Primary Structure of Human Deoxycytidylate Deaminase and Overexpression of Its Functional Protein in *Escherichia coli* 

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The cDNA encoding human dCMP deaminase was isolated from an XZAPII expression library using an antibody generated against highly purified HeLa cell dCMP deaminase. The cloned cDNA consists of 1856 base pairs and encodes a protein of 178 amino acids with a calculated molecular mass of 19,986 daltons. The sequence of several cyanogen bromide-cleaved peptides derived from HeLa cell dCMP deaminase are all contained within the deduced amino acid sequence. A zinc binding region is present in the enzyme, similar to that reported for cytidine deaminase (Yang, E. C., Carlow, D., Wolfenden, R., and Short, S. A. (1992) Biochemistry 31, 4168–4174). Northern blot analysis revealed a predominant messenger RNA species of 1.9 kilobases. Expression of the active protein to about 10% of *Escherichia coli*'s total protein was achieved by subcloning the open reading frame into a high expression system using the polymerase chain reaction. Polyacrylamide gel electrophoresis revealed a prominent protein band which comigrated with affinity purified HeLa dCMP deaminase, while Western blot analysis yielded an immunoreactive band which comigrated with the single immunoreactive affinity column purified dCMP deaminase band. The enzyme which possesses a $k_{cat}$ of $1.02 \times 10^4 \text{ s}^{-1}$ was purified to homogeneity in over 60% yield. The overexpression of dCMP deaminase should permit more exacting studies on the regulation of this important allosteric enzyme which provides substrate for DNA synthesis.

Deoxycytidylate deaminase (EC 3.5.4.12) catalyzes the deamination of dCMP to dUMP, thus providing the nucleotide substrate for thymidylate synthase. Control of deaminase activity at this juncture in deoxynucleotide metabolism is determined by the ratio of dCTP to dTTP in the cell, since the enzyme is allosterically activated by dCTP and inhibited by dTTP (1). Evidence in support of this thesis was obtained recently by Xu and Plunkett (2) using an in situ assay. A consequence of this nucleotide interplay is that the amount of dUMP available for thymidylate synthase is finely controlled by the end products of the pyrimidine deoxynucleotide pathway.

Several observations indicate the potential importance of dCMP deaminase in DNA replication. First, the activity of the deaminase is elevated in such rapidly dividing tissues as chick and rat embryo (3), regenerating liver (4), and rat hepatomas (5). Second, HeLa cell dCMP deaminase activity is highest in late S phase and subsequently declines in the following G2 phase (6). Finally, the absence of dCMP deaminase activity in mammalian cells induces an imbalance in deoxynucleotide pools such that dTTP levels decrease while dCTP levels increase, resulting in enhanced mutagenesis (7). Evidence has also been presented by Jackson et al. (8) and Chiu et al. (9) that dCMP deaminase is a major contributor of dUMP for thymidylate synthase. In the past several years, dCMP deaminase has been purified to homogeneity from a variety of sources including chick embryo (10), donkey spleen (11), T2r bacteriophage-infected *Escherichia coli* (12), and HeLa cells (13). However, due to the low amounts of the enzyme present in mammalian cells, purification of the enzyme to homogeneity has proven difficult, thus limiting the availability of pure enzyme for biochemical analysis.

Recently, a dCMP deaminase gene was cloned and sequenced from T4 bacteriophage (14) and compared with the complete amino acid sequence of the enzyme isolated from T2 bacteriophage-infected *E. coli* (15). A homology comparison of the latter with a deduced sequence of dCMP deaminase from *Saccharomyces cerevisiae* has been made (16), and several regions of similarity were found. In this paper, we describe the cloning and expression of dCMP deaminase from HeLa cells, providing for the first time the amino acid sequence from a mammalian source.

**EXPERIMENTAL PROCEDURES**

**Materials**

The HeLa cell XZAPII cDNA library and picoblue immunoscreening kit were purchased from Stratagene (Palio Alto, CA). The nitrocellulose filters used were from Schleicher and Schuell. Hybond-N nylon membranes, $[^{32}P]dCTP$ and $[^{35}S]dATP$ were obtained from Amersham Corp. Cyanogen bromide was purchased from Pierce Chemical Co. All solvents were prepared with Millipore nanopure water. Oligonucleotide primers were synthesized in this institution using a Millipore model 8750 DNA Synthesizer.

