Differential Transcription-Coupled Translational Inhibition of Human p53 Expression: A Potentially Important Mechanism of Regulating p53 Expression in Normal versus Tumor Tissue

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Abstract
p53 protein accumulation is triggered following exposure to potentially carcinogenic DNA-damaging agents and other physiological processes. Here we show that although p53 mRNA transcribed from the downstream P1 transcription start site was the only p53 transcript detected in human cell lines and tumor specimens, p53 transcripts initiated at the upstream P0 and P2 start sites were primary in normal human tissues, with P0-initiated p53 transcripts comprising approximately 50% of total p53 transcripts. P1-initiated p53 mRNA was not detected in most normal human tissues examined. Decreased translational efficiency was observed for mRNAs containing p53 5′ untranslated region sequences located between P0 and P1 in rabbit reticulocyte lysates and in cell lines; no inhibitory activity was observed for sequences located downstream of the P1 start site. These data suggest that a transcriptional switch from P0/P2- to P1-initiated p53 mRNA could be an important mechanism by which cells regulate p53 expression.

Introduction
The p53 tumor suppressor gene encodes a multifunctional transcription factor that has been strongly implicated in the carcinogenic process (1, 2). Wild-type p53 protein has been observed to rapidly accumulate following certain types of DNA damage and plays a major role in inhibiting cell cycle progression as well as the induction of apoptosis (3, 4). p53 protein accumulation has been shown to be triggered following exposure to potentially carcinogenic DNA-damaging agents including γ-irradiation (5, 6) and UV light (7, 8). p53 protein levels also increase in response to various other potentially non-genotoxic stresses including hypoxia (9) and ribonucleotide pool depletion (10) and during a variety of physiological processes including growth stimulation from quiescence (11, 12), differentiation (13, 14), and senescence (15). Since many diverse cellular insults and physiological processes cause p53 protein to accumulate (3), p53 expression may be regulated by a variety of transcriptional, translational, and post-translational mechanisms (16–18). For example, post-translational stabilization of endogenous p53 protein has been observed in response to cellular insult (19, 20). In addition, ongoing translation may also be required for this response because the protein synthesis inhibitor cyclohexamide prevents p53 protein accumulation and partially abrogates growth arrest following cellular exposure to genotoxic agents (5, 21, 22).

Several previous observations have lent support to the possibility that human p53 exon 1 sequences may play an important role in the regulation of p53 gene expression at the translational level. The 5′ untranslated region (UTR) of human p53 mRNA is primarily derived from exon 1 sequences that are separated from the first coding exon (in exon 2) by a distance of greater than 10 kb (23). Similar to that described for other translationally regulated mRNAs, the p53 5′ UTR sequence encodes a relatively long GC-rich span of 5′ untranslated sequence that is highly conserved among species (24, 25). The human p53 gene was originally shown to encode a p53 mRNA species containing a 248-nucleotide (nt) 5′ UTR that was transcribed at a transcription start site termed P0 (23). Several transcription start sites were subsequently identified for the p53 gene including a cluster of start sites (termed P1) located approximately 104 bp downstream of the P0 site (26). Previous investigators have suggested that the p53 5′ UTR plays an important role in regulating p53 expression (23, 24, 27). Human p53 mRNA was postulated to contain secondary structure in the region spanning P0-P1 that could potentially play a role in regulating the translational efficiency of P0-initiated p53 transcripts (23, 24). No such secondary structure was predicted to occur in the region downstream of P1 (23, 26) and it was suggested that P1-initiated p53 mRNA would be more readily translated than P0-initiated transcripts (23, 26). Since human P0- and P1-initiated p53 transcripts may have non-identical translational efficiencies, differential expression of these transcripts could affect the level of newly synthesized p53 protein. However, in SV40-transformed fibroblasts, the majority of
human p53 gene transcription is initiated from the P1 transcription start site (23, 26), suggesting that in these cells, a P0-specific translational regulatory mechanism may be less relevant than previously suggested.

The goal of the present study was to examine the potential importance of a 5' UTR-mediated translational regulatory mechanism in controlling p53 expression. Described are studies assessing the relative level of P0 versus P1-initiated p53 mRNAs in various human tissues and cell lines and examining the translational efficiency of mRNAs containing p53 5' UTRs corresponding to P0 versus P1-initiated p53 sequences. Results from these experiments implicate a transcription-coupled 5' UTR-mediated translational regulatory mechanism in the control of cellular p53 expression.

Results

p53 Transcriptional Start Sites

Primer extension analysis was used to determine the length of the human p53 5' UTR and assess the start site(s) used to initiate p53 transcription in immortalized and tumorigenic cell lines derived from a variety of human tissues. In all cell lines tested, we observed an extended band corresponding to p53 mRNA initiated at the P1 start site (~158 nt; Fig. 1A). This band was observed in both non-tumorigenic, immortalized cells (BEAS-2B, lane 7) as well as tumorigenic cells (MCF-7, LNCaP, H460; lanes 8 – 10). Similar results were obtained for the tumorigenic oral carcinoma-derived cell lines 1386 and 1483, and for the non-tumorigenic, immortalized MSK-Leuk1 cell line (results not shown). As expected, no p53 extension product was observed in the p53-null H358 cell line (Fig. 1A, lane 11). Although an upper band at ~210 nt and a lower band at ~85 nt could be delineated for some of the cell lines, they were also observed in the p53-null H358 cell line, suggesting that they were not p53-specific. Extension of β-actin mRNA of the expected size (~112 nt) was observed for all cell lines tested (Fig. 1A, lanes 1 – 5).

