Transforming growth factor-βs (TGF-βs) are potent inhibitors of cell proliferation, and disruption of components of the TGF-β signaling pathway leads to tumorigenesis. Mutations of transmembrane receptors and Smads mediating intracellular signaling have been reported in various cancers. To identify transcriptional targets of TGF-β, we conducted an expression profile analysis. HaCaT cells derived from human keratinocytes and highly sensitive to TGF-β were treated with TGF-β in the absence or presence of cycloheximide (CHX). mRNAs extracted from the HaCaT cells were used for hybridization of oligonucleotide arrays representing approximately 5600 human genes. TGF-β increased the expression of PAI-1, junB, p21 cdk inhibitor, Smad7, IG-H3, and involucrin that have been reported to be up-regulated by TGF-β, validating the usefulness of this approach. The induction of IG-H3 by TGF-β was completely abolished by CHX, suggesting that the transcription of IG-H3 is not directly regulated by TGF-β. Unexpectedly, we identified more genes down-regulated by TGF-β than up-regulated ones. TGF-β repressed the expression of epithelial specific Ets that may be involved in breast and lung tumorigenesis, which could contribute to tumor suppression by TGF-β. Among a panel of cell cycle regulators, TGF-β induced the expression of p21 cdk inhibitor; however, the induction of other cdk inhibitors was not significant in the present study. Taken together, the results suggest that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

Key words: TGF-β — DNA chip — HaCaT — p21 — Ets

Transforming growth factor-βs (TGF-βs) belong to a large family of secreted polypeptides that include activins, bone morphogenetic proteins (BMPs), and other ligands. Members of the TGF-β superfamily exert a wide variety of biological activities, and govern cell fate, such as growth, apoptosis, and differentiation.1) TGF-βs invoke varying cellular responses depending upon the cell type and environment. TGF-βs inhibit cell growth and arrest cells at the G1/S boundary in the cell cycle.2) Thus, TGF-βs are negative regulators of cell growth and suppress tumorigenesis.3) In a different context, however, TGF-βs promote cell proliferation. This is thought to be an indirect effect via induction of secretion of other growth factors. TGF-β1 was originally identified as a factor that induces anchorage-independent growth of normal cells. Thus once tumor cells are rendered insensitive to TGF-β, TGF-β may support tumor invasion through promotion of cell adhesion, angiogenesis, and immunosuppression.4)
deacetylase.11–14) p300 and CBP neutralize the positive charge of histones and loosen chromatin structure, resulting in activation of transcription. In contrast, histone deacetylases tighten chromatin structure and repress transcription. Thus Smads are involved in both positive and negative regulation of transcription by the TGF-β superfamily members. Inhibitory Smads (I-Smads) antagonize signaling by R-Smads and Co-Smads at least by inhibiting phosphorylation of R-Smads.

Eight mammalian Smads have been identified.5, 6) Smad2 and Smad3 are activated by TGF-β and activin type I receptors. Smad1, Smad5, and Smad8 mediate BMP signaling. Smad4 is the only Co-Smad found in mammals. Smad4 was originally identified as DPC4, a tumor suppressor gene product in pancreas cancers.15) Smad6 and Smad7 are I-Smads. Smad6 preferentially inhibits BMP signaling, whereas Smad7 antagonizes TGF-βs, activins and BMPs.

Components of the TGF-β signaling pathway are altered in cancer cells.3) The TGF-β type II receptor gene contains a consecutive stretch of 10 adenines that correspond to amino acids 125–128 within the extracellular region of the receptor. In cases of hereditary non-polyposis colorectal cancer (HNPCC) with mismatch repair defect, this adenine stretch is frequently mutated to give rise to truncated receptors.16) It was also reported that a case of HNPCC without mismatch repair defect suffers from a germline mutation in the TGF-β type II receptor gene.17) Repression of TGF-β type II receptor was shown to be responsible for oncogenesis of Ewing sarcomas.18) Although the number is less than the type II receptor, alterations of the TGF-β type I receptor have been reported.3, 19) As mentioned above, Smad4 was identified as a tumor suppressor in pancreas cancers. Mutations of Smad4 are also found in colon, lung, and other cancers.20) Smad2 was found to be mutated in colon and lung cancers.21, 22) In an animal model, heterozygotic compound mutation of APC and Smad4 gave rise to invasive colon cancers.23) Polyps with loss of heterozygosity of the Smad4 gene grew in mice with heterozygous loss of Smad4.24) Smad3 knock-out mice frequently developed invasive colon cancers.25) Mice with heterozygous deletion of the TGF-β1 gene exhibited accelerated tumorigenesis by chemical carcinogens compared to wild type mice.26) All of these observations are consistent with the idea that TGF-β is a tumor suppressor.

It is thus important to identify targets of TGF-β in growth regulation. Recent advances in the DNA chip technology have enabled comprehensive survey of such target genes. We conducted oligonucleotide microarray analysis using HaCaT cells derived from human keratinocytes. TGF-β increased the expression of p21 cdk inhibitor. On the other hand, TGF-β repressed the expression of epithelial specific Ets that may be involved in breast and lung tumorigenesis.27, 28) Our results indicate that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

**MATERIALS AND METHODS**

**Cell culture** HaCaT cells were provided by Nobert E. Fusenig (DKFZ, Heidelberg, Germany), and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics. Mv1Lu cells were obtained from American Type Culture Collection (Bethesda, MD), and cultured in DMEM with 10% FBS and antibiotics.

