The transforming growth factor βs (TGF-βs) are a group of multifunctional growth factors that inhibit cell cycle progression in many cell types. The TGF-β-induced cell cycle arrest has been partially attributed to the regulatory effects of TGF-β on both the levels and activities of the G1 cyclins and their cyclin-dependent kinase partners. The ability of TGF-β to inhibit the activity of these kinase complexes derives in part from its regulatory effects on the cyclin-dependent kinase inhibitors, p21/WAF1/Cip1, p27kip1, and p15. Upon treatment of cells with TGF-β, these three inhibitors bind to and block the activities of specific cyclin-cyclin-dependent kinase complexes to cause cell cycle arrest. Little is known, however, on the mechanism through which TGF-β activates these cyclin-dependent kinase inhibitors. In the case of p21, TGF-β treatment leads to an increase in p21 mRNA. This increase in p21 mRNA is partly due to transcriptional activation of the p21 promoter by TGF-β. To further define the signaling pathways through which TGF-β induces p21, we have performed a detailed functional analysis on the p21 promoter. Through both deletion and mutation analysis of the p21 promoter, we have defined a 10-base pair sequence that is required for the activation of the p21 promoter by TGF-β. In addition, this sequence is sufficient to drive TGF-β-mediated transcription from a previously nonresponsive promoter. Preliminary gel shift assays demonstrate that this TGF-β responsive element binds specifically to several proteins in vitro. Two of these proteins are the transcription factors Sp-1 and Sp-3. These studies represent the initial steps toward defining the signaling pathways involved in TGF-β-mediated transcriptional activation of p21.

The transforming growth factor βs (TGF-βs),1 a group of protein hormones that regulate many cellular functions, inhibit cell proliferation by causing growth arrest in the G1 phase of the cell cycle (1–4). Progression through G1 is dependent on the sequential formation, activation, and subsequent inactivation of cyclin-cyclin-dependent kinase complexes, primarily cyclin D-cyclin-dependent kinase 4 and cyclin E-cyclin-dependent kinase 2 complexes (5, 6). The TGF-β-induced G1 cell cycle arrest has been attributed to the regulatory effects of TGF-β on both the levels and activities of these G1 cyclins and cyclin-dependent kinases (7–9). The inhibition of G1 cyclin-cyclin-dependent kinase complex activity by TGF-β is mediated in part through several members of a recently described family of low molecular weight cyclin dependent kinase inhibitors. These cyclin-dependent kinase inhibitors, which include p21/WAF1/Cip1, p27kip1, p57kip2, p16, p15, and p17, physically associate with their target cyclins, cyclin-dependent kinases, or cyclin-cyclin-dependent kinase complexes to inhibit their activities (reviewed in Refs. 10–13). TGF-β regulates the activities of three of these cyclin-dependent kinase inhibitor family members: p27kip1, p15, and p21 (reviewed in Refs. 10 and 11).

p21 was first cloned and characterized as an important effector that acts to block cyclin E-cyclin-dependent kinase 2 complex kinase activity in p53-mediated cell cycle arrest induced by DNA damage (14–21). p21 has subsequently been shown to be induced by TGF-β (22–24) and has been implicated as an effector of the TGF-β growth inhibitory signaling pathway (22). The increase in p21 seen on TGF-β treatment is mediated, in part, through a transcriptional activation of the p21 promoter by TGF-β via a p53-independent mechanism (22).

Although great strides have been made in understanding the mechanisms through which TGF-β inhibits the G1 cyclin-cyclin-dependent kinase complexes, the signaling pathways between the TGF-β receptor complex and these downstream targets remain unknown. The finding that TGF-β transcriptionally activates the p21 promoter opens an opportunity to study these signaling pathways. We describe here a detailed functional analysis of the p21 promoter as a first step in defining the pathway through which TGF-β activates p21 expression and causes cell cycle arrest.

**MATERIALS AND METHODS**

Tissue Culture—HaCaT cells were the gift of Dr. Baukamp and Dr. Fusenig (25). HaCaT were maintained in 10% fetal bovine serum (Life Technologies, Inc.) in α-MEM (Life Technologies, Inc.)

