Structure and Regulation of the Mouse ing1 Gene

THREE ALTERNATIVE TRANSCRIPTS ENCODE TWO PHD FINGER PROTEINS THAT HAVE OPPOSITE EFFECTS ON p53 FUNCTION*

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The human ING1 gene encodes nuclear protein p33ING1, previously shown to cooperate with p53 in cell growth control (Garkavtsev, I., Grigorian, I. A., Ossovskyaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998) Nature 391, 295–298). p33ING1 belongs to a small family of proteins from human, mouse, and yeast of approximately the same size that show significant similarity to one another within the C-terminal PHD finger domain and also contain an additional N-terminal region with subtle but reliably detectable sequence conservation. Mouse ing1 is transcribed from three differently regulated promoters localized within a 4-kilobase pair region of genomic DNA. The resulting transcripts share a long common region encoded by a common exon and differ in their 5′-exon sequences. Two transcripts are translated into the same protein of 185 amino acids, the mouse equivalent of the human p33ING1, while the third transcript encodes a longer protein that has 94 additional N-terminal amino acids. Overexpression of the longer protein interferes with the accumulation of p53 protein and activation of p53-responsive promoters after DNA damage. Between the two products of ing1, only the longer one forms a complex with p53 detectable by immunoprecipitation. These results indicate that a single gene, ing1, encodes both p53-suppressing and p53-activating proteins that are regulated by alternative promoters.

The ING1 gene was identified as a result of a functional screening of genes, the suppression of which is associated with neoplastic transformation (1). Inhibition of ING1 expression by antisense RNA promotes anchorage-independent growth in mouse breast epithelial cells, increases the frequency of focus formation in NIH 3T3 cells, and prolongs the life span of diploid human fibroblasts in culture. ING1 expression is up-regulated in senescent human fibroblasts (2), and ectopic expression of ING1 cDNA leads to G1 arrest or promotes apoptosis in several cell types (3). The accumulated observations indicating ING1 participation in the negative regulation of cell proliferation, control of cellular aging, and apoptosis have defined ING1 as a candidate tumor suppressor gene.

We have recently found that the biological effects of ING1 and p53 are interrelated and require the activity of both genes. Neither of the two genes can, on its own, cause growth inhibition when the other one is suppressed (4). Furthermore, activation of transcription from the p21/WAF1 promoter, a key mechanism of p53-mediated growth control, depends on the expression of ING1. A physical association between p33ING1 and p53 proteins is detected by immunoprecipitation. These results indicates that p33ING1 is a component of the p53 signaling pathway and that p33ING1 cooperates with p53 in negative regulation of cell proliferation by modulating p53-dependent transcriptional activation.

Despite the apparent importance of ING1 in the control of cell proliferation, our knowledge of expression, regulation, and function of this gene remains incomplete. Moreover, GenBankTM contains two ING1 mRNA sequences differing in their 5′-ends. The origin of these differences is unknown and requires explanation. So far, all of the information about the function of p33ING1 was obtained from in vitro experiments that involved ectopic expression of ING1 cDNA or its suppression by antisense RNA. The analysis of the structure of the ING1 gene and its regulation in vivo are essential steps toward the understanding of its function and involvement in developmental and physiological processes. This is particularly important due to the cooperation between ING1 and p53, which suggests that the functioning of the p53 signaling pathway could be dependent on the regulation of ING1 expression.

In the present work, we used the mouse ortholog of the human ING1 gene for detailed structural and expression studies, with the goal of subsequently utilizing it as a system for extensive genetic analysis. ing1 was found to be a highly evolutionarily conserved gene with complex regulation, which involves generation of alternative transcripts initiated from different promoters and translated into proteins that differ in structure and expression patterns. Moreover, these proteins have opposite effects on p53-regulated transcription, indicating that ing1 encodes two products with the properties of a putative tumor suppressor and a putative oncogene.

