

Phosphorylation of Deoxycytidine Analog Monophosphates by UMP-CMP Kinase: Molecular Characterization of the Human Enzyme¹

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ABSTRACT

Phosphorylation of deoxycytidine analogs by cellular enzymes is a prerequisite for the activity of these compounds. We have investigated the kinetic parameters for the phosphorylation of 1- β -D-arabinofuranosylcytosine (araC) and 2',2'-difluorodeoxycytidine (dFdC) to their diphosphate forms catalyzed by human UMP-CMP kinase. We cloned the cDNA of this enzyme to enable characterization of the recombinant protein, determine its expression in different tissues, and determine the chromosome location of the gene. We showed that the recombinant UMP-CMP kinase phosphorylated CMP, dCMP, and UMP with highest efficiency and dUMP, AMP, and dAMP with lower efficiency. The monophosphates of araC and dFdC were shown to be phosphorylated with similar efficiency as dCMP and CMP.

We further showed, in a combined enzymatic assay, that human deoxycytidine kinase and UMP-CMP kinase together phosphorylated araC, dFdC, and 2',3'-dideoxycytidine to their diphosphate forms. Northern blot analysis showed that the UMP-CMP kinase mRNA was ubiquitously present in human tissues as a 3.9-kb transcript with highest levels in pancreas, skeletal muscle, and liver. The human UMP-CMP kinase gene was localized to chromosome 1p34.1-1p33 by radiation hybrid analysis. We further expressed the UMP-CMP kinase as a fusion protein to the green fluorescent protein in Chinese hamster ovary cells, and showed that the fusion protein was located in the cytosol and nucleus.

Deoxycytidine (dCyd) analogs that are used in chemotherapy of cancer and virus infections require phosphorylation by cellular enzymes to become active. The phosphorylation of dCyd analogs by deoxycytidine kinase (dCK) is regarded to be the rate-limiting step in the activation of the compounds; the further phosphorylation to the di- and triphosphates has not been as thoroughly studied as the initial phosphorylation step. However, it is important to identify all enzymes involved in the activation of nucleoside analogs for a rational use of these compounds as single drugs or in combination therapy. Among the monophosphate kinases, only thymidylate kinase has been carefully studied. The anti-HIV nucleoside analog 3'-azido-2',3'-dideoxythymidine (AZT) is a poor substrate for thymidylate kinase, which results in AZT-monophosphate accumulation in cells (Furman et al., 1986; Lavie et al., 1997). AZT-monophosphate is inactive in inhib-

iting retroviral replication and may cause adverse effects (Tornevik et al., 1995).

There are two pyrimidine nucleoside monophosphate kinases identified in mammalian cells: the thymidylate kinase and the UMP-CMP kinase. Thymidylate kinase phosphorylates dTMP and dUMP, whereas UMP-CMP kinase phosphorylates CMP and UMP as well as dCMP and dUMP (Arima et al., 1977). Both thymidylate kinase and UMP-CMP kinase are known to phosphorylate structural analogs of pyrimidine nucleosides that are used in the treatment of cancer and virus infections. Similar to AZT, there are cytidine and uridine nucleoside analogs that accumulate at the monophosphate level (Vilpo and Vilpo, 1993; Schinazi et al., 1994), and the UMP-CMP kinase may accordingly be limiting for the phosphorylation of pharmacologically important nucleoside analogs. UMP-CMP kinase further phosphorylates (S)-1-(3-hydroxy-2-phosphonomethoxypropyl)cytosine (cidofovir), an acyclic nucleotide analog used in treatment of cytomegalovirus retinitis (Cihlar and Chen, 1996). The formation of the diphosphate form may be the rate-limiting step in the phosphorylation of this compound.

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¹ The DNA sequence reported in this paper has been deposited in the GenBank database (accession no. AF070416).

ABBREVIATIONS: dCyd, deoxycytidine; dCK, deoxycytidine kinase; GST, glutathione-S-transferase; GFP, green fluorescent protein; araC, 1- β -D-arabinofuranosylcytosine; dFdC, 2',2'-difluorodeoxycytidine; ddC, 2',3'-dideoxycytidine; AZT, 3'-azido-2',3'-dideoxythymidine; PCR, polymerase chain reaction; TLC, thin-layer chromatography; dAMP, deoxyadenosine monophosphate; dCMP, deoxycytidine monophosphate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; MP, monophosphate.

The majority of studies on mammalian UMP-CMP kinase have been performed on partially purified enzymes from different cells and tissues (Maness and Orengo, 1975; Arima et al., 1977; Hande and Chabner, 1978; Scott and Wright, 1979; Seagrave and Reyes, 1987). In recent years UMP-CMP kinases have been cloned from the yeast *Saccharomyces cerevisiae* and the slime mold *Dictyostelium discoideum* as well as from several prokaryotes (Liljelund and Lacroute, 1986; Wiesmüller et al., 1990; Jong et al., 1993; Bucurenci et al., 1996). Recently, the first cloning of a mammalian UMP-CMP kinase, from pig, was reported (Okajima et al., 1995). The pig UMP-CMP kinase was recombinantly expressed, but no thorough characterization of the enzyme in regard to nucleotide and nucleotide analog phosphorylation was performed. We have in the present study cloned the cDNA of the human homolog of UMP-CMP kinase, expressed and purified the recombinant protein, and studied phosphorylation of both natural as well as structurally modified nucleotides. Our study includes the first characterization of the enzymatic conversion of the clinically important dCyd analog 2',2'-difluorodeoxycytidine (dFdC) to its diphosphate form.

