p53 is a tumor suppressor protein that induces apoptosis at least in part through its ability to act as a sequence-specific transactivator. This work reports that intron 1 of the mouse Fas death receptor gene contains a p53-responsive element (p53RE) that matches the p53 consensus sequence and that is located between nucleotides +1704 and +1723 from the transcription initiation site. This element is specifically bound by p53 and functions as a p53-dependent enhancer in mammalian or in yeast reporter gene assays. Contrary to bax, another known pro-apoptotic p53-target gene, both mouse and human FAS p53REs are still activated by the discriminatory p53 mutants Pro-175 and Ala-143, a class of mutants unable to induce apoptosis. We propose that p53-dependent up-regulation of Fas does not induce apoptosis per se but sensitizes the cell to other pro-apoptotic signal(s). The functional conservation of p53-dependent Fas up-regulation argues strongly in favor of its biological importance and suggests that murine models may be used to study further the in vivo role of Fas in the p53 response.

Inactivation of the p53 tumor suppressor gene occurs in over half of all human tumors, implying that loss of the functional protein represents a key event in promoting tumoral pathogenesis (1, 2). p53 is an inducible phosphoprotein acting as a "guardian of the genome" (3), mainly by mediating cell cycle arrest function have been described (28, 29). Interestingly, these mutants, so-called discriminatory mutants, present a differential ability to transactivate target cellular promoters; they retain the ability to activate the expression of WAF1 but fail to activate BAX or IGFBP3 p53RE (29–31). Implication of PIG3 and IGFBP3 in p53-dependent apoptosis has been proposed but not demonstrated. It is therefore difficult to assign a precise role for each of these genes during p53-dependent apoptosis.

Several p53 mutants that have lost apoptotic but not cell-cycle arrest function have been described (28, 29). Interestingly, these mutants, so-called discriminatory mutants, present a differential ability to transactivate target cellular promoters; they retain the ability to activate the expression of WAF1 but fail to activate BAX or IGFBP3 p53RE (29–31). Therefore, these mutants are unique molecular tools to study the implication of p53 target genes in apoptosis. We were interested in studying the involvement of Fas during p53-dependent apoptosis. At the beginning of this work, Fas was not yet characterized as a direct target of p53, and we therefore looked for a p53-responsive element (p53RE) in the mouse Fas gene. We then employed p53 discriminatory mutants to characterize further the p53-dependent regulation of Fas. We report here that, like its human counterpart, the murine Fas gene contains a functional p53RE located in the first intron. In addition, we show that p53 discriminatory mutants are able to activate Fas p53RE in contrast to p53REs derived from other pro-apoptotic genes, suggesting a distinct function of the Fas gene in the course of p53-dependent apoptosis.
**Fas Is Activated by p53 Mutants**

**RESULTS**

**Putative p53REs Are Located in the Intron 1 of the Mouse Fas Gene**—To search for a p53RE in the mouse Fas gene, we have employed a multistep approach, based on sequence analysis and reporter gene assay. Sequence analysis of the 800 bp upstream of the transcription initiation site did not reveal any p53 consensus sequence as previously defined. Furthermore, when inserted upstream of the luciferase gene in the pGL3-basic vector, this fragment was not able to induce expression of the reporter gene in a p53-dependent manner in H1299 or in SAOS-Val-135 cells (data not shown). To test whether a p53RE could be present further upstream of the transcription initiation site, an EcoRI fragment spanning from −3250 to −385 (starting from the transcription initiation site) was inserted in both orientations in pPy upstream of the polyoma virus minimal promoter driving the expression of the luciferase gene. When transfected either in H1299 or in SAOS-Val-135 cells, the luciferase assay was performed using the Dual-lucerase reporter assay system (Promega), and the activity of the reporter gene was measured with a Microlumat LB 960 (EG & G Berthold). The Renilla luciferase was used to normalize the transfection efficiency. For each reporter vector, fold activation was calculated as the ratio between the experimental luciferase activity and the corresponding value obtained without p53-expressing vector. Results shown are the mean of at least three experiments, and the standard deviations are indicated.

**Electrophoretic Mobility Shift Assay (EMSAs)**—EMSAs was performed as described (14) without competitor DNA unless specified. Oligomers Aa and Ab (as described above) were annealed and labeled as described (14) and were used as probes. DNA protein complexes were separated by electrophoresis at 4 °C on 4–15% PhastGel Gradient with PhastGel Native Buffer Strips on LKB-PhastSystem (Amersham Pharmacia Biotech) using the following steps: step 1, 400 V, 10 mA, 2.5 watts, 2 V-h; and step 3, 400 V, 10 mA, 2.5 watts, 450 V-h.