MATERIALS AND METHODS

Plasmids and Libraries—Retroviral vector pLXSN, used for the introduction of mouse and human ING1 cDNA in NMuMG, 10(1), and ConA cells, was provided by A. Dusty Miller (5). Retroviral vector pLXIG was constructed on the basis of pLXSN vector by substituting SV40 promoter and neo sequences with the internal ribosome entry site

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of encephalomyocarditis virus (6) followed by the enhanced green fluorescent protein sequence (7). This vector permits us to translate both the gene of interest (cloned upstream of the internal ribosome entry site) and enhanced green fluorescent protein from a single bicistronic mRNA transcribed from Moloney murine leukemia virus long terminal repeat (LTR) DNA Library. Sequence-specific mRNA in fibroblasts was constructed using SuperScript system (Life Technologies, Inc.) according to the manufacturer’s protocol and cloned into the p phage λ g22A vector. Stratagene’s 129SVJ mouse genomic cloning into the λ FIX II vector was used for the isolation of the mouse genomic, ing1-containing clones.

Cell Lines—cultures of mouse embryonic fibroblasts were obtained from 10-day-old embryos. 10(1) cell line, a derivative of Balb/c 3T3 cells 12-1 (8) with wild type p53, was described earlier (10). It carries the lacZ gene encoding β-galactosidase Escherichia coli under the control of the p53-dependent promoter and therefore allows monitoring p53 transcriptional activation by a routine X-gal staining. All cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplement and were boiled in the same solution containing 1–5 mg/ml RNase A at 37 °C for 30 min, in 2X SSC at room temperature for 20 min, in 2X SSC, 0.04 µg/ml RNase A at 37 °C for 30 min, in 2X SSC at 37 °C for 30 min, in 1X SSC at 60 °C for 15–30 min, and finally in 0.1X SSC at 60 °C for 15–30 min and exposed to x-ray film for 2–7 days.

RESULTS

Multiple Transcripts of the Mouse ing1 Gene Differ in Their 5’-End Sequences—In order to isolate the mouse ortholog of the ING1 gene, we screened a cDNA library prepared from senescent mouse embryonic fibroblasts, using human ING1 as a probe. The choice of the library was determined by the fact that ING1 is expressed at higher levels in senescent, compared with normal, human fibroblasts (2). As a result, several clones were isolated, and the two longest were sequenced. The clones were identical to each other and highly similar to human ING1 through most of their length, except for the 5’-ends, which were different and not homologous to the human gene (Fig. 1A). This observation could be an indication of alternative splicing of mouse ing1; however, it could also be potentially explained by cloning artifacts that occurred during the cDNA library preparation.

To determine the structure of 5’-end sequences of ing1, we used a 5’-RACE technique for the isolation of cDNA sequences corresponding to the 5’-termini of ing1 mRNA. cDNA was synthesized from mouse spleen and brain mRNA and ligated to synthetic adaptors. The cDNA was amplified by PCR using a sense primer specific for the adaptor and an antisense primer specific for the common part of ing1 located close to the divergent region. Two fragments of different size were subsequently cloned, sequenced, and compared with the clones iso-

The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactosidase.
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Fig. 1. Cloning of different variants of mouse ing1 cDNA and identification of their corresponding mRNA transcripts. A, schematic alignment of the mouse ing1 cDNA clones and 5'-RACE products with human ING1 cDNA. 1a and 1b represent mouse ing1 CDAs isolated from the library of senescent mouse embryonic fibroblasts. A 5'-RACE reaction gave two products, one identical to 1b and another shown as 1c. The position of the gene-specific PCR primers used for the RACE reaction is indicated. Mouse ing1 sequences are aligned with human ING1 cDNA. The open reading frame for the human ING1 clone is indicated. B, analysis of ing1 transcription by Northern hybridization. ing1 expression in mouse liver, heart, and testis was analyzed using a multiple-tissue Northern blot (CLONTECH Laboratories), which was hybridized with the probe corresponding to the common part of ing1 (lane 1), the 5'-end of isoform 1a (lane 2), the 5'-end of isoform 1b (lane 3), or the 5'-end of isoform 1c (lane 4). Probes were obtained by PCR using ing1-specific primers. An arrow shows the position of the RNA marker, 2.37 kilobases in size.

lateral from the cDNA library. The results of this comparison are schematically presented in Fig. 1A. One of the 5'-RACE products was identical to one of the previously isolated cDNA clones. Another product revealed the third variant of ing1 cDNA, which again consisted of common and unique regions, with the junction located at exactly the same position as in the other sequences. Fig. 1A shows schematic alignment of the isolated mouse ing1 cDNA clones and the 5'-RACE products. All variants are identical to each other (and homologous to human ING1) up to the same nucleotide and differ only in their 5'-ends.

