In Vivo Cloning and Characterization of a New Growth Suppressor Protein TOE1 as a Direct Target Gene of Egr1*

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Egr1, an immediate early transcription factor, responds to diverse stimuli and affects gene transcription to accomplish its biological effects. One important effect of Egr1 expression is to decrease the growth and tumorigenic potential of several tumor cell types. To identify important Egr1 target genes, we have adapted a methodology involving formaldehyde-induced protein-DNA cross-linking, chromatin immunoprecipitation, and multiplex PCR. Using this approach, we report the cloning of a new Egr1 target gene that is able to account, at least in part, for the growth inhibitory activity of Egr1. We have named this new protein TOE1 for target of Egr1.

A common feature associated with the expression of immediate-early genes is their rapid, transient response to a diverse variety of extracellular signals. We have been studying the properties of the early growth response gene, Egr1, which can be transcriptionally induced by a wide spectrum of stimuli including growth factors, cytokines, stresses, depolarizing stimuli, phorbol esters, vascular injury, and irradiation, both ionizing and nonionizing, in a rapid and transient manner with kinetics mirroring those of c-fos (1). We have previously presented evidence suggesting a role for Egr1 in suppressing tumor cell growth (2, 3). Specifically, we demonstrated that overexpression of Egr1 in transformed cells suppresses growth in soft agar as well as inhibits their tumor formation in nude mice. Furthermore, it was shown that the DNA-binding domain of Egr1 is necessary for its ability to suppress tumor formation, highlighting the importance of its transactivation of downstream genes in this process (4). Together these results indicate that transformed cells can be induced to revert to normal growth patterns following the re-expression of Egr1. These studies suggest that the loss of Egr1 may result in the loss of cellular homeostasis because of a deficit in Egr1-responsive genes and that this may play a pivotal role in tumorigenesis. Clearly, the identification of a genetic profile of Egr1-responsive genes would constitute a significant step in understanding the different activities associated with Egr1, including its role in cellular growth control. Over the past several years there have been numerous studies identifying various individual Egr1 target genes in diverse cell and tissue types. Reported Egr1 targets include TGF-β1, platelet-derived growth factors A and B, basic fibroblast growth factor, tissue factor, interleukin 2, and CD44 to mention only a few (reviewed in Ref. 5). These studies have focused on the in vitro analysis of an individual target gene in a specific cell type under a defined set of experimental conditions. As a step toward a more complete understanding of the biological role for a transcription factor, it would be informative to be able to identify in vivo target genes.

Currently, few techniques are available to address this issue. Both differential display and subtractive hybridization analyses are aimed at isolating messages that are up- or down-regulated from pools of RNA isolated from cells or tissues either positive or negative for the gene in question. One clear drawback with both of these techniques is that they select for any RNA message that shows a change in expression pattern. Therefore, when screening for changes in gene expression induced by a transcription factor, these methods do not select purely for direct targets. Recently we and others have described a method for the direct isolation of protein-bound DNA involving formaldehyde followed by immunoprecipitation from chromatin (ChiP). This method was successfully used in applications ranging from examining chromatin structures surrounding the polycistron group proteins during Drosophila development (6) and the identification of nuclear matrix attachment sites (7) to the isolation of DNA sequences bound by Egr1 (8). In addition, the same cross-linking method has been used to examine nucleosomal structure, transcription factor occupancy of promoter sites, regions of histone acetylation, and mapping of telomere silencing protein binding, illustrating its broad application utility (9–12). Recently, coupling the ChiP approach with hybridization to genomic or promoter region DNA microarrays has allowed a comprehensive characterization of in vivo transcription factor DNA binding patterns (13–16).

In this report we have extended ChiP technology, allowing gene discovery of Egr1 target genes by multiplex PCR. Moreover, we present the cloning of a newly identified gene, called TOE1, as an Egr1 target gene. We have characterized TOE1 as a cell growth inhibitor by altering the cell cycle through the induction of p21. Furthermore, we show that the increase in the p21 level is consistent with a mechanism involving TGF-β1.