**Growth inhibition assay** Cells were seeded in 24-well plates at a density of 5×10⁴ cells per well, and treated with various concentrations of TGF-β. [³H]Thymidine incorporation was assayed as previously described.29)

**RNA extraction and northern blotting** HaCaT cells were treated with 400 pM of TGF-β for the indicated time periods. When cells were cultured in the presence of cycloheximide (Sigma, St. Louis, MI), 20 µg/ml of the drug was added to the medium 1 h before the addition of TGF-β. Total RNA was extracted from the cells with Isogen (Wako, Osaka). Ten micrograms of RNA was electrophoresed and blotted onto a membrane. Radioactive probes were made using Ready-To-Go Kit (Amersham Biosciences).
Transcriptional Targets of TGF-β

Pharmacia Biotech, Piscataway, NJ). Membranes were hybridized, washed, and subjected to Fuji BAS imaging as described.\(^{30}\) mRNA was purified from total RNA using Oligotex dT-30 Super latex beads (TaKaRa Biochemicals, Tokyo). Northern blotting was performed to monitor the quality of mRNA (unpublished results).

**Oligonucleotide microarray analysis**

Oligonucleotide microarray “GeneChip” (Affymetrix, Santa Clara, CA) analysis was performed essentially as described.\(^{31}\) Aliquots of the mRNA carefully examined by northern blotting were used for the preparation of biotinylated probes. The first strand cDNA was synthesized from 2 \(\mu\)g of mRNA with an oligo(dT) primer containing a T7 RNA polymerase promoter sequence at its 5' end using SuperScript Choice System (Gibco BRL, Rockville, MD). The second strand cDNA was synthesized by *Escherichia coli* DNA polymerase I and ligase. One microgram of cDNA was used for the following *in vitro* transcription. The reaction was performed in the presence of biotinylated ribonucleotides using EnZo BioArray High Yield RNA Transcript Labelling Kit (Affymetrix). Synthesized cRNA was cleaned with RNeasy (Qiagen, Valencia, CA), and fragmented by incubation at 94°C for 35 min in buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate. Hybridization of a GeneChip array (HuGeneFL) was performed for 16 h. Washing and staining were done as described.\(^{31}\) GeneChip arrays were scanned by a confocal scanner.

The data collected from scanning were processed by using GeneChip software supplied by Affymetrix,\(^{32, 33}\) and “Average Difference” intensities and fold changes were calculated. Note that fold change does not necessarily match the ratio of intensities because the formula for fold induction is not the simple ratio of intensities, but takes other factors into consideration. In extracting genes that show significant change (Tables I and II, but not Table III), we set the criterion that fold change is greater than or equal to 3 at 2 or 6 h of TGF-β treatment. In addition, we excluded genes whose intensity is lower than the background level after increase or before decrease.

**Fig. 2.** Northern blotting of TGF-β-inducible genes. HaCaT cells were treated with 400 \(\mu\)M of TGF-β for the time periods indicated. Total RNA was extracted from the cells, and subjected to northern blotting. The probes used were PAI-1 (A) and junB (C). In the experiment (A), cells were cultured in the absence or presence of 20 \(\mu\)g/ml cycloheximide (CHX). In the experiment (C), CHX was not added. The intensities of the bands were quantified for PAI-1 (B) and junB (D). The values were normalized against the intensity at time 0 in the absence of CHX. (B) ○ CHX (-), ◆ CHX (+).
RESULTS AND DISCUSSION

Responses of HaCaT cells to TGF-β To identify genes transcriptionally regulated by TGF-β, we used HaCaT cells derived from human keratinocytes. We confirmed the inhibitory effect of TGF-β on the growth of HaCaT cells in comparison with Mv1Lu mink lung epithelial cells as a reference (Fig. 1). The DNA synthesis of HaCaT cells was almost completely inhibited by TGF-β at the concentration of 100 pM. Thus, HaCaT cells are highly sensitive to TGF-β, at least in growth inhibition assay. We used cycloheximide (CHX) in an attempt to identify genes directly regulated by TGF-β. In a previous study, 10 µg/ml of CHX was used to inhibit de novo protein synthesis. We used 20 µg/ml of CHX. This concentration of CHX caused no morphological change of HaCaT cells for 24 h (unpublished results). We next performed northern blotting to see the time course of expression of TGF-β-inducible genes (Fig. 2). The expression of plasminogen activator inhibitor-1 (PAI-1) continuously increased for at least 6 h, and CHX treatment caused a slight decrease at 6 h. On the other hand, junB transcripts reached a peak at around 2 h, and then decreased at 4 h as reported previously in NRK cells. Based upon these observations, we treated HaCaT cells with 400 pM TGF-β for 2 and 6 h in the presence or absence of CHX, and extracted mRNA.

Genes up-regulated by TGF-β We conducted an expression profile analysis using oligonucleotide arrays, the GeneChip system developed by Affymetrix. We first monitored the quality of the extracted mRNAs using test chips containing control genes such as glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) and β-actin (unpublished results), and confirmed that the mRNAs are intact enough to perform hybridization of oligonucleotide arrays of approximately 5600 human genes. Global expression patterns of 2 and 6 h TGF-β treatment are shown in Fig. 3.

Genes up-regulated by TGF-β are listed in Table I. In selecting the genes, we employed a relatively stringent criterion. According to the manufacturer’s specification, a 2-fold difference of hybridization intensity can be significant. We adopted 3-fold difference as the cut-off threshold either at 2 or 6 h, and identified 32 genes that account for 0.6 % of the 5600 genes examined. When we took a 2-fold change as the threshold, approximately 200 genes were selected (unpublished results). As previously reported, PAI-1, junB, p21 cdk inhibitor, Smad7, βIG-H3, and involucrin exhibited increase. gadd45, which was shown to be induced by TGF-β, also increased 2.7 fold at 2 h in the absence of CHX (unpublished results). The induction of PAI-1 in GeneChip analysis correlated well with the result of northern blotting both in the absence and presence of CHX (Fig. 2B). Besides these genes, it was revealed that expression of many other genes was also induced upon TGF-β stimulation. Although the relevance of these genes to the action of TGF-β is not clear at present, future studies should shed light on this subject.