**Methods**

**Peptide Sequencing—** Human (HeLa cell) dCMP deaminase was purified as described previously (13) and was homogeneous as determined by SDS-PAGE. 1 Approximately 400 μg of the purified enzyme was mixed with 2 nmol acrylamide and subjected to electrophoresis; then an acrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CD, DNA insert encoding dCMP deaminase; PCR, polymerase chain reaction, AP, affinity column purified; kb, kilobase(s).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CD, DNA insert encoding dCMP deaminase; PCR, polymerase chain reaction, AP, affinity column purified; kb, kilobase(s).
was cleaved with cyanogen bromide (200-fold excess over methionine) in 70% formic acid at room temperature in the dark (17). After 18 h, the reaction mixture was diluted 20-fold with water and lyophilized. The cleavage products were dissolved in 400 μl of 0.1% trifluoroacetic acid (TFA) containing 0.1% methionine sulfone, added to a C8 aquagel column (Beckman, Palo Alto, CA) in a reverse-phase column (0.4 x 25 cm) using a Beckman model 110A system equipped with a Spectra-Physics model 7400 dual-wavelength detector and a Shimadzu CR-3A data processor (Shimadzu, Kyoto, Japan). The column was eluted at a flow rate of 0.7 ml/min with a linear gradient of 0.1% trifluoroacetic acid to 60% of a solution containing 0.1% trifluoroacetic acid in 80% acetonitrile. Individual fractions were collected, and those selected for sequencing were concentrated in a Savant Speed-Vac to a small volume. Peptide sequences were determined by automated Edman degradation with a 477A Applied Biosystem sequencer/120A phenylthiohydantoin amino acid analyzer.

**Immunoscreening for Detection of dCMP Deaminase Antigen—** Screening of the HeLa cell library (2 x 10⁶ recombinants) was performed according to the procedure of Young and Davis (18) with minor modifications as described in Stratagene's (Stratagene Cloning Systems, La Jolla, CA) polyclonal immunoscreening protocol. The antisera, rabbit anti-dCMP deaminase, was prepared as described previously using homogeneous HeLa dCMP deaminase as the immunogen (13). The antisera was protein A purified and preclared of nonspecific antibodies at a dilution of 1:100 by pseudoscreening with the AZAP II library (19). The preclared dCMP deaminase antiserum reacted only marginally with an E. coli phage lysate. For immunoscreening, the anti-dCMP deaminase serum was used at a 1:1000 dilution in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, and 0.05% Tween-20, pH 8.0).

All procedures following plaque lifts were performed at room temperature. IPTG-soaked nitrocellulose filters were lifted and briefly rinsed in TBST. The filters were then blocked overnight in 1% gelatin-TBS. Afterward, the filters were washed two times for 10 min each in TBST, followed by incubation for 2 h with the deaminase antiserum. The filters were then washed three times for 10 min each and incubated with goat antirabbit-IgG-alkaline phosphatase-conjugated antibody (Promega, Madison, WI) at a 1:1000 dilution in TBST. The samples were then developed with 100 μl of buffer (10 mM Tris base, 100 mM NaCl, 0.05% Tween-20, pH 8.0). The samples were washed and incubated with goat antirabbit-IgG-alkaline phosphatase-conjugated antibody for 1 h at a 1:2000 dilution in TBST. Color development was initiated by adding the substrate solution containing 0.1 mg/ml p-nitrophenyl phosphate, 50 mM Tris-HCl, 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂. Under these conditions, approximately 2 ng of purified HeLa dCMP deaminase could be detected by dot blotting.

