Abstract

The role that the p53 tumor suppressor gene product plays in cellular differentiation remains controversial. However, recent evidence indicates that p53 is required for proper embryogenesis. We have studied the effect of p53 on the expression mediated by the promoter of the rat muscle-specific phosphoglycerate mutase gene (M-PGAM), a marker for cardiac and skeletal muscle differentiation. Experiments involving transient transfection, mobility shift assay, and site-directed mutagenesis demonstrated that p53 specifically binds and transactivates the M-PGAM promoter. The p53-related proteins p51A and p73L also transactivated M-PGAM. Moreover, stable expression of a p53 dominant mutant in C2C12 cells blocked the induction of M-PGAM expression during the myoblast to myotube transition and the ability of p53, p51A, and p73L to transactivate the M-PGAM promoter. In addition, impaired expression of M-PGAM was observed in a subset of p53-null animals in heart and muscle tissues of anterior-ventral location. These results demonstrate that p53 is a transcriptional activator of M-PGAM that contributes in vivo to the control of its cardiac expression. These data support previous findings indicating a role for p53 in cellular differentiation.

Introduction

The differentiation of cardiac muscle cells is a process that is beginning to be understood in detail. Cardiogenesis begins with a commitment of mesodermally derived progenitor cells to the myocyte lineage in response to endodermal signals, followed by the formation of the primordial heart tube (1, 2). Organogenesis then proceeds through a series of involutions of the heart tube and the onset of septation, chamber formation, and the acquisition of regional-specific properties of atrial, ventricular, and conduction system cells (3). This process progresses during the embryonic life and is completed early after birth. Each step in cellular differentiation is characterized by the expression of a specific set of molecular markers. The transcriptional control of these genes depends upon the synchronized action of cardiac-specific and ubiquitous transcription factors (3).

We isolated previously the rat M-PGAM subunit (4, 5). M-PGAM encodes a dimeric metabolic enzyme and resembles the MCK gene in its timing and pattern of developmental expression (6). It is, therefore, not surprising that both genes contain similar DNA regulatory elements that control their specific expression in skeletal and cardiac muscle (4, 5). Previous studies have shown that MCK also contains p53-responsive elements (7–10). An MCK p53 site was shown to mediate p53-responsiveness when subcloned into a heterologous minimal promoter (8). Transactivation of MCK by p53 can be inhibited by MDM2 (11), a protein frequently amplified in human sarcomas (12). Importantly, although it has been shown that p53 binds and transactivates the MCK promoter, little is presently known about the role of p53 in the activation of this or other muscle-specific genes during myocyte differentiation.

We have investigated the ability of p53 to regulate transcription from the M-PGAM promoter in rat neonatal cardiocytes, C2C12 cells, and SAOS cells. We show that p53 and p53-related proteins transactivate the rat M-PGAM promoter. Moreover, we identified a p53-responsive element in the M-PGAM promoter. This DNA element contains a consensus p53 DNA binding site that is highly homologous (86% identity) to that located in the MCK enhancer. Mobility shift assays detected binding of endogenous rat cardiac p53 and purified human p53 to the M-PGAM site. In addition, mutagenesis of the M-PGAM p53 site blocked the transactivation of the M-PGAM promoter by p53 in C2C12 cells and decreased its expression in rat neonatal cardiocytes. Importantly, reduced M-PGAM expression was observed in situ hybridization and Northern analysis in a subset of p53-null animals. Strikingly, differences were observed specifically in muscle tissues of anterior and ventral location, such as tongue or heart. These results demonstrate that p53 directly

The abbreviations used are: M-PGAM, muscle-specific phosphoglycerate mutase gene; MCK, muscle creatine kinase gene; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; ANF, atrial natriuretic factor gene; MLC2v, myosin light chain 2v gene; EMSA, electrophoretic mobility-shift assay; p.c., postcoitum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
interacts with and transactivates the M-PGAM promoter and support a role for this protein as a regulator of gene expression during muscle differentiation.

