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Genomic structure and chromosomal localization of the human deoxycytidine kinase gene

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ABSTRACT **Deoxycytidine kinase (NTP:deoxycytidine 5'-phosphotransferase, EC 2.7.1.74) is an enzyme that catalyzes phosphorylation of deoxyribonucleosides and a number of nucleoside analogs that are important in antiviral and cancer chemotherapy. Deficiency of this enzyme activity is associated with resistance to these agents, whereas increased enzyme activity is associated with increased activation of such compounds to cytotoxic nucleoside triphosphate derivatives. To characterize the regulation of expression of this gene, we have isolated genomic clones encompassing its entire coding and 5' flanking regions and delineated all the exon/intron boundaries. The gene extends over more than 34 kilobases on chromosome 4 and the coding region is composed of 7 exons ranging in size from 90 to 1544 base pairs (bp). The 5' flanking region is highly G+C-rich and contains four regions that are potential Sp1 binding sites. A 697-bp fragment encompassing 386 bp of 5' upstream region, the 250-bp first exon, and 61 bp of the first intron was demonstrated to promote chloramphenicol acetyltransferase activity in a T-lymphoblast cell line and to have >6-fold greater activity in a Jurkat T-lymphoblast than in a Raji B-lymphoblast cell line. Our data suggest that these 5' sequences may contain elements that are important for the tissue-specific differences in deoxycytidine kinase expression.**

Deoxycytidine kinase (dCyd kinase; NTP:deoxycytidine 5'-phosphotransferase, EC 2.7.1.74) is an enzyme that phosphorylates 2'-deoxyadenosine, 2'-deoxyguanosine, and 2'-deoxycytidine to their corresponding 5'-monophosphates and thus plays an important role in overall cellular deoxyribonucleotide metabolism (1). In addition, a wide variety of nucleoside analogs such as 1-β-D-arabinofuranosylcytosine and 2-chlorodeoxyadenosine that are important in the chemotherapy of hematologic malignancies are substrates for this enzyme and require phosphorylation as a rate-limiting step for their initial activation (2, 3). Several studies have demonstrated that dCyd kinase is expressed at variable levels in different tissues. In particular, the levels of dCyd kinase activity in the thymus gland and in leukemic T lymphoblasts have been demonstrated to be higher than those in other cell types (4–6), a finding that in part explains the high susceptibility of these cells to deoxyribonucleoside-induced cytotoxicity (7). Because of the potential role for this enzyme in mediating the tissue-specific cytotoxicity of chemotherapeutic agents for both hematologic and nonhematologic malignancies, further delineation of the factors regulating dCyd kinase expression is warranted.

We have recently cloned the cDNA for human dCyd kinase and have demonstrated that its steady-state mRNA levels in human Molt-4 and Jurkat T lymphoblasts are 5- to 10-fold higher than in Epstein–Barr virus-transformed B-lymphoblast cell lines (7). We have proceeded to use this cDNA to obtain genomic clones of the dCyd kinase gene in order to characterize its structure and obtain further insights into the regulation of its expression.¶

EXPERIMENTAL PROCEDURES

Isolation of Human Genomic dCyd Kinase Clones. Human genomic libraries constructed in either Lambda DASH (gift of Michael Clarke, University of Michigan, Ann Arbor) or Lambda FIX (Stratagene; gift of John Lowe, University of Michigan, Ann Arbor) were screened for dCyd kinase genomic DNA sequences with a ^{32}P-labeled 2.4-kilobase (kb) cDNA containing the entire dCyd kinase coding region, 160 base pairs (bp) of 5' untranslated region, and 1530 bp of 3' untranslated region (7). Positive clones were plaque-purified and characterized by restriction endonuclease mapping, Southern blot analysis, and DNA sequencing.

DNA Sequence Analysis. Appropriate restriction enzyme fragments from genomic clones were subcloned directly into pUC19 plasmid vectors (Boehringer Mannheim) for sequence analysis. DNA sequencing was performed on both strands using double-stranded plasmid DNA as template and synthetic 17-mer oligonucleotide primers. Sequence analysis was carried out by the dideoxynucleotide chain-termination method (8).

