., 1993). At the molecular level, it was associated with alterations in the expression of certain keratins, and down-regulation of EGF-R. Hyperkeratosis of the epidermis was pronounced in juveniles, but also observed in adults that expressed particularly high levels of the transgene. As in the previously cited study, spontaneous papillomas occurred at sites of wounding, but most were prone to regression.

Results from these various transgenic studies show that, although TGFα is not generally oncogenic in vivo, it does produce growth abnormalities in certain contexts. The latter can extend to neoplastic transformation, and as described, overexpression of TGFα results in the appearance of malignant tumors in liver and mammary gland, and benign tumors in skin. Thus, acting alone, TGFα has limited oncogenic potential. However, a series of investigations have established that the growth factor can collaborate with both viral and cellular oncogenes to accelerate tumorigenesis in multiple tissues. The latter include examples of tissues that are not transformed by TGFα alone. Thus, co-expression of a TGFα transgene, together with that of transgenes encoding either simian virus 40 T-antigen or c-*myc*, dramatically accelerated tumor growth in mouse liver or pancreas compared with that observed in response to either oncogene (or TGFα) alone (Sandgren et al., 1993; Murakami et al., 1993). The mean survival times of the dual-positive transgenic mice were also significantly decreased relative to those of the corresponding single-positive transgenic mice. In both tissues, accelerated tumorigenesis was accompanied by an increased incidence of preneoplastic lesions, and tumors arising in TGFα × c-*myc* mice were more malignant in appearance than those produced by c-*myc* alone. Interestingly, in liver (but not pancreas), TGFα transgene expression was markedly higher in tumors than in adjacent uninvolved tissue. Moreover, the growth of tumors from either tissue transplanted into nontransgenic host mice was dependent on induced expression of the MT-TGFα transgene. These results point to the apparent importance of the growth advantage conferred by overexpression of TGFα.

Consistent with the preceding results, TGFα overexpression also dramatically accelerated chemically-induced hepatocarcinogenesis in transgenic males but not females (Takagi et al., 1993). Both genotoxic (diethylnitrosamine and dimethylnitrosamine) and nongenotoxic (phenobarbitol) chemicals produced malignant liver tumors with a high incidence several months before the time at which tumors normally developed in the untreated TGFα transgenic mice.

5. EGF-R as a therapeutic target. A logical deduction from findings described in the preceding sections is that disruption of TGFα/EGF-R interactions might slow tumor growth and, therefore, be of therapeutic benefit. In principle, this could be accomplished through the delivery of peptide antagonists that bind, but do not activate, receptor. The finding that all three disulfide-bonded loops are required for receptor binding suggests that it may be difficult to design effective antagonists. This concern notwithstanding, it is interesting to note that a truncated, nonglycosylated form of VGF that included only the actual EGF-like sequence bound EGF-R with high affinity, but only weakly stimulated mitogenesis and colony formation by NRK clone 49F and NIH-3T3 cells (Lin et al., 1990). In contrast, this synthetic VGF was either a partial or full agonist for other cell types. It remains to be seen whether this novel form of VGF has any utility as a therapeutic agent.

An alternate strategy is based on efforts to selectively kill tumor cells that express relatively high levels of EGF-R. Chaudhary et al. (1987) synthesized a novel gene that fused mature TGFα coding sequences with those specifying a modified *Pseudomonas* toxin that lacked its usual cell-recognition domain. The resulting chimeric protein retained TGFα's EGF-R binding activity and efficiently killed human tumor-derived A431 and KB cells that expressed roughly 10^6 and 10^5 EGF-R

molecules per cell, respectively. In contrast, human MCF-7 breast cancer cells, which harbored less than 10^4 receptor molecules on their surface, retained viability. Extending these observations, Heimbrook et al. (1990) reported that delivery of a TGFα-*Pseudomonas* exotoxin fusion protein doubled the survival time of nude mice injected with EGF-R-positive tumor cells but had no effect on that of mice injected with EGF-R-negative cells. Adverse effects produced by the recombinant toxin were minimal, and tissue damage was limited to slight hepatomegaly and mild periportal necrosis. In vivo delivery of the TGFα toxin also delayed the growth of implanted tumors formed from epidermoid and prostate carcinoma cells (Pai et al., 1991). More recent reports (Debinski et al., 1991; Theuer et al., 1992; Theuer et al., 1993) have emphasized the design of variant TGFα-toxin molecules, including one in which sequences responsible for the heparin-binding property of HB-EGF were fused to the TGFα domain. The resulting toxin bound heparin, and as an apparent consequence, its ability to kill cells that express relatively low levels of EGF-R was enhanced (Mesri et al., 1993).