Results

p53 Transactivates the Rat M-PGAM Gene Promoter. To test the effect of p53 on the transcription mediated by the M-PGAM promoter, we transfected primary rat neonatal cardiocytes, C2C12 cells, and SAOS cells with a reporter plasmid containing −400- to +5-bp sequences of the M-PGAM promoter subcloned upstream of the CAT gene (plasmid M-PGAM CAT). We have previously shown that this promoter fragment accounts for most of the M-PGAM promoter strength and mediates its muscle-specific expression (5). M-PGAM CAT was cotransfected in combination with CMV-driven expression vectors containing no insert, human wild-type p53, or a dominant mutant p53 cDNA sequences. Cotransfection of M-PGAM CAT with the wild-type p53 expression vector did not alter M-PGAM CAT expression in neonatal cardiocytes. However, cotransfection of M-PGAM CAT with the mutant p53 expression vector in neonatal cardiocytes originated a 60% decrease in CAT activity. In contrast, cotransfection of M-PGAM CAT with the wild-type p53 expression vector in C2C12 and SAOS cells resulted in strong activation of M-PGAM CAT activity. No transactivation was observed when M-PGAM CAT was cotransfected with a mutant p53 expression plasmid in C2C12 cells (Fig. 1A). Titration experiments indicated that maximal transactivation by p53 was reached using 3 μg of the CMV-p53 expression plasmid (Fig. 1B). As a whole, these data indicated that the transcriptional activity mediated by the M-PGAM promoter is regulated by p53. The lack of transactivation of M-PGAM by wild-type p53 in neonatal cardiocytes indicates that the level of endogenous p53 protein in neonatal cardiocytes saturates a putative M-PGAM p53-responsive promoter element. The inhibition of M-PGAM CAT activity obtained by the overexpression of the mutant p53 form supports this hypothesis. It has previously been shown that structural p53 mutant proteins may work as dominant negative mutants, inhibiting the transcriptional activity of the wild-type protein (8, 15–17). Also, an additional imperfect pentamer, TGCCA, was transactivated with the wild-type p53 expression vector. Because the effect of p53 on M-PGAM in primary neonatal cardiocytes may be masked by the presence of endogenous p53 protein, these assays were performed in SAOS cells. The result of these experiments is shown in Fig. 1D. The M-PGAM CAT deletion mutants defined a promoter fragment between −172- and −87-bp sequences that mediates responsiveness to p53. Similar results were obtained with C2C12 cells (Fig. 1E). Inspection of the −172- to −87-bp promoter sequences revealed the presence of a consensus p53 binding site at positions −116- to −90-bp. Interestingly, this site is strikingly homologous to the MCK and RGC p53 sites (Fig. 2) and contains two TGGCT (pentamers) motifs (28–30). Also, an additional imperfect pentamer, TGCCA, was found five nucleotides upstream of this site (data not shown). This finding strongly suggested that p53 directly interacts with the M-PGAM promoter.

p53 Directly Interacts with the M-PGAM Gene Promoter. A specific interaction of p53 with the M-PGAM promoter was investigated by EMSA using the oligonucleotide probe (duplex) tgcagCTGAGTTGCCTCGCTGCTGAGCTG (M-PGAM, pentamer motifs underlined) and nuclear extracts prepared from neonatal rat hearts. One major nucleoprotein complex band was observed that was effectively competed by a mass excess of an oligonucleotide containing the p53 MCK site but not by oligonucleotides containing Sp1- or nuclear factor K binding sites (Fig. 3A, p53, arrowhead). Thus, the formation of this complex was p53 site specific. The presence of p53 in this band was then confirmed using anti-p53 monoclonal antibodies. A supershift was observed with the addition to the EMSA reactions of the anti-p53 antibodies PAb 421 (31) and DO-1 (32), which recognize wild-type p53 associated with DNA (Ref. 33; Fig. 3B). However, PAb 240, a monoclonal antibody that recognizes mutant p53 (34), had no effect (Fig. 3B). In summary, these experiments demonstrated that endogenous rat heart p53 binds specifically to the M-PGAM p53 consensus site. A small amount of nucleoprotein complex was not supershifted by the PAb 421 and DO-1 antibodies. This result suggested that either some of the p53 in the complex was not recognized by the antibodies, as has been shown by others (33), or other proteins were present in this band. However, because
most of the complex was supershifted by the anti-p53 antibodies, we can conclude that, at least in neonatal cardiac cells, p53 is the major factor binding the M-PGAM p53 consensus site.

