

# Baculovirus-Mediated Expression of Rat Liver CTP:Phosphocholine Cytidylyltransferase

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**We describe herein the expression and purification of milligram quantities of rat liver CTP:phosphocholine cytidylyltransferase in recombinant baculovirus-infected insect cells. The enzyme was purified by incorporating modifications to a previously published procedure (P. A. Weinhold and D. A. Feldman, 1992, in "Methods in Enzymology" (E. A. Dennis and D. E. Vance, Eds.), Vol. 209, pp. 248-258, Academic Press, San Diego, CA). Like cytidylyltransferase purified from rat liver, the purified recombinant cytidylyltransferase has the same molecular weight (42 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and reacts to antibodies generated against the N and C termini of rat liver cytidylyltransferase. In addition, like the purified rat liver enzyme, the recombinant cytidylyltransferase is activated by lipids. We also expressed rat liver cytidylyltransferase as a fusion protein with glutathione-S-transferase and with a thrombin cleavage site between the two enzymes for rapid isolation of cytidylyltransferase. Thrombin cleavage was, however, incomplete and prolonged exposure to thrombin resulted in the degradation of cytidylyltransferase itself.** © 1993 Academic Press, Inc.

In animal cells the majority of phosphatidylcholine is synthesized *de novo* via the CDP-choline pathway; CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (1) is rate-limiting and regulatory for this pathway. Activation of cytidylyltransferase appears to be dependent upon its association with lipids either *in vitro* by the addition of lipid vesicles or *in vivo* by the interconvertible translocation of the enzyme from the cytosol, where it is mostly inactive, to membranes (1), or by the seques-

tration of lipids from intracellular membranes to form a high-molecular-weight protein-lipid aggregate (2,3). Cytidylyltransferase is a 42-kDa phosphoprotein (4) and changes in its activity and intracellular localization are accompanied by changes in the phosphorylation state of the enzyme (5). None of these phosphorylation sites have been mapped and it remains to be seen which kinases and phosphatases are involved in the regulation of cytidylyltransferase.

Cytidylyltransferase has been purified from rat liver (6,7); however, the process is laborious and the final yield is low (about 11  $\mu$ g of homogeneous protein from six to eight rat livers). Such low yields put enormous constraints on the study of cytidylyltransferase with respect to protein chemistry. The cDNA encoding cytidylyltransferase has been cloned (8) and it would be advantageous to overexpress this clone in a fashion that would allow for the purification of large amounts of enzyme.

The baculovirus expression system represents a powerful tool for the expression of moderately large amounts of target proteins in eukaryotic cells (9-12). In this system the gene to be expressed is placed under the control of the strong polyhedrin promoter in the insect baculovirus *Autographa californica* nuclear polyhedrosis virus. The polyhedrin protein is produced in very large amounts during late infection and is involved in embedding the mature virus particles in the nucleus, but is not necessary for lateral transmission of the virus under laboratory conditions (11,12). Recombinant virus particles arise by homologous recombination when insect cells are cotransfected with wild-type virus DNA and a plasmid containing the polyhedrin promoter, the target gene, and sequences which are homologous to those surrounding the polyhedrin gene in the viral chromosome (11,12). In this paper we report the expression of milligram quantities of rat liver cytidylyltransferase in recombinant baculovirus-infected insect cells.

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## MATERIALS AND METHODS

### Materials

The baculovirus cloning vector pBlueBac was obtained from Invitrogen. Other vectors pAc401 and pAc373, the virus AcNPV and Sf-9 insect cells were obtained with permission of Dr. Max Summers from Dr. N. Nielsen (Department of Agronomy, Purdue University). Grace's insect cell culture medium, yeastolate solution, lactalbumin hydrolysate solution, and fetal bovine serum were obtained from GIBCO-BRL. Oligonucleotides were synthesized at the core facility, Purdue University. Restriction endonucleases were from New England Biolabs and GIBCO-BRL. The cloning vector pBluescript was from Stratagene. [<sup>14</sup>C]Phosphocholine (55.6 mCi/mmol) and [<sup>32</sup>P]dCTP (>6000 mCi/mmol) were from Amersham. Octylglucoside was from Calbiochem. All other chemicals and reagents were from Sigma.

### Maintenance of Cell Lines

*Spodoptera frugiperda* (Sf-9) cells were used throughout and were maintained in both suspension and monolayers at 27°C in Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and 10% fetal bovine serum (11,12).

### Construction of Cytidylyltransferase Vectors

All plasmids were propagated in the *rec A*<sup>-</sup> *Escherichia coli* strain DH5 $\alpha$ . The 1.1-kb cDNA encoding cytidylyltransferase was isolated from rat liver mRNA using the polymerase chain reaction and primers corresponding to specific sequences within the coding region (8) and was subcloned into the *Sal*I, *Xba*I restriction sites of pBluescript. The orientation of cytidylyltransferase insertion was 5'-*Sal*I, 3'-*Xba*I. The plasmid was designated pBCT-7.