Intriguingly, nma is a human homolog of BAMBI. BAMBI was identified in Xenopus, and was shown to form inactive complexes with receptors for members of the TGF-β superfamily. nma thus may act as a negative feedback component in TGF-β signaling.
Table 1. Genes Up-regulated by TGF-β (400 pM)\textsuperscript{a,b}

| Genbank accession no. | Description | Function | Control intensity (0 h) | TGF-β (2 h) | TGF-β (6 h) |
|-----------------------|-------------|----------|-------------------------|-------------|-------------|
|                        |             |          | CHX - | CHX + | CHX - | CHX + | CHX - | CHX + | CHX - | CHX + | CHX - | CHX + | CHX - | CHX + |
| J03764                 | PAI-1       | extracellular matrix | 52 | 96 | 466 | 520 | 1074 | 682 | 20.5 | 7.1 |
| L07919                 | Dhr-2       | transcription factor | 2 | 5 | 46 | 57 | >6.9 | >4.4 | >6.9 | >4.4 | >6.9 | >4.4 |
| U62800                 | cystatin M  | proteinase inhibitor | -13 | -9 | 37 | 49 | >6.2 | >4.8 | >5.8 | >16.1 |
| U23070                 | nma (Bambu homologue) | TGF-β-family pseudoreceptor | 25 | 66 | 98 | 98 | 4.9 | 1.5 | 148 | 159 | 5.2 | 2.4 |
| U20734                 | junB        | proto-oncogene, transcription factor | 43 | 209 | 172 | 660 | 4.5 | 3.2 | 89 | 650 | 2.4 | 3.1 |
| X16707                 | fra-1       | transcription factor | 38 | 253 | 161 | 643 | 4.2 | 2.5 | >100 | 1212 | <10.7 | 4.8 |
| L43821                 | HEF1        | docking protein | -3 | 18 | 25 | 44 | >3.8 | >2.4 | 15 | 51 | >1.8 | 2.8 |
| U67784                 | RDC1        | G protein-coupled receptor | 12 | 8 | 48 | 8 | >3.7 | >1.2 | 23 | 20 | >1.7 | >2.1 |
| U90546                 | BTF4        | glycoprotein | 12 | 6 | 44 | 14 | 3.7 | >1.4 | 36 | 0 | >2.2 | >1.1 |
| L22846                 | EZF-2       | transcription factor | 36 | 52 | 43 | 63 | 3.6 | 1.2 | 42 | 53 | >2.4 | 1.0 |
| D13540                 | SHP-2       | tyrosine phosphatase | -13 | 10 | 11 | -1 | >3.5 | <1.6 | 2 | 1 | >1.7 | <1.6 |
| U73936                 | Jagged1     | Notch ligand | 11 | 28 | 31 | 39 | >3.4 | >1.4 | 25 | 57 | >1.8 | >2.1 |
| M16364                 | creatine kinase-B | protein kinase | -6 | -16 | 17 | -37 | >3.4 | <2.1 | -8 | 24 | <1.1 | >3.8 |
| J04102                 | ets-2       | proto-oncogene, transcription factor | 30 | 50 | 62 | 187 | 3.3 | 3.2 | 131 | 211 | 4.4 | 4.3 |
| Z79693                 | protein tyrosine phosphatase receptor type R | receptor | 1 | 17 | 24 | 21 | >3.3 | >1.3 | 3 | 56 | >1.1 | 3.4 |
| M16750                 | pim-1       | oncogene, serine/threonine kinase | 38 | 77 | 81 | 62 | 3.3 | 1.4 | 61 | 169 | 1.6 | 2.5 |
| J04111                 | c-jun       | proto-oncogene, transcription factor | 9 | 82 | 31 | 177 | >3.2 | 2.2 | 36 | 122 | >2.0 | 1.5 |
| X02612                 | cytochrome P-450 | cytochrome | 22 | 40 | 60 | 194 | 3.2 | 5.9 | 30 | 1727 | 1.4 | 43 |
| AF010193               | Smad7       | TGF-β signaling inhibitor | 87 | 291 | 290 | 644 | 3.1 | 2.7 | 251 | 408 | 2.9 | 1.4 |
| U09579                 | p21         | CDK inhibitor | 109 | 182 | 334 | 349 | 3.1 | 2.5 | 216 | 496 | 2.5 | 3.5 |
| M93143                 | plasminogen-like protein | extracellular matrix | 11 | 20 | 36 | 28 | 3.1 | 1.4 | 19 | 32 | >1.4 | 1.6 |
| X17025                 | human homolog of yeast IPP isomerase | biosynthetic enzyme | 43 | 57 | 129 | 82 | 3.0 | 1.4 | 137 | 135 | 1.6 | 1.6 |
| L27624                 | tissue factor pathway inhibitor-2 | proteinase inhibitor | 22 | 50 | 66 | 8 | 3.0 | <3.1 | 43 | 22 | 1.9 | 2.2 |
| L29219                 | CDC-42 kinase 1 | protein kinase | 4 | 15 | 24 | 24 | >3.0 | >1.4 | 20 | 5 | >1.5 | <1.7 |
| L13286                 | mitochondrial 1,25-dihydroxyvitamin D3 24-hydroxylase | mitochondrial protein | 0 | 10 | 24 | 27 | >2.5 | >1.9 | 55 | 26 | >3.1 | >2.6 |
| M24351                 | parathyroid hormone-like protein A (PTHHLH) | parathyroid hormone | 130 | 226 | 280 | 139 | 2.1 | 1.6 | 501 | 135 | 3.8 | -1.7 |
| M77349                 | βIG-H3 (TGF-β induced gene product) | unknown | 1389 | 1804 | 2491 | 1657 | 1.8 | 1.0 | 5698 | 1755 | 4.1 | 1.1 |
| M13903                 | involucrin | membrane-bound protein | 314 | 322 | 518 | 540 | 1.7 | 1.7 | 1207 | 530 | 3.8 | 1.6 |
| M62324                 | modulator recognition factor 1 (MRF-1) | unknown | 19 | 41 | 32 | 24 | 1.7 | 1.1 | 85 | 31 | >4.2 | 1.4 |
| M63262                 | 5-lipoxygenase activating protein (FLAP) | biosynthetic enzyme | 8 | 20 | 29 | 8 | 1.7 | 1.2 | 53 | 36 | >3.2 | 1.8 |
| Z37976                 | latent transforming growth factor-β binding protein (LTBP-2) | extracellular matrix | 28 | 44 | 36 | 55 | 1.3 | 1.6 | 116 | 73 | 3.5 | 2.1 |
| X80822                 | ribosomal protein L18a | ribosomal protein | 1372 | 2474 | 1702 | 3619 | 1.2 | 1.5 | 4219 | 3165 | 3.1 | 1.3 |

