Structural Organization of the Human Dihydropyrimidine Dehydrogenase Gene

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Abstract

Deficiency of the pyrimidine catabolic enzyme, dihydropyrimidine dehydrogenase (DPD), has been shown to be responsible for a pharmacogenetic syndrome in which administration of 5-fluorouracil is associated with severe and potentially life-threatening toxicity. Following the recent availability of the cDNA for DPD, there were initial reports of several molecular defects (point mutations, deletions due to exon skipping) that were suggested as a potential molecular basis for DPD deficiency, even before the complete physical structure of the DPD gene was known. To understand the mechanism responsible for DPD deficiency, we have determined the genomic structure and organization of the human DPD gene. The gene is approximately 150 kb in length, and it consists of 23 exons, ranging in size from 69 to 1404 bp. The sequences of intronic regions flanking the exon boundaries have been determined. The physical map of the DPD gene should permit development of rapid assays to detect point mutations or small deletions in the DPD gene associated with 5-fluorouracil toxicity.

Introduction

5-fluorouracil (FURA)4, synthesized in 1957 and studied extensively by Heidelberger et al. (1), today continues to be widely used in the management of several common malignancies, including cancers of the colon, breast, and skin. The anticancer effects and toxicity of FURA are directly related to anabolism of FURA into its nucleotides, which can then produce cytotoxicity through inhibition of thymidylate synthase activity or incorporation into RNA and/or DNA (2). In humans, more than 85% of administered FURA is degraded through the catabolic pathway (3). DPD (EC 1.3.1.2; also known as dihydouracil dehydrogenase, dihydrothymine dehydrogenase, and uracil reductase) catalyzes the initial and rate-limiting step in pyrimidine catabolism, the reduction of uracil or thymine to 5,6-dihydropyrimidine (4). Thus, DPD regulates the availability of FURA for anabolism, thereby affecting its pharmacokinetics, toxicity, and efficacy (5).

Several recent studies have described a pharmacogenetic disorder in which individuals with absent or significantly decreased DPD activity develop life-threatening toxicity following exposure to FURA (6–10). Administration of standard doses of FURA to DPD-deficient patients has resulted in severe life-threatening toxicity, including mucositis, granulo-cytopenia, neuropathy, and even death. Since the initial reports several years ago, there have been an increasing number of cases reported suggesting that this disorder may be more frequent than initially thought (7, 11). Population studies in 124 healthy volunteers (7) and 185 cancer patients (11) have demonstrated that approximately 3% of the individuals tested were partially DPD deficient, having enzyme activity below the 95% distribution range of DPD enzyme activity (below 0.064 nmol/min/mg). The recent availability of the DPD cDNA (12, 13), has allowed investigators to examine, at the molecular level, patients who were previously phenotyped (by enzyme assay) as DPD deficient. These studies have demonstrated polymorphisms (14) and exon skipping (15, 16) as possible bases for this pharmacogenetic syndrome.

These observations have raised the possibility that there are multiple causes for DPD deficiency. An understanding of the structure and organization of the DPD gene should enhance our ability to identify mutations and alternatively spliced regions of this gene. Here, we report the genomic structure and organization for the human DPD gene.

Materials and Methods

Library Screening. Clones representing the human genomic locus of the DPD gene were isolated from a titered P1 human genomic library (Genome System Inc., St. Louis, MO). The library was screened using PCR and the following primers: sense, TAGGAAAACAGCTCAGTACCTTGG, and antisense, CTGCTAGCCGAAATCTACAGGTCTAG. Specific cycling conditions were evaluated prior to screening the P1 library using purified genomic DNA. The conditions used to screen the library were 10 μm each primer, 60 mm Tris-HCl (pH 8.5), 15 mm (NH4)2SO4, 2.5 mm MgCl2, 2.5 mm each dNTP, and 1 unit of AmpliTaq DNA polymerase in a total volume of 50 μl. The samples were amplified in an MJ model PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) programmed for a temperature-step cycle of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. This cycle was repeated for a total of 30 cycles.

