Transcriptional Regulation by p53 through Intrinsic DNA/Chromatin Binding and Site-Directed Cofactor Recruitment

Joaquin M. Espinosa and Beverly M. Emerson
Regulatory Biology Laboratory
The Salk Institute for Biological Studies
10010 North Torrey Pines Road
La Jolla, California 92037

Summary
The tumor suppressor protein, p53, plays a critical role in mediating cellular response to stress signals by regulating genes involved in cell cycle arrest and apoptosis. p53 is believed to be inactive for DNA binding unless its C terminus is modified or structurally altered. We show that unmodified p53 actively binds to two sites at −1.4 and −2.3 kb within the chromatin-assembled p21 promoter and requires the C terminus and the histone acetyltransferase, p300, for transcription. Acetylation of the C terminus by p300 is not necessary for binding or promoter activation. Instead, p300 acetylates p53-bound nucleosomes in the p21 promoter with spreading to the TATA box. Thus, p53 is an active DNA and chromatin binding protein that may selectively regulate its target genes by recruitment of specific cofactors to structurally distinct binding sites.

Introduction
The p53 tumor suppressor gene is the most frequent target for genetic alterations in cancer, with mutations occurring in approximately 50% of all human tumors. The importance of p53 in cancer prevention results from its ability to regulate such critical processes as cell cycle progression and apoptosis (recent reviews Levine, 1997; Prives and Hall, 1998; Vogelstein et al., 2000). p53 is a sequence-specific DNA binding protein that has been shown to activate transcription of a number of target genes, including p21, Mdm2, GADD45, Bax, and cyclin G. Induction of p21 results in cell cycle arrest in response to DNA damage by inhibiting cyclin-dependent kinase activity (El-Deiry et al., 1993; Xiong et al., 1993). Activation of Bax and other genes by p53 promotes apoptosis (Miyashita and Reed, 1995). The regulation of cell cycle arrest and apoptosis by p53 are mechanistically distinct processes (Attardi et al., 1996). Recently, oligonucleotide microarray analysis has identified a broad spectrum of genes that are controlled by p53 in a positive or negative manner, and whose functions fall into categories that reflect the role of p53 as an integrator of diverse cellular signals (Zhao et al., 2000).

The 393 amino acid p53 protein consists of two N-terminal activation domains (amino acids 1–42; 42–83), a sequence-specific DNA binding domain (102–292), and a C-terminal oligomerization domain (324–355). The DNA binding activity of p53 is thought to be under negative constitutive regulation through two regions within its inhibitory C-terminal domain (290–325 and 356–393) (Hupp et al., 1992). This inhibition can be relieved by acetylation, phosphorylation, or protease cleavage. After DNA damage, p53 is phosphorylated and acetylated at a number of sites within its N- and C-termini. Phosphorylation within the N-terminal activation domain most likely affects its interactions with Mdm2, which controls p53 stability, and components of the transcription initiation machinery (Prives, 1998). Acetylation of p53 by the histone acetyltransferases CBP/p300 and PCAF activates DNA binding in vitro and each HAT can coactivate p53-dependent transcription in transient expression experiments (Gu and Roeder, 1997; Avantaggiati et al., 1997; Lill et al., 1997; Scolnick et al., 1997; Liu et al., 1999).

Although a wealth of information exists concerning p53, little is known about the actual mechanism by which this critical tumor suppressor protein directly interacts with its target genes and regulates their expression. We have begun to address these issues by developing a p53-dependent in vitro transcription system using chromatin-assembled p21 promoter-driven genes. The p21 gene is a natural and direct target of p53 and is regulated in an inducible (El-Deiry et al., 1993) and a constitutive basal level manner (Tang et al., 1998) through two consensus p53 promoter binding sites at −2.3 kb (5′) and −1.4 kb (3′). The p53 activation domain interacts with CBP/p300, and both CBP/p300 and PCAF increase the ability of p53 to activate p21 gene expression in vivo (Scolnick et al., 1997).

Using the natural p21 promoter, we show that p53 functions synergistically with p300 to activate transcription through chromatin from a distance of at least 1.4 kb. Interestingly, p300 mediates p53-dependent transcription without increasing p53 binding to the chromatin template. Instead, p53 associates with its nucleosomal target sites in the absence of chromatin remodeling or modifying complexes. Chromatin-bound p53 then recruits p300 to the p21 promoter, resulting in localized nucleosome acetylation with regional spreading to the TATA box. p300 mediates p21 gene expression by p53-targeted nucleosomal acetylation rather than through p53 acetylation, which does not affect its transcriptional activity in vitro.