Creation of p21 Promoter Reporter Constructs—The human p21 promoter construct, WWP-Iuc, was a gift of Dr. Bert Vogelstein (15). The 2.4-kilobase pair genomic fragment containing the p21 cDNA start site at its 3′ end was subcloned into the HindIII site of the luciferase reporter vector, pGL2-basic (Promega), to create p21P. To create p21PΔp53, p21P was digested with ScaI and religated to remove approximately 250 bases from the 5′ end of the p21 promoter, including a p53 consensus site (15). The deletion panel p21PΔ400 to p21PΔ2.3 was created using the exonuclease III based system, Erase-a-Base (Promega). The extent of 5′ deletion was determined by restriction site and sequence analysis. p21PSma1 was created by digesting p21P with Smal and religation, p21PSma5 was created by cloning the 50-base pair Sma fragment of the p21 promoter into p21PSma1, as shown in Fig. 1. p21PSma5Δ was created by performing a Sma partial digest of p21P and religating. To create p21P 93-S, complementary oligonucleotides corresponding to the sequence between –49 and –93 of the p21 promoter, relative to the transcriptional initiation site (15), were synthesized, annealed, and cloned into the EcoRV site in the cloning vector pBSK (Stratagene). The resulting construct was digested with Smal, and the 50-base pair Sma fragment was then subcloned into

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1 The abbreviations used are: TGF-β, transforming growth factor β; EMSA, electrophoretic mobility shift assay; TpRE, TGF-β responsive element; RCE, Rb control elements.
p21Psmal1.

Mutagenesis of p21 P 93-S was performed using the Muta-Gen M13 In Vitro mutagenesis kit (Bio-Rad). The oligonucleotides used in the mutagenesis reactions are as follows: p21P 93-S mut#1, CCGCGCCGCGTCAAGGATCCGGGACC; p21P 93-S mut#2, CCGCGCCGCGTCAAGGATCCGGGACC; p21P 93-S mut#3, CCGCGCCGCGTCAAGGATCCGGGACC; p21P 93-S mut#4, CCGCGCCGCGTCAAGGATCCGGGACC; p21P 93-S mut#5, CCGCGCCGCGTCAAGGATCCGGGACC; p21P 93-S mut#6, CCGCGCCGCGTCAAGGATCCGGGACC.

Electrophoretic Mobility Shift Assays—Complementary oligonucleotides representing the sequences in the p21 promoter were synthesized and annealed into pGL2-basic. The number and orientation of the inserts was determined by sequence analysis. The DNA construct P1634, which contains a consensus TATA box was chosen as a gift from Dr. S. Smale (26). The consensus TATA and INR sequences contained on a BglII–HindII fragment were subcloned into pGL2-basic to create pGL2-T 1. The resulting construct was subcloned into pGL2-basic. The number and orientation of the inserts was determined by sequence analysis.

Luciferase Assay—HaCaT cells were plated into 6-well plates at a density of 200,000 cells/well in α-MEM and 10% fetal bovine serum and grown overnight. Cells were transfected with 6 μg of plasmid reporter construct using a standard DEAE-dextran transfection protocol (22). h after transfection cells were incubated in α-MEM and 10% fetal bovine serum in the presence or absence of 100 pm TGF-β1 for 20 h. Cells were lysed, and luciferase activity in the lysates was assayed by incubating the lysate with a Berthold luminometer. The luciferase activities were normalized based on protein concentrations.

RESULTS

Deletion Analysis of the p21 Promoter—The first step in defining the signaling pathway through which TGF-β induces the expression of p21 is to determine the regions of the p21 promoter responsible for the transcriptional activation by TGF-β. To this end, a series of progressive 5′ promoter deletion mutants, spaced at 300–400 base pairs, of the p21 promoter were created (Fig. 1). One additional deletion construct was created, p21Psmal1, which contains only 61 base pairs proximal to the transcriptional initiation site. These constructs were transfected into HaCaT, a human keratinocyte cell line that up-regulates p21 and is growth inhibited in response to TGF-β (22), and TGF-β-induced luciferase activity was measured. The
Functional Analysis of the p21 Promoter

Fig. 2. Mutation analysis of the p21 promoter. Mutants #6–#11 are identical to the wild type p21P 93-S sequence with the exception of the sequences shown for each mutant construct. p21P 93-S mut#2 and mut#3 are identical to the wild type p21P 93-S with the exception of the underlined bases. These constructs were transfected into HaCaT and TGF-β1-induced luciferase activity was measured. Luciferase activity was normalized to protein concentration. Fold induction was calculated by comparing the luciferase activity of cells treated with TGF-β1 and untreated controls. Transfection were done in duplicate in each experiment, and these results are those of a representative experiment. The region of the p21 promoter found to be necessary for induction by TGF-β1 has been underlined and labeled TβRE. Several putative Sp-1 sites are also indicated.

full-length promoter construct, p21P, was activated 10-fold by TGF-β1, whereas the minimal promoter construct, p21P 93-S, was not induced by TGF-β1. When the panel of deletion mutants were assayed, the region of the promoter responsible for induction by TGF-β1 was determined to be in a 150-base pair region near the transcriptional initiation site (Fig. 1).