Primer-Extension Analysis. A 32-bp reverse complementary primer extending from bp −13 to +19 of the sequence in Fig. 1 was end-labeled with [^{32}P]ATP and sequentially annealed to 7 μg of poly(A)$^+$ mRNA from Jurkat cells at 95°C for 5 min, 70°C for 10 min, and 60°C for 10 min. Primer extension was carried out using 5 units of avian myeloblastosis virus reverse transcriptase and 0.5 mM dNTPs for 40 min at 42°C. After ethanol precipitation, the reaction product was run on a 6% polyacrylamide gel. A sequencing reaction with the same primer and p697 plasmid DNA was run in an adjacent lane.

Functional Analysis of the 5' Regulatory Region. The 697-bp *Hin*dIII fragment encompassing the first exon and 5' flanking regions (Fig. 1) was cloned into the *Hin*dIII site of the pCAT-Enhancer plasmid (Promega) in both orientations. To determine whether the 697-bp fragment mediated tissue-specific gene expression, 30 μg of plasmid DNA and 20 μg of a vector containing the β-galactosidase cDNA under the control of a cytomegalovirus (CMV) (9) or a Rous sarcoma virus (RSV) promoter (10) were coelectroporated into either Jurkat T lymphoblasts or Raji B lymphoblasts. Electroporation was performed on 10^7 cells in a total vol of 500 μl of serum-free RPMI 1640 medium using a Bio-Rad Gene Pulser

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Abbreviations: dCyd kinase, 2'-deoxycytidine kinase; ADA, adenosine deaminase; DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; RSV, Rous sarcoma virus; TdT, terminal deoxynucleotidytransferase.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07485).

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-545  AAGCTTTAAGTCTATCCAGTTCTGTCCAGACGCGTCTGTTCATCTCCAACA
      GGCCCACTAGAGAGGCGGGTTTTCCAAAGCCTGCACGGCCCACTGCAGGT
-444 GACGCCCTCTGCCCTCCAGCGCCCTCAGGCCTCTGGGGTCCACCCTTCCT
      CCCCACCCGACTCCGGAACCTCTTCCGCGCTGCCCGGGCGCCTGGCTGCT
-344 TGGGGTAGAGGCCTTCCGCCACACGCGCGGCCCCGCCCCGGCCTTCACGT
      GACCTGCGTGCGGAGCGCGCAGCGGGAACCCGCGCTGGAGGCGGGCGAGG
-244 GCCGAGGGGCAGCTAGGGAGCGCGGCTTGAGGAGGGCGGGGCCGCCCCGC
      AGGCCCGCCAGTGTCCTCAGCTGCCTCCGCGCGCCAAAGTCAAACCCCGA
-144 CACCGCGGCGGGCCGGTGAGCTCACTAGCTGACCCGGCAGGTCAGGATCT
      GGCTTAGCGGCGCCGCGAGCTCCAGTGCGCGCACCCGTGGCCGCCTCCCA
-44  GCCCTCTTTGCCGGACGAGCTCTGGGCCGCCACAAGACTAAGGAATGGCC
      ACCCCGCCCAAGAGAAGCTGCCCGTCTTTCTCAGCCAGCTCTGAGGGGAC
+57  CCGCATCAAGAAAATCTCCATCGAAGGGAACATCGgtaaggagcctccggaaatg
      tgggacgcaaggctggggtgtcgcggcagtggctgaagctt
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FIG. 1. Sequence of the 697-bp genomic *Hin*dIII fragment containing the 5′ flanking sequence and the first exon of the dCyd kinase gene. Sequence is numbered from A of the ATG initiation codon (+1). Potential Sp1 binding sites are underlined. The 9-bp sequence with sequence identity to the E2F binding site is indicated in italics. The major transcription initiation site is indicated in boldface at bp −146. Smaller letters indicate the first intron sequence.