\textsuperscript{a} Genes were listed according to the magnitude of the fold increase at 2 h in the absence of CHX. Upward and downward arrows represent increase and decrease, respectively.

\textsuperscript{b} A greater than sign (>) indicates that the fold change likely represents an overestimation, since the intensity of the gene was below a certain threshold in the TGF-β-untreated control sample, and, consequently, the fold change was increased to an arbitrary, low value by the GeneChip software.

\textsuperscript{c} A less than sign (<) indicates that the fold change likely represents an underestimation, since the intensity of the gene was below a certain threshold in the TGF-β-untreated control sample, and, consequently, the fold change was decreased to an arbitrary, high value by the GeneChip software.

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Table II. Genes Down-regulated by TGF-β (400 pM)\(^a\)

| Genbank accession no. | Description | Function | CHX | TGF-β (2 h) | TGF-β (6 h) |
|-----------------------|-------------|----------|-----|-------------|-------------|
|                       |             |          | Intensity | Fold change | Intensity | Fold change |
| X04500                | promonterleukin 1β | cytokine | 142 | 13.2 \(\downarrow\) | 24 | 6.0 \(\downarrow\) |
| U41163                | creatine transporter (SLC6A10) | transporter | 16 | -11 | 14 | 3.4 \(\uparrow\) |
| M29550                | calcineurin A1 | protein phosphatase | 67 | 7 | 35 | 1.2 \(\uparrow\) |
| X57522                | RING4 | transporter | 50 | 6 | 35 | 5.5 \(\uparrow\) |
| U60276                | arsenite-stimulated human ATPase | anion-transporting ATPase | 56 | 2 | 13 | 4.3 \(\uparrow\) |
| U90543                | butyrophilin (BTF1) | glycoprotein | -6 | -58 | -31 | 2.2 \(\uparrow\) |
| U38451                | epithelial-specific Ets (ESE-1b) | transcription factor | 240 | 46 | 21 | 2.9 \(\uparrow\) |
| D38037                | FKBP-12 | peptidyl-prolyl cis-trans isomerase | 1 | -31 | -54 | 3.6 \(\uparrow\) |
| M27492                | interleukin-1 receptor | cytokine receptor | 36 | 17 | 18 | 1.9 \(\uparrow\) |
| L40386                | DP-2 | transcription factor | 33 | 20 | 35 | 1.6 \(\uparrow\) |
| D86961                | KIAA0206 gene | unknown | 32 | 6 | 33 | 1.8 \(\uparrow\) |
| L60203                | sterol regulatory element binding protein-2 | transcription factor | 15 | 13 | 42 | 4.1 \(\uparrow\) |
| M30703                | ampharelulin | growth factor | 30 | 3 | 28 | 3.1 \(\uparrow\) |
| L25270                | XI-E169 | unknown | -7 | -20 | 25 | 1.9 \(\uparrow\) |
| X86163                | B2-bradykinin receptor 3 | G-protein coupled receptor | 27 | 10 | 19 | 1.4 \(\uparrow\) |
| U94836                | ERPROT 213-21 | unknown | 52 | 34 | 3 | 3.4 \(\uparrow\) |
| U30313                | diadenosine tetraphosphate synthase | nucleotide pyrophosphatase | 26 | 1 | 17 | 3.0 \(\uparrow\) |
| M31525                | MHC class II lymphocyte antigen (HLA-DNA) | lymphocyte antigen | 132 | 159 | 170 | 1.3 \(\uparrow\) |
| U53003                | GT335 | unknown | 32 | 9 | 6 | 2.2 \(\uparrow\) |
| X74795                | cdc46 | DNA replication licensing factor | 521 | 568 | 558 | 1.3 \(\uparrow\) |
| U72661                | ninjurin 1 | adhesion molecule | 47 | 125 | 39 | 3.8 \(\uparrow\) |
| D49490                | protein disulfide isomerase related protein (PDIR) | oxidoreductase | 14 | 17 | 41 | 2.3 \(\uparrow\) |
| X04325                | gap junction protein | unknown | 19 | 2 | 25 | 3.1 \(\uparrow\) |
| Y11215                | SKAP55 | Src kinase-associated phosphoprotein | 23 | 31 | 27 | 1.2 \(\uparrow\) |
| D84307                | phosphoethanolamine cytidylyltransferase | biosynthetic enzyme | 67 | 29 | 73 | 1.3 \(\uparrow\) |
| X55448                | glucose-6-phosphate dehydrogenase | biosynthetic enzyme | 83 | 60 | 73 | 1.4 \(\uparrow\) |
| L13720                | procathebin 43 | adhesion molecule | 24 | 6 | 21 | 1.2 \(\uparrow\) |
| U38864                | C2H2-150 | transcription factor | -9 | -26 | 7 | 4.2 \(\uparrow\) |
| L26081                | semaphorin-III (Hsema-1) | ligand | 37 | 15 | 5 | 3.2 \(\uparrow\) |
| M55621                | N-acetylgalactosaminyltransferase I (GlcNAc-TI) | biosynthetic enzyme | 85 | 88 | 23 | 3.6 \(\uparrow\) |
| AB003698              | Cdc7-related kinase | protein kinase | 58 | 33 | 20 | 3.4 \(\uparrow\) |
| D85418                | phosphatidylinositol-glycan-class C (PIG-C) | biosynthetic enzyme | 80 | 86 | 7 | 4.5 \(\uparrow\) |
| U77664                | RNaseP protein p38 (RP3P8) | nucleotide processing enzyme | 73 | 86 | 12 | 3.5 \(\uparrow\) |
| U90549                | non-histone chromosomal protein (NHC) | chromosomal protein | 68 | 49 | 15 | 3.6 \(\uparrow\) |