**Putative dsREs**

**Materials and Methods**

**Library Screening—**A C3H/HeJ mouse genomic DNA library (32) was screened under high stringency conditions (33) using a DNA fragment carrying the mouse Fas cDNA. A recombinant DNA (pMFS8) that contains the 5’ part of the Fas gene was subcloned into pBlueScript II (Stratagene). Two plasmids were further used as follows: pMFS8-IX that contains an XhoI DNA fragment encompassing exon 1 (approximately 4500 to +3890 starting from the transcription initiation site) and pMFS8-2X that contains the 3’ adjacent part of the intron 1 (from nucleotide +3900 to +10,000).

**Cell Lines, Media, and Drugs—**All cells were routinely maintained in a water-saturated 5% CO2, 95% air atmosphere. All media used were supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 1% of penicillin/streptomycin (Life Technologies, Inc.). M1-S6 (expressing the mouse ts Val-135 p53 mutant) was maintained at 38 °C in RPMI 1640 medium (Life Technologies, Inc.). H1299 (expressing no p53) and SAOS-Val-135 (expressing the mouse ts Val-135 p53 mutant) cell lines were maintained in Dulbecco’s modified medium (Life Technologies, Inc.) at 37 and 38 °C, respectively. Actinomycin D was purchased from Sigma, and human interleukin-6 (IL-6) was from Life Technologies, Inc.

**DNA Manipulations and Sequence Determination—**Plasmids were constructed using standard procedures (33). DNA sequences were determined using a T7 sequencing kit (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

**Northern Blot Analysis—**Total cellular RNAs were extracted using the Trizol LS reagent (Life Technologies) according to the manufacturer’s recommendations. Electrophoresis and Northern blotting were performed as described previously (34). A 0.9-kb EcoRI-BamHI DNA fragment corresponding to the cDNA of the mouse Fas gene and a 1.3-kb Pol I cDNA fragment corresponding to the rat GAPDH gene were used as probes for northern blot analysis. The relative level of the hybridization signal of specific mRNAs was evaluated with a Bio-Rad GS 363 molecular imager.

**RESULTS**

**Putative p53REs Are Located in the Intron 1 of the Mouse Fas Gene—**To search for a p53RE in the mouse Fas gene, we have employed a multistep approach, based on sequence analysis and reporter gene assay. Sequence analysis of the 800 bp upstream of the transcription initiation site did not reveal any p53 consensus sequence as previously defined. Furthermore, when inserted upstream of the luciferase gene in the pGL3-basic vector, this fragment was not able to induce expression of the reporter gene in a p53-dependent manner in H1299 or in SAOS-Val-135 cells (data not shown). To test whether a p53RE could be present further upstream of the transcription initiation site, an EcoRI fragment spanning from −3250 to −385 (starting from the transcription initiation site) was inserted in both orientations in pPy upstream of the polyoma virus minimal promoter driving the expression of the luciferase gene. When transfected either in H1299 or in SAOS-Val-135 cells, the luciferase assay was performed using the Dual-lucerase reporter assay system (Promega), and the activity of the reporter gene was measured with a Microlumat LB 960 (EG & G Berthold). The Renilla luciferase was used to normalize the transfection efficiency. For each reporter vector, fold activation was calculated as the ratio between the experimental luciferase activity and the corresponding value obtained without p53-expressing vector. Results shown are the mean of at least three experiments, and the standard deviations are indicated.
this fragment did not lead to a p53-dependent up-regulation of the reporter gene (data not shown). From these results, we conclude that the mouse Fas promoter region does not contain a p53RE. We therefore decided to look for the presence of a p53RE downstream of the transcription initiation site. The sequence between nucleotide +1 and +4150 (encompassing exon 1 and part of intron 1) was determined and searched for a p53 consensus sequence, two PuPuPuC(A/T)(A/T)GPyPyPy decamers separated by 0–13 nucleotides (11). Compared with the above consensus sequence, some mismatches do not alter the functionality of the p53-binding site (11–14). According to this notion we have accepted a maximum of three variations compared with the consensus sequence proposed by El-Deiry et al. (11) with no more than one error in a single decamer if it is located in one of the two central nucleotides of a decamer. The C in position 4 and the G in position 7 were considered as invariant positions. Fig. 1 presents the position and sequence of the four putative p53REs found, named A, B, C, and D. These p53REs are located between nucleotides +1600 and +4000 from the transcriptional initiation site. Putative p53RE D and p53RE A contain one and two additional decamers, respectively.