Primer extension analysis was subsequently performed using total RNA purified from various human tumor specimens. These tumor specimens were derived from different organ sites including larynx, oral cavity (floor of mouth, OT-1), breast, liver, and lung. Results of a typical primer extension analysis of tumor RNA specimens are shown in (Fig. 1B, lanes 3 – 9). Similar to that observed in cell lines including BEAS-2B (Fig. 1B, lane 10), only one extended p53 product was observed in tumor RNAs. This product again corresponded in length to that expected for P1-initiated p53 mRNA (~158 nt).

Since the regulation of p53 gene expression may be altered in immortalized and tumorigenic cells, primer extension analysis was used to determine the major start sites for p53 gene transcription in a variety of normal, disease-free, human tissues. Analysis performed for total RNA samples purified from normal lung or liver tissue specimens revealed two different extended p53 products (see Fig. 1B, lanes 1 and 2). One of these products corresponded in length to that expected for P1-initiated p53 mRNA (~261 nt), while the second extended product may be derived from p53 mRNA initiated from a transcriptional start site located between the P0 and P1 start sites as described previously by Tuck and Crawford (26), a transcription start site we have designated as “P2.” The approximate length of the P2-initiated p53 mRNA-extended product was 190 nt. Neither of the normal tissue RNA specimens examined by primer extension analysis exhibited an extended band of 158 nt corresponding to the P1 transcription start site.

To better examine p53 transcription start sites in normal tissues, similar primer extension experiments were performed with mRNA samples purified from a variety of normal human tissues purchased from Clontech. As shown in Fig. 1C, only P0- and P2-initiated p53 transcripts were observed by primer extension analysis of mRNA from normal human stomach (lane 8), liver (lane 9), lung (lane 10), and testis (lane 11). The primer-extended band corresponding to P1-initiated mRNA observed in LNCaP cells (lane 12) was not detectable in these normal tissues. In addition to primer-extended bands corresponding to P0- and P2-initiated mRNA, primer extension analysis of mRNA from mammary tissue exhibited another primer-extended product corresponding to that expected for P1-initiated p53 mRNA (Fig. 1C, lane 13). Densitometric analysis of primer extension autoradiographs revealed that P0-initiated p53 transcripts constituted approximately 50% of the total p53 mRNA in the majority of the normal human tissues tested (see Fig. 1B, lanes 1 and 2, and Fig. 1C). Extension of β-actin mRNA of the expected size (112 nt) was observed for mRNA from all tissues tested (see Fig. 1C, lanes 1 – 6).

To confirm the results obtained by primer extension analysis, a multiplex reverse transcription (RT)-PCR strategy was employed using several sense primers homologous to different sequences within the p53 5' UTR in combination with a p53 exon 2-specific antisense primer, and sense and antisense 5' UTR sequences within the p53 5' UTR in combination with a p53 sense primer (179 bp) were observed for RNA samples from Clontech (Fig. 2). In this assay, the presence of P1 primer-generated products could be derived from P1-, P2-, or P0-initiated p53 mRNAs, P2 primer-generated products from P1- or P0-initiated p53 mRNAs, and P0 primer-generated products only from P0-initiated p53 mRNA. Successful RT-PCR amplification (282 bp) for the P0-specific primer (homologous to p53 sequences +1 – 21 relative to the P0 transcriptional start site) was only observed in reactions containing RNA from normal tissues (panel A, lanes 5 and 6) but not tumor specimens (lanes 1 – 4). Similar results were observed in RT-PCRs using p53 sense primers homologous to p53 sequences +22 – 40 and +41 – 58 (relative to the P0 transcriptional start site; results not shown). In contrast, successful RT-PCR amplifications using the P2-specific p53 sense primer (179 bp) were observed for RNA samples from both normal tissues and tumor specimens (panel C, lanes 1 – 6). Successful RT-PCR amplification was also observed for all RNA samples tested using the P2-specific p53 sense primer (224 bp), although the degree of amplification were routinely less for RNAs from most of the tumor specimens (panel B, lanes 1 – 3) as compared to normal tissue (lanes 5 and 6). β-Actin sequences were successfully amplified (202 bp) in all RT-PCRs. These data are consistent with the results obtained from primer extension analysis demonstrating a differential usage of p53 transcriptional start sites in normal versus tumor tissue.
FIGURE 1. Primer extension of p53 transcripts. Primer extension analysis was performed using a $^{32}$P-labeled probe homologous to the 5'-end of p53 exon 2 for total RNA isolated from, (A) various human cell lines, (B) normal or tumor tissues (human), or (C) normal human tissue mRNAs purchased from Clontech (Palo Alto, CA). As a positive control, a $^{32}$P-labeled probe homologous to $\beta$-actin was used for primer extension analysis of cell lines (panel A) and Clontech normal human mRNAs (panel C) as indicated. p53 transcription start sites $P_0$, $P_1$, or $P_2$, corresponding to each of the extended products, are indicated. $M$ indicates $^{32}$P-labeled RNA marker; corresponding RNA marker sizes are indicated on left of each panel.
To better assess the major start sites for \( p53 \) gene transcription in a variety of normal human tissues, a human mRNA slot-blot from Clontech containing normalized mRNAs purified from 26 normal adult tissues and 7 normal fetal tissues was screened for \( P_0 \)- versus \( P_1 \)-initiated \( p53 \) mRNA. This blot was initially hybridized with probe A, a \( 32\text{P} \)-labeled probe specific for \( p53 \) mRNA initiated upstream of the \( P_1 \) transcriptional start site. This probe was used to detect both \( P_0 \)- and \( P_2 \)-initiated \( p53 \) mRNAs because it specifically spanned the \( P_0\text{-}P_1 \) \( 5' \) UTR region inclusive of the \( P_2 \)-initiated \( p53 \) transcriptional start site. After densitometric analysis, the blot was stripped and then hybridized to probe B, a \( 32\text{P} \)-labeled probe homologous to the 117 nt of \( p53 \) \( 5' \) UTR sequence located immediately downstream of the \( P_1 \) start sites (i.e., representative of total \( p53 \) mRNA). A comparison of densitometric results between the two probes indicated that, similar to the results obtained from the primer extension and multiplex RT-PCR analysis, the combined level of \( P_0 \)- and \( P_2 \)-initiated transcripts comprised a large portion of total \( p53 \) mRNA in all normal human tissues tested. For example, the vast majority of \( p53 \) mRNA was shown to be transcribed from the \( P_0 \) or \( P_2 \) start sites in brain, heart, lung, and liver (Fig. 3), which is consistent with that observed for both lung and liver by primer extension analysis (Fig. 1). For some tissues (such as spleen and thymus), \( p53 \) transcription was observed to be more heterogeneous, with \( P_0\text{-}P_2\)-specific probe binding decreased to 35–65% that observed for the \( P_1 \)-specific probe for both tissues.