Finally, at least one report has described improved tumor eradication in response to combined application of a chemotherapeutic agent (doxorubicin) and EGF-R-blockading monoclonal antibodies (Baselga et al., 1993).

VII. The TGFα Gene

A. Gene Structure

1. Chromosomal location. The TGFα gene is located in a linkage group on the short arm of human chromosome 2 (p11 to p13 region) (Brissenden et al., 1985; Tricoli et al., 1986), that is conserved in the 35-cM region of mouse chromosome 6 (Fowler et al., 1993; Barrow et al., 1994). This linkage group includes Igk-Ef1 and Igk-J (markers of the Igk complex), Ly-2 and Ly-3 (chains that form the murine T-cell CD8 glycoprotein), FABP1 (a liver protein), e.g., r4 (a nerve growth factor-induced, zinc-finger transcription factor), and Anx4 (a member of the lipocortin family). Recent studies have suggested a weak association between the human TGFα locus and genetic susceptibility to cleft lip with or without cleft palate (Chenevix-Trench et al., 1992; Stoll et al., 1993; Sassani et al., 1993; Farrall et al., 1993). The position of the human TGFα gene is close to the breakpoint of the Burkitt's lymphoma t(2;8) variant, but no significance has yet been attached to this observation.

2. Gene organization. ProTGFα is encoded by a 4.5 to 4.8 kb mRNA (see fig. 3). The proTGFα coding sequence, which is preceded by a relatively short 5′ UT region, is contained within the first 0.7 kb. The remainder of the transcript corresponds to a large 3′ UT sequence. In the rat, this 3′ UT contains an ACA repeat and multiple A+T motifs, but no role(s) for these have yet been established. The use of an alternate polyadenylation site located roughly midway through the 3′ UT sequence results in the occasional production of a 2.3 kb transcript that was fortuitously cloned as a complementary DNA by Lee and coworkers (1985a).

The rat TGFα mRNA is derived from six exons that collectively span approximately 90 kb of genomic DNA (Blasband et al., 1990b) (fig. 3). Exon 1 encodes the short 5′ UT region together with the NH_2-terminal portion of the signal peptide. The remainder of the signal peptide is encoded by exon 2. Exon 3 specifies the first two disulfide-bonded loops of the mature EGF-like sequence, whereas exon 4 encodes the third disulfide-bonded loop together with the transmembrane domain. The cytoplasmic sequence of proTGFα is specified by exon 5, and the entire 3′ UT portion of TGFα mRNA is encoded by a relatively large sixth exon. Exons 1 to 3 are separated by two large introns of roughly 35 to 40 kb each, whereas exons 3 to 6 are clustered in a region of 10 to 15 kb. Interestingly, the presence of a tandem CAG donor sequence at the 3′ end of the second intron results in micro-heterogeneity of splicing and the production of both 159- and 160-amino-acid proTGFα species in the same cell (Kudlow et al., 1989; Blasband et al., 1990b). The larger form contains an additional alanine residue in the short stretch between the signal peptide and the EGF-like sequence, but no significance has yet been ascribed to this heterogeneity. Although it has not been characterized in as much detail, the human TGFα gene seems to be similarly organized (Derynck et al., 1987).

