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Characterization Of The Human Transforming Growth Factor-Alpha Promoter.

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Characterization of the human transforming growth factor-α promoter

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CHARACTERIZATION OF THE HUMAN TRANSFORMING GROWTH FACTOR-α PROMOTER

by

TAE HO SHIN

A DISSERTATION

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of Doctor of Philosophy in the Department of Cell Biology
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ABSTRACT OF DISSERTATION
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Title Characterization of the human transforming growth factor-α promoter

A proximal region of the human TGFα promoter was studied for potential DNA elements and transcription factors that may interact with these elements. Here, we define four DNA elements in the first 350-bp region of the promoter that are important for efficient transcription. Each element binds a specific protein in nuclear extracts, and these bindings correlate with strength of the promoter. One element, the initiator, is required for both high level transcription and accurate transcription initiation directed at a unique start site. The elements we have identified are: 1) Sp1-binding sites, 2) a TBP-binding site or a non-consensus TATA box, 3) the initiator and 4) a p53-binding site. These studies illustrate basic organization of the TGFα trans-cription unit and imply potential regulatory mechanisms. Studies from other laboratories indicate that a group of growth-controlling genes utilizes a promoter similar to that of the TGFα gene. Coordinated regulation of these genes may be accomplished through the common promoter structure to ensure orchestrated cell growth control. Finally, interaction between the p53 tumor suppressor gene and the TGFα gene is of interest since both genes are implicated in formation of breast and other cancers.

Abstract Approved by: Committee Chairman J E Cleaver

Program Director John R Couchman

Date 8/24/94 Dean of Graduate School Joan S Logan

DEDICATION

This dissertation is dedicated to my parents, Yoon Shik Shin and Kyung Hee Kim. It is my duty as well as gratification to recognize that this accomplishment would have never been realized without their unabridged support and understanding.

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LIST OF ABBREVIATIONS

AdMLP	adenovirus major late promoter
5-azaC	5-azacytidine
cAMP	adenosine 3':5'-cyclic monophosphate
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
Cdk	cyclin-dependent kinase
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CSF-1	colony stimulating factor-1
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV-1	human immunodeficiency virus-1
Inr	initiator
MMLV	murine Molony leukemia virus
MMTV	mouse mammary tumor virus
NBT	nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

RACE	rapid amplification of cDNA ends
SCF	stem cell factor
TBE	tris-borate-EDTA
TdT	terminal deoxynucleotidyl transferase
TGFα	transforming growth factor-α
TGFβ	transforming growth factor-β
TPA	12-*O*-tetradecanoyl phorbol 13-acetate (phorbol 12-myristrate 13-acetate)

INTRODUCTION

An important question in developmental and cancer biology is how expression of growth factor genes are regulated. The overall aim of the studies described in this dissertation is to characterize the promoter for the human transforming growth factor-α (TGFα) gene and to examine potential mechanisms regulating its transcription. The dissertation contains materials either submitted or published and consists of three parts: the study of a role of DNA methylation in TGFα transcription, identification and characterization of the TGFα initiator and the study of transcriptional regulation by p53.

Growth factors and growth factor receptors

Cell-cell interaction is essential for proper development of multicellular organisms (Horvitz and Sternberg, 1991; Sternberg and Horvitz, 1991; Greenwald and Rubin, 1992; Jessell and Melton, 1992). One means of such communication is provided by polypeptide growth factors and their cognate receptors. This system is widely adapted by a variety of organisms, including nematode, fruit fly, rodent and human. TGFα and epidermal growth factor (EGF) are two of the best-characterized growth factors, both of which bind and activate the common receptor, the epidermal growth factor receptor (EGFR) (Ullrich and Schlessinger, 1990; Egan et al., 1993). Genetic and biochemical studies have identified a number of proteins that participate in the signal transduction pathway involving the activated EGFR. For instance, a family of small GTPases Ras proteins (Barbacid, 1987; Bourne et al., 1990; Downward, 1992; Boguski and McCormick, 1993; Schlessinger, 1993), function

downstream of EGFR in *C. elegans* (Han and Sternberg, 1990; Beitel et al., 1990), *Drosophila* (Simon et al., 1991; Bonfini et al., 1992) and mammals (Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Medema et al., 1993; Moodie et al., 1993; Vojtek et al., 1993). The striking conservation among various species probably indicates the versatility of this system. Indeed, depending on circumstances, cells respond to TGFα/EGF in a variety of different manners, including accelerated proliferation, changes in cell morphology, induced motility and the promotion of differentiation. A consequence of these cellular responses is an orchestrated development and maturation of tissues and organs.

The *ras* gene is a protooncogene; that is, mutant Ras proteins contribute to a number of transformed cellular properties. Constitutively activated EGFR can also lead to sarcomas and erythroleukemia (Fung et al., 1983; Nilsen et al., 1985; Pelley et al., 1989). Furthermore, overexpression of growth factors and their receptors is frequently associated with malignant tumors and appears to cooperate with other transforming agents such as activated Ras (Sandgren et al., 1993). These observations indicate that, in addition to their roles in developing embryo, growth factors and their interacting molecules are crucial in the maintenance of proper cell status in adult tissues.

Developmental roles of TGFα

TGFα is expressed in a wide spectrum of normal adult tissues, including anterior pituitary gland, brain, skin, deducia, kidney, fat, normal and regenerating liver, gastrointestinal mucosa and activated macrophages (for a review, see Luetteke et al., 1993). A potential involvement of TGFα in early development is inferred based on its expression patterns in embryonic tissues. In mice, TGFα mRNA has been detected in unfertilized oocyte, preimplantation embryo (Rappolee et al., 1988) and several tissues of day 9 and day 10

embryos (Wilcox and Derynck, 1988). More direct evidence in support of the developmental role of TGFα stems from genetic studies in *C. elegans* and *Drosophila*. For example, *lin-3* is essential for induction of certain cell types during vulval development of *C. elegans*. The lin-3 protein is closely related to mammalian TGFα and acts through let-23, *C. elegans* EGFR (Hill and Sternberg, 1992). Similarly, in *Drosophila* embryo, products of maternal genes *gurken* (*grk*) and *torpedo/DER* (*top/DER*) cooperate in establishing dorsoventral axis (Schupbach et al., 1991). The *grk* mRNA is expressed and localized dorsally in the oocyte and encodes a TGFα-like protein (Neuman-Silberberg and Schupbach, 1993). Top/DER, a *Drosophila* homologue of EGFR (Price et al., 1989; Schejter and Shilo, 1989), is expressed in somatic follicle cells in egg chambers surrounding an oocyte. Thus, in both invertebrates, proteins homologous to TGFα play clearly an important role during early development.

In higher organisms, however, the situation is much more complex. Mice homozygous for a disrupted TGFα gene display pronounced disorganization of hair follicles and various eye abnormalities (Luetteke et al., 1993; Mann et al., 1993). Rather surprisingly, however, these mice otherwise develop normally and are fertile. A lack of more profound abnormalities in the TGFα null mice is assumed in part due to functional redundancy. TGFα belongs to a family of growth factors that are structurally related and all are a ligand for the EGFR (Carpenter and Cohen, 1979; Ciccodicola et al., 1989; Shoyab et al., 1989; Higashiyama et al., 1991). Furthermore, strong mutant alleles of *grk* but not those of *top* are nonlethal, suggesting that *Drosophila* EGFR interacts with a second ligand at later stages of development (Neuman-Silberberg and Schupbach, 1993). Conceivably, in the null mice, a loss of TGFα is compensated by other related molecules expressed in early embryo.

TGFα and neoplastic transformation

TGFα is overexpressed in a number of primary tumors and transformed cell lines, including glioblastomas, melanomas and carcinomas of kidney, breast and liver (Derynck et al., 1987; Nister et al., 1988; Reiss et al., 1991). Deregulated production of TGFα is thought to contribute to the initiation and progression of neoplastic transformation. Direct evidence in support of this view comes from studies with transgenic mice in which overexpression of TGFα results in a variety of neoplastic lesions (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990). For instance, animals with an MMTV (mouse mammary tumor virus) promoter-TGFα transgene express high levels of TGFα in the breast and develop mammary hyperplasia and carcinoma (Matsui et al., 1990). This observation is of particular interest since in normal adults TGFα is expressed at low levels in developing mammary gland (Liscia et al., 1990; Snedeker et al., 1991) but at much higher levels in transformed cells from breast epithelium . Similar lesions in the breast were observed when TGFα was expressed from the metalothionine promoter (Jhappan et al., 1990; Sandgren et al., 1990). Other abnormalities associated with the TGFα transgenic mice include epithelial hyperplasia and/or neoplasia in pancreas (Jhappan et al., 1990; Sangren et al., 1990), liver (Jhappan et al., 1990), colon (Sandgren et al., 1990) and skin (Vassar and Fuchs, 1991). These studies are consistent with the notion that abnormally regulated TGFα expression can contribute to certain types of cancer.

Regulation of TGFα synthesis

Cells achieve proper expression of TGFα by controlling two major steps of its synthesis, transcription and posttranslational processing. TGFα is synthesized as a larger precursor protein, proTGFα (Derynck et al., 1984; Lee et al., 1985; Bringman et al., 1987; Teixido et al., 1987). proTGFα is an integral

membrane protein and, upon proteolytic cleavage within the extracellular domain, is converted into a diffusable 50 amino acid growth factor (Ignotz et al., 1986; Teixido et al., 1990). This process is known to be controlled; agents such as TPA (12-*O*-tetradecanoyl phorbol 13-acetate) and calcium ionophores promote the release of soluble TGFα (Pandiella and Massague, 1991a, 1991b; Bosenberg et al., 1992). A valine at the C-terminus of the cytoplasmic domain is required for TPA-induced processing and is conserved among other transmembrane growth factor precursors such as steel/stem cell factor (SCF) and CSF-1 (Bosenberg et al., 1992).

The second regulatory step in the TGFα production is its transcription (Coffey et al., 1987; Bjorge et al., 1989; Mueller et al., 1989; Pittelkow et al., 1989; Raymond et al., 1989). Hormones such as estrogen (Dickson et al., 1986; Bates et al., 1988) and TGFα/EGF itself (Coffey et al., 1987; Bjorge et al., 1989; Mueller et al., 1989) induce a steady state level of TGFα mRNA in both normal and neoplastic cells. An activated Ras (Dickson et al., 1987; Salomon et al, 1987; Godwin and Lieberman, 1990) and TPA (Mueller et al., 1989; Pittelkow et al., 1989; Raymond et al., 1989) are also inducers of TGFα mRNA. The mRNA induction is largely due to the increased transcriptional activity of the TGFα promoter (Raymond et al., 1989; Raja et al., 1991; Paterson and Kudlow, 1994). For example, activity of the human TGFα promoter reaches the maximum 6-8 hours after EGF stimulation and gradually declines to a basal level by 24 hours (Paterson and Kudlow, 1994). In a transfection assay, a proximal 313-bp region of the human promoter responds to both TPA and EGF to a similar degree with the endogenous mRNA (Raja et al., 1991). In addition, the stability of TGFα mRNA is unchanged, indicating that these agents affect mainly transcription rates of the TGFα promoter (Bjorge et al., 1989; Paterson and Kudlow, 1994).

The TGFα promoter

Promoter regions for the human and rat TGFα genes have been cloned (Jakobovits et al., 1988; Blasband et al., 1990). Two most notable features of the TGFα promoters are a lack of conventional TATA boxes and the presence of multiple GC boxes, a potential binding site for a transcription factor Sp1. Indeed, Sp1 can bind a subset of GC boxes in these promoters and appears to be important for efficient transcription (Chen et al., 1992; Shin et al., 1992). Interestingly, a similar promoter organization is associated with a subset of genes that are involved in cell growth control and DNA synthesis. These include genes for the EGFR (Hudson et al., 1990), transforming growth factor-β (TGFβ) (Kim et al., 1989), Ras (Lee and Keller, 1991), Yes (Matsuzawa et al., 1991), dihydrofolate reductase (DHFR) (Chen et al., 1984) and DNA polymerase-α (Sudo et al., 1992). It is not known if these similarities reflect a common regulatory mechanism coordinating expression of genes that are crucial for cell growth.

The characterization of the human TGFα promoter

To study potential mechanisms regulating the human TGFα promoter, we cloned a 5'-regulatory region of the gene extending approximately 3.5 kb from the transcription initiation site (Raja et al., 1991). By utilizing various molecular and cell biology techniques, we have attempted to characterize this promoter in detail. In this dissertation, three sets of results will be discussed: 1) CpG dinucleotide and potential regulation by DNA methylation, 2) an initiator element and 3) potential regulation by p53.

DNA methylation and regulation of gene expression

In vertebrates, methylation of DNA occurs at 5 position of a cytosine within CpG dinucleotides. A large body of evidence indicates that transcription is not compatible with extensive DNA methylation at or near the promoter

(Cedar, 1988; Cedar and Razin, 1990; Bird, 1992). For example, tissue-specific genes are highly methylated in cells that do not express them but left under-methylated in expressing cells (Waalwijk and Flavell, 1978; Shemer et al., 1990; Antequera et al., 1990). Similarly, X chromosome-associated genes are densely methylated and kept transcriptionally silent on the inactive chromosome, while the other allele is transcriptionally active and largely unmethylated (Hansen et al., 1988; Grant and Chapman, 1988; Riggs and Pfeifer, 1992). Recently, proteins that specifically interact with methylated DNA have been identified (Boyes and Bird, 1991; Lewis et al., 1992). It was proposed that these methyl-CpG binding proteins mediate transcription repression imposed by DNA methylation. Alternatively, a methylcytosine in CpG sequences can directly interfere with binding of transcription factors if it is located within the recognition motifs (Kovesdi et al., 1987; Iguchi-Ariga and Shaffner, 1989; Prendergast et al., 1991).

Transcription suppression by CpG methylation is thought be important during development (Cedar, 1988; Cedar and Razin, 1990; Bird, 1992). A genome-wide methylation pattern is established during gametogenesis and in early developing embryos (Monk et al., 1987; Chaillet et al., 1991; Kafri et al., 1992) and transmitted by clonal inheritance (Wigler et al., 1981). A unique enzyme, DNA methyltransferase, is responsible for all CpG methylation in mammals (Li et al., 1992). A homozygous null mutation of the DNA methyltransferase gene results in death of developing mice between day 9 and day 11 of gastulation, indicating a role of CpG methylation during or prior to this period (Li et al., 1992). Finally, an inhibitor of DNA methylation, 5-azacytidine, induces differentiation of fibroblastic 10T1/2 cells into myocytes and other cell types (Taylor and Jones, 1979; Lasser et al., 1986; Davis et al., 1987). This myogenic conversion is due to inappropriately activated expression of MyoD, a transcription factor important for muscle formation (Davis et al.,

1987; Weintraub, 1993). These results are consistent with the idea that DNA methylation maintains terminally differentiated cell status by suppressing transciption of key regulators, thereby preventing an alternative cell fate.

That the human TGFα promoter has unusually high contents of CpG dinucleotides suggests that its expression may be under negative control by DNA methylation. Consistent with this notion, most, if not all, of the CpG sites in the TGFα promoter are methylated in HA-A cells, a human melanoma cell line which expresses only very low levels of TGFα mRNA. 5-Azacytidine treatment of HA-A cells leads to demethylation of the TGFα promoter and a concomitant increase in the level of TGFα mRNA. In this dissertation, experiments concerning a potential regulatory role of CpG methylation in TGFα transcription will be described (Shin et al., 1992).

An initiator element

A majority of TATA-less genes are transcribed from a cluster of transcription initiation sites spread over a large region of a promoter. In contrast, transcription from the human TGFα promoter occurs at a unique initiation site (Jakobovits et al., 1988). A similar situation exists for the rat TGFα promoter which uses predominantly a single site for transcription initiation, although several minor nucleotides are also used (Blasband et al., 1990). Both the human and rat promoters lack a consensus TATA box, a major determinant of a transcription initiation site. Thus, it is likely that an alternative element exists in the TGFα promoters, which substitute for the function usually carried out by a TATA element.

The murine terminal deoxynucleotidyl transferase (TdT) promoter is another example of TATA-less promoter with a unique transcription initiation site (Smale and Baltimore, 1989). Close investigation of the TdT promoter lead to identification of an essential element, mutations of which severely

reduce the transcription levels and lead to initiation at sites not normally used (Smale and Baltimore, 1989). This element, the initiator, is approximately 17-bp long and contains within itself the transcription initiation site. Similar elements were later found in several different pro-moters of both TATA box-containing and TATA-less types (for reviews, see Weis and Reinberg, 1992; Kollmer and Farnham, 1993; Smale, 1994). In each of these promoters examined, an initiator element overlaps a transcription initiation site and is required for both accurate and efficient transcription initiation.

The human TGFα promoter was examined in the search for a potential element that could support accurate initiation in the absence of a TATA box. We found an element that functionally related to the TdT initiator (Shin and Kudlow, 1994a). Limited sequence similarities are also noticed between the TdT initiator and the element in the TGFα promoter. Based on these results, we call this element the TGFα initiator. We also suggest that the TGFα initiator may function by interacting with a 105 kD protein, TIBP-1. These results will be descussed in this dissertation

The transcriptional regulation by p53

A tumor suppresser protein p53 is known to act as a sequence-specific transcription activator (for review, see El-Deiry et al., 1993). However, only a handful of the p53-target genes are known to date (El-Deiry et al., 1993, and references therein). A most relevant p53-target so far identified is a gene called WAF1. WAF1 mRNA is highly induced by p53 (El-Deiry et al., 1993), and an encoded 21 kD protein is a potent inhibitor of cyclin-dependent kinases (Cdks) (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). These findings are consistent with a role of p53 in a cell cycle blockage at G1/S transition (Dulic et al., 1994) and its tumor suppressing activity in general (Hollstein et al., 1991; Levine et al., 1991).

Point mutations, deletions, allelic loss and rearrangement in the p53 gene are frequently associated with cancers of organs such as colon, brain and breast (Hollstein et al., 1991; Levine et al., 1991). Since a spectrum of these p53-sensitive tissues significantly overlaps with tissues where TGFα is implicated in normal development as well as neoplastic transformation, we have examined if the transcription of the TGFα gene is regulated by p53. We have identified a p53-binding site in a proximal region of the TGFα promoter (Shin and Kudlow, 1994b). This sequence is necessary for the transcriptional activation by wild-type but not mutant p53. Furthermore, once multimerized, this site confers strong p53 responsiveness to the minimal adenovirus major late promoter. These and other experiments will be described in the dissertation.

