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Identification of a putative p53 binding sequence within the human mitochondrial genome

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Abstract A small fraction of the total cellular amount of nuclear transcription factor p53 seems to be located at and within mitochondria. Transcription factors of the steroid receptor superfamily that, like p53, lack a classical mitochondrial leader sequence are nonetheless imported into mitochondria where they regulate mtDNA transcription through binding to specific recognition sequences. Here, we examined seven candidate sequences from the human mitochondrial genome with similarity to the consensus p53 binding motif. Two imperfect half-sites at coordinate 1553 with homology to the nuclear IGF-BP3 box A binding sequence are demonstrated to confer responsivity to p53 and the p53 relatives p73α and β in the context of the cell nucleus. Mitochondrial p53 may thus bind directly to mtDNA and, perhaps, be involved in the regulation of mitochondrial transcription/replication.

Keywords: p53; Mitochondrion; Transcription; Transactivation

1. Introduction

p53 is a predominantly nuclear, homotetrameric transcription factor that is activated by various stresses (reviewed in [1]). Up until recently, it was thought that activated p53 acts mainly via the transcriptional regulation of genes whose products exert primarily one of two functions: they either arrest the cell cycle, with the p21Waf inhibitor of cyclin-dependent kinases as a major player, or they contribute to forcing cells into apoptosis, with the BH (Bcl-2 homology domain) 123 protein Bax and the BH3-only proteins PUMA and Noxa as examples [2–5]. It is now known that p53 protein itself can be present at the mitochondria and trigger the mitochondria-mediated death machinery [6–10]. Several studies have furthermore suggested p53 to be present even within the mitochondrial matrix, the location where up to 10 mitochondrial (mt) DNA genomes are transcribed and replicated [6,11]. Since there are several precedents for the regulation of the mt DNA by nuclear transcription factors which, like p53, lack a classical mitochondrial import signal, it was interesting to ask whether there might be p53-recognition motifs within the human mitochondrial genome.

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p53 binds specifically to sequences composed of two 10-mers (half-sites) with the degenerate consensus 5′-r r r-C(A/T)(A/T)G-y y y-3′ (r, purines; y, pyrimidines) and spaced by 0–13 bp [12]. Numerous studies have indicated that conformity to consensus, the numbers of half-sites in close proximity and perhaps the presence of non-linear, stem-loop or bent DNA, all constitute important parameters of binding (reviewed in [13]). Furthermore, novel sequences with low conformity to the consensus but high attraction for p53 have recently been identified [14], and the protein has been shown to be able to directly associate with mitochondrial transcription factor A (mtTFA or TFAM) [15]. Here, we identify a sequence within the human mitochondrial 16S rDNA region that can function as a p53 binding motif in the context of the cell nucleus.

2. Materials and methods

2.1. Cell culture, plasmids and transfection

Saos-2 and H1299 cells were cultured in DMEM, supplemented with 10% FCS and grown in a humidified 7% CO_2 atmosphere at 37 °C. HCT116 cells and the p53−/− derivatives were cultured in McCoy's medium under identical conditions. The human mitochondrial DNA fragments were PCR-amplified and inserted into the pGA50-7 luciferase expression vector upstream of a β-globin minimal promoter and luc gene. The used primer sequences will be provided upon request. p21Waf-luc contained the p53-responsive p21Waf promoter in front of a luc gene and served as a positive control. p53-recognition sequence 1553 was mutated from 5′agaCAAGtcg–taaCATGgta3′ to 5′agaTAAAtcg–taaTATTgta3′ by PCR mutagenesis; the promoter sequence deletions shown in Fig. 3 were made by PCR cloning. For transient transfection, exponentially growing cells in 12-well dishes were incubated for 32 h with 0.4 μg of total DNA, of which 0.1 μg was reporter vector, 0.1 μg was the green fluorescence protein expressing plasmid pEGFP-C3 (Clontech, Palo Alto, USA) included to normalize for transfection efficiency in flow cytometry analyses, 0.1 μg was one of the CMV-driven effector plasmids producing wt p53, p53 mutant 22/23, p53 mutant 273H, p73α or p73β, and finally 0.1 μg was the anti-apoptotic plasmid pCMV-E1B19K that prevented apoptosis induction by p53 expression. As transfection reagent, effectene from Qiagen (Hilden, Germany) was employed according to the manufacturer's recommendation for 12-well dishes. Luciferase assays were carried out using the "Luciferase Assay System" (Promega, Mannheim, Germany) as specified by the manufacturer.