Exon 6 of the rat TGFα gene also encodes a small antisense transcript of approximately 0.2 kb that is derived from sequence immediately upstream of the alternate 5′ polyadenylation site (Blasband et al., 1990b). This transcript is predicted to encode a 51-amino-acid polypeptide that lacks an apparent signal peptide but contains both an unusual asparagine-glutamine-$(\text{asparagine})_7$ motif and a potential glycosylation site. Inasmuch as this small transcript was constitutively expressed in tissues and cell lines that contained markedly different levels of TGFα mRNA, it is unlikely to function as an antisense regulator. On the other hand, a 0.35 kb transcript that similarly derives from the noncoding strand in the sixth exon of the human TGFα gene was inversely expressed relative to TGFα mRNA in a limited survey (Jakowlew et al., 1988).

B. Promoter Characterization

1. Transcriptional initiation. The human and rat TGFα promoters resemble those of so-called "housekeeping" genes in that both are embedded in GC-rich regions (the rat promoter is approximately 80% G+C) lacking TATA and CAAT motifs. In light of this observation, it is not surprising that transcription of the rat gene initiates from multiple sites. Using primer extension methodology, Blasband and colleagues (1990b) mapped two prominent transcription start sites to -58 and -188 (+1 corresponds to the first position of the translational initiation codon). Additional, minor start sites were mapped to the

region from -284 to -150 by both primer extension and S1 nuclease analyses, and their existence was indirectly supported by the finding that the 5′ ends of 6 independent rat TGFα complementary DNA clones corresponded to sites from -258 to -143. Inasmuch as these transcription start sites are roughly clustered in two regions (around -58 and -188) that collectively span over 200 base pairs of DNA, it may be appropriate to describe the rat gene as having two promoters, even though there is no evidence that the two are differentially utilized (fig. 6). Interestingly, despite the fact that the 5′ flanking regions are highly conserved (Lee et al., 1993), transcription was reported to initiate from a single site in the human TGFα promoter (Jakobovits et al., 1988). The position of this single site (at -62) is analogous to that of the rat downstream (-58) start site.

The functional activity of the human and rat promoters was established by demonstrating their ability to direct expression of reporter genes in transient transfection assays (Jakobovits et al., 1988; Blasband et al., 1990b). Additionally, the rat promoter was shown to direct specific transcription from the -58 and -188 start sites in vitro in the presence of crude nuclear extracts (Blasband et al., 1990b). An observation common to both the human and rat promoters was that constructs containing larger segments of 5′ flanking sequence showed reduced ability to direct reporter gene expression compared with proximal promoter elements containing 500 to 1000 base pairs of 5′ flanking sequence (Jakobovits et al., 1988; Blasband et al., 1990b). This could indicate the presence of upstream transcriptional repressor elements.

2. Transcription factor requirements. The TGFα promoter contains multiple sequence elements with greater than 70% homology to a ten-base-pair Sp1 consensus binding site (Chen et al. 1992). Transfection of a rat TGFα promoter-reporter construct into *Drosophila* Schneider cells, which normally lack endogenous Sp1 activity, suggested that at least some of these are functionally important. When transfected alone, the TGFα promoter was unable to direct significant expression of the reporter gene, but when cotransfected with an Sp1 expression vector, the reporter gene product was readily detected (Chen et al., 1992). The functionality of particular Sp1 binding sites from the region upstream of the -58 start site was corroborated by the finding that corresponding oligonucleotides displayed gel mobility shifts in the presence of purified Sp1 protein. Both the gel mobility shifts, as well as transcription in vitro from the -58 start site, were inhibited in the presence of an excess of unlabeled oligonucleotide containing a bona fide Sp1 consensus binding sequence. Finally, the sequential mutagenesis of individual Sp1 binding sites also inhibited transcription, with the mutation of multiple sites producing a greater degree of inhibition. The latter observation suggests that, consistent with results from other Sp1-dependent promoters, interactions between multiple Sp1 sites are required for maximal expression from the TGFα promoter. In recent work from our laboratory, the application of in vivo footprinting methodology has revealed evidence of protein binding to Sp1 binding sites in the rat TGFα promoter upstream of the -188 start site (Chen et al., 1994). (The -58 region was not well-resolved by this approach.) A synthetic oligonucleotide containing the footprinted sequence displayed a gel-mobility shift in the presence of Sp1 protein, and deliberate mutation of overlapping Sp1 sites in the footprinted sequence reduced transcription from the -188 site relative to that from the -58 site. These results suggest that transcription from the -58 and -188 regions may be differentially controlled by two separate clusters of Sp1 binding sites (fig. 6).