In contrast with current views, we find that p53 is not a latent but an active DNA binding protein that does not require modification of the C-terminal domain by acetylation or antibody binding to interact with either DNA or chromatin. Our studies indicate that the C terminus is not inhibitory for p53 binding and is, in fact, required for p53 association with particular target sites. p53 interacts with distinct types of nucleosomal binding sites within the p21 promoter, as shown by different behaviors upon response to C terminus perturbations. In addition, p53 associates with chromatin at a higher affinity than with DNA in the absence of cofactors or protein modifications. Thus, transcriptional regulation by p53 may be a complex property of chromatin structure, DNA topology, and recruitment of specific cofactors to allosterically-regulated binding sites.
Synergistic Transcriptional Activation of the Chromatin-Assembled p21 Promoter by p53 and p300

We expressed full-length human p53 and full-length human p300 in insect cells using the baculovirus expression system. Flag-tagged p53 and His-tagged p300 were purified by affinity chromatography to apparent homogeneity (Figure 1A). The full-length size of recombinant p300 was confirmed by comparative Western blot, using native p300 as control (Figure 1B). When p300 acetyltransferase activity was assayed using core histones and p53 as substrates, p300 efficiently acetylated p53 and histones H3 and H4 in solution and histones H2A and H2B to a lesser extent (Figure 1C). This experiment shows that full-length p300 can acetylate p53, as previously reported for its histone acetyltransferase (HAT) domain alone (Gu and Roeder, 1997).

The p53 protein obtained from insect cells was mainly inactive for specific DNA binding activity when measured by EMSA using a 25 bp oligonucleotide harboring the 5′ site in the p21 promoter. In agreement with previous experiments using only the catalytic domain of p300, the DNA binding activity of p53 was dramatically improved by acetylation using full-length p300 (Figure 1D). The efficiency of activation by p300-mediated acetylation was comparable to that observed with other known activating stimuli, such as binding of the antibody PAb421 to the C-terminal region of the protein or deletion of the C terminus itself (see Figures 4B and 5B). Our analyses demonstrate that the p53 protein produced in insect cells is mainly nonacetylated, an observation that is further supported by Western blot assays using antibodies that specifically recognize the acetylated form of p53 (see Figure 3C). This experiment also suggests that other modifications previously reported to activate p53 DNA binding activity are absent in these preparations.

To analyze the mechanism of p53-dependent transcriptional activation of chromatin-assembled p21 promoters, we used a DNA construct containing a 2.4 kb fragment of the human p21 promoter. This includes two binding sites for p53, which are centered around positions −2270 (5′ site) and −1380 (3′ site), respectively (Figure 2A). This promoter fragment drives the transcription of a luciferase reporter gene (WWP-LUC or p21-LUC). This 8 kb plasmid has been extensively used to study p53-dependent transcription of the p21 promoter by transient expression experiments, and the requirement of at least one of these two binding sites for p53-dependent transactivation has been amply demonstrated (El-Deiry et al., 1995).

Chromatin assembly of this template DNA was performed using Drosophila embryo cell-free extracts and purified core histones as previously described (Bulger and Kadonaga, 1994). The quality of the reconstituted chromatin was assessed by transcriptional repression and structural analysis by micrococcal nuclease digestion (Figure 2C, lanes 5–6, and Figure 2D). The micrococcal nuclease digestion pattern of the in vitro reconstituted chromatin showed the presence of physiologically spaced nucleosomes and was correlated with a complete repression of p21 promoter activity.
Figure 2. p53 and p300 Can Direct Synergistic Transcriptional Activation from Chromatin-Assembled p21 Promoters

(A) Schematic diagram of the p21 promoter. Relative positions to the transcription initiation site and sequences of the p53 binding sites are indicated.

(B) Schematic representation of the in vitro chromatin assembly and transcription protocol.

(C) p53-dependent transcription of chromatin-assembled p21 promoters by addition of p53/H11006 p300/H9251-globin is shown as an internal control.

(D) Analysis of p21-LUC chromatin structure by micrococcal nuclease digestion.

The p21-LUC chromatin template was subjected to in vitro transcription reactions using HeLa cell nuclear extracts as a source of basal initiation components in the presence or absence of p53 and/or p300, as described in the protocol depicted in Figure 2B. Both factors were added after chromatin assembly was complete ("post assembly") and incubated with the template prior to in vitro transcription. Addition of p53 alone produced very modest transcriptional activation (Figure 2C, lane 7). However, coincubation with p300 gave a strong synergistic de-repression of the chromatin template (lane 8). Neither p53 nor p300 appreciably influenced transcription of naked DNA templates (lanes 1–4). Identical results were obtained using extracts from another cell line, erythroid K562 (data not shown). Thus, transcriptional activation from the chromatin-assembled p21 promoter in vitro requires p53, acting at a distance of at least 1.38 kb, in combination with a histone acetyl transferase cofactor, p300. Interestingly, other putative cofactors, such as PCAF and human SWI/SNF, do not facilitate p53-dependent transcription in these assays even though they both interact with p53 (our unpublished data).

p300 Can Mediate p53-Dependent Transcription without Increasing p53 Binding to the Chromatin Template

To analyze the mechanism by which p300 mediates p53-dependent activation, we performed DNase I chromatin
Molecular Cell 60

Figure 3. p300 Mediates p53-Dependent Transcription without Enhancing Its Interaction with Chromatin

After incubation of chromatin with p53 and p300, the sample was split into three aliquots and tested in separate assays:

(A) DNase I chromatin footprinting of the 5' and 3' binding sites in the p21 promoter. Bars at the left indicate the position of the corresponding binding sites. Bars at the right indicate regions of protection (+) or hypersensitivity (−) to DNase I digestion.