In addition, the direct interaction of p53 with M-PGAM promoter sequences was investigated by EMSA using a purified baculovirus-expressed human p53 protein. Fig. 3C shows that human p53 binds to the rat M-PGAM probe with
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PGAM  GGTGCTGCTGCTGCTGCTGTTA
MCK   GCCGGCTGCTGCTGCTGCTGCTG
RGC   GGGCTGCTGACTGGCTGCTG

Fig. 2. Consensus p53 sites in the M-PGAM, MCK, and RGC genes. M-PGAM sequences were from the rat M-PGAM gene positions -116 to -90 bp. MCK and RGC sequences were as described previously (8, 15).

A similar affinity than to other oligonucleotide probes containing consensus p53 sites from the RGC or MCK genes. Moreover, binding of p53 to the M-PGAM probe was competed by a mass excess of an oligonucleotide containing a consensus p53 site (MCK) but not by an unrelated sequence (Fig. 3D). In summary, these experiments confirmed that p53 specifically interacts with the M-PGAM promoter.

p53 Is Required for Full Activation of the M-PGAM Promoter in Rat Cardiocytes. To determine whether the M-PGAM p53 consensus binding site was responsible for the transactivation of the M-PGAM promoter by wild-type p53, we created an M-PGAM reporter construct with point mutations at the two consensus p53 pentamer motifs, namely ∆p53 M-PGAM CAT. The native and mutant M-PGAM CAT plasmids were transfected in rat neonatal cardiocytes and cells incubated and processed for the assay of CAT activity. Mutation of the consensus pentamer motifs decreased the transcriptional activity mediated by the M-PGAM promoter in neonatal cardiocytes by ~65% (Fig. 4A). In addition, we cotransfected C2C12 cells with the native or mutant M-PGAM CAT reporters and wild-type or mutant p53 expression vectors. The results of these experiments, shown in Fig. 4B, indicated that mutation of the M-PGAM p53 binding site blocks the transactivation of this promoter by wild-type p53. Thus, these experiments confirmed that the M-PGAM promoter contains a consensus p53 binding site that mediates the transactivation of this promoter by p53. Moreover, these experiments demonstrate that the M-PGAM-responsive element is constitutively active in primary rat neonatal cardiocytes but not in C2C12 myoblasts.

p53 Regulates the Expression of the M-PGAM Gene Promoter in Vivo. The experiments described above suggested that p53 function might be important for the regulation of M-PGAM expression in vivo. To begin to elucidate the role that p53 may play in the transcriptional control of M-PGAM in vivo, we investigated the effect of the expression of a dominant mutant p53 protein on M-PGAM expression in C2C12 cells. For that purpose, we generated a cell line of mutant p53-expressing C2C12 cells. These experiments showed that the myoblast to myotube transition in C2C12 cells. However, experiments of gel shift assay showed no changes in protein binding to the M-PGAM p53 site during this period (data not shown). Thus, other processes, such as protein-protein interaction, may be implicated in the regulation of M-PGAM transactivation by p53 and related factors during myocyte differentiation.