Experimental Procedures

Cloning and Expression of Human UMP-CMP Kinase cDNA. We searched the expressed sequence tag library of the GenBank database at the National Institute for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) with the Basic Local Alignment Search Tool (BLAST) to identify a human homolog of the pig UMP-CMP kinase. The expressed sequence tag cDNA clones were obtained from Research Genetics Inc. The DNA sequences of the plasmids were determined with the automatic laser fluorescent (A.L.F.) sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

We expressed the cDNA encoded protein in *Escherichia coli* as a fusion protein to glutathione-S-transferase (GST). Two oligonucleotide primers that flanked the open reading frame of the cDNA were designed with *Bam*HI and *Sal*I restriction enzyme sites (5'-GGG-GATCCGATGCTGAGCCGCTGCCGCA and 5'-ATGTCGACT-TAGCCTTCCTTGTCAAAAATCTG). The oligonucleotides were used in a polymerase chain reaction (PCR) and the amplified DNA fragment was cloned in the *Bam*HI to *Sal*I sites of the pGEX-5X-1 plasmid vector (Amersham Pharmacia Biotech).

The plasmid was transformed into the *Escherichia coli* strain BL21(DE3)pLysS (Stratagene, Inc., La Jolla, CA). Protein expression was induced at 27°C with 1 mM isopropyl β -D-thiogalactoside for 12 h and purification was performed as described (Johansson and Karlsson, 1997a). The purified recombinant protein was eluted in 50 mM Tris pH 8.0 supplemented with 10 mM reduced glutathione (Sigma Chemical Co., St. Louis, MO). The size and purity of the recombinant protein was determined by SDS-polyacrylamide gel electrophoresis (Phast system; Amersham Pharmacia Biotech). The protein concentration was determined with Bradford Protein Assay (Bio-Rad, Hercules, CA) and BSA was used as the concentration standard.

Enzyme Assays. The nucleoside monophosphates and triphosphates were obtained from Sigma. [γ -³²P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. All assays were performed in 50 mM Tris-HCl pH 8.0, 5 mM MgCl₂, and indicated concentrations of nucleotides and enzyme. The enzymatic assays with radiolabeled ATP were performed in 10 μ l with 1 mM ribonucleoside monophosphate or deoxyribonucleoside monophosphate and 2.5 μ Ci/ μ l [γ -³²P]ATP. The reaction mixtures were incubated 30 min at 37°C. The reaction products were separated by thin-layer chromatography (TLC) on poly(ethyleneimine)-cellulose F chromatography sheets (Merck & Co., Inc., Whitehouse Station, NJ) in 0.5 M ammonium formate pH 3.5 (Lust and Sahud, 1972). The TLC sheets

were soaked in methanol before use. The TLC sheets were autoradiographed using phosphorimaging plates (BAS 1000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

The kinetic determinations were performed by ion-pair reversed phased HPLC (5 μ m Hypersil ODS 250 \times 4.6 mm column; Hypersil, Cheshire, UK) using 0.1 M triethylamine phosphate buffer (pH 7) and methanol (95:5, v/v) (Lim and Peters, 1989; Uesugi et al., 1997). Chromatography was performed on Gilson 506C HPLC system with 1 ml/min flow rate and detection of nucleotides at 254 nm. The assays for kinetic determinations were performed with 1 mM ATP and different concentrations of the nucleoside monophosphates ranging from 0.008 to 10 mM. The reaction mixtures were incubated at 37°C for 30 min, and stopped by boiling for 5 min before HPLC analysis.

Assays of Nucleoside Analog Phosphorylation. Human dCK cDNA (Chottiner et al., 1991) was cloned into the pGEX-5X-1 vector and recombinant dCK-GST was expressed as described (Johansson and Karlsson, 1997a). One microgram of dCK, 1 μ g UMP-CMP kinase, 1 μ Ci/ μ l [γ -³²P]ATP, 1 mM unlabeled ATP, and 1 mM either dCyd (Sigma), 1- β -D-arabinofuranosylcytosine (araC; Sigma), 2',3'-dideoxycytidine (ddC; Sigma), or dFdC (Eli-Lilly) were used in the assays. The reaction mixtures were incubated at 37°C for 2 h, and the reaction products were separated by TLC as described above. Kinetic determinations for araC-MP and dFdC-MP were performed with the HPLC method described above. araC-MP was obtained from Sigma and dFdC-MP was enzymatically produced with recombinant *Drosophila melanogaster* deoxyribonucleoside kinase (M.J., A.R.V.R., B. Degrève, J. Balzarini and A.K., unpublished data).

Green Fluorescent Protein (GFP) Fusion Protein. We used the pEGFP-N1 vector (Clontech, Palo Alto, CA) to express the enzyme as a fusion protein with the GFP. Oligonucleotides containing *Hind*III and *Sal*I restriction enzyme sites (5'-CGAAGCTTGTGTATGCTGAGCCGCTGCCGCA and 5'-ATGTCGACCAGCCTTCCTTGTCAAAAATCT) were used to clone the open reading frame of UMP-CMP kinase cDNA into the *Hind*III-*Sal*I sites of the pEGFP-N1 vector. The Chinese hamster ovary cell line was obtained from American Type Culture Collection. The cytosolic and nuclear dCK-GFP constructs, cell culture, transfection, and fluorescence microscopy were performed as described (Johansson et al., 1997b).

Northern Blot. A human multiple tissue Northern blot was purchased from Clontech. The Northern blot contains approximately 2 μ g of poly A⁺ RNA per lane from eight different human tissues. The complete UMP-CMP kinase cDNA was labeled with [α -³²P]dCTP (6000 Ci/mmol; QuickPrime, Amersham Pharmacia Biotech) and the probe was hybridized using the ExpressHyb hybridization solution (Clontech) as described in the manufacturer's protocol. As a quality control the Northern blot was afterward probed with radioactively labeled human β -actin cDNA control probe.

Chromosome Mapping. The GeneBridge 4 (Gyapay et al., 1996) human/hamster radiation hybrid panel was obtained from Research Genetics Inc. (Huntsville, AL). Two oligonucleotide primers located in the untranslated 3' region of the UMP-CMP kinase cDNA (bp 2593-2803) were designed (5'-GGCAACATTGAGTTTTGGAGTTGATT-GA and 5'-AGGCCACAGCACAAAATTTATTGAGA). PCR amplifications were performed as described (Stewart et al., 1997), and the PCR products were analyzed by agarose gel electrophoresis. The results of the GeneBridge 4 radiation hybrid panels were analyzed via Internet at <http://www-genome.wi.mit.edu>. The cytogenetic location was calculated using MapView software at <http://www.gdb.org/>.