The structure of the TGFα promoter

Through these and other studies, an overall design of the TGFα promoter is beginning to emerge. Some of the features are common to other growth-regulating genes such as the neu and cyclin D1 genes, suggesting coordinated transcriptional control of these genes. Conceivably, the activity of a common key regulator(s) is modulated in response to proliferative and/or differentiating cues that, in turn, ensure orchestrated cell growth control. Perhaps most importantly, this and other questions are now more accessible to us since many of the players in the TGFα transcription unit have been identified.

5-AZACYTIDINE TREATMENT OF HA-A MELANOMA CELLS INDUCES SP1 ACTIVITY AND CONCOMITANT TRANSFORMING GROWTH FACTOR α EXPRESSION

by

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ABSTRACT

Evidence indicates that DNA methylation is a part of the regulatory machinery controlling mammalian gene expression. The human melanoma cell line HA-A expresses low levels of transforming growth factor α (TGFα). TGFα mRNA accumulated, however, in response to DNA demethylation induced by a nucleoside analog 5-azacytidine (5-azaC). The importance of DNA methylation in the TGFα promoter region was examined by transient transfection assay using luciferase reporter plasmids containing a portion of the TGFα promoter. 5-azaC treatment of HA-A cells prior to the transfection caused a significant increase in the luciferase activity. Since input plasmids were confirmed to remain unmethylated, DNA demethylation of the TGFα promoter itself does not account for the observed increase in TGFα mRNA. Using an electrophoretic mobility shift assay (EMSA), the enhanced formation of protein-TGFα promoter complex was detected in response to 5-azaC treatment. This 5-azaC-induced complex was shown to contain the transcription factor, Sp1, by the following criteria: the protein-DNA complex formed on the TGFα promoter contained immunoreactive Sp1; the mobility of the complex in an EMSA was similar to that formed by recombinant Sp1; and DNAase I footprinting analysis demonstrated that the 5-azaC-induced complex produced a footprint on the TGFα promoter identical to that of authentic Sp1. These observations suggest that 5-azaC induces TGFα expression by augmenting the Sp1 activity. However, neither the Sp1 mRNA nor protein was induced by 5-azaC. These results suggest that, in HA-A cells, TGFα expression is down-modulated by DNA methylation. In addition, this process may involve the specific regulation of Sp1 activity without altering the amount of the transcription factor.

INTRODUCTION

In mammalian cells, modification of DNA is confined almost exclusively to CpG dinucleotides, where cytosine is methylated at the 5 position. The CpG dinucleotide occurs at a frequency 4-5 fold lower than expected from random distribution, and the majority of this sequence contains 5-methylcytosine. DNA methylation is thought to play a role in gene expression (6, 7), and in general, increased methylation correlates with decreased gene expression. This is especially prominent in tissue-specific genes that appear to be hypermethylated except in those tissues where the gene is expressed (43, 50). In addition to this negative correlation, DNA methylation clearly suppresses gene expression in transient transfection assays. When γ- and β-globin genes were transfected into mouse fibroblasts, the unmethylated genes were expressed, while the *in vitro* methylated counterparts were silent (5, 53). A more recent study with the cAMP response element (CRE) revealed that methylation of this sequence abolishes both specific factor binding and CRE-dependent transcription *in vitro* and *in vivo* (17). This observation is particularly important because it provides direct linkage between DNA methylation and the action of a transcription factor.

DNA methylation also appears to be important in development and differentiation. Specific methylation patterns occurring during mouse embryonic development could provide a basis for developmental stage- and tissue-specific gene expression (26, 35). The striking evidence for this model is the conversion of 10T1/2 fibroblasts to myocytes, chondrocytes or adipocytes following exposure of the cells to a DNA demethylating reagent, 5-azacytidine (5-azaC) (10, 31, 48). At the morula and blastula stages in the mouse embryo, global DNA demethylation occurs, perhaps allowing generalized expression of tissue specific genes (26). In concert with this idea,

Rupp and Weintraub recently reported that a muscle-specific transcription factor MyoD is transiently expressed at the midblastula transition throughout the Xenopus embryo (39). These observations support the idea that proper development requires specific patterns of DNA methylation in order to achieve spatial and temporal regulation of gene expression.

Peptide growth factors play an important role in multicellular organisms by providing a means of paracrine communication between cells. Despite the nomenclature, the responses to growth factors are not confined to cell growth; indeed, depending on the target cells, they also may inhibit proliferation, promote differentiation and maturation, and facilitate highly specific cellular functions such as cytokine production. Growth factors are also implicated in developmental processes, where a highly coordinated mechanism of cell-cell interaction is essential (9, 51). Transforming growth factor α (TGFα), a member of the epidermal growth factor (EGF) family and a ligand for the EGF receptor is expressed in a developmentally regulated fashion with higher expression in the embryo than in the adult. Since TGFα stimulates its own expression and that of its receptor (8, 29), small alterations in the expression of the growth factor could be amplified. This property of TGFα and other autocrine growth factors necessitates tight regulation of the expression of this class of genes, which if disregulated could lead to temporally and spatially aberrant patterns of cell growth and differentiation. Recently, we and others have cloned and started the characterization of the human TGFα promoter region (20, 38). The 310 bp 5'-flanking region of the promoter has been shown to confer basal activity and responsiveness to various hormones (38). The most notable features of this sequence are the absence of a TATA box and the presence of multiple GC boxes (Fig. 1). Sp1 is a cellular transcription factor which specifically binds a GC box and is

required for the transcription of a number of genes (24). Sp1 is ubiquitously expressed, although its expression shows some tissue-specificity (41). The importance of this transcription factor in TGFα transcription, however, has not been established.

In this paper, the possible involvement of DNA methylation in the TGFα expression was examined. In the human melanoma cell line, HA-A, TGFα is expressed at a low level. We have noticed that the TGFα promoter region is highly methylated in these cells. 5-AzaC treatment of HA-A cells induced DNA demethylation with a concomitant increase in the TGFα mRNA level. Unexpectedly, however, DNA demethylation at the TGFα promoter is not required for the action of 5-azaC because the unmethylated promoter placed in reporter plasmids responded to the drug to a similar degree as did the endogenous gene. Instead, we were able to detect enhanced Sp1 binding to the TGFα promoter region. These results are consistent with the idea that DNA methylation suppresses gene expression in mammalian cells and that Sp1 could be a limiting factor for gene expression in certain situations (40).

MATERIALS AND METHODS

Plasmids

Constructions of the luciferase reporter plasmids pXP1/1.1 and pXP1/310 were as previously described (38). These plasmids harbor a portion of the TGFα 5'-flanking region of approximately 1.1 kb and 310 bp in length, respectively, followed by the coding sequence of the luciferase gene. In some experiments, pXP1/1.1 was methylated *in vitro* by Sss I methylase (New England Biolabs) according to the manufacturer's procedure. The plasmid pKKSp1-F enabled the expression of human Sp1 in *E. coli* and was used in order to facilitate antibody production. The construction of this

plasmid was as follows: a 3 kb fragment containing the almost full-length Sp1 coding sequence was isolated from pSVSp1-F (generously provided by Dr. J. D. Saffer, 40) by first digesting with Eco RI followed by a Klenow fill-in reaction of the restriction ends and a second restriction digestion with Hind III. This fragment was ligated into pKK233-2 (Pharmacia) between Nco I (blunt ended with Klenow fragment) and Hind III sites. This manipulation allowed the Sp1 sequence to be in frame relative to a translation initiation codon provided by the vector. For a stable transfection of HA-A cells, pSV2-neo (45) was used as a selectable marker plasmid.

Cell manipulations

A human melanoma cell line HA-A was maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% newborn bovine serum and antibiotics (100 μg/ml penicillin, 50 μg/ml gentamicin). For experiments where 5-azacytidine was used, cells were plated at a density of $2x10^5$ cells per 15cm culture dish (day 0). 5-azacytidine (Sigma) was added to the growth medium (2 μg/ml, unless indicated otherwise) in two 24 hour pulses on days 2 and 5. Cells were harvested on day 8 for Northern and Southern blot analyses or for transfections with various plasmids. Where indicated, 50nM TPA (phorbol 12-myristate 13-acetate, Sigma) was included in the medium 6 hours prior to the assays.

Northern and Southern blotting

Electrophoresis and blotting procedures for both Northern and Southern blotting were essentially according to Sambrook et al. (42). For Northern blot analysis, RNA was harvested by the guanidine isothiocyanate method as previously described (30). Total RNA was hybridized with a 900 bp fragment of the human TGFα cDNA (30) for the detection of the TGFα mRNA, while a 1.1 kb Bam HI-Hind III fragment of the human Sp1 cDNA (25) was used for

the Sp1 mRNA. Southern blot analyses shown in Figs. 2A and 3A were performed using the 1.1 kb 5'-flanking region of the TGFα gene as a probe. For the experiment shown in Fig. 2A, genomic DNA was isolated from 5-azaC treated or untreated cells (42), digested with Eco RI and subjected to Southern blot analysis. The plasmid pXP1/1.1 used in the experiment shown in Fig. 3A was either left untreated, methylated *in vitro* with SssI methylase, or recovered from transfected cells (see below). These samples were digested with Hpa II or Msp I or left untreated and analyzed by the Southern blotting technique.

Recovery of plasmid from transfected cells

At 48 hours post-transfection, plasmid was extracted from transfected cells by the alkali lysis protocol (42) commonly used for the isolation of plasmids from *E. coli*. The plasmid was further purified by digestion of the extract with RNase A and proteinase K followed by organic extractions. This sample was passed through a gel filtration column (Chroma Spin-1000, Clontech) to remove remaining RNA, and pure plasmid DNA was obtained by ethanol precipitation.

Transfections and luciferase assays

Forty-eight hours after the removal of 5-azaC, $2x10^7$ cells were electroporated with 20μg of pXP1/1.1 or pXP1/310 as previously described (38). Cells were lysed, and luciferase activity was measured at various time points using reagents described in the Promega technical bulletin (No. 101). The light emission from luciferase reactions was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory) for 10 seconds. The outputs (relative luciferase units) were normalized by protein concentrations in the cell lysates determined by the Bradford assay. The stably transfected HA-A cells were obtained by cotransfecting 10μg of pXP1/1.1 and 1μg of pSV2-neo,

and grown in the presence of 400μg/ml G418. Four weeks after transfection, surviving colonies were trypsinized, and individual clones were replated in the fresh medium containing G418. The luciferase activity in cell lysates was measured when these cells reached 80% confluency, and clones with moderate luciferase activity were retained.

Antiserum

The Sp1-expressing plasmid, pKKSp1-F, was transformed into a strain of *E. coli*, DH5α, and grown overnight in LB broth. More than 90% of the bacterially produced Sp1 protein was found in inclusion bodies from which the protein was subsequently purified according to methods described by Sambrook et al. (42). Partially purified protein was subjected to SDS/PAGE and transferred to a nitrocellulose membrane. Proteins on the resultant blot were visualized by Ponceau-S staining. A portion of the blot containing the Sp1 protein was excised and treated with sequentially diminishing concentrations of guanidium hydrochloride solutions, as done in the standard Southwestern technique, in an attempt to obtain the renatured protein. The membrane was thoroughly dried, solubilized in DMSO and injected into rabbits every two weeks. Sera positive for Sp1 were obtained after a period of 8 weeks.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared essentially according to the method of Dignam et al. (13) except that proteinase inhibitors (leupeptin, pepstatin and aprotinin, Boehringer Manheim) were included in each solution at the concentrations suggested by the manufacturer. The probe used for electrophoretic mobility shift assays (EMSAs) was a 130 bp segment of the TGFα 5'-flanking region. The Bam HI end of the probe was filled in with Taq DNA polymerase in the presence of ^{32}P-α-dATP. The reaction, containing 4μg of

nuclear proteins and 1ng of radiolabeled probe in binding buffer (20mM HEPES (pH 7.9), 100mM KCl, 1mM DTT, 2μg poly dI-dC and 20% glycerol), was incubated on ice for 20 minutes prior to electrophoresis. Electrophoresis through a native gel (4% polyacrylamide, 0.5x TBE and 2% glycerol) was performed at 4°C with 0.5x TBE as running buffer. In some experiments, the following competitors were included in the reaction: the unlabeled 130 bp fragment (indicated as 130 in Fig. 5), the 130 bp fragment methylated *in vitro* with SssI methylase (met130), an oligonucleotide CACCCCCATCGGGGCG-GGAGGGGGGGGTCA and its complementary DNA (I), an oligonucleotide CGCTCAGCGTCCCGCCCTTACCCCAAC and its complementary DNA (II), an oligonucleotide GATCCCAGGTCGCCCCGCCCAGCAGCCCGC and its complementary DNA (III), and an oligonucleotide GATCCCAGGT-CGCCGGGCCCAGCAGCCCGC and its complementary DNA (IIIm). While double-stranded oligonucleotides I-III represent GC boxes in the 130 bp region of the TGFα gene, IIIm contains two nucleotide substitutions in the conserved hexanucleotide region of GC box. All oligonucleotides were synthesized on a Cyclone Plus DNA Synthesizer (Milligen/Biosearch). In one experiment, purified human Sp1 (0.3 ng, Promega) was mixed with nuclear extract from untreated HA-A cells and subjected to an EMSA.

Western blotting

For the experiment shown in Fig. 6, a preparative scale EMSA was performed with nuclear extract from 5-azaC treated cells as described above. As a positive control, purified Sp1 was used in a separate assay. After electrophoresis, retarded bands and free probe were localized by exposing wet gels to a X-ray film for 3 hours at 4°C. Portions of the polyacrylamide gels containing the 5-azaC-specific or Sp1-DNA complex, or free probe were excised, and proteins were eluted in elution buffer (50mM NH_4HCO_3 pH 8.0,

0.5mM EDTA and 0.1% SDS) for 3 hours at 37°C with agitation. After precipitation with cold acetone, these proteins were then subjected to SDS/PAGE and Western blotting procedures. The Sp1 protein was immunoblotted with anti-Sp1 serum and visualized by the use of alkaline phosphatase and nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) as substrates.

DNase I footprinting assay

DNase I footprinting assay was performed as described by Johnson et al. (21). The 130 bp sequence of the TGFα gene was uniquely end radio-labeled on the upperstrand with T4 polynucleotide kinase and ^{32}P-γ-ATP. Twenty or forty micrograms of nuclear extract or 60ng of purified Sp1 was used.

RESULTS

5-AzaC treatment induces the TGFα mRNA

The 5'-flanking region of the TGFα gene contains numerous CpG dinucleotides (Fig. 1A). To test if the state of DNA methylation influences TGFα expression, we used a DNA demethylating reagent, 5-azacytidine (5-azaC) (22, 23). This reagent has been shown to substitute for cytidine in replicating DNA where it causes hypomethylation of DNA. Additionally, evidence suggests that the incorporated drug depletes DNA methyltransferase activity by forming a covalent complex with the enzyme (49). The effect of 5-azaC on DNA methylation was confirmed by Southern blotting using a set of methylation sensitive restriction enzymes. An example of such experiments is shown in Fig. 2A. When HA-A cells were treated with two pulses of 5-azaC each at the concentration of 2 μg/ml, partial DNA demethylation was detected in the otherwise extensively methylated 5'-flanking region of the TGFα gene. At this concentration of the drug, cells replicated

somewhat more slowly than the control cells. We did not observe morphological changes associated with the treatment. Use of 4 μg/ml 5-azaC failed to intro-duce further DNA demethylation in this region of the gene, while cell growth was attenuated by approximately 50%. Higher doses of the drug induced more complete demethylation but also caused inhibition of cell growth and, ultimately, cell death (data not shown). Based on these observations, a concentration of 2 μg/ml of 5-azaC was used in subsequent experiments. The effect of 5-azaC treatment on the level of TGFα mRNA was then assessed by Northern blot analysis. Treatment with 5-azaC resulted in a significant increase in TGFα mRNA (Fig. 2B). Interestingly, 5-azaC treatment also resulted in the sensitization of the TGFα mRNA level to 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment. Although TPA has been shown to stimulate TGFα expression in other cells (4, 36, 38), this was not the case for HA-A cells in the absence of 5-azaC. The effect of 5-azaC treatment on TGFα expression was relatively specific since the abundance of the Sp1 transcript was not similarly affected. Thus, these observations are consistent with the idea that DNA methylation negatively regulates TGFα expression in a specific manner.

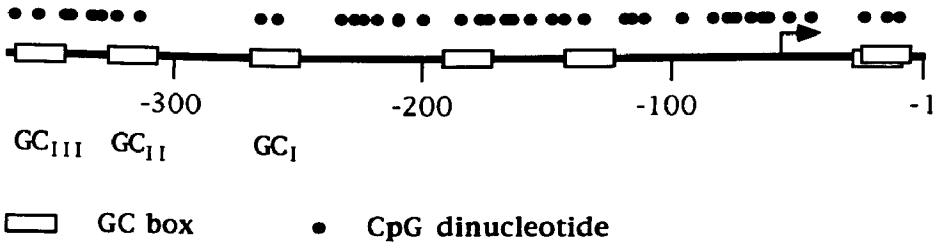
Unmethylated TGFα promoter responds to 5-azaC

The increase in TGFα mRNA in response to 5-azaC is likely a result of DNA demethylation (7), although this drug could potentially affect other cellular functions. However, the locus (loci) of DNA demethylation responsible for the increased TGFα expression was not determined by the experiments described above. While DNA demethylation of the TGFα gene itself could have caused the observed effect, demethylation in other gene(s) could also have been responsible. In order to distinguish these possibilities, segments of the TGFα promoter linked to a luciferase reporter gene were

Figure 1. A. Schematic representation of the TGFα promoter region. The promoter region from -373 to -1 is shown, where A of the AUG initiation codon is +1. GC boxes are indicated as open boxes. Three of these sequences are located in the distal 130 bp region (-373 to -243) and denoted as GC_I-GC_{III}. CpG dinucleotides are represented as small closed circles. The bent arrow indicates a transcription initiation site described by Jakobovitz et al. (20).

B. Sequence comparisons of the consensus GC box and three GC boxes in the 130 bp region of the TGFα promoter. Nucleotides identical to the consensus are shown in uppercase, while diverged nucleotides are in lowercase.