Subsequent experiments investigated the role that p53 plays in the control of M-PGAM expression in vivo using Northern analysis and in situ hybridization of gene transcripts. Control and p53 null mouse embryos at day 13.5 p.c. were employed in these studies. This stage was selected because it represents the period of maximal expression of p53 in mouse embryonic development (36–38). Crosses between mice heterozygous for a targeted mutation in exon 5 of p53 on the inbred 129/sv genetic background (129/Sv-Trp53tm1Tyj mice) yielded 17% homozygous mutant offspring (6 of 34 embryos). A preliminary Northern analysis revealed no significant differences in the level of whole-animal M-PGAM expression between a control and a p53-null progeny (data not shown). M-PGAM expression was then investigated by in situ hybridization. This technique was used because it provided the advantage to study M-PGAM expression in an individual and tissue-specific fashion. Fig. 6A shows a typical muscle-specific distribution of M-PGAM transcripts found in control embryos. M-PGAM mRNA was enriched in heart cavities, eluding the valvular system, and diaphragm, whereas no expression was found in bone or lung. Similarly, M-PGAM transcripts accumulated at tongue, limb, and intercostal muscles (data not shown). Control experiments using a sense M-PGAM riboprobe resulted in background hybridization levels over these tissue sections (data not shown). The expression of M-PGAM in p53-null mice was characterized by a similar muscle-specific distribution, indicating that p53 does not affect the tissue specificity of this transcript (Fig. 6, B–D, and data not shown). Three of five p53-null embryos studied had no alterations in M-PGAM expression (60%). However, in two of these embryos (40%), reduced M-PGAM expression was detected. Strikingly, lower levels of M-PGAM expression were evidenced in muscle organs of anterior-ventral but not in lateral locations. Although a small reduction (~30%) in M-PGAM expression was observed in embryonic hindlimb muscles (Fig. 6, B and E), M-PGAM hybridization was reduced by 40 and 65%, respectively, at heart and tongue muscles related to their wild type p53 littermates (Fig. 6, C, D, and F). This defective pattern of M-PGAM expression was not observed in any of the control embryos studied (six in total; data not shown). Importantly, the lower M-PGAM expression in these p53 null animals was not due to organ hypoplasia. Nuclei
counts per optical field were similar in day 13.5 p.c. hearts and tongues of control and p53 null embryos with low M-PGAM expression (not shown). In addition, M-PGAM expression was first detected in control animals at day 11 p.c. in heart and tail muscles (Fig. 6E). This localized expression spread to lateral muscles by day 13.5 p.c. (Figure 6B). Therefore, because all p53-null animals demonstrated relatively normal M-PGAM expression in hindlimb muscles, it is highly unlikely that a defective M-PGAM expression in heart was originated by deferred development.

To confirm these results, new crosses between heterozygous 129/Sv-Trp53tmTyj mice were carried out yielding a 16.6% homozygous mutant offspring (3 of a total of 18 embryos at day 13.5 p.c.). Northern analysis of whole embryo M-PGAM transcripts demonstrated a significant reduction in M-PGAM expression in one of three p53-null embryos obtained (Fig. 7). No change in M-PGAM expression was found in six heterozygous p53 mutant embryos studied (data not shown). In summary, these experiments showed a reduced M-PGAM expression in a subset of p53-null animals. These data demonstrated that p53 contributes to the regulation of M-PGAM expression in vivo and support previous observations that indicate a role for p53 in mouse embryogenesis (37, 39).

The fact that partial penetrance was observed in p53 null animals whereas a more dramatic decrease in M-PGAM expression was observed in C2C12 cells that stably express a mutant p53 protein, prompted us to investigate the possibility that mutant p53, in addition to inactivate wild-type p53, may interfere with the ability of other p53-related proteins to

Fig. 3. p53 binds the rat M-PGAM promoter. A. EMSA showing the binding of neonatal rat heart p53 to an M-PGAM promoter probe. Nuclear extracts were prepared from rat neonatal cardiocytes as indicated in "Materials and Methods." Five µg of nuclear extracts were assayed by EMSA using 0.2 ng of a M-PGAM p53 site oligonucleotide probe and 10 µg of poly (dI·dC): (dI·dC) in buffer B. Extracts were incubated for 30 min with 40 ng of the indicated oligonucleotides prior to the addition of the M-PGAM probe. B. Supershift assay identifying p53 bound to an M-PGAM probe. EMSA reactions were as above. One µg of the respective antibodies was added to the reaction, and extracts were incubated for 1 h at 37°C. C. EMSA of purified human baculovirus expressed human p53 using a series of DNA probes. Binding reactions were prepared as in A. D. EMSA of purified human baculovirus-expressed human p53 using 0.2 ng of the M-PGAM probe and 0, 0.1, 1, or 10 ng of the indicated oligonucleotide competitor. For a description of these oligonucleotides probes and other experimental details, see "Materials and Methods." Figures show experiments that are representative of at least two assays.
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p53 site on the transactivation of the M-PGAM promoter in rat neonatal cardiocytes. Cells were transfected with 1 μg of CMV-Luc and 10 μg of plasmids M-PGAM p53Δ53 CAT (WT) or M-PGAMp53Δ3CAT (ΔS3), incubated for 48 h and processed for the assay of CAT activity, as indicated in “Materials and Methods.” B, transient transfection assay showing the effect of the mutagenesis of the p53 site on the transactivation of the M-PGAM promoter by p53. C2C12 cells were cotransfected with 1 μg of CMV-Luc, 10 μg of reporter plasmids M-PGAM CAT (WT), or M-PGAMp53Δ3CAT (ΔS3) and 3 μg of expression plasmids CMV (CMV), CMV-p53 (CMV-p53), or CMV-p53 Δ143A (CMV-p53Δm). Cells were incubated for 48 h and processed for the assay of CAT activity, as indicated in “Materials and Methods.” Column, means of three experiments; bars, SD.

