0026-895X/93/030380-08$03.00/0

MOLECULAR PHARMACOLOGY, 43:380–387

A First Step in the Development of Gene Therapy for Colorectal Carcinoma: Cloning, Sequencing, and Expression of *Escherichia coli* Cytosine Deaminase

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Received September 2, 1992; Accepted December 29, 1992

SUMMARY

We have developed a new approach involving gene therapy for the treatment of primary and metastatic tumors in the liver. As a first step toward the development of this gene therapy treatment for metastatic colorectal carcinoma, the *Escherichia coli* gene that encodes cytosine deaminase (CD) (EC 3.5.4.1) has been cloned. By using positive genetic selection, a plasmid carrying a 10.8-kilobase *Bam*HI/*Eco*RI DNA insert was isolated that had CD enzymatic activity. Genetic screening, followed by enzymatic assays, identified a 3-kilobase DNA fragment that retained CD activity. Deamination of cytosine and 5-fluorocytosine (5-FC) by cloned CD was demonstrated. DNA and protein sequencing identified an open reading frame of 427 amino acids that encodes CD. To demonstrate that expression of CD in eukaryotic cells allows metabolism of the nontoxic prodrug 5-FC to the toxic metabolite 5-fluorouracil, CD was cloned into a eukaryotic expression vector and transfected into a human colorectal carcinoma cell line. Growth inhibition studies showed a shift in the IC_{50} for 5-FC from 17,000 μM in the parental cell line to 30 μM in cells expressing CD.

Despite intensive effort, the overall 5-year survival rate for patients with CRC has not meaningfully improved in the last three decades. At the time of primary diagnosis, approximately 50% of patients with CRC show lymphatic system involvement, with approximately 25% having disseminated disease (1–3). The liver is the most common site for distant metastasis and, in approximately 30% of patients, the sole initial site of tumor recurrence after successful resection of the primary colon cancer (1). Hepatic metastases are the most common cause of death in patients with CRC (4) and, if present, reduce the median survival time to approximately 5 months (5). Obviously, early detection and management of regional disease are only two critical aspects in the complete management of patients with CRC. Significant therapeutic advances must be made in the treatment of CRC hepatic metastasis if the overall 5-year survival rate is to improve.

The treatment of choice for the majority of patients with hepatic CRC metastases is systemic or regional chemotherapy using 5-FU alone or in combination with other agents such as leviamasole (for review, see Ref. 1). 5-FU alone or in combination has consistently proven to be the most efficacious chemotherapeutic agent and to produce the highest overall partial response rates for hepatic CRC metastasis. Despite extensive effort, there is still no satisfactory treatment for hepatic CRC metastasis. New innovative approaches must be developed if the overall 5-year survival rate is to be meaningfully improved upon.

We have developed an innovative approach using gene transfer technology for the treatment of primary and metastatic tumors in the liver (6). We now describe the first step in the development of this approach for the treatment of hepatic CRC metastasis. CEA is a tumor-associated marker that is expressed in a large percentage of primary and metastatic CRC cells and is widely used as an important diagnostic tool for postoperative surveillance, chemotherapy efficacy determinations, immunolocalization, and immunotherapy (for review, see Refs. 7–12). In our gene therapy approach, the final step will be to create a chimeric gene composed of the 5′ transcriptional regulatory sequences of the CEA gene and the coding domain of the CD gene. Thus, transcription of CD from this chimeric gene will be restricted to CEA-positive hepatic CRC metastases and it will not be expressed in normal liver cells. CD, which is present in microbes and fungi but absent in higher eukaryotes, catalyzes the hydrolytic deamination of cytosine and 5-FC to uracil and 5-FU, respectively. By placing the expression of the gene encoding CD under the transcriptional control of the CRC-associated marker gene CEA, the nontoxic compound 5-FC can be metabolically activated to 5-FU selectively in hepatic CRC cells. This approach is illustrated in Fig. 1.

As a first step in this approach, we describe the cloning,

ABBREVIATIONS: CRC, colorectal carcinoma; CD, cytosine deaminase; CEA, carcinoembryonic antigen; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); LB, Luria-Bertani.