The plasmid used to transfer cytidylyltransferase to the viral genome was constructed as follows. Two oligonucleotides (5'-TTT GCG GCC GCG GAT CCA CCA CCA TGG ATG CAC AGA GTT CAG CTA AAG-3' and 5'-ATC GAT GTC GAC GAC AAT GCG GG TGA TGA TGT CTG ATG TGG-3') were synthesized. The former oligonucleotide contains a short leader sequence containing eukaryotic translational signals (13) as well as the first 25 nucleotides of rat liver cytidylyltransferase (8). In addition, *Not*I and *Bam*HI restriction sites are present at the 5' end. The latter oligonucleotide is an antisense sequence complementary to nucleotides 602-631 in the cytidylyltransferase coding region (8) and contains a *Sal*I restriction site for subcloning.

A fragment corresponding to the first 631 nucleotides of the cytidylyltransferase coding region was amplified by PCR using the primers shown above and cloned into the *Sal*I, *Not*I restriction sites of pBluescript. The re-

sulting plasmid (pBCT-5bac) was cut with *Sal*I, blunt ended with Klenow, and further digested with *Acc*I to give a fragment consisting of pBluescript and the first 423 nucleotides of the cytidylyltransferase coding region (8). The plasmid pBCT-7 was then digested with *Not*I, the end was made blunt with Klenow, and the 681-nucleotide 3' end of cytidylyltransferase was then excised by digestion with *Acc*I and cloned into the *Acc*I, blunt end sites of pBCT-5bac to give a plasmid designated pBCT-bac containing the full-length coding sequence. The plasmid pBCT-bac also contains a unique *Xba*I restriction site just 6 bases downstream from the cytidylyltransferase stop codon. Cloning cytidylyltransferase in segments was necessitated by the fact that we were unable to generate the full-length sequence via PCR using primers complimentary to the extreme 5' and 3' regions.

The 5' PCR primer was originally synthesized with a view to clone cytidylyltransferase into the baculovirus vector pAc373; however, we later decided to use pBlueBac as the latter plasmid contains a  $\beta$ -galactosidase gene for rapid identification of recombinants plated in the presence of X-Gal.<sup>2</sup> Since pBlueBac utilizes an *Nhe*I cloning site in place of the *Bam*HI site of pAc373 we replaced the 5' *Bam*HI restriction site in cytidylyltransferase (pBCT-bac) with an *Nhe*I site. Following this, cytidylyltransferase was liberated from pBCT-bac by digestion with *Nhe*I and *Xba*I and cloned into the *Nhe*I site of pBlueBac. The resulting construct was designated pBBCT (Fig. 1). The orientation of insertion was verified by digestion with *Eco*RV.

### Generation of Recombinant Baculovirus

Generation of recombinant baculovirus clones was carried out as previously described using Sf-9 insect cells (11,12). In the case of clones derived from pBBCT, plaques were identified chromogenically by the addition of X-Gal (150  $\mu$ g/ml) in the agarose. Isolation of baculovirus DNA was as described (12). Southern blotting of viral DNA was performed using standard procedures (14). Clonal recombinants expressing cytidylyltransferase were identified by Western blotting of infected cell extracts as described previously (5), using polyclonal antibodies generated against synthetic peptides corresponding to the amino and carboxy termini ("N"- and "C"-antibodies) respectively of rat liver cytidylyltransferase. Details regarding the N-antibody have been presented elsewhere (5). The C-antibody was generated against the peptide KAVTCDSEDEED, which corresponds to the carboxy-terminal 12 amino acids of rat liver cytidylyltransferase (8). Peptide synthesis and an-

<sup>2</sup> Abbreviations used: X-Gal, 5-brom-4-chlor-3-indolyl- $\beta$ -D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; CHO, Chinese hamster ovary.

tibody preparation were according to Watkins and Kent (15) except that the structure of the peptide was verified by fast atom bombardment-mass spectroscopy.

#### *Purification of Recombinant Cytidylyltransferase*

Sf-9 cells were grown in suspension in spinner flasks (Bellco) to high density ( $2-3 \times 10^6$  cells/ml). The cells were then pelleted and resuspended in an equal volume of complete insect medium and returned to freshly sterilized 250-ml spinner flasks. Recombinant baculovirus (5-10 ml virus preparation/250 ml cells; virus titer was about  $2 \times 10^8$  plaque-forming units/ml) was added to these flasks and infection was allowed to continue for 3 days. A typical enzyme preparation would involve three spinner flasks containing a total of 750 ml.