**Translational Efficiencies of \( p53 \) \( 5' \) UTR-Containing Messenger RNAs in Vitro**

Rabbit reticulocyte lysates were used to evaluate the effect of different segments of the \( p53 \) \( 5' \) UTR on translational efficiency. Initially, the constructs pcDNA3.1/p\( p53(1–103)\)-luc, pcDNA3.1/p\( p53(104–220)\)-luc, and pcDNA3.1/p\( p53(1–220)\)-luc were generated so as to examine how different \( p53 \) \( 5' \) UTR segments would affect the translational efficiency of the luciferase reporter gene (see Fig. 4). The vector pcDNA3.1/\( Mcmyc(1–360)\)-luc, containing the mouse \( c\text{-}myc \) \( 5' \) UTR, was used as a positive control because it had been previously shown to inhibit mRNA translation in rabbit reticulocyte lysates (28–30), while the vector pcDNA3.1/DHSP(1–228)-luc, containing the DHSP \( 5' \) UTR, was used as a negative control because it had been shown not to inhibit translation (29–31). \( 5' \) UTR-luc mRNAs (including the control-luc mRNA without gene-specific \( 5' \) UTR sequences) were tran-
Translational Efficiency of the Human p53 5’ UTR on transla-
tional efficiency.

while the DHSP 5’ UTR had no effect on luciferase mRNA
significantly (P < 0.001) decreased luciferase protein synthesis
for both mRNAs). In contrast, no decrease in translation was
observed for plasmids coding for mRNAs containing p53 5’ UTR
sequences 1–220 and 1–103 (P > 0.001) as compared to that
observed for plasmids containing P0-specific p53 5’ UTR sequences
present in all p53 mRNA). A significant decrease in luciferase
expression was also observed for the last 117 nt of exon 1
(known as exon 1 sequences 104–220). These data suggest
that p53 5’ UTR sequences derived exclusively from
the 5’ UTR exhibited significant decreases in luciferase expression in 1299 cells
when compared to those observed for the first 103 nt of human p53 exon 1
(present only in p53 mRNAs initiated upstream of the P1 transcription start site) on translational efficiency. Plasmid
coding for CAT mRNAs containing p53 5’ UTR sequences were of similar length to mRNAs transcribed from
the newly constructed pcDNA1(HE)-CAT plasmid that transcribed luciferase mRNA containing P1-specific p53 5’ UTR sequences.

Translational Efficiency of the Human p53 5’ UTR in Vivo

To assess the ability of the human p53 5’ UTR to decrease translation in vitro, transient co-transfection experiments were performed using pcDNA3.1/5’ UTR-luc plasmids in combination with a ß-galactosidase (ß-gal)-containing plasmid (used for transfection efficiency determinations and normalization). Transient transfections into the p53-null 1299 cell line revealed significant decreases in luciferase expression for pcDNA3.1/5’ UTR-luc plasmids transcribing for mRNAs containing P0-initiated p53 5’ UTR exons 1 sequences 1–103 and 1–220 (P < 0.001 for both mRNAs) as compared to that observed for the pcDNA3.1/luc control (Table 1). In contrast, the pcDNA3.1/p53(104–220)-luc plasmid transcribing for luciferase mRNA containing P1-initiated p53 5’ UTR sequences 104–220 exhibited no significant effect on luciferase expression in 1299 cells. This pattern of inhibition for p53 5’ UTR sequences is consistent with that observed in vitro in rabbit reticulocyte lysates. Similar to that observed previously in vivo (28, 32), the mouse c-myc 5’ UTR exhibited no significant effect on luciferase expression in 1299 cells (Table 1).

Significant decreases in luciferase expression were also observed for plasmids coding for mRNAs containing p53 5’ UTR sequences 1–220 and 1–103 (P < 0.01 for both mRNAs) in both JEG-3 and BEAS-2B cells (Table 1). No effect on luciferase expression was observed for the pcDNA3.1/p53(104–220)-luc plasmid that transcribed luciferase mRNA containing P1-specific p53 5’ UTR sequences. These results, obtained from wild-type p53-containing cell
lines, further demonstrate that P0-specific p53 5’ UTR sequences may be involved in regulating p53 expression and are consistent with that observed in the p53-null 1299 cell line and in rabbit reticulocyte lysates.