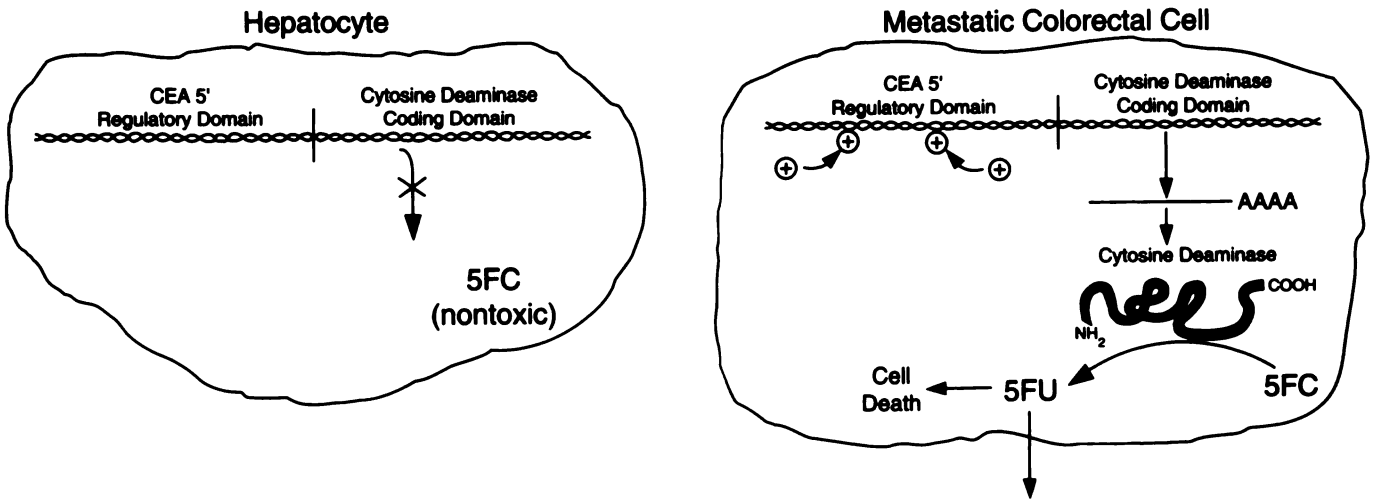


Fig. 1. Schematic representation of selective expression of the chimeric CEA/CD gene in metastatic CRC cells. After expression of the chimeric gene, treatment with 5-FC leads to cell death as the result of metabolism of 5-FC to 5-FU by CD.

sequencing, and expression of the *Escherichia coli* gene encoding CD. The *E. coli* CD gene was chosen because the enzyme is thermostable at 40° for months in the pH range of 7–9 (13). In contrast, previous work by Katsuragi *et al.* (14) with purified CD from bakers yeast had found it to be thermolabile. In addition, the wide variety of genetic techniques available for studies with *E. coli* allowed the design of a positive genetic selection scheme for the cloning of the CD gene.

Materials and Methods

Bacterial strains and growth conditions. The *E. coli* strain χ82 [*lacZ53*(Am), λ⁻, *trpC60*, *pyrF287*, *hisG1*, *rpsL8*] was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). The *E. coli* strains MM294 [F⁻, *endA1*, *hsdR17* (r_k^- m_k^+), *supE44*, *thi-1*] and MBM7007 [F⁻, *ara*Cam, *araD*Δ(*argF-lac*)U169, *trp*am, *mal*Bam, *rpsL*, *relA*, *thi*] and the P1*vir* lysate were kindly provided by W. Dallas (Wellcome Research Labs, Research Triangle Park, NC). The Kohara λ phage lysates were kindly provided by Y. Kohara (National Institute of Genetics, Sizuoka-Ken Japan). Strains were grown either in LB or in minimal medium (15) supplemented with the appropriate amino acids (20 μg/ml), 0.2% glucose, and thiamine. Ampicillin was added at 100 μg/ml.

Transduction. Generalized transduction was used to move DNA between various bacterial strains, following the method of Miller (16). P1*vir* lysates were grown on the strain carrying a marker of interest. After harvesting of the lysate, the P1*vir* lysate was used to move the marker into a new strain. The genetic properties of the resulting strain were verified using selective media.

Isolation of λ DNA. λ Phage particles were isolated by polyethylene glycol precipitation followed by CsCl block gradient centrifugation. DNA was isolated from the purified phage particles by dialysis against 50% formamide, followed by dialysis against TE, 10 mM Tris-HCl, 1 mM EDTA pH 7.5 (17).

Transformation. Ligation reaction products were initially amplified by transforming DH5α according to the supplier's specifications (BRL Life Technologies Inc., Gaithersburg, MD). Plasmid DNA was isolated from transformed cultures grown overnight under ampicillin-selective pressure. Isolated plasmid DNA was used to transform BA101. Cells competent for uptake of DNA were prepared using $CaCl_2$.

CD enzymatic assay. The ability of cell extracts to deaminate cytosine to uracil or 5-FC to 5-FU was assayed spectrophotometrically as described by West and O'Donovan (18). Cell cultures were grown overnight at 37° in LB or minimal medium containing the appropriate supplements. Density of the cultures was determined before 10^{10} cells were harvested. Cell pellets were washed twice with cold 0.9% NaCl and then resuspended in 0.75 ml of cold 50 mM Tris·HCl, pH 7.3. Cell suspensions were sonicated using a Branson 450 sonifier. Whole cells and cellular debris were removed by centrifugation at 14,000 rpm for 30 min. Protein concentrations were determined using a Bio-Rad protein determination kit (Bio-Rad, Richmond, CA). CD activity was measured in a 1-ml assay mixture containing 50 mM Tris·HCl, pH 7.3, 10 μl of cell extract, and 0.5 mM cytosine or 5-FC. Decreases in absorbance at 285 nm were measured over time. Product was estimated by using a molar extinction coefficient of 1.038×10^3 M^{-1} cm^{-1} for cytosine to uracil and 1.96 mM^{-1} cm^{-1} for 5-FC to 5-FU.