MATERIALS AND METHODS

Cells, Transfection, Antibodies, and Growth Assays—Both the H4 clone derived from the human fibrosarcoma cell line HT1080 and the

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† The abbreviations used are: TGF, transforming growth factor; ChiP, chromatin immunoprecipitation; RT, reverse transcriptase.

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Egr1 stably transfected H4 subclone E9 have been previously described (4). 293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. All of the DNA transfections were performed using LipofectAMINE 2000 (Invitrogen), following the manufacturer’s instructions. Antibodies against cdc2, phospho-cdc2( Y15), and phospho-p38 (S165) were from Cell Signaling Technology. Antibodies against cyclin B1, p21, and p53 were from Santa Cruz Biotechnology. Anti-actin and the M2 monoclonal anti-FLAG antibody were from Sigma. For cell growth assays 20 × 10^6 control and TOE1 expressing 293 selected and pooled clones were seeded into 96-well plates in triplicate. At the indicated times, cell growth was determined using the CellTiter Cell Proliferation Assay (Promega).

In Vivo Formaldehyde Cross-linking and Chromatin Immunoprecipitation—In order to identify and chromatin immunoprecipitation were performed as previously described (6, 8). Briefly, the cells were grown in 150-mm plates to 80% confluence and then cross-linked by the addition of buffered formaldehyde to a final concentration of 1%. Following exposure to formaldehyde at room temperature for a period of 30 min, the cells were lysed by sonication and chromatin purified by centrifugation through a 5–8 mL gradient in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Purified chromatin was dialyzed against 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5% glycerol to remove the urea. Samples of 30–60 µg of chromatin were digested with 10 units of EcoRI overnight at 37°C and then precleared by the addition of nonimmune rabbit serum and protein A-Sepharose beads. The precleared samples were incubated with affinity purified anti-Egr1 antibodies and protein A-Sepharose beads (17). DNA fragments cross-linked and co-precipitating with Egr1 were purified and ligated to EGR1 linkers consisting of 5′-AATCCGAAGTTGGATCCGACG-3′ and 5′-CTGGCTGGATCAGCTTTG-3′. Following ligation, the products were amplified using the 20-mer as primer. Amplification conditions were 95°C for 30 s, 65°C for 30 s, and 72°C for 4 min for 30 cycles. For direct amplification of the ChiP samples, no linker ligation was performed, and direct amplification from the Egr1 immunoprecipitates was done using specific primers for TOE1 (see below), TGF-β, and cyclophilin. The TGF-β primers used were 5′-GGGCTAGGAAGGGACCCCTCCT-3′ and 5′-TCTCGGGGACTCTTCTCCT-3′. The cyclophilin primers used were 5′-GTCGTAGTTTGGTCTGTCAG-3′ and 5′-CACCACATGCT- GCAATC-3′.

Library Multiplex PCR and TOE1 cDNA Cloning—Following amplification of linker-ligated products as described above, the linkers were removed by EcoRI digestion, and the products were purified using a PCR product purification kit (Roche Molecular Biochemicals). Multiplex PCR was performed using 100 ng of PCR products as the 5′ primer mix and a T7 oligonucleotide as the 3′ primer, with 100 ng of an undifferentiated NT2 cell cDNA library (Stratagene). 30 cycles of hot start PCR were performed using the following parameters: 95°C for 1 min for 25 cycles. Following the RT reaction for 30 min, the PCR conditions were 95°C for 45 s, 65°C for 30 s, and 72°C for 2 min. A 2- kilobase pair band derived from the multiplex PCR was excised from the gel, eluted, cloned into the pGEMT vector (Promega), and sequenced. Data base homology searches were performed using the BLAST program to confirm the full-length TOE1 cDNA. TOE1 was amplified using the forward multiplex PCR reaction for TOE1 expression vector was generated by PCR using the following primers: 5′-CCGAGACCTTGTGATTA- CAAGGACGACGACGAAATGCGCAGTG-3′ incorporating the FLAG epitope tag and 5′-CCGGATATCTACGGATCCCGACAGTGC-3′. PCR was performed for 30 cycles of 95°C for 45 s, 62°C for 30 s, and 72°C for 2 min. The PCR product was digested with HindIII/EcoRI and cloned into the same site in pcDNAIII. All of the constructs were sequenced.