2.2. Antibodies and immunoblot analysis

The p53 monoclonal antibody DO-1 was purchased from Calbiochem (San Diego, USA); the anti-HA-tag antibody recognizing HA-tagged p73 was from Sigma–Aldrich (Taufkirchen, Germany). Cells from 10 cm dishes were lysed in 150 μl of a lysis buffer heated to 85 °C and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20% glycerol. Samples containing 15 μg of total cellular pro-

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tein were subjected to 12% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, USA). Signals were detected upon overnight incubation of the membranes with antibodies followed by a final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, USA), performed as specified by the supplier.

2.3. Southwestern blotting

0.3 μg of each DNA sample was mounted onto a nitrocellulose membrane (Schleicher & Schuell, Germany) and crosslinked with 120 J/m^2 of UVC light. Membranes were washed twice in PBS and blocked with 5% skimmed milk in PBS for 3 h. Cellular extracts were generated as described [16], diluted to a protein concentration of 1 mg/ml in gel-shift buffer (12.5 mM Tris–HCl, pH 7.9; 3.1 mM $MgCl_2$; 25 mM KCl, 0.5 mM DTT; 10% glycerol; 0.25 mM EDTA; 0.2 mM PMSF; 10 μg/ml leupeptin; 1 μg/ml pepstatin; 10 μg/ml aprotinin and 1 mg/ml of pGL2-Basic plasmid DNA (Promega) for blocking) and incubated overnight at 4 °C with the membrane, followed by 3 × 5 min washes in PBS–Tween (0.05%). The membrane was next incubated for 1 h with antibody DO-1, followed by 3 × 5 min washes in PBS–Tween. A final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents was performed as specified by the supplier.

3. Results

When the human 16569 bp mitochondrial genome (Acc# NC 001807; and [17]) was inspected for putative p53-recognition sequences, a total of seven candidate sequences were detected (Fig. 1): 1553, 1809, 2397 and 2903 positioned within the 12S and 16S rDNA region downstream of the heavy strand promoter, HSP; 4637 situated within NADH dehydrogenase subunit 2 gene; 12230 positioned between NADH dehydrogenase subunit 4 and 5 genes; and finally 16190 lying within the regulatory D-loop, downstream of the light strand promoter, LSP (the numbers denote the position of the first recognition sequence nucleotide). Previous studies have shown that a certain degree of discordance with the consensus is tolerated as long as the core invariant bases of the half-sites are maintained, and the alignments in Fig. 1 document that there is an appreciable degree of homology between the putative mitochondrial p53 binding sites and previously identified recognition sequences from well-established nuclear p53-responsive genes. One half-site, the second of sequence 2397, perfectly matches the consensus. Furthermore, the first half-site of 1553, the second of 2903, and the second of 16190, display just one deviation from consensus at relaxed positions. However, previous observations have strongly indicated that even half-sites with two or three deviations at relaxed position (with intact core invariant bases) may be functional when this deficiency is compensated by the presence of additional (imperfect) half-sites in proximity [18]. Notable in this respect is 1553 which may consist of five imperfect half-sites, spaced by zero to three basepairs. It is unclear at present whether conditions exist under which spacer sequences of more than (the allowed) 13 bp can be bridged, perhaps through the formation of secondary structures.