Analysis of Plasmids Coding for p53 5’ UTR-Containing CAT Messenger RNAs

Plasmids containing 5’ UTR sequences located upstream of the CAT reporter gene were constructed to determine whether the regulatory effects observed with p53 5’ UTR P0-P1 sequences were affected by other transcribed sequences located either upstream or downstream of the 5’ UTR. 5’ UTR-containing CAT plasmids were constructed using a different vector backbone [pcDNA1(HE)] than that used for the construction of the luciferase plasmids described above (pcDNA3.1). Different in transcribed polylinker sequence and reporter gene, the resulting 5’ UTRs of mRNAs transcribed from the newly constructed pcDNA1(HE)/5’ UTR-CAT plasmids were of similar length to mRNAs transcribed from the pcDNA3.1-based plasmids. As shown in Fig 5B, a virtually identical pattern of inhibition was observed for pcDNA1(HE)/5’ UTR-CAT plasmids when compared to pcDNA3.1/5’ UTR-luc plasmids in 1299 cells, with significant decreases in luciferase expression observed for plasmids coding for CAT mRNAs containing p53 5’ UTR sequences 1–220 and 1–103 but not p53 5’ UTR sequences 104–220. A similar pattern was also observed for in vitro-synthesized p53

FIGURE 4. Schematic representation of p53 5’ UTR-containing plasmids gener-
at for analysis of the effects of the p53 5’ UTR on translational efficiency. Plasmid
nomenclature is indicated on left of panel. Reporter genes are either chloramphenicol
acyetyltransferase (CAT) or luciferase (luc).
Regulating p53 Expression in Normal Versus Tumor Tissue

Stability of p53 5' UTR-Containing Messenger RNAs

To assess whether the observed decreases in translational efficiency were due to differences in mRNA stability, 32P-labeled p53 5' UTR-containing CAT mRNAs derived from pcDNA(HE)-CAT plasmids were incubated in rabbit reticulocyte lysates at 37°C for up to 90 min, purified, electrophoresed in formaldehyde-PAGE, autoradiographed, and analyzed visually and by densitometry. No significant differences in mRNA stability were observed for p53 5' UTR-containing CAT 32P-labeled mRNAs for up to at least 60 min incubations (Fig. 6A). To further assess p53 5' UTR-containing mRNA stability, equimolar amounts of either control-luc or human p53(1–220)-luc mRNAs were incubated in rabbit reticulocyte lysates, and luciferase activity was subsequently measured at 30 min time intervals. Although p53(1–220)-luc mRNA exhibited decreased activity as compared to the control-luc mRNA at all times examined, linear increases in luciferase activity were observed from 30 to 90 min for both mRNAs (results not shown), a pattern consistent with the two mRNA species being translated with different efficiencies but exhibiting no differences in stability. To assess p53 5' UTR-containing mRNA stability in vivo, Northern analysis was performed for p53 5' UTR-containing luciferase mRNAs after transfection of p53 5' UTR-containing luciferase plasmids into cell lines. As shown for JEG-3 and 1299 cells (Fig. 6B), no significant difference in luciferase mRNA levels were observed for any of the cell lines tested. Together, these data strongly suggest that the translational inhibition observed with p53 P0-initiated sequences is not associated with differences in mRNA stability.

Discussion

Human p53 gene transcription is initiated from a number of transcription start sites located within the first half of exon 1 (23, 26). Initial reports suggested that p53 gene transcription began at a site (termed P0), 220 bp upstream of the 3' end of exon 1 (23). In SV40-transformed human fibroblasts, the majority of p53 gene transcription was later shown to be initiated from a cluster of sites (termed P1) located approximately 117 bp upstream of the 3' end of p53 exon 1 (26). p53 transcripts initiated at the P0 transcriptional start site were

Table 1. Fold Inhibition of Luciferase Expressiona for Human p53 5' UTR-Containing Luciferase Plasmids After Transfection Into Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Human p53 5' UTR Sequences</th>
<th>Mouse c-myc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1–220b</td>
<td>1–103</td>
</tr>
<tr>
<td>1299</td>
<td>6.2 ± 0.9e</td>
<td>5.9 ± 0.5f</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>3.3 ± 0.5f</td>
<td>4.0 ± 1.1f</td>
</tr>
<tr>
<td>JEG-3</td>
<td>7.1 ± 0.8e</td>
<td>8.1 ± 2.5f</td>
</tr>
</tbody>
</table>

aFold inhibition of luciferase activity expressed relative to that observed for control-luc mRNA. Data are presented as mean ± SE of a minimum of three experiments.

Numbers indicate nucleotide relative to the p53 P0 transcription start site.

Significant fold inhibition, P < 0.001.

Significant fold inhibition, P < 0.01.
undetectable by primer extension analysis in this cell line. In the present study, results from primer extension, RT-PCR, and slot-blot analysis demonstrate that heterogeneous populations of p53 transcripts differing in 5' UTR composition are detectable at significant levels in normal tissues. Specifically, transcription start sites upstream of P1 are the primary start sites in most normal human tissues. As shown in the present study, P1-initiated p53 transcripts were undetectable in many of the normal human tissues tested, including oral cavity, stomach, liver, lung, and testis. However, in several normal tissues including mammary as well as spleen and thymus, the P1 start site appears to be used in addition to the upstream P0 and P2 sites, indicative of the heterogeneous nature of human p53 gene transcription in normal tissues. In contrast, P1-initiated p53 transcripts were the only detectable p53 transcript observed by primer extension analysis in tumor specimens from multiple human tissue sites including larynx, oral cavity, breast, liver, and lung. The fact that P2-initiated transcripts were detected by RT-PCR but not primer extension analysis for tumor RNA suggests that P2-initiated p53 transcripts may be present at low levels in tumor cells, an observation also made for SV40-transformed fibroblasts in previous studies (26). In addition, only P1-initiated p53 transcripts were detected by primer extension analysis in all cell lines tested in this study, which included both tumorigenic as well as non-tumorigenic, immortalized lines. This pattern is again consistent with previous studies demonstrating that the P1 start site is the primary transcription start site in SV40-transformed human fibroblasts (26).