\(^a\) Intensity \((p=0.01)\) and fold change (\(\times 10^{-1}\)) are indicated. \(\text{CHX} = \text{control} + \text{CHX}\). \(\text{TGF-β (2 h)} = \text{TGF-β} + \text{2 h}\). \(\text{TGF-β (6 h)} = \text{TGF-β} + \text{6 h}\).
| Genbank accession no. | Description                  | Function                                  | Control intensity (CHX) | TGF-β (2 h) | TGF-β (6 h) |
|-----------------------|------------------------------|-------------------------------------------|-------------------------|-------------|-------------|
|                       |                              | (0 h)                                      | Intensity               | Fold change | Intensity   | Fold change |
|                       |                              | -                                         | -                       | -           | -           | -           |
| X99720                | TPRC                         | unknown                                   | 69                      | 53          | 35          | 47          | 2.0↓ 1.1↓ | 10          | 54          | <3.8↓ 1.0↓ |
| L20859                | leukemia virus receptor 1     | transporter                                | 66                      | 119         | 35          | 126         | 1.9↓ 1.1↑ | 20          | 97          | <3.2↓ 1.2↓ |
| M58286                | tumor necrosis factor receptor | cytokine receptor                         | 130                     | 133         | 69          | 170         | 1.9↓ 1.3↑ | 98          | 45          | 3.4↓ 3.0↓ |
| M83667                | NF-IL6-β protein             | transcription factor                       | 67                      | 94          | 52          | 84          | 1.9↓ 1.2↑ | 21          | 373         | 3.2↓ 4.0↑ |
| U80034                | mitochondrial intermediate peptide precursor | mitochondrial protein | 52                      | 54          | 31          | 36          | 1.7↓ 1.0 | -14         | 18          | <3.1↓ 1.2↓ |
| D78586                | CAD                          | biosynthetic enzyme                        | 139                     | 110         | 86          | 85          | 1.6↓ 1.3↓ | 45          | 16          | 3.1↓ 6.9↓ |
| U52513                | RIG-G                        | unknown                                   | 234                     | 189         | 143         | 139         | 1.6↓ 1.1↑ | 45          | 185         | 4.1↓ 1.0  |
| M59371                | protein tyrosine kinase      | protein kinase                             | 66                      | 125         | 40          | 84          | 1.5↓ 2.1↑ | -8          | 264         | <4.5↓ 2.5↑ |
| U35113                | metastasis-associated mta1   | unknown                                   | 37                      | 29          | 25          | 49          | 1.5↓ 2.3↑ | 20          | 32          | <4.5↓ 1.8↓ |
| L19871                | ATF3                         | transcription factor                       | 109                     | 229         | 60          | 451         | 1.4↓ 2.0↑ | -2          | 421         | <4.1↓ 1.8↑ |
| M24594                | interferon-inducible 56 Kd protein | unknown                                 | 128                     | 138         | 92          | 89          | 1.4↓ 1.3↓ | 19          | 50          | <5.1↓ 2.4↓ |
| U26266                | deoxyhypusine synthase       | biosynthetic enzyme                        | 88                      | 50          | 64          | 63          | 1.4↓ 1.3↑ | 19          | 104         | <4.3↓ 2.1↑ |
| D86973                | KIAA0219 gene (GCNI human homolog) | transcription factor                    | 115                     | 51          | 91          | 60          | 1.3↓ 1.2↑ | -1          | 36          | <6.6↓ 1.4↓ |
| D87120                | cancellous bone osteoblast   | unknown                                   | 94                      | 65          | 56          | 27          | 1.3↓ 2.4↓ | 21          | 7           | <3.5↓ <5.0↓ |
| X63417                | iriB                         | unknown                                   | 60                      | 48          | 46          | 68          | 1.3↓ 1.4↑ | 1           | 70          | <3.5↓ 1.5↑ |
| U15641                | E2F-4                        | transcription factor                       | 107                     | 101         | 84          | 89          | 1.3↓ 1.1↓ | 25          | 78          | 4.3↓ 1.3↓ |