Using two convenient Smal sites in this region, the deletion construct p21P 93-S was created, which contains the p21 promoter sequences from base −111 through the transcriptional initiation site (Fig. 1). This construct was activated by TGF-β1 to a level similar to that of the full-length p21P, further defining the TGF-β1 responsive element to a 50-base pair region between −62 and −111. In addition to decreasing TGF-β1-mediated activation of transcription, removal of this 50 base pair region also decreases basal promoter activity. When this 50-base pair region was removed from the full-length p21P promoter, the TGF-β1-induced luciferase activity of the resulting construct, p21P 93-S mut#1, was significantly less than that of the wild type promoter (Fig. 1). These results suggest that the 2.3-kilobase pair S′ end of the p21 promoter proximal to the Smal site at −111 is not required nor sufficient for induction of the promoter by TGF-β1. Finally, the construct p21P 93-S, which contains the promoter sequences between −62 and −93, was activated by TGF-β1 to a level similar to that of the wild type promoter (Fig. 2). Thus, the TGF-β1 responsive element is harbored in a 32-base pair region between −93 and −62.

Mutational Analysis of the p21 Promoter—To precisely define the regions of the p21 promoter necessary for induction by TGF-β1, a serial site-directed mutagenesis of the p21 promoter between bases −93 and −34 was performed. Six mutant promoter constructs were created, each containing 10 consecutive mutated bases (Fig. 2). These mutants, which spanned the 60-base pair stretch between −93 and −34 in the promoter construct p21P 93-S (Fig. 2), were transfected into HaCaT, and TGF-β1-induced luciferase was activity measured. All the mutant constructs p21P 93-S mut#4–#6 had a decreased basal promoter activity in the absence of TGF-β1, p21P 93-S mut#8, mut#10, and mut#11, however, retained the ability to be induced by TGF-β1 in a manner similar to that of the wild type promoter construct p21P 93-S. Only one of these constructs, p21P 93-S mut#2, had completely lost the ability to be activated by TGF-β1 (Fig. 2). p21P 93-S mut#4 had a reduced ability to be activated by TGF-β1, but it was consistently induced 2.5–3.5-fold on TGF-β1 treatment. From these studies we concluded that the sequences of the p21 promoter between −74 and −83 are essential for activation by TGF-β1. The promoter sequence between −54 and −63 may also play an important role in induction by TGF-β1, because mutation of this region decreases both the ability of the promoter to be induced by TGF-β1 and basal promoter activity. The effect of mutations in this region, however, is less dramatic than the mutation of the sequences between −74 and −83. For this reason, further studies were performed on the sequences between −74 and −83.

To more precisely map this specific region of the p21 promoter, two additional mutant constructs were created. p21P 93-S mut#2.2 has a mutation of bases −78 and −79 from CC to AG, and p21P 93-S mut#2.3 has a mutation of bases −76 and −77 from CT to GG (Fig. 2). These two mutant constructs have a significantly reduced ability to be activated by TGF-β1 (Fig. 2). Not only do these mutations provide insight into the bases that are essential in binding the factors driving TGF-β1-mediated transcription, but they serve as a useful control in the electrophoretic mobility shift assays (EMSAs) studies described below.