PAGE. Cell extracts prepared for CD enzymatic assays were also analyzed by SDS-PAGE as described by Laemmli (19). Thirty-five micrograms of protein were loaded in each lane of a 12% SDS-polyacrylamide gel and electrophoresed under denaturing conditions.

DNA and protein sequencing. DNA sequence was determined using double-stranded templates and custom oligonucleotides purchased from Synthecell, Inc. (Rockville, MD). Sequencing reactions were carried out according to manufacturer's recommendations, using either the BRL dsDNA Cycle Sequencing System (Life Technologies, Inc., Gaithersburg, MD) or the United States Biochemicals Sequenase kit (United States Biochemicals, Cleveland, OH).

Protein sequence determination was done using an ABI 477A protein sequencer. Fifty micrograms of cell extract were electrophoresed on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were electroblotted onto Immobilon-P (Millipore, Inc., Bedford, MA). The membrane was then stained with 0.1% Coomassie Brilliant Blue/50% methanol to visualize the proteins. The M_r 52,000 band was excised and the amino-terminal sequence was determined.

Construction of mammalian expression vector. Oligonucleotide site-directed mutagenesis was used to change the start codon of the CD gene from GTG to ATG and to introduce a *Hind*III site 5′ to the start codon. The mutagenesis was carried out according to manufacturer's instructions (Amersham, Inc., Arlington Heights, IL). Site-directed mutagenesis was performed on the 201-base pair *Pst*I fragment (see restriction map in Fig. 3). The presence of the *Hind*III site and of the ATG start codon were confirmed by DNA sequencing. The resulting 80-base pair *Hind*III/*Pst*I fragment was ligated to *Pst*I/*Hind*III-digested pEA006 to reconstruct the entire CD gene (pEA017). The mutagenized CD gene was subcloned into pBS^+ as a *Hind*III/*Pvu*II fragment (pEA018). The vector pRc/CMV (Invitrogen Corp., San Diego, CA) was linearized with *Bst*XI. Blunt ends were created by treatment with T4 polymerase. The vector was then cut with *Hind*III and ligated with

the *Hind*III/*Sma*I CD fragment isolated from pEA018, to create pCMV/CD-1.

Cell culture. WiDr cells, a human CRC cell line, were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 0.02 mg/ml gentamicin. Linearized pCMV/CD-1 was transfected into the cells using Lipofectin (GIBCO BRL, Bethesda, MD). Forty-eight hours after transfection, cells were selected with medium containing 1 mg/ml G418. After G418 selection, inhibition of cell growth in the presence of 5-FC or 5-FU was determined as described previously (6).

Results

Construction of BA101. The *E. coli* gene encoding CD was chosen for cloning because of the well characterized genome of *E. coli* and the genetic techniques available for studies with *E. coli*. CD converts cytosine to uracil as part of the pyrimidine salvage pathway (Fig. 2) and is the only means by which *E. coli* can metabolize cytosine. CD functions as a multimer of identical subunits. Studies with purified holoenzyme have determined that the enzyme has an apparent molecular weight of 210,000 and is thermostable between pH 7 and 9 (20). Mapping studies have determined that the gene encoding CD, *codA*, lies between 6 and 8 min on the *E. coli* chromosome (21, 22), with a gene order of *lac-codAB-pro*. The *codA* gene is thought to form part of an operon that includes *codA* and *codB*, a permease responsible for transporting cytosine across the cytoplasmic membrane (23).

A bacterial strain, BA101, was constructed that could utilize cytosine as a sole pyrimidine source only when CD was provided *in trans*. BA101 was constructed to carry both a deletion of the *codAB* operon and a *pyrF* mutation. The *pyrF* mutation, which confers a pyrimidine growth requirement, was isolated from the *E. coli* strain χ82 and transduced into the strain MBM7007. MBM7007 carries a chromosomal deletion from *lac* to *argF* that includes the *codAB* operon. The resulting strain, BA101, is able to grow on minimal medium supplemented with uracil but is unable to utilize cytosine as the sole pyrimidine source. BA101 is also resistant to 5-FC, because of the absence of *codA*. The growth characteristics of strains carrying these mutations are illustrated in Fig. 2.

Cloning of *codA*. The construction of BA101 provided a positive selection method for the cloning of *codA*. A subset of the Kohara λ phage (24) that contains DNA inserts spanning the 6–8-min region of the *E. coli* chromosome was screened for the ability to allow growth of BA101 in medium containing cytosine as the sole pyrimidine source. Of the six phage lysates tested, only two clones, 137 and 138, allowed BA101 to utilize cytosine as a pyrimidine source. The physical map of the chromosomal DNA inserts carried by these phage showed that these inserts were partially overlapping.