Identification of Exon-Intron Boundaries. Approximately 10% of the exon-intron boundaries were identified by direct cycle sequencing (New England Biolabs) of the P1 genomic clone (17) using primers designed from DPD cDNA. The remaining boundaries were determined using suppression PCR, as follows (Clontech Laboratories, Inc., Palo Alto, CA). Genomic DNA was digested with EcoRV, ScaI, DraI, PvuII, and SphI separately. An adaptor was ligated to the ends of the DNA fragments, and a small aliquot of each digested-ligated DNA was used as a template for PCR using adaptor primers and gene-specific primers designed from DPD cDNA (18). Amplified products were subcloned into the pcRII vector (Invitrogen) and sequenced using the universal T7 and M13 primers bordering the multiple cloning site. DNA sequencing was accomplished using the dyeoxy chain termination procedure (Sequenase Version 2, United States Biochemical Corp.). Exon-intron boundaries were identified by the presence of consensus splice junctions at sites where the sequence of the genomic product differed from the published DPD cDNA sequence. The computer programs MacVector and AssemblyLIGN were used for comparison of DNA sequences.

Sizes of introns were determined by exon-exon PCR amplification products using LA PCR (Takara Shuzo Co., Kyoto, Japan) with a buffer supplied by the manufacturer. The final reaction volume was 50 μl and contained 10 μm each primer and 50 ng of genomic DNA as a template. Cycling conditions were as follows: denaturation at 98°C for 20 s and annealing and extension at 68°C for 15 min. Following the first 14 cycles, an autosense extension of 15 s per...
cycle (at the annealing and extension step temperature of 68°C) was added for the remaining 16 cycles (19, 20). Most runs included an initial 15-min hold at 94°C and a final 10-min hold at 72°C. All samples were run using a manual "hot start" technique in which Mg²⁺ was withheld until samples had been incubated at 94°C. A positive control that amplified a region of the human β-globin cluster (21.5 kb) was run with each reaction and used the following primers: sense, ACATGATFAGCAAAAGGGCCTAGCTFGGACTCAGA, and antisense, TGCACCTGCTCFGTGAUATGACTATCCCACAGTC.

**Results and Discussion**

In the initial phase of this study, we screened a titered P1 human genomic library with specific primers (designed within exon 22 of the DPD DNA sequence). Clones representing the human genomic locus of the DPD gene were identified by amplification of a 135-bp product. Three independent P1 clones were isolated, and the boundaries for exons 21–23 were identified by direct thermal cycle sequencing using exon-specific primers. Unfortunately, PCR and hybridization analysis of the three P1 clones demonstrated that they did not contain exons 1–20. The relatively large size of the introns isolated for the DPD gene from the P1 screening (23), along with previous reports describing difficulties in screening phage libraries (15), suggested that the average insert size of most commercial λ phage libraries (14–20 kb) would be too small to be useful for this particular gene. We therefore elected to use a recently described technique known as suppression PCR (18, 24, 25). This method had a particular advantage over traditional library screening with regard to the DPD gene in that only the boundary of each splice site junction is amplified from genomic DNA and subcloned. Following amplification of each splice site, the PCR products were purified on low melting point agarose and subcloned into the pCRII vector for sequence analysis.

Previous studies have mapped the DPD gene to chromosome 1p22 (26). We now report the organization and structure of the DPD gene, which consists of 23 exons spanning a region approximately 150 kb in length. The size and sequence of intronic regions flanking the exon boundaries has also been determined and is summarized in Table 1. All intron-exon boundaries were found to conform to the canonical GT-AG rule. A physical map encompassing the entire coding region of the DPD gene is shown in Fig. 1. Exons are numbered 1–23, with exon 15 (69 bp) being the smallest and exon 23 (1404 bp) being the largest. The translation start site (ATG) was located in exon 1. Exon 23 contained 168 nucleotides of coding sequence, followed by the translation stop codon (TAA) and a 1236-nucleotide 3' untranslated region. The largest. The translation start site (ATG) was located in exon 1. Exon 23 contained 168 nucleotides of coding sequence, followed by the translation stop codon (TAA) and a 1236-nucleotide 3' untranslated region. The human liver DPD cDNA sequence published by Yokota et al. (12) did not contain the complete 3' untranslated region. Sub-
sequent studies in our laboratory identified the complete human lymphocytic DPD cDNA sequence (GenBank accession no. U20938), which contained this region and was used in our comparison of genomic and cDNA sequence searching for consensus splice junctions where the sequence of the genomic product differed from the lymphocytic DPD cDNA sequence.