Next, the identified sequences plus approximately 80 bp upstream and downstream were inserted in a reporter plasmid harboring a β-globin minimal promoter and a luciferase gene to study the responsiveness of these sequences in transient transfections, in the context of the cell nucleus. The generated reporter plasmids, a plasmid carrying the promoter sequences of the p53-responsive p21Waf gene as positive control, and the empty vector as a further control, were then each co-transfected into the p53-negative human osteosarcoma cell line Saos-2 along with either a wt p53-expressing effector plasmid or the

half-site										half-site										half-site											
r	*r*	*r*	*C*	*W*	*W*	*C*	*y*	*y*	*y*	*n*	*r*	*r*	*r*	*C*	*W*	*W*	*C*	*y*	*y*	*y*	*n*									*n*	*CON*
a	g	a	C	A	A	G	t	c	**g***	-	**t***	a	a	C	A	T	G	**g***	t	**a***	3	g	**t***	a	C	T	**G***	G	**a***	**a***	**a***	2	1553
g	**c***	a	C	T	T	G	**g***	**a***	c	-	g	a	a	C	**C***	A	G	**a***	**g***	t												
a	a	a	C	A	A	G	c	c	**a***	1	**c***	a	a	C	A	T	G	c	t	t											BP3
a	a	**c***	C	A	A	G	c	**a***	t	4	**t***	a	g	C	A	A	G	**g***	**a***	c											1809
g	**c***	**t***	C	A	A	G	t	**g***	t	6	a	g	**c***	C	A	T	G	c	t	c											PERP
c*	a	a	C	A	A	G	t	c	**a***	27	a	g	g	C	A	T	G	c	t	c	110	a	**c***	a	C	A	T	G	t	t	t		2397
g	a	g	C	A	A	G	c	c	c	14	g	g	g	C	A	T	G	c	t	c												PTEN
t*	a	a	C	T	T	G	**a***	c	c	4	g	a	a	C	A	A	G	t	t	**a***											2903
t*	**c***	a	C	A	A	G	**a***	c	c	1	a	g	a	C	A	A	G	c	c	t											Bax
c*	a	**t***	C	A	A	G	t	**a***	t	6	a	**c***	g	C	A	A	G	c	**a***	**a***											4637
g	**c***	**t***	C	A	A	G	t	**g***	t	6	a	g	**c***	C	A	T	G	c	t	c											PERP
a	**c***	**t***	C	A	T	G	c	c	c	-			**c***	C	A	T	G	t	c	t	2	**c***	a	a	C	A	T	G	**g***	c	t		12230
g	**c***	**t***	C	A	A	G	t	**g***	t	6												a	g	**c***	C	A	T	G	c	t	c		PERP
c*	**c***	**c***	C	A	T	G	c	t	t	2	a	a	g	C	A	A	G	t	**a***	c											16190
t*	**c***	**t***	C	T	T	G	c	c	c	-	g	g	g	C	T	T	G	t	c	**g***											AIP

Fig. 1. Human mitochondrial genome sequences with similarities to the consensus p53 binding motif (CON). The numbers denote the coordinates of the first recognition sequence nucleotides (derived from human mtDNA sequence NC 001807). Each putative motif is aligned with the most closely related p53 binding site of a previously identified nuclear p53-responsive gene (BP3, IGF-BP3 box A; Bax, Bax box A; PERP, PERP 2; and AIP, p53AIP1). *n* is the number of spacing nucleotides. The arrows indicate p53 monomer binding sites; two of these oriented head-to-head form a half-site. 1553 consists of five degenerate half-sites, spaced by zero to three bases. The invariant bases in capital letters are separated from the purine (r) and pyrimidine (y) tract nucleotides, and the bases that deviate from consensus are boldface and marked with an asterisk.

empty effector plasmid. In a further set of transfections, the p53-expression plasmid was replaced with similar plasmids producing the p53 relatives p73α or p73β [19]. Immunoblot analyses had revealed that all effector genes, driven by the CMV promoter, were expressed at comparable levels (data not shown). When luciferase assays were performed at 32 h after transfection, the p21Waf promoter produced a strong signal indicative of p53 responsiveness, as expected. Surprisingly, the mitochondrial sequence 1553, but none of the other sequences, also responded to p53, albeit much weaker than the p21Waf promoter (Fig. 2(a)), indicating that 1553 harbors a binding site with moderate to low affinity for p53. A comparable transactivation was observed upon transfection of the p53-deficient human H1299 lung adenocarcinoma cells, documenting independence of cell type (Fig. 2(b)). Furthermore, 1553 was approximately twofold inducible by the two p73 proteins, which were not only less effective than wt p53 on this sequence but also on the p21Waf promoter, as expected (Fig. 2(c)).