Restriction enzymes were used to generate DNA fragments of 10–12 kb from λ 137 (9F1) and λ 138 (10A6). These fragments were ligated with linearized pBR322 and amplified in DH5α before being used to transform BA101. Analysis of plasmid DNA isolated from BA101 transformants that grew on cytosine identified two inserts. One of the plasmids, pEA001, contained a 10.8-kb *Eco*RI/*Bam*HI insert that completely overlapped the approximately 11.5-kb *Eco*RI/*Hind*III insert of the other isolate, pEA002 (Fig. 3). These plasmids were used to transform BA101 again, to confirm that the ability of these plasmids to allow growth of BA101 with cytosine was the result of the presence of a plasmid insert encoding CD and not a chromosomal mutation in response to selective pressure.

Deletion analysis. A number of deletion derivatives (Fig. 3) were created from the plasmid pEA001. The results of the deletion analysis indicate that the minimum DNA fragment capable of CD activity lies between the *Bgl*II sites and extends past the proximal *Cla*I site. The presence or absence of *codA* was determined by genetic screening of the deletion derivative plasmids by transformation of BA101 followed by selection for growth on medium containing cytosine as the sole pyrimidine source. Deletion of DNA sequences between the *Bam*HI site and the *Bgl*II site (pEA003 and pEA006) did not affect the ability of the plasmid to complement the *codA* defect of BA101. Likewise, deletions from the right that removed the *Cla*I/*Eco*RI DNA fragment (pEA009 and pEA013) still allowed growth of BA101 in medium containing cytosine. However, any deletions that removed DNA sequences between the *Bgl*II site and the distal *Sna*BI site (pEA004, pEA005, pEA007, pEA010, and pEA014) eliminated CD activity.

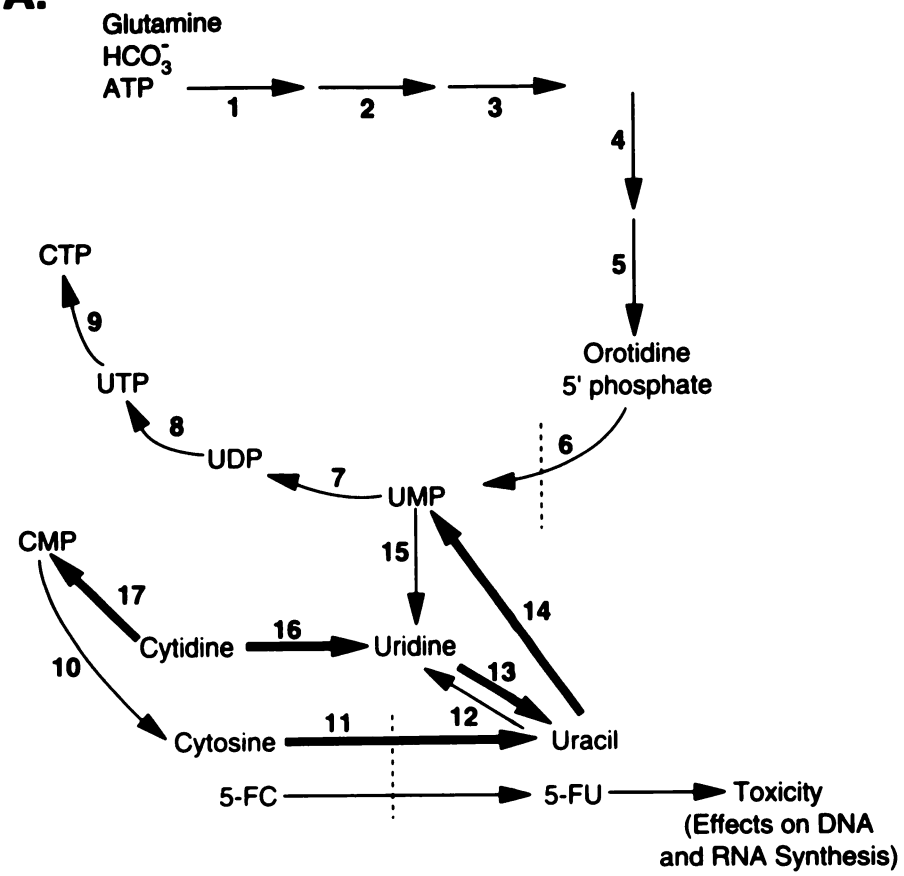
The presence of CD in cells carrying plasmids encoding *codA* should result in the conversion of 5-FC to 5-FU and, thus, toxicity to the cells. This was verified by testing the ability of BA101 transformed with various plasmids to grow on minimal medium containing 5-FC. The plasmids that allowed growth of BA101 with cytosine did not allow growth of BA101 in medium containing 5-FC and uracil. Likewise, plasmids that did not complement the *codA* defect of BA101 did allow growth of BA101 in medium containing 5-FC and uracil (Fig. 3). These results confirmed the presence of CD activity in these transformants.

Enzymatic verification of CD activity. The genetic results suggesting the presence or absence of *codA* on a plasmid were confirmed using cell extracts in a spectrophotometric assay that measures the deamination of cytosine to uracil. The extracts were prepared from cells grown in either rich medium, LB, or minimal medium supplemented with glucose and uracil. The level of CD activity measured with cell extracts prepared from cultures grown in minimal medium was much higher for most plasmids tested (Fig. 3). Previous studies (25, 26) had shown that expression of the *cod* operon was repressed in rich media and induced in minimal medium; our results support this observation. The differences in activity observed with pEA001, pEA002, and pEA003 versus pEA006 are thought to reflect the differences in plasmid copy number between the vectors pBR322 and pBS$^+$, respectively. In every case, the results of the genetic screening agreed with the presence or absence of CD enzymatic activity in cell extracts prepared from the appropriate strain.