In order to verify that the observed cDNA structure reflects naturally existing transcripts, we analyzed ing1 mRNA species by Northern hybridization using probes that represent common or specific regions of the isolated cDNAs. As shown in Fig. 1B, the probe for the common region revealed multiple transcripts in RNA isolated from mouse liver, heart, and testis. Probes specific for individual transcripts, however, showed more simple hybridization patterns, which in combination covered the whole set of transcripts found by hybridization with the common probe. These results indicated that the cloned sequences represent the majority of multiple transcripts of ing1 synthesized in the thymus and spleen.

Mapping of Coding Regions of ing1 in Mouse Genomic DNA—Comparison of sequences of individual cDNA clones showed differences in their 5'-regions, suggesting that ing1 has multiple alternative 5'-exons (Fig. 1). To verify this hypothesis, we determined the structure of the mouse ing1 gene. We isolated phage clones carrying sequences homologous to the mouse ing1 cDNA by hybridization screening of a mouse genomic library. These clones were mapped by restriction digestion analysis, in combination with Southern blot hybridization, with the probes corresponding to the different ing1 parts. The interpretation of the results obtained is shown in Fig. 2A.

Comparison of genomic and cDNA sequences of ing1 using PCR with different ing1-specific primers (data not shown) as well as with the sequencing data showed that most of the transcribed sequences of the ing1 gene come from a single exon. Alternative 5'-ends are encoded by different exons positioned upstream from the common exon. Comparison of the sequences of cDNA and genomic clones, as well as 5'-RACE products, revealed three isoforms of mouse ing1 that differ from each other only in their 5'-ends, which also indicates that each isoform is most probably expressed from its own promoter. Southern blot hybridization analysis of ing1-related sequences in the mouse genome indicated that ing1 is a single gene with no obvious close family members (data not shown).

The length of the isolated cDNA clones appeared to be significantly shorter than that of the mRNA species detected by Northern hybridization (Fig. 1B), suggesting that part of the transcribed sequences were missing from the isolated cDNAs. To determine the exact start sites of ing1 transcription, we used a new procedure called “SMART-based 5'-RACE” as described under “Materials and Methods.” Using this method, we were able to extend the cDNA sequences for the 5'-end of isoform 1b. This exon contains an extremely G/C-rich region that blocked DNA elongation during the original 5'-RACE reaction (Figs. 2B and 3) and complicated sequencing of the final, long 5'-RACE product. Therefore, the transcription start site of isoform 1b was not precisely identified, since it was estimated based on the size of the 5'-RACE product and the analysis of the genomic sequence.

Comparing the 3'-ends of human and mouse ING1 cDNAs showed that although the mouse transcript was flanked by a poly(A) stretch, its 3'-untranslated region was significantly shorter than the human one. Analysis of the genomic clone with the mouse ing1 sequence revealed the presence of a long poly(A) stretch that could potentially be used as a primer-binding sequence for reverse transcription initiated from oligo(dT) primers (Fig. 3C). Moreover, the alignment with the 3'-end of the human ING1 cDNA sequence resumes downstream from this genomic poly(A) stretch. The exact end of ing1 transcription in mice was determined using the same method applied for the generation of 5'-sequences. Mouse ing1 cDNA was, in fact, found to be longer than was originally thought (Figs. 2B and 3). Sequence comparison showed that mouse and human ING1 transcripts terminate at the same point and share significant levels of similarity up to the very 3'-ends.