(B) In vitro transcription of p21 chromatin with p53 and p300.

(C) Western blot analysis of chromatin samples containing p53 and p300. Membranes were probed with antibodies against anti-acetyl p53 (top) or p53 N-terminal domain (bottom).

footprinting assays to examine the occupancy of the 5' and 3' sites of the p21 promoter under conditions of transcriptional coactivation. Chromatin assembly and factor incubation steps were performed as described for Figure 2 but the samples were split into three aliquots just before the in vitro transcription reaction. One aliquot was subjected to DNase I digestion, the second was transcribed, and the third was analyzed by Western blot to determine the acetylation status of p53. Surprisingly, p53 alone was able to generate a clear footprinting pattern at both the 5' and 3' binding sites within the p21 promoter (Figure 3A, lanes 2 and 6). Addition of p300 did not significantly change the footprinting pattern produced by p53, even though it clearly enhanced transcription from the p21 promoter (Figure 3B, lane 4). In a parallel experiment using the same proteins and reagents, p300 clearly activated p53 DNA binding activity in EMSA (data not shown, similar to Figure 1D).

The acetylation status of both untreated and previously acetylated p53 was preserved during incubation with the chromatin template as determined by Western blot analyses using antibodies that specifically recognize only the acetylated form of p53 (Figure 3C). These antibodies did not recognize native p53, whereas the p300-acetylated p53 isoform gave a strong signal. Thus, native p53 remained unacetylated, and previously acetylated p53 was not deacetylated during the chromatin assembly incubation. We conclude from this experiment that p300 can mediate p53 transactivation without affecting the ability of p53 to bind to its DNA recognition sequences in chromatin.

These observations raised some interesting questions about the coactivation mechanism of p300 and the DNA/chromatin binding properties of p53. For example, is unmodified p53 able to interact with its chromatin binding sites as well as the acetylated isoform in contrast to what is observed in EMSA for short pieces of naked DNA? Can p53 interact with its chromatin binding sites on its own or does it require chromatin modifying or remodeling activities present in the assembly extract?

p53 Does Not Require Acetylation or Remodeling Complexes to Interact with Chromatin

To answer these questions, we performed footprinting experiments with chromatin-assembled p21 promoters after ATP depletion and purification by gel filtration using nonsaturating amounts of p53. These chromatin samples are devoid of endogenous ATP-dependent nucleosome remodeling activities, small molecules like ATP or acetyl CoA, and the majority of proteins from the chromatin assembly extract (Mizuguchi and Wu, 1999). As shown in Figure 4A, both native and previously acetylated p53 are able to interact with the 5' and 3' binding sites in the purified p21 promoter chromatin with similar affinities.

From this experiment, we draw three main conclusions. First, although native p53 possesses an almost undetectable DNA binding activity when assayed on short oligonucleotides, it binds very efficiently to the same sites in a larger promoter context when assembled into chromatin. Second, whereas acetylation of p53 by p300 dramatically increases its affinity for short DNA
Figure 4. Interaction of p53 with Its Chromatin Binding Sites in the p21 Promoter Is Not Enhanced by Acetylation or by Antibodies Directed against the C-Terminal Domain

Chromatin reactions were depleted of ATP by treatment with apyrase and further purified by gel filtration prior to DNaseI footprinting.

(A) Chromatin templates were incubated with increasing subsaturating concentrations of untreated or previously acetylated p53 (between 1 and 20 nM) and processed as described.

(B) EMSA analysis of different p53 isoforms. The binding of p53 to a 25 bp oligonucleotide (5’ site) was tested with the antibody PAb421. A control with p300-acetylated p53 was also included.