Enzymatic assays also identified a third class of plasmids that expressed very low CD activity (8–20 nmol of uracil formed/min/mg of protein). This included pEA009 and pEA013, both of which terminate at the *Cla*I site. Cell extracts prepared from wild-type *E. coli* showed low activity (32 nmol of uracil formed/min/mg of protein) comparable to that of pEA009 and pEA013. This is consistent with *codA* being a single-copy gene on the *E. coli* chromosome. Extracts prepared from the selective strain BA101 were also tested for CD activity and were negative with both cytosine and 5-FC.

Enzymatic assays of cell extracts using 5-FC as a substrate were also done to confirm that 5-FC could serve as a substrate

A.



B.

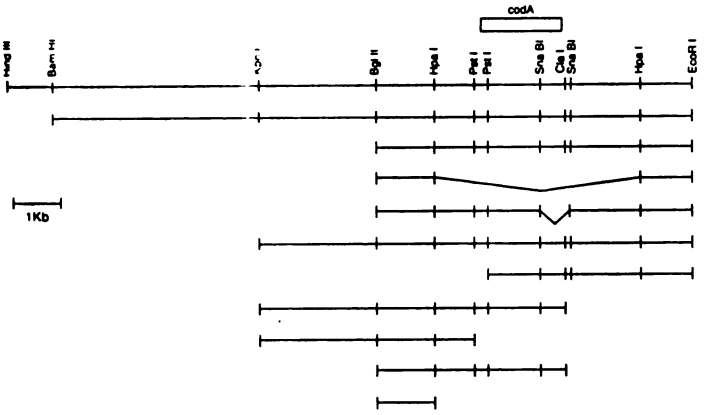
Chromosomal Mutation	Growth Patterns			
	Cytosine	5-FC	5-FU	Uracil
codA (Cytosine deaminase, 11)	+	+	−	+
pyrF (OMP decarboxylase, 6)	+	−	−	+
codA, pyrF	−	+	−	+
wild-type	+	−	−	+

Fig. 2. A, Illustration of the pyrimidine *de novo* synthesis and salvage pathways of *E. coli*. The enzymes involved at each step are as follows: *1*, carbamoylphosphate synthase; *2*, aspartate carbamoyltransferase; *3*, dihydroorotase; *4*, dihydroorotate oxidase; *5*, orotate phosphoribosyltransferase; *6*, orotidine 5′-phosphate decarboxylase; *7*, UMP kinase; *8*, nucleoside diphosphokinase; *9*, CTP synthetase; *10*, ribonucleotide glycosylase; *11*, CD; *12* and *13*, uridine phosphorylase; *14*, uracil phosphoribosyltransferase; *15*, uridine kinase; *16*, cytidine deaminase, *17*, cytidine kinase. B, The growth characteristics of relevant bacterial strains, illustrating the basis for the selection scheme described in the text. *E. coli* strains carrying a mutation in *codA*, the gene encoding CD, are unable to metabolize cytosine. A strain carring a mutation, such as *pyrF*, in the pyrimidine *de novo* synthesis pathway is dependent on an external source of pyrimidines. The presence of both mutations results in a strain that is unable to utilize cytosine as the sole pyrimidine source unless the gene encoding CD is provided *in trans*.

for CD. The specific activities observed using 5-FC were lower than those observed with cytosine as the substrate. This is consistent with previous reports (27). In the case of those plasmids with low activity with cytosine (pEA009 and pEA013), the activity with 5-FC was below the threshold of detection of the spectrophotometric assay. Wild-type *E. coli* cell extracts also had no detectable CD activity using 5-FC as a substrate. Cell extracts that had no CD activity with cytosine as the substrate exhibited no activity with 5-FC.

Visualization of CD on PAGE. Cell extracts prepared from CD-positive and CD-negative BA101 transformants were analyzed by SDS-PAGE (Fig. 4). A band migrating with an apparent molecular weight of approximately 52,000 was observed in cell extracts prepared from CD-positive cells (Fig. 4, *lanes 2, 3, 5, 6,* and *8-11*). In cell extracts prepared from transformants with low CD enzymatic activity (Fig. 4, *lanes 10* and *11*) this band was much fainter than in extracts prepared from transformants with higher CD activity (Fig. 4, *lanes 2, 3, 5, 6, 8,* and *9*). Fig. 4, *lanes 1, 4, 7,* and *12*, shows cell extracts prepared from CD-negative cells. There was also less CD in cell extracts prepared from cultures grown in LB than in those prepared from cultures grown in minimal medium (compare Fig. 4, *lanes 2* and *3* with *lane 9*). This is consistent with the lower level of CD expression that occurs in rich medium (25, 26).