Results

cDNA Cloning and Expression of the Human UMP-CMP Kinase. Human expressed sequence tag cDNA clones homologous to the cDNA of pig UMP-CMP kinase were identified in GenBank. The identified clones were deposited by

the Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) consortium (Lennon et al., 1996) (I.M.A.G.E. clone ID 83173, 545355, 140570, 595781, 171759, and 813072). The complete 2.9-kilobase pair cDNA sequence was determined (Fig. 1). The longest open reading frame encoded a 228-amino-acid residue protein with a predicted molecular mass of 26 kDa. The open reading frame of the human UMP-CMP kinase was 93% identical with the pig UMP-CMP kinase sequence at the nucleotide level and 98% identical at the amino acid level. The open reading frame of the pig UMP-CMP kinase lacked the first 32 amino acids present in the open reading frame of the human enzyme. N-terminal peptide sequences of native pig and human UMP-CMP kinase suggest, however, that the second methionine in the human cDNA sequence is the translation start (Hughes

et al., 1993; Okajima et al., 1995). The function of these first 32 amino acids in the human UMP-CMP kinase are not known. Alignment of the human enzyme with UMP-CMP kinase cloned from other species showed that the enzyme was 43% similar to yeast *Saccharomyces cerevisiae* UMP kinase, 50% similar to *Dictyostelium discoideum* UMP-CMP kinase, and 15% similar to the *Escherichia coli* CMP kinase (Fig. 2). The UMP-CMP kinases are also related to the adenylate kinase enzyme family and the human UMP-CMP kinase was 21 to 39% similar to the mammalian adenylate kinases. Three functional domains have been described in the primary structure of nucleoside monophosphate kinases: 1) the nucleoside triphosphate binding glycine rich region, 2) the nucleoside monophosphate binding site, and 3) the lid domain that closes over the substrate upon binding (Scheff