**DNA Sequence Analysis—** The dCMP deaminase cDNA insert was excised from the AZAP II vector and subcloned into pBluescript KS-. The nucleotide sequence of both strands of the double-stranded dCMP deaminase cDNA was determined by the Sanger dideoxy nucleotide chain termination method (20) using Sequenase (United States Biochemical Corp.). The ends of the cDNA were sequenced using a primer, 5'-AGCACCAGTGATCGACATATGAAGTGGCTGTTTCC-3', which introduces a HindIII site and encodes the first 5 amino acids of dCMP deaminase (underlined). The dCMP deaminase cDNA insert was equilibrated with 0.1 M Tris-HCl, pH 7.5, was washed with 150 ml of buffer A at pH 7.1, prior to addition to the column. After the sample was adsorbed, the column was washed with buffer A until the A₄₀₀/A₂₈₀ ratio decreased and the eluate was clear. The potassium phosphate concentration of buffer A was increased to 100 mM, and 50 ml was applied to a first phosphocellulose column. The sample was centrifuged at 12,000 g for 20 min at 4 °C, the supernatant was collected, and the residue was dissolved in buffer A containing 20 mM potassium phosphate, pH 7.5. The enzyme was eluted in the last buffer in a volume of about 200 ml and was precipitated with solid ammonium sulfate to a final concentration of 60% saturation. The precipitate was collected by centrifugation at 45,000 x g for 20 min and stored frozen at -50 °C.

**Phosphocellulose Chromatography—** The precipitate was thawed, dissolved in buffer A, and dialyzed overnight against two 2-liter changes of buffer A.

**DE52 Chromatography—** A column of DE52 (3.4 x 10 cm) pre-equilibrated with 10 mM potassium phosphate, pH 7.5, was washed with 200 ml of buffer A prior to addition of the sample. The dialyzed sample was sonicated 10 times for 1 min each (to further reduce associated nucleic acid in size) with 150 min of cooling between each sonication and then applied to the column. After the sample was adsorbed, the column was washed with buffer A until the A₄₀₀/A₂₈₀ ratio decreased and the eluate was clear. The potassium phosphate concentration of buffer A was increased to 100 mM, and 50 ml was applied to the column, followed by 300 ml of buffer A containing 300 mM potassium phosphate, pH 7.5. The enzyme was eluted in the last buffer in a volume of about 200 ml and was precipitated with solid ammonium sulfate to a final concentration of 80% saturation. The precipitate was collected by centrifugation at 45,000 x g for 20 min and stored frozen at -50 °C.
**Retinal Pigment Epithelium**

**RESULTS**

Isolation and Sequence Analysis of a HeLa Cell dCMP Deaminase cDNA—Screening of approximately 2 × 10^6 recombinants from a HeLa cell XZAPII cDNA library with protein A-purified dCMP deaminase antiserum yielded three positive clones. All three were digested with EcoRI and analyzed by Southern blot analysis.

**DISCUSSION**

The results indicate that the HeLa cell line contains a single copy of the dCMP deaminase gene. The deduced amino acid sequence of the cDNA clone is consistent with the established protein structure and enzymatic properties of dCMP deaminase.

**Fig. 1.** Nucleotide and deduced amino acid sequence of human dCMP deaminase. Residue numbers are shown at left. The sequence of peptides obtained from automated Edman degradation of CNBr fragments of HeLa cell dCMP deaminase are underlined. The boxed residues indicate a putative polyadenylation signal.

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**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—SDS-PAGE was performed as described by Laemmli (28) using a Mini-Protein II Dual slab cell (Bio-Rad). The samples were electrophoresed using a 12.5% (w/v) resolving gel and a 4% stacking gel, each containing 0.1% SDS, at 30 V for 25 min followed by 200 V for an additional 50 min. After electrophoresis the proteins in the gel were stained with Coomassie Brilliant Blue R-250 or subjected to Western blot analysis.

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**Nucleotide and deduced amino acid sequence of human dCMP deaminase.** The sequence of peptides obtained from automated Edman degradation of CNBr fragments of HeLa cell dCMP deaminase are underlined. The boxed residues indicate a putative polyadenylation signal.
lyzed by agarose gel electrophoresis (data not shown). One of the clones, pCD12, which contained a 1.8-kb insert was selected for further analysis, since it hybridized with degenerate oligonucleotides based on the partial amino acid sequence of the clones, pCD12, which contained a 1.8-kb insert was selected for further analysis, since it hybridized with degenerate oligonucleotides based on the partial amino acid sequence of dCMP deaminase. The complete nucleotide sequence of both strands of pCD12 was obtained by the dideoxy chain termination method (20) and is presented with the deduced amino acid sequence of dCMP deaminase.