Sequence determination. The DNA sequence extending from the *Bgl*II site past the distal *Sna*BI site was determined using double-stranded DNA templates. The DNA sequence was determined for both DNA strands. Computer analysis identified an open reading frame of approximately 1280 nucleotides (Fig. 5), beginning between the *Pst*I sites and extending past the *Cla*I site. This is consistent with the genetic results indicated in Fig. 3.



Plasmid[a]	Cytosine Phenotype[b]	Cytosine Specific Activity[c]		5-Fluorocytosine Phenotype	5-Fluorocytosine Specific Activity	
		MG	1890		MG	29
pEA002	+	LB	555	–	LB	15
		MG	3919		MG	46
pEA001	+	LB	1618	–	LB	36
		MG	2043		MG	36
pEA003	+	LB	584	–	LB	15
		MG	0		MG	0
pEA004	–	LB	0	+	LB	0
		MG	0		MG	0
pEA005	–	LB	0	+	LB	0
		MG	17407		MG	284
pEA006	+	LB	4914	–	LB	9
		MG	0		MG	0
pEA007	–	LB	0	+	LB	0
		MG	16		MG	B.T.
pEA009	+	LB	19	–	LB	B.T.
		MG	0		MG	0
pEA010	–	LB	0	+	LB	0
		MG	8		MG	B.T.
pEA013	+	LB	20	–	LB	B.T.
		MG	0		MG	0
pEA014	–	LB	0	+	LB	0

Fig. 3. Restriction maps of plasmid DNA inserts and their phenotypic characteristics and enzymatic activities. The coding region of *codA* is indicated at the *top*. *a*, Plasmids pEA001–005 are cloned into pBR322, whereas plasmids pEA006–0014 are cloned into pBS$^+$. *b*, Phenotype refers to the ability of a plasmid to allow BA101 to utilize cytosine as a sole pyrimidine source. *c*, Specific activity is defined as nmol of cytosine or 5-FC deaminated/mg of protein/min. Specific activity was measured spectrophotometrically as a decrease in absorbance at 285 nm, in a 1-ml assay mixture containing cell extract in 50 mM Tris·HCl, pH 7.3, and 0.5 mM cytosine or 5-FC.

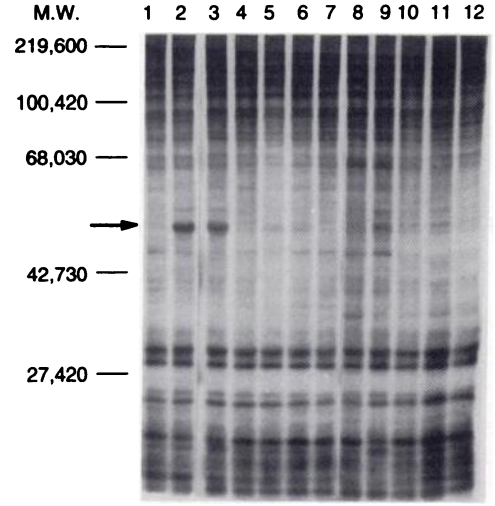


Fig. 4. PAGE analysis of cell extracts prepared from cultures of BA101 transformed with various plasmids. *Lane 1*, pBR322; *lanes 2* and *3*, pEA006; *lane 4*, pEA005; *lane 5*, pEA001; *lane 6*, pEA003; *lane 7*, pEA004; *lane 8*, pEA001; *lane 9*, pEA006; *lane 10*, pEA009; *lane 11*, pEA013; *lane 12*, pEA014. The extracts in *lanes 1-7* were prepared from cultures grown in minimal medium, whereas those in *lanes 8-12* were prepared from cultures grown in LB. *Arrow*, CD band; molecular weight markers are indicated on the *left*.

A reading frame of this size is sufficient to encode a protein of approximately 427 amino acids. The first 30 amino acids of gel-purified CD protein were determined using an automated protein sequencer. Additional protein sequences were obtained from cyanogen bromide fragments generated from the gel-purified protein and analyzed with the protein sequencer, to generate amino acid sequences within the CD coding region. The DNA and protein sequencing results confirm that a protein that migrates with an apparent molecular weight of 52,000 on SDS-PAGE and is significantly expressed in cell extracts with significant levels of CD enzymatic activity is the *E. coli* CD protein encoded by *codA*.

Growth inhibition. The plasmid pCMV/CD-1 was transfected into the human CRC cell line WiDr. Stable G418-resistant cells were used for growth inhibition studies in the presence of 5-FC or 5-FU. The results of these studies are illustrated in Fig. 6. Seven days after the addition of 5-FC or 5-FU, growth inhibition in the presence of varying concentrations of drug (4.6–30,000 μM) was measured. In cells expressing CD, the IC_{50} for 5-FC was shifted to approximately 30 μM, compared with an IC_{50} of approximately 17,000 μM in parental WiDr cells. Expression of CD had no detectable effect on 5-FU toxicity.