A

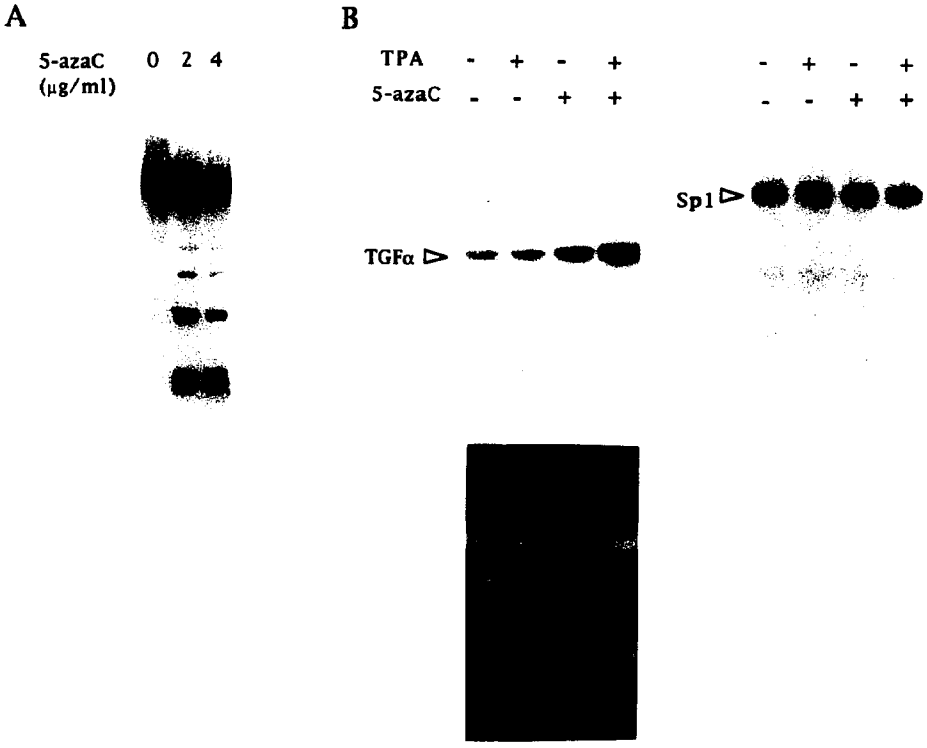


B

consensus GC box	:	G G G G C G G G G C
GC_I	:	G G G G C G G G a g
GC_{II}	:	G G G G C G G G G a
GC_{III}	:	t G G G C G G G G C

Figure 2. A. 5-AzaC induces DNA demethylation at the promoter region of the TGFα gene. Genomic DNA was isolated from HA-A cells treated with indicated amounts of 5-azaC, where "0" indicates the absence of the treatment. The state of DNA methylation was assayed using a restriction enzyme Hha I, which cleaves DNA at GCGC sequences only when the central cytidines are unmethylated. Digested DNA samples were separated by electrophoresis, and hybridized to a ^{32}P-labeled 1.1 kb segment of the TGFα gene 5'-flanking region by the standard Southern blotting technique. Small fragments generated by Hha I digestion are indicative of cytosine demethylation at GCGC sequences.

B. 5-AzaC treatment induces the TGFα mRNA. RNA was isolated from HA-A cells treated with or without 5-azaC and/or TPA as indicated, and subjected to Northern blotting using the ^{32}P-labeled human TGFα cDNA as a probe. A single message corresponding to 4.8 kb was detected. Reprobing of the same blot with a cDNA probe for human Sp1 is also shown, where a major species of 8.2 kb long is seen. Ethidium bromide staining of the original agarose gel, indicating equal loadings of RNA samples in each lane, is shown in the bottom panel.


A
5-azaC
(μg/ml)
0 2 4
B
TPA - + - +
5-azaC - - + +
TGFα
- + - +
- - + +
Sp1

used in a transient transfection assay. Plasmid constructs used in this study have been previously shown to confer basal transcriptional activity as well as responsiveness to various hormones (38). The bacterially propagated plasmids are totally unmethylated at cytidine residue in CpG dinucleotides and should remain so after transfection into eukaryotic cells where they do not replicate (see Fig. 3A). Therefore, if the effect of 5-azaC results solely from demethylation of the TGFα promoter, luciferase activity should be independent of the drug treatment. On the contrary, if pretreatment of cells with 5-azaC results in, for example, the enhanced expression of transcription factor(s) involved in TGFα expression, luciferase activity should be higher in 5-azaC treated cells than in untreated cells. HA-A cells were treated with 5-azaC as before and transfected with reporter constructs. First, we determined whether the transfected plasmid retained its unmethylated state. As expected, the plasmid recovered from cells 48 hours after transfection was fully susceptible to digestion with a methylation sensitive restriction enzyme, Hpa II (Fig. 3A) indicating that the plasmid was not methylated *de novo* even in the cells not treated with 5-azaC. The luciferase activity in extracts of cells treated with or without 5-azaC was then compared (Fig. 3B). For each of the reporter constructs tested, 5-azaC pretreatment resulted in a significant stimulation of basal transcription. Furthermore, a significant response to TPA was detected only in 5-azaC treated cells. These results agree with the responses seen by Northern blotting and demonstrate that the induction of TGFα mRNA results mainly from the transcriptional activation of the TGFα promoter. They also indicate that for this activation, demethylation of the TGFα promoter is not required. Instead, it appears that demethylation must occur in another gene to permit increased TGFα gene transcription. Nevertheless, the state of methylation of the TGFα promoter may also contribute to this effect. To

examine this possibility, the reporter plasmid was first methylated *in vitro* at every cytidine residue in CpG dinucleotides and subsequently transfected into HA-A cells. The luciferase activity was undetectable regardless of the treatment with or without 5-azaC (data not shown). This observation may indicate that the TGFα promoter is suppressed when fully methylated at CpG dinucleotides. Alternatively, since the plasmid was methylated indiscriminately in its entirety, it may have been inactivated by a mechanism(s) distinct from that responsible for the low level of TGFα expression in HA-A cells. For the reasons discussed below, we believe that the latter is more likely.

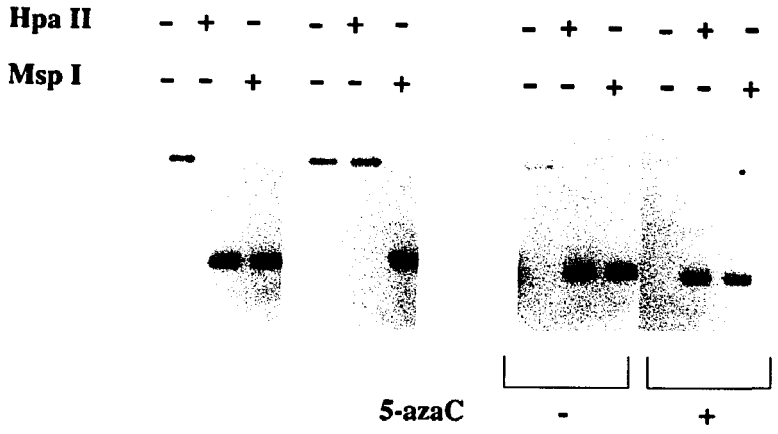
Transient and reversible nature of the 5-azaC effect

In somatic cells, the state of DNA methylation is usually maintained over cell generations (27, 46, 52). 5-AzaC-induced DNA demethylation could be maintained, resulting in prolonged expression of TGFα. However, we noticed that the effect of 5-azaC on TGFα mRNA was highly time-dependent. To examine this observation in more detail, a time course of the 5-azaC effect was studied. We stably transfected HA-A cells with pXP1/1.1, a reporter plasmid containing the 1.1kb TGFα promoter and isolated clones expressing moderate levels of luciferase activity. Stable transfection eliminated the ambiguity associated with plasmid degradation in the transiently transfected cells. When the selected clones were treated with 5-azaC, the luciferase activity in cell extract increased by 6-10 fold (Fig. 4). The maximal level of activation was reached approximately 24 hours after the second pulse of the drug, followed by a gradual decrease to near basal level. When an additional pulse of 5-azaC was applied, the luciferase activity increased to a level equal to or greater than that achieved after the second pulse. This was again followed by a gradual decrease in the luciferase activity. Thus, the effect of 5-azaC on the TGFα promoter was highly transient and reversible. Note that

Figure 3. A. Plasmid transfected into HA-A cells remained unmethylated regardless of 5-azaC treatment. For the experiment shown on the left panel, pXP1/1.1 was either methylated *in vitro* with SssI methylase or left untreated. Sss I methylase methylates cytidines in every CpG dinucleotide. The plasmids were then digested with Hpa II or Msp I or left untreated as indicated. Both enzymes cleave DNA at CCGG sequence, but only Msp I does so at methylated sites. The samples were subjected to Southern blot analysis. As seen in the figure, the methylated plasmid is cleavable with Msp I but not with Hpa II, while the unmethylated counterpart was digested with both enzymes. For the experiments shown in the right panel, pXP1/1.1 was transfected into HA-A cells which were previously treated with or without 5-azaC as indicated. Forty eight hours following transfection, plasmids were recovered from the transfected cells as described in Materials and Methods. These samples were digested with Msp I or Hpa II or left untreated and subjected to Southern blotting.

B. 5-AzaC stimulates the TGFα promoter-driven expression of the luciferase reporter gene. Cells transfected with either pXP1/1.1 or pXP1/310 were assayed for luciferase activity 48 hours after transfection. In some experiments, TPA (50nM) was added to the growth medium 6 hours prior to the assay. Results from two independent assays were averaged, and the inductions by various treatments were plotted as fold-increases over unstimulated samples.

A


Hpa II
Msp I
5-azaC

B

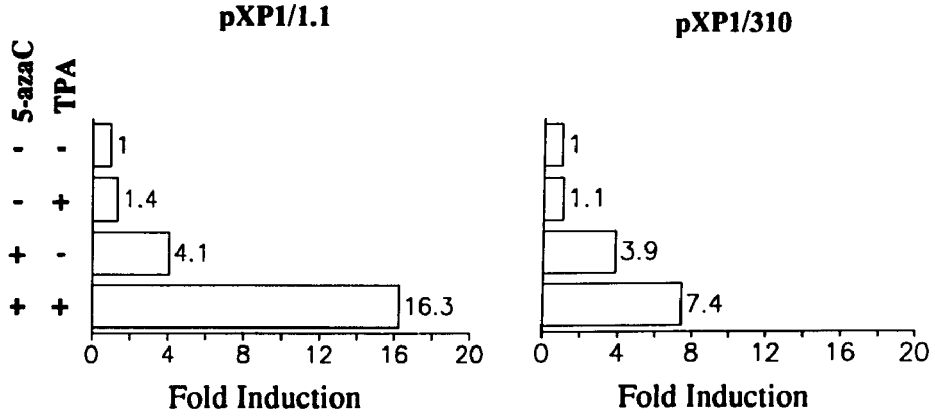
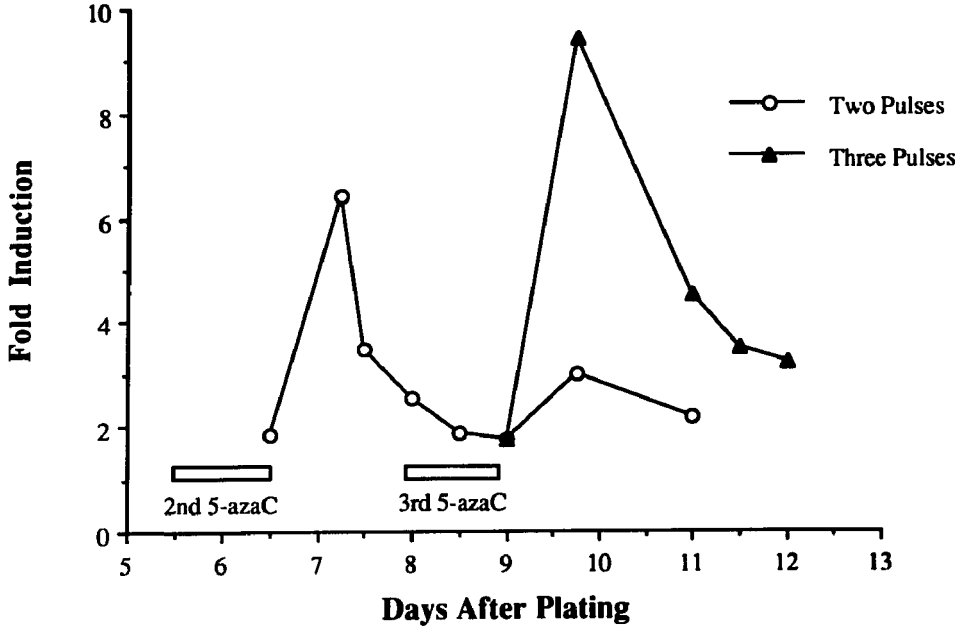

pXP1/1.1
pXP1/310
5-azaC
TPA
1
1.4
4.1
16.3
1.1
3.9
7.4
0
4
8
12
16
20
Fold Induction

Figure 4. Time course study of the 5-azaC effect on luciferase activity. HA-A cells were stably transfected with pXP1/1.1 as described in Materials and Methods. These cells were treated with 2 μg/ml of 5-azaC as done in previous experiments. In addition, on day 8, some of the cells received an additional dose of the drug. Luciferase activities in the cell extracts were measured at various time points, and fold inductions by 5-azaC treatment were determined. Open circles: cells were treated with two doses of 5-azaC. Closed triangles: cells were treated with three doses of 5-azaC. Result obtained with one of three independent clones is shown. The responses seen with the other two clones are similar to the one shown here (data not shown).


Fold Induction
10
8
6
4
2
0
5
6
7
8
9
10
11
12
13
Days After Plating
2nd 5-azaC
3rd 5-azaC
Two Pulses
Three Pulses

we do not know the location into which the TGFα-luciferase chimeric DNA was incorporated in the genome. It is possible that, to a certain degree, DNA demethylation of surrounding sequence contributes to the observed response in the luciferase activity. However, this is unlikely because the response was similar among three independent clones (data not shown). The transient activation of the TGFα promoter may be explained by proposing the presence of *de novo* methylase activity in these cells (1).

Increased formation of a protein-DNA complex on the TGFα promoter correlates with the effect of 5-azaC

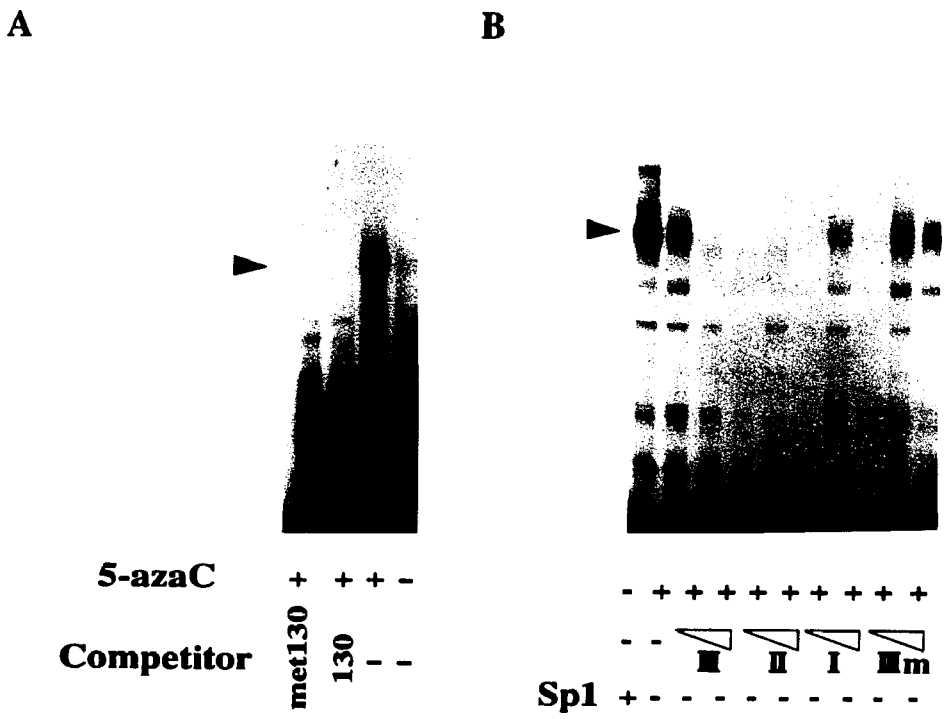
The above results imply that DNA demethylation at an undetermined locus (loci) may be responsible for the increased TGFα mRNA resulting from 5-azaC treatment. This demethylation could result in increased expression of a gene product encoded by this locus. A likely candidate for this gene product(s) is a transcription factor involved in TGFα expression. We, therefore, compared DNA binding activities present in nuclear extracts from 5-azaC treated and untreated cells by electrophoretic mobility shift assay (EMSA). The probe used for this assay was the 130 bp segment of the gene (-373 to -243 relative to the A in the translation initiation codon), which consists of the distal half of the 310 bp region used in the transient transfection assays shown in Fig. 3B. This DNA segment, in conjunction with the minimal thymidine kinase promoter from herpes simplex virus, has been shown to confer responsiveness to various hormones in MDA468 human carcinoma cells (38). As shown in Fig. 5A, a marked enhancement of a protein-DNA complex was observed with nuclear extract from 5-azaC treated cells. Specificity of this complex was established by using the same segment of DNA as a competitor. In some experiments, the enhancement of this complex was smaller than that shown in Fig. 5A. However, there was generally a good correlation between

increases in the TGFα promoter activity and the formation of the protein-DNA complex following 5-azaC treatment. Interestingly, this complex was formed on and competed by unmethylated and methylated forms of the 130 bp DNA fragment equally well (Fig. 5A and data not shown). This result again suggests that the DNA methylation status of the TGFα promoter may have little to do with the observed increase in TGFα mRNA. Instead, it appears that 5-azaC-induced DNA demethylation enhances the binding activity of a transcription factor, which is able to bind to the TGFα promoter even when it is methylated.

Sp1 activity is induced by 5-azaC treatment

The 130 bp segment of the TGFα gene contains three recognizable GC boxes (Fig. 1), putative binding sites for the transcription factor Sp1. In *in vitro* transcription assays, Sp1 is essential for the transcription from promoters lacking a TATA box (37, 44). Since the promoter for the TGFα gene belongs to such a TATA-less promoter family, Sp1 may also be important for the expression of the growth factor. The possibility that Sp1 could be a component of the 5-azaC-induced DNA binding activity was examined. First, double-stranded oligonucleotides each representing one of the GC boxes in the 130 bp segment were used as competitors in gel shift assays. All of these oligonucleotides competed the 5-azaC-specific retarded band (Fig. 5B). Interestingly, the two distal GC boxes competed noticeably better than the most proximal one, which appears to be the least favorable Sp1 binding site among the three. Another oligonucleotide representing the most distal GC box having the conserved hexanucleotide, CCGCCC, mutated to GGGCCC, failed to compete this complex (Fig. 5B). Furthermore, addition of Sp1 to untreated HA-A cell nuclear extract resulted in the formation of a protein-DNA complex having similar mobility to the complex formed from the 5-azaC treated

Figure 5. Electrophoretic mobility shift assay of nuclear extracts from HA-A cells treated with or without 5-azaC. Nuclear extracts were prepared essentially according to a method described by Dignam et al. (13). EMSAs were performed using a 130 bp segment of the TGFα promoter region as a probe. In some cases, various competitors were included in the reaction. The following competitors were used: 130; the unlabeled 130 bp fragment, met130; the 130 bp fragment methylated *in vitro* with Sss I methylase, I-III; double-stranded oligonucleotides representing three GC boxes present in the 130 bp sequence, GC_I-GC_{III} (see Fig. 1), IIIm; a mutated form of III with the sequence GGGCCC instead of GGGCGG. In the experiment shown in panel A, a 20-fold excess of each competitor was used. In panel B, the competitors were 5-fold excess at the small end of the triangle and 20-fold excess at the large end. Where indicated, purified human Sp1 was added to the reaction together with nuclear extract from 5-azaC untreated cells.