Cloning of the TOE1 Proximal Promoter and Luciferase Assays—The proximal region of the TOE1 cDNA sequence was cloned from human genomic DNA using the Advantage-GC genomic PCR kit (Clontech). Primers used for PCR were 5′-GGCGGAGACGCTTACACC-3′ and 5′-GGGTTAAGACGCGCCATG-3′. TOE1 PCR parameters were 95°C for 45 s, 60°C for 30 s, and 72°C for 1 min for a total of 30 cycles. This generated a 580-bp product immediately 5′ of the initiation codon. The PCR product was digested with KpnI and Hpal and cloned into the KpnI and SacI sites of pGL3basic (Promega). 293 cells were transfected in 12-well plates with a total of 500 ng of DNA using LipofectAMINE 2000 (Invitrogen). Transfected DNA consisted of expression vector DNA, 200 ng of reporter DNA, and 100 ng of cytomegalovirus-β-galactosidase DNA for normalization. 24 h after transfection, the luciferase assays were performed as described (8).

Mutagenesis—to generate the TOE1 expression construct without the putative nuclear localization signal, QuikChange mutagenesis (Stratagene) was performed. The primers used were 5′-GGCGGAGAGGAGGTATTAGACACTA-3′ and 5′-TAGGTCTCATTAAAGGCCTTC-CTGCCGC-3′. Construction of the correct deletion was confirmed by sequencing.

Gel Shift—The gel shift assay was performed as previously described (8) using the 580-bp radiolabeled TOE1 promoter region described above as a recombinant Egr1 protein.

Confocal Microscopy—Control and TOE1 expressing H4 cells were dually stained with rabbit anti-FLAG (Affinity Bioreagents) and mouse antibody to nucleolin (Santa Cruz Biotechnology) antibodies. Secondary labeling was performed using fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch).

Flow Cytometry—The cells were harvested and fixed in 70% methanol and stored at −20°C until all of the samples were collected. The cells were collected by centrifugation at 2000 × g for 3 min, and the cell pellets were suspended in phosphate-buffered saline, digested with RNase A, and stained with propidium iodide.

Northern Blotting—A human Multiple Tissue Northern blot (Clontech) was hybridized with a PCR-generated TOE1-specific 32P-labeled probe using the primers 5′-AACCGGAGACCAGGCAGCC-3′ and 5′-GTTAGGGTTACATGCTGCC-3′ following the manufacturer’s instructions.

RT-PCR—To detect TOE1 expression following Egr1 transfection, total RNA was harvested from transfected cells using Tri Reagent (Molecular Research Center). Following DNase I treatment, 2 µg of RNA was used for reverse transcription using Moloney murine leukemia virus reverse transcriptase (New England Biolabs). TOE1 expression was then assessed by PCR using the same primers described above for Northern probe preparation, and glyceraldehyde-3-phosphate dehydrogenase expression was determined as a loading control using the primers 5′-AACATGAGAATGTCGACAC-3′ and 5′-GTCATACCGAGAATTGACCT-3′. Expression of the p21 gene was determined using the primers 5′-CTCAATACGTCGAGCCTCT-3′ and 5′-ACAGCTCATGTTGGAAAACGGA-3′. TGF-β1 expression was assessed using the primers 5′-GCGCTGGACACTATGTGC-3′ and 5′-AGGCTCGAATTGAGGAGCAG-3′, and cyclophilin A was amplified using the primers 5′-CTCCTTTGTGCGGTTCCTG-3′ and 5′-CACCACATGCTGTCACCT-3′. PCR conditions were 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 25 cycles.

Real time PCR reactions were performed using the one-step RT-PCR SYBR green kit from Roche using a Roche Light Cycler instrument. Following the RT reaction for 30 min, the PCR conditions were 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s for 40 cycles. mRNA quantitation was performed by measuring cyclophilin mRNA levels against a standard curve measurement of cyclophilin mRNA from a control sample. The primers used are described above.

In Vitro Kinase Assay—In vitro phosphorylation was performed as described (18).