The Sequences Between −71 and −86 Are Sufficient to Mediate TGF-β1-Induced Transcription—To determine if the region of p21 promoter defined above is sufficient for induction by TGF-β1, the sequences between −71 and −86 were used in an attempt to confer TGF-β1 inducibility to a nonresponsive promoter. A luciferase construct containing a consensus TATA box and initiator sequence, pGL2T+1, was nonresponsive to TGF-β1 (Fig. 3A). However, when one, two, or four copies of the p21 promoter sequence between −71 and −86 were inserted 5′ of the TATA box, this promoter became responsive to TGF-β1 (Fig. 3B). Thus, the sequence of this region of the p21 promoter is sufficient to confer TGF-β1 responsiveness to a previously nonresponsive promoter. This region has been termed a TGF-β1 responsive element (TβRE). Notably, the presence of this element also significantly increase the basal transcription activity, further supporting the conclusion from mutational analysis.
of the p21 promoter that the transcription factor(s) binding to this site may be involved in both TGF-β-mediated transcription and basal promoter activity (Fig. 2).

Initial Characterization of Proteins That Interact with the TβRE—To determine if any proteins could be detected that specifically interact with the TβRE sequence, EMSAs were performed with the wild type TβRE sequence and the mutant TβREs created in p21P 93-S mut2.2 and p21P 93-S mut2.3. Nuclear extracts were prepared from both TGF-β-treated and untreated HaCaT cells. Gel shift assays performed with these extracts and 32P end-labeled wild type TβRE probe revealed three specific bands of retarded mobility, TβRE-1, TβRE-2, and TβRE-3 (Fig. 4A). The binding of these retarded proteins could be competed away by an excess of unlabeled TβRE DNA (Fig. 4A). EMSA was next performed with mutant TβREs, which have dramatically reduced ability of being activated by TGF-β. A competition analysis of labeled wild type TβRE with an excess of unlabeled mutant TβREs revealed that the mutant TβREs were unable to compete for the binding of the retarded proteins (Fig. 4A). In addition, when the mutant TβREs were labeled and used in a gel shift analysis, no specific bands were seen (Fig. 4B). These analyses also revealed no change in the pattern or the intensity of the retarded bands when nuclear extract from either TGF-β-treated or untreated cells was used.

Sp-1 and Sp-3 Bind to the TβRE—As shown in Fig. 2, the region of the p21 promoter that is necessary and sufficient for induction of the p21 promoter by TGF-β contains a nearly consensus binding site for the transcription factor, Sp-1. To determine if any of the retarded bands on EMSA represent the binding of Sp-1 or Sp-3, a member of the Sp-1 family, gel shift assays were performed in the presence of specific anti-Sp-1 or anti-Sp-3 antibodies. These studies revealed that the retarded band, TβRE-1, consists of two distinct shifted complexes and is represented by the binding of Sp-1 and Sp-3, respectively. In the presence of Sp-1-specific antibodies, the top band in the TβRE-1 complex is supershifted, whereas the lower band is supershifted in the presence of Sp-3-specific antibodies (Fig. 4C). Furthermore, Sp-3-specific antibodies eliminated the binding of the TβRE-2 complex (Fig. 4C). This suggests that the lower bands of the TβRE-1 complex and the TβRE-2 complex represent the binding of proteins antigenically related to Sp-3. These possibly represent Sp-3 and a recently described alternatively translated product of the full-length Sp-3 (28). The TβRE-3 complex was not affected by Sp-1- or Sp-3-specific antibodies.

DISCUSSION

To investigate the mechanism through which TGF-β induces the expression of p21, we have performed a detailed functional analysis of the p21 promoter. Through both deletion and mutation analysis, we have defined the sequences required for TGF-β-activated transcription and demonstrated that the sequences could confer TGF-β inducibility to a minimal basal promoter. We have termed this sequence a TβRE, TGF-β-responsive element.

EMSA with the wild type and mutant TβRE sequences and nuclear extracts from HaCaT revealed three specific bands of retarded mobility. The presence of these three retarded bands is correlated functionally with the ability of the TβRE to drive TGF-β-mediated transcription. No change in the pattern or
intensity of the retarded bands was observed when nuclear extract from TGF-β-treated HaCaT was used. Thus, TGF-β is not activating transcription by increasing the binding of these proteins to the TβRE sequence. Consistent with this observation, the induction of p21 mRNA by TGF-β was not blocked by pretreatment of cells with cyclohexamide, a protein synthesis inhibitor (data not shown). Therefore, the factors responsible for the induction of p21 by TGF-β are likely to be pre-existing. These factors may become phosphorylated or interact with proteins that are modified or phosphorylated upon TGF-β treatment to activate transcription.