Stimulation of genes by p53 requires intact transactivation and core DNA binding domains. When Saos-2 cells were transfected as before and the p53-expression plasmid was replaced by vectors producing either the p53 transactivation domain mutant Q22/S23 or the DNA binding-defective mutant 273H, no transactivation of the p21Waf or 1553 reporter plasmids was observed (Fig. 3(a)), indicating that DNA contact (through the core DNA binding domain) and the interaction of p53 with factors of the basal transcription machinery (through the transactivation domain) are necessary for the transactivation of 1553. To further investigate precisely which sequences within the 288 bp fragment that was denoted 1553 (containing the mitochondrial DNA sequences from position 1485 to 1772) were mediating the p53 responsiveness, reporter plasmids with mutated fragments were generated. As is summarized in Fig. 3(b), p53 responsiveness was maintained as long as the first binding motif of 1553 (comprising the first two half-sites of 1553 with zero bp spacer in Fig. 1) was intact. Mutation of the four invariant bases within the 20-mer abrogated the response to p53. A second putative binding motif, comprising the two half-sites between positions 1588 and 1607 (half-sites 4 and 5 of 1553 in Fig. 1) was not functional. As in Fig. 2(c), the p73 proteins were less active on the full-length regulatory sequences when compared with p53; however, their activity was increased when the sequences between 1573 and 1772 were eliminated (Fig. 3b), pointing to the possibility that a negative regulatory element is present within this fragment. We thus conclude that the first binding motif within the mitochondrial 1553 sequence (1553–1572) can confer p53 responsivity to a reporter plasmid when transfected into the cell nucleus.

To further study whether p53 can associate with 1553, an in vitro protein–DNA binding assay based on the Southwestern blotting procedure [16] was employed. For this purpose, the p21Waf plasmid as a positive control, the 1553-luc plasmid, and the luciferase plasmid pGA50-7 lacking the p21Waf and mitochondrial sequences were immobilized on nitrocellulose membranes. These were then incubated with nuclear extracts from human wt p53-proficient HCT116 colon carcinoma cells treated with the DNA-damaging (p53 activating) drug ADR (0.34 μM; Sigma), or with extracts from similarly treated p53-deficient HCT116 derivatives, and were exposed to anti-p53 antibody. As documented in Fig. 4, the extracts containing