The first three bases of the insert appear to code for the amino-terminal methionine of dCMP deaminase, but since the amino terminus of the isolated HeLa dCMP deaminase was found to be blocked, this residue could not be verified by amino acid sequencing. Interestingly, the pCD12 insert contains a very long 3'-untranslated region of 1.3 kb, which includes a polyadenylation signal at nucleotides 902-907, two putative AU-rich motifs at nucleotides 738-742 and 1639-1643, as well as the poly(A) tail (residues 1832-1856).

Northern blot Analysis of HeLa Cell RNA—A 32P-labeled EcoRI/HindIII fragment of pCD12, which spans the entire coding region was used as a hybridization probe to determine the size of dCMP deaminase mRNA. Northern blot analysis of total RNA (Fig. 2, lane 1) and poly(A)+ mRNA (lane 2) isolated from HeLa cells revealed the labeled DNA fragment to hybridize with a major RNA band of 1.9 kb in length, which is about the size expected for a transcript of the 1.86-kb insert of pCD12. It is clearly seen from the intensity of the band in lane 2 relative to lane 1 that the deaminase mRNA is greatly enriched in the latter. Hybridization of the EcoRI/HindIII fragment to a minor band of the poly(A)+ mRNA at 0.7 kb, which is not seen with total RNA (lane 1), may be due to a degradation product of the full-length mRNA encoding dCMP deaminase.

Expression of a Functional HeLa dCMP Deaminase in E. coli—To determine the extent to which pCD12 expresses dCMP deaminase in E. coli, 0.4 mM IPTG was used to promote the synthesis of this enzyme from the lac promoter preceding the insert. At best only 0.06 units/mg protein of dCMP deaminase activity was obtained, which was comparable to that found in crude HeLa cell extracts (13). Because of this low level of deaminase expression the pBluescript plasmid was obviously not useful as a vector to amplify the deaminase. This effect may be due to the apparent toxicity of dCMP deaminase when expressed at high levels in continuously growing cells (14). A more controlled system was sought, therefore, which could be turned on as desired and was found in the pET-3xc vector developed by Studier and co-workers (26, 31, 32), where protein synthesis is under the control of bacteriophage T7 transcription and translation signals. The PCR procedure was used to amplify the open reading frame of pCD12 with NdeI and BamHI sites conveniently placed at the 5' and 3' ends, respectively. This DNA fragment was then

FIG. 2. Northern blot analysis of total and poly(A)+ RNA from HeLa cells. RNA was fractionated on a 1.2% agarose/formaldehyde gel, blotted onto nylon, and hybridized with a 32P-labeled 0.53-kb EcoRI/HindIII fragment of pCD12, which spans the protein coding region of dCMP deaminase. RNA molecular weight markers were used to estimate the size of the transcript. Lane 1, 2.5 µg of total RNA; lane 2, 2 µg of poly(A)+ RNA. Arrows indicate positions of 18 and 28 S ribosomal RNAs. The asterisk (*) indicates the position of dCMP deaminase mRNA.

FIG. 1. Alignment of the complete DNA sequence of the pCD12 insert (underlined in Fig. 1) with the amino acid sequence of the dCMP deaminase.

FIG. 3. Bacterial expression of dCMP deaminase in E. coli. Strain BL21(DE3)pLysS harboring pET-3xc or pETCD plasmids were grown and induced with 0.4 mM IPTG as described under “Experimental Procedures,” followed by SDS-PAGE of various protein extracts. Panel A, Coomassie Blue staining of gel. Panel B, Western blot analysis of protein extracts similar to those in panel A after electrophoretically transferring nitrocellulose and immunostaining with rabbit anti-dCMP deaminase as described under “Experimental Procedures.” Lane 1, standard molecular mass markers in kDa; lane 2, plasmid pET-3xc (+IPTG); lane 3, plasmid pETCD (--IPTG); lane 4, plasmid pETCD (+IPTG); lane 5, AP-dCMP deaminase. The amount of protein applied to lanes 2-5 of panel A was 5 and 1 µg to lane 5; for panel B, 1 µg was applied to lanes 2-4, while 0.2 µg was added to lane 5.
Sequence of Human Deoxyctydylate Deaminase