Because the TGF-β-responsive site in the p21 promoter contains a nearly consensus Sp-1 binding site, we investigated whether the retarded bands on EMSA represent the binding of the transcription factor Sp-1 or other Sp-1 family members. Using Sp-1- and Sp-3-specific polyclonal antibodies, we were able to show that both Sp-1 and Sp-3 are capable of binding to the p21 TβRE sequence. This result alone does not demonstrate that Sp-1 and its family members are involved in TGF-β-mediated signal transduction. Other unidentified proteins, capable of binding to this sequence, may be responsible for TGF-β-activated transcription. An important role for Sp-1, however, is supported by our recent finding that TGF-β activates transcription from the promoter of the p15 gene, another TGF-β-activated cyclin-dependent kinase inhibitor, through a consensus Sp-1 site (36).2 In a previous study of the mouse α2 collagen promoter, an Sp-1 site was also found to be a critical component of the TGF-β-inducible element (29). Taken together, these studies suggest that a subset of TGF-β-inducible genes may contain a similar TGF-β responsive element that may be activated by TGF-β through Sp-1 or Sp-1-like factors capable of recognizing an Sp-1 consensus site.

Although the functionally defined TβRE is capable of activating transcription by itself, the region of the p21 promoter between bases −54 and −63, which contains an Sp-1 binding site, also appears to play an important role in TGF-β-mediated transcription. Mutations in this site reduce the TGF-β-mediated induction of the p21 promoter by half, in contrast to mutations in the TβRE Sp-1 site, which eliminates TGF-β-mediated induction. This suggests that these Sp-1 sites are functionally different. This may be due to differences in their spacing from the transcriptional initiation site or due to subtle base pair differences in their sequence.

Sp-1 binding sites have been described in many promoters, and consequently Sp-1 has fallen into the stereotype of being a “house keeping” transcription factor, whose activity is necessary solely for the basal transcription of many genes. This simple model for Sp-1 was complicated by the identification of a number of Sp-1-related proteins (28,30–33). All of these Sp-1 family members are capable of interacting with a consensus Sp-1 site. Several family members are expressed in a cell type-specific manner (31–33). These findings suggest that the presence of an Sp-1 binding site is not simply to ensure adequate

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TβRE DNA probe corresponding to regions −71 to −86 of the wild type p21 promoter sequence. In each panel an increasing amount of unlabeled specific competitor was used corresponding to the sequences between −71 and −86 of the wild type p21 promoter, p21P93-S mut#2.2, or p21P93-S mut#2.3. B, DNA probes corresponding to the bases −71 through −86 of the wild type p21 promoter, p21P93-S mut#2.2, and p21P93-S mut#2.3 were end-labeled and used in an EMSA with both TGF-β-treated and untreated HaCaT nuclear extract. Each EMSA was performed in the presence and the absence of a 100-fold excess of unlabeled DNA probe as a specific competitor. C, EMSA was performed using the wild type TβRE probe as in A with the inclusion of polyclonal antibodies to either Sp-1, Sp-3, or preimmune serum.
basal transcription but may serve as a site for the interplay of several differentially expressed transcription factors.

Perhaps the most suggestive work demonstrating that Sp-1 may play an important role in regulated transcription is the demonstration that Sp-1 is critical in a factor regulating transcription mediated by the retinoblastoma protein, Rb. Several groups have shown that Rb overexpression can lead to the activation of a number of genes including c-fos and TGF-β1 (27, 34, 35). The Rb control elements (RCEs) in these promoters have been defined and found to interact with Sp-1 (27, 34, 35). Although no direct association between Rb and Sp-1 could be detected and no specific regulation through protein association or phosphorylation has been observed, the effect of Rb overexpression on this element is believed to be through Sp-1. Experiments have shown that the binding of Sp-1 alone is both necessary and sufficient for the ability of these Rb-responsive promoters to be activated by Rb (35).

A potential connection may be made between the TGF-β-responsive element defined here and the previously described RCE. Like the RCE, the p21 TβRE is capable of binding Sp-1. The p21 TβRE, therefore, may represent an RCE. By maintaining Rb in an hypophosphorylated state, TGF-β may be exerting its effects on the p21 promoter through Rb. Induction of p21 would in turn lead to a further increase in the hypophosphorylated form of Rb, thus establishing a positive feedback loop between p21 and Rb, ensuring an effective G1 cell cycle arrest. It should be noted that although the model of positive feedback is attractive, it remains to be determined if the TβRE in the p21 promoter can function as the previously described RCEs.

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