Discussion

The *E. coli* gene encoding CD has been cloned using a positive selection scheme that is based on the inability of *E. coli* to metabolize cytosine except through CD. An *E. coli* strain, BA101, was constructed that could not utilize cytosine as a sole pyrimidine source unless a plasmid-borne copy of CD was present. Using this approach, two plasmid clones were isolated that allowed BA101 to metabolize cytosine, which suggests that they carried the gene encoding CD.

The smaller of these plasmid clones (pEA001) contained an approximately 10.8-kb insert. Based on restriction enzyme mapping studies, DNA fragments were deleted from the pEA001 insert. These studies localized the *codA* gene to a 4.8-kb *Bgl*II/*Eco*RI fragment. The resulting plasmids were screened for the ability to allow BA101 to metabolize cytosine. The presence or absence of CD was verified by assaying the ability of cell extracts to deaminate cytosine or 5-FC to uracil or 5-FU, respectively.

Cell extracts that were CD positive by genetic and enzymatic assays also contained a protein that migrated on SDS-PAGE with an apparent molecular weight of 52,000. The intensity of this protein band reflected the amount of CD enzymatic activity; cell extracts with high enzymatic activity had a more intense CD protein band present, whereas low activity cell extracts had much a fainter CD band. Protein sequencing of the amino terminus of the gel-purified CD band identified the first 30 amino acids. Sequence determination of both strands of a 3-kb region of the DNA fragment containing the *codA* gene identified an open reading frame that started with the amino acids determined by protein sequencing. Site-directed mutagenesis was performed to change the translational start codon of

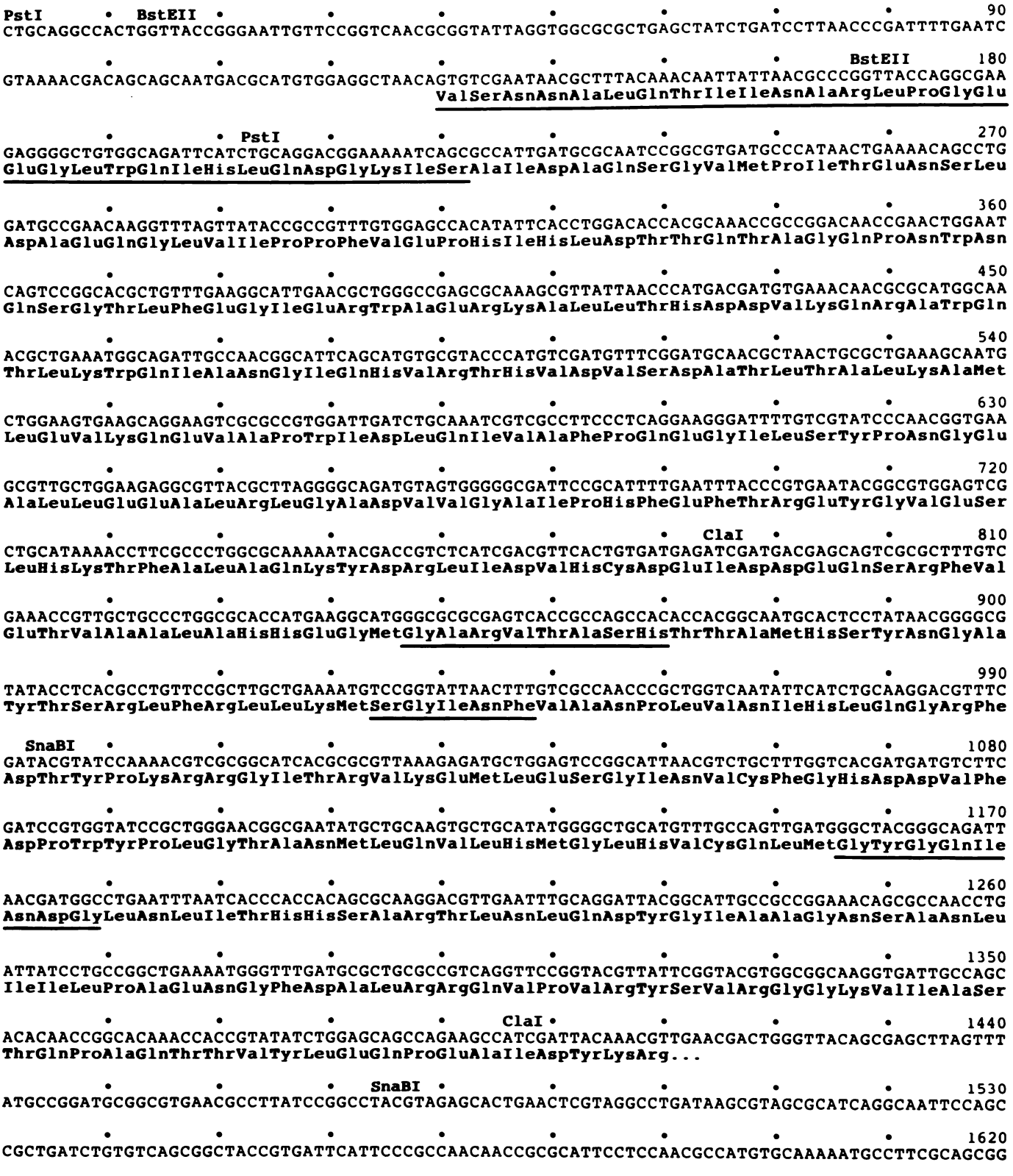


Fig. 5. Sequence of *codA* extending from the *Pst*I site to the *Pvu*II site. The coding region is translated underneath the DNA sequence; the amino acids verified by protein sequencing are *underlined*.