(C) Binding of PAb421 antibody to the C-terminal domain of p53 inhibits its ability to occupy the 3’ p21 promoter chromatin binding site.
duplexes, it does not increase its affinity for the same sequences in p21 promoter chromatin. Third, native p53 interacts efficiently with its nucleosomal binding sites in the absence of chromatin modifying or remodeling activities.

p53 Possesses Distinct Types of Chromatin and DNA Binding Sites

To gain more insight into the interaction of p53 with chromatin, we made use of a different tool commonly employed to activate its dormant DNA binding activity. Previous reports have concluded that the C-terminal domain of p53 inhibits its ability to interact with DNA. Such inhibition can be artificially relieved by binding a monoclonal antibody to the C-terminal domain (Hupp et al., 1992). Interestingly, the binding of the monoclonal antibody PAb241 does not increase the affinity of p53 for all DNA binding sites tested. Moreover, the affinity for some binding sites is significantly decreased, whereas it remains unchanged for others. Similar behavior has been observed for other “activating” modifications, suggesting the existence of different types of DNA binding sites for p53. In this regard, the two p53 recognition sequences in the p21 promoter fall into different categories: binding to the 5’ site is strongly enhanced by treatment with PAb421, whereas binding to the 3’ site is inhibited when measured by EMSA (Resnick-Silverman et al., 1998). Therefore, we decided to test the effect of PAb421 on p53 binding to these two binding sites when assembled into chromatin.

As shown in Figure 4B, the addition of PAb421 to native p53 increases its DNA binding activity for the 5’ site to a similar extent as that observed by p300-dependent acetylation when examined by EMSA. As expected, the protein-DNA complex was supershifted by the presence of the antibody. However, when examined by chromatin footprinting (Figure 4C), treatment with PAb421 did not increase the affinity of p53 for the 5’ site, similar to our previous observations with acetylation by p300 (Figure 4A). Interestingly, PAb421 clearly blocked the binding of p53 to the 3’ site (Figure 4C, bottom). Thus, using chromatin-assembled p21 promoters, the antibody retained its inhibitory effect on p53 interaction with the 3’ site, but it lacked the stimulatory effect on the 5’ site, in agreement with previous reports (Kim et al., 1997; Cain et al., 2000).

This chromatin footprinting analysis allows us to make two major conclusions. First, two different putative activating modifications of native p53, namely acetylation and binding of the antibody PAb421, fail to improve the ability of p53 to interact with the chromatin-assembled p21 promoter. Second, different types of p53 binding sites exist within this promoter as revealed by the selective inhibition of binding to only the 3’ site by PAb421.

The C-Terminal Domain of p53 Is Not Inhibitory and Is Differentially Required for Binding to Distinct DNA Sequences

The observation that two modifications of the C-terminal domain, acetylation and binding of the antibody PAb421, do not enhance the interaction of p53 with the p21 promoter argues against the idea that the C-terminal domain inhibits the DNA binding activity of p53. To gain insight into this issue, we examined a C-terminal deleted form of p53, lacking the last 30 amino acid residues (p53 ΔC30). After production in insect cells and comparative quantification with wild-type p53 (Figure 5A), we tested the binding activity of this protein in both EMSA and DNase I footprinting. As expected according to previous reports, the ΔC30 mutant was strongly active in the EMSA analysis as compared with the acetylated form of wild-type p53 (Figure 5B). By contrast, when the ΔC30 mutant was assayed by DNase I footprinting on p21 promoter DNA or chromatin, it showed a weaker binding to the 5’ site and no binding to the 3’ site (Figure 5C). We conclude that the C-terminal domain is not inhibitory for DNA binding and that it is required for interaction with both the 5’ and 3’ p21 promoter sites (to different extents) when these are present in larger molecules of DNA or chromatin. This is consistent with a functional analysis showing that the C-terminal domain is required for in vitro transcription from the chromatin-assembled p21 promoter (Figure 5D).

The Affinity of p53 for the 5’ p21 Promoter Binding Site Increases as a Function of Target DNA Size

There are many differences between the two DNA binding assays performed in this study, EMSA and chromatin or DNA plasmid footprinting. First, the size of the template DNA is much longer in the footprinting experiments (25 bp for EMSA versus 8 kb for footprinting). Second, protein-DNA binding occurs in solution in footprinting reactions, whereas binding continues inside the polyacrylamide gel in EMSA, which can affect the formation or stability of the complex.

To discriminate between these alternatives, we performed EMSA with native and acetylated p53 using pieces of double-stranded naked DNA of increasing size. Probes were generated that contained a centered 5’ site of the p21 promoter with increasing flanking regions from the same promoter which are devoid of any other p53 binding sites. A typical result is shown in Figure 6A in which binding of p53 to a 25 bp oligonucleotide was increased 12-fold by acetylation, whereas binding to a 160 bp DNA molecule was increased only 1.5-fold. This decrease in the influence of acetylation was due to the fact that unacetylated p53 had increased affinity for the longer piece of DNA. The presence of additional p53 binding sites in the longer molecule is unlikely because we detected only one shifted complex in EMSA and only one protected site within this region in a parallel footprinting experiment (Figure 6B). The lack of effect of acetylation on p53 binding is most apparent when analyzed on the p21 promoter within an 8 kb plasmid. In this case, native and acetylated isoforms of p53 showed indistinguishable affinities for the 5’ binding site as determined by DNase I footprinting (Figure 6B).
Figure 5. The C-Terminal Domain of p53 Is Not Inhibitory and Is Differentially Required for Binding to Distinct DNA Sites

(A) Protein purification of recombinant wild-type (WT) and C-terminal deletion mutant of p53 (∆C30).
(B) EMSA analysis of ∆C30 mutant.
(C) DNase I footprinting comparing the binding of wild-type p53 and ∆C30 to both the 5’ site (top) and 3’ site (bottom) of the p21 promoter as naked DNA (left column) or chromatin (right column).
(D) In vitro transcription of p21 chromatin comparing wild-type p53 versus ∆C30.