| D38305                | Tob                          | tumor suppressor                          | 50                      | 47          | 42          | 36          | 1.2↓ 1.3↓ | -1          | 28          | <3.4↓ 1.7↓ |
| U10324                | nuclear factor NF90          | transcription factor                       | 93                      | 137         | 121         | 39          | 1.2↓ 1.5↑ | -8          | 46          | <5.9↓ 1.4↓ |
| D43947                | KIAA0100 gene                | unknown                                   | 53                      | 54          | 58          | 30          | 1.1↓ <2.9↓ | 11          | 16          | <3.0↓ <5.0↓ |
| L08238                | Mg44                         | unknown                                   | 87                      | 137         | 82          | 77          | 1.1↓ <2.3↓ | -451         | -213        | <16.9↓ <3.6↓ |
| U37408                | CbBP                        | transcription factor                       | 61                      | 80          | 56          | 20          | 1.1↓ 4.0↑ | 33           | 8           | <4.4↓ <3.7↓ |
| U84720                | RAE1                        | transporter                                | 189                     | 175         | 171         | 145         | 1.1↓ 1.2↓ | 49          | 277         | 3.8↓ 1.6↑ |
| Z24724                | polyA site DNA               | unknown                                   | 60                      | 40          | 56          | 35          | 1.1↓ 1.2↓ | 15          | 15          | <3.1↓ 2.7↓ |
| Y12711                | putative progesterone binding protein | steroid membrane receptor             | 88                      | 92          | 88          | 42          | 1.0   | 2.2↓ 2.5 | 25          | 28          | 3.0↓ 3.2↓ |
| D42400                | KIAA9001 gene                | unknown                                   | 84                      | 340         | 81          | 189         | 1.0   | 1.4↓ 1.2 | 12          | 211         | <4.5↓ 1.3↓ |
| U12128                | tyrosine phosphatase 1       | protein phosphatase                       | 60                      | 57          | 59          | 61          | 1.0   | 1.1↑ 1.9 | 19          | 20          | <3.0↓ 2.9↓ |
| L08488                | inositol polyphosphate 1 phosphatase | biosynthetic enzyme                   | 103                     | 111         | 106         | 94          | 1.0   | 1.5↓ 50 | 104         | 3.8↓ 1.1↓ |
| X77366                | HBZ17                        | transcription factor                       | 99                      | 102         | 103         | 94          | 1.0   | 1.1↓ 22 | 109         | 4.2↓ 1.1↑ |
| X04470                | antileukoprotease (ALP)      | protease inhibitor                        | 235                     | 129         | 239         | 209         | 1.0   | 1.6↑ 20 | 276         | <8.2↓ 1.5↑ |
| D42053                | KIAA0091 gene                | unknown                                   | 73                      | 81          | 83          | 52          | 1.1   | 1.7↓ 39 | 62          | <4.9↓ 1.4↓ |
| L77213                | phosphomethylcarbon kinase  | metabolic enzyme                          | 54                      | 106         | 118         | 8           | 1.1   | <3.5↓ 11 | 31          | <3.1↓ 1.8↓ |
| U03688                | dioxin- inducible cytochrome P450 (CYP1B1) | cytochrome                             | 81                      | 87          | 100         | 109         | 1.2   | 1.6↑ 23 | 229         | 3.5↓ 3.3↓ |
| X74262                | Rhap48                       | chromosomal protein                      | 102                     | 111         | 123         | 72          | 1.2   | 1.8↑ 33 | 53          | 3.1↓ 2.4↓ |
| U16799                | Na,K-ATPase β-1 subunit      | biosynthetic enzyme                       | 110                     | 89          | 167         | 82          | 1.5   | 1.7↓ 37 | 122         | 3.0↓ 1.4↓ |
| M21388                | unproductively rearranged Ig mu-chain mRNA | V-region                     | 548                     | 285         | 833         | 407         | 1.5   | 1.4↑ 167 | -13         | 33.2 <1.3↓ |
| X16707                | fra-1                        | transcription factor                      | 38                      | 253         | 161         | 643         | 4.2↑ 2.5↑ | -100        | 1212       | <10.7↓ 4.8↑ |