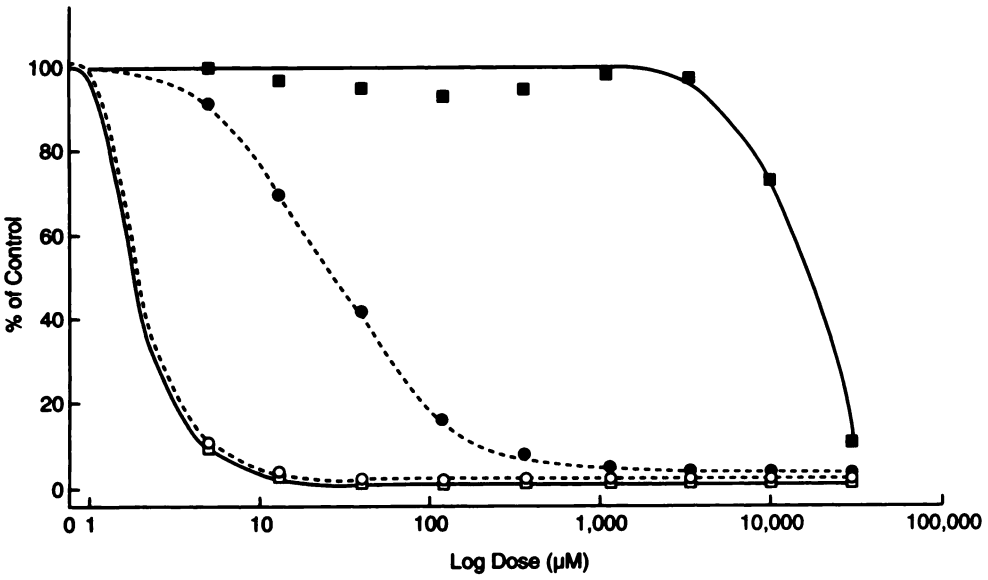


Fig. 6. Logarithmic dose-response curves showing inhibition of growth of WiDr cells by various concentrations of 5-FC and 5-FU. Each point represents eight individual determinations. ■ and □, Values for WiDr cells in the presence of 5-FC and 5-FU, respectively; ● and ○, values for WiDr/pCMV/CD-1 cells in the presence of 5-FC and 5-FU, respectively. The *x*-axis shows increasing concentrations of drug; the *y*-axis shows growth inhibition expressed as percentage of control.

the CD gene from GTG to ATG and to introduce a convenient restriction site. The mutagenized CD gene was cloned into the eukaryotic expression vector pRc/CMV, resulting in the plasmid pCMV/CD-1. Growth inhibition studies of human CRC cells stably transfected with pCMV/CD-1 showed an approximately 560-fold increase in the toxicity of 5-FC, compared with the parental cell line. This demonstrates that bacterial CD can be expressed in eukaryotic cells, allowing metabolism of 5-FC to 5-FU and leading to cell death. In addition, while this manuscript was in preparation a recent publication by Mullen *et al.* (28) demonstrated that the *E. coli* gene encoding CD could be expressed in tissue culture cells under control of the Moloney murine sarcoma virus long terminal repeat. In clonogenic assays with these cells, treatment with 5-FC resulted in an 85–97% decrease in colony formation (28).

We suggest that tissue-specific expression of CD should allow a high local concentration of 5-FU to be achieved after administration of the nontoxic prodrug 5-FC (see Fig. 1). By placing expression of CD under the control of the CEA transcriptional regulatory sequences, cell-specific expression of CD should occur, thus allowing targeted killing of CRC cells. A concern might be that 5-FC is not metabolized as efficiently as cytosine by CD. However, the observation that the very low level of CD activity in wild-type *E. coli* is sufficient to kill cells treated with 5-FC suggests that 5-FC is converted to 5-FU by CD at a sufficient rate for efficient cell killing. Further support for this hypothesis comes from previous studies with purified CD isolated from *E. coli* (20). The purified enzyme was placed in dialysis tubing that was subsequently surgically implanted in rats bearing subcutaneous tumors of glioma cells. After treatment with 5-FC for 30 days, a reduction in tumor size was observed. The next step in our approach is the creation of a chimeric CEA/CD gene contained in a retroviral vector system. The effect of 5-FC treatment can then be assessed in several CEA-positive and CEA-negative cell lines transfected with the CEA/CD chimera.

Acknowledgments

We thank C. Dow and A. Brier for excellent technical assistance. We thank D. Porter and B. Merrill (Wellcome Research Labs, Research Triangle Park, NC) for assistance with CD enzymatic assays and for protein sequencing, respectively. We also thank C. Richards and P. Condreay for helpful discussions (Wellcome Research Labs).

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