Transactivation of the M-PGAM promoter. To test this hypothesis, C2C12 cells were cotransfected with the M-PGAM CAT reporter plasmids and CMV-driven expression vectors containing p53, p51A, or p73L sequences, alone or in the presence of a p53 143A expression vector. Interestingly, mutant p53 blocked in part the transactivation of the M-PGAM promoter by p53, p51A, and p73L (Fig. 8A). Moreover, these proteins failed to transactivate the M-PGAM promoter in C2C12 cells stably expressing p53 143A (Fig. 8B). These results demonstrate that, at least in transient transfection assays, mutant p53 may interfere with the ability of p51A and p73L to activate M-PGAM expression. These results are in agreement with recent data demonstrating that mutant p53 blocks the transcriptional activity of p73 isoforms (24). These data suggest the possibility that p53-related proteins may compensate for some p53 functions in p53 null animals and may reconcile some of the differences observed between C2C12 cells expressing mutant p53 and p53-null embryos.

Discussion

We have found that the expression mediated by the rat M-PGAM promoter is regulated by p53. The activation by p53 of the activity of an M-PGAM CAT reporter construct was demonstrated in C2C12 and SAOS cells (Fig. 1A). Transfection of wild-type p53 in rat neonatal cardiocytes did not alter M-PGAM CAT activity (Fig. 1A). However, the expression of a dominant mutant p53 form in these cells inhibited M-PGAM-mediated expression. The fact that some mutant p53 proteins behave in a dominant negative fashion, inhibiting the transactivation of coexpressed or endogenous wild-type p53, is well documented (40–42). In view of these data, we interpreted that the overexpression of mutant p53 protein in neonatal cardiocytes inhibited the endogenous wild-type p53 that constitutively transactivates the M-PGAM promoter. This hypothesis was supported by the reduction of the transactivation activity mediated by the M-PGAM promoter in neonatal cardiocytes by the mutagenesis of the p53 site (Fig. 4, see below). Moreover, current experiments in our laboratory indicate that overexpression of the p53 transcriptional inhibitor protein MDM2 (11) in neonatal cardiocytes represses M-PGAM expression in a p53 site-dependent manner (data not shown). The fact that p53 protein is expressed in neonatal cardiocytes has been shown previously (36). Also, gel shift assays demonstrated that p53 is the major factor in neonatal hearts binding a p53 consensus site in the M-PGAM promoter (Fig. 3).

Maximal transactivation of M-PGAM in C2C12 was observed with 1–3 μg of a p53 expression vector (Fig. 1B). Similar concentrations of p51A and p73L expression vectors were required for maximal M-PGAM promoter activity. Lower amounts of p53 and p53-related proteins have been shown to transactivate p53 gene targets in other cell types (24). However, in agreement with our results, relatively large concentrations of p53 were used by others to determine the ability of p53 to transactivate gene targets in muscle cells (7, 8). We have seen also that 1–3 μg of a p53 expression vector were required in C2C12 to obtain maximal transactivation of reporter plasmids containing the RGC p53 site (PG13) or a p21 promoter fragment (not shown). These results indicate that M-PGAM is as responsive to p53 transactivation as other p53 targets. We hypothesize that the differences observed with the results obtained in other cell types are originated by the lower transfection efficiency of muscle cells.