5'-CTCCCTCAGCGTCCGCCGAGGCGCGGTGT																			
ATG	CTG	AGC	CGC	TGC	CGC	AGC	GGG	CTG	CTC	CAC	GTC	CTG	GGC	CTT	AGC	TTC	CTG	CTG	57
M	L	S	R	C	R	S	G	L	L	H	V	L	G	L	S	F	L	L	19
CAG	ACC	CGC	CGG	CCG	ATT	CTC	CTC	TGC	TCT	CCA	CGT	CTC	ATG	AAG	CCG	CTG	GTC	GTG	114
Q	T	R	R	P	I	L	L	C	S	P	R	L	M	K	P	L	V	V	38
TTC	GTC	CTC	GGC	GGC	CCC	GGC	GCC	GGC	AAG	GGG	ACC	CAG	TGC	GCC	CGC	ATC	GTC	GAG	171
F	V	L	G	G	P	G	A	G	K	G	T	Q	C	A	R	I	V	E	57
AAA	TAT	GGC	TAC	ACA	CAC	CTT	TCT	GCA	GGA	GAG	CTG	CTT	CGT	GAT	GAA	AGG	AAG	AAC	228
K	Y	G	Y	T	H	L	S	A	G	E	L	L	R	D	E	R	K	N	76
CCA	GAT	TCA	CAG	TAT	GGT	GAA	CTT	ATT	GAA	AAG	TAC	ATT	AAA	GAA	GGA	AAG	ATT	GTA	285
P	D	S	Q	Y	G	E	L	I	E	K	Y	I	K	E	G	K	I	V	95
CCA	GTT	GAG	ATA	ACC	ATC	AGT	TTA	TTA	AAG	AGG	GAA	ATG	GAT	CAG	ACA	ATG	GCT	GCC	342
P	V	E	I	T	I	S	L	L	K	R	E	M	D	Q	T	M	A	A	114
AAT	GCT	CAG	AAG	AAT	AAA	TTC	TTG	ATT	GAT	GGG	TTT	CCA	AGA	AAT	CAA	GAC	AAC	CTT	399
N	A	Q	K	N	K	F	L	I	D	G	F	P	R	N	Q	D	N	L	133
CAA	GGA	TGG	AAC	AAG	ACC	ATG	GAT	GGG	AAG	GCA	GAT	GTA	TCT	TTC	GTT	CTC	TTT	TTT	456
Q	G	W	N	K	T	M	D	G	K	A	D	V	S	F	V	L	F	F	152
GAC	TGT	AAT	AAT	GAG	ATT	TGT	ATT	GAA	CGA	TGT	GAG	AGG	GGA	AAG	AGT	AGT	GGT		513
D	C	N	N	E	I	C	I	E	R	C	L	E	R	G	K	S	S	G	171
AGG	AGT	GAT	GAC	AAC	AGA	GAG	AGC	TTG	GAA	AAG	AGA	ATT	CAG	ACC	TAC	CTT	CAG	TCA	570
R	S	D	D	N	R	E	S	L	E	K	R	I	Q	T	Y	L	Q	S	190
ACA	AAG	CCA	ATT	ATT	GAC	TTA	TAT	GAA	GAA	ATG	GGG	AAA	GTC	AAG	AAA	ATA	GAT	GCT	627
T	K	P	I	I	D	L	Y	E	E	M	G	K	V	K	K	I	D	A	209
TCT	AAA	TCT	GTT	GAT	GAA	GTT	TTT	GAT	GAA	GTT	GTG	CAG	ATT	TTT	GAC	AAG	GAA	GGC	684
S	K	S	V	D	E	V	F	D	E	V	V	Q	I	F	D	K	E	G	228
TAA	TTCTAAACCTGAAAGCATCCTTGAAATCATGCTTGAATATTGCTTTGATAGTCTGCTATCATGACCCCTTTT	758																	
TAAGGCAATTCTAATCTTTCATAACTACATCTCAATTAGTGGCTGGAAAGTACATGGTAAAAACAAAGTAAATTTT	833																		
TTTATGTTCTTTTTTGGTCACAGGAGTAGACAGTGAATTCAGGTTTAACTTCACTTGTATGTTGTTGCTCACCA	908																		
AACGAAGGGTATCAGCTATTTTTTTTTTAAATTCAAAAAGAAATATCCCTTTTATAGTTTGTGCCTTCTGTGAGCAA	983																		
AACTTTTTAGTACGCGTATATATCCCTCTAGTAATCACAACATTTTAGGATTAGGGATACCTGCTTCTCTCTTTT	1058																		
TCTTGCAAGTTTTTAAATTTTCCAACTTAAAGTGAATTTGTGGACCAAAATTTCAAAGGAATTTTGTGTAGTCAGT	1133																		
TCTTGCACAATGTGTTTGGTAAACAACTCAAAATGGATTCTTAGGAGCATTTTAGTGTTTATTAATAAATGATGAC	1208																		
CATTGCTGTAGAAAGATGAGAAACTTAAGCTTTGTTTCTACTACAACCTGTACAAAGTGTATGACAGGGCATA	1283																		
TTCTTTGCTTCCAAAGATTTGGGTTGGGGGCACTAGGGGTTGAGAGCCTGGCAGAATTGTGAGCTTTAGTCTGACA	1358																		
TAATCTAAGGGTATGGGGCAAGGATCAGTCTAATGCTTGTGTCCTTATCTATTATATAGTGTATTATCATGA	1433																		
TTGAGCTGATCTTAAACAAATTCGTAGCAGTGGAACTTGAATGCAATGTGGCTAGATTATGCTAAAAATGATTC	1508																		
TCAGTTAGCATTTTAGTAACACTTCAAGGTTTTTTTTTGTGTTTCTAGACTTAATAAAAGCTTAGGATTAA	1583																		
TTAGAAGAAGCAATCTAGTTAAATTTCCCATTTGTATTTTATTTTCTTGAATACTTTTTTCATAGTTATTTGTTT	1658																		
AAAAAGATTTAAAAATCATTGCACTTTGGTCAGAAAAATAATAAATATATCTTATAAATGTTTGATTCCCTTCCT	1733																		
TGCTATTTTATTAGTAGATTTTGTGTTGGCATCATGTTGAAGCACCAGAAAGATAAATGATTTTAAAGGCTA	1808																		
TAGAGTCCAAAGGAATATTCTTTACACCAATCTCTCTTTAAAAATCTCTGAGGAATTTGTTTTCGCCTTACTT	1883																		
TTTTTTCTTCTGTCACAAATGCTAAGTGGTATCCGAGGTTCTTAATATGAGATTAAAAATCTAAAAATGTTTCTTA	1958																		
TTTTTTCAGCACTTACATCATTTGGTACACAGGTCAGTATAGGGCAAAATAATTTTGTCTTTGTATAATAGATTGTAT	2033																		
ATTTAAAGTCACTGGAATAGGACAGTTAATGGATGTTTTTATATTTAATAGAATCATTTATTTCTATGTGTT	2108																		
ATGAAATTCACCTTAATGATAAATTTTCAACATACTTGCCATTAGAAAAACAAAGTATTGCTAAGTACTATAACAT	2183																		
ATTGGCCACTAAAAATTCATATTGAGATTATCTTGGTTTCTTGGAAAGATAGGAATGAGTTCTTATCTAGTGTG	2258																		
CAGGCCAGCAAAATACAGAGGTGGTTTAAATCAACAGCTCTAGTATGAAGCAAGATAAAGACTAAGGTTTCGAGA	2333																		
GCATTCCTACTCACATAAGTGAAGAAATCTGTGAGATAGGAATCTAAATTTTATAGTGAATGTGAAAGCAAC	2408																		
CTTAAAGTTTGAAGAAGCTGATGAGACTAGGTGCTTTGCTTCTTTCATCAGGTATCTTCTGTGGCATTGTA	2483																		
GAACAGAAACCAAGAAACATGGTAATTAATTAATGAGGCTTTGCTTTTGTGTTTGAAGTAGAAAAACA	2558																		
TGTTGGCAACCATTTGAGTTTGGAGTTGATTGAGATAATATGACTTAAGTATTTTGTCAATCCCTTTTAAAGA	2633																		
TACAGTCACCAAGAATGTTTGTGTTTTTGAAGACCCCAATTTAAGCCTTGCTTATTTTAAATATTTCCAT	2708																		
TCAGTGATGTTGGATGTATATCAGTTATTTAGTAAATAATCTCAATAAATTTTGTGCTGTGGCCTTTGCTAAAAA	2783																		
AA	2858																		

Fig. 1. The cDNA and predicted amino acid sequence of human UMP-CMP kinase. The amino acid and nucleotide sequence are numbered from the first methionine.

fzek et al., 1996). The alignment showed a highly conserved glycine-rich region at amino acid residues 42 to 50 corresponding to the binding site of the phosphate donor. Amino acid residues 65 to 95 are similar to the nucleoside monophosphate binding site. The lid domain contains many of the catalytically important residues and the corresponding domain was present in human UMP-CMP kinase at amino acid residues 166 to 175.

We expressed the human UMP-CMP kinase as a fusion protein to GST to facilitate purification of the recombinant protein. A major band of ≈ 50 kDa was detected after purification, which corresponded to the 52-kDa molecular mass of GST and UMP-CMP kinase.

Enzymatic Properties of the Recombinant Enzyme.

To verify enzymatic activity of the recombinant enzyme, we tested the naturally occurring ribo- and deoxyribonucleoside monophosphates for activity (Fig. 3). We used ATP as phosphate donor in the assays because studies on UMP-CMP kinase purified from rat liver indicate that the preferred donors are ATP and dATP although the enzyme can use several other phosphate donors (Maness and Orengo, 1975). The ribonucleotides UMP and CMP were both efficiently phosphorylated by the enzyme. However, lower levels of activity was also detected for dCMP, dUMP, AMP, and dAMP. No phosphorylation of GMP, IMP, dGMP, dIMP, or dTMP was detected. The relative activity of UMP and CMP phosphorylation was similar. dCMP phosphorylation was 12%

and dUMP, AMP, and dAMP were 3 to 5%, compared with the activity of UMP and CMP.