Sequence Analysis of ing1—Nucleotide and predicted amino acid sequences of the three mouse ing1 cDNA isoforms and the promoter regions for all three alternative transcripts are shown in Fig. 3. It was found that ing1 isoforms 1a and 1c have several
FIG. 2. Mapping of mRNA-coding regions within the mouse ing1 locus. A, map of the mouse genomic region containing ing1 cDNA-related sequences. Three ing1-positive genomic clones were isolated from the mouse genomic library using ing1 cDNA as a probe (G2, G9, and G10), and analyzed by restriction digestion and Southern blot hybridization. This analysis, in combination with the PCR data and with partial sequencing of the genomic clones, allowed us to determine the positions of the ing1 exons and the structure of ing1 cDNA clones as demonstrated after cDNA library screening and 5'-RACE analysis. The structure of two known human ING1 cDNA isoforms in relation to mouse ing1 sequences is shown above the genomic DNA. The lower panel demonstrates the final structure of the ing1 gene determined as a result of precise mapping of transcription initiation and termination sites. In the upper panel, the position of the G/C-rich region in the 5'-end of clone 1b and the poly(A) region in the 3' part of the ing1 transcripts are indicated, which interfered with the polymerase chain reaction during the original 5'-RACE.

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Tightly clustered transcription start sites. Sequences upstream of the initiation sites (putative promoters) lack TATA boxes, but they do contain a sequence corresponding to the loose initiator consensus PyPyAN(T/A)PyPy that includes the transcription start sites (Fig. 3). In both cases, areas upstream from the initiator are extremely GC-rich with multiple Sp1-binding sites. All of these features are typical of TATA-less promoters (18, 19). Binding sites of inducible factors that are usually present further upstream in the promoter area (e.g. CAAT box, Oct-1 and Oct-2 binding site, NF-kB, or ATF binding site) were not found in these promoters. Transcription of ing1 isoform 1b is estimated to start approximately 30 nucleotides downstream from the TATA-like box. A putative CAAT box is present 60 nucleotides upstream from the TATA-like sequence. Since the transcription initiation sites for isoforms 1a and 1b are only about 200 base pairs apart, there is a possibility that their promoters share some of the regulatory sequences including, for example, a number of Sp1 binding sites located upstream of this area.

All three alternative transcripts of mouse ing1 contain the same long open reading frame, although the sizes of the predicted proteins are different. While isoform 1b encodes a protein of 279 amino acids, the other two isoforms are predicted to encode a shorter protein product of 185 amino acids, which lacks 94 N-terminal amino acids. Translation of these two products is expected to start from the initiation codon, which is located at the beginning of the common exon. Alignment of the predicted amino acid sequences with that of human p33ING1 (GenBank™ accession number AF044076) revealed high similarity between the mouse and human proteins (89% sequence identity) (Fig. 4).

The nonredundant protein sequence data base at NCBI was searched using the ing1-encoded protein sequence as a query, and a highly significant sequence similarity was detected with a human paralog of p33ING1. These included three uncharacterized proteins from the budding yeast Saccharomyces cerevisiae and their homologue from fission yeast Schizosaccharomyces pombe (probability of the similarity being observed by chance <10^-12). All of these proteins are approximately the same size and contain a C-terminal PHD finger domain (20, 21). The sequence conservation in the PHD domain in these six protein sequences is striking. There are 13 invariant residues in addition to the eight metal-chelating cysteines and histidines that are conserved in all PHD fingers (Fig. 4B). Multiple alignment analysis resulted in the delineation of an additional N-terminal region that is conserved in these proteins (Fig. 4B). This region consists of approximately 100 amino acid residues, includes two distinct conserved motifs, and shows a fairly subtle similarity that was not statistically significant in the context of the screening of the complete data base, except for the conservation between the two human paralogs. However, in the reduced search space defined by the presence of the PHD finger, it was shown that the probability of finding this level of similarity by chance was less than 10^-13 for the distal motif and 10^-5 for the proximal motif.