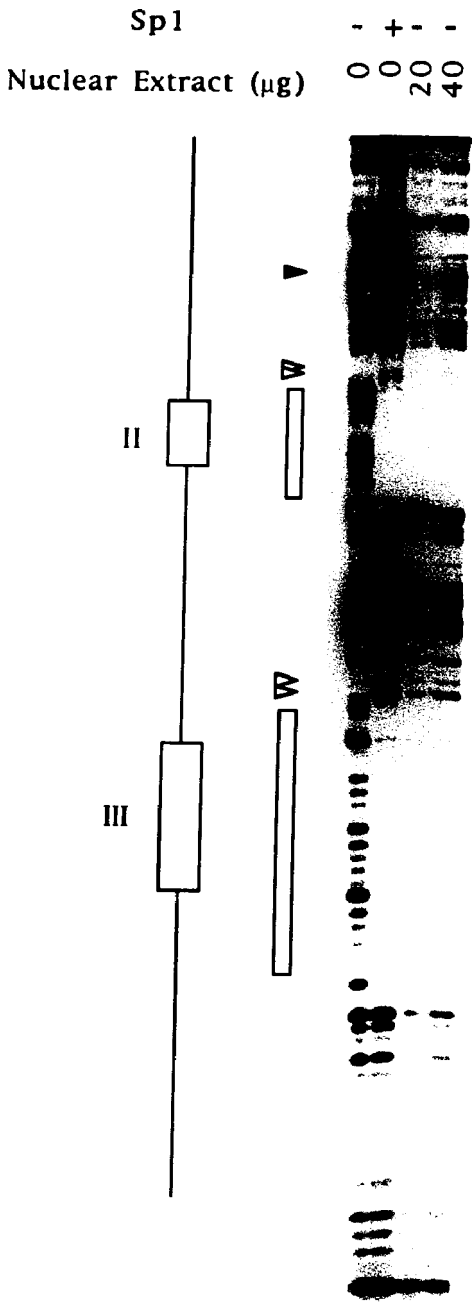

A
B
5-azaC
Competitor
met130
130
Sp1
III
II
I
IIIm

extract (Fig. 5B). As mentioned earlier, the 5-azaC-specific complex was insensitive to methylation of the 130 bp sequence (see Fig. 5A). Consistent with this observation, previous studies by other groups have demonstrated that binding of Sp1 to a GC box is not affected by the methylation status of the sequence (2, 15, 16), further suggesting that Sp1 may be a part of the DNA binding activity induced by 5-azaC. To more directly assess this possibility, we obtained immunological criteria. A preparative scale gel shift assay was performed as in Fig. 5, and the portion of the polyacrylamide gel containing the specific complex was excised. The DNA binding protein(s) was eluted from the gel slice and subjected to a Western blot analysis with anti-Sp1 serum. A band of appropriate molecular weight was detected (Fig. 6), indicating Sp1 is actually a component of the 5-azaC-induced DNA binding activity. Furthermore, a DNase I footprinting assay demonstrated that footprinting patterns made by purified Sp1 and nuclear extract from 5-azaC treated cells were almost indistinguishable from each other (Fig. 7). This could mean either that Sp1 is the only component of the 5-azaC-induced DNA binding activity or that another component(s) of the activity does not appreciably alter the Sp1 footprint. Taken together, the simplest explanation for the 5-azaC effect on TGFα mRNA would be that the expression of Sp1 is stimulated by 5-azaC-induced DNA demethylation, which, in turn, results in the transcriptional activation of the TGFα gene. To examine this possibility, the abundance of the Sp1 mRNA was assessed by Northern blot analysis (Fig. 2B). Unexpectedly, however, the steady state level of Sp1 mRNA was not affected by 5-azaC treatment. Western blotting with anti-Sp1 serum also detected no difference in the amount of Sp1 protein between 5-azaC treated and untreated nuclear extracts (data not shown). These observations demonstrate that 5-azaC did not increase the concentration of Sp1, yet enhanced the

Figure 6. Sp1 is a component of the 5-azaC-specific protein-DNA complex. The 5-azaC-specific complex shown in Fig. 4 (denoted here as complex A), and the complex between purified Sp1 and the 130 bp probe (Sp1) were eluted from native polyacrylamide gels as described in Materials and Methods. These samples were subjected to Western blotting using anti-Sp1 serum. The antiserum detected a somewhat diffused band of approximately 105kD in the lane corresponding to Sp1. A single 105 kD band was also detected in a lane corresponding to the complex A. The antiserum detected no protein in the sample obtained from the polyacrylamide gel containing free probe. Positions of molecular weight standards are indicated in kD on the left side of the panel.

Free Probe
Complex A
Sp1
200 —
97 —
68 —
43 —

Figure 7. DNase I footprinting assay of the TGFα 5'-flanking region using nuclear extract from 5-azaC treated cells. The 130 bp segment of TGFα promoter, identical to the one used in the experiments Fig. 5, was labeled at the 5'-end of the upper strand. DNase I footprinting assays were performed using either purified Sp1 or different amounts of nuclear extract from 5-azaC treated cells as indicated on the figure. A digestion pattern of the probe in the absence of added protein was shown in the left most lane. The positions and extents of regions protected from DNase I (narrow boxes) are identical when purified Sp1 and nuclear extract were used. Furthermore, hypersensitive sites juxtaposing these protected sites (open arrowheads) are also identical. Note, however, that there are hypersensitive sites unique to purified Sp1 (closed arrowheads). Two of the GC boxes on this region of the gene (see Fig. 1) are also shown as wide boxes.


Sp1
Nuclear Extract (μg)
- + - -
0 0 20 40
II
III

binding activity of Sp1 to the TGFα promoter. We hypothesize the existence of a yet undetermined activity that augments the DNA binding activity of Sp1 to the TGFα promoter and is inducible by 5-azaC treatment.

DISCUSSION

The concept that DNA methylation regulates mammalian gene expression has been gaining experimental support (6, 7). The results described here are compatible with and expand this idea. In HA-A cells, the low level of TGFα expression can be up-regulated by 5-azaC treatment. Thus, TGFα expression appears to be negatively influenced by DNA methylation. This observation may have relevance both to normal development and to the pathogenesis of cancer. In development, the spatial and temporal control of growth factor expression is thought to be critical for the process of organogenesis. TGFα expression is high in embryonic tissues and becomes down-modulated in adult tissues. The requirement for such developmental stage-specific expression could be fulfilled in part by a mechanism involving DNA methylation. The results presented here imply the existence of a locus (loci) whose DNA methylation status could influence TGFα expression. The progressive down-modulation of TGFα expression during development could result from developmentally specific DNA methylation at this locus. In this view, it should be noted that individual genes undergo specific and dynamic changes in the state of DNA methylation during embryonic development (26, 47). Such dynamic alterations would provide a basis for developmental regulation of the growth factor expression. In contrast to the germ line and early embryonic cells, DNA methylation is static in adult organisms (27, 46, 52). Tissue-specific genes appear to be methylated in cells that do not express these genes, while housekeeping genes are unmethylated in all cells (3). Thus, TGFα expression could be attenuated by DNA methylation in some

somatic cells. Aberrant DNA demethylation in such cells could result in uncontrolled expression of the growth factor. Overexpression of TGFα and other autocrine growth factors could lead to growth factor-independent cell growth (9, 11, 12). In carcinogenesis, DNA demethylation at a specific locus could contribute to tumor progression by leading to the derepression of growth factor expression.

Gene regulation by DNA methylation could occur in each member of the set of genes repressed in a given tissue. Alternatively, only a few "master genes" may be subjected to methylation repression. The mouse fibroblast cell line 10T1/2 was converted to myoblasts following exposure of these cells to 5-azaC (28, 31). The high frequency of this event suggested that demethylation of only a few genetic loci is necessary for the alteration of cell phenotype. Indeed, the gene responsible for the myogenic conversion was cloned by the subtraction hybridization technique between 5-azaC treated and untreated cells. The isolated gene, a now well-characterized transcription factor MyoD, was shown to be sufficient for the myogenic conversion of 10T1/2 cells when reintroduced into these cells (10). Thus, DNA methylation at a single transcription factor gene locus appears to be sufficient for the suppression of the entire myogenic differentiation program. The finding that Sp1 activity is inducible by 5-azaC may provide another example of DNA methylation regulating a transcription factor αctivity. Since Sp1 is essential for transcription from TATA-less promoters (37, 44), the down-regulation of Sp1 activity could account for the low level expression of TGFα in HA-A cells. In transient transfection assays, deletion of the 130 bp TGFα sequence, which contains at least two Sp1 binding sites (see Fig. 7), resulted in an undetectable level of luciferase activity (data not shown). We do not know if the same or similar mechanism operates on other cellular genes or in other cells.

However, GC boxes are present in numerous cellular and viral genes (24, 41) and, in a few cases, have been demonstrated to be required for optimal transcription of these genes (14, 33, 34). Furthermore, Sp1 appears to be a limiting factor in the transcription of SV40 early genes (40).

The mechanism by which 5-azaC enhanced DNA binding activity of Sp1 is unknown. That 5-azaC treatment did not increase the concentration of Sp1 indicates that in HA-A cells, the Sp1 gene is not repressed by DNA methylation. It is possible that 5-azaC affects properties of Sp1 indirectly without involving alterations in DNA methylation status. However, a more likely explanation is that 5-azaC induces DNA demethylation at yet another genetic locus (loci) and alters gene expression there. A protein encoded by this gene would then facilitate Sp1 binding to the TGFα promoter. This factor may directly bind Sp1 and modulate DNA binding activity of the transcription factor. A precedent for this model is found in the cAMP-CREB system, where a hepatitis B virus protein forms a complex with CREB and recruits the transcriptional activator to the viral enhancer (32). A similar factor could exist for Sp1, but, of note, there was no change in the mobility of the protein-DNA complex in the EMSA (see Fig. 5B) nor the footprinting pattern in the DNase I footprinting assay (see Fig. 7). An alternate candidate for a modulator of Sp1 activity is a group of protein modification enzymes. Sp1 is known to be post-translationally modified, including O-glycosylation (18) and phosphorylation (19). It is conceivable that these modifications alter the affinity or specificity of Sp1 binding to DNA. For example, Sp1 is phosphorylated upon its binding to a GC box (19). This phosphorylation may stabilize the transcription factor-DNA complex shifting the equilibrium in favor of increased DNA occupancy.

In HA-A cells, TPA has only a minimal effect on TGFα expression. 5-AzaC apparently induces the ability of TGFα promoter to respond to this stimulus. As is the case for basal expression, the TPA response occurs without DNA demethylation of the TGFα promoter. There are three potential explanations for these observations: first, a putative TPA-responsive factor(s) may be induced by 5-azaC (either directly or mediated by Sp1), second, the action of the TPA-responsive factor may require Sp1 activity, and third, Sp1 itself may mediate the signal initiated by TPA.

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IDENTIFICATION AND CHARACTERIZATION OF THE HUMAN TRANSFORMING GROWTH FACTOR-α INITIATOR

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ABSTRACT

Eukaryotic transcription requires promoter proximal elements. For class II promoters, two such elements are the TATA box and the initiator (Inr). The promoter for the human transforming growth factor-α (TGFα) gene has been shown to lack a TATA box yet initiate transcription at a unique site. We have identified an approximately 13-bp sequence between -5 and +8 as a new promoter element. We call this element the TGFα Inr based on the following observations; 1) it is located at the transcription initiation site. 2) The promoter activity is largely reduced by either deletion or mutation of the element. 3) Mutations result in initiation upstream of the authentic start site; the TGFα Inr directs site-specific initiation. An electrophoretic mobility shift assay demonstrated that a protein(s) in nuclear extracts forms complexes with the TGFα Inr. This interaction is sequence-specific and depends on nucleotides that are critical for the promoter activity in vivo. Two polypeptides, 105 and 95 kD, were detected by Southwestern blot analysis on the basis that they specifically interact with the TGFα Inr. The larger polypeptide, TIBP-1, was subsequently purified by a matrix-immobilized TGFα Inr sequence and was shown to possess the TGFα Inr-specific mobility shift activity and an ability to interact with the Inr when immobilized on a membrane. In summary, we identified and characterized the TGFα Inr, a proximal element that is important for the accurate transcription of the TGFα gene, and the 105 kD protein that interacts with this element. We speculate that TIBP-1 may be a transcription factor that functions through the TGFα Inr.

INTRODUCTION

Transforming growth factor-α (TGFα) is a ligand for epidermal growth factor receptor. Its biological effects include modulation of signal transducing molecules, changes in cell morphology, induction of gene transcription,

cell-cycle progression and cell differentiation (1, 2). TGFα is expressed in a wide variety of neoplastic tumors as well as in normal tissues. In normal physiology, the expression of this growth factor is tissue- and developmental stage-specific and regulated by various hormones including TGFα itself (3-5). Studies have indicated that this hormonal regulation acts mainly at a transcriptional level and requires the 5'-regulatory region proximal to the promoter (5). The TGFα promoter lacks a TATA box and contains multiple binding sites for the transcription factor, Sp1 (6, 7). Unlike many other TATA-less promoters, transcription initiation from the TGFα promoter occurs predominantly at a single nucleotide rather than at clustered sites (8).

Recently, the list of TATA-less promoters has been growing rapidly. The TATA box serves as a promoter element that is recognized by RNA polymerase II transcription machinery or, more specifically, by a general transcription factor, TFIID (9-14). A lack of such motifs in some promoters, therefore, has suggested that the assembly of the transcription initiation complex can be achieved through an alternative pathway. The second core promoter element, the initiator (Inr), was initially defined in a TATA-less promoter of the terminal deoxynucleotidyl transferase (TdT) gene, which directs transcription initiation from a unique site (15). It is now known that an Inr is located at the transcription start site of several promoters, both TATA-containing and TATA-less (16, 17). Deletions or mutations of this element cause transcription rates to decrease significantly both in vitro and in vivo. In addition to these quantitative changes, such mutations also result in aberrant selection of the transcription initiation site resulting in the use of cryptic sites. Thus, one function of the Inr is to position the transcription initiation complex in a proper context and promote site-specific initiation. This positioning effect is partly intrinsic to RNA polymerase II itself, since highly purified

enzyme can recognize the Inr and transcribe RNA from a specific nucleotide (18).

The Inr for the adenovirus major late (AdML) promoter has been extensively studied (19). This promoter contains both a TATA box and an Inr, which can each independently lead to successful initiation. The TATA box is directly recognized by TFIID, but this reaction is strongly facilitated by another general factor, TFIIA. Once a stable TFIID/-A complex is formed, the rest of general factors, along with RNA polymerase II, successively assemble into a larger complex that is transcriptionally competent. Alternatively, a 120 kD protein, TFII-I, can direct transcription initiation through its interaction with the AdML Inr. TFII-I can bypass the requirement for TFIIA by complexing with and recruiting TFIID to the Inr (20). This latter pathway does not require a functional TATA box. Thus, the Inr is also a binding site for specialized transcription factors which, by interacting with the general factors, promote assembly of the initiation complex independently from the TATA box-mediated mechanism.

Several cellular proteins which interact with Inrs have been identified. Each of these Inr-binding factors is specific to a particular type of Inr. For example, TFII-I also binds the TdT Inr and the Inr for human immunodeficiency virus-1 (HIV-1) promoter (19), whereas the dihydrofolate reductase (DHFR) Inr is recognized by distinct proteins, HIP1 and E2F (21-23). Unlike the AdML promoter, no TATA boxes are found in the DHFR promoter. HIP1, a 180 kD protein, also binds the transcription start site region of several housekeeping genes, which are also TATA-less (22). It is postulated that HIP1 controls transcription of these genes coordinately by interacting with their Inrs. The DHFR promoter is activated at the onset of S-phase, and this induction requires the E2F binding site within the Inr (16). The E2F activity

oscillates during cell-cycles (24). Expression of the wild-type but not mutant E2F in quiescent cells induces transcription from the DHFR promoter (25). Thus, for the DHFR gene, E2F is a modulator of the cell-cycle regulation that functions through the Inr. YY1 is another transcription factor that binds a subset of Inrs. YY1-binding sites are found in the Inrs for the adeno-associated virus p5 (26) and cytochrome C oxidase subunit Vb (27) promoters. Transcription from both promoters depends on the presence of YY1. Thus, various Inrs are uniquely recognized by a set of distinct transcription factors, and this interaction is required for Inr function.

In this communication, we report the identification and characterization of an Inr element in the TGFα promoter. Efficient and accurate transcription of the gene depends on the integrity of the TGFα Inr. Furthermore, the TGFα Inr interacts with nuclear proteins that appear to be different from previously identified Inr-binding proteins. Mutations in the Inr that interfere with protein-binding also reduce the promoter activity, suggesting the importance of the protein-DNA interaction.

MATERIALS AND METHODS

Plasmids

The deletion mutants of the TGFα promoter were derived from the previously described constructs containing the TGFα promoter sequence between -311 and +4 or between -181 and +4 in pXP1. Plasmids were digested with SacII at -12 in the TGFα promoter and HindIII within the multiple cloning sites of the vector. This manipulation removed the promoter sequence downstream of -12. To extend the TGFα promoter to +10, a double-stranded oligonucleotide containing the promoter sequence between -11 and +10 (5'-GGCGCCGCTCCGCCACTCGGGTGGA-3' and 5'-AGCTTCCACCCGAG-TGGCGGAGCGGCGCCGC-3') was ligated into the SacII/HindIII digested

plasmids. Similarly, extension of the promoter to +38 was achieved by inserting another double-stranded oligonucleotide 5'-GGCGCCGCTCCGCCAC-TCGGGCACAGGTAGGGCAGGAGGCTGGAGA-3' and 5'-AGCTTCTCC-AGCCTCCTGCCCTACCTGTGCCCGAGTGGCGGAGCGGCGCCGC-3'. Finally, each of the promoters was excised from pXP1 by digestion with BamHI and HindIII and ligated into a luciferase reporter plasmid pGL2 (Promega) through BglII and HindIII sites. For constructing saturation mutant plasmids, oligonucleotide-mediated site directed mutagenesis was used (47). Single-stranded DNA was made from -181/+38 construct in pGL2. Since these mutations involved introduction of restriction site, screening for mutant clones was facilitated. Each mutant construct was sequenced through the entire TGFα promoter to confirm that any unintended mutations had not occurred. The mutated promoters were excised and recloned into pGL2 assuring the identical background for each mutant.

