Molecular Cloning and Functional Characterization of the Upstream Promoter Region of the Human p73 Gene

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Abstract

The p73 gene encodes a protein that shares structural and functional homologies with the p53 tumor suppressor protein. To investigate the mechanism of transcriptional regulation of the p73 gene, we isolated a genomic DNA fragment spanning the 5' upstream region of the human p73 gene and characterized the promoter region. Unlike the p53 gene promoter, the human p73 gene promoter contained a putative TATA-box, and did not exhibit any extended homology to the p53 gene. Two CpG islands were located in the 5' upstream region. Transient transfection assays using progressive truncations of the p73 promoter showed that deletion from -119 to +19 relative to exon 1 resulted in a 13- to 20-fold reduction in the p73 promoter activity, suggesting that the elements for basal promoter activity exist in this region, where putative Spl, AP-2 and Egr-1, 2, 3 sites are located and CpG dinucleotides are especially concentrated.

Key words: p73; Promoter; Luciferase assay; CpG island

The p73 gene is considered an important gene in tumorigenesis because it encodes a protein with remarkable sequence similarity to the p53 tumor-suppressor gene in the DNA binding, oligomerization, and transactivation domains. Moreover, overexpression of p73 in cells can activate p21, a well known p53 target gene, and induce both cell cycle arrest and apoptosis. Therefore, p73 is a structural and functional homologue of p53, which led us to consider the possibility that p53 function in p53-inactivated carcinomas may be restored by activating p73. In this context, it is important to gain insight into the mechanisms regulating p73 expression.

However, mutational and expression analysis of the p73 gene in various cell lines and cancers show that p73 is not mutated in many carcinomas, and overexpression or loss of imprinting (activation of the silent allele) of the p73 gene is associated with the development of cancers. In this context, it is also important to reveal the regulatory mechanism of the p73 gene and the mechanisms that lead to abnormal expression in cancers.

In this study, we report the molecular cloning of the upstream promoter region of the human p73 gene and structural characterization of the upstream sequence of the human p73 gene in comparison with that of the human p53 gene, as well as the functional characterization of the upstream promoter region of the human p73 gene.

The upstream promoter region of the human p73 gene was cloned from a λPS1 genomic phage library (see Fig. 1A legend). One positive clone, which spans about 20 kb, was selected for characterization. The positive clone was digested with various restriction enzymes and analyzed by Southern blotting using the same probe. This fragment contained the 5' flanking region, the first exon and a part of first intron (data not shown).

An approximately 6-kb Kpn I/EcoRI fragment that contained the 5' upstream region of p73 was subcloned into the pSP72 vector (Promega), and the nucleotide sequence was determined. Computer searching using MalignInspector V2.2 at the TRANSFAC WWW site revealed that there could be multiple transcription factor binding sites such as E2F, Egr, c-Myb, AP-2, and Sp1 that might regulate the transcription of human p73. We could not find a typical TATA box within 0.3 kb of the 5' upstream region relative to the published p73 cDNA, but a TATA signal prediction program identified TATAAA
We plotted the (G + C) percentage and the observed/expected CpG ratio using a 200-bp window moving along the sequence at 1-bp intervals. Whereas bulk genomic DNA has a GC content of 40% and an observed/expected CpG ratio of 0.25, CpG islands were defined as areas larger than 200 bp that show a GC content at least 60% and an observed/expected CpG ratio above 0.6. There was a large CpG island spanning from -1934 to exon 1 with a GC content greater than 70% and an observed/expected CpG ratio of >0.6 (Fig. 1B). An additional smaller CpG island with a GC content of >60% and an observed/expected CpG ratio of >0.6 was located further upstream at position -2309 to -2663 (Fig. 1B).

We compared the human p73 and p53 promoter sequences and found that there is no similarity in the sequence and potential transcription factor binding sites (data not shown). We concluded that human p73 and p53 have different features in their promoter although they share functional and structural similarities in their coding sequences. This is consistent with a previous report that they have a different genomic organization.

To analyze the promoter activity of the 5' flanking region of p73, several promoter fragments were linked to a luciferase reporter gene in pGVB2 (see Fig. 2 legend). The luciferase activity of each construct was determined by transient transfection into the human breast carcinoma cell line, MCF-7, which had been shown to express the endogenous human p73 gene. As shown in Fig. 2, the plasmid construct p73PF conferred luciferase activity 23-fold higher than the activity of the parental vector, pGVB2. The deletion mutants p73Nru I, p73Sac I, p73Pst I, p73Pvu II, p73Not I, and p73Not I-Apa I showed similar or higher activities than that of p73PF. However, in the p73PsrA I construct, removal of the sequence from -119 to +19 relative to the first base of the published p73 cDNA led to a 13-fold loss of promoter activity compared with that of p73Not I. These results suggest that a sequence approximately 138 bp between -119 to +19 relative to the first base of published p73 exon 1 is sufficient to confer the maximal basal activity of the p73 promoter. As CpG dinucleotides are especially concentrated in this region, and the p73 is reported to be an imprinting gene, it is intriguing to think that methylation of this region may be associated with transcriptional inactivation of p73, and may be involved in the imprinting mechanism of p73.