a) Genes were listed according to the magnitude of the fold decrease at 2 h in the absence of CHX. Upward and downward arrows represent increase and decrease, respectively.

b) A less than sign (<) indicates that the fold change likely represents an underestimation as described in Table I.

c) A greater than sign (>) indicates that the fold change likely represents an overestimation as described in Table I.
lated Dlx-2 whose expression is regulated by BMP-4 as well.46) TGF-β transiently induced the expression of Fra-1, a Fos-related gene,47) with kinetics similar to that of junB. These two proteins belong to the AP-1 family, and may mediate early responses to TGF-β.

### Effect of CHX

In a number of cases, CHX itself exhibited moderate induction of mRNA, as exemplified in the induction of PAI-1. CHX may inhibit synthesis of proteins involved in mRNA degradation. HaCaT cells treated with CHX exhibited a higher level of PAI-1 at 0 and 2 h than in control

### Table III. Transcriptional Regulation of Cell Cycle Regulators by TGF-β

| Genbank accession no. | Description | Control intensity (0 h) | TGF-β (2 h) | TGF-β (6 h) |
|------------------------|-------------|-------------------------|-------------|-------------|
| X05360                 | CDC2        | 128 112 133 72 1.0 1.3↓ | 95 57 1.2 1.6↓ | |
| M37712                 | CDC2 like 1, (PITSLRE) | 23 55 47 12 2.1↑ <2.6↓ | 32 11 1.4↑ <2.5↓ | |
| U77949                 | CDC6        | 142 82 71 85 1.7↑ 1.0 | 44 72 2.8↓ 1.2↓ | |
| AB003698               | CDC7        | 58 33 33 31 2.1↓ 1.1↓ | 20 27 <3.4↓ 1.2↓ | |
| U18291                 | CDC16       | 73 69 54 56 1.3↓ 1.2↓ | 52 50 2.2↓ 1.4↓ | |
| M81933                 | CDC25A      | 67 74 44 59 1.5↓ 1.3↓ | 46 59 1.4↓ 1.6↓ | |
| S78187                 | CDC25B      | 671 497 476 481 1.4↓ 1.0↓ | 364 477 1.8↓ 1.0 | |
| L26584                 | CDC25C      | 10 13 25 48 2.5↑ >2.0↓ | 48 46 >2.8↑ >2.1↑ | |
| L10844                 | CDC42       | -37 -52 -47 -29 <2.1↓ >2.2↑ | -64 -42 <2.3↓ >1.7↑ | |
| X51688                 | cyclin A    | 147 67 147 73 1.0 <1.2↓ | 128 21 1.1↑ 5.5↓ | |
| M25753                 | cyclin B1   | 353 323 318 224 1.1↓ <1.4↓ | 286 193 1.2↓ 1.7↓ | |
| M74091                 | cyclin C    | 9 10 6 12 <1.3↓ <1.3↓ | 4 -1 <1.3↓ <1.8↓ | |
| X59798                 | cyclin D1   | 862 782 931 1150 1.2↑ 1.6↑ | 1028 1531 1.3↑ 2.0↑ | |
| D13639                 | cyclin D2   | 461 552 175 455 2.4↑ 1.0 | 325 498 1.3↓ 1.1↓ | |
| M92287                 | cyclin D3   | 158 129 108 113 1.3↓ 1.1↓ | 109 87 1.2↓ 1.2↓ | |
| X95406                 | cyclin E1   | -64 -66 -41 -30 <2.1↓ <2.1↓ | -51 -64 <2.7↓ >1.1↑ | |
| Z36714                 | cyclin F    | 101 90 39 154 2.6↑ 1.1↑ | 167 113 1.1↓ 1.4↓ | |
| X77794                 | cyclin G1   | 116 65 91 52 1.3↓ 1.3↓ | 40 42 2.1↓ 1.5↓ | |
| U11791                 | cyclin H    | 216 202 166 167 1.3↓ 1.1↓ | 162 176 1.2↓ 1.2↓ | |
| D50310                 | cyclin I    | 487 349 472 367 1.0 1.0 | 505 212 1.0 1.6↓ | |
| M68520                 | CDK2        | 115 69 81 47 1.0 1.5↓ | 55 49 1.5↓ 1.4↓ | |
| U37022                 | CDK4        | 354 291 279 256 1.3↓ 1.0 | 212 154 1.5↓ 1.4↓ | |
| X66365                 | CDK6        | -16 -59 -92 -86 <8.7↓ <2.4↓ | -110 -80 <5.5↓ <2.5↓ | |
| L36844                 | p15/ink4b   | 41 51 43 66 1.1↑ 1.3↑ | 53 60 1.9↓ 1.2↓ | |
| U26727                 | p16/ink4a   | 107 90 93 123 1.1↓ 1.3↑ | 218 124 2.0↑ 1.4↑ | |
| U40343                 | p19/ink4d   | 43 68 35 47 1.3↓ 1.5↓ | 31 88 1.4↓ 1.0 | |
| U90579                 | p21         | 109 182 334 349 3.1↑ 2.5↑ | 216 496 2.5↑ 3.5↑ | |
| U10096                 | p27/Kip1    | -11 34 -10 22 <1.3↓ <2.1↓ | -15 25 <2.5↓ <1.9↓ | |
| X80343                 | p35 regulatory subunit of cdk5 kinase | -24 -71 -49 -53 <3.5↓ >1.9↑ | -73 -49 <3.3↓ >2.5↑ | |
| U22398                 | p57/Kip2    | -7 -8 -7 -19 <1.2↓ <1.2↓ | 0 -2 1.0 >1.3↑ | |
| M22898                 | p53         | 394 372 327 335 1.2↓ 1.1↓ | 351 325 1.1↓ 1.1↓ | |
| L41870                 | RB          | 43 33 37 32 1.2↓ 1.0 | 45 11 1.1↓ <1.9↓ | |
| L14812                 | p107       | 40 46 45 29 1.1↑ 1.6↓ | 40 29 1.0 1.6↓ | |
| X76061                 | p130       | 8 12 16 2 >1.9↑ <1.5↓ | 14 4 >1.3↓ <1.6↓ | |
| U47677                 | E2F-1      | 8 9 26 45 <2.8↑ 1.0 | 66 48 >1.1↓ >1.2↓ | |
| L22846                 | E2F-2      | 36 52 43 63 3.6↓ 1.2↑ | 42 53 >2.4↑ 1.0 | |
| D38550                 | E2F-3      | 94 72 72 46 1.3↓ 1.0 | 64 28 1.3↓ 2.6↓ | |
| U15641                 | E2F-4      | 107 101 84 89 1.3↓ 1.1↓ | 25 78 4.3↓ 1.3↓ | |
| U31556                 | E2F-5      | 16 20 2 22 1.3↓ 1.1↓ | -14 35 <1.8↓ 1.7↓ | |
| L23959                 | DP-1       | -6 9 22 -6 >3.9↑ <4.5↓ | -52 -67 <3.2↓ <10.0↓ | |
| L40386                 | DP-2       | 33 2 3 20 <4.1↑ >1.9↑ | 35 -7 1.6↓ <1.6↓ | |
| M15759                 | PCNA       | 455 429 313 312 1.5↓ 1.4↑ | 489 133 1.1↓ 3.2↓ | |
| L00058                 | c-Myc      | 129 78 48 92 2.7↓ 1.2↓ | 54 57 2.4↓ 1.7↓ | |
| U92436                 | PTEN       | 21 36 18 35 1.2↑ 1.0 | 31 23 1.1↑ 1.3↓ | |
| X62048                 | Weel       | 56 51 47 32 1.2↓ 1.4↓ | 7 37 <2.7↓ 1.4↓ | |