RESULTS

Cloning of TOE1—We have previously characterized a clone of HT1080 cells, called H4, as a cell line that does not express either basal or UV-induced Egr1. We have also described a series of stable transfected Egr1 clones (19). We used the clone with the maximum expression of Egr1, termed E9, to isolate and identify in vivo Egr1 target genes. We performed formaldehyde cross-linking on untreated and UV-stimulated cells followed by chromatin immunoprecipitation as described earlier (8). Because it is generally accepted that Egr1-binding sites usually occur within the proximal promoter region of genes, our immunoprecipitated Egr1-bound sequences are likely to consist of predominantly promoter regions with extensions into the 5′-untranslated region and even into the coding region. To identify target gene sequences we performed multiplex PCR using our immunoprecipitated Egr1-bound DNA sequences as 5′ multiplex primers. As the template we selected a cDNA library and used a T7 primer that anneals 3′ to all cDNAs permitting full-length cDNA amplification. Using DNA captured from E9 cell Egr1 immunoprecipitates, we found that multiplex PCR-amplified products only in the presence of the multiplex primers, cDNA library, and the 3′ T7 primer (Fig. 1A, lane 2). When multiplex primers derived from UV-treated E9 cells were used, on occa-
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We focused on the distinct DNA band amplified using primers isolated from E9 cells and migrating with an approximate size of 2 kb (Fig. 1A, lane 4). Cloning and sequencing of this DNA revealed an open reading frame coding for a predicted polypeptide of 510 amino acids and with a predicted molecular mass of ~58 kDa. To confirm that this clone represented a full-length cDNA, we performed 5’ rapid amplification of cDNA ends. Sequencing results confirmed that the captured sequence represented a full-length cDNA clone. A database homology search of the DNA sequence identified the chromosomal map position on human chromosome 1 (1p34.1–35.3). Comparison of the sequence of this region of chromosome 1 to our cloned cDNA identified an 8-exon gene. BLAST homology searches (20) revealed no extended homology with any known protein. However, a potential single zinc finger was noted as well as a possible nuclear localization signal.

To show that the clone represented an expressed gene, a multiple tissue Northern blot was hybridized and showed intense hybridization to a 2-kb mRNA species in six of the 12 tissues with the highest level of expression in placenta, liver, and kidney (Fig. 1B). We cloned the open reading frame of the cDNA, together with a FLAG epitope tag, into a mammalian expression vector and transfected the construct into H4 cells. Western analysis of cells transfected with the FLAG-tagged expression vector and anti-FLAG antibodies showed that the expressed protein migrated on SDS-PAGE with a molecular mass of ~60 kDa, in close agreement with its predicted mass of 58 kDa (data not shown).

TOE1 Is a Target for Egr1 Binding and Transactivation—To confirm the specificity of Egr1 binding to TOE1 in vivo, DNA recovered from immunoprecipitates was PCR-amplified to detect the 5’ region of TOE1. As shown in Fig. 2A we were able to amplify TOE1 from E9 but not from H4 immunoprecipitates. We did, however, confirm the presence of the TOE1 gene in the total chromatin fraction, thus ruling out the formal possibility that the TOE1 gene is deleted in H4 cells. Further, the known Egr1 target gene TGF-β was also amplified from E9 cells (21). The lack of amplification of cyclophilin sequence served as a negative control. This provided evidence that TOE1 was indeed a target of Egr1 in these cells and that the immunoprecipitated