We determined the Michaelis-Menten kinetic parameters for CMP, UMP, dCMP, and dUMP (Table 1). The activity of UMP-CMP kinase purified from tissues has been shown to be regulated by reducing agents (Maness and Orengo, 1976a,b), and we therefore determined kinetic properties of the recombinant enzyme both in the presence and absence of 10 mM DTT. CMP exhibited the lowest K_m of 0.5 mM whereas UMP and dCMP had 3-fold higher K_m values and dUMP showed a 12-fold higher K_m . In the absence of DTT, the V_{max} of CMP and UMP were similar, 2.1 and 1.6 $\mu\text{mol/mg/min}$, whereas the V_{max} of dCMP and dUMP both were 7-fold lower. However, addition of DTT to the assay buffer increased the V_{max} of dCMP 21-fold, whereas there were no major changes in velocity for UMP, CMP, or dUMP phosphorylation. In spite of the large increase in dCMP V_{max} in the presence of DTT, the K_m remained unaffected (data not shown). In summary, the efficiency of the different substrates, calculated as V_{max}/K_m , showed that CMP was the best enzyme substrate. Compared with CMP, the efficiency of dCMP phosphorylation was 2-fold lower, UMP phosphorylation was 8-fold lower, and dUMP phosphorylation was 420-fold lower.

In addition to the natural substrates, we determined the kinetic parameters of the monophosphate derivatives of the anticancer nucleoside analogs araC and dFdC. Both nucleoside analogs were efficiently phosphorylated by UMP-CMP

Human UMP-CMPK	MLSRCRSGLLHVLGLSFLLOTRRPILLCSPRIMKPLVVFVLGGPGAGKGTQCARI	56
Pig UMP-CMPK	MRPKVVFVLGGPGAGKGTQCARI	24
Yeast UMPK	MTAATTSQPAFSPDQVSVIFVLGGPGAGKGTQCEKLV	37
Dicty. UMP-CMPK	MEKSKFNVVFVLGGPGSGKGTQCANIV	27
E.coli CMPK	MTAIPVITIDGPSGAGKGTCKAMA	26
Glycine-rich loop		
Human UMP-CMPK	EKYGYTHLSAGELLR-----DERKNPDSQYGELEIKYIK	90
Pig UMP-CMPK	EKYGYTHLSAGELLR-----DERKNPDSQYGELEIKYIK	58
Yeast UMPK	KDYSEFVHLSAGELLR-----AEQGRAGSQYGELEIKNCIK	71
Dicty. UMP-CMPK	RDEGVVHLSAGELLR-----QEQQSGSKD-GEMTATMIK	60
E.coli CMPK	EALQWHLLDSCAIYRVLALAAHHHVDVASEDALVPLASHLDVRFVSTNGNLEVL	82
NMPbind domain		
Human UMP-CMPK	EGKIVPVEITISLLKREMDQMAANAQKNKFLIDGFPRNQDNLQGWNKTM-----	140
Pig UMP-CMPK	DGKIVPVEITISLLRREMDQMAANAQKNKFLIDGFPRNQDNLQGWNKTM-----	108
Yeast UMPK	EGQIVPQEITLALLRNAISDNVANK--FKFLIDGFPRKMDQAISFER-----	117
Dicty. UMP-CMPK	NGEIVPSIVTVKLLKNAIDANQG-----KNFLVDGFPNENNNNSWEENMK-----	106
E.coli CMPK	EGEDVSGEIRTQEVANAASQVAA-----FPRVREALRRQRAFRELPLGL	126
LID domain		
Human UMP-CMPK	-----DGKADVSVLFFDCNNEICIERCLERGKSSGRSDDNRESLEKRIQTY	187
Pig UMP-CMPK	-----DGKADVSVLFFDCNNEICIERCLERGKSSGRSDDNRESLEKRIQTY	155
Yeast UMPK	-----DIV-ESKEILFFDCPEDIMLERLIERGKTSGRSDDNIESIKRENTF	163
Dicty. UMP-CMPK	-----DFV-DTKEVLFVDFCPPEEVMTCRLIKRGESSGRSDDNIESIKRENTF	152
E.coli CMPK	IADGRDMGTVVFDAPVKIFLDASSEERAHRRMLQLQKEG-----FSVNFERL	174
LID domain		
Human UMP-CMPK	LQSTKPIIDLIEEMGKVKKIDASKSVDEVFDEVVQIFDKEG	228
Pig UMP-CMPK	LQSTKPIIDLIEEMGKVKKIDASKSVDEVFDEVVQIFDKEG	196
Yeast UMPK	KETSMIEVIEYEETKSKVVRVRCDSVEDVYKDVQDAIRDSL	204
Dicty. UMP-CMPK	NVQTKLVIDHYNKFVKVLIIPANRDVNEVYNDVENLEKSMGF	194
E.coli CMPK	LAETKERDDRRNRAVAPLVPAADALVLDSTLSIEQVIEKALQYARQKLALA	227

Fig. 2. Alignment of the predicted amino acid sequences of human UMP-CMP kinase and UMP-CMP kinases from pig, *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, and CMP kinase from *Escherichia coli*. Black boxes indicate conserved amino acid residues compared with human UMP-CMP kinase. NMPbind, nucleoside monophosphate binding.

kinase, with an efficiency similar to the efficiency of dCMP and CMP phosphorylation (Table 1). The 1.4-mM K_m of araC-MP was similar to the K_m of dCMP and these two substrates also exhibited similar V_{max} . dFdC-MP had a 3-fold lower K_m than araC-MP. The kinetic parameters of dFdC-MP were accordingly similar to those of CMP.

Two-Step Phosphorylation of Nucleoside Analogs In Vitro. We decided to test if we could study phosphorylation of the pyrimidine nucleoside analogs to their diphosphate derivative in vitro by combining the nucleoside kinase dCK with UMP-CMP kinase. dFdC, araC, and ddC were all phosphorylated to their diphosphate derivative in the presence of dCK and UMP-CMP kinase (Fig. 4).