These experiments indicate that p53 has very low affinity for its recognition sequences when present in the form of short oligonucleotides, and that the binding improves significantly when the flanking regions are extended. This type of behavior is very suggestive of the presence of secondary structures in the binding sites (Kim et al., 1997). Such structures cannot be adopted by short oligonucleotides, but are firmly stabilized by the addition of adjacent duplex regions. An alternative explanation is the possible requirement of DNA bending at the p53 binding sites for high affinity protein interaction (Nagaich et al., 1999). Interestingly, as the affinity of p53 increases for DNA of a particular length and topology, it becomes indistinguishable from the acetylated form.

p53 Binds with Higher Affinity to Chromatin than to DNA

We next assessed the relative binding affinities of p53 for its two sites within the p21 promoter as DNA or chromatin. The interaction of some DNA binding proteins is impeded by nucleosomes, and specific remodeling complexes are required to facilitate their association (Armstrong et al., 1998; Kadam et al., 2000). To address this issue for p53, we performed extensive protein titrations in parallel experiments using DNA or CL4B-purified chromatin. Surprisingly, these experiments revealed that p53 binds with higher affinity to nucleosomal templates, most significantly at the 5’ site (Figure 6C, lanes 2–10, compare top and bottom). Binding conditions were exactly the same for both DNA and chromatin templates, and protein concentrations were equalized with purified BSA to avoid “protein carrier” effects. DNA concentration was also the same in all reactions as assessed by electrophoresis in agarose gels. Because DNA supercoiling strongly influences p53 binding within the MDM2 promoter (Kim et al., 1999), we examined whether the relaxation introduced by nucleosome assembly could explain the difference in the affinity for naked DNA compared to chromatin. However, after relaxing supercoiled p21-LUC plasmids by treatment with topoisomerase I, no differences in p53 binding to the 5’ or 3’ site were observed between relaxed and supercoiled templates (data not shown).

Quantification of the occupancy of both 5’ and 3’ p21 promoter sites was performed by densitometric analysis of bands marked by asterisks (Figure 6C). This demonstrated a clear difference in the kinetics of p53 binding to DNA or chromatin templates at both sites. Whereas p53 interaction with chromatin increased almost logarithmically linear with protein concentration, binding to DNA showed a clear “threshold” effect with a dramatic change in occupancy within a narrow window of protein concentration. This difference was more apparent for the 3’ site (lanes 13–21). Taken together, these data suggest that the structure of the two p21 promoter bind-
Figure 6. p53 Sequence-Specific DNA Binding Affinity and Kinetics Are Sensitive to Target DNA Size and Chromatin Assembly

(A) EMSA analysis of p53 binding using DNA probes of different size. 25 and 160 bp oligonucleotides containing one centered copy of the p21 promoter 5' site were incubated with untreated or acetylated p53.

(B) DNase I footprinting of p53 on naked DNA. Supercoiled p21-LUC plasmids were incubated with increasing concentrations of untreated or acetylated p53 and processed as described.

(C) p21-LUC plasmid DNA (top row) or CL4B-purified chromatin (bottom row) were used as templates for extensive titrations of wild-type p53 on both the 5' and 3' sites of the p21 promoter. Binding conditions were identical for both templates, and the concentration of DNA in both types of samples was equivalent.

Acetylation of the C-Terminal Domain of p53 Does Not Influence Its Transcriptional Activity

Gene regulation by p53 occurs in discrete stages: first, interaction with its binding sites in chromatinized target promoters; and second, transactivation of the transcriptional machinery. After observing that p300 is required to mediate p53-dependent activation of chromatin-assembled p21 promoters (Figure 2) but does not facilitate the binding of p53 (Figures 3 and 4), we investigated whether acetylation of p53 played an important role at another step in the transcription process. The acetylation pattern of p53 has been previously determined, and mutations of lysine residues that are targeted by p300 have been generated and analyzed elsewhere (Gu and Roeder, 1997; Avantaggiati et al., 1997; Scolnick et al.,...
Transcriptional Regulation of Chromatin by p53