Additional evidence of a role for Sp1 was unexpectedly derived from studies designed to investigate the effect of DNA methylation on TGFα promoter activity. Shin and colleagues (1992) observed that exposure of a human melanoma cell line (HA-A) to the demethylating agent, 5-azacytidine, induced expression of TGFα mRNA. In follow-up experiments, they found that previous exposure of transfected HA-A cells to 5-azacytidine increased expression of the TGFα promoter/reporter gene without altering the methylation status of the transfected promoter sequences. Instead, 5-azacytidine treatment enhanced the formation of a promoter DNA-protein complex that contained immunoreactive Sp1 protein, and DNase1 footprinting of this complex revealed a footprint

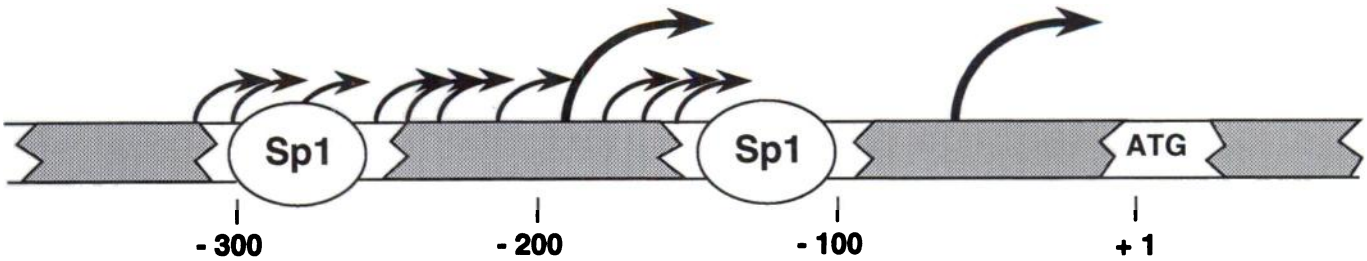

FIG. 6. Schematic of the rat TGFα promoter. Two major transcription start sites at -58 and -188 are indicated by large arrows, and multiple minor start sites are designated by small arrows. The relative positions of two clusters of Sp1-like binding sites shown to be required for maximal expression of the predominant start sites are also indicated. Numbering is relative to the first position of the initiating ATG codon.

indistinguishable from that formed with pure Sp1 protein. Additionally, gel mobility shifts carried out with extracts from 5-azacytidine-treated HA-A cells revealed a shifted complex that comigrated with a complex produced by recombinant Sp1 protein. The additional finding that 5-azacytidine did not alter Sp1 mRNA or protein levels was interpreted to suggest that the drug enhanced Sp1's accessibility to binding sites within the TGFα promoter region.

3. Repression by GC factor. Kitadai et al. (1993) recently reported that GC factor, which was originally identified as an activity that bound to G+C-rich sequences and repressed EGF-R promoter function, inhibited expression of endogenous TGFα mRNA when overexpressed from a vector transfected into human gastric carcinoma cell lines. Transient assays in which GC factor and TGFα promoter-reporter constructs were cotransfected confirmed the the ability of GC factor to repress TGFα promoter activity.

C. Gene Regulation

1. Transformation-associated induction. Numerous reports have described increased expression of TGFα protein and/or mRNA in cells transformed by retroviruses (Twardzik et al., 1982c, 1983), chemicals (Luetteke et al., 1988; Liu et al., 1988; L. W. Lee et al., 1991), and activated oncogenes (Salomon et al., 1987; Godwin and Lieberman, 1990). In some cases, it has been possible to directly compare the levels of TGFα produced by transformed progeny versus their normal, parental counterparts. For example, L. W. Lee and coworkers (1991) found consistent induction (up to 60-fold) of TGFα mRNA in 16 transformed rat liver epithelial cell clones that were derived by exposing the normal parental cell line, WB-F344 (WB), to the carcinogen N-methyl-N′-nitro-N-nitrosoguanidine. Recent work has shown that induction of TGFα mRNA in several of these transformed clones results from increased transcription of the gene, although the stability of resulting transcripts may also be enhanced to a modest extent (E. A. Berkowitz and D. C. Lee, unpublished observations).