Subcellular Location. The mammalian adenylate kinases are located in either the cytosol or the mitochondria. Sequence analysis of the UMP-CMP kinase primary structure did not reveal any known signal for targeting the protein to any subcellular compartment. To verify that UMP-CMP kinase was located in the cytosol, we decided to express the enzyme as a fusion protein to GFP and visualize the protein in cultured cells. The cells transfected with GFP alone showed as expected green fluorescence both in the cytosol and nucleus (Fig. 5). Although GFP lacks a nuclear import signal, the small size of the protein allows it to passively enter the nucleus through the nuclear envelope pores. As controls, we transfected the cells with a wild-type nuclear dCK-GFP and a mutant cytosolic dCK-GFP construct (Johansson et al., 1997b). Nuclear dCK showed strong fluorescence in the nucleus and weak fluorescence in the cytosol, whereas the cytosolic mutant of dCK showed strong fluores-

cence in the cytosol but no fluorescence in the nucleus. The cells transfected with the plasmids encoding the UMP-CMP kinase-GFP fusion showed fluorescence in both the cytosol and nucleus, similar to the cells transfected with wild-type GFP. We conclude that the UMP-CMP kinase-GFP fusion lacks subcellular targeting signals and that the relative small molecular mass of the fusion protein allows it to passively diffuse between the cytosol and nucleus.

Northern Blot Analysis. We used a multiple tissue Northern blot with mRNA from eight different human tis-

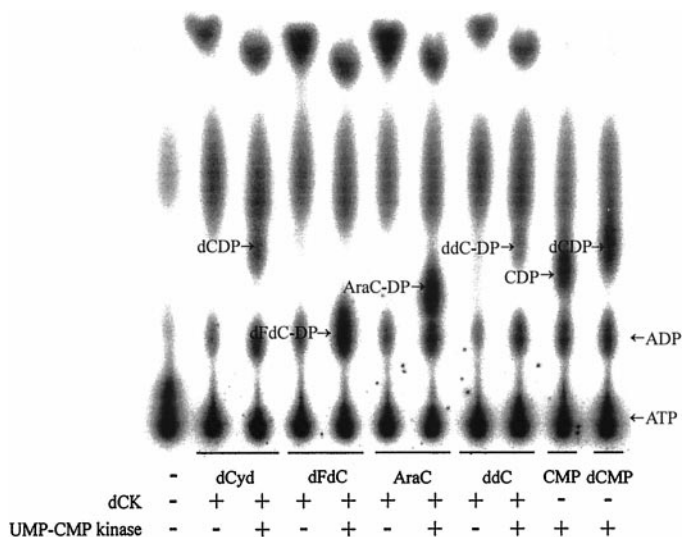


Fig. 4. A coupled assay using dCK and UMP-CMP kinase to phosphorylate the nucleoside analogs dFdC, araC, and ddC. dCyd, CMP, and dCMP were used as controls. +, reaction with enzyme present; -, reaction without enzyme added.

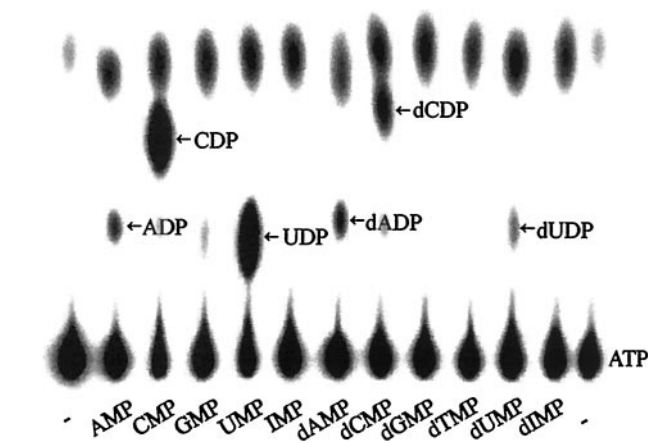


Fig. 3. Screening of substrate specificity of human recombinant UMP-CMP kinase. The substrate concentration was 1 mM and ATP was used as phosphate donor.

TABLE 1

Kinetic properties of the recombinant human UMP-CMP kinase with ATP as phosphate donor

Substrate	K_m	V_{max}		V_{max}/K_m	
		-	+	-	+
	mM	$\mu\text{mol}/\text{mg}/\text{min}$			
CMP	0.50	2.1	4.2	4.3	8.4
UMP	1.6	1.6	1.6	1.0	1.0
dCMP	1.6	0.30	6.2	0.19	3.9
dUMP	5.9	0.23	0.12	0.04	0.02
araC-MP	1.4	N.D.	5.2	N.D.	3.7
dFdC-MP	0.45	N.D.	3.6	N.D.	8.0

-, no DTT; +, with DTT.

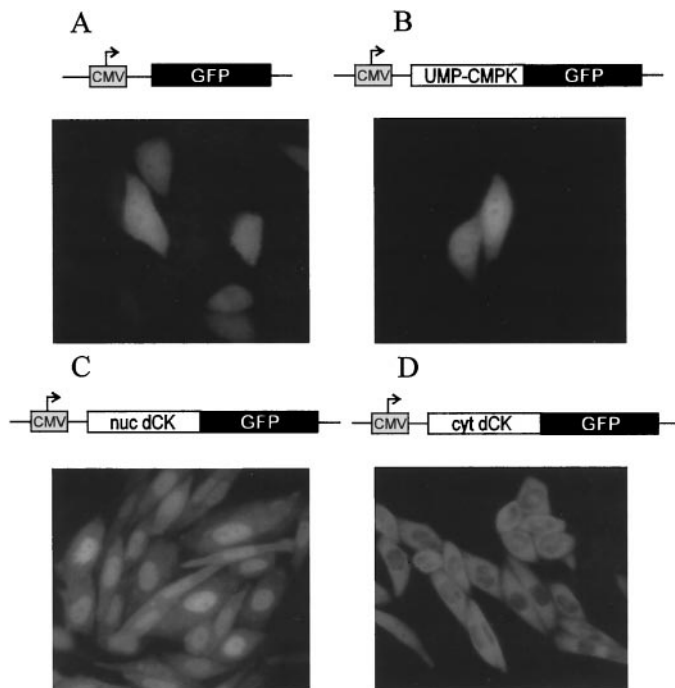


Fig. 5. Fluorescence microscopy images of Chinese hamster ovary cells transfected with plasmids encoding GFP and UMP-CMP kinase-GFP fusion protein. A, wild-type GFP; B, UMP-CMP kinase-GFP; C, nuclear dCK-GFP (nucdCK-GFP); D, cytosolic dCK-GFP (cytdCK-GFP). CMV, cytomegalovirus promoter.

