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Transforming Growth Factor α: an Aromatic Side Chain at Position 38 Is Essential for Biological Activity

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Site-directed mutagenesis has been performed in the human transforming growth factor α gene. When tyrosine 38 is mutated into phenylalanine or tryptophane, biological activity is retained. In contrast, other alterations between cysteine 34 and cysteine 43 and disruption of disulfide bonds 8 to 21 and 34 to 43 resulted in loss of activities. The presence of an aromatic side chain at position 38 of transforming growth factor α seems to be essential for its activity.

Transforming growth factor α (TGF-α) is a 50-amino-acid polypeptide. First isolated from a retrovirus-transformed mouse cell line (10), it has subsequently been found in human tumor cells (11, 31), in early rat embryo cells (19), in cell cultures from the bovine pituitary gland (25), and normal keratinocytes from human adults (8). Although the function of TGF-α is not yet clear, roles for TGF-α have been proposed in transformation (1, 33, 24), wound healing (29), bone resorption (15), angiogenesis (28), and cell migration (2). TGF-α is related to epidermal growth factor (EGF) both structurally and functionally (1, 20, 21). Little is known about the amino acids involved in their binding to the receptor. We have previously described (18) mutations carried out at residues Asp-47 and Leu-48 and shown that Leu-48 is critical for the function of TGF-α. Here we describe mutations performed in other parts of the molecule.

Mutations performed on TGF-α. We performed mutagenesis as shown in Fig. 1. The most mutations were performed on amino acids located between cysteines 34 and 43 that were conserved in the EGF-like family of peptides. Conservation of these residues suggests that they are important either structurally or functionally. Tyr-38, Gly-40, and Arg-42 are conserved among all EGF-like peptides (4, 7, 12, 14, 20, 27, 30, 32). The fact that three conserved amino acids are located close to each other is striking; the only other amino acids conserved among all EGF-like peptides (apart from the six cysteines) are Gly-19 and Leu-48. The crucial importance of Leu-48 for the activity of TGF-α has been described (18).

We focused our attention on Tyr-38, which is a large polar amino acid and well conserved. Tyr-38 was mutated into Ala (small, nonpolar), Ser and Thr (small, with a hydroxyl group), His (large, with an imidazole ring), and Trp and Phe (aromatic amino acids like Tyr) (Fig. 1). Of special interest is the mutation of Tyr-38 into Phe, because Phe differs from Tyr only in the lack of a hydroxyl group on the aromatic ring.

Detection and biological activity of yeast-secreted proteins of mutant TGF-α. The mutations shown in Fig. 1 were performed by site-directed mutagenesis of a human TGF-α coding sequence cloned in M13mp18 (12). The sequence of the mutated gene has been determined (26) along the entire sequence of TGF-α for each mutant. The mutated sequences were inserted into the yeast shuttle vector pyTE1 (18) and expressed in *Saccharomyces cerevisiae* 20B-12 (*MATα trp1 pep4-3*) (16). Selected clones were grown to saturation, and the yeast media were dialyzed thoroughly against 1 M acetic acid in 3,000-molecular-weight cutoff dialysis tubing. The presence of mutant TGF-α proteins was verified by radioimmunoassay in nondenaturing conditions by using a polyclonal antibody (34D) raised against the recombinant human TGF-α molecule (6). Biological activity of the mutant proteins was also tested in radioreceptor and colony formation assays (1). All of the tested mutant TGF-α proteins were detected by the radioimmunoassay, proving that they were all expressed and secreted into the yeast media. The amounts of mutant TGF-α proteins detected varied between 50 and 500 ng/ml. The dialyzed yeast media were lyophilized and concentrated 20 or 100 times, and radioreceptor and colony-forming abilities were measured on these concentrated media. The only active mutants were two mutants in position 38: [Trp-38]-TGF-α and [Phe-38]-TGF-α.