with a capacitance extender at 240 V per 960 μF. Cells were resuspended in 30 ml of RPMI 1640 medium containing 10% fetal calf serum and cultured for 48 hr at 37°C. Cells were washed twice in divalent cation-free phosphate-buffered saline and extracted in 150 μl of 0.25 M Tris·HCl (pH 8.0) by freeze–thawing three times and spun at 12,000 × *g* for 10 min. Supernatants were stored at −70°C without heating and assayed for chloramphenicol acetyltransferase (CAT) activity by liquid scintillation counting after heating to 60°C for 10 min by the Promega protocol (11). β-Galactosidase activity was measured in unheated extracts by a modification of a published technique (12) using chlorophenol red β-D-galactopyranoside (Boehringer Mannheim) as a substrate. Reactions were performed at room temperature and were linear with time. Activity was based on values obtained with the crystalline enzyme (Boehringer Mannheim). Protein was assayed in unheated extracts with Bradford reagent (Bio-Rad).

Chromosome Assignment. A human–hamster hybrid commercial mapping panel (Bios, New Haven, CT) was screened for dCyd kinase genomic sequences by PCR and genomic primers flanking the 193-bp third exon of the dCyd kinase gene (5′ primer, TAAATAGCCCTATTGACCATTAAT; 3′ reverse complement primer, CAAATGGCCACGTACAAGCCA). PCR analysis was also performed on murine–human hybrid cell lines containing human chromosomes 11 and 4 (HDM-18), human chromosome 11 alone [10T½(11n)-5] (obtained with the kind permission of Mathew Thayer, Fred Hutchinson Cancer Center, Seattle, WA; ref. 13), and human chromosome 4 alone [HA(4)A] (obtained from Ann Killary, M. D. Anderson Hospital, Houston, TX). In each instance, the PCR was carried out on 250–500 ng of genomic DNA using a 55°C annealing temperature and a 72°C extension for 1 min each for a total of 30 cycles.

RESULTS

Isolation of Genomic Clones. A 2.4-kb cDNA clone of dCyd kinase previously isolated in our laboratory (7) was used to screen a human genomic library in Lambda DASH and two positive clones containing identical 18.2-kb inserts were identified and plaque-purified. Southern blot analysis of *Hin*dIII-digested DNA from one of these clones (N5) revealed two bands of 697 bp and 5.4 kb, which were identical in size to two of the five bands identified on Southern blots of *Hin*dIII-digested genomic DNA (data not shown; Fig. 2). The screening of a second library constructed in the Lambda FIX vector yielded two genomic clones, one of which contained the identical 697-bp *Hin*dIII fragment and 18 kb of DNA 5′ to this region (T11). The second clone (T16) contained internal *Hin*dIII fragments of 2.2, 1.8, 1.5, and 0.95 kb and *Hin*dIII/*Sal* I fragments of 9.0 and 4.4 kb at the 5′ and 3′ termini, respectively. RNA probes generated from the 5′ T3 and 3′ T7 RNA polymerase binding sites of the N5 clone did not hybridize to T16 DNA, nor did probes from T16 hybridize to N5. Thus, these clones do not overlap.

Structure of the dCyd Kinase Gene. The 697-bp *Hin*dIII fragment from the N5 clone was found to contain the translation initiation site and this fragment was completely sequenced, as shown in Fig. 1. This clone contains 386 bp of sequence 5′ to the published cDNA sequence that is high in G+C content (71%) and contains three consensus sequences for Sp1 binding (14). An additional potential Sp1 binding site was identified within the transcribed region, whereas no consensus CAAT or TATA elements were identified. Also of note is the CGCGCCAAA sequence at bp −165 relative to the ATG codon. This sequence represents 9 of the 12 bp of E2F transcription factor consensus binding site (TTTCGCGCCAAA) (15). Analysis of this clone using a transcription factor data base (16) did not reveal any other significant transcription factor binding sites. Primer-extension analysis was performed to identify the transcription initiation site(s) within this sequence. A single major band was identified at bp −146 (Fig. 3), with several considerably fainter bands located at −130, −123, −115, and −109 (data not shown).