A Functional p53RE Is Located between Nucleotides +1704 and +1723 within the Mouse Fas Intron 1—To explore the functionality of the putative p53REs found in intron 1 of the mouse Fas gene, we first used a yeast reporter gene assay. Pairs of decamers (corresponding to the p3 DNA-binding site (11)) found in the four putative Fas p53REs were subcloned into pLGΔ178 upstream of a truncated CYC1 promoter regulating lacZ reporter gene expression (35). The reporter plasmids obtained were used to co-transform yeast strain W303 incubated with a double strand oligonucleotide corresponding to the Fas p53RE (nucleotides 1704 to +1723), complexes leading to band shifts were detected (Fig. 3, lane 2). To identify the specific protein-DNA complex in lane 2, competition assays were performed. Addition of a 100-fold excess of non-radioactive oligonucleotide devoid of a p53-binding site did not affect the presumably specific complex (lane 4), whereas the formation of this complex was inhibited by 100-fold excess of unlabelled homologue probe or an alternative p53 consensus sequence (lanes 5 and 6). Finally, the mobility of this specific complex was retarded in the presence of an antibody directed against p53 (lane 3), confirming the presence of p53 in the complex. Taken together these results demonstrate that p53 specifically binds to the Fas p53RE.

p53 Activates the Transcription Rate of the Mouse Fas Gene

![Figure 1. Structure and sequence analysis of 5 kb of the mouse Fas gene encompassing part of the promoter, exon 1, and part of intron 1. Schematic representation of the sequenced 5' part of the mouse Fas gene is shown. Exon 1 is indicated as an open box and part of the promoter and intron 1 are shown as lines. Nucleotide numeration starts at the transcription initiation site (determined by primer extension, R. Watanabe-Fukunaga and S. Nagata, unpublished data.), and location of the initiation codon (ATG) is illustrated. Each of the putative p53REs found is indicated by a black box, and nucleotide sequence is shown. Each decamer PuPuPuC(A/T)(A/T)GPyPyPy is boxed. Variant nucleotides compared with the perfect consensus sequence are underlined, and the total number of errors for each decamer is indicated above the box. Bold lines and corresponding lowercase letters indicate the decamer pairs tested in the yeast reporter assay (see text below).](image)
derived cell lines, we induced Fas were used to obtain the control Fas indicated below the graph were subcloned upstream of a truncated promoter in the pPy luciferase reporter gene plasmid and transfected into SAOS-Val-135 cells, or co-transfected into H1299 cells with the human p53 expression vector pSVE-hump53. p53-dependent fold activation was calculated for both cell types as described under "Materials and Methods." DNA fragments of p53RE A indicated below the graph were subcloned upstream of the E1B minimal promoter in the pGL3-E1B/TATA luciferase reporter plasmid and co-transfected into H1299 cells with pSVE-hump53. p53-dependent fold activation was calculated as described under "Materials and Methods."

![Fig. 2. The mouse Fas gene contains a p53RE located between nucleotides +1704 and +1723. A, the DNA fragments of Fas intron 1 indicated below the graph were subcloned upstream of a truncated promoter in the pPy luciferase reporter gene plasmid and transfected into SAOS-Val-135 cells, or co-transfected into H1299 cells with the human p53 expression vector pSVE-hump53. p53-dependent fold activation was calculated for both cell types as described under "Materials and Methods." B, DNA fragments of p53RE A indicated below the graph were subcloned upstream of the E1B minimal promoter in the pGL3-E1B/TATA luciferase reporter plasmid and co-transfected into H1299 cells with pSVE-hump53. p53-dependent fold activation was calculated as described under "Materials and Methods."](image)

![Fig. 3. p53 binds in vitro to the Fas p53RE. Binding of purified baculovirus-produced human p53 (44) to the Fas p53RE is shown by EMSA. A DNA probe corresponding to the core of the p53RE A (from nucleotide +1703 to +1723) was incubated with p53 and, when indicated, with anti-p53 VJO1 (a monoclonal antibody directed against the N-terminal part of human p53, E. May and P. May, personal communication.). Competitions were performed by using 100-fold molar excess of unlabeled double-stranded oligonucleotides: either nonspecific (containing no p53 consensus sequence: CGGTATCCACCGTGTCGGACGCCATGCC) or homologous (the p53RE A core) or specific (containing a synthetic p53 consensus sequence: GTGGACGGACGCCATGCCC).