Mutations of TYR-38 not equally tolerated for biological activity. Mutants of Tyr-38 were further purified as described previously (18). The elution profiles on a Biogel P30 column for the mutant TGF-α proteins were identical to that shown for wild-type TGF-α in a previous paper (18). The wild-type and mutant TGF-α activities were always found between the two peaks of A_{280}, in an area were few proteins were present. The maximum activities in both radioreceptor and soft-agar assays were found in the same fraction, called the peak fraction. The activities were expressed in terms of EGF equivalence as reported previously (18). The peak fractions were also tested in thymidine incorporation assays and in radioimmunoassays (with the Biotope polyclonal antibody in denaturing conditions). All of the partially purified mutant TGF-α proteins were recognized, under denaturing conditions, by this polyclonal antibody, which was raised against the native TGF-α. Nevertheless, our radioimmunoassay,

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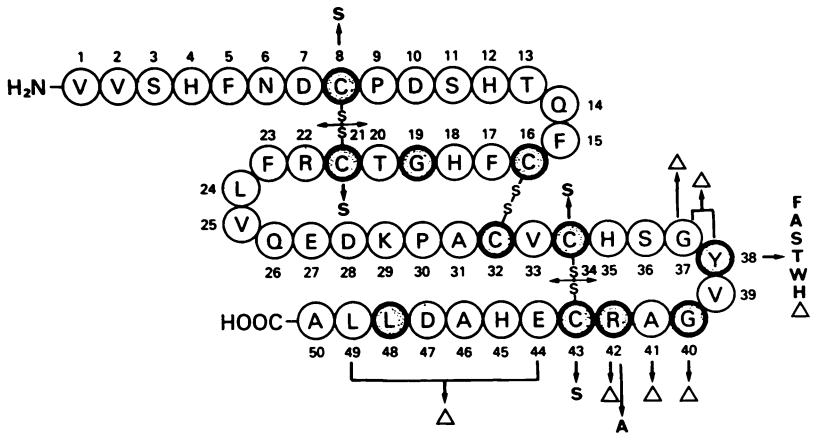

FIG. 1. Mutations in human TGF-α. The amino acids conserved in all of the family of EGF-like growth factors (human and murine EGFs and TGF-αs), as well as the gene products of the vaccinia virus (vaccinia growth factor), the Shope fibroma virus (Shope fibroma growth factor), and the myxoma virus (myxoma growth factor), are enclosed in bold circles. The mutations of amino acids at positions 8, 21, 34, 38, 42, and 43 are indicated by arrows. The deletions at positions 37, 38, 40, 41, 42, and 44 to 49 are symbolized by Δ. Symbols: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

which is carried out under conditions for which TGF-α is partially or fully denatured, does not discriminate between correctly and improperly folded TGF-α molecules. The results of the bioassays are summarized in Table 1. As a general feature, mutants showing very little activity in the radioreceptor assay (less than 2 ng/ml, EGF equivalence) exhibit more activity in the soft-agar assay. This discrepancy has been observed for other TGF-α mutants (18). The amounts of protein present in the peak fraction of the biologically active mutants were measured by a Bradford (5) micromethod assay and were (μg/ml) 1.8 for wild-type TGF-α, 1.5 for [Phe-38]-TGF-α, and 0.5 for [Trp-38]-TGF-α. Dilution curves of the peak fractions (Fig. 2) were used to quantitate the biological activities and to check whether wild-type TGF-α and the active mutant proteins [Phe-38]-TGF-α and [Trp-38]-TGF-α behave in a similar way. The slopes of these curves in the three assays performed (binding competition, Fig. 2A; colony formation, Fig. 2B; and thymidine incorporation, Fig. 2C) were parallel to the ones established in the same assay with recombinant TGF-α and with EGF used as standards. In Table 2, activities of the mutants were normalized in protein amounts and expressed as percentages of those obtained with the wild type. Although [Trp-38]-TGF-α showed similar relative potencies in all assays, [Phe-38]-TGF-α showed only 50% activity relative to wild-type TGF-α in the radioreceptor assay and showed 100% activity relative to wild-type TGF-α in colony formation and thymidine incorporation assays. The differences between the relative binding and colony-forming activities for [Trp-38]-TGF-α suggest that this mutant acts as a super-