Some investigations have focused on the link between *ras* transformation and TGFα expression. An early study showed that rat cells transformed with a temperature-sensitive mutant of Kirsten murine sarcoma virus (harboring Ki-v-*ras*) secreted TGFα only when the cells were phenotypically transformed at the permissive temperature (Kaplan et al., 1982). More recently, Godwin and Lieberman (1990) showed the levels of TGFα mRNA to be increased in Rat-1 fibroblast clones that expressed a stably integrated c-Ha-*ras* gene under the control of the metal-inducible MT promoter. The rise in TGFα mRNA was delayed relative to that of some other gene products, and the fold induction was also relatively small, suggesting that TGFα induction may not have been a direct *ras* response in this cell line. However, it should be noted that the rat TGFα promoter region contains the sequence GTGACTAA (around -460) which has been implicated as a *ras* response element in the polyoma virus enhancer (Imler et al., 1988). Induction of TGFα by *ras* is not limited to fibroblast lines but has also been described for mammary (Salomon et al., 1987; Ciardiello et al., 1988) and intestinal (Filmus et al., 1993) epithelial cells. Finally, in related observations, Ciardiello et al. (1990c) described increased levels of TGFα transcripts in NIH-3T3 cell clones that were transformed by a variety of oncogenes including *met*, *mos*, *src*, *fms*, *fes*, and *trk*. Although the levels of induced transcript varied in the different cell lines, it is not possible to correlate the degree of induction with particular oncogenes, inasmuch as only one clone was examined in each case.

Although a frequent association between cellular transformation and enhanced TGFα gene activity has been established, the mechanisms that link the two phenomena are unknown. The fact that Sp1 (or an Sp1-like factor) is required for maximal TGFα promoter activity raises the possibility that TGFα promoter activity could be induced in transformed cells by enhanced Sp1 expression or activity. However, compared with levels in the normal, parental cell line, Sp1 mRNA and protein were not significantly increased in several transformed rat liver epithelial cell clones that overexpressed the TGFα gene (Chen et al., 1994). This observation is consistent with the finding that other Sp1-dependent genes (e.g., dihydrofolate reductase) were not overexpressed in transformed cells that showed induced TGFα expression. However, it should be noted that the activity of Sp1 toward specific promoters could be selectively enhanced through modification of either the Sp1 protein itself (e.g., by phosphorylation) or the target sequences (e.g., by changes in methylation status), or the differential expression of Sp1-interacting proteins.

2. Regulation by defined agents. Expression of TGFα mRNA is regulated by a number of agents, as described below.

EGF-R LIGANDS AND PHORBOL ESTERS. TGFα mRNA is induced in a variety of cell types by EGF and TGFα (autoinduction), as well as by phorbol esters (e.g., TPA) (Coffey et al., 1987a; Mueller et al., 1989; Bjorge et al., 1989a; Pittelkow et al., 1989; Raymond et al., 1989). Interestingly, expression of EGF-R is also regulated by these same agents (Davis et al., 1985; Bjorge and Kudlow, 1987). In response to TPA, which typically produces a greater-fold induction than the EGF-R ligands, TGFα mRNA levels generally rose to a peak within 6 to 12 h and then declined significantly (though generally not to background levels) by 24 h. Nuclear run-on analyses indicated that the TPA response was mediated via increased transcription of the gene in rat liver epithelial (Raymond et al., 1989) and human colon cancer (Coffey et al., 1992) cell lines. In contrast, measurements of mRNA decay in the presence of actinomycin D suggested that TPA induction in human keratinocytes resulted