We examined a mutant version of p53 in which lysines 370, 372, 373, 381, and 382 within the C-terminal domain have been replaced by arginine (p53KR, Luo et al., 2000). p53KR was expressed in insect cells, purified, and analyzed for its ability to activate transcription compared with wild-type p53. As shown in Figure 7A, the mutant protein is still coactivated by p300 to a similar extent as wild-type p53 (compare lanes 4 and 6). This indicates that acetylation of the C-terminal domain does not play a major role in either chromatin binding or subsequent steps in the p300-mediated activation process. A Western blot analysis confirms that p53KR is not recognized by antibodies specific to acetylated p53 (Figure 7B). These results are in agreement with in vivo transient expression experiments, which demonstrate that mutations in acetylatable lysines result in only a slight or no decrease in p53-dependent transcription (Scolnick et al., 1997, Nakamura et al., 2000).

p53 Recruits p300 and Directs Nucleosome Acetylation of the p21 Promoter

Our studies indicate that p300 does not coactivate p53 function by either facilitating its interaction with chromatin or increasing its transactivation potential. We, therefore, explored other mechanisms by which p300 may mediate p53-dependent transcription. Particularly whether p53 recruits p300 to acetylate nucleosomal histones within the p21 promoter. For this purpose, we assembled the p21 promoter into chromatin using defined factors instead of Drosophila embryo extracts. This recombinant chromatin assembly system, developed by Kadonaga and colleagues, consists of the ACF complex (dACF/H11001/dISWI) and the histone chaperone NAP-1, which together catalyze the assembly of free histones into nucleosomes in the presence of ATP. This system has been used successfully to obtain regularly spaced nucleosome arrays and allows a higher degree of manipulation compared to the embryo extracts (Ito et al., 1999). For our purposes, use of recombinant factors enabled us to detect de novo acetylation of the chromatin-assembled p21 promoter by incorporation of exogenously added 3H-acetyl CoA. Under the optimal histone:DNA ratio, the majority of histones in the assembly reaction are incorporated into a nucleosomal array. This is critical because it has been demonstrated recently that p300 alone can acetylate free histones but not nucleosomes unless recruited by the activator GAL4-VP16 (Ito et al., 2000).

The quality of p21 promoter chromatin assembled in this purified system was determined by micrococcal nuclease digestion, as for embryo extracts, with similar results (data not shown). The templates were then incu-
bated with p53 and/or p300 in the presence of \(^3\)H-acetyl CoA and the extent of acetylation monitored by incorporation of radioactive activity when analyzed by SDS-PAGE and fluorography (Figure 7C). When both p53 and p300 were present in the reaction, a strong acetylation of all four nucleosomal histones was easily detected (lane 3), whereas very weak acetylation was observed by p300 alone (lane 4). A control reaction with equivalent amounts of free histones and p300 was included (lane 5). Acetylation of p53 and autoacetylation of p300 was also detected (lanes 3–4). This experiment clearly demonstrates that p53 can recruit the HAT activity of its coactivator p300 to the chromatin template and mediate de novo acetylation of nucleosomal histones.

To determine whether p53 influenced the localization of nucleosome acetylation on the p21 promoter by direct recruitment of p300, we performed in vitro chromatin immunoprecipitation assays. p21 promoters were assembled into chromatin using purified components and incubated with p53 and/or p300. Chromatin was digested with micrococcal nuclease and acetylated oligonucleosomes were immunoprecipitated using anti-acetyl-H4 antibodies. Specific p21 plasmid sequences in the immunoprecipitates were detected by PCR amplification (Figure 7D) or Southern slot blot (data not shown). Identical results were obtained from both assays. As expected, the amount of DNA precipitated was greatly enhanced when both p53 and p300 were incubated with the chromatin template (lane 11). The extent of increased acetylation in different regions of the p21-LUC plasmid varied from 3.4-fold near the 3' p53 binding site to 1.4-fold in a distal region located 2 kb downstream of the proximal promoter. Interestingly, nucleosome acetylation also occurs several hundred base pairs from the 3' site to reach the proximal promoter (TATA box). We consistently obtained the following decreasing levels of acetylation: 3' site > TATA box > 5' site >> +2 kb.

These data indicate that in the presence of p53, p300 acetylates nucleosomes in a targeted manner within chromatin-assembled p21 promoter-containing plasmids (lane 11). In the absence of p53, only negligible nucleosome acetylation by p300 is apparent (lane 12). Interestingly, acetylation is highest when p300 is recruited to the promoter proximal 3' p53 binding site and then apparently spreads to encompass the TATA box. Thus, the probable mechanism by which p300 coactivates p53-dependent transcription is by targeted nucleosomal acetylation of the proximal promoter when recruited by bound p53 at a distance of at least 1.4 kb.