Transient transfection assays

MDA468 cells were maintain in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% newborn calf serum. HeLa cells were grown in the DMEM containing 5% fetal bovine serum. Cells were harvested and transfected with the TGFα-luciferase reporter plasmid by electroporation as described previously (5). A reference plasmid, pCMV-βgal, was also transfected for correction for transfection efficiency. Twenty four hours after transfection, luciferase (7) and β-galactosidase (47) activities were determined. The luciferase activity in each cell lysate was normalized to the activity of β-galactosidase. Typically, the activities of the -311/+38 and -181/+38 constructs were $1.0x10^5$ and $6.0x10^4$ RLUs (relative light units) per 200μl lysate, respectively. Protein concentrations in the cell lysate differed only slightly (less than 10%) regardless of the transfected plasmid, indicating

that there is no plasmid-specific cell toxicity. Three to five independent transfection assays were performed for each construct tested, and the results were expressed as a mean value.

Nuclear extracts and an electrophoretic mobility shift assay

Nuclear extracts were prepared according to the method described by Dignam et. al. (48) from an 80-90% confluent monolayer of MDA468 cells or approximately $1.0x10^6$ cells/ml HeLa S3 suspension cells. MDA468 cells were grown as described for transient transfection assays. HeLa S3 cells were maintained in Joklik's modified minimal essential medium (GIBCO) with 5% donor horse serum (Intergen). The probe for the mobility shift assay was a double-stranded oligonucleotide containing the TGFα sequence between -12 and +10 (5'-GGCGCCGCTCCGCCACTCGGGTGGAGATCTA-3' for the untranscribed strand and 5'-AGCTTAGATCTCCACCCGAGTGGCGGAGC-GGCGCCGC-3' for the transcribed strand). The annealed oligonucleotide was radiolabeled with the Klenow fragment of DNA polymerase I and [^{32}P]-α-dATP. The radiolabeled DNA was analyzed by nondenaturing gel electrophoresis, and found predominantly in a double-stranded form. The conditions for EMSA were previously described (7). For binding competition assays, various oligonucleotides were used as a competitor. The oligonucleotide AdMLP contains the AdML promoter sequence between -13 and +11 (5'-GGCCGCGTTCGTCCTCACTCTCTTCCGA-3' and 5'-AGCTTCGGA-AGAGAGTGAGGACGAACGC-3'). The "random" oligonucleotide is the TGFα Inr in which nucleotides are scrambled (5'-TCGACGGCGCCGCTC-CGATCCGCCGAGCC-3' and 5'-TCGAGGCTCGGCGGATCGGAGCG-GCGCCG-3'). The AP1 oligonucleotide contains an AP1 binding site from the human collagenase promoter (5'-CTAGATGCTGACTCATTG-3' and

5'-TCGACAATGAGTCAGCAT-3'). The c/EBP oligonucleotide is a synthetic binding site for the transcription factor c/EBP (5'-AATTCAATTGG-GCAATCAGG-3' and 5'-AATTCCTGATTGCCCAATTG-3'). Mutant Inr oligonucleotides mt1, mt2, mt3 and mt5 are identical to the wild-type TGFα Inr except for mutations indicated in the figures.

Southwestern blotting

Nuclear extracts or fractions from the DNA-affinity column were subjected to SDS/polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and treated according to a standard Southwestern blotting procedure (49). The binding reaction was performed at room temperature with the TGFα Inr oligonucleotide probe in a buffer containing 40mM HEPES (pH7.9), 50mM KCl, 7.5mM $MgCl_2$, 1mM DTT and 20% (v/v) glycerol. Subsequently the blot was washed for 15 min with two buffer exchanges either in the binding buffer alone or in the binding buffer plus 0.02% Sarkosyl (Sigma).

Primer extension assay

Total RNA was isolated from approximately $2x10^7$ MDA468 cells that had been transfected with the indicated TGFα-luciferase plasmid. Poly(A)-tailed RNA was enriched by oligo(dT)-cellulose chromatography and used for a primer extension assay using a luciferase-specific primier, 5'-AATGG-CGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATT-3', and murine Molony leukemia virus (MMLV) reverse transcriptase. Products of the reverse transcriptase reaction were amplified by PCR using 5'-RACE (50) system according to the manufacturer's instruction (BRL). This step was necessary to obtain sufficient signals from the plasmid-driven reporter mRNA. For PCR, two nested luciferase-specific primers were used for additional specificities; 5'-TTGGCGTCTTCCATTTTACCAACA-3' and

5'-CCATTTTACCAACAG-TACCGGAATG-3'. The second PCR was done with a primer that had been phosphorylated with [^{32}P]-γ-ATP and T4 polynucleotide kinase. The radioactive products were analyzed by denaturing electrophoresis through urea/polyacrylamide gel. The expected product for the properly initiated transcripts is 70-nucleotide long.

Purification of TIBP

For an Inr DNA-affinity column (51), the oligonucleotides 5'-GATCCC-GGCGCCGCTCCGCCACTCGGGCA-3' and 5'-GATCTGCCCGAGTGGCG-GAGCGGCGCGGG-3' were annealed and ligated. The concatamers were digested with BamHI and BglII to remove head-to-head ligation products. An approximately 180-bp DNA, which contained six tandemly repeated Inr sequences, was isolated and cloned into pGL2 that had been digested with the same set of enzymes. After propagation in an *E. coli* strain, DH5α F'IQ, the plasmid (15μg) was digested with BamHI and XhoI, and a 200-bp insert was isolated by Sephacryl S-1000 (Pharmacia) gel filtration chromatography (C26/1m). The isolated DNA was cross-linked to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instruction. Nuclear proteins from $5x10^9$ to $8x10^9$ HeLa S3 cells were adjusted to TM(0.1) (40mM Tris-HCl (pH7.9), 0.1M KCl, 12.5mM $MgCl_2$, 0.5mM DTT, 0.2mM PMSF, 20% (v/v) glycerol and 0.1% (v/v) NP-40) and loaded onto a 5ml heparin sulfate-Sepharose CL-6B column (Pharmacia). The column was washed with 5 column volumes of TM(0.1) and then eluted successively with TM(0.2), TM(0.4) and TM(1.0) (identical to TM(0.1) except for the concentration of KCl which is indicated in parentheses). The fraction eluted at 0.4M KCl contained the specific mobility shift activity. This fraction was diluted four times with TM containing no KCl and applied to the Inr DNA-affinity column. After an extensive wash with TM(0.1), proteins were eluted by a linear salt gradient

elution. Fractions eluted at approximately 0.4 to 0.65M KCl were positive in the mobility shift activity. These fractions were pooled and anlyzed by SDS/polyacrylamide gel electrophoresis and Southwestern blot analysis.

RESULTS

Deletional analysis of the TGFα promoter

The TGFα promoter sequence between -311 and +38 (+1 is the major transcription initiation site; 8) was cloned into a luciferase reporter plasmid, pGL2. This construct (-311/+38) was transcriptionally active when examined by a transient transfection assay in human mammary carcinoma MDA468 cells (approximately $1.0x10^5$ RLU (relative light unit) per measurement). Subsequently various regions of the promoter were deleted, and transcriptional activity was determined by a transient transfection assay (Fig. 1). The -181/+38 construct retained 94% of the full-length promoter activity indicating that the sequence between -311 and -181 is not necessary for the basal promoter activity. Deletion of a sequence between +11 and +38 (-181/+10) moderately reduced the transcriptional activity (by 26%) indicat-ing that this sequence contributes somewhat to the promoter activity. In clear contrast, deletion of only 6 more nucleotides, CTCGGG, at the 3'-end, yielded a construct (-181/+4) with only 1% of the activity of the full-length promoter. Finally, the -311/+4 construct, which lacked the same 6-bp sequence also displayed much decreased promoter activity (by 73%). Thus, efficient transcription of the TGFα gene requires a promoter sequence between +4 and +10. Since this sequence contributes to a promoter element that is functionally similar to the reported Inrs (see below), we will refer to it as the TGFα Inr.

Mapping the TGFα Inr by site-directed mutagenesis

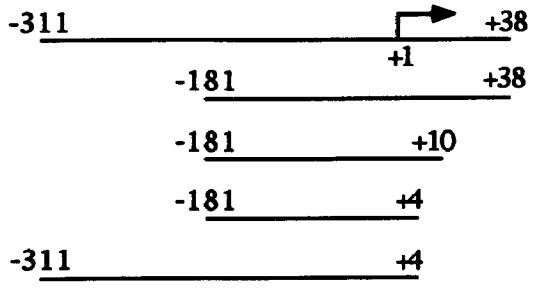
Site-directed mutagenesis was used to define the TGFα Inr more precisely. The -181/+38 promoter was mutagenized in the proximal region

between -18 and +12 by replacing various short sequences (1 to 4 bp) with restriction sites (Fig. 2, left panel). Transcriptional activity of each mutant promoter was determined by a transient transfection assay in MDA468 cells and is summarized in Fig. 2. The first three constructs, LS-18, LS-14 and LS-8, showed essentially wild-type promoter activity. Each of these plasmids contained a 4-nucleotide mutation in the region between -18 and -5; thus, this 14-bp sequence contributes little to TGFα promoter activity. In contrast, the next three constructs, LS-5, LS-3 and LS+2, which contained mutations downstream of -5, displayed a 40% decrease in transcriptional activity. This data places the 5'-boundary of the TGFα Inr at approximately -5. The promoter activity was even more severely suppressed, by 70-80%, with mutations located between +3 and +8 (LS+3, LS+5 and LS+8), indicating the particular importance of this region of the promoter. This result is consistent with the deletional analysis which also indicated the functional importance of the sequence between +4 and +10. Finally, the LS+9 construct, with nucleotides +9 to +12 substituted, retained more than 80% of the wild-type activity, thus placing the 3'-boundary of the Inr at around +8. These results were confirmed using HeLa cells (data not shown). Thus, the TGFα Inr is an approximately 13-bp element consisting of the nucleotide sequence, GCTCCGCCACTCG (the initiating nucleotide is underlined). A specific sequence within this element is required for efficient transcription of the TGFα gene.

Mutations in the TGFα Inr result in spatially aberrant transcription initiation

A central property of an Inr is that it positions the assembly of the transcription initiation complex at a specific site. Thus, disruption of the Inr not only reduces the number of transcripts initiated at the proper start site but often allows the use of cryptic initiation sites elsewhere. We examined the

Figure 1. Deletional analysis of the TGFα promoter. Various portions of the TGFα promoter were cloned upstream of the luciferase gene in the plasmid, pGL2. Schematic representation of these promoters are shown on the left. Coordinates are relative to the position of the major transcription initiation site of the gene (+1). Each construct was transfected into MDA468 cells, and the luciferase activity in the cell lysates was measured. The results are summarized on the right as the activity relative to that of the -311/+38 construct.

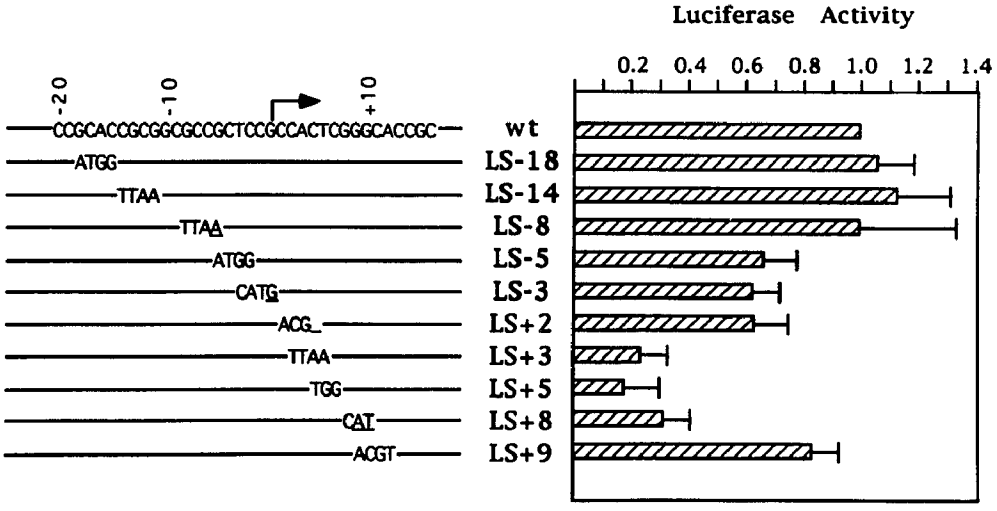


Construct	RelativeActivity
-311/+38	1
-181/+38	0.94
-181/+10	0.74
-181/+4	0.01
-311/+4	0.27

Figure 2. The TGFα Inr requires a specific 13-bp sequence.

(Left) Nucleotide sequence of the wild-type TGFα promoter (-20 to +16) is shown on the top line (WT). For the mutant constructs, indicated are the nucleotides changed relative to the wild-type promoter sequence. Each mutant plasmid is designated by the 5'-position of the mutant sequence which replaces the endogenous TGFα sequence. For example, LS-18 has nucleotide substitutions starting at -18 (to -15). Nucleotide insertions are indicated by underlined letters. A bar in LS+2 indicates deletion of a single nucleotide at the position.

(Right) Activities of LS mutant promoters in MDA468 cells. Cells were cotransfected with one of the TGFα promoter reporter plasmids and pCMVβgal. For each transfection assay, the luciferase activity was normalized by the activity of β-galactosidase in order to account for variations in transfection efficiency. The normalized values were averaged over three (for LS-18, -14, -8 and +9) or five (for LS-5, -3, +2, +3, +5, +8 and WT) independent transfection assays. Promoter activity of each construct is expressed relative to that of the wild-type plasmid. Bars indicate standard deviations.

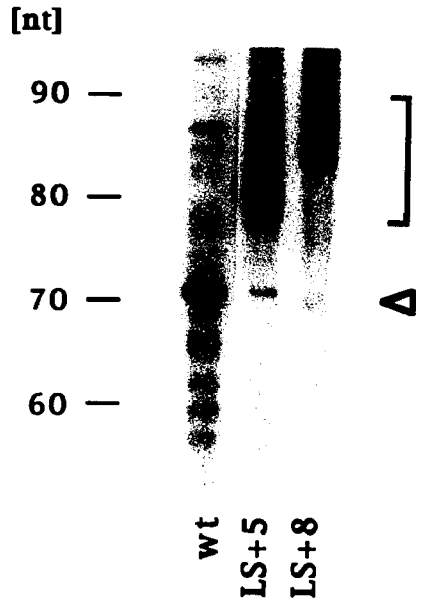

Luciferase Activity
0.2
0.4
0.6
0.8
1.0
1.2
1.4
-20
-10
+10
CCGCACCGCGGCGCCGCTCCGCCACTCGGGCACCGC
wt
ATGG
LS-18
TTAA
LS-14
TTAA
LS-8
ATGG
LS-5
CATG
LS-3
ACG
LS+2
TTAA
LS+3
TGG
LS+5
CAT
LS+8
ACGT
LS+9

possibility that transcription start site selection might be similarly affected by mutations in the TGFα Inr, particularly in those mutations within the +3 to +8 region, which most markedly attenuated the promoter activity (see Fig. 2). The initiation site was determined by directly assessing the 5'-end of the luciferase transcript in cells transiently transfected with a chimeric TGFα-luciferase reporter construct. Primer extension analysis using a luciferase-specific primer, of RNA isolated from cells that had been transfected with the wild-type plasmid (-181/+38) yielded a major reaction product that migrated at a size expected for transcripts initiated at the endogenous start site (Fig. 3). This finding demonstrates that this promoter region contains sufficient information for a site-specific initiation. On the other hand, when the initiation site was analyzed in cells that had been transfected with either LS+5 or LS+8 (see Fig. 2), a cluster of bands were observed that migrated more slowly than the wild-type product, indicating that these mutant constructs direct transcription initiation to multiple sites upstream of the authentic site. Based on these observations, we conclude that the TGFα sequence, that we have defined as the Inr, directs transcription initiation at a specific nucleotide in a manner similar to other Inrs.

MDA468 nuclear extracts contain
TGFα Inr-binding activities

An electrophoretic mobility shift assay was used to study whether nuclear proteins from MDA468 cells can form a complex with the TGFα Inr. Using an oligonucleotide probe containing the TGFα sequence between -12 and +10, seven specific shifts (Fig. 4A; a bracket) were detected. These shifts could be competed by excess amounts of the wild-type Inr oligonucleotide (Fig. 4A) but not by an Inr whose nucleotide sequence was scrambled (Fig. 4B; random). Furthermore, oligonucleotides containing the binding sites for the

Figure 3. Disruptions of the TGFα Inr cause transcription initiation at a cluster of cryptic sites. Transcription start sites in the wild-type promoter plasmid (wt) or two mutant plasmids (LS+5 and LS+8) were determined by a primer extension assay utilizing the 5'-RACE system. A band corresponding to the major wild-type initiation site is indicated with an arrowhead. Clusters of bands indicated by a bracket are specific to the mutants and represent transcripts initiated upstream of the wild-type site.


[nt]
90
80
70
60
wt
LS+5
LS+8
Δ

transcription factors AP1 or c/EBP did not compete the TGFα Inr complexes at 200-fold excess (Fig. 4B). Similar results were obtained using HeLa nuclear extracts (data not shown). Thus, we conclude that MDA468 and HeLa cell nuclear extracts contain one or more sequence-specific TGFα Inr-binding proteins (TIBP). We do not know how many distinct TIBPs exist. There could be as many as seven separate polypeptides, each capable of forming a specific complex with the Inr. Alternatively, some of the complexes may reflect protein posttranslational modifications, proteolytic cleavages or the association of auxiliary proteins.