A wild-type p53-responsive promoter fragment in the M-PGAM promoter was defined by cotransfection of a wild-type p53 expression vector and a series of M-PGAM CAT deletion mutants in C2C12 and SAOS cells (Fig. 1, B and C). Inspection of this DNA sequence revealed the presence of two TGGCT pentamers motifs at positions −116 to −90 bp with an additional imperfect pentamer TGGCA 5 nucleotides upstream. This putative p53 binding site was found to be strikingly homologous to the MCK and RGC p53 sites (Fig. 2). Importantly, mutagenesis of this site decreased the activity of M-PGAM in neonatal cardiocytes and blocked the transactivation of the M-PGAM promoter by wild-type p53 in C2C12 cells (Fig. 4). These experiments demonstrated that the rat M-PGAM promoter contains a wild-type p53 consensus site that mediates the transactivation of M-PGAM by this protein. A similar sequence, with 13 of 17 identical nucleotides, was found in the human M-PGAM promoter, suggesting that this element is well conserved (data not shown). Whether the human M-PGAM promoter is also responsive to p53 should be the object of further investigation. EMSA detected the binding of endogenous rat cardiac p53 and purified baculovirus-expressed human p53 to the p53 M-PGAM site (Fig. 3). Importantly, these experiments demonstrated that p53 directly interacts with M-PGAM promoter sequences. The fact that rat and human p53 are both able to bind with high affinity the rat M-PGAM promoter in a specific...
manner indicated that the interaction between p53 and M-
PGAM is well conserved.

Finally, a role for p53 in the transcriptional regulation of M-
PGAM was demonstrated in C2C12 cells (Fig. 5) and in
mouse embryos (Figs. 6 and 7). Strikingly, a decrease in
M-PGAM expression was observed in p53-null mouse em-
bryos in heart and tongue but not at limb muscles, indicating
that p53 contributes to the control of M-PGAM expression in
muscle tissues of anterior-ventral location. Studies of p53
expression during mouse embryogenesis indicated high lev-
els of p53 mRNA in all tissues (38). At late stages of devel-
opment, p53 expression becomes more pronounced in cells
undergoing differentiation (38). A similar scenario has
been observed during chicken embryogenesis (43), supporting the
hypothesis that p53 plays a role in tissue-specific differenti-
ation. Multiple studies have implicated the p53 tumor sup-
pressor gene during differentiation in vitro (13, 14, 44–50).
These results were disputed by the absence of developmen-
tal alterations initially reported in p53 null animals (51, 52).
However, recent evidence has indicated the presence of
neural tube and cranio-facial malformations in a subset of
p53-null animals (37, 39, 53). These data underscore the fact
that p53 may be important in normal development as well as
in tumorigenesis. Sah et al. (37) hypothesized that, during

Fig. 5. Expression of a mutant p53 form blocks the increase in M-PGAM expression during the terminal differentiation of C2C12 cells. A, immunopre-
cipitation of mutant p53 with antibody PAb 240 in C2C12 cells infected with vector pBabe (no insert, pBabe) or vector pBabe p53 143A (p53143A). Cells
were incubated with radiolabeled methionine, harvested, and lysed; PAb 240-reactive p53 was immunoprecipitated; and immunoprecipitates were resolved
by SDS-PAGE and exposed to a PhosphorImager screen (Molecular Dynamics). B, Northern analysis of M-PGAM expression in C2C12 stable transfected
with plasmids pBabe (pBabe, no insert) or pBabe p53 143A (p53143A). Cells (3 x 104 cells/cm2) were incubated in serum-free DMEM supplemented with
10 μg/ml insulin and 5 μg/ml transferrin for 2–4 days (13). RNA was isolated and processed for Northern analysis, as indicated in “Materials and Methods.”
RNA integrity was verified by re-probing with a GAPDH sequence (American Type Culture Collection). The figure is representative of two experiments.
C, Northern analysis of MCK expression in the C2C12 RNA extracts used in part B. MCK sequence probe was from American Type Culture Collection. D, effect
of the mutagenesis of the p53 M-PGAM site on the activation of M-PGAM CAT during the terminal differentiation of C2C12 cells. C2C12 cells were
cotransfected with 1 μg of CMV-Luc and 10 μg of reporter plasmids M-PGAM CAT (WT) or M-PGAMp53Δ CAT (Δ53). Cells were incubated for 72 h in DMEM
with 10% fetal bovine serum (10% Serum) or in serum-free DMEM supplemented with 10 and 5 μg/ml transferrin (No Serum), harvested, and processed
for the assay of CAT activity as indicated in “Materials and Methods.” Column, means of three experiments; bars, SD.