Studies performed both in vitro (rabbit reticulocyte lysates) and in vivo (cell lines) suggest that sequences between P0 and P1 in the p53 5' UTR (nucleotides 1–103) significantly decrease mRNA translational efficiency. Similar to that observed for MCF-7 cells (33), no significant effect on

**FIGURE 6.** Stability of human p53 5' UTR-containing mRNAs. A. Stability of human p53 5' UTR-containing CAT mRNAs in rabbit reticulocyte lysates. Representative autoradiographs of formaldehyde agarose gel-electrophoresed purified 32P-labeled human p53 5' UTR CAT mRNAs after incubations in rabbit reticulocyte lysates for 0, 30, and 60 min. Equal counts per minute (cpm) of 32P-labeled mRNAs were loaded onto gels before electrophoresis. B. Stability of human p53 5' UTR-containing luciferase mRNAs after transfection into JEG-3 or 1299 cells. Representative Northern analysis of luciferase (top panels) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; lower panels) mRNAs after co-transfection of pluc vectors in a 5:1 ratio with pCMV-β-gal into 1299 or JEG-3 cells. The Northern analysis of GAPDH was performed for the same membranes used to probe for luciferase mRNA. Relative densitometric ratios of luciferase:GAPDH probe binding are shown, with the luciferase:GAPDH ratio for cells co-transfected with the pcDNA3.1-luc and pCMV-β-gal plasmids designated as 1.0. Lane 1, cells transfected with pCMV-β-gal only; lane 2, cells co-transfected with the pcDNA3.1-luc control plasmid and pCMV-β-gal; lane 3, cells co-transfected with the pcDNA3.1/Hp53(1–103)-luc plasmid and pCMV-β-gal; lane 4, cells co-transfected with the pcDNA3.1/Hp53(104–220)-luc plasmid and pCMV-β-gal; lane 5, cells co-transfected with the pcDNA3.1/Hp53(1–220)-luc plasmid and pCMV-β-gal. Lanes for JEG-3 and 1299 cells are identical.
translational efficiency was observed for P1-specific sequences (p53 5′ UTR nucleotides 104–220) in any of the experiments performed in these studies. Therefore, these results suggest a potentially important regulatory role for sequences between P0 and P1 within the p53 5′ UTR and, together with the observed differential expression of p53 transcripts in normal versus tumor tissue, suggest that a 5′ UTR-mediated regulatory mechanism may play an important role in the regulation of p53 expression.

A coupled transcription/translation regulatory mechanism where protein levels are altered due to the switching of transcriptional start sites to produce a mRNA with or without 5′ UTR regulatory sequences has been described for other important regulatory proteins including mdm2 (34, 35) and the cyclin-dependent kinase, p18INK4C (36). Therefore, this type of regulatory mechanism may be important in the control of cell proliferation and tumorigenesis. However, the exact mechanism of how p53 P0-specific sequences regulate translation is presently unclear. One potential mechanism is the binding of a possible repressor protein to a P0/P2-specific regulatory sequence, similar to that occurring for ferritin mRNA (37). Alternatively, previous investigators have suggested that the human P0-initiated p53 5′ UTR may form stable secondary structure of sufficient strength to decrease translation of the P0-initiated mRNA (23, 24). However, similar predicted secondary structures are observed for the 103-nt P0-P1 sequence (ΔG = −29.4 kcal/mol) as compared to the 117-nt P1-specific sequence (ΔG = −33.2 kcal/mol) by computer modeling (38). In addition, translational inhibition was observed with p53 P0-specific sequences in cell lines despite the fact that the mouse c-myc 5′ UTR, postulated to contain significant secondary structure, does not inhibit translation in cell lines (present studies, Refs. 28, 32). Furthermore, it has been suggested that a helicase capable of unwinding RNA secondary structure may be overexpressed in cell lines (28, 39, 40), suggesting that secondary structure is not playing a major role in P0-induced inhibition of p53 translation. A third possibility is the presence of upstream AUG (uAUG) codon sequences and open reading frames (uORFs). Several genes such as S-adenosylmethionine decarboxylase (41), mdm2 (42), Fli-1 (43), and AML1 (44) code for mRNAs that encode uORFs that cause ribosomal stalling and result in decreased translational efficiency for the downstream protein-encoding ORF. On analysis of the p53 gene, there are two in-frame upstream ATG sequences located between P0 and P1. In contrast, no upstream ATGs are located downstream of P1. Due to the presence of an in-frame upstream translation TAG stop codon (GTG) downstream of the P1 transcription start site, the P0/P2-specific upstream AUGs result in two uORFs within the p53 5′-UTR that could play a role in overall p53 mRNA translational efficiency. The possibility that uAUGs and uORFs may play a role in p53 5′ UTR-mediated translational regulation and the question of whether both P0- and P2-initiated transcripts contain translationally repressive sequences are currently being examined in our laboratory.