from a stabilization of TGFα transcripts (Coffey et al., 1992). Several reports described the fact that exposure of cells to either prolonged doses of TPA (Pittelkow et al., 1989) or protein kinase C inhibitors (Bjorge et al., 1989b; Coffey et al., 1992) blunted induction by either phorbol ester or growth factor, but in one study, a protein kinase C down-regulator (bryostatin) was found to abrogate the phorbol ester response without affecting growth factor-mediated autoinduction (Klein et al., 1992). Consistent with these results, TGFα mRNA levels were induced in human keratinocytes by a synthetic diacylglycerol (Pittelkow et al., 1989) and in a rat hepatic epithelial cell line by several hormones (angiotensin, epinephrine, bradykinin) that activate protein kinase C in this cell type (Raymond et al., 1989). The effects of RNA and protein synthesis inhibitors, on the other hand, have depended on the cell type. Thus, the RNA synthesis inhibitor, actinomycin D, abrogated the induction by phorbol ester in human keratinocytes (Pittelkow et al., 1989) but induced TGFα mRNA in human pancreatic carcinoma cells, even in the absence of TPA (Glinsmann-Gibson and Korc, 1991). The protein synthesis inhibitor, cycloheximide, did not prevent induction by TPA in keratinocytes: in fact, when added alone, it produced a significant increase in TGFα mRNA accumulation (Pittelkow et al., 1989). On the other hand, concurrent addition of cycloheximide blocked TPA induction in rat liver epithelial cells, but if TPA was present for at least 2 h before its addition, cycloheximide enhanced the TPA response (Raymond et al., 1989). Finally, another type of tumor promoter, dioxin, was found to increase TGFα mRNA levels in human keratinocytes (Choi et al., 1991), probably as a result of transcript stabilization (Gaido et al., 1992). Taken together, these data indicate that, although there may be mechanistic differences between various cell types, production of TGFα can be regulated and autoregulated through protein kinase C-dependent pathways. They also suggest that tumor-promoting agents may contribute to neoplastic progression, in part by inducing expression of this growth factor. Finally, it should be noted that Raja et al. (1991) have, at least partially, reproduced phorbol ester and growth factor regulation of the TGFα promoter by transfecting human mammary carcinoma cells with a TGFα promoter/luciferase reporter gene construct containing 300 base pairs of 5′ flanking sequence.

STEROIDS. Increased levels of TGFα protein and/or mRNA have coincided with estrogen-induced growth responses in uterus (Nelson et al., 1992) and anterior pituitary (Borgundvaag et al., 1992). Estrogen-induction of TGFα was inhibited by a dopamine receptor agonist in anterior pituitary but not in the uterus. Numerous reports have also described three- to five-fold inductions of TGFα protein and mRNA in estrogen- and progesterone-treated human breast cancer cells (Dickson et al., 1987; Arteaga et al., 1988a; Bates et al., 1988; Murphy and Dotzlaw, 1989; Musgrove et al., 1991; Murphy et al., 1991), with the induction in MCF-7 cells being largely inhibited by retinoic acid (Fontana et al., 1992). Furthermore, estrogen regulation of TGFα promoter/reporter constructs in transfected human breast cancer cells has been demonstrated (Saeki et al., 1991). An 1140-basic protein fragment of the human TGFα 5′ flanking region directed a two- to ten-fold increase in chloramphenicol acetyltransferase activity in estrogen-responsive cells, with no increase observed in estrogen-unresponsive cells. The same fragment directed 30- to 300-fold inductions of luciferase reporter gene expression, surprisingly robust responses given the modest induction of the endogenous TGFα gene. Elements responsible for estrogen induction were localized to the region between -77 and -343 by deletion analyses. Finally, TGFα mRNA levels were decreased in human endometrial adenocarcinoma cell lines treated with the progestin, medroxyprogesterone acetate (Gong et al., 1991).