neural tube closure, p53 could have a role in mediating cell
cycle arrest to limit cell proliferation or to prepare cells for a
differentiation event. Our results support the hypothesis that
p53 may have a role as a positive regulator of muscle cell
differentiation in vivo. These results are not in contradiction
to the well-known cell cycle regulatory properties of p53. The
growth suppressor properties of p53 are well substantiated
by the ability of this protein to inhibit the proliferation of
cultured tumor cells (54), prevent neoplastic transformation
in vitro (55–59), and inhibit the formation of tumors in animal
models (24). Importantly, unlike in skeletal muscle, where
cellular proliferation and a differentiated phenotype are mu-
tually exclusive (60), the increase in cardiac mass during
embryonic life arises predominantly from the proliferation of
mononucleated differentiated cardiomyocytes (61). Terminal
differentiation, with irreversible withdrawal from the cell cy-
cle, does not take place in cardiomyocytes until shortly after
birth (62). Our results indicate that p53 may function in the
heart as a regulator of a specific set of genes associated with
phenotypic differentiation rather than as a growth suppres-
sor. This conclusion is supported by recent data of Soddu
et al. (13) indicating that, in the C2C12 model, inactivation of
p53 function affects cell differentiation but not cell cycle
arrest.
In situ hybridization of M-PGAM transcripts in normal and p53-null embryos. Homozygous p53 +/+ and −/− null mouse embryos were generated from heterozygous, 129-Trp53 p53 (+/−) intercrosses and identified by PCR genotypic analysis using primers designed against the neo gene, as described by Jacks et al. (52). Day 13.5 p.c. embryos were sacrificed, fixed in cold 4% paraformaldehyde, dehydrated through graded ethanol series, and embedded in paraffin wax. Seven-μm-thick paraffin sections were processed, as indicated in “Materials and Methods.” Plasmid Sm2 containing M-PGAM cDNA sequences was digested with NcoI and a 35S-UTP-labeled riboprobe was generated using T3 RNA polymerase. In situ hybridizations were carried out for 16 h at 60°C. Slides were washed and exposed to autoradiographic emulsion for 5 weeks, developed in D19 Kodak solution, counterstained with toluidine blue.
bars in tissue sections of p53 transcripts in a sagittal section of an 11-day p.c. p53 of response to exposure to γ radiation. The fact that p53 may exert a localized developmental role may explain the limited in vivo data. The reasons for a selective pattern of transcriptional regulation by p53 are unknown. In addition, molecular redundancy could compensate for the lack of p53 function at posterior-lateral locations. Molecular redundancy has been shown to underlie the paucity of developmental alterations observed in knockout animals lacking the expression of major transcriptional regulators of muscle-specific expression (64, 65).

As indicated above, several p53 homologs have been recently shown to transactivate p53 gene targets (18–25). We show that p51A and p73L may both transactivate the M-PGAM promoter (Fig. 1B). The results of our EMSA suggest that p53 is the major factor interacting with the M-PGAM p53 consensus site in neonatal cardiocytes (Fig. 3B). However, p53-related proteins may play a more important role in the regulation of the promoter activity of M-PGAM in other muscle tissues. Interestingly, it has been shown that p51 is expressed at high levels in skeletal muscle (19). The physiological role of p51 is yet unknown. However, its ability to transactivate M-PGAM and other p53 targets in skeletal muscle deserves further investigation.