Nucleotides critical for the TGFα promoter activity are also required for the TIBP-binding

If the interaction between TIBP and the Inr occurs in vivo and if this interaction is important for the functions of the Inr, then mutations that reduce the promoter activity should also weaken the protein-binding. We tested the ability of mutant Inrs to compete with the wild-type Inr for TIBP binding in a mobility shift assay. Mutations in these competitors corresponded to the constructs LS-3, LS+5 and LS+8 (mt3, mt5 and mt2 in Fig. 4B, respectively), which had been shown to reduce the promoter activity by approximately 40, 80 and 70%, respectively (see Fig. 2). Each competitor was added to the reaction at a 200-fold excess over the probe (Fig. 4B). All of the mutant Inrs displayed reduced ability to compete with the wild-type probe for complex formation with TIBP indicating that the nucleotides critical for the promoter activity are also necessary for high affinity binding of TIBP to the Inr.

Evidence that the TGFα Inr is distinct from the AdML-class Inrs

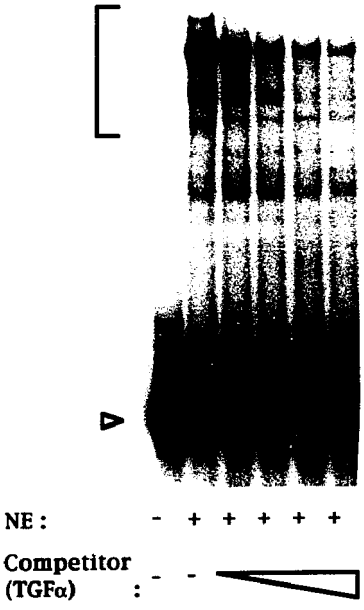
Sequence comparisons with other known Inrs suggested that the TGFα Inr may be most closely related to the Inr element in the AdML promoter

Figure 4. Electrophoretic mobility shift assay of the TGFα Inr and nuclear extracts from MDA468 cells.

A. MDA468 cell nuclear extract was incubated with a radiolabeled oligonucleotide probe spanning the TGFα promoter sequence from -12 to +10. Specific complexes are indicated by a bracket. Increasing amounts of an unlabeled oligonucleotide (identical to the probe) were added as a competitor. The competitor was in 10-, 20-, 50- or 100-fold excess over the probe (from the closed to open ends of a triangle). The arrowhead indicates the free probe.

B. Unrelated sequences or mutant Inr sequences do not compete the TGFα Inr complexes. The following competitors were added to the binding reaction at a 200-fold excess over the TGFα Inr probe: wt (the wild-type TGFα Inr), mt2 (the TGFα Inr with mutations identical to those in LS+8; see Fig. 2), mt3 (the TGFα Inr with mutations identical to those in LS-3), mt5 (the TGFα Inr with mutations identical to those in LS+5), random (a scrambled TGFα Inr), AP1 (an AP1 binding site from the human collagenase gene) and c/EBP (a synthetic c/EBP binding site).

A



NE : - + + + + +

Competitor (TGFα) : - - ▶

B

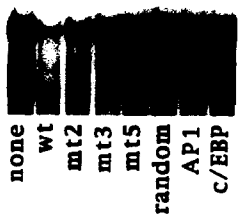


Figure 5. A TFII-I binding motif is neither necessary nor sufficient for the TGFα Inr complex formation.

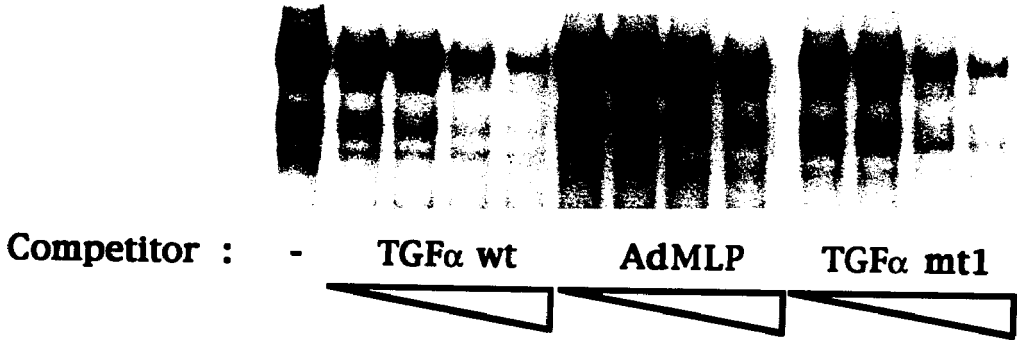
A. A sequence comparison between the AdML and the TGFα Inrs. Underlined A and G indicate the major transcription initiation sites for the AdML and the TGFα promoters, respectively. The nucleotide sequence of the TGFα mt1 oligonucleotide is also shown. A part of the TFII-I-like motif is mutated in this oligonucleotide and indicated with outlined letters.

B. TGFα mt1 but not AdMLP competes the TGFα Inr complexes. Unlabeled competitors were the wild-type TGFα Inr (TGFα wt), the AdML promoter (AdMLP; sequence between -13 and +11) and the mt1 oligonucleotide. Each competitor was added at a 10-, 20-, 50-, or 100-fold excess over the probe.

A

AdML	G T C C T C A C T C T C
TGFα wt	T C C G C C A C T C G G
TGFα mt1	T C C G C C A G A T G G

B



(Fig. 5A). The AdML Inr is directly recognized by a 120 kD transcription factor, TFII-I (19). Interestingly, the similarities between the TGFα and AdML elements include a motif, CACTC (-1 to +4 in the AdML promoter; see Fig. 5A) which contributes to the TFII-I binding site. To test the possibility that the TGFα Inr interacts with TFII-I, two competitors were used in a mobility shift assay. The first competitor was the wild-type AdML Inr (AdMLP; -13 to +11) containing a TFII-I binding site. The second competitor, mt1, was a mutant TGFα Inr in which the CACTC motif was replaced by the sequence, CAGAT. Previously, these same nucleotide substitutions had been used to abolish TFII-I binding to the AdML Inr (19). Thus, if the TGFα Inr interacts with TFII-I, this binding would be blocked by large excesses of the AdMLP but not the mt1 competitor. Contrary to this prediction, none of the complexes were competed by AdMLP (Fig. 5B). Instead, mt1 competed nearly as efficiently as the wild-type TGFα Inr, indicating that the TFII-I binding motif is dispensable for the TIBP-Inr interaction. Based on these results and results from Southwestern blot analysis (see below), we conclude that the TGFα Inr interacts with proteins that are distinct from TFII-I and, therefore, the TGFα Inr does not belong to the AdML Inr family.

Southwestern blot analysis indicates that 105 and 95 kD proteins interact specifically with the TGFα Inr

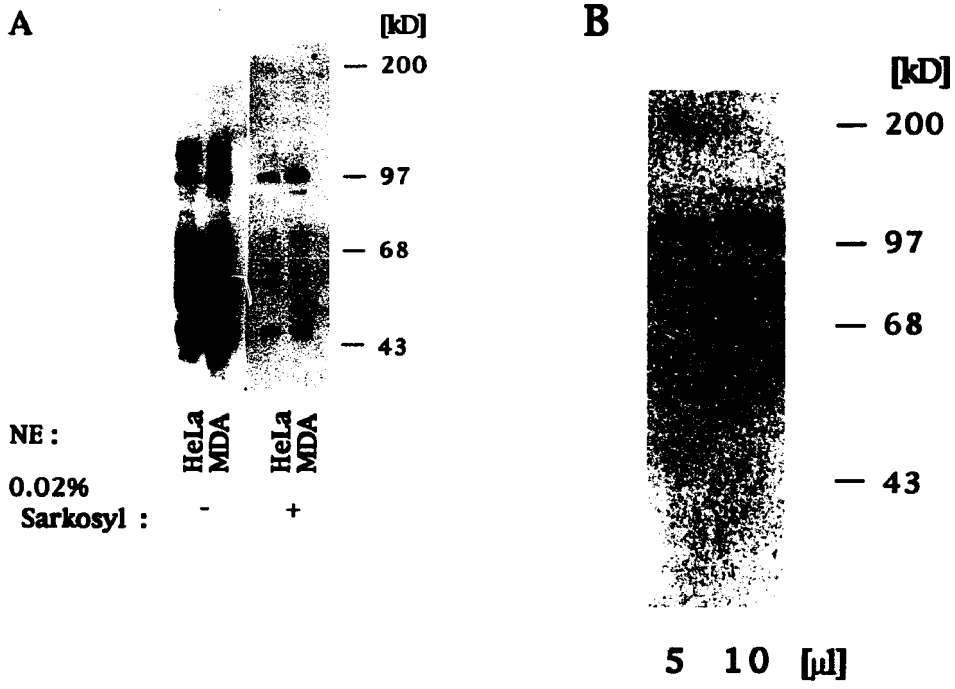
The TIBP was analyzed by Southwestern blotting. Following SDS/polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane and tested for their ability to interact with the TGFα Inr probe. Under nonstringent conditions (5mM $MgCl_2$ and 50mM KCl), the radioactive probe was found to interact with many polypeptides on the membrane (Fig. 6A, left). We believe that the probe bound to many of these proteins as a result of nonspecific interaction involving salt bridge formation

between the phosphate backbone of DNA and positively charged residues on the proteins. In an attempt to reduce such electrostatic interactions without disturbing specific base recognition by DNA-binding proteins, which involves hydrogen bonds and van der Waals interactions, we used a low concentration anionic detergent. Sarkosyl was chosen for this purpose since it has been shown not to perturb protein conformation (28). First, we tested if Sarkosyl interferes with the formation of the TIBP-Inr complexes in a mobility shift assay. At a concentration of 0.06% or less, the detergent was found to have little to no effect on complex assembly. Next we analyzed a recombinant c/EBP protein by Southwestern blotting. After incubation with the c/EBP binding site probe, the blot was washed with a buffer containing 0.02% Sarkosyl. Although the detergent treatment reduced background signals significantly, the ability of the immobilized c/EBP to interact with its binding site was not affected. Following these preliminary studies, TIBP were reanalyzed using the 0.02% Sarkosyl wash. As expected, this treatment markedly decreased the intensity of most of the bands (Fig. 6A, right). However, the intensity of the bands corresponding to the 105 and 95 kD polypeptides remained constant. Thus, it is likely that these detergent-resistant bands represent proteins that specifically recognize the TGFα Inr. Similar results were obtained with the HeLa cell nuclear extracts except that the 95 kD band was not detectable in these extracts. The mt3 oligonucleotide contains mutations that reduce both protein-binding (see Fig. 4B) and transcriptional activity (see Fig. 2). When this oligonucleotide was used as a probe in Southwestern blotting, the intensity of both the 105 and 95 kD bands was much reduced (data not shown). Taken all together, we believe that both proteins specifically interact with the TGFα Inr.

Figure 6. 105 and 95 kD proteins specifically bind the TGFα Inr.

A. Southwestern blot analysis of nuclear proteins in the absence and presence of Sarkosyl. Approximately equal amounts of proteins from HeLa or MDA468 nuclear extracts were separated by SDS/polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and incubated with the TGFα Inr probe. Blots were washed in a buffer not containing (-) or containing (+) 0.02% Sarkosyl. Bands corresponding to the 105 and 95 kD polypeptides were resistant to the detergent wash. Migration positions of molecular size standards are shown on the right.

B. Southwestern blot analysis of materials partially purified by Inr DNA-affinity column chromatography. HeLa nuclear extracts were subjected to DNA-affinity chromatography using an agarose-immobilized TGFα Inr. Indicated amounts of partially purified material were analyzed as described for panel A with a Sarkosyl wash. Note that HeLa nuclear extracts do not contain detectable amounts of the 95 kD protein.


A
[kD]
200
97
68
43
NE :
HeLa
MDA
HeLa
MDA
0.02%
Sarkosyl :
-
+
B
[kD]
200
97
68
43
5 10 [µl]

The 105 kD protein can be partially purified by DNA-affinity chromatography.

To further characterize the 105 kD protein, HeLa nuclear extracts were fractionated over a heparin sulfate-agarose column. The eluted fractions were tested for TIBP using a mobility shift assay, and fractions with a TIBP activity were pooled and applied to a DNA-affinity column. This column consisted of tandemly repeated TGFα Inr coupled to CNBr-activated agarose beads. After an extensive wash, the bound proteins were eluted from the column with a linear KCl gradient. The specific mobility shift activity was found in the fractions that eluted between 0.4 to 0.65M KCl. Proteins in these fractions were analyzed by Southwestern blotting. As shown in Fig. 6B, a single major band corresponding to 105 kD was detected by the TGFα Inr probe. Silver staining of a duplicate gel also showed a protein of similar size, highly enriched in these fractions (data not shown). Thus, the 105 kD protein can bind the TGFα Inr in a soluble form and a sufficient fraction of the protein can survive the denaturation and renaturation process necessary for detection by Southwestern blotting. This result further supports the idea that the 105 kD protein is a specific TIBP, which we call TIBP-1.

DISCUSSION

The Inr is a core promoter element that is essential for transcription initiation at several promoters (16, 17). Inr-containing promoters include the major late (19) and IVa promoters (18) of adenovirus 2, the P5 promoter of adeno-associated virus type 2 (26), the ribosomal protein S16 promoter (29), the porphobilinogen deaminase promoter (30), the TdT promoter (15) and the DHFR promoter (22). Here we show that a similar element also controls transcription of the TGFα gene. This element conforms to the definition of an initiator based on its location and function in the TGFα gene. That is, the

TGFα Inr is located between -5 and +8, which is consistent with other Inrs (for example, the AdML Inr is between -6 and +11 and the DHFR Inr is -11 and +9). Furthermore, the TGFα Inr is important both for efficient transcription initiation and for selection of a specific initiation site.

The initiator sequences, where investigated in other genes, have been shown to be recognized by transcription factors. We have shown that the TGFα Inr is recognized by at least one but possibly two proteins that specifically interact with this element. The specificity of this binding was demonstrated by showing that mutations that interfere with the protein-binding also reduce the promoter activity. While these TIBPs were investigated using the TGFα Inr, it is possible that they are used by a subset of genes that possess a similar Inr element. That is; the TGFα promoter contains multiple Sp1 binding sites and lacks a TATA box, and this is similar to the promoter organization observed in a number of growth regulating genes. These genes include the genes for growth factors and their receptors (31-33), cytoplasmic signal transducing molecules (34, 35), cyclins (36) and transcription factors (37, 38). An intriguing possibility is that the proper regulation of cell growth may require the function of the Inr. In this regard, it is noteworthy that the protein product of the c-myc oncogene interacts with TFII-I and inhibits transcription from an Inr-containing promoter (39).

By a Southwestern analysis of membrane immobilized proteins, two TGFα Inr-binding proteins (TIBP) were identified. Although their functional roles are currently not known, at least one of them (TIBP-1) can be partially purified by DNA-affinity chromatography, indicating that its interaction with the TGFα Inr is not merely an artifact intrinsic to the blotting technique. The apparent sizes of TIBP (105 and 95 kD) suggest that they are different from the previously identified Inr-binding factors. Consistent with this view, a

mobility shift assay indicates that the TIBPs do not recognize the AdML Inr which is a target for TFII-I (Fig. 5B). Furthermore, other Inrs, including those from the TdT, DHFR and ribosomal protein S16 genes, fail to compete the TGFα Inr complexes in a mobility shift assay (data not shown). Based on these observations, we believe that TIBP may be a new entry to a growing list of Inr-binding factors.

The Inr functions as a positioning element to direct the transcription initiation complex to the proper start site. This function appears to be served by a sequence motif common to all Inrs (16, 40). Consistent with this view, a sequence homologous to the 8-bp consensus (G/T)C<u>A</u>(C/G/T)(A/C/T)(C/T)(C/G/T)(C/T) (initiation site underlined) is also found in the TGFα Inr (CCACTCGG between +2 and +10). The position of this motif relative to the transcription start site is different in the TGFα Inr from the other Inrs. Although this discrepancy could be due to inaccuracy of the primer extension technique, we did map the start site of the TGFα-luciferase chimeric gene and found that this initiating nucleotide was identical to the one used by the endogenous TGFα promoter (8). This feature of the TGFα Inr again suggests that it is distinct from the previously defined Inrs.

The mechanism of Inr-mediated initiation is not well understood. However, studies with the TdT and AdML Inrs have demonstrated that TFIID is an integral component of the initiation complex, even in the absence of a TATA box (18, 41-43). In this regard, it is interesting that the known Inr-binding proteins can also act as transactivators. For example, E2F binding sites in a number of promoters are important for transcription but not necessarily located at the initiation site (25, 27, 44). YY1 is another Inr-binding activity that also activates transcription from distal binding sites (45, 46). Targets of these transactivators are likely to be general transcription factors, in

particular TFIID. Thus, a generalized function of Inr-binding factors appears to be to recruit TFIID to a TATA-less promoter through the Inr. A recent experiment which supports this idea demonstrated that TBP and TFII-I cooperatively interact with the AdML promoter in the absence of a TATA box (20). We speculate that TIBP may also interact with and recruit TFIID to the TGFα promoter. As a preliminary result, however, we failed to detect TBP in the Inr complexes using a specific antibody. Nor could the immobilized TGFα Inr co-purify the two activities (data not shown). Further investigations on a potential interaction between TIBP and TFIID (and other general factors) are required, but they may await a molecular cloning of the gene encoding TIBP.

ACKNOWLEDGMENTS

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THE HUMAN TGFα PROMOTER IS A TARGET OF THE TRANSCRIPTION FACTOR P53

by

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ABSTRACT

The recent demonstration of p53 as a sequence-specific transcription factor has provoked a vigorous search for its target genes. Since certain types of cancers appear to have a higher incidence of p53 mutations, we considered the possibility that genes important in formation of these cancers might be targeted by the p53 transcription factor. In breast cancer, pathways involving the epidermal growth factor receptor (EGFR) and the product of the *neu* proto-oncogene appear to play important roles. We found potential binding sites for p53 in the transforming growth factor-α (TGFα), a ligand for the EGFR, and neu promoters. Here we report identification of functional p53 binding sites in the human TGFα gene. Transcription activity of the 1.1 kb TGFα promoter increases by approximately 2.5-fold with the expression of p53 in MDA468 cells. Two potential p53-binding sites were found in this region of the promoter. The proximal site (site I, -53/-34) binds p53 in vitro and is necessary and sufficient for the p53-induced transcription in transfection assays. The multimerized site I sequence confers strong p53-responsiveness to a reporter plasmid containing the adenovirus major late promoter. These transcriptional responses required wild-type p53. Furthermore, contrary to the previous assumption, we show that the TGFα promoter contains a non-consensus but functional TBP-binding site approximately 30-bp upstream of the transcription start site. This non-consensus TATA motif does not mediate p53-transcription suppression, unlike other consensus TATA boxes, suggesting that the negative p53-TBP interaction depends on the context of TFIID-binding sites. Identification of the TGFα gene as a p53 target should facilitate future investigations on the physiological roles of p53, as well as TGFα, in normal and cancer biology.