Expression of ing1 Transcripts in Adult and Embryonic Mouse Tissues—We analyzed the expression of ing1 mRNA in the organs of adult mice and mouse embryos at different stages of development by Northern blot hybridization. The probe representing the common exon of the ing1 gene revealed multiple mRNA size classes that represent alternative transcripts of ing1 (Fig. 5A). Overall ing1 mRNA expression is most abundant in thymus and testis. Much lower levels were detected in the rest of the tissues tested, and they also differed in the content and relative intensity of the hybridizing bands. The same pattern of ing1 mRNA expression was observed in p53-
FIG. 3. Sequences of the putative promoter areas of the ing1 gene, ing1 cDNAs, and their predicted protein products. A, sequence of the genomic region with the putative promoter areas of cDNA isoforms 1a and 1b; the start sites of transcription of these two variants are marked by arrows (the start site of isoform 1b was estimated based on the size of the 5'-RACE product and the analysis of genomic sequence). The initiator sequence of isoform 1a, including multiple initiation start sites, is underlined; TATA-like sequence for isoform 1b is shown in boldface type. The underlined boldface italic sequence is the CAAT box positioned about 100 base pairs upstream from the 1b transcript initiation site. Sp1 binding sites are displayed in boldface italic type.

B, sequence of the putative promoter area of isoform 1c, with the underlined initiator sequence.
deficient mice, which showed no direct effect by p53 on ing1 regulation (data not shown). In embryos, the highest expression was found on the 11th day of development and was characterized by changes in the relative expression of different classes of mRNA transcripts.

Patterns of ing1 expression in embryogenesis were also checked by in situ hybridization. We used the histoblotting technique, in which embryonic sections were fixed on a nitrocellulose membrane and then hybridized with the RNA probes corresponding to the common part of the ing1 cDNA. Besides sense and antisense ing1-specific RNA probes, we also used an antisense probe for β-actin, a gene with ubiquitous expression, as an internal standard. Sections were prepared from 10-, 12-, 16-, and 18-day embryos. Results of in situ hybridization were shown in Fig. 5C. ing1 is uniformly expressed in the whole mouse embryo at all stages of development examined. This is in agreement with the result obtained from the adult mouse tissues, where ing1 is expressed ubiquitously and at similar levels in all organs analyzed. However, in day 10 embryos, higher expression levels were observed in the yolk sac, while at day 16 and 18 of development, higher levels of expression were detected in inner compartments of bones and probably match areas of ongoing ossification.

In order to determine patterns of expression of different ing1 transcripts in mouse tissues and during embryogenesis, Northern blots were hybridized with probes corresponding to the alternative start sites of ing1 (Fig. 5, A and B). Isoforms 1b and 1c were expressed ubiquitously in all adult mouse tissues tested, with the highest levels in thymus. Both isoforms were expressed in the embryos at all stages of development analyzed, with the highest levels at day 7 in the case of isoform 1c or day 11 in the case of isoform 1b. Of all adult tissues overlapping the two transcription start sites. Multiple Sp1 binding sites are also indicated. C, sequences of ing1 cDNAs and their predicted protein products. Sequences of the alternative 1a, 1b, and 1c first exons as well as the common ing1 exon are shown. The first ATG codon of isoform 1b is underlined as well as the ATG codon in the common exon that is used as the initiation codon for 1a and 1c translation. Stop codons, indicating the end of translation, are also underlined.

FIG. 4. Protein sequence analysis of p37ING1. A, alignment of amino acid sequences of mouse ing1 1b isoform and its human ortholog. Numbers of amino acids are indicated; numbers in parenthesis indicate the size of the truncated protein product of 1a and 1c ing1 isoforms. The underlined methionine in the mouse sequence indicates the beginning of the protein product encoded by isoforms 1a and 1c. The asterisks indicate identical amino acids, while dots indicate conserved changes in amino acid sequence. B, a multiple alignment of p33ING1, its yeast homologs, and additional PHD finger-containing proteins. The aligned conserved blocks are separated by variable spacers whose lengths are indicated by numbers; for the N-terminal block, the distance to the protein N termini is indicated (the sequence of the human paralog of p33ING1 is incomplete). Consensus1 shows amino acid conservation in the p33ING1 family of proteins; Consensus2 shows the conservation in an expanded set of PHD finger proteins (in addition to the six proteins of the p33ING1 family, the sequences that aligned with p33ING1 with a probability of a random match below 10^-14 in the first iteration of the PSI-BLAST analysis were included). Each consensus shows amino acid residues conserved in all sequences of the respective set; h indicates a hydrophobic residue, p indicates a polar residue, s indicates a small residue, and a indicates an aromatic residue. The metal-chelating residues in the PHD finger domain are shown by white type on black. Each protein is identified by a gene name followed by species abbreviation and the gene identification number from the nonredundant protein data base at the NCBI. p33ING1p is the human paralog of p33ING1, Mi-2 is a human autoantigen, and CHD3 is a chromatin-associated helicase of the SNF2 family; the remaining proteins are uncharacterized gene products designated by their systematic gene names. Species abbreviations are as follows. Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Ce, Caenorhabditis elegans.
analyzed, mRNA for isoform 1a was expressed only in testis and also in the 11-day embryo. While there is no detectable signal with the 1a-specific probe in the 7-day embryo, traces of hybridization could be detected in mRNA from day 15 and day 17 embryos, which indicates an extremely low expression level at these points of mouse embryogenesis.