Recently two groups reported that the 5' CpG island around exon 1 of the p73 gene was methylated aberrantly in approximately 30% of primary acute lymphoblastic leukemias and Burkitt's lymphomas, and that methylation was associated with loss of transcription of the p73 gene, suggesting that hypermethylation may be a critical alternative mechanism for inactivation of this gene in a tumor-specific way. It has been reported that monoallelic expression of p73 is observed in several tissues or cell lines, such as normal lymphocytes, normal kidney or neuroblastoma cell lines, which was consistent with imprinting. However, unexpectedly, the 5' region around exon 1 of the p73 gene was not hypermethylated. On the other hand, in some cases aberrant methylation occurs at a distant region. Therefore, it might be worth examining the methylation status further upstream of the p73 promoter, which we have identified in the present study.

We believe that further investigation of the p73 promoter will provide useful information for the mechanism of monoallelic expression and the involvement of p73 expression in tumorogenesis. Furthermore it might provide useful information for the development of agents that promote transcription of the p73 gene in a therapeutic or chemopreventive context, which we have termed as gene-regulating chemotherapy or chemoprevention.

**Figure 1.** Nucleotide sequence and structural features of the p73 promoter. A. Putative regulatory sequences in the p73 promoter. To isolate the human p73 gene, a human genomic leukocyte library in the λPS1 phage (Mo Bi Tec, Göttingen, Germany) was screened essentially according to the instructions of the manufacturer. About 1 x 10⁷ phage plaques were transferred onto Hybond N membrane (Amerham Pharmacia Biotech) and hybridized with end-labeled oligonucleotide corresponding to bp 1-72 of the published human p73 cDNA (GenBank Accession number Y11416). One positive plaque was purified through three rounds of re-screening, and this phage DNA was converted to plasmid DNA by transduction into a Cre-recombinase-expressing bacterial strain according to the instructions of the manufacturer. The positive genomic clone was mapped by single and multiple restriction enzyme digestion followed by Southern blot analysis using standard procedures. An approximately 6-kb *Kpn I/ EcoRI* fragment that contains the region hybridizing to bp 1-77 of the human p73 cDNA was subcloned into pSP72 (Promega). The Kilo-sequence Deletion Kit (TaKaRa, Tokyo, Japan) was used to generate various deletions, and DNA sequencing was performed using an automated DNA sequencing system from PE-Applied Bioystem (Foster City, CA). A search for putative transcription factor binding sites was carried out using the MathInspector V2.2 at the TRANSFAC WWW site. TATA signal prediction was carried out using the HCtata program. Exon 1 (77 bp) and 1873 bp of the 5'-flanking p73 gene relative to the first base of exon 1 of the p73 gene and 5 bp of intron 1 are illustrated. The first nucleotide relative to the published p73 gene cDNA is indicated by a diamond (*) and indicated as +1. Putative regulatory sequences within 373 bp upstream of exon 1 are indicated by boxes. An additional putative E2F binding site further upstream of the promoter region is also indicated by a box. B. CpG island in the p73 promoter. Using a 200-bp window moving along the sequence at 1-bp intervals, the observed/expected CpG ratio and GC percentage were determined. The gray line in the histogram represents the observed/expected ratio of CpG dinucleotides across about 2.7 kb of exon 1 and the 5'-flanking region of the p73 gene. The GC content is indicated by black lines. Map plots below the histogram compare the densities of CpG and GpC dinucleotides over the same region. CpG islands were defined as being greater than 200 bp, having a GC content of at least 60%, and having an observed/expected CpG ratio above 0.6. The identified CpG islands are indicated as A (-2309 to -2663) and B (-1934 to exon 1).
Deletion mutants of the p73 promoter

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<th>Mutation</th>
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<td>pGVB2</td>
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Figure 2. Determination of the minimal p73 promoter sequence required for essential basal activity. An approximately 2.8-kb Not I-Pst I fragment between −2713 and +77 relative to the first base of already known p73 cDNA was subcloned into luciferase reporter plasmid pGVB2 (Nippon Gene, Tokyo, Japan). This human p73-luciferase fusion plasmid was termed p73PF (−2713/+77). To generate promoter deletion mutants, p73PF was digested separately with Nru I, Sac I, Pst I, Pvu II, Not I, or Apa I. These enzyme sites were blunted with T4 DNA polymerase and then self-ligated (DNA Ligation Kit, TaKaRa, Japan). These plasmids were termed p73Nru I (−1210/+77), p73Sac I (−883/+77), p73Pst I (−299/+77), p73Pvu II (−220/+77), p73Not I (−119/+77), and p73Apa I (+20/+77), respectively. To generate the deletion mutant, p73Not I-Apal (−119/+19), p73Not I was digested with Apa I and HindIII, then blunted and self-ligated. All the constructs in this series of deletions were confirmed by sequencing. Human breast cancer MCF-7 cells were cultured in Dulbecco’s modified Eagle medium (DMEM, GIBCO-BRL) supplemented with 10% fetal bovine serum (GIBCO-BRL). Next, 5×10⁴ cells were seeded into 3.5-cm-diameter tissue culture dishes. After 24 hr, 2 µg of reporter construct and 1 µg of pACT/β-gal (a kind gift from Dr. S. Ishii), a plasmid containing β-galactosidase driven by the actin promoter for normalizing the transfection efficiency, were co-transfected into cells using the calcium phosphate co-precipitation method using the CellPhect reagent (Amersham Pharmacia Biotech). Cells were harvested 48 hr after transfection. The luciferase activity of each cell lysate was measured by the luciferase assay system (PicoGene Luminescence Kit, Nippon Gene), and was normalized by β-galactosidase activity in the cell lysates as described previously. The transfection assays were repeated three times. Each construct is shown schematically on the left, and exon 1 is indicated by a box. Data are shown as means (bars, standard error) (n=3). *p<0.004, **p<0.002.

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References