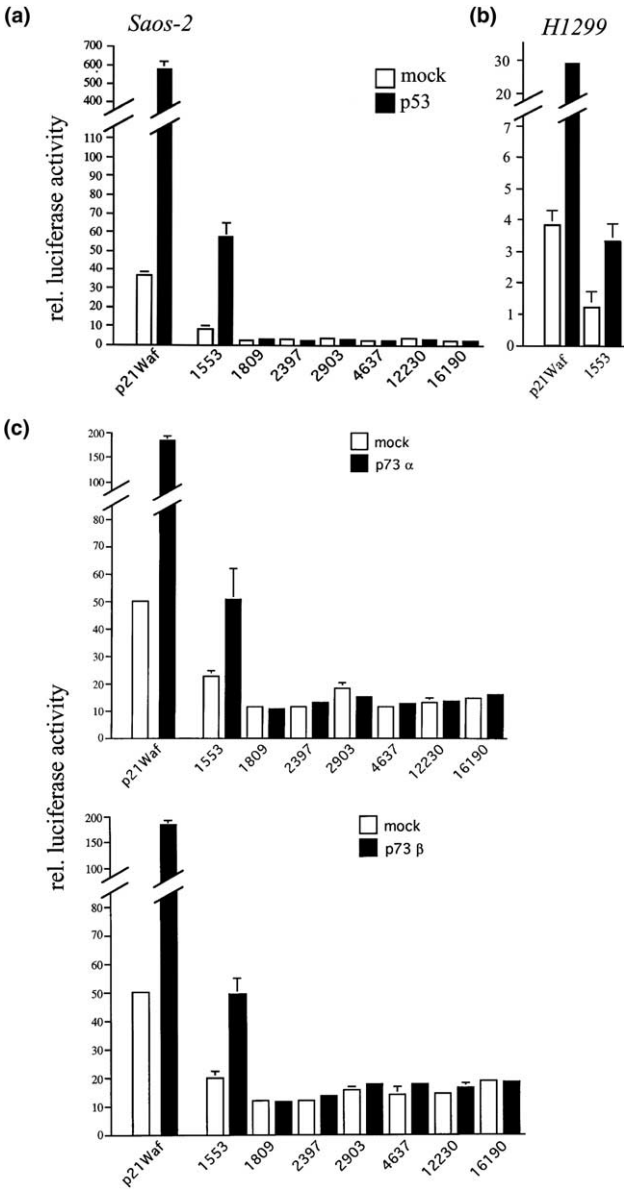

Fig. 2. p53 responsiveness of mtDNA-luc-reporter constructs upon transient transfection of Saos-2 and H1299 cells. (a) Exponentially growing Saos-2 cultures were transfected for 32 h with 0.1 μg reporter vector, 0.1 μg pEGFP-C3 to normalize for transfection efficiency in flow cytometry analyses, 0.1 μg pCMV-E1B19K to inhibit p53-meditated apoptosis, and 0.1 μg of either pCMV-p53 effector plasmid or empty vector (mock). Total cell extracts were prepared to determine luciferase activity (fold-over-background). Reporter plasmid p21Waf-luc served as positive control for p53 responsiveness. Error bars denote SD derived from at least three experiments. (b) Transient transfection of p21Waf-luc or 1553-luc as detailed in (a), with exponentially growing H1299 cultures as target. (c) Transfection of Saos-2 cultures as in (a), but with pCMV-p73α or pCMV-p73β as effector plasmids. Expression of the effector proteins has been verified by Western blotting (not shown).

p53 produced a signal with the p21Waf-luc plasmid but not with the pGA50-7 vector, while extracts from p53-null cells failed to generate any signal, indicating that p53 associates with known target sequences but not with vector in this experimental setup. 1553-luc was also recognized by p53, in accord with the