**Indications of Proliferation-dependent Regulation of ing1 Expression**—To check whether ing1 expression was proliferation-dependent, Northern blot hybridization was performed on total mouse RNAs isolated from various organs (A). Similarly, Northern blot analyses were conducted on RNA from mouse embryos at different stages of development (B). In situ histoblot hybridization was also performed on mouse embryos (C).
that cooperates with p53 in transcriptional activation of p53-responsive genes. Its mouse homologue, p31ING1, has a similar biological effect (data not shown) that is consistent with the high degree of identity between the mouse and the human proteins (see Fig. 4). To analyze the influence of the longer product of ing1 on p53 function, we overexpressed cDNA for p37ING1 in mouse cells maintaining wild type p53. To effectively monitor p53-dependent transcriptional activation, we previously characterized ConA cells (see "Materials and Methods"), carrying the p53-responsive bacterial lacZ gene. We transduced ConA cells with retrovirus expressing p37ING1 and enhanced green fluorescent protein from a bicistronic mRNA and generated cell populations with different levels of overexpression of p37ING1 by sorting cells differing in the levels of enhanced green fluorescent protein fluorescence. These cells were compared with ConA cells transduced with either the empty pLXIG vector or with retroviral vector expressing the short protein product of ing1. The expression of introduced constructs was confirmed by Western immunoblotting using an anti-p33ING1 antibody that recognizes both human and mouse proteins (16) (Fig. 7B). p53-dependent expression of lacZ was determined by X-gal staining of ConA cells treated with UV light (Fig. 7A). The expression of p53 protein and p53-responsive p21ING1 protein in untreated and in UV-irradiated cells was estimated by Western immunoblotting (Fig. 7B). A decreased intensity of the p21 band by Western blot and a weaker X-gal staining of ConA cells treated with UV light indicate that p37ING1 clearly inhibited UV-induced accumulation of both p53-inducible proteins. Moreover, overexpression of p37ING1 strongly attenuated accumulation of p53 in response to UV light (Fig. 7B). These observations demonstrate that p37ING1 has a suppressive effect on p53-dependent transcriptional activation supposedly by attenuating p53 accumulation in response to DNA damage. This means that the two proteins encoded by the ing1 gene have opposite effects on p53 function.

It was previously shown that p33ING1 can be detected in cells in a complex with p53 by co-immunoprecipitation (4). Since at that time it was not known that ING1 encodes two different proteins, it was unclear which of the two products of ING1 was detected in these experiments. We therefore compared the ability of short and long ING1-encoded proteins to form a complex with p53 by using immunoprecipitation. Since the protocol of immunoprecipitation was optimized for human proteins, we analyzed human variants of ING1 proteins expressed in human cells by transient transfection. p53-deficient Saos-2 cells were used in these experiments that have both alleles of the p53 gene deleted and presumably retain an intact p53 pathway. They were co-transfected in different combinations with the expression plasmids encoding wild type p53 and the short or long products of ING1. Cell lysates were treated with anti-p53 antibodies (see "Materials and Methods"), and the presence of ING1-encoded proteins in precipitates was detected by Western immunoblotting (Fig. 7C). The results obtained showed that although p33ING1 is co-precipitated with p53, the shorter ING1 product is not detectable in the p53 precipitate. This observation suggests that differences in the effect of the two ING1 products on p53 function could be a reflection of differences in their interaction with p53 in the cell.