TABLE 1. Biological and biochemical activities of partially purified mutant TGF-α proteins

Insert in yeast expression vector	Clone no.	EGF equivalence (ng/ml) in[a]:		TGF-α equivalence (ng/ml) in radioimmunoassay
		Radioreceptor assay	Soft-agar assay	
Wild-type TGF-α	27	167 (a)	173 (a)	320
		350 (b)	240 (b)	2,400
	215	450 (c)	540 (c)	2,000
None	32	0	0	0
[Phe-38]-TGF-α	36	87 (a)–178 (b)	101 (b)	800
	39	41 (a)–73 (b)	60 (b)	300
[Trp-38]-TGF-α	125	50 (b)	128 (b)	600
	126	40 (c)	220 (c)	900
[His-38]-TGF-α	42	2 (a)	3.3 (b)	320
	44	1.4 (a)	1.8 (b)	770
[Thr-38]-TGF-α	1	2.3 (b)	4.8 (b)	1,600
	3	1 (b)	2.1 (b)	2,400
[Ser-38]-TGF-α	7	1.6 (b)	4 (b)	1,400
	9	1 (b)	2.3 (b)	2,000
[Δ Tyr-38]-TGF-α	11	0.1 (b)	0 (b)	600
[Ala-38]-TGF-α	27	0.3 (b)	5.6 (b)	860
	30	0.4 (b)	5.6 (b)	900
[Δ Ala-41]-TGF-α		0	0	900
[Δ Arg-42]-TGF-α		0	0	700
[Ala-42]-TGF-α		0	0	360

[a] a, b, and c refer to different series of experiments.