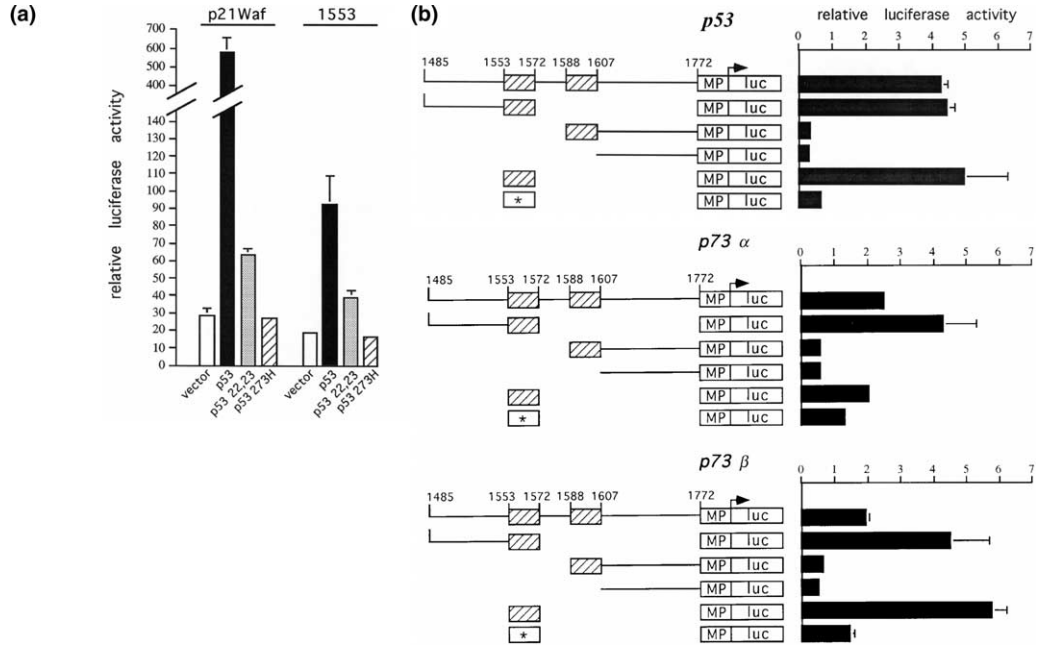

Fig. 3. Characterization of the p53 functional domains and the 1553 sequence motifs required for p53 responsiveness. (a) Transient transfection of Saos-2 cultures as detailed in Fig. 2(a), with p21Waf-luc or 1553-luc as reporters, and either empty vector, pCMV-p53, pCMV-p53-22/23, or pCMV-p53-273H as effector plasmids. (b) Transfection of Saos-2 cells with p53, p73α or p73β effector plasmids, together with one of the indicated reporter plasmids. Box 1553–1572 denotes the first half-site as detailed in Fig. 1, box 1588–1507 the fourth and fifth half-sites (the third was omitted because of mutation of the third invariant base). The box marked with an asterisk depicts half-site 1553–1572 with invariant bases mutated (see Section 2 for detail).

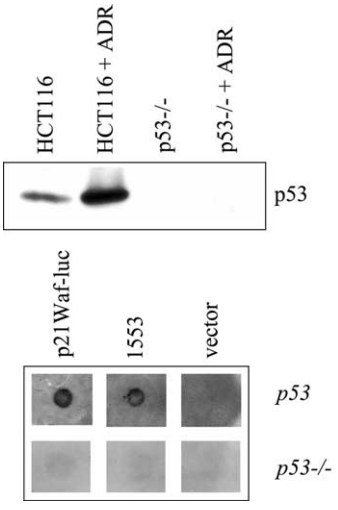

Fig. 4. In vitro-binding of p53 to immobilized plasmids with or without p53-recognition sequences. The upper panel shows a Western blot of extracts from HCT116 cells and HCT116 p53−/− cells either mock-treated or treated with ADR (0.34 μM) for 12 h. The p53 antibody DO-1 was used at a dilution of 1:1000. The lower panel shows the result of Southwestern blots of membranes carrying 0.3 μg of p21Waf-luc, 1553-luc or luciferase plasmid pGA50-7 (vector). The membranes were incubated with extracts from ADR-treated p53-positive or negative cells (italic), and were stained with p53 antibody DO-1 at 1:1000 and a peroxidase-conjugated mouse secondary antibody at 1:3000.

transient transfection experiments. Although previous work has suggested a small fraction of p53 to be located within the mitochondrial matrix of apoptotic [6] and even unstressed cells [11], we have so far failed to detect p53-mitochondrial DNA complexes in chromatin immunoprecipitation (ChIP) assays.

4. Discussion

Transcription of the circular mitochondrial genome is initiated in opposite directions from two promoters (the HSP and LSP) situated in close proximity to an approximately 1 kb triple DNA structure, the displacement (D) loop. Transcription is accomplished by a bacteriophage-homologous, nuclear gene-encoded mitochondrial RNA polymerase, and is required for the initiation of replication through RNA priming. Mitochondrial DNA replication is independent of the cell cycle. Transcription results in the formation of three polycistronic RNAs (two from HSP and one from LSP) that are eventually cleaved to produce separate mature RNAs (for review, see [20]).