Fig. 2. Egr1 binds to the 5′ region of TOE1 and activates its expression. A. PCR amplification of the TOE1 5′ region from cross-linked chromatin. Either total cross-linked chromatin (Input) or Egr1 immunoprecipitates (Egr1 i.p.) were screened directly for the presence of TOE1 5′ sequences by PCR using primers designed to amplify a 580-bp fragment 5′ of the initiation codon. The same samples were also used for amplifications using primers for TGF-β1 and cyclophilin A. The same primers were used to analyze Egr1 immunoprecipitates from untreated or 12-O-tetradecanoylphorbol-13-acetate-treated MCF7 cells. B. Egr1 expression activates TOE1 expression. RT-PCR amplification of TOE1 from Egr1 transfected H4 cells. Increasing amounts of Egr1 (shown above the lane) were transfected into H4 cells, and total RNA was prepared 24 h later to perform RT-PCR for TOE1. Primers within the coding sequence of TOE1 were designed to amplify a 454-bp product. An equal RNA loading in the RT-PCR reaction was determined using primers amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH). C. Egr1 binds directly to the TOE1 5′ region. The 580-bp region upstream of the initiation codon of TOE1 was used as a probe in the gel shift assay. Increasing amounts of recombinant Egr1 showed the binding to this region. Specific binding was determined by adding either unlabeled homologous probe DNA or nonspecific DNA at a 50-fold molar excess. The positions of the free probe (FP) and Egr1 shift (Egr1) are indicated. D. Egr1 transactivates expression from the TOE1 5′ region. The same 580-bp 5′ sequence from TOE1 was cloned into the pGL3Basic luciferase reporter. Empty reporter vector or the TOE1 reporter in the presence or absence of co-transfected Egr1 expression vector were transfected into 293 cells. 24 h later the cells were harvested and analyzed for luciferase activity. The results have been normalized for transfection efficiency as determined by β-galactosidase measurements. The results are plotted as the average values ± standard deviations. The experiment was repeated three times with similar results.
DNA included the 5’ region of the gene. Because E9 cells constitutively overexpress Egr1, we sought to determine whether TOE1 is an Egr1 target in an alternate cell type upon transient Egr1 induction. MCF7 cells were stimulated to express Egr1 by 12-O-tetradecanoylphorbol-13-acetate treatment, and then the ChiP assay was performed on untreated or 12-O-tetradecanoylphorbol-13-acetate-treated cells. The results shown in Fig. 2A, TOE1 was also an Egr1 target gene in these cells. To determine the role of Egr1 in regulating the transcription of TOE1, we used RT-PCR following transfection with an increasing amount of an Egr1 expression vector and found a proportional increase in TOE1 expression (Fig. 2B).

Direct binding of Egr1 to the TOE1 promoter region was assessed by a gel shift analysis using as probe a region spanning 580 bp upstream of the translation start. Using recombinant Egr1 we found specific binding to the probe (Fig. 2C). When oligonucleotides representing the consensus Egr1-binding site were used as competitor, effective competition was also observed (data not shown). As a test of the functional properties of the complex we inserted the same 580-bp 5’ region upstream of a luciferase reporter. We observed that this region responds to Egr1 expression by activating transcription (Fig. 2D). Together, these results are consistent with in vivo binding of Egr1 to and transactivation of the TOE1 gene.

**Subcellular Localization of TOE1**—To determine the intracellular localization of TOE1, a FLAG-tagged expression construct was transfected into H4 cells. As shown in Fig. 3, following immunostaining for the FLAG epitope, the subcellular localization of TOE1 was distinctly nuclear. Transfection and staining of H4 and 293 cells (not shown) showed patterns of concentrated localization within the nucleus. These sites of concentration appeared to correspond to nucleoli. Dual staining using anti-FLAG and anti-nucleolin antibodies followed by confocal microscopy (Fig. 3) showed that most of the expressed TOE1 co-localized with nucleolin, indicating a predominant nuclear location for TOE1. In addition to its nuclear localization we observed intense staining for TOE1 as multiple nucleolar speckles. As noted above, data base homology searches identified a putative nuclear localization sequence consisting of KRRRRRRRREKRKR located at positions 335–347 in the 510-amino acid protein. Deleting the putative nuclear localization basic stretch of amino acids resulted in the cytoplasmic localization of TOE1 (Fig. 4), suggesting that this sequence is responsible for TOE1 nuclear targeting.

**TOE1 Expression Affects the Growth of 293 and H4 Cells**—To test whether TOE1 might be involved in mediating the growth effects of Egr1, we measured the growth rate of cells stably transfected with a TOE1 expression vector. Fig. 5A shows that the growth rate of TOE1-expressing cells was severely reduced in comparison with empty vector control cells. The doubling time for control cells was ~24 h, whereas a pool of TOE1 expressing clones required 40 h to double in number. Transfection of the same vector expressing the calcium binding protein calbindin had no effect on cell growth (data not shown), suggesting that inhibition by TOE1 was not a nonspecific effect of protein over expression. Similar results were obtained in H4 cells (data not shown).