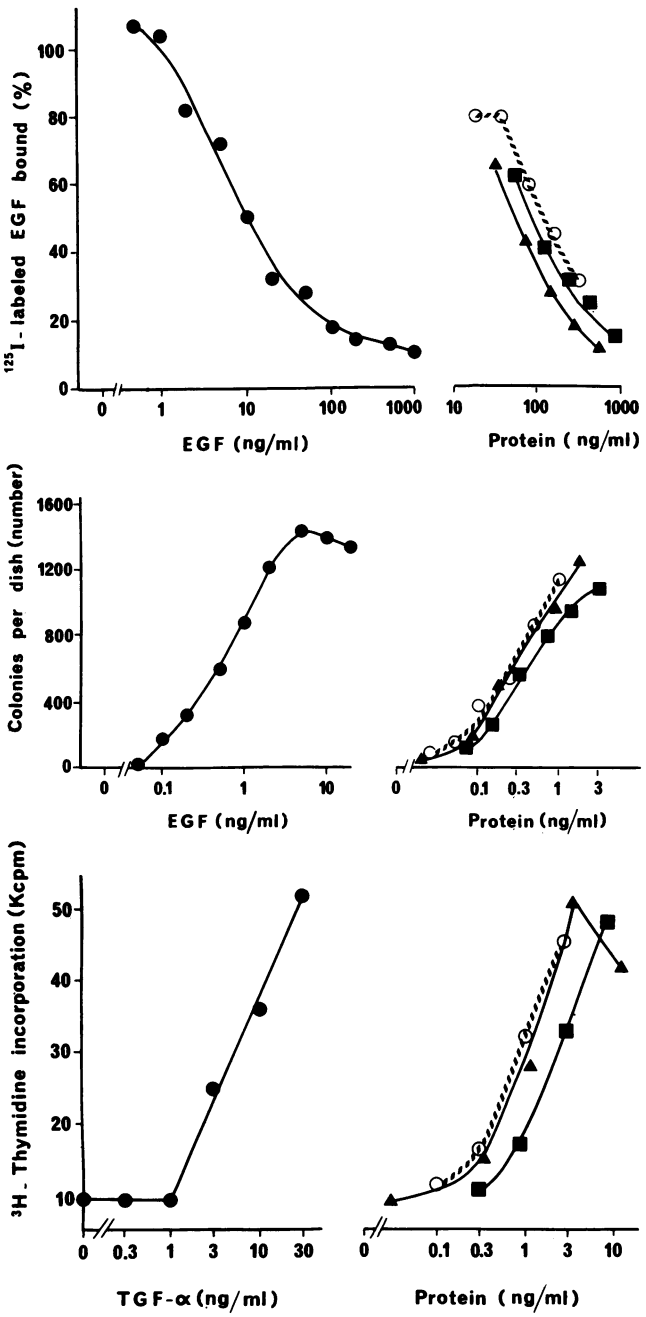

FIG. 2. Biological activities of wild-type TGF-α, [Phe-38]-TGF-α, and [Trp-38]-TGF-α. Biological activities of the peak fraction from the P30 columns were measured and normalized to protein amounts (4). Dilutions of the peak fractions were used to establish the curves. Symbols: ▲, wild-type TGF-α; ■, [Phe-38]-TGF-α; ○, [Trp-38]-TGF-α. (A) EGF radioreceptor assay. ●, EGF standard curve. (B) Colony-forming assay. Colonies (62 μm) were counted. ●, EGF standard curve. (C) [^{3}H]thymidine incorporation assay. ●, TGF-α standard curve.

agonist and that the molecular basis of TGF-α binding might be distinguishable from its transforming activity.

[His-38]-TGF-α seemed to be an interesting mutant to be compared with wild-type TGF-α because of the nature of the side chain of His. This mutant has an aromatic imidazole side chain at position 38. Nevertheless, all its biological activities are low, and [His-38]-TGF-α does not bind to the receptor. It seems that the nature of the aromatic side chain at position 38 is critical for the binding of TGF-α to its receptor.

TABLE 2. Relative bioactivities of partially purified TGF-α proteins

Insert in the yeast expression vector	Bioactivities expressed as % of those of the wild type		
	Radioreceptor assay	Colony formation	[^{3}H]thymidine incorporation
Wild-type TGF-α	100	100	100
No TGF-α	0	0	0
[Phe-38]-TGF-α	60	60	50
[Trp-38]-TGF-α	50	100	100
[His-38]-TGF-α	1	1	4

A few other mutant TGF-α proteins, [ΔAla-41]-TGF-α, [ΔArg-42]-TGF-α, and [Ala-42]-TGF-α, were also partially purified on a Biogel P30 column. The expression of these TGF-α mutants was confirmed by radioimmunoassay, and the peak fraction was defined in this case as the fraction containing the highest amount of immunoreactive TGF-α. Despite further purification, none of these mutants which were inactive in the initial screening (above) displayed biological activity (Table 1), either in the radioreceptor or in the colony-forming assay.

We wished to study the relationship between structure and function of TGF-α by means of site-directed mutagenesis of TGF-α. Our first results showed the critical role that Leu-48 plays in the function of TGF-α (18). In this paper, we report that disruption of the disulfide bonds 8 to 21 or 34 to 43, as well as deletion of six amino acids (positions 44 to 49) at the carboxyl terminus of TGF-α, leads to the loss of activity (data not shown). Changes in amino acids located between Cys-34 and Cys-43 showed that deletion of Gly-37, Gly-40, Ala-41, and Arg-42 and changing Arg-42 into Ala-42 also resulted in loss of biological activity.

We focused our attention on Tyr-38, which is highly conserved among EGF-like peptides. When Tyr-38 is deleted or mutated into Ala, Ser, His, and Thr, the mutant proteins have no activity or insignificant activity. For biologically inactive mutants, we cannot distinguish effects of mutation on the folding of TGF-α from effects on the biological activity due to alterations of the side-chain functional groups. Structural physical data such as circular dichroism or nuclear magnetic resonance would be necessary to distinguish these effects. Interestingly, mutation of Tyr-38 into Phe or Trp leads to mutants displaying binding ability. Phe and Trp both have an aromatic side chain, suggesting that the presence of an aromatic side chain at residue 38 of TGF-α is essential for the interaction with the receptor. However, while [Phe-38]-TGF-α is equipotent in binding, colony-forming, and mitogenic assays, [Trp-38]-TGF-α is more potent in the colony-forming and mitogenic assays than in binding. This result suggests that it might be possible to dissociate binding from signal transduction.