**Discussion**

Our studies demonstrate that p53 activates transcription from a natural target promoter, p21, when bound at a distance of at least 1.4 kb in a chromatin environment. Transcriptional activation requires the histone acetyltransferase, p300. Surprisingly, p300 does not function by facilitating p53 binding to its DNA recognition sites within chromatin. Instead, p300 acts at a later step in the transcription process by acetylating nucleosomes within the proximal and distal p21 promoter when targeted by bound p53. This presumably renders the nucleosomes sufficiently fluid to allow interaction with other components of the transcription machinery. p300-mediated transcriptional activation has been described for other chromatin-assembled genes (Kraus et al., 1999; Dilworth et al., 1999). Our experiments demonstrate that a mechanism by which p300 can regulate the activity of natural promoters is by acetylating chromatin over a long-range when recruited by a distal transcription factor. Targeted nucleosome acetylation by chromatin-bound GAL4-VP16 has been described recently (Ito et al., 2000). Previous studies in yeast revealed a localized factor-directed acetylation encompassing 1–2 nucleosomes within the proximal promoters (Rundlett et al., 1998; Struhl et al., 1998). In the absence of p53, p300 cannot acetylate nucleosomes due to lack of template targeting, and the p21 promoter remains inactive. We find that p53 proteins containing mutations in lysine residues acetylated by p300 are as active as wild-type p53 in regulating p21 transcription in vitro. This indicates that acetylation of p53 does not contribute to its transcriptional activation potential, and that p300 does not mediate transcription by this mechanism in our biochemical assays. This conclusion is in agreement with previous in vivo analyses in which p53 mutants lacking these lysine residues did not show a significant decrease in transcriptional activity (Scolnick et al., 1997; Nakamura et al., 2000). However, p53 acetylation may play a role in protein stabilization or subnuclear localization (Nakamura et al., 2000; Pearson et al., 2000).

Numerous studies have focused on the DNA binding properties of p53 and the role of distinct protein domains in this process. This issue is especially germane because the majority of p53 mutations found in human cancers occur within the DNA binding domain. Thus, the inability of p53 to interact with its target genes and regulate transcription would be expected to contribute significantly to cancer development and progression. Previous experiments have led to the conclusion that p53 is a latent DNA binding protein which contains an inhibitory C-terminal domain. Using primarily EMSA analyses, latent DNA binding of p53 has been shown to be activated in several ways: posttranslational modifications of the C terminus, such as acetylation or phosphorylation; association with the monoclonal antibody PAb421; or deletion of the C-terminal domain (reviewed in Prives and Hall, 1999). In contrast to these observations, our experiments demonstrate that unmodified, full-length p53 binds very efficiently to its natural recognition sequences within both DNA and chromatin. This occurs in the absence of p53 modification by acetyl transferases or chromatin disruption by ATP-dependent remodeling complexes. In fact, perturbations of the C-terminal domain reveal the distinct nature of individual p53 binding sites. For example, deletion of the C terminus or association of this domain with PAb421 actually abolishes p53 interaction with the 3' site within the p21 promoter. Conversely, both modifications result in the switch from latent to active DNA binding by p53 when analyzed by EMSA. The discrepancy between p53 binding results obtained with EMSA using short oligonucleotides and DNase footprinting using larger promoter fragments can be resolved by increasing the length of the oligonucleotides so that it presumably adopts a secondary structure to which p53 can stably bind. Importantly, Cain et al. (2000) have shown that modifications of the
N terminus greatly affect p53 binding to the p21 promoter (5′ site) as measured in footprinting assays. This is especially significant because the N terminus is the site of multiple posttranslational modifications and can associate with critical cofactors such as MDM2, TAFs, and TRAPs (Cain et al., 2000 and references therein).

Seminal experiments by Kim and colleagues demonstrated that cruciforms or other non-B-DNA structures are normal recognition elements for p53 (Kim et al., 1997, 1999). This would explain why unmodified p53 fails to bind to a short DNA oligonucleotide in EMSA, because this sequence cannot readily form the secondary structure it would usually adopt in a larger piece of DNA. Thus, modification by acetylation, antibody association, or deletion of the C terminus might be required for p53 to bind to a DNA structure that the native protein normally does not recognize. This interpretation by Kim et al (1997, 1999) is entirely consistent with our results. More recently, it was demonstrated that p53 can be specifically directed to cruciform structures, even though the sequences forming the cruciform do not fit the p53 consensus binding site (Jett et al., 2000). Taken together, these studies emphasize the importance of using DNA recognition sequences within an appropriate context when examining the effect of both C- and N-terminal modifications on p53 binding. Moreover, the results may differ depending upon the specific target gene and p53 recognition sequence used.