Cell growth inhibition in TOE1-expressing cells was also examined by performing colony forming assays. Control cells formed numerous rapidly growing colonies, whereas TOE1-expressing cells were only able to form 30% as many colonies (data not shown). To determine whether the decrease in cell growth of TOE1-expressing cells represented a generalized slowing of growth or a cell cycle stage-specific slowing, we performed flow cytometry on log phase cells. We found a significant increase in the fraction of cells present in the G2/M phases of the cell cycle in TOE1-expressing cells (27%), compared with the control cells, with 13% of the cells in this fraction (Fig. 5B). We found no difference between the mitotic index of control and TOE1-expressing cells, suggesting that TOE1 was pausing the cells in the G2 phase (data not shown). In addition, it should be noted that we found TOE1 expression to be highly influenced by the growth state of the cells. Specifically, we have found TOE1 expression to be regulated by cell culture density, possibly indicating a form of activation caused by contact inhibition.2 The expression of TOE1 in dense cell cultures occurred even in cells that cannot express Egr1, indicating that although Egr1 can activate expression of TOE1, the

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2 I. de Belle and J.-X. Wu, unpublished observation.
gene must be subject to additional forms of regulation.

TOE1 Causes an Increase in p21 Expression in H4 Cells—To investigate the mechanism of TOE1 induced G2 phase delay, we performed Western blotting on several G2 cell cycle markers. Fig. 6A shows that there was no significant change in cyclin B1, cdc2, or phospho-cdc2 levels between control, TOE1, and mutant TOE1-expressing cells (with the nuclear localization deleted). This suggested that the activation potential of the G2-specific CDK complex was unaffected by the expression of TOE1. We therefore examined the possibility that the activity of the complex might be modulated by its known inhibitor p21. The level of p21 was dramatically up-regulated in TOE1-expressing cells but not in either control or TOE1 mutant cells. Because p53 is a known transactivator of the p21 gene, we examined the level and activation of p53 in our cells. We were unable to find a significant induction or activation of p53, at least insofar as serine 15 phosphorylation is concerned. Further exploration of the induction of p21 using RT-PCR showed that TOE1-expressing cells up-regulated p21 at the mRNA level (Fig. 6B). This activation was not seen in cells expressing non-nuclear mutant TOE1. To demonstrate that the increase in p21 was functionally associated with an effect on cdc2 activity, we immunoprecipitated cyclin B1 and measured the associated kinase activity in vitro with histone H1 as substrate. Fig. 6C shows a significant decrease in kinase activity only in TOE1-expressing cells, correlating with increased p21 expression in those cells.

Increased TGF-β1 in TOE1-expressing cells—Because Egr1 expression is known to affect TGF-β1 levels (21), we sought to determine whether the increase in p21 levels might be mediated by TGF-β1. Using real time quantitative PCR, we examined the TGF-β1 levels in cells transfected with a TOE1 expression vector. As shown in Fig. 7, using both MCF7 and H4 cells lines, we noted an increase in the level of TGF-β1 mRNA in TOE1 transfected cells compared with control transfected cells.

DISCUSSION

With these studies we report, for the first time, the application of chromatin immunoprecipitation to cDNA cloning using a form of multiplex PCR. We have demonstrated that this technique was successful not only in cloning transcription factor target genes but also in the identification of a new target for Egr1. Together our results indicated that the multiplex amplification produced a genuine cDNA and that the cloned DNA represented an expressed gene. This newly cloned gene encodes
a 510-amino acid protein that we have shown to be an authentic Egr1 target gene. To confirm that the gene codes for an endogenously expressed protein, we have recently raised a polyclonal antibody using a synthetic peptide epitope derived from the predicted amino acid sequence. Preliminary testing has shown reactivity against both recombinant and an endogenous protein of identical molecular mass, suggesting that the cDNA is expressed at both the mRNA and protein level.