The second exon was identified in the 5.4-kb *Hin*dIII fragment from the N5 clone, while exons 3–7 were present in the T16 clone (Fig. 2). Exon sizes ranged from 90 to 1544 bp and exon/intron boundaries conformed to standard splice donor and acceptor recognition sequences (Table 1). The second intron extended 12 kb 3′ of the second exon in N5 and included 7 kb of 5′ sequence from the T16 clone, leaving a gap of unknown size in this region. Attempts to obtain additional clones from two genomic libraries by using a third exon probe were unsuccessful. Southern blots of genomic DNA with a variety of restriction enzymes were performed in an effort to

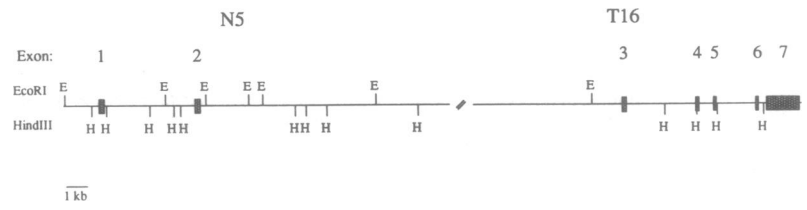

FIG. 2. Schematic representation of the seven exons of the human dCyd kinase gene. Clone N5 contains an 18.2-kb insert and T16 contains a 19.8-kb insert. The terminal 4.4-kb *Hin*dIII/*Sal* I fragment of the T16 clone does not contain coding sequence and is not shown.

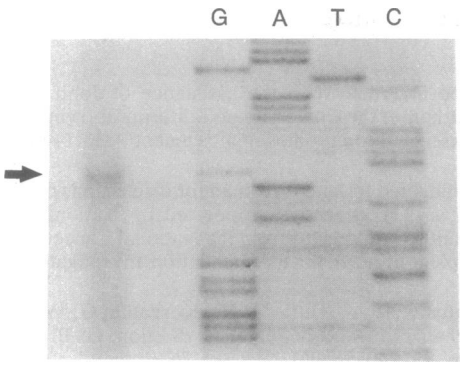

FIG. 3. Primer-extension analysis of the 5′ end of dCyd kinase mRNA. A 30-bp primer was annealed to 7 μg of Jurkat cell poly(A)$^+$ RNA and extended as described. The major product is shown by the arrow, with the corresponding genomic sequence on the right.

identify the size of the missing intron fragment. While a probe from the N5 clone identified several discrete bands, probes from the 5′ end of the T16 clone contained repetitive elements that could not be blocked by competition with total genomic DNA. Therefore, the absolute size of the second intron has not been determined.

Chromosomal Assignment. To determine the chromosomal location of the dCyd kinase gene, we screened a commercial hybrid mapping panel by using PCR primers flanking exon 3. These studies suggested that the gene resided on chromosome 4 or 11. We therefore analyzed mouse–human hybrids containing chromosomes 4 and/or 11 (Fig. 4). A PCR product of the expected 193 bp was obtained from human lymphoblast genomic DNA (lane 2), HA(4)A DNA containing human chromosome 4 alone (lane 4), the MRC5 human diploid fibroblast cell line (lane 6), and the HDM-18 cell line containing both human chromosomes 11 and 4 (lane 9). No PCR product was obtained in the two mouse fibroblast parental cell lines 3TG and 10T½ (lanes 7 and 8) or in two murine cell lines containing chromosome 11 alone [MCH 556.1 c5 (lane 3) and 10T½(11n)-5 (lane 10)]. These data demonstrate the presence of the dCyd kinase gene on human chromosome 4.