INTRODUCTION

It is now well accepted that the tumor suppressor protein, p53, acts as a sequence-specific transcription activator (for a review, see 1). Induction of transcription by wild-type but not mutant p53 has been shown both in vitro and in vivo. This trans-activation requires binding of p53 to specific DNA sites (consensus; PuPuPuC(A/T)(T/A)GPyPyPy) (2, 3), to date identified only in a limited set of genes (1 and references therein). On the other hand, p53 can also suppress transcription from promoters containing a TATA box without binding directly to DNA (4). Interaction between p53 and TATA box-binding protein (TBP) (and TFIID) has been demonstrated and presumably is responsible for both the negative and positive regulation (5-9).

That p53 inhibits the cell cycle in G1 (10 and references therein) suggests that genes which are induced by p53 may participate in the blockage of the G1/S transition. Identification of GADD45, a gene implicated in growth arrest in response to ionizing radiation, as a p53-target gene is consistent with this view (10). More recently, another p53-inducible gene called WAF1 was isolated (1). The protein product of the gene, $p21^{CIP1/WAF1}$, binds to and thereby inhibits the activity of G1-specific cyclin-dependent kinases (11-14), thus preventing cell cycle progression through G1. These findings are consistent with the tumor suppressor role of p53; a loss of the p53 functions renders cells insensitive to negative regulation at a cell cycle checkpoint. However, apart from this handful of examples, other physiological targets of the p53 transcription factor are currently unknown.

Despite the progress in understanding the mechanism by which p53 is a tumor suppressor, its role in normal physiology remains unclear. A transgenic mouse line deficient in p53 exhibits an increased rate of spontaneous tumor formation but appears to be otherwise normal in development (15).

Recently, involvement of p53 in the process of apoptosis has been demonstrated in certain cell types (16-19). Given the function of p53 in cell cycle control and apoptosis, it was anticipated that the null mutation of both p53 alleles would lead to profound defects in development of many tissues as a result of inappropriately regulated cell growth, proliferation and death. The lack of developmental abnormalities may mean that p53 does not play a role during embryo- and organogenesis. Alternatively, it may reflect functional redundancy in this complex biological system. In the latter case, gene knock-out experiments may turn out to be without much success.

An alternative approach for investigating the normal functions of p53 might come from observations that certain tissues are more susceptible to neoplastic transformation when the p53 activity is lost. Point mutations, deletions, allelic loss and rearrangement in the p53 gene are most frequently associated with cancers of organs such as colon, brain and breast (20, 21). In addition to the universal role in the cell cycle control, p53 may also elicit more specialized functions affecting only limited cell types at a particular time of development. For example, p53-regulated production of a growth factor would have a biological significance only in those tissues able to produce both the growth factor and its cognate receptor at the same time. Such tissues might be more sensitive to a loss of p53. Those genes implicated in the establishment and maintenance of neoplasia in such p53-susceptible organs could then be good candidates as targets of the p53 transcription factor. A systematic search based on this concept might facilitate the identification of p53 target genes that are otherwise difficult to find. Moreover, this approach would shed more light on the normal physiological roles of this tumor suppressor protein.

Transforming growth factor-α (TGFα) stimulates epithelial cell growth (22, 23) by binding to and activating the epidermal growth factor receptor (EGFR) (for a recent review, see 24). Other responses to TGFα include the induction of cell motility, changes in cell morphology and the regulation of differentiation. TGFα is expressed in a variety of adult tissues, including gastric mucosa and mammary epithelium (for reviews on the expression pattern, see 22, 23), and also transiently in the developing embryo (25). TGFα has been implicated in physiological as well as pathological conditions of breast and colon. Overexpression of TGFα in transgenic mice leads to profound epithelial hyperplasia (and in some cases neoplasia) in several organs, including the mammary gland and colon (26-29). In both cases, TGFα expression is associated with more branching and complex ducts, suggesting its role in the formation of the exocrine gland. Furthermore, epidermal growth factor (EGF), another ligand for the EGFR, is a potent mitogen for gastrointestinal epithelial cells in both tissue culture and living animals. In addition, stromal cells in the mammary gland also express EGFR. TGFα may participate in maturation of the gland by acting on the stroma as a mitogen and/or morphogen (26).

Based on the observations that both p53 and TGFα have been linked to neoplasia in many of the same tissues, we examined the possibility of a relationship between TGFα and p53. In this report we demonstrate that p53 acts as a sequence-specific transcription activator of the TGFα promoter. Identification of the TGFα gene as a p53-target may contribute to our understanding of the role of p53 in the development of the mammary gland, colon and other organs.

MATERIALS AND METHODS.

Plasmids

Two p53-expression vectors (effector plasmids) were used in this study. pC53-SN_3 (30) encodes the wild-type p53 protein and was a generous gift from Drs. L. A. Laimins and B. Vogelstein. pC53-248_3 encodes the 248 Arg to Trp mutant and was kindly provided by Dr. A. J. Levine. All reporter plasmids contain the coding sequence for the firefly luciferase gene and a sequence from the TGFα promoter or the AdMLP. The 1.1 kb promoter construct derives from pXP1/1.1 (31) with an extension of the promoter to +38. Two 220-bp promoter constructs were used in this study. pG-181/+38 was described previously (32). pG-181/+38AAN is identical to pG-181/+38, except that restriction sites for ApaI, AvrII and NcoI were engineered at -65, -40 and -19 of the promoter. Activities of the two constructs are nearly identical in transfection assays. For construction of TATA-replacement vectors, the following double-stranded oligonucleotides were inserted between AvrII and NcoI sites of pG-181/+38AAN: 5'-CTAGGCTGGGGCTATAAAAAGG-3' and 5'-CATGCCTTTTATAGCCCCACG-3' for the "good" TATA box and 5'-CTAGGTCTGCGTCGGCCGGTC-3' and 5'-CATGGACCGGCCGACGCAG-AC-3' for the "poor" TATA box. For deletion of p53-binding site I, the following double-stranded oligonucleotide was inserted between ApaI and AvrII sites in pG-181/+38AAN: 5'-CTCCCGCGCGGATCC-3' and 5'-CTAG-GGATCCGCGCGGGAGCCGG-3'. pAdMLP1 contains a sequence between -38 and +11 of the AdMLP in XhoI and BglII sites of a luciferase vector, pXP2. pAdEamI derives from pAdMLP1 and contains four copies of the site I sequence in a head-to-tail array at a HindIII site upstream of the AdMLP. Since each site I sequence consists of two binding motifs in tandem, there are total of

eight p53-binding sites in this plasmid. pSVK3 (Pharmacia, Piscataway, NJ) was used to balance the amounts of plasmids in transfection assays.

Transfection assays

MDA468 cells were electroporated with a total of 35 μg plasmid DNA containing 20 μg of a reporter plasmid, 0-5 μg of an effector plasmid, 10 μg pCMVβgal and appropriate amounts of the balancer plasmid. Conditions and procedures for electroporation and enzymatic (luciferase and β-galactosidase) assays were described previously (33). Cells were harvested at 36-43 hours after transfection. All experiments were done in triplicate and repeated at least three times. The luciferase activity was normalized by the activity of the β-galactosidase in the same lysate.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from approximately $1x10^7$ transfected or mock-transfected MDA468 cells according to a method by Shreiber et al. (34). The probe used for the study of p53-binding contains the sequence from -65 to -15 of the TGFα promoter. EMSA was done under a standard condition (33). Monoclonal antibodies (PAb421 and PAb1801 for p53 and C36 for Rb) were obtained from Oncogene Science (Uniondale, NY). Recombinant TBP protein was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). EMSA using TBP was performed according to Peterson et. al. (35) with a probe containing the TGFα promoter sequence between -40 and -20. Twenty-five-fold excesses of TATA competitors (sequences are shown above) were added to selected reactions.

Western blot analysis

Ten microliters of nuclear extracts were subjected to SDS/poly-acrylamide gel electrophoresis (PAGE). A nitrocellulose blot was prepared by standard Western blotting procedures and probed with the monoclonal

antibody PAb421, followed by a goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Pierce, Rockford, IL).

RESULTS

The TGFα promoter activity is induced by the expression of wild-type but not mutant p53 in MDA468 cells

To investigate potential effects of p53 on TGFα transcription, we co-transfected p53 expression vectors into MDA468 cells, along with a reporter plasmid containing 1.1-kb of the promoter region of the human TGFα gene. The MDA468 cell line is derived from a human mammary carcinoma harboring a single p53 allele which encodes a protein with a missense mutation at codon 273 (Arg to His) (36). This p53 protein does not behave as a dominant negative mutant in transcription assays (37). Cotransfection of the wild-type p53 expression plasmid (pC53-SN3) resulted in the induction of TGFα promoter activity in a dose-dependent manner (Fig. 1A). This induction was not seen, however, when the mutant p53 expression vector was used. The protein encoded by this plasmid contains a missense mutation at codon 248 (Trp instead of Arg) and is frequently expressed in cancers of colon and breast (20, 21). The lack of induction by the 248 mutant did not result from failure of p53 protein expression. As shown in Fig. 1B, the 248 mutant protein was expressed at a comparable level to that of the wild-type p53 protein in nuclear extracts prepared from the transfected cells.

Examination of the 1.1-kb sequence of the TGFα promoter revealed two potential binding sites for p53 (Fig. 1C). One element is located approximately 600 bp upstream of the transcription start site (site II), while the other element (site I) is located proximally between -53 and -34. p53-binding sites have been identified in several genes (1 and references therein). These sites are located one or more kilobases away from the transcription start site in

either a 5'- or a 3'-direction, except for a sequence in the GLN retrovirus-like element (38). As in other p53-binding sites, both sites in the TGFα promoter consist of two tandemly repeated 10-bp sequences, each closely resembling the consensus binding motif (17 and 16 matches out of 20, respectively).

The p53-dependent transcription induction requires p53-binding sites

The activation of the TGFα promoter could be an indirect consequence of p53 expression. To examine this possibility, we used a proximal 220-bp version of the TGFα promoter which lacks site II but retains the more proximal site I. This promoter again responded to the expression of wild-type p53 (approximately 2-fold) but to a slightly lesser degree than the 1.1 kb promoter (Fig. 2). This induction depends on the presence of site I because the deletion of this sequence almost completely eliminated the p53-dependent activation (1.15-fold) (Fig. 2). These data suggest direct involvement of p53 in the induction of TGFα promoter activity.

Wild-type but not mutant p53 binds site I

To assess if site I is a bona fide p53-binding site, we conducted an electrophoretic mobility shift assay (EMSA). A band with a retarded mobility was detected in nuclear extracts from MDA468 cells transfected with pC53-SN3 but not from mock-transfected cells (Fig. 3). This complex contained the p53 protein, as demonstrated by the appearance of a supershift by monoclonal antibodies against p53 but not by an antibody against the Rb protein. In accordance with earlier studies using the 248 mutant (for example, see 37), expression of the mutant protein did not lead to the formation of the p53-containing complex, and the p53-specific antibody had no effects. Since the p53 protein is abundantly expressed in nuclear extracts (see Fig. 1B), the failure to detect the p53-containing complex is due to the inability of the 248

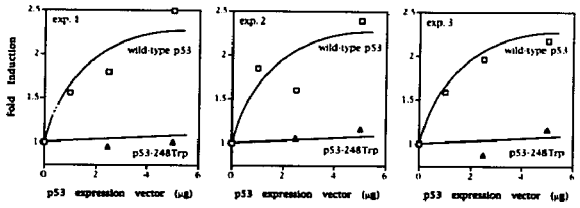
Figure 1. The TGFα promoter activity is induced by the expression of wild-type but not mutant p53.

A. A 1.1 kb TGFα promoter-luciferase reporter plasmid was transfected into MDA468 cells, along with 0, 2.5 or 5 μg of either a wild-type (squares, pC53-SN_3) or a mutant p53 expression vector (triangles, pC53-248_3). Shown are the fold-inductions of the luciferase activity with the indicated amounts of p53 expression plasmids. All values were normalized against the activity of β-galactosidase expressed from a cotransfected pCMVßgal.

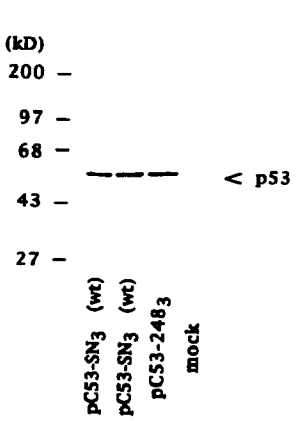
B. A Western blot showing expression of p53 proteins in nuclear extracts. Nuclear extracts were prepared from MDA468 cells that had been either mock-transfected or transfected with pC53-SN_3 or pC53-248_3. The Western blot was probed with the monoclonal antibody pAb421.

C. Potential p53-binding sites in the TGFα promoter. The consensus p53-binding motif is shown. Numbers on the TGFα sequence are nucleotide positions relative to the transcription start site.

A



B



C

Consensus : P P P CWWGY Y Y
(P= G or A, R= C or T, W= A or T)

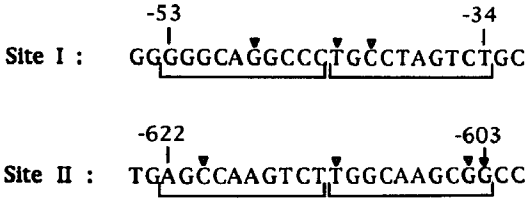
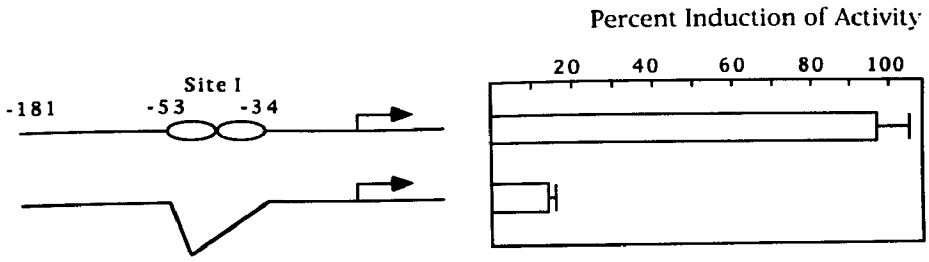


Figure 2. Site I is required for the induction of the TGFα promoter activity by p53. Reporter plasmids (schematically drawn on the left) contained the proximal 220-bp TGFα promoter either with or without the site I p53-binding motifs (ovals). These were cotransfected into MDA468 cells with 0 or 5 μg of pC53-SN_3 (wild-type). The percentage increases in the normalized luciferase activity, averaged from three experiments, in the presence of pC53-SN_3 are shown on the right side of each reporter construct, along with standard deviations.

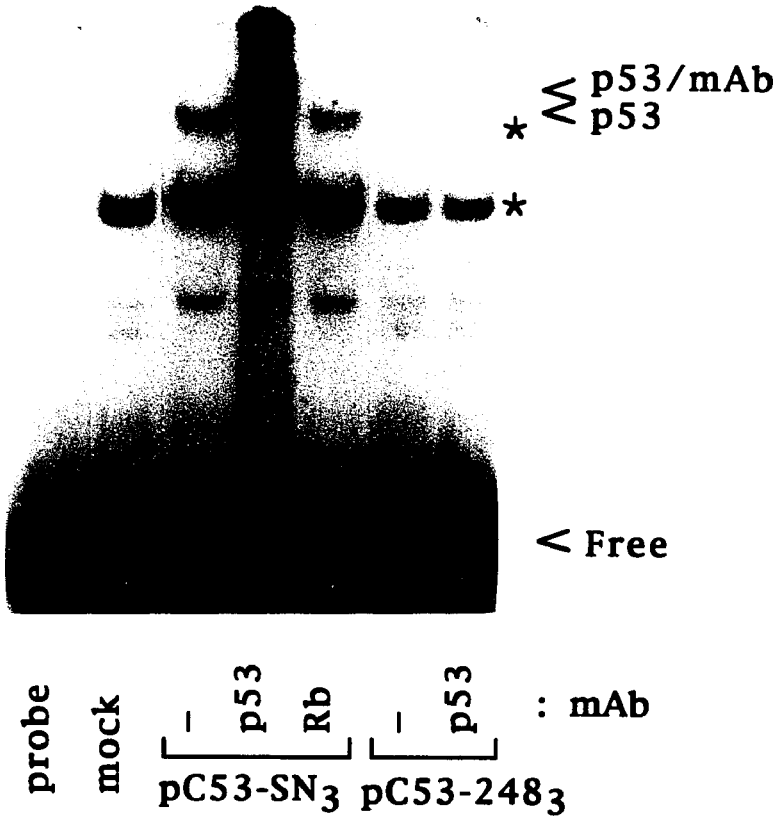

Percent Induction of Activity
20
40
60
80
100
Site I
-181
-53
-34

mutant to bind the site I sequence. These results further support the notion that wild-type p53 induces TGFα transcription by directly interacting with the promoter.