Finally, we have seen that a p53 mutant protein was able to block the ability of p53, p51A, and p73L to transactivate M-PGAM (Fig. 8). These results are in agreement with a recent report by Di Como et al. (24) that mutant p53 proteins may interfere with the transcriptional activity of p53-related proteins. This is not surprising, considering the high degree of sequence homology between the members of this family. As stated above, these data may reconcile some of the discrepancies observed between our C2C12 and p53 null embryo experiments (Figs. 5 and 6). In addition, it is well-known that mutant p53 proteins may display gain-of-function properties that cannot be completely explained by the inactivation of wild-type p53 (66). Mutant p53 proteins may work as transcriptional activators by a mechanism that do not require the inactivation of wild-type p53 (67, 68).

Materials and Methods

Animals. Heterozygous 129Sv-Trp53<sup>tm1Tyj</sup> mice were purchased from The Jackson Laboratory. Control (+/+) and homozygous p53 null (−/−) mouse embryos were generated from heterozygous intercrosses and identified by PCR genotypic analysis using primers designed for the neo gene, as described previously by Jacks et al. (52).

Cell Culture, Cell Extracts, Transfections, and Reporter Assays. Rat neonatal cardiocyte cultures were prepared as described previously (13). C2C12 and SAOS cells were from American Type Culture Collection.
Regulation of Muscle Gene Expression by p53

Digestion of plasmid CMV-p53 143A into the retroviral-based vector were transfected by the calcium phosphate method as described (5). As an additional control, pBABE p53 143A plasmids containing p53 mutant sequences and a puromycin selectable marker was generated by Refs. 74 and 75. The pBabe p53 143A plasmid containing p53 mutant gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD; T 4 polynucleotide kinase (NEB) and 32P-labeled gDNA for 4 h at 37°C in 5 ml of new medium with 2.5 mcI of [32P]methionine (1175 Ci/mmol; NEN). Cells were collected by centrifugation and lysed in 1 ml of immunoprecipitation buffer; PBS containing 1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 1500 × g for 10 min. Cell lysates were incubated in the presence of 1 µg of the corresponding antibody at 4°C for 4 h followed by incubation for 1 h with protein A/G-agarose (Santa Cruz Biotechnology). Immunoprecipitates were collected by centrifugation at 5000 × g for 10 min, washed five times with 1 ml of immunoprecipitation buffer, resuspended in SDS-PAGE sample buffer, boiled for 5 min, and electrophoresed by 15% PAGE. Dried gels were exposed to a PhosphorImager screen and analyzed using ImageQuant software (Molecular Dynamics). Northern Blot Analysis. RNA was isolated from 13.5 p.c. mouse embryos or C2C12 cells using Trizol reagent (Life Technologies, Inc.). For Northern analysis, 30 µg of total RNA were electrophoresed in a 1.3% agarose/formaldehyde gel, visualized using ethidium bromide, transferred to nitrocellulose filters (Amersham), fixed by UV cross-linking, and baked at 80°C for 1 h. For hybridization, 10 6 cpm of a random primed 32P-labeled Nco I/Sma I sequences used for the generation of probes was as described previously (73). The MCK and GAPDH sequences used for the generation of probes were from American Type Culture Collection. The ANF probe was as described previously (71). Filters were washed in 2 x SSC, 0.1% SDS, and exposed to a PhosphorImager screen and analyzed using ImageQuant software (Molecular Dynamics). In Situ Hybridizations. On day 13.5 p.c., embryos were sacrificed, fixed in 4% paraformaldehyde, dehydrated through graded ethanol series, and embedded in paraffin wax. Seven-µm-thick paraffin sections were mounted in polylysine-pretreated slides. Tissue sections were then dewaxed, rehydrated, and treated with acetic anhydride. Specimens were then dehydrated and dried. In situ hybridizations were performed according to the method described in Lyons et al. (79). A Sm2 genomic fragment of M-PGAM (5) was digested with NcoI and a 32P-labeled riboprobe was generated using T3 RNA polymerase. Hybridizations were carried out for 16 h at 60°C. Slides were then washed and exposed to Ilford autoradiography film. Radioactive bands were also quantified using a PhosphorImager screen and ImageQuant software (Molecular Dynamics).

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