**TABLE I**

| Step       | Volume (ml) | Protein (mg/ml) | Specific activity (μmol/mg) | Yield (%) |
|------------|-------------|-----------------|----------------------------|-----------|
| 1. Extract | 103         | 5.50            | 77                         | 100       |
| 2. Ammonium sulfate | 57       | 9.25            | 58                         | 71        |
| 3. DE52    | 220         | 1.30            | 108                        | 70        |
| 4. Phosphocellulose | 224     | 0.28            | 509                        | 64        |

FIG. 4. Purification of recombinant HeLa dCMP deaminase as revealed by SDS-PAGE. Samples of protein were placed in a denaturing buffer as described under “Experimental Procedures,” and aliquots were placed in the wells of a 12.5% SDS-PAGE minigel apparatus (Bio-Rad) and electrophoresed as described. Lane 1, standard molecular mass markers in kDa; lane 2, 15 μg of protein from a crude extract of HeLa cells (Table 1); lane 3, 15 μg of protein from step 3; lane 4, 3 μg of protein from step 4; and lane 5, 2 μg of affinity column purified enzyme (13) from step 5.

This paper describes for the first time the amino acid sequence of a human dCMP deaminase as deduced from its corresponding cDNA. The only other eukaryote dCMP deaminase sequence available to date is that from *S. cerevisiae* (16), but in view of its unusual size (312 amino acids) relative to the human enzyme (178 amino acids) and most other deaminases described to date, its structure should be verified. Most of the homology between the sequence of the yeast deaminase and the T4 phage and human deaminases resides in 150 amino acids of the yeast enzyme’s carboxyl-terminal end and of these, about 50 amino acid residues are related and fall into the linear peptide segments presented in Fig. 5. Since the yeast deaminase has not been purified it is not clear how many subunits constitute this protein or what its true size is. It would be surprising though if this enzyme turns out to be as different from the T4 phage and human dCMP deaminases, as the deduced sequence suggests (16).

Another deaminase with an anomalous molecular mass has been purified to apparent homogeneity from human spleen (33), one with an apparent molecular mass of 106 kDa. Unlike the other dCMP deaminases, which contain six subunits, this enzyme is composed of two subunits and possesses a specific activity that is 40-50 times lower than the HeLa cell deaminase. However, the spleen enzyme is still allosterically regulated by dCTP and dTTP. Whether the spleen deaminase represents an organ-specific variant of the human dCMP deaminase described in this paper remains to be determined.

There is little doubt that the cDNA isolated from the HeLa cell library encodes the same protein purified to homogeneity from HeLa cell extracts (13). Thus, several CNBr peptides isolated from the purified enzyme were identified within the cDNA sequence of the deaminase, the M, values of the isolated HeLa cell and recombinant proteins were identical, and both gave positive Western blots with antibody to the HeLa deaminase at the same migration distance following SDS-PAGE. In addition both were inhibited with the same K, by the transition state analogue, 2'-β-D-deoxyribose-pyrimidin-2-one 5'-phosphate. The only difference between the two proteins resides in the fact that the recombinant protein’s amino end is free, while that from HeLa cells appears to be
Sequence of Human Deoxycytidylate Deaminase

Human
\[ ^{37}Y - G - A - C - I - V \]
T4-Phage
\[ ^{34}Y - G - A - V - I - E \]
Yeast
\[ ^{43}Y - G - C - V - I - V \]

Human
\[ ^{47}K - I - V - G - I - G - Y - N - G - M - P \]
T4-Phage
\[ ^{39}R - I - I - S - T - G - Y - N - G - S - P \]
Yeast
\[ ^{196}R - V - I - A - T - G - Y - N - G - S - P \]

Human
\[ ^{205}H - A - E - L - N - A - I \]
T4-Phage
\[ ^{198}H - A - E - L - N - A - I - L \]
Yeast
\[ ^{223}L - H - A - E - E - N - A - I - L \]
E. coli (cdd)
\[ ^{102}H - A - E \]

Human
\[ ^{109}G - C - S - M - Y - V - A - L - F \]
T4-Phage
\[ ^{122}G - A - T - M - Y - V - T - L - S \]
Yeast
\[ ^{251}A - T - L - Y - C - D - T - C \]
E. coli (cdd)
\[ ^{130}P - C - G - H - C \]

Fig. 5. Comparison of homology regions of human, T4 phage, and yeast dCMP deaminases. The superscripts locate the position of the indicated amino acid from the amino end of the respective proteins. cdd is the deduced sequence from cytidine deaminase (38). The boxed regions indicate the location of the zinc binding sites which involve mainly histidine and 2 cysteines.

blocked, which is a common feature of proteins isolated from mammalian sources.