Previous studies have demonstrated that p53 has high affinity for RNA (45), has been detected in polysome preparations (46), and has been shown to decrease the translational efficiency of its own miRNA (47) as well as that of cdk4 (48) and FGF-2 (49) by a direct interaction. Mosner et al. (47) have proposed that mouse p53 protein may bind to the mouse p53 5′ UTR and repress its own translation. It was proposed that endogenous mouse p53 is translocated into the nucleus following cellular insult, relieving the repression of p53 synthesis and therefore enabling newly synthesized p53 to rapidly accumulate (47). In the present study, human p53 5′ UTR sequences 1–220 and 1–103 both repress translation in the p53-null 1299 lung carcinoma cell line. The ability of these sequences to decrease translational efficiency in BEAS-2B and JEG-3 cells is not enhanced by the presence of endogenous wild-type p53. While future studies will assess if human p53 protein interacts with the full-length human P0-initiated p53 5′ UTR, the results from the present study demonstrate that p53-independent mechanisms exist to repress p53 protein synthesis from P0-initiated transcripts.

In normal tissues, p53 levels are low, but p53 protein accumulates after exposure to DNA-damaging agents or during the onset of various physiological processes (3). This accumulation may be facilitated in part by decreased transcription of P0/P2-initiated p53 mRNA and subsequent increased expression of non-5′ UTR-regulated P1-initiated transcripts. We hypothesize that to better resist challenges to genome integrity, normal human cells may attempt to increase p53 protein levels by switching to the P1 transcriptional start site to bypass translational repression of p53 P0-initiated transcripts. Interestingly, the normal tissues examined in the present study were all isolated from disease-free, healthy individuals. In preliminary studies, only P1-initiated p53 transcripts have been observed by primer extension analysis in “normal” tissue specimens obtained from cancer patients during surgery (unpublished results). In addition, only P1-initiated p53 transcripts were observed in immortalized, non-tumorigenic cell lines. Because the immortalization process required to generate a non-tumorigenic cell line may cause sufficient insult to the genome of a normal cell to precipitate a switch from the P0/P2 sites to P1, a protective switch to P1-initiated p53 transcription may be a very early event in the tumorigenic process and may be why it has been difficult for us to establish a cell line system where p53 transcriptional start sites may be modulated (unpublished results). Once an in vitro system is established, further studies examining the timing and regulation of this transcriptional switching mechanism will better enable us to understand this process.

Materials and Methods

Cell Culture

The LNCaP (prostate adenocarcinoma), H460 and H358 (non-small cell lung adenocarcinoma), and MCF-7 (breast adenocarcinoma) cell lines were purchased from the American Type Culture Collection (Rockville, MD). The 1386 (oral squamous cell carcinoma), 1483 (oral squamous cell carcinoma, and MSK-Leuk-1 (oral leukoplakia) cell lines were kind gifts from Peter Sacks (Memorial Sloan-Kettering Cancer Center, New York City, NY); the 1299 lung carcinoma and JEG-3 choriocarcinoma cell lines were kindly provided by Dale Haines (Temple University, Philadelphia, PA) while the BEAS-2B immortalized tracheal epithelial cell line was kindly
supplied by Anders Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). The LNCaP, H460, and H358 lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum; MCF-7, 1299, and JEG-3 cells were grown in DMEM supplemented with 10% fetal bovine serum; BEAS-2B cells were cultured in LHC-9 medium (Biofluids, Inc., Rockville, MD); and MSK-Leuk-1 cells were cultured in Keratinocyte growth medium (complete, modified MCDB 153) supplemented with 15 mg bovine pituitary extract. All cell lines were cultured at 37°C in 5% CO2.

**Primer Extension Analysis**

To evaluate p53 transcriptional start sites by primer extension analysis, total RNA was purified from cell lines as well as normal and tumor tissue specimens purchased from the Cooperative Human Tissue Network (Philadelphia, PA) using established guanidinium isothiocyanate/cesium chloride gradient methodologies (50). p53 transcriptional start sites were also analyzed in selected normal human tissue mRNAs purchased from Clontech. Primer extension was performed using the AMV reverse transcriptase primer extension system (Promega, Madison, WI; protocol supplied by manufacturer) using 20 μg total RNA from cell lines or fresh-frozen normal or tumor tissue or 1 μg Clontech normal tissue mRNA, with antisense primers specific for sequences at the 5' end of exon 2 of human p53 (p53as; 5'-GGCGGTCCTCCATGGCGATGGACCAGGAGGCA-3'; nucleotides +230–261 relative to the P0 transcriptional start site) or human β-actin (β-actinas; 5'-AAGCGTGTCACCGT-3' nucleotides +196–220 relative to the β-actin transcription start site). Oligonucleotide probes were end-labeled with γ-32P-ATP (3000 Ci/mmOL, New England Nuclear, Boston, MA) before primer extension using T4 polynucleotide kinase. These probes were used in separate RT reactions and the 32P-labeled fragments were32P-labeled by random priming using the Ambion DECAprime kit (Ambion Corp., Austin, TX; protocol supplied by manufacturer). After overnight hybridization of the 32P-labeled probes specific for different regions of the p53 5' UTR. Probes were generated for different regions of the p53 5' UTR exon 1 (probe A, representing p53 mRNAs initiated upstream of the P1 transcriptional start site) as well as the 117 bp located at the 3' end of exon 1 (probe B, representing total p53 mRNA). These exon 1 sequences were isolated by EcoRI digestion of the pCRII-p53(1–103) [probe A] and pCRII-p53(104–220) [probe B] vectors, respectively (see below for vector construction), and were purified by electrophoresis in low melting point agarose (LMPA) followed by digestion in 1 unit of AgarACE (Promega)/200 mg LMPA. Purified exon 1 fragments were 32P-labeled by random priming using the Ambion DECAprime kit (Ambion Corp., Austin, TX; protocol supplied by manufacturer). After overnight hybridization of probe A to the mRNA blot at 65°C, the blot was washed for 20 min in 2× SSC (150 mM NaCl/15 mM sodium citrate), 1% SDS at 65°C. This wash was repeated four times followed by two additional 20-min washes in 0.1× SSC, 0.5% SDS at 55°C. Following visualization by autoradiography, the blot was stripped and then hybridized overnight at 65°C to probe B as described above for probe A. Densitometric analysis of scanned autoradiographs was performed using Alphaimager software (Alpha Innotech, San Leandro, CA). Values were normalized relative to the signal detected for human DNA controls present on the blot.