sues to study the expression pattern of UMP-CMP kinase mRNA. The Northern blot analysis showed that the mRNA transcript of UMP-CMP kinase was detected as a ≈ 3.9 -kilobase band ubiquitously expressed in human tissues (Fig. 6). Among the eight investigated tissues, the mRNA was present in all investigated tissues with the highest level of expression in pancreas, skeletal muscle, and liver. The β -actin cDNA showed the presence of a single 2.0-kb band in all lanes and a second band in the heart (1.8-kb). The highest level of β -actin was in skeletal muscle, lung, and heart. Expression of β -actin mRNA varies between different tissues, and the mRNA level of actin in skeletal muscle is higher than in other tissues. We therefore cannot draw any conclusions with regard to the level of expression of the UMP-CMP kinase in skeletal muscle. We are presently generating antibodies against the recombinant UMP-CMP kinase protein to study the tissue distribution in detail.

Chromosome Mapping. We used the GeneBridge4 human-hamster radiation hybrid panel to determine the chromosome location of the UMP-CMP kinase gene. The cell hybrid clones were screened for the presence or absence of a PCR amplifiable marker for the UMP-CMP kinase locus. The radiation hybrid analysis indicated that the UMP-CMP kinase gene was located 3.77 centiRay distal to marker D1S2134 (GeneBridge 4 positive clones: B2, C11, D7, E2, E6, F13, I1, I4, K9, K12, P11, Q4, R3, S6, S12, V7, Y4, Y8). This marker is located at chromosome 1p34.1–1p33.

Discussion

We study the enzymes involved in the activation of nucleoside analogs in an attempt to reconstitute their pathways of activation. In the present investigation we have focused on the phosphorylation of clinically important dCyd analogs to their corresponding diphosphates. We have cloned, expressed, and characterized the human UMP-CMP kinase. Because this is the first report on recombinant human UMP-CMP kinase we performed a careful characterization of the enzyme using natural substrates, in addition to the nucleoside analogs. Our data showed that CMP, dCMP, and UMP

were the best substrates for the enzyme, whereas dUMP was poorly phosphorylated. The physiological basis of this discrepancy is probably that dUTP is harmful to the cells and low efficiency of dUMP phosphorylation by UMP-CMP kinase may contribute to keeping the cellular dUTP levels low. The pattern of substrate specificity of the recombinant UMP-CMP kinase is generally in agreement to that reported for the tissue-purified mammalian enzymes (Maness and Orengo, 1975; Arima et al., 1977; Scott and Wright, 1979; Okajima et al., 1995). However, previous reports on the kinetic parameters of UMP-CMP kinases are partially conflicting. The K_m for UMP and CMP of purified enzyme from mammalian tissues is in most studies reported to be ≈ 10 -fold lower than the K_m values of the recombinant enzyme (Sugino et al., 1966; Maness and Orengo, 1975, 1976a; Teng et al., 1976; Hande and Chabner, 1978). However, there are studies on tissue-purified UMP-CMP kinase that report similar affinity of UMP and CMP as for the recombinant enzyme (Scott and Wright, 1979; Seagrave and Reyes, 1987). The reasons for these discrepancies are not yet clear. In regard to the affinity to dCMP and dUMP, most reports are in agreement with the kinetic data of the recombinant enzyme (Sugino et al., 1966; Maness and Orengo, 1975, 1976a; Arima et al., 1977; Hande and Chabner, 1978). The majority of studies on mammalian UMP-CMP kinases have been performed on partially purified enzyme and it is therefore difficult to compare the specific enzymatic activity of different enzyme preparations, but a study on highly purified rat UMP-CMP kinase shows a V_{max} in the same range as for the recombinant human enzyme (Maness and Orengo, 1976a). The intracellular levels of UMP and CMP are in most tissues 10-fold lower than the K_m of UMP-CMP kinase and the corresponding deoxyribonucleotide levels are more than 1000-fold lower than the K_m determined for the recombinant UMP-CMP kinase (Traut, 1994). These data, together with the kinetic parameters of the recombinant enzyme, indicate that UMP-CMP kinase would preferentially phosphorylate ribonucleotides in vivo.

UMP-CMP kinase activity requires the presence of reducing agents and dCMP phosphorylation shows the highest sensitivity (Sugino et al., 1966; Maness and Orengo, 1975, 1976a). Inactive oxidized UMP-CMP kinase can regain full activity by incubation with DTT (Sugino et al., 1966; Maness and Orengo, 1976a,b). The mechanism of activation involves cleavage of inter- and intramolecular disulfide bonds that cause the enzyme to change its quaternary structure (Maness and Orengo, 1976b). It is currently not known which amino acids in the enzyme are involved in formation of the disulfide bonds. The recombinant human enzyme was active both in the presence and absence of DTT. However, dCMP phosphorylation was markedly increased by addition of DTT, whereas phosphorylation of other substrates were not affected. It is possible that the fusion of the recombinant enzyme to GST affects the enzyme's ability to form disulfide bonds and thereby make the enzyme less dependent of a reducing environment. It is possible that dCMP phosphorylation catalyzed by the enzyme in vivo is redox-regulated. However, there is at present time no experimental support of the hypothesis.