Primer extension experiments were undertaken to determine the precise size of exon 1. Freshly isolated RNA from peripheral blood mononuclear cells was used for this analysis. The band indicated by the arrow (Fig. 2) is the reverse-transcribed product, whereas the other bands represent known DNA sequences that were included for determination of molecular size. Comparison of the nucleotides in the known sequence with the mobility of the DPD mRNA lane demonstrates that the distance from the primer to the 5' end of the message coding for DPD is 104 nucleotides. This result suggested that there were approximately 20–25 additional nucleotides in the mRNA sequence that had not been identified previously by cDNA cloning (12, 13). We used 5'-RACE to clone and sequence these additional nucleotides located upstream from the published DPD cDNA sequence.

PCR with an anchor primer and an internally nested primer located 193 bp from the 5' end of the published human DPD cDNA sequence resulted in the amplification of a cDNA approximately 214 bp in length. Following subcloning, 10 clones were selected and sequenced. All of the inserts examined contained the human DPD cDNA sequence reported previously; however, 8 of the 10 clones extended further upstream for an additional 21 nucleotides. These data suggest that exon 1 is 140 nucleotides long and contains 101 nucleotides of 5' untranslated region, followed by the initiating methionine and 36 nucleotides of coding sequence.

Intron sizes were determined by PCR amplification using genomic DNA as a template with gene-specific primer pairs from adjacent exons. The use of nested PCR allowed verification of the sizes of the introns. Only PCRs that gave a single well-defined product (Fig. 3) were used for estimation of intron sizes. Introns 5 and 10 were the largest, with sizes greater than 20 kb, whereas introns 9 and 17 were the smallest.

The recent examination of two unrelated DPD-deficient patients has suggested exon skipping (15, 16) as a possible mechanism for DPD deficiency. These studies demonstrated a 165-bp deletion in the mRNA of both patients. Genomic DNA analysis of these patients revealed a G to A mutation in the GT 5' splicing recognition sequence of the intron preceding the mutation. It is now clear from the current study that exon 14 represents the skipped exon and that the G to A point mutation in the invariant GT splice donor site is located at the 5' end of intron 14. However, our examination of this region of the DPD gene in two additional well-characterized DPD-deficient patients has demonstrated that neither patient exhibits this particular G to A mutation.

The aim of this study was to determine the structure and organization of the DPD gene. These data could then be used as a foundation for rapidly scanning the DPD gene for mutations in genomic DNAs in an attempt to define the molecular basis of DPD enzyme deficiency. The data presented in this study should allow the development of genetic assays that could clarify the role of the DPD gene in deficient patients. The clinical diagnosis of DPD deficiency is difficult because the appearance of life-threatening toxicity secondary to exposure to FUra is the first symptom of this pharmacogenetic syndrome. Until now, only phenotypic DPD enzyme assays have been available to diagnose patients as DPD deficient. These assays are expensive and labor intensive and are not available in most cancer treatment centers. Thus, there is a great need for a more direct diagnostic method,
preferably available to the patient prior to receiving FUra. The characterization of the genomic structure of the DPD gene provides the basis for the development of simple genetic tests. To date, all DPD-deficient patients have been analyzed without a complete understanding of the DPD gene structure and organization. The stability of DNA in comparison to RNA along with the ease with which DNA samples may be extracted and purified represents a clear diagnostic advantage. In addition, the development of genomic DNA-based assays would allow analysis of archive paraffin-embedded samples from deceased DPD-deficient patients. These samples would allow researchers to build pedigrees on families (including determination of heterozygotes), who, until now, were impossible to study. Although the size and complexity of the DPD gene makes scanning the gene for mutations a challenge, we believe that recent advances in the field of molecular biology (such as single-stranded conformational polymorphism) may be used for identifying additional mutations in the DPD gene.

References
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