**DISCUSSION**

**Multiple Products of the ing1 Gene—ING1** was originally described as a gene whose suppression promotes neoplastic transformation (1). Consistent with that, ectopic expression of the first isolated human cDNA was growth-suppressive for different cell lines. **ING1** was found to encode a nuclear protein termed p33ING1 that was shown to functionally and physically interact with p53 in cell growth regulation (4, 22). Now, after

**FIG. 6. ing1 mRNA and protein expression varies depending on cell growth conditions.** A, RNA was isolated from dividing 10(1) (lane a) and NMuMG (lane c) cells, as well as from the dividing young mouse embryonic fibroblasts (lane h). RNA was also isolated from contact-inhibited 10(1) (lane b) and NMuMG cells (lane f); serum-starved 10(1) cells (lane c) (64 h at 0.5% FBS); γ-irradiated 10(1) cells (lane d) and NMuMG cells (lane g); and senescent MEFs (lane i). Filters were probed with total ing1 probe. The bottom panels show RNA gels from which Northern blots were made. The arrows indicate positions of the 2.37-kilobase RNA marker (upper panels) or 28 and 18 S rRNAs. B, detection of ing1-encoded proteins by Western immunoblotting, using antibodies against p33ING1. Cell lysates were prepared using radioimmune precipitation buffer from the cells treated in the same way as explained above. As a control, cell lysates were prepared from 293 cells transfected with the plasmids expressing mouse 1b ing1 variant (lane a) or the human homologue of p31ING1 that produces the protein product of the same size as mouse 1c and 1a variants.
we characterized the structure and expression of the mouse ing1 gene, it became clear that ing1 regulation is more complicated than was originally thought. ing1 is transcribed from at least three differently regulated promoters, and the resulting transcripts encode at least two different proteins.

A similar type of regulation (alternative initiation leading to variability of 5′-exons) found in other tumor suppressor genes, namely BRCA1, APC, and INK4 (23–26), is associated with the generation of proteins with different functions (in the case of INK4, different reading frames are translated in the alternative transcripts). Consistently, the two products of ing1 have opposite effects on p53-dependent transcription regulation; one acts as a p53 cooperator, while the other acts as a p53 suppressor. All of the transcripts share a common region encoded by a common exon but differ in their 5′-exons. Two of these alternative exons do not contain protein-coding sequences (isoforms 1a and 1c), while the third one does (isoform 1b). Consistently, one of the ing1 transcripts encodes a 37-kDa protein (p37ING1), while two others are translated into a shorter protein of 24 kDa that surprisingly runs as if it was 31 kDa (p31ING1). Structures of mouse and human ing1 genes are likely to be similar, considering the high degree of evolutionary conservation of ing1 sequences and the fact that the two versions of human ING1 that are currently available in GenBank™ also share a large common part but have different N termini. It is noteworthy that one of the human variants of ING1 (GenBank™ accession number AF001964) does not have a homologue with similar 5′-sequences among the identified mouse ing1 isoforms, which raises the possibility that there could be more variants of mouse ing1 transcripts that so far have not been isolated.

p33ING1 and its homologs in such a distantly related species as yeast contain a remarkably conserved PHD finger domain and an additional, weakly conserved domain of unknown function. PHD fingers have been shown to bind DNA (21, 27), but to our knowledge there is no evidence that they mediate protein-protein interactions. Therefore, it seems likely that the C-terminal PHD domain in the p33ING1 family of proteins is involved in specific DNA binding that may be important for transcription regulation. Given the outstanding conservation of this domain across the large phylogenetic distance that separates humans and yeast, the specificity of DNA binding with respect to particular binding sites is expected to be conserved either in terms of DNA sequence, distinct features of chromatin structure, or both. It may be further surmised that the conserved N-terminal domain is involved in specific protein-protein interactions that couple transcription regulation by p33ING1 and its homologs to other elements of cell cycle control. The conservation of domain organization in p33ING1 and its yeast homologs is particularly notable given that yeast does not have any homologs of p53. Thus, it appears that p33ING1 is an ancient cell cycle regulator whose interaction with p53 is a later evolutionary addition.