TGFβ. TGFβ has been observed to both increase and decrease TGFα expression, depending on the cell type. Mueller and Kudlow (1991) found that TGFα mRNA levels were decreased 70% in bovine anterior pituitary cells cultured in the presence of 1 ng/ml TGFβ for 4 days, an effect that coincided with decreased proliferation of the cells. In contrast, two recent reports described from two- to several-fold inductions of TGFα mRNA (and protein) in human colon carcinoma cells treated with either TGFβ1 or dimethylformamide (Zipfel et al., 1993; Lynch et al., 1993). Paradoxically, both agents coincidentally inhibited cellular proliferation. The TGFβ1 response was reproduced by transfecting human colon adenocarcinoma cells with TGFα promoter/luciferase gene constructs (Lynch et al., 1993). The same study localized the TGFβ1 responsive element(s) to the region from -77 to -201.

OTHER AGENTS. Induction of both TGFα and EGF-R mRNAs was recently observed in several human pancreatic cell lines treated with TNF-α (Schmiegel et al., 1993). Induction of EGF-R mRNA, which was accompanied by increased transcription of the gene, was dependent on protein synthesis as well as a pathway that may involve phospholipase A_2. Interestingly, the effects of TNF-α on TGFα and EGF-R expression may be mediated via different TNF receptors. Whereas TNF regulation of TGFα was dependent on the p75 TNF-receptor, the induction of EGF-R expression was mediated by the p55 TNF-receptor (Kalthoff et al., 1993). Finally, expression of TGFα promoter-reporter constructs and/or endogenous TGFα mRNA were also induced by interleukin-1 in human keratinocytes (S. W. Lee et al., 1991), by γ-interferon in MDA468 human breast carcinoma cells (Hamburger and Pinnamaneni, 1993), by glucose or glucosamine in vascular smooth muscle cells (McClain et al., 1992), by cell density in human keratinocytes (Pittelkow et al., 1993), and by the human immunodeficiency virus-1 Tat protein in human breast carcinoma cells, especially when Tat was present in combination

with EGF (Nabell et al., 1994). For the most part, these inductions were relatively small, and both their underlying mechanisms and physiological relevance remain to be established.

VIII. Conclusions

Since the time of its initial discovery, TGFα has emerged as one of the best-characterized polypeptide growth factors. A wealth of studies from a large number of laboratories have analyzed its structure, synthesis and processing, patterns of expression, and interactions with EGF receptor. In addition, animal models of TGFα overexpression and deficiency have been created. As a result of these investigations, our understanding of TGFα biology has become considerably more sophisticated. We now appreciate that TGFα is expressed not only in embryos and fetuses but also in a wide variety of normal adult tissues. In fact, based on comparative patterns of expression, TGFα is likely to be a more common ligand for the EGF-R than is EGF. On the other hand, a considerable body of data supports the notion that induction of TGFα is a frequent consequence of neoplastic progression, and studies in animal models indicate that its overexpression can markedly influence the course of neoplastic disease. In some instances, this influence may be independent of its growth-promoting activities. Moreover, although TGFα is a powerful epithelial mitogen in vivo, it can nevertheless inhibit the growth and/or development of some tissues in transgenic animals. Hence, it seems appropriate to view TGFα as another example of a bifunctional or multifunctional growth factor. Finally, TGFα is the best-studied example of a subset of polypeptide growth factors defined by the release of soluble forms from integral membrane proteins.

Despite the fact that a large body of information has been published on TGFα, fundamental issues still must be resolved. For example, its precise physiological roles are not known. Because this is also true of the several other EGF-R ligands, we cannot yet appreciate the extent to which their roles may overlap or be distinct. Indeed, even a thorough comparison of the relative patterns of expression of the various EGF-R ligands has not yet been reported. Additionally, although there are clear distinctions in the biological responses that are evoked by the different EGF family ligands, we have only a vague understanding of the underlying mechanisms. An important point is that we do not yet appreciate the relative importance in vivo of membrane-anchored versus soluble forms of these growth factors. Given our current state of knowledge, it is entirely possible that the integral membrane forms are the physiologically important species, with soluble forms arising in response to cell stress or death. Finally, the mechanisms that regulate expression of TGFα at both the pre- or post-translational levels, and particularly those responsible for induced TGFα expression in tumorigenesis, largely remain to be elucidated. Hopefully, these and other issues will have been explored in much greater detail before too many more reviews are written on this subject.

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