It is intriguing that p53 binds to the p21 promoter with higher affinity and with different kinetics when assembled into chromatin than it does to DNA. Particularly because this occurs in the absence of chromatin remodeling or modifying complexes and is not observed with other transcription factors that can also bind to nucleosomes, such as Gal4-VP16 (Pazin et al., 1998). This could be explained if bending of the DNA, when wrapped around a nucleosome, generates a secondary structure that is more stable for p53 binding. Indeed, previous studies determined the importance of DNA bending for p53 high-affinity binding and predicted that some p53 binding sites would be exposed and accessible when incorporated into a nucleosome (Nagaich et al., 1999). Importantly, our findings demonstrate that the structure recognized by p53 in p21 promoter DNA is preserved and improved or stabilized in chromatin. The physiological significance of the distinct kinetics of p53 occupancy observed on the p21 promoter as chromatin or DNA is unclear. The linear rate of association of p53 with chromatin may indicate that lower concentrations of p53 are required to fully occupy binding sites in vivo than the cooperative binding to DNA would indicate. This could be significant if the cell has to respond efficiently to activate p53-responsive pathways without waiting for a critical threshold of p53 concentration to be reached. It should be emphasized, however, that the nature of p53 binding to chromatin and the requirements for remodeling/modifying activities may vary with individual target promoters.

One can surmise that the variable nature of DNA consensus sequences recognized by p53 is a critical regulatory feature, because it enables diverse structures to be generated within target promoters and manipulated by a variety of cellular signals. In fact, the ability of p53 to function as an activator or repressor and differentially recruit cofactors may be determined by the conformation it assumes when bound to DNA/chromatin sites having specific topologies. Such allosteric regulation has been demonstrated for the transcription factor, Pit-1, which can switch from an activator to a repressor by a two base pair change in its binding sites (Scully et al., 2000). For this reason, the use of an optimized consensus DNA sequence for p53 interaction may not be appropriate because it does not represent the physiological complexity of p53 function.

It will be of great interest in the future to examine the distinct mechanisms employed by p53 to choose among its numerous target genes to regulate specific pathways in response to cellular stress.

Experimental Procedures

Plasmids and Expression of Recombinant Proteins

The WWP-LUC (p21-LUC) plasmid was constructed as described in El-Deiry et al. (1995) and was a generous gift of Dr. Bert Vogelstein.

Full-length human Flag-tagged p53 was expressed in Sf9 cells and purified from total cell extracts by affinity to anti-Flag M2 affinity gel (Sigma) according to the protocol used to purify Flag-tagged dACF described by Ito et al. (1999). p53 baculovirus was a gift from Dr. Ingrid Grummt and Dr. Renate Voit. Baculoviruses for expression vector (Bac-to-Bac System, Gibco BRL), and the protein was purified by similar means. Recombinant AC complex (Flag-tagged dACF + untagged dISWI) was produced in Sf9 cells and purified by anti-Flag M2 (Sigma) affinity chromatography as described by Ito et al. (1999). Histidine-tagged NAP-1 was purified from baculovirus-infected Sf9 cells by Ni-NTA (Qiagen) affinity chromatography, followed by a conventional Mono-Q chromatographic step. Full-length human Histidine-tagged p300 was expressed in Sf9 cells and purified as previously described by Kraus et al. (1999). ACF, NAP-1, and p300 baculoviruses were a generous gift of Dr. James Kadonaga.

Electrophoretic Mobility Shift Assay

Acetylation of p53 by p300 was performed as described by Gu and Roeder (1997). EMSA was carried out essentially as described by Jayaraman, et al. (1998). The 25 bp double-stranded oligonucleotide harboring the 5′ site of the p21 promoter was generated by annealing the single-stranded oligonucleotides 5′-caggaatctccccacagtg gaa-3′ and 5′-tcatcactggtggcagctgct-3′. The 160 bp region of the p21 promoter containing a centered 5′ site was generated by PCR from the plasmid p21-LUC, using the primers 5′-cgcgggtgcaatggattatac-3′ (sense) and 5′-ccatctccctctacacactgata-3′ (antisense). Both probes were 32P end-labeled using T4 polynucleotide kinase and further purified by 12%–15% polyacrylamide native gels.

Chromatin Assembly and In Vitro Transcription

Chromatin was reconstituted using Drosophila embryonic extracts as described (Bulger and Kadonaga, 1994). After assembly, the chromatin template (500 ng of DNA in 50 μl) was incubated with p53 and/or p300 (typically between 0.2–10 pmol of p53 and 0.2–0.5 pmol of p300), for 30 min at 27°C. For transcription, 20 μl of HeLa cell nuclear extract (typically 8 mg/ml) was added and incubated on ice for 10 min, then reactions proceeded as described (Kadam et al., 2000). The purified RNA was analyzed by primer extension analysis with primers specific to luciferase and ß-globin gene (used as an internal control) sequences.

DNase I Footprinting of Chromatin and DNA

DNase I footprinting was done using three different templates: crude chromatin-assembled mixtures, CL48-purified chromatin, and naked DNA. For purification of chromatin assembled with Drosophila embryonic extracts, we proceeded as previously described (Meu-