It is interesting to note that, in two homeotic gene products, the *Drosophila* Notch gene product (17, 34) and the *lin-12* gene product of the nematode *Caenorhabditis elegans* (13), as well as in repeat units from the EGF precursor (3), there are EGF-like sequences where the residue corresponding to Tyr-38 of TGF-α is in most cases a tyrosine or a phenylalanine. At the DNA level, the preferred codon for Tyr is TAC and for Phe it is TTC, which allows the mutation

of Tyr into Phe by only one base substitution. The solution structures of murine and human EGFs determined by nuclear magnetic resonance analysis, show, that Tyr-37 of EGF (which is the equivalent of Tyr-38 of TGF-α) is exposed in the molecule, making it a possible candidate for interaction with the receptor (9, 22, 23). Montelione et al. proposed that Tyr-37 and Leu-47 belong to a cluster of amino acids present at the surface of the EGF. We have shown previously that Leu-48 of TGF-α is crucial for the activity of the molecule.

Our present data show that Tyr-38 can be mutated into Phe or Trp, both being amino acids with aromatic side chains. We suggest that Leu-48 and Tyr-38, which are close to each other in solution and are exposed in the molecule, are both involved in the function of TGF-α. They may interact and provide a proper conformation to TGF-α or bind directly to the receptor.