Functional Analysis of 5′ Flanking Region. A pCAT-Enhancer construct containing the 697-bp *Hin*dIII 5′ flanking region (p697CAT) was used to determine whether this region contained a functional dCyd kinase promoter and whether this region might confer specificity of expression in human T lymphoblasts. The p697CAT construct was electroporated into Jurkat T lymphoblasts and Raji B lymphoblasts in conjunction with either a CMV or a RSV β-galactosidase vector as a control for electroporation efficiency. As shown in Table 2, the expression of p697CAT was 6- to 8-fold higher in the Jurkat T lymphoblasts than in Raji B lymphoblasts in both the 5′ → 3′ and 3′ → 5′ orientations. Expression of β-galactosidase activity was uniformly higher in Raji than in Jurkat cells by 2- to 3-fold with both promoters, suggesting that differences in electroporation efficiency are not responsible for the observed differences. Identical T- and B-lymphoblast differences were observed in other experiments comparing Molt-4 T lymphoblasts and MGL-8 B lymphoblasts, although the electroporation efficiencies were significantly lower in these cell types (data not shown).

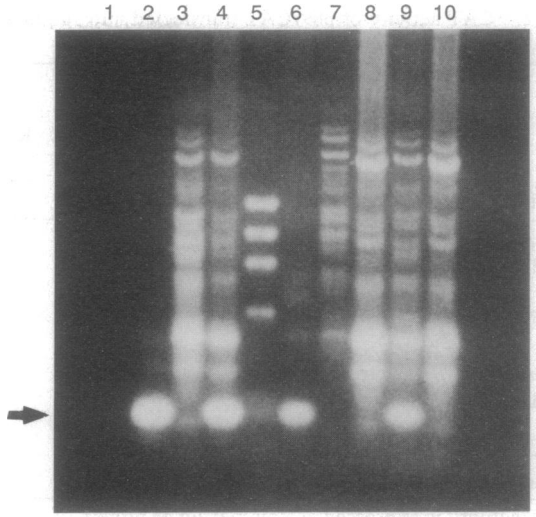

FIG. 4. Chromosomal assignment of the dCyd kinase gene. DNA templates for PCR are as follows: lane 1, water; lane 2, human B-lymphoblast genomic DNA; lane 3, MCH 556.1 c5 mouse hybrid containing human chromosome 11; lane 4, HA(4)A mouse hybrid with human chromosome 4; lane 5, Phi X markers; lane 6, MRC5 human fibroblast DNA; lane 7, 3TG mouse fibroblast DNA; lane 8, 10T½ mouse fibroblast DNA; lane 9, HDM-18 mouse hybrid with human chromosomes 11 and 4; lane 10, 10T½(11n)-5 mouse hybrid with human chromosome 11. Arrow indicates expected 193-bp fragment from primers flanking exon 3.

DISCUSSION

We have determined that the human dCyd kinase gene is composed of seven exons distributed over >34 kb of DNA on chromosome 4. The relatively large size of the second intron of this gene has precluded a precise delineation of its size, although it is >19 kb long. Of interest is the fact that two other enzymes involved in deoxyribonucleotide metabolism, adenosine deaminase (ADA; ref. 17) and terminal deoxynucleotidyltransferase (TdT; refs. 18 and 19) both have large introns at the 5′ end of their respective genes (12.8 and 13 kb, respectively). In the case of ADA, a core region within a 1.3-kb segment of the first intron has been shown to play an important role in the high level of expression of this enzyme in the thymus and T lymphoblasts (20, 21). dCyd kinase, like ADA and TdT, is expressed at the highest levels in thymocytes and leukemic T lymphoblasts and expression is down-regulated during T-cell development (22). It will be of considerable interest to determine the factors contributing to the tissue-specific regulation of these three genes.

dCyd kinase is an enzyme that is expressed in all normal tissues examined. Hence, the finding that the dCyd kinase

Table 1. Exon/intron boundaries in the human deoxycytidine kinase gene

Exon	Position in cDNA sequence	Size of exon, bp	Intron	Sequence of exon/intron junctions* (5′-splice donor–intron–3′-splice acceptor)
1	1–250	250	1	ACATCGgtaagg ..... caacagCTGCAG
2	251–366	115	2	TTTGAGgtatga ..... aaatagGAACTT
3	367–560	193	3	TGACAGgtatgt ..... ttttagGTATAT
4	561–708	146	4	CCAGAGgtaaaa ..... tttagACATGC
5	709–824	115	5	ACTGAAgtaaga ..... aacagAACCAA
6	825–915	90	6	GAAAAGgtagat ..... cctcagGTCAAA
7	916–2460	1544		

*Capital letters signify exon sequence and lowercase letters signify intron sequence.