During the course of these studies an unpublished and unnamed cDNA generated through a library sequencing effort was deposited in the GenBank™ data base that was identical to our cloned cDNA (nucleotide accession number AK024011). Based on the sum of our observations, we have called this cDNA the HUGO approved name and symbol TOE1 for target of Egr1. Expression of TOE1 was detected in all of the adult human tissues examined but at varying levels, indicating that the regulation of this gene may vary depending on cell or tissue type.

Examination of the sequence of TOE1 did not reveal conserved domain structures apart from a single potential zinc finger and a possible nuclear localization signal. Immunostaining confirmed that TOE1 was found localized to the nucleoplasm and nucleolus. Despite the absence of a recognized DNA-binding domain, we have examined the possibility that TOE1 might participate in transcriptional regulation. However, TOE1 cloned as a GAL4 fusion failed to activate a GAL4-binding site reporter, suggesting that TOE1 alone is not sufficient for transcriptional regulation. The possibility remains that TOE1 can participate in transcriptional regulation through protein interactions and indirect DNA association not recapitulated in the GAL4 fusion experiments. Although no extended homology to any known gene was noted by BLAST searches, a limited region of homology to poly(A)-specific deadenylase was revealed. We are currently investigating the possibility that TOE1 may function as a nuclease.

To better understand the biological role of TOE1, we examined the effects of its expression and noted a dramatic decrease in both the growth rate and colony growth of H4 cells. We found that this was not the result of a general decrease in growth rate but rather was due to a G2 cell cycle phase delay. Furthermore, the G2-specific cell cycle delay correlated with an increase in the expression of the cyclin-dependent kinase inhibitor p21. Deletion of the nuclear localization signal abrogated this effect, suggesting not only that TOE1 could induce cell cycle-specific G2 pausing but also that its nuclear/nucleolar localization was critical for this function. The localization of TOE1 in the nucleolus may provide further evidence for a role in cell cycle regulation because it has been found that many important cell cycle proteins can be found in the nucleolus as a means of sequestration, thereby limiting their function until the appropriate time (22-24).

Because p21 is also able to inhibit cyclin-dependent kinase activities controlling passage through the G1 restriction point, it would be predicted that the TOE1-directed increase in p21 levels would also display a G1 phase pausing. Although we did not see this in log phase growing cells, when cells were synchronized in the M phase and then released to pass through G1, we noted a marked delay in the TOE1-expressing cells (data not shown). This suggested that the increase in p21 levels was also active at the G1 check point, but this was only seen if cells had been synchronized outside of the G2 phase. Although p21 is well known for its activity in G1 phase pausing, its role in G2 is being increasingly recognized (25, 26). These results suggest that the mechanism by which TOE1 affects cell growth is through transcriptional up-regulation of the p21 gene. We have not, however, formally ruled out the possibility that the increase in p21 levels might be due to an increase in transcript stability rather than increased expression. Also, we have not completely ruled out a contributing role for p53 in the up-regulation of p21 but have demonstrated that p53 levels and serine 15 phosphorylation were not altered. Further, we have provided evidence that TOE1-dependent TGF-β1 activation may participate in the increase in p21. However, it also remains possible that TOE1 and p53 cooperate in the transactivation of p21 either directly or indirectly. We have preliminary evidence that TOE1 and p53 are able to interact physically, but the significance and specificity of this interaction remain to be analyzed.3 Although the precise mechanism of action remains to be studied, our results have shown that expression of TOE1

3. I. de Belle, unpublished observation.
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leads to growth inhibition as well as a decrease in colony forming ability, likely involving the activation of p21. Given that these same features are seen following expression of Egr1, we expect that the downstream target TOE1 plays an important role in executing this physiological function of Egr1 in its proposed role as a tumor suppressor.

Finally, it is intriguing to note that the chromosomal location of TOE1 maps to 1p34.1–35.3. Deletion of the distal portion of 1p accounts for a significant proportion of chromosome 1 aberrations and has been observed in brain, breast, ovarian, colorectal, and other tumor types (27–29). Combined data suggest that chromosome 1p likely harbors one and possibly multiple tumor suppressor genes, and given the growth inhibitory effect of TOE1, we are currently investigating the possibility that TOE1 may also function in this capacity.

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