**Generation of 5' UTR Fragments**

Human p53 exon 1 (220 bp) was PCR-amplified from genomic DNA (previously isolated from a blood sample from a healthy subject) using 300 ng (40–50 pmol) of primers Hp53P0 (sense) and Hp53P220 (antisense; 3'-CCAAATCCAGGGAACCGTGTCACCCG-3'; nucleotides +196–220 relative to

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**Master Blot Screening**

To perform semiquantitative analysis of individual p53 mRNA species in a variety of human tissues, a human mRNA blot (Clontech) was hybridized to 32P-labeled probes specific for different regions of the p53 5' UTR. Probes were generated from both the 103 bp located at the 5' end of human p53 exon 1 (probe A, representing p53 mRNAs initiated upstream of the P1 transcriptional start site) as well as the 117 bp located at the 3' end of exon 1 (probe B, representing total p53 mRNA). These exon 1 sequences were isolated by EcoRI digestion of the pCRII-p53(1–103) [probe A] and pCRII-p53(104–220) [probe B] vectors, respectively (see below for vector construction), and were purified by electrophoresis in low melting point agarose (LMPA) followed by digestion in 1 unit of AgarACE (Promega)/200 mg LMPA. Purified exon 1 fragments were 32P-labeled by random priming using the Ambion DECAprime kit (Ambion Corp., Austin, TX; protocol supplied by manufacturer). After overnight hybridization of probe A to the mRNA blot at 65°C, the blot was washed for 20 min in 2× SSC (150 mM NaCl/15 mM sodium citrate), 1% SDS at 65°C. This wash was repeated four times followed by two additional 20-min washes in 0.1× SSC, 0.5% SDS at 55°C. Following visualization by autoradiography, the blot was stripped and then hybridized overnight at 65°C to probe B as described above for probe A. Densitometric analysis of scanned autoradiographs was performed using Alphaimager software (Alpha Innotech, San Leandro, CA). Values were normalized relative to the signal detected for human DNA controls present on the blot.
the P0 transcriptional start site). Human p53 5′ UTR P0-P1 sequences (base pairs 1–103) were PCR-amplified using primers Hp53P0 (sense) and Hp53P103 (antisense; 5′-CTCAAAACCTTTTAGCCGAGC-3′; nucleotides +84–103 relative to the P0 transcriptional start site) while P1-specific sequences (base pairs 104–220) were amplified using primers Hp53P1 (sense; 5′-CCTCCGAAAGTCCTAGGCC-3′; nucleotides +104–122 relative to the P0 transcriptional start site) and Hp53P220. PCRs were performed as described above in a 100-μl reaction volume containing 100 ng of normal genomic DNA, 20 pmol of both sense and antisense primers, and 1.5 units of Taq DNA polymerase (Roche Biochemicals) with incubations at 100°C for 2 min, 32 cycles of 94°C for 15 s, 58°C for 20 s, 72°C for 20 s, and a final cycle at 72°C for 10 min. Drosophila heat shock protein (DHSP) 5′ UTR sequences were PCR-amplified as described above using the DHSP-containing vector pDM301 (a kind gift from Susan Linquist, University of Chicago; see Ref. 31) with primers DHSPI (sense; 5′-CAAATTGAAATCAACAGCAA-3′) and DHSP2 (antisense; 5′-TGTTTTAGAATCTCTTCTTCT-3′) at an annealing temperature of 48°C. PCR bands were visualized and quantified over UV light after electrophoresis on an 8% polyacrylamide gel and ethidium bromide staining. The sequences of all purified PCR-amplified fragments were determined by direct dideoxy sequencing (52). All 5′ UTR PCR products were independently cloned into the pcP- vector using the TA cloning system (Invitrogen) and then cloned into the pCR II vector using T4 DNA ligase. All 5′ UTR inserts were located immediately downstream of the T7 promoter. All cloned sequences were confirmed by dideoxy sequencing (52).

**Plasmid Construction**

The pcDNA3.1/luc vector was synthesized by unidirectional subcloning of the firefly luciferase gene from the pGEM/luc vector (Promega) into the pcDNA3.1 (+) vector at the NotI/XhoI restriction enzyme sites. To generate p5′ UTR/luc vectors, 5′ UTR sequences (PCR-amplified and subcloned as described above) were unidirectionally subcloned from the pcDNA1(HE) 5′ UTR insert-containing plasmids into the pcDNA3.1/luc vector at the HindIII/BstXI restriction enzyme sites located upstream of the luciferase gene. 5′ UTR-containing pcDNA1(HE)-CAT vectors were generated by unidirectional subcloning of the CAT gene from pCR II-CAT (previously generated by subcloning of the CAT gene from pKSCAT [a kind gift of Dr. Nahum Sonenberg, McGill University, Montreal, Canada] into the SpeI/KpnI restriction enzyme sites of a circularized pCR II plasmid) into the 5′ UTR-containing pcDNA1(HE) vectors described above at the EcoRV/NsiI restriction enzyme sites located downstream of the PCR inserts. Representative control vectors for the 5′ UTR-containing pcDNA3.1-luc and pcDNA1(HE)-CAT constructs contained the reporter gene without gene-specific 5′ UTR sequences.