Both HSP and LSP are regulated by mitochondrial transcription factor A (mtTFA or TFAM) belonging to the high mobility group (HMG)-box family, and by the transcription factors TFBM1 and 2 that contact the core RNA polymerase [21]. Remarkable recent findings furthermore document that nuclear transcription factors of the steroid receptor superfamily may be directly involved in the regulation of mitochondrial transcription without harboring typical amino-terminal mitochondrial leader sequences. An N-terminally truncated form of the nuclear triiodothyronine receptor c-ErbAα1 has been shown to recognize, in dependence of T3 ligand, mtDNA D-loop sequences with similarity to nuclear T3 response elements, and to stimulate mitochondrial protein synthesis [22,23]. Truncated c-ErbAα1 protein, like p53, fails to bind

efficiently to DNA as a monomer, suggesting that either the mitochondrial import mechanism allows retention of the multimeric structure of c-ErbAα1, or that multimers can re-form in the matrix of the organelle. Later observations have indicated that a truncated version of the retinoic acid X receptor (RXR) α is also located in the mitochondrial matrix and able to regulate mitochondrial transcription, either through heterodimerization with variant c-ErbAα1 or as homodimers and in dependence of ligand [24]. Not least, the estrogen receptors ERα and β have been located within the mitochondria, and estrogen response elements have been identified in the mtDNA [25,26].

While the mitochondrial estrogen receptor levels may account for more than 10% of the total cellular ER levels [25], p53 seems to associate with the organelle in appreciable quantities only during apoptosis [6,8,9]. In unstressed cells, p53 levels are usually very low and the p53 levels that are detectable at the mitochondria are even proportionately lower [11]. Is p53 involved in the regulation of mitochondrial transcription/replication, and if so, how? A first hint implicating p53 in mitochondrial function was provided by the finding that p53-deficient mouse embryos show a secondary reduction in mitochondrial 16S RNA transcript levels during embryonal switch from anaerobic to aerobic metabolism [27]. A recent in vitro extension of that work, employing a mitochondria-targeted dominant-negative p53 miniprotein, has correlated bona fide absence of functional mitochondrial p53 with inhibition of 16S rRNA expression in murine NIH3T3 cells [28].

The human mitochondrial sequence 1553 identified here to be p53 responsive is located within the 16S rDNA locus, not too far upstream of the LSP and downstream of the HSP regulatory sequences. However, the analogous coordinates in the mouse mt genome (AJ512208; position 976 corresponds to human 1553) show a C–T replacement in the critical first invariant base position, rendering unlikely a direct connection between the previous findings (in the p53-knockout mice and murine NIH3T3 cells, respectively) and our findings in human cells. Of further interest in this context, the human sequence 1553 examined here is known to be polymorphic (http://www.mitomap.org). The polymorphisms comprise (i) a G–A replacement at position 1598 within the purine tract of putative half-site 1588–1607 (see Figs. 1 and 3(b)), which is neutral, and (ii) an A–G replacement at position 1603, which mutates an invariant base of the same half-site. However, Fig. 3(b) documents that this latter polymorphic half-site does not contribute to the p53 responsivity of sequence 1553 in transfection assays. The critical half-site 1553–1572, by contrast, is conserved within the primate lineage. Further work is required to clear up whether 1553 is actually targeted by p53 in vivo in the mitochondrion.

Finally, it should be noted that for p53 to regulate mitochondrial transcription or replication there need not be direct DNA contact. The mitochondrial transcription factor mtTFA (TFAM) has recently been documented to interact directly with p53 [15]. Interestingly, this interaction apparently requires a domain within p53′s C-terminus that is modified by acetylation, and we have recently presented data suggesting that mitochondrial p53, in contrast to nuclear p53, is not acetylated [11]. The further observation by Yoshida and colleagues that p53 can enhance the binding of mtTFA to cisplatin-damaged DNA 10- to 20-fold [15] might even point to a role of p53 in some forms of mtDNA damage repair. Future studies will address whether p53 – or perhaps the p53-relative p73 – can regulate mitochondrial DNA directly, via specific recognition sequences such as sequence 1553 identified here, or through interaction with mtTFA.

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