A non-consensus TATA box is located immediately downstream of a p53-binding site (site I)

p53 can selectively suppress transcription from TATA box-containing promoters but not transcription directed by an initiator (4). This repression does not require direct binding of p53 to the promoter and appears to be mediated by interaction of p53 with TBP. The TGFα promoter lacks a consensus TATA motif and uses an initiator for the accurate transcription initiation (32). However, it has not been determined whether the TGFα promoter truly lacks a functional TATA box, in that a non-consensus TATA motif may mediate the binding of TFIID (39). Interestingly, we noticed a region of extensive homology between the human and rat TGFα promoters positioned approximately 30 bp upstream of the transcription start site (Fig. 4A) (40-42). In this region, not only is a contiguous 18-bp sequence almost perfectly conserved, but the distance between the start site and the conserved region is nearly identical. Furthermore, within this 18-bp stretch is a motif, TTTTTCCCCC, that is somewhat reminiscent of the T-rich strand of the TATA box contained in the adenovirus major late promoter (AdMLP). To assess if this motif functions as a TATA box and to address a potential relationship to the neighboring p53-binding site, we replaced this native sequence with either a consensus TATA motif (from the AdMLP) or an unrelated sequence (Fig. 4B). Substitution with the "good" (AdMLP) TATA box increased the promoter activity by about 2.5-fold, while the promoter with the unrelated sequence was only half as active as the wild-type promoter (Fig. 4C). This result is consistent with the idea that this motif in the TGFα promoter is required for

Figure 3. p53 binds site I in an electrophoretic mobility shift assay. An EMSA was conducted with nuclear extracts prepared from cells transfected with no effector plasmid, pC53-SN3 (wild-type) or pC53-2483 (mutant). The probe contained the sequence of site I as well as short sequences from the flanking regions. In selected reactions, monoclonal antibodies against p53 or Rb were coincubated with the nuclear extracts. The slower migrating bands seen in the presence of the p53 antibody represent supershifted p53-containing complexes.


p53/mAb
p53
Free
probe
mock
-
p53
Rb
-
p53
: mAb
pC53-SN3
pC53-2483

interacting with TFIID. Indeed, recombinant TBP protein is capable of directly binding to an oligonucleotide containing the TGFα motif (Fig. 4D). This binding was inhibited by both the TTTTTCCCCC sequence itself and the AdMLP TATA box but not by the "poor" TATA box that also functioned poorly in the transfection assay. These results indicate that the TGFα promoter contains a non-consensus TATA motif that is, nevertheless, recognized by TBP.

p53-dependent transcription suppression is not supported by the TTTTTCCCCC motif of the TGFα promoter

In TATA containing promoters that lack a p53 binding site, p53 suppresses transcription. By deletion of the p53 binding sites in the TGFα promoter, we could test whether the TTTTTCCCCC motif could support transcription suppression by p53. As noted in Fig. 2, deletion of the p53 binding sites results in a promoter which is practically uninducible by p53 (see Fig. 2). We have noted that this version of the promoter occasionally responds to wild-type p53 with a slight induction (not more than 17%) but never with transcriptional suppression. This observation indicates that the non-consensus TATA box in the TGFα promoter does not support p53-dependent suppression. This result was also observed in HeLa cells and in experiments with the same promoter in a different plasmid background (data not shown). Instead, the suppression requires a consensus TATA box, in that p53 expression suppressed transcription from a promoter containing the AdMLP TATA box (Table 1) (4). These results indicate that TBP bound to the nonconsensus site in the TGFα promoter is inaccessible to p53 for repression. This, in turn, implies that the TFIID complex can take two distinct conformations depending on the context of its binding site.

Figure 4. A non-consensus TATA box is present at approximately 30 bp upstream of the transcription start site.

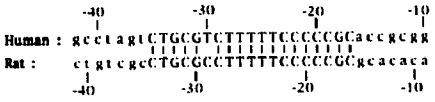
A. A sequence comparison of the human and rat TGFα promoters at the -30 region. A 18-bp conserved region is shown by uppercase letters with small vertical lines indicating identical nucleotides in the two sequences. Nucleotide positions relative to the respective transcription start sites are also shown.

B. Sequences of TATA motifs used in the following set of experiments. The "good" TATA motif is that of the AdMLP.

C. Activities of the TATA-replacement promoters, each containing one of the TATA motifs shown in panel B. Sequences of the three promoters are identical, except in the region around -30. The normalized luciferase activities are shown (an average of three experiments), along with standard deviations.

D. Recombinant TBP binds the TGFα TATA motif in an EMSA. The probe for the EMSA is a double-stranded oligonucleotide containing the TATA-like motif of the TGFα promoter. Competitors were the TATA motifs shown in panel B at a 25-fold molar excess over the probe.

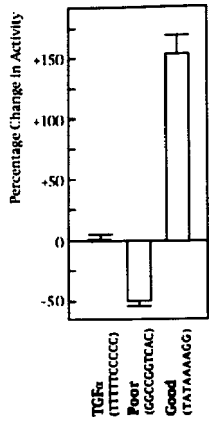
A



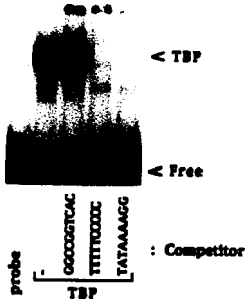
B

TGFα :	CGTCTTTTTCCCCC
Good (AdMLP) :	GGGCTATAAAAGGC
Poor :	CGTCGGCCGGTCAC

C



D



Site I supports the transcription induction by p53 independent of the promoter context

In the TGFα promoter, site I is required for the p53-dependent transcription induction and site I can be recognized by p53 in vitro in an EMSA. Further evidence that site I directly supports the induction by p53 was obtained by demonstrating that site I is able to confer the p53-responsiveness to a heterologous promoter (Table 1). Four copies of the site I sequence (total 8 binding sites, see Fig. 1C) were cloned in front of the AdMLP in a luciferase reporter plasmid. As shown in Table 1, this reporter construct was very strongly induced (200- to 500-fold) by wild-type p53 but not the 248 mutant form of this transcription factor.

DISCUSSION

Potential roles of the p53-regulated TGFα transcription

The experiments described here provide compelling evidence that the TGFα promoter is subject to regulation by p53. That is, wild-type but not mutant p53 binds to consensus sites in the TGFα promoter and induces promoter activity, both in the context of the TGFα gene and with a heterologous promoter. While these results suggest the likelihood that p53 regulates the TGFα gene in living organisms, the significance of this regulation in normal physiology and development remains unknown. In particular, because p53 is a tumor suppressor, whereas TGFα is associated with tumor development, we expected p53 to suppress TGFα gene transcription. Surprisingly, p53 induces the activity of the TGFα promoter. This induction might play an important role in the skin, where TGFα is highly expressed. Ionizing and ultra-violet radiation induces p53 activity in certain cell types (14 and references therein). In skin injuries caused by such radiation, the p53-induced expression of TGFα could facilitate wound healing by promoting

Table 1.

Activities of the AdMLP and the AdMLP containing four copies of the site I sequence.

PLASMIDS	EXP. 1	EXP. 2	EXP. 3
AdMLP alone	1.1 $x10^3$	2.8 $x10^3$	1.4 $x10^3$
AdMLP + pC53-248_3	1.0 $x10^3$	2.7 $x10^3$	-
AdMLP + pC53-SN3	$0.44x10^3$	$0.82x10^3$	$0.50x10^3$
AdMLP(SITE I)$_4$ alone	$2.2x10^3$	$9.6x10^3$	$3.3x10^3$
AdMLP(SITE I)$_4$ + pC53-248_3	$2.0x10^3$	$9.1x10^3$	-
AdMLP(SITE I)$_4$ + pC53-SN3	$5.6x10^5$	$2.2x10^6$	$1.3x10^6$

Cells were cotransfected with one of the p53-expression plasmids and a luciferase reporter plasmid containing either the AdMLP alone or the AdMLP plus four copies of the site I sequence in a tandem array. Luciferase activities were normalized against the activity of β-galactosidase expressed from cotransfected pCMVβgal. Each value was obtained as relative light units of the luciferase activity divided by arbitary units of the β-galactosidase activity.

epidermal cell proliferation, squamous epithelial hypertrophy and/or migration of keratinocytes to the site of injury (29).

The role of p53 induction of TGFα gene transcription in mammary epithelium remains unclear. Overexpression of TGFα in transgenic mice results in mammary epithelial hyperplasia, increased ductal branching, reduced lactation and a marked increase in the incidence of mammary carcinoma. In human mammary carcinoma, there is a high incidence of p53 mutations. This convergence of TGFα and p53 in the mammary gland suggested the possibility that the TGFα gene might be regulated, probably in a negative manner, by p53. Indeed, this reasoning also led us to examine another gene implicated in the pathogenesis of mammary carcinoma, c-neu. Interestingly, we found a potential p53-binding site in the 5'-promoter region of the human c-neu gene (43; sequence 650-669, GenBank accession number: J05264), although we have yet to test the activity of p53 on the expression of this gene. That p53 stimulates TGFα gene expression is difficult to reconcile with its known tumor suppressor activity. However, physiological levels, timing and localization of TGFα gene expression in the mammary gland may play a more subtle role than is predicted by the overexpression of this growth factor in transgenic animals. For example, localized delivery of EGF to rapidly proliferating mammary ducts is inhibitory to ductal growth (44). In addition, stromal cells are also known to express EGFR; thus, TGFα may influence mammary morphology by acting on these cells. A physiological signal that induces p53 activity could block progression of mammary epithelia through the cell cycle but allow the p53 induced growth factor to signal cell hypertrophy and differentiation. Since p53 contributes to the control of TGFα gene transcription, it could play a role in the normal development of the mammary

gland, particularly if p53 activity is regulated in a physiological or developmental manner.

Transcription suppression by p53

Previously, the TGFα promoter was classified as a TATA-less promoter. However, our experiments suggest that efficient transcription of this gene requires a non-consensus TATA motif present approximately 30-bp upstream of the transcription start site. Our data demonstrate that TBP directly binds to this motif in the TGFα gene. Furthermore, transfection studies demonstrated a correlation between the strength of the TATA motifs as a TBP-binding site and its promoter activity.

Transcription suppression by p53 has been observed only with promoters containing a TATA box but no p53 binding sites (4). Our observation that the TGFα promoter is not suppressed by p53 when the p53 binding sites are removed, suggests that the mere presence of a TBP-binding site is not sufficient for the repression. The TGFα promoter also contains an initiator element (32). Presumably, this initiator recruits a TBP-containing protein complex in collaboration with the weak upstream TATA box. We believe that although TBP can directly interact with the TGFα promoter at the -30 bp region, it is in a configuration and/or association with a distinct set of proteins (e.g., TAFs) which distinguishes it from TBP bound to a consensus TATA box. In this model, the p53-interaction domain of TBP may not be exposed to free p53 molecules. Evidence exists in support of multiple forms of TBP-containing complexes (45). Alternatively, TBP may not be involved in transcription from the TGFα promoter in vivo. This is unlikely, however, in light of our results, as well as recent reports indicating that TFIID itself can interact with initiator elements (46, 47).

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SUMMARY

Both during embryonic development and in adult tissues, growth factor genes are subjected to tight transcriptional regulation. Abnormalities in growth factor production could affect a number of cellular functions, including proliferation, cell shape, motility, differentiation and maintainance of mature functions. Our overall goal is to understand how the human TGFα promoter is regulated in response to various environmental cues. In addition to the results discussed in this dissertation, studies currently undertaken in the laboratory include identification and characterization of two novel protein binding sites in the TGFα promoter. Also, a potential binding site for a transcription factor STAT1 has been noted and currently is being investigated in the laboratory. Upon completion, these studies should help us understand a basic design of the TGFα promoter (i.e. cis-acting DNA elements and interacting transcription factor proteins). In turn, by altering these specific protein-protein and protein-DNA interactions, cells can achieve specific timings and rates of the growth factor transcription.

The studies discussed in this dissertation demonstrate that the TGFα promoter directly interacts with three transcription factors, Sp1, TBP and p53. In addition, we have identified the TGFα initiator that is required for efficient and accurate transcription initiation. The experimental results suggest that a 105 kD protein, TIBP-1, may participate in one or both of these processes by interacting with the initiator.

Sp1

Sp1 is a glutamine-rich transcription factor which binds a consensus sequence CCGCCC (Kadonaga et al., 1986). Binding sites for this transcription factor have been identified in a number of promoters, usually in the vicinity of a transcription initiation site. By locating as such, Sp1 apparently helps interactions between general transcription factors bound near the initiation site and transcription activators which bind distal enhancer elements (Gerber et al., 1994). Genes containing Sp1-binding sites include those expressed only in a limited number of tissues (tissue-specific genes) and those expressed in many different cell types (housekeeping genes). Expression of Sp1 itself is ubiquitous (Saffer et al., 1991). Thus, Sp1 appears to have a general importance in transcription per se but not necessarily reflect a particular phenotype of specific cells.

TGFα is expressed in a number of different cell types (for reviews, see Luetekke et al., 1993; Mann et al., 1993). Sp1 binds at least two sites in the TGFα promoter, and this binding correlates with the promoter activity (Shin et al., 1992). In light of its general transcription role, Sp1 is likely to be responsible for the widespread expression of the growth factor. Cells not expressing TGFα, in turn, may contain an antagonistic activity that can overcome the positive influence of Sp1. Interestingly, we have found that Sp1 DNA-binding activity in HA-A cells is induced by 5-azacytidine, an inhibitor of the DNA methyltransferase. This finding suggests that CpG methylation may negatively control the activity of Sp1. These results, however, should be interpreted carefully since we do not know the nature of induced Sp1 activity. For example, neither Sp1 mRNA nor Sp1 protein increases upon 5-azacytidine treatment, suggesting that yet another gene is under the control of CpG methylation. Conceivably, it is a product of this second gene that regulates

the Sp1 activity. However, it is also possible that 5-azacytidine is affecting other cellular functions besides DNA methylation (Jones, 1985). Further experiments are necessary to rule out this possibility. One such experiment may be to express an antisense RNA to the DNA methyltransferase in HA-A cells, thereby reducing the amount of the unique CpG methylation enzyme (Li et al., 1992) without disturbing other cellular processes. Similar strategies have been succussfully used for a number of systems, including one for the DNA methyltransferase itself (Szyf et al., 1992). Despite a lack of such experiments, however, our observations clearly indicate that the activity of the TGFα promoter is very sensitive to the availability of Sp1.

TBP

In contrast to a previous assessment, our results indicate that the TGFα promoter contain a weak TATA box (Shin and Kudlow, 1994b). First, a motif within the -30 bp region of the promoter can directly interact with a recombinant TBP protein in vitro. Second, a mutant sequence which fails to bind TBP acts poorly in a transfection assay. Although TBP has been previously shown to bind and mediate transcription through various non-consensus sequences (Hahn et al., 1989; Singer et al., 1990), the motif in the TGFα promoter (TTTTTCCCCC) is certainly one of the most divergent.

The sequence tolerence within TBP-binding sites can be explained in part by a special mode of TBP-DNA interaction (Kim et al., 1993a; Kim et al., 1993b). Unlike most other sequence-specific DNA-binding proteins, TBP recognizes its target sequece through a minor groove where nucleotide bases are not readily distinguishable. In this manner, five contiguous A-T base-pairings in the TGFα motif may be quite permissive to an approaching TBP molecule. Nevertheless, the affinity of TFIID to the TGFα motif appears to be somewhat reduced in vivo. This is reflected by an approximately 2.5-fold

gain in the promoter activity by substituting the native motif with a consensus TATA box (Shin and Kudlow, 1994b). Thus, a non-consensus sequence in the TGFα promoter is permissive but not efficient for recruiting TFIID to the promoter region. The inefficient TATA box is, in turn, compensated by the downstream initiator element (Shin and Kudlow, 1994a). Indeed, a mutation in the initiator reduces the promoter activity to a much greater degree than that in the TATA motif itself, indicating that the initiator plays a major role in stabilizing TFIID-DNA interaction.

The initiator and TIBP-1

A 105 kD protein, TIBP-1, specifically interacts with the TGFα initiator (Shin and Kudlow, 1994b). The studies using various mutant TGFα sequence indicates that the promoter activity correlates with the strength of the protein binding. However, whether TIBP-1 is actually important in transcription initiation is not known. Several transcription factors are known to function through various initiators and include TFII-I, E2F and YY1 (for reviews, see Weis and Reinberg, 1992; Kollmer and Farnham, 1993; Smale, 1994). These proteins are required for transcription in vitro using a promoter containing a respective initiator. A proof that TIBP-1 acts as a transcription factor has to come from a molecular cloning of the gene and direct demonstration of its activity in vitro.

Recently, direct interaction between TFIID itself and an initiator was demonstrated (Kaufmann and Smale, 1994; Purnell et al., 1994; Verrijzer et al., 1994). An initiator sequence that is preferred by *Drosophila* TFIID has been determined (Purnell et al., 1994). This sequence resembles not only a loose initiator consensus in fruit fly and human but also a sequence required for accurate transcription initiation determined using human components (PyPy-AN(T/A)PyPy) (Javahery et al., 1994). Thus, one function of an initiator

element is to position TFIID over a transcription start site by directly interacting with it. On the other hand, initiator binding of the above mentioned transcription factors correlates more with the level of transcription than with the accuracy (Javahery et al., 1994). Thus, this class of transcription factors may not necessarily influence initiation site selection but rather simply recruit TFIID to the vicinity of a start site. Interestingly, the TGFα initiator contains a sequence CCACTCG that is similar to the preferred TFIID-binding sequence (Purnell et al., 1994), suggesting that this element, too, interacts with TFIID. Mutations within this 7-bp motif severely reduce the promoter activity (Shin and Kudlow, 1994a). However, mutations outside of the motif also negatively influence transcription levels, suggesting that the initiator element extends beyond this sequence. Thus, the TGFα initiator may also consist of two elements, a transcription factor-binding site and a TFIID contacting sequence.

p53

Only a handful of genes are known to be regulated by p53 (El-Deiry et al., 1993), and even in these cases, a physiological relevance of such regulation is mostly unclear. Given the tumor suppressor activity of p53, it is assumed that genes involved in cell cycle control, DNA synthesis and DNA repair may be the p53-targets. Consistent with this idea, p53 was recently shown to induce transcription of the WAF1/CIP1 gene, a gene encoding an inhibitor of cyclin-dependent kinases (Cdks) (El-Deiry et al., 1993). Similarly, the gadd45 gene is implicated in DNA repair, and under certain circumstances its mRNA is induced by p53 (Kastan et al., 1992). Other potential targets of p53 include genes encoding a growth factor and a growth factor receptor. Our finding that the TGFα promoter contains a functional p53-binding site is consistent with this notion (Shin and Kudlow, 1994b). We have also noticed a potential

p53-binding site in a promoter region of the *neu* gene, a gene encoding an EGFR-related tyrosine kinase receptor (for recent reviews, see Luttrell et al., 1994; Qian et al., 1994). Although the physiological importance of these findings is currently unknown, TGFα and the Neu receptor are implicated in normal development as well as neoplastic transformation of mammary gland. Loss of p53 is frequently observed in breast cancer and may result in altered expression of the critical growth factor and growth factor receptor. A similar situation exists in the skin tissues, where p53 may induce TGFα transcription upon injuries induced by ultra-violet radiation (Vassar and Fuchs, 1991; Dulic et al., 1994).

Recently, we have generated transgenic mice, which express bacterial β-galactosidase protein under the control of a 3.5 kb human TGFα promoter. In this transgenic mouse line, expression of the marker protein appears to well correlate with that of endogenous TGFα, indicating that this promoter contains sufficient information for tissue-specific expression. By genetically crossing these TGFα-β-galactosidase mice with animals made deficient for p53 (Donehoewer et al., 1992), we can directly assess the importance of p53 in regulation of TGFα synthesis. This experiment would prove if our findings with cultured cells are indeed occurring in living organisms. Moreover, it should help understanding interactions between the tumor suppressor gene and the growth factor gene during development, as well as tumorigenesis.

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