**In Vitro Transcription**

All 5′-UTR-CAT and 5′-UTR-luc plasmids were linearized with XhoI before in vitro transcription using T7 RNA polymerase. In vitro transcription reactions were performed in the presence of 0.5 mM m7GpppG (Amersham-Pharmacia Biotech, Piscataway, NJ) and carried out as previously described (53), except that the GTP concentration was raised to 100 μM. Transcripts were labeled internally with [5-3H]-CTP (Amersham-Pharmacia Biotech), purified and quantitated as previously described (53), and diluted to 0.2 μg/ml. The integrity of labeled transcripts was routinely verified by electrophoresis under denaturing conditions in formaldehyde-agarose gels followed by autoradiography. All species of mRNA were found to consist of only one species of the expected size.

**In Vitro Translation**

Equimolar amounts of mRNAs were translated in rabbit reticulocyte lysates (DuPont NEN, Boston, MA) as outlined in the manufacturer’s protocol (200 ng 5′ UTR-CAT mRNA/10 μl lysate; 5 ng 5′ UTR-luc mRNA/5 μl lysate). For CAT mRNAs, the amount of CAT protein produced was determined by assaying for CAT enzymatic activity as previously described (54) using [14C]-l-deoxychloramphenicol (Amersham-Pharmacia Biotech) and n-butyryl-CoA (Promega) as substrate and co-substrate, respectively, with incubations at 37°C. For luciferase mRNAs, the amount of luciferase protein produced was determined using a luciferase assay system (Promega, protocol supplied by manufacturer) with a single-tube luminometer with incubations at 37°C for up to 90 min. The activities of gene-specific 5′ UTR-containing CAT or luc mRNAs were expressed relative to the activity observed for the control-CAT or control-luc mRNAs, respectively.

**DNA Transfections**

All cell lines were grown at 37°C in 5% CO2 and plated 24 h before transfection at a density of 0.5 × 106 cells per 100-mm-diameter culture plate. pCAT or pluc vectors (5–12.5 μg) were co-transfected in a 5:1 ratio with a plasmid coding for β-galactosidase (1–2.5 μg pCMV-β-gal; Stratagene, Palo Alto, CA) into 1299 and BEAS-2B cells. Transfections were performed by either calcium phosphate precipitation (BEAS-2B and 1299 cells; Ref. 50), addition of 25 μl of Lipofectamine (Life Technologies; protocol supplied by manufacturer) with a 6-h Lipofectamine exposure period (JEG-3 cells), or electroporation (250 mV/960 μF) in a GenPulsar electroporator (Bio-Rad, Hercules, CA; 1299 and JEG-3 cells). After transfection, cells were incubated at 37°C in 5% CO2 for 3 days, pelleted, and lysed in Reporter Lysis Buffer (Promega). β-Galactosidase activity was assayed using the Galacto-star luminometric...
assay (Tropix, Bedford, MA; protocol supplied by manufacturer). Estimates of transfection efficiency were based on β-galactosidase activity for each plate of transfected cells. Following normalization of homogenate protein levels based on β-galactosidase activity, CAT or luciferase activities were determined as described above with CAT assays performed at 37°C for up to 30 min. The activities of gene-specific 5’ UTR-containing CAT or luc mRNAs were expressed relative to the activity observed for the control-CAT or control-luc mRNAs, respectively.

**Messenger RNA Stability Assays**

To determine the stability of CAT transcripts in rabbit reticulocyte lysates, transcription reactions were carried out in the presence of α-32P-ATP instead of [5'-32P]CTP as previously described (29, 55). After quantification of mRNA transcript levels, equal amounts (20,000 cpm) of 32P-labeled mRNAs were incubated in lysate and purified at various time-points post-incubation by phenol-chloroform extraction (50). For each post-incubation mRNA species, 10,000 cpm of extracted radioactivity were electrophoresed in formaldehyde-agarose gels that were subsequently dried, and mRNA band integrity was examined over time by autoradiography.

To determine the steady state level of luciferase transcripts in cell lines, separate plates were cultured and co-transfected with pluc vectors plus the pCMV-β-gal plasmid (as described above) for determination of luciferase mRNA levels for all transfectants. Overexpressed luciferase mRNA levels were monitored by Northern analysis using a luciferase-specific probe synthesized after restriction enzyme digestion isolation and agarose gel purification of luciferase sequences from the pcDNA3.1-luc plasmid. Probes were 32P-labeled by random priming using the Ambion DECAprime II kit (Ambion, protocol supplied by manufacturer) (50). DNA template for the synthesis of a GAPDH probe was provided by the manufacturer (Ambion) and 32P-labeled GAPDH probe was synthesized according to the manufacturer’s protocols. After overnight hybridization of the luciferase probe to the Northern blot at 65°C, the blot was washed for 20 min in 20 mM sodium phosphate (pH7.4)/0.1% SDS at 65°C. This wash was repeated two times at 23°C. Following visualization using a phosphoimager (Storm 860, Molecular Dynamics, Sunnyvale, CA), the autoradiograph was stripped and then hybridized overnight at 65°C for RNA lanes from cells transfected with the pcDNA3.1-luc plasmid.

**Statistical Analysis**

All comparative activity analysis was performed using the Student t test (two-sided).

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**References**


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