in the M1-LTR13 Cells—Since we demonstrated the presence of a functional p53RE in the mouse Fas gene, it was of interest to test whether p53 regulates the transcription of this gene. M1-S6 is a mouse myeloid leukemia cell line that does not express p53. M1-LTR13 is a derived clone stably transfected with a plasmid that allows expression of the mouse temperature-sensitive (ts) Val-135 mutant of p53. In this cell line activation of p53 by temperature shift (32.5 °C) induces apoptosis (45). Our laboratory has recently shown that activation of p53 in the M1-LTR13 cells leads to an accumulation of the Fas mRNA. We tested whether this accumulation resulted from transcriptional activation or mRNA stabilization. To this end, the transcription inhibitor actinomycin D was employed to measure the half-life of Fas mRNA induced by p53. M1-S6 cells were used to obtain the control Fas mRNA population. Since the basal level of Fas mRNA is not easily detectable in M1-derived cell lines, we induced Fas transcription with IL-6. IL-6 action is mediated through the transcription factor NF-IL-6 which induces Fas transcription without modifying the stability of the Fas mRNA (46, 47). To analyze Fas mRNA stability, RNAs were sequentially harvested from M1-LTR13 cells cultured at 32.5 °C and from M1-S6 cells cultured in presence of IL-6 at 32.5 °C after addition of actinomycin D. As shown in Fig. 4, the kinetics of Fas mRNA degradation in cells treated with actinomycin D was quite similar in both cell lines, whether Fas was induced by p53 or by IL-6. Thus, as expected from the characterization of a p53RE in intron 1 of the mouse Fas gene, p53 activates the transcription rate of this gene.

Fas p53RE Is Activated by p53 Discriminatory Mutants—Concomitantly with the loss of apoptotic but not growth arrest functions, p53 discriminatory mutants retain the ability to activate transcription of the cell cycle inhibitor WAF1 gene but fail to activate p53-responsive sequences derived from the pro-apoptotic BAX or IGFBP3 genes (30, 31). To investigate the role of Fas in p53-mediated apoptosis, we tested whether the mouse Fas p53-responsive sequence is activated by such p53 mutants. In H1299 cells we confirmed that the Pro-175 discriminatory mutant transactivated the WAF1 p53-responsive promoter as well as wt p53, although this mutant failed to transactivate the BAX p53-responsive promoter (30) (Fig. 5A). Under the same experimental conditions, a 210-bp DNA fragment containing the mouse Fas p53RE placed in front of a polyoma virus minimal promoter was significantly activated by the Pro-175 p53. Since h-BAX p53REs failed to activate p53RE derived from h-WAF1 (42), h-BAX (18), m-Fas, and h-FAS (21) in the same minimal promoter context by discriminatory Pro-175 and ts Ala-143 mutants. Since h-IGF3 was suggested to be implicated in p53-mediated apoptosis (20), we also tested in this system the response of its p53RE. Surprisingly, under the conditions used in our reporter system, wt p53 was unable to activate the p53RE derived from h-IGF3 (data not shown). Pro-175 and Ala-143 p53 discriminatory mutants were able to activate efficiently p53REs from h-WAF1, m-Fas, and h-FAS genes but did not, or very weakly, activate p53RE derived from h-BAX gene (Fig. 5B). We conclude that mouse and human FAS p53REs have a unique feature among p53RE derived from pro-apoptotic genes; they can be activated by p53 mutants unable to induce cell death.

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3 C. Choisy-Rossi et al., submitted for publication.
**DISCUSSION**

**Mouse Fas Gene Contains a p53RE**—In this report we show that the mouse Fas gene contains a functional p53RE. The minimal element is located in the first intron between nucleotides +1704 and +1723 (from the transcription initiation site) and is composed of two decamers that match the p53 consensus sequence (score 1820). Evidence for p53-dependent activity of this cis-acting sequence is based on EMSA experiments, functional assays with reporter gene constructs and on the analysis of Fas gene transcription in p53-overexpressing M1 cells. Two additional decamers that show limited homology to the p53 consensus sequence (score 1620) are located eight nucleotides upstream of this minimal element. These two decamers are not necessary for the activity of the minimal p53RE at least under the conditions used in our reporter systems. Whether they are necessary for the p53-dependent regulation of the mouse Fas gene in the chromosomal context cannot be completely ruled out.

It has been shown recently that the human FAS gene also contains a p53RE composed of two decamers located within the first intron (21). To check whether the murine and human FAS p53RE are homologous, the DNA sequence of the mouse intron 1 was aligned with the published human p53RE DNA sequence fragment. One region of significant homology with the human sequence was found in the mouse intron 1, but this mouse sequence did not contain any counterpart of the human p53RE (result not shown). In addition, sequence alignment of the human and mouse p53REs did not reveal a high degree of similarity. These results suggest that both p53REs may have a distinct origin. A more conclusive analysis would require a complete sequencing of the human FAS intron 1. The conservation of the p53-dependent regulation of Fas between the two species argues against a coincidental event and emphasizes the requirement for this regulation in the control of mammalian homeostasis. Indeed, it has been shown in several human cell lines that increased Fas expression is necessary for a full p53-dependent apoptotic response following genotoxic stress (21, 24).