a) Upward and downward arrows represent increase and decrease, respectively.
b) A less than sign (<) indicates that the fold change likely represents an underestimation as described in Table I.
c) A greater than sign (>) indicates that the fold change likely represents an overestimation as described in Table I.
the absence of CHX. At 6 h, however, the level of PAI-1 was less in the presence of CHX than that in the absence of CHX. The result reflects complex regulation of the transcription of PAI-1. In an earlier phase, the induction of PAI-1 may not require de novo protein synthesis, whereas the expression at a later phase may depend on protein synthesis. junB showed transient induction by TGF-β, as was found by northern blotting (Fig. 2, C and D). CHX almost completely suppressed the marked decrease of junB expression from 2 to 6 h (note the changes of the intensities in Table I). A similar pattern was observed with Dlx-2 and Fra-1 that are also transiently induced by TGF-β. The expression of βIG-H3 increased for 6 h in the absence of CHX, whereas the induction by TGF-β was completely abolished by the presence of CHX. Thus βIG-H3 is unlikely to be a direct target of TGF-β, and the induction requires synthesis of other protein(s).

**Genes down-regulated by TGF-β** Ununexpectedly, we observed many genes down-regulated by TGF-β (Table II). This could be due to the induction of proteases. The number of the repressed genes is 70, which is 1.3% of the genes examined. When we took 2-fold change as the threshold, approximately 700 genes were selected (unpublished results). TGF-β repressed the expression of proinflammatory cytokines, which was antagonized by CHX. Interleukin-1β, on the other hand, induces the expression of Smad7, thereby inhibiting TGF-β signaling. Interestingly, TGF-β also down-regulated interleukin-1 receptor. TGF-β repressed expression of genes induced by interferon, RIG-G and 56 kd protein. Thus, TGF-β seems to affect the actions of various cytokines through transcriptional regulation.

TGF-β markedly repressed expression of epithelial specific Ets (ESX/ELF3/ESE-1/ERT). ESX was shown to be overexpressed at an early stage of human breast cancer development. Furthermore, ELF3 expression was shown to be induced in lung carcinoma. Thus, the repression of ESX/ELF3/ESE-1/ERT may contribute to the tumor suppressive activity of TGF-β. ERT, however, was identified as a transcription factor that induces the expression of TGF-β type II receptor, and loss of ERT may be responsible for oncogenesis in a different context. Smad2 and Smad3 interact with transcriptional coactivators such as p300 and CBP. Recently, however, Smad2 and Smad3 have been shown to associate with TGFIF and c-Ski that recruit histone deacetylase. Thus, TGF-β seems to both activate and repress transcription through Smad proteins, depending on cellular conditions. Taken together, the results indicate that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

**Transcription of cell cycle regulators** TGF-β is a potent inhibitor of cell growth. The transcriptional regulation of various cell cycle regulators by TGF-β is summarized in Table III. It has been reported that TGF-β induces the expression of p15 and p21 cdk inhibitors in HaCaT cells. TGF-β, on the other hand, represses the expression of c-myc, cdk4, and cdc25A. It has been suggested that targets of growth inhibition by TGF-β may vary depending on the cell type. In our analysis, p21 increased 3.1 fold at 2 h upon treatment by TGF-β. p15, however, increased only 1.1 and 1.9 fold at 2 and 6 h, respectively. Northern blot analysis showed a more significant increase of p15 (unpublished results). The reason for the discrepancy between the northern blotting and the GeneChip analysis is not clear at present. The intensity of Cdk4 decreased from 354 to 279 and 212 at 2 and 6 h, respectively. Cdk6 exhibited a more marked decrease, which may contribute to cell cycle arrest by TGF-β. p16 increased 2.0 fold at 6 h. The levels of p19, p27 and p57 cdk inhibitors remained rather constant. p18 is not contained in the DNA chip. It was reported that TGF-β does not directly affect the expression of cyclin D’s, whereas it inhibits increase of cyclin E and A in cycling HaCaT cells. In the present analysis, the intensity of cyclin E1 decreased moderately at 2 and 6 h, whereas the levels of cyclin A and cyclin B1 remained almost constant. The reason for this is probably that most of the cells were still cycling and did not reach the G1/S arrest, which would eventually be caused by TGF-β treatment during the relatively short TGF-β treatment used in our experiment. The result also suggests that cyclins are unlikely to be direct targets of growth arrest by TGF-β, c-myc and cdc25A decreased about 2.7 and 1.5 fold at 2 h, respectively. Interestingly, TGF-β exerted varying effects on members of the E2F family. TGF-β down-regulated E2F-4 and DP-2, whereas it up-regulated E2F-2.

**Targets of TGF-β** The identification of previously reported TGF-β inducible genes in the present study validates the usefulness of the GeneChip analysis in the investigation of transcriptional regulation by TGF-β. We have identified many genes that have not yet been reported to be regulated by TGF-β. The results provide important clues about the mechanisms of the biological activities of this pleiotropic growth/differentiation factor. The oligonucleotide arrays contain approximately 5600 genes, but the human genome is thought to code approximately 30 000 genes. The DNA microarray analysis of the uncharacterized genes will almost certainly reveal novel targets of TGF-β, which may play critical roles in tumor suppression by the factor.

**ACKNOWLEDGMENTS**

M. K. is supported by the Princess Takamatsu Cancer Research Foundation.

Received October 11, 2000/Revised November 28, 2000/Accepted December 4, 2000)
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