Although UMP-CMP kinases from yeast and *D. discoideum* are closely sequence-related to the mammalian kinases, these enzymes exhibit different patterns of substrate

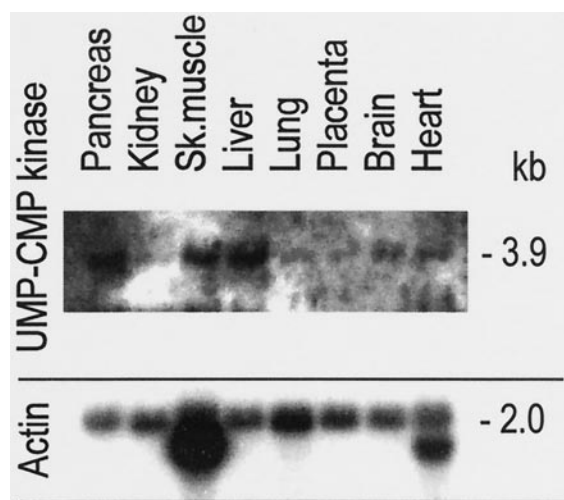


Fig. 6. Northern blot analysis of human UMP-CMP kinase mRNA expression in human tissues. The UMP-CMP kinase cDNA probe hybridized with a single mRNA band at 3.9 kilobases in all tissues. An actin probe was used as a control of the amount of mRNA immobilized on the membrane.

specificity. The yeast enzyme is predominantly a UMP kinase and it phosphorylates CMP poorly (Jong et al., 1993), whereas the *D. discoideum* enzyme phosphorylates CMP better than UMP (Wiesmüller et al., 1990). Bacterial enzymes, such as the CMP kinase of *E. coli*, phosphorylates CMP and dCMP equally efficiently whereas UMP is a poor substrate for the enzyme (Bucurenci et al., 1996). Recently, structural studies of both prokaryotic as well as eukaryotic UMP-CMP kinases have been reported, and these studies will contribute to the understanding of the difference in substrate recognition between the enzymes (Scheffzek et al., 1996; Briozzo et al., 1998).

In addition to phosphorylation of the pyrimidine nucleoside monophosphates, we also detected low levels of AMP and dAMP phosphorylation catalyzed by the recombinant enzyme. UMP-CMP kinases from other species, such as yeast, have been reported to phosphorylate other nucleotides, such as AMP and dTMP, but the activity is generally low (Jong et al., 1993). However, complementation studies performed in thymidylate or adenylate kinase-deficient yeast cells show that the yeast UMP-CMP kinase is a dominant suppressor of these deficiencies (Choi et al., 1989; Schricker et al., 1992). These experiments suggest, at least in the absence of other nucleoside monophosphate kinases, that the yeast UMP-CMP kinase can be important for dTMP and AMP phosphorylation. However, the physiological role of the human UMP-CMP kinase for these alternative substrates remains to be shown.

Biochemical studies on cell extracts suggest that human cells contain a single UMP-CMP kinase (Sugino et al., 1966; Arima et al., 1977). Subcellular fractionation has further shown that the major UMP-CMP kinase activity is present in the cytosol (Shiosaka et al., 1975). The cloned human enzyme did not appear to contain a signal for transport to another subcellular compartment and the absence of a subcellular targeting sequence was further supported by the predominantly cytosolic location of the UMP-CMP kinase-GFP fusion protein. However, cellular nucleotide metabolism is separated into two compartments: the cytosolic/nuclear and the mitochondrial. The mitochondria inner membrane constitutes a barrier that prevents free nucleotide transport between the cytosol and the mitochondria. The mitochondria contains several nucleoside and nucleotide kinases that phosphorylate pyrimidines, but no mitochondrial pyrimidine nucleoside monophosphate kinase has yet been identified. Recently, a mitochondrial dCTP import system was identified, and it may explain the apparent absence of a CMP kinase in the mitochondria (Bridges et al., 1999).

The chromosome location of a human UMP-CMP kinase has been determined to 1p32 using low-resolution somatic cell hybrid analysis and family studies (Cook and Hamerton, 1979). The basis for these studies is the identification of three different UMP-CMP kinase alleles that can be distinguished by their different biochemical properties. The 1p32 location is adjacent to 1p34.1–1p33, where the gene of the cloned UMP-CMP kinase was located. Accordingly, we conclude that the cloned UMP-CMP kinase gene is the same as the one previously mapped to 1p32. In a study on the incidence of bacterial infection, the UMP-CMP kinase allele 2 was linked to prolonged respiratory infections (Giblett et al., 1974) and patients homozygous for UMP-CMP kinase allele 3 showed increased incidence of invasive *Hemophilus influenzae* type B

infections (Petersen et al., 1985). These studies included, however, few patients, and the potential involvement of UMP-CMP kinases in an immunodeficiency disorder is still not clarified. Several types of solid tumors frequently exhibit 1p deletions and the UMP-CMP kinase gene has been suggested to be included in these deletions. However, there is no evidence that these tumors have changed levels of UMP-CMP kinase enzyme activity (Bravard et al., 1991).

Phosphorylation of nucleoside analogs is a prerequisite for pharmacological activity of these compounds. Although a few nucleoside analogs exert their pharmacological effects in the monophosphate form, the majority of compounds require phosphorylation to di- and triphosphates. Pyrimidine nucleoside analogs, such as araC and dFdC, are widely used in chemotherapy of malignancies. araC is used in the treatment of several hematological malignancies and dFdC is used in the treatment of solid tumors such as pancreatic cancer and nonsmall cell lung cancer. We showed that the monophosphate derivatives of araC and dFdC were efficient substrates of human UMP-CMP kinase. We also showed that the anti-HIV nucleoside analog ddC, at the monophosphate level, is a substrate of the human UMP-CMP kinase. Northern blot analysis showed that UMP-CMP kinase mRNA is present in all investigated tissues with the highest levels in pancreas, liver, and skeletal muscle. There are, however, no data suggesting that these tissues would be more sensitive to cytotoxic pyrimidine nucleoside analogs phosphorylated by UMP-CMP kinase.

We have further shown that nucleoside analogs incubated with both the nucleoside kinase dCK and the UMP-CMP kinase can be phosphorylated to their diphosphate derivatives in vitro. Assays of nucleoside analog phosphorylation using multiple kinases to reconstitute the pathways involved in the activation of the compounds will be a useful method for the screening of novel nucleoside analogs.

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