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LITERATURE CITED

1. **Anzano, M. A., A. B. Roberts, J. M. Smith, M. B. Sporn, and J. E. DeLarco.** 1983. Sarcoma growth factor from conditioned medium or virally transformed cells is composed of both type α and type β transforming growth factors. Proc. Natl. Acad. Sci. USA **80**:6264–6268.
2. **Barrandon, Y., and H. Green.** 1987. Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-α and epidermal growth factor. Cell **50**:1131–1137.
3. **Bell, G. I., N. M. Fong, M. M. Stempien, M. A. Wormsted, D. Caput, L. Ku, M. S. Urdea, L. B. Rall, and R. Sanchez-Pescador.** 1986. Human epidermal growth factor precursor: cDNA sequence, expression in vitro and gene organization. Nucleic Acids Res. **14**:8427–8446.
4. **Blomquist, M. C., L. T. Hunt, and W. C. Barker.** 1984. Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. Proc. Natl. Acad. Sci. USA **81**:7363–7367.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Bringman, T. S., P. B. Lindquist, and R. Derynck.** 1987. Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell **48**:429–440.
7. **Chang, W., C. Upton, S. Hu, A. F. Purchio, and G. McFadden.** 1987. The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similar to those encoding epidermal growth factor and transforming growth factor α. Mol. Cell. Biol. **7**:535–540.
8. **Coffey, R. J., R. Derynck, J. N. Wilcox, T. S. Bringman, A. S. Goustin, H. L. Moses, and M. R. Pittelkow.** 1987. Production and auto-induction of transforming growth factor-α in human keratinocytes. Nature (London) **328**:817–820.
9. **Cooke, R. M., A. J. Wilkinson, M. Baron, A. Pastore, M. J. Tappin, I. D. Campbell, H. Gregory, and B. Sheard.** 1987. The solution structure of human epidermal growth factor. Nature (London) **327**:339–341.
10. **DeLarco, J. E., and G. J. Todaro.** 1978. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA **75**:4001–4005.
11. **Derynck, R., D. V. Goeddel, A. Ullrich, J. U. Gutterman, R. D. Williams, T. S. Bringman, and W. H. Berger.** 1987. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res. **47**:707–712.
12. **Derynck, R., A. B. Roberts, M. E. Winkler, E. Y. Chen, and D. V. Goeddel.** 1984. Human transforming growth factor-α: precursor structure and expression in *E. coli*. Cell **38**:287–297.
13. **Greenwald, I.** 1985. Lin-12, a nematode homeotic gene, is homologous to a set of mammalian proteins that include epidermal growth factor. Cell **43**:583–590.
14. **Gregory, H.** 1975. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature (London) **257**: 325–327.
15. **Ibbotson, K. J., D. R. Twardzik, S. M. D'Souza, W. R. Hargreaves, G. J. Todaro, and G. R. Mundy.** 1985. Stimulation of bone resorption *in vitro* by synthetic transforming growth factor-α. Science **228**:1007–1009.
16. **Jones, E.** 1976. Proteinase mutants of *Saccharomyces cerevisiae*. Genetics **85**:23–30.
17. **Kidd, S., M. R. Kelley, and M. W. Young.** 1986. Sequence of the Notch locus of *Drosophila melanogaster*: relationship of the encoded protein to mammalian clotting and growth factors. Mol. Cell. Biol. **6**:3094–3108.
18. **Lazar, E., S. Watanabe, S. Dalton, and M. B. Sporn.** 1988. Transforming growth factor-α: mutation of aspartic acid 47 and leucine 48 results in different biological activities. Mol. Cell. Biol. **8**:1247–1252.
19. **Lee, D. C., R. Rochford, G. J. Todaro, and L. P. Villarreal.** 1985. Developmental expression of rat transforming growth factor-α mRNA. Mol. Cell. Biol. **5**:3644–3646.
20. **Marquardt, H., H. W. Hunkapiller, L. E. Hood, and G. J. Todaro.** 1984. Rat transforming growth factor type 1: structure and relation to epidermal growth factor. Science **233**:1079–1082.
21. **Massague, J.** 1983. Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. **258**:13614–13620.
22. **Montelione, G. T., K. Wuthrich, E. C. Nice, A. W. Burgess, and H. A. Scheraga.** 1986. Identification of two anti-parallel beta-sheet conformations in the solution structure of murine epidermal growth factor by proton magnetic resonance. Proc. Natl. Acad. Sci. USA **83**:8594–8598.
23. **Montelione, G. T., K. Wuthrich, E. C. Nice, A. W. Burgess, and H. A. Scheraga.** 1987. Solution structure of murine epidermal growth factor: determination of the polypeptide backbone chain-fold by nuclear magnetic resonance and distance geometry. Proc. Natl. Acad. Sci. USA **84**:5226–5230.
24. **Rosenthal, A., P. B. Lindquist, T. B. Bringman, D. V. Goeddel, and R. Derynck.** 1986. Expression in rat fibroblast of a human transforming growth factor-α cDNA results in transformation. Cell **46**:301–309.
25. **Samsoondar, J., M. S. Kobrin, and J. E. Kudlow.** 1986. α-transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. J. Biol. Chem. **261**:14408–14413.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5466.
27. **Savage, C. R., Jr., J. H. Hash, and S. Cohen.** 1973. Epidermal growth factor: location of disulfide bonds. J. Biol. Chem. **248**:7669–7672.
28. **Schreiber, A. B., M. J. Winkler, and R. Derynck.** 1986. Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor. Science **232**:1250–1253.
29. **Schultz, G. S., M. White, R. Mitchell, G. Brown, J. Lynch, D. R. Twardzik, and G. J. Todaro.** 1987. Epithelial wound healing enhanced by transforming growth factor-α and vaccinia growth factor. Science **235**:350–352.
30. **Simpson, R. J., J. A. Smith, R. L. Moritz, M. J. O'Hare, P. S. Rudland, J. R. Morrison, C. J. Lloyd, B. Grego, A. W. Burgess, and E. C. Nice.** 1985. Rat epidermal growth factor: complete amino sequence. Eur. J. Biochem. **153**:629–637.
31. **Todaro, G. J., C. Fryling, and J. E. DeLarco.** 1980. Transforming growth factors produced by certain human tumor cells:

polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. USA **77**:5258–5262.
32. **Upton, C., J. L. Macen, and G. McFadden.** 1987. Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. J. Virol. **61**:1271–1275.
33. **Watanabe, S., E. Lazar, and M. B. Sporn.** 1987. Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type α transforming growth factor gene. Proc. Natl. Acad. Sci. USA **84**:1258–1262.
34. **Wharton, K., K. M. Johansen, T. Xu, and S. Artavanis-Tsakonas.** 1985. Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell **43**:567–581.