Since this regulation is conserved, the murine model is a relevant experimental system to study the precise in vivo role of Fas in p53-dependent apoptosis.

**Fas p53REs Are Activated by p53 Mutants Unable to Induce Apoptosis**

**Mouse and human FAS p53REs are activated by p53 discriminatory mutants.** A, reporter gene plasmids containing the region of the murine Fas p53RE (pPymFA), the bax promoter (pbox-luc), and the WAF1 promoter (pWFP-luc) were used to co-transfect the H1299 cells with pCMV-hump53 plasmids expressing the wt or the indicated p53 mutant (mut, His-175 p53 mutant; wt, wild type p53; and dis, discriminatory Pro-175 p53 mutant). p53-dependent fold activation was calculated as indicated under “Materials and Methods.” B, pE1B-hWAF1, pE1B-mFas, pE1B-bFAS, and pE1B-hBAX plasmids, containing p53REs derived from the indicated genes and cloned upstream the E1B minimal promoter in pGL3-E1bTATA, were used to co-transfect H1299 cells with or without pCMV-hump53 plasmids expressing wt p53, His-175 p53 mutant, or Pro-175 or Ala-143 discriminatory mutants. Luciferase assay was performed after 24 h of incubation at 37 °C for Pro-175 mutant, and 6 h at 37 °C and 18 h at 32 °C for the ts-Ala-143 discriminatory mutant. Results are presented as percent of activation relative to wt p53 used under the same experimental conditions. His-175 mutant never activates more than twice the corresponding p53RE (data not shown).

**Apoptosis**—Several lines of evidence have suggested that the ability of p53 to induce apoptosis is separable from its role in cell growth arrest. Thus, studying the mechanism(s) of p53-induced apoptosis would require specific tools, and such a tool is provided by a particular set of p53 mutants, the so-called discriminatory mutants. These mutants are able to induce growth arrest but not apoptosis (28, 31). Concomitantly, the discriminatory mutants are capable of activating the WAF1 gene encoding a cell cycle inhibitor but are no longer able to activate promoters containing p53REs from the BAX or IGFBP3 pro-apoptotic genes (29–31). Interestingly, we demonstrate that both the human and mouse Fas p53REs are activated by the discriminatory mutants. This is the first report on p53REs from pro-apoptotic genes that are still activated by p53 discriminatory mutants. This activation does not depend on adjacent gene sequences or on the structure of the natural promoter, since it is effective in a synthetic construction containing only the sequence corresponding to the p53RE. In the same DNA context, WAF1 p53RE is activated by the discriminatory mutants, whereas BAX p53RE is not. Since activation of p53REs by the p53 discriminatory mutants is an intrinsic property of these elements, this activation should be effective on the endogenous Fas gene. Indeed, we observed that the Ala-143 ts p53 discriminatory mutant was able to activate Fas expression in two different cellular models (21, 48). The Fas gene is therefore activated by p53 mutants able to induce growth arrest but unable to induce cell death. Thus, the death...
receptor Fas may have a yet unknown function in cell cycle regulation. Alternatively, up-regulation of Fas by p53 may not be sufficient to induce apoptosis. The second hypothesis is supported by the death receptor function of Fas; activation of the Fas receptor requires the interaction with its specific ligand (FasL). In this context, a recent study has identified another death receptor, DR5 (KILLER), as a p53 target that is up-regulated during genotoxic stress-induced apoptosis (49, 50).

In light of our results, it would be of interest to test whether DR5 presents the same regulation by the discriminatory mutants.

**Fas in the Transcription-dependent p53 Response, a Model**—Since particular p53 mutants can discriminate between the variable p53REs, it is tempting to speculate that wt p53 is also able to do so. Several lines of evidence suggest that this could indeed be the case. First, as for discriminatory mutants, cell cycle arrest and apoptosis activities of wt p53 can be separated as follows: low expression of wt p53 induces cell cycle arrest, and high expression preferentially results in apoptosis (51).

Second, activation of p53 target genes in normal thymocytes following irradiation was shown to have different kinetics for the different target genes as follows: WAF1 is already fully activated 2 h after irradiation, activation of Fas begins, while Bax is hardly induced at this time (26). Third, it has been shown that wt p53 binds in vitro more efficiently to WAF1 p53RE than to p53RE of the BAX gene (31). Finally, activation of different p53REs under the same synthetic promoter context by a modulatory mutants for invaluable discussions.

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