**Regulation of ing1 Expression**—The results of expression analysis indicated that mouse ing1 is a subject of regulation both at the protein and mRNA levels and that different isoforms have different expression patterns. Thus, p37ING1 is ubiquitously expressed in all tissues analyzed with elevated mRNA expression levels in the thymus, while levels of the p31ING1 protein vary dramatically among organs and cell lines.

Sizes of the ing1 mRNA transcripts detected by Northern hybridization correlate well with the length of cDNA sequences isolated from all of the tissues except testis. Probes specific for each of the identified splice variants reveal multiple RNA
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bands, indicating a variability of the ing1 transcripts in this organ. It is likely that in testis ing1 mRNAs are either terminated or processed differently from the rest of the tissues.

There is a clear link between the proliferation rate of the cell and ing1 expression both at the protein and mRNA levels. This observation may reflect cell cycle dependence of ing1 transcription detected earlier for the human ING1 gene; the biological significance of this regulation is not obvious so far. We failed to observe up-regulation of mouse ing1 in senescent cells as it has been reported for human ING1 (8), which suggests that regulation patterns of mouse and human ing1 genes may not always be the same.

ING1 and p53: Cooperators or Antagonists?—Human ING1 has been known as a cooperator of p53 involved in negative growth regulation (1, 4), apoptosis (3), and senescence (2). This has been known as a cooperator of p53 involved in negative ways be the same.

This gene rearrangement in one neuroblastoma cell line, allow ING1 to be defined as a candidate tumor suppressor gene (p53 inhibitor). This means that a simplified view of the potential role of ING1 in cancer should be revised. In the case of ING1, we may have a new genetic mechanism of promoting cancer that involves an imbalance between the two products of one gene. Further characterization of the biological activity of the long product of ING1, p37ING1, indicated that it can affect the whole spectrum of cell properties known to be controlled by p53, including sensitivity to apoptosis, replicative senescence, cooperation with dominant oncogenes, and drug response.

Thus, overexpression of the longer product of ING1, which functionally acts as a p53 suppressor, could be the mechanism of attenuation of p53 activity in tumors that do not require mutations in p53 itself.

A large scale analysis of clinical samples is needed to conclude whether this hypothesis is correct or not. However, any attempts to investigate the potential cancer relevance of ING1 should involve separate analysis of the alternative isoforms of this gene. It has already become obvious that a direct measurement of the overall mRNA expression in tumors cannot be used as an approach to judge the involvement of ING1 in carcinogenesis. The ratio between expression levels of the two ING1 products seems to provide much more meaningful information. It is therefore essential to establish such assays that would distinguish between the two protein products or among the alternative ING1 transcripts.

Although the mechanism of p53 modulation by the two products of the ING1 gene remains unknown, we hope that the two new observations described in this work will shed light on this problem. We found that (i) p53 is found in a complex with the long but not with the short product of ing1 and (ii) overexpression of the longer product inhibits accumulation of p53 protein after DNA damage. Both properties of p37ING1 clearly resemble those of the natural p53 antagonist, Mdm2, which was shown to affect nuclear localization of p53 (28, 29) and to promote its proteasomal degradation (30, 31). All of these possibilities should be tested for p37ING1 as well as a potential interdependence of the biological activity of these two proteins.

Is the function of ING1 limited to its interaction with the p53 signaling pathway? It seems doubtful, considering the high evolutionary conservation of ING1. In fact, ING1 homologues found in yeast (Fig. 4), which does not have detectable p53-related genes, suggest some important p53-independent role for ING1. The phenotypes associated with the knockout of ING1 in yeast and mice may provide a lead toward its currently unknown cellular function. It is noteworthy that a gene knockout approach will be complicated by the necessity to separately inactivate expression of each of the two products of ING1.

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