The human dCMP deaminase mRNA contains an unusually long 3′-untranslated region, which is about 1.3 kb in length. Transferrin receptor mRNA also contains a long 3′-untranslated region, which appears to be involved in the iron-dependent regulation of mRNA stability (34, 35). Whether a similar role can be attributed to the 3′-untranslated region of dCMP deaminase mRNA remains to be determined.

Several regions of common identity have been noted on comparison of the human, T4-phage and S. cerevisiae dCMP deaminases (Fig. 5). One of particular interest is that most likely involved in the catalytic site, which is similar to that described for cytidine deaminase (36). The latter has been shown to contain a region that could be one of high affinity for the chelation of zinc. The residues in this site believed to be involved are 1 histidine and 2 cysteines (see boxed regions in Fig. 5). This is in contrast to the case of adenosine deaminase, where it has been shown recently that the phage deaminase not only contains a zinc atom in the above-mentioned catalytic site, but also one in another site that appears to be a unique zinc finger in which the zinc is coordinated between 3 histidines and 1 cysteine (38). Evidence has been obtained for the T4-dCMP deaminase to support the role of zinc as a promoter of hydrolysis similar to that found for the cytidine and adenosine deaminases, in that replacement of histidine 105 or cysteines 132 or 135 of T4 phage dCMP deaminase by other amino acids, results in the loss of zinc and coincidentally deaminase activity. It is of interest to note that the addition of Zn\(^{2+}\) to the HeLa deaminase affects neither activity nor allosteric activation or inhibition. In these cases Mg\(^{2+}\) appears to play a major role. However, in the case of the Bacillus subtilis dCMP deaminase Zn\(^{2+}\) is absolutely required for both effects and could not be replaced by Mg\(^{2+}\) (39). The role of zinc in such biological processes as transcription and enzyme activity is becoming increasingly obvious. It would be of interest to determine whether a mutational deficiency in Zn\(^{2+}\) is deleterious to these processes, in particular dCMP deaminase activity.

\(^{2}\) J. T. Moore, G. F. Maley, and F. Maley, manuscript in preparation.
The importance of dCMP deaminase in DNA replication, through its provision of dUMP for thymidylate synthase, has become evident through studies with cells that are deficient in the enzyme. These cells show imbalances in their intracellular dCTP and dTTP pools (6, 40, 41), which can lead to increased mutation rates during DNA replication (42-44). Thus, the relative importance of dCMP deaminase in DNA replication heralds its importance as a potential target for chemotherapeutic agents. However, due to the relative difficulty in obtaining the active human enzyme until now, most studies on the deaminase have been performed on non-human sources. Our ability to overexpress the active enzyme will now enable the isolation of sufficient deaminase to probe its structure and to undertake the development of potential chemotherapeutic agents specifically targeted to the deaminase. It should be determined whether their use in combination with inhibitors of thymidylate synthase can potentiate the effectiveness of thymidylate synthase inhibitors.

Clinically, dCMP deaminase levels have been found to be elevated in the sera from patients with various disease states (45) and, therefore, have the potential of being used for the early detection of such diseases. As an example, several studies have clearly demonstrated that pregnant women with pre-eclampsia show increases in serum dCMP deaminase activity early on in the onset of the disease (46-49). In another case, that of myocardial infarction (50, 51), the enzyme slowly but significantly increases in serum. The assays employed in these studies, however, are too complex or too insensitive to be clinically useful, and in addition, determine only the amount of active deaminase in serum. Now, through the large scale production of the active recombinant enzyme, we can develop a highly sensitive immunoassay, which could determine more accurately the true serum levels of dCMP deaminase protein both active and inactive.

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