Table 2. CAT activity of p697CAT transfected into Jurkat T and Raji B lymphoblasts

Construct	CAT activity J	CAT activity R	Relative ratio J/R	β-Galactosidase activity J	β-Galactosidase activity R	Relative ratio J/R
p697CAT CMV β-galactosidase						
5′ → 3′	108	23	6.3	9	31	0.4
	136	16		10	21	
3′ → 5′	97	17	6.0	9	16	0.5
	132	21		12	28	
p697CAT RSV β-galactosidase						
5′ → 3′	788	71	7.7	5	9	0.4
	514	98		4	13	
pCAT-	0.3	0.0				
Enhancer	0.0	0.1				

CAT activity is expressed as cpm per μg of protein of individual assays. Each set of experiments was performed in duplicate. β-Galactosidase activity is expressed as units/μg × 10^{-2}. Baseline β-galactosidase activity in cell extracts alone was 0.3 unit/μg × 10^{-2} for both Jurkat (J) and Raji (R) cell lines.

gene is regulated by a G+C-rich promoter typical of genes that are constitutively expressed in a variety of cell types is not surprising and renders it similar to a number of other human genes encoding enzymes in the nucleotide metabolic pathway such as dihydrofolate reductase (DHFR) (23, 24), ADA (17, 25), and hypoxanthine guanine phosphoribosyltransferase (26). The identification of four potential Sp1 binding sites in the 5′ region makes it likely that Sp1 plays a role in constitutive expression of the gene, as has been well documented for DHFR (23). The finding of a 9-bp region of identity to the 12-bp E2F dyad binding site is of considerable interest, given the association of E2F sites with genes involved in DNA replication, including the human, hamster, and mouse DHFR genes (27, 28); thymidine kinase; and DNA polymerase α (29). It has been demonstrated that constructs in which the TTT at the 5′ end of the E2F binding site are substituted retain the ability to respond to E2F (15). It is thus possible that the CGCGCCAAA sequence of the dCyd kinase promoter, which is identical to the remainder of the E2F binding site, contributes to regulation of gene expression.

Of interest is the fact that there appears to be some specificity for T-lymphoblast cell lines in the expression of the 697-bp fragment in human lymphoid cell lines. A search of a transcription factor data base (16) did not reveal any lymphoid-specific binding sites, nor does this sequence contain the recently described 5′ LyF-1 enhancer sequence, which appears to play a major role in the transcription of murine TdT in lymphoid cells (30). On the one hand, it is possible that as yet unidentified elements lie within this region to account for the increased expression of dCyd kinase in T lymphoblasts. It is also possible, however, that other cell-specific elements could modulate Sp1-mediated transcription of this gene. There is increasing evidence that additional transcription factors may interact with Sp1 to further enhance transcription, as specifically evidenced by the role of the E2F factor in DHFR regulation and of the retinoblastoma gene product in insulin-like growth factor II expression (31). Such modulation may also occur during cellular differentiation (32) and could potentially explain the differences we have observed in our transient expression experiments. In view of the important role dCyd kinase plays in dictating the sensitivity of hematopoietic malignancies to a wide range of chemotherapeutic agents, it will be important to further define the elements in the gene responsible for tissue- and/or differentiation-related differences in expression.

Note Added in Proof: With the assistance of John Wasmuth and Michael Altherr of the University of California at Irvine, dCyd kinase has been assigned to chromosomal segment 4q13.1–4q21.1.

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