All procedures were performed on ice or at 4°C. The infected cells were pelleted by low-speed centrifugation, washed once with phosphate-buffered saline, and resuspended in 20 ml lysis buffer (10 mM Tris-Cl, pH 7.5, 30 mM NaCl, 0.2 mM EDTA, 2 mM DTT, 2.5 µg/ml leupeptin, 2.0 µg/ml chymostatin and pepstatin, 1.0 µg/ml antipain, 10 µg/ml each *p*-aminobenzamidine and benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were disrupted by Dounce homogenization and the NaCl concentration was brought up to 150 mM. The homogenate was stirred on ice for 10 min, following which the soluble and particulate fractions were separated by ultracentrifugation for 1 h at 100,000g in a Beckman 70 Ti rotor. The pellet was either resuspended in 5 ml buffer A (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, and protease inhibitors as above) or discarded.

The rest of the purification procedure was essentially the same as that outlined previously (6,7) with some modifications. Cytidylyltransferase was recovered from the cytosolic fraction by the addition of phosphatidylcholine:oleic acid vesicles (5 mM phosphatidylcholine, 10 mM oleic acid in buffer A minus protease inhibitors, sonicated for at least 1 h directly before addition to the cytosol; 450 nmol phosphatidylcholine/mg cytosolic protein) followed by the dropwise addition of 1.0 M acetic acid to a pH of 5.0. The mixture was stirred on ice for an additional 10 min and then centrifuged at 10,000 rpm for 25 min in a Beckman JM-1 centrifuge. The pellet was resuspended in 30 ml buffer A with protease inhibitors and recentrifuged as above. The pellet was resuspended in 20 ml buffer A with protease inhibitors and 20 mM octylglucoside, stirred on ice for 30 min, and recentrifuged as above. Greater than 80% of cytidylyltransferase activity was recovered in the supernatant liquid.

All chromatography buffers contained 2.5 µg/ml leupeptin and 2.0 µg/ml each of chymostatin and pepstatin. A 2.5 × 18-cm column was packed with DEAE-Sepharose Cl-6B and equilibrated with buffer B (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT). To

the octylglucoside extract was added one-tenth volume of sonicated phosphatidylcholine:oleic acid vesicles (prepared as above) and the preparation was loaded on the column at a flow rate of 0.5 ml/min. The column was washed sequentially at a flow rate of 1.0 ml/min with 75 ml equilibration buffer, 150 ml buffer C (50 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2 mM DTT), and 100 ml buffer D (50 mM Tris-Cl, pH 7.5, 400 mM NaCl, 2 mM DTT, 1 mM EDTA, 50 mM octylglucoside). Protein was monitored throughout by measuring the absorbance at 280 nm. Cytidylyltransferase was eluted in buffer D.

The peak fractions containing cytidylyltransferase were pooled, diluted threefold with buffer B, and applied to a column of hydroxylapatite which had previously been equilibrated with buffer B. The column was loaded at a flow rate of 0.5 ml/min. The column was washed sequentially at a flow rate of 1.0 ml/min with 100 ml buffer B, 150 ml buffer E (buffer B with 200 mM potassium phosphate), and 150 ml buffer F (buffer E with 50 mM octylglucoside). Cytidylyltransferase was eluted by washing the column with 150 ml buffer G (buffer E with 0.03% Triton X-100). Fractions expressing peak cytidylyltransferase were pooled, divided into aliquots, and stored in siliconized glass test tubes at -70°C.

#### *Assays*

Cytidylyltransferase activity was assayed as previously described (4). The reaction mixture contained 20 mM Tris-succinate, pH 7.8, 6 mM MgCl<sub>2</sub>, 5 mM CTP, 0.2 mM phosphatidylcholine:oleate (1:1 molar ratio sonicated in buffer A), 4 mM [<sup>14</sup>C]phosphocholine (sp act 0.2 µCi/umol), and enzyme protein in a final volume of 50 µl. The reaction was stopped by the addition of 150 µl of 100 mM phosphocholine in 7.5% trichloroacetic acid and 500 µl of 10 mg/ml activated charcoal and washed twice with water and radioactivity as [<sup>14</sup>C]CDP-choline was quantitated by liquid scintillation counting. Protein was determined by the Bradford method (16) using reagent purchased from Bio-Rad. Bovine serum albumin was used as standard. Polyacrylamide gel electrophoresis was performed as described by Laemmli (17).

#### RESULTS

Cytidylyltransferase was cloned from rat liver poly(A) RNA using the polymerase chain reaction and primers derived from the published sequence (8). We report here two base changes in the coding region of the clone we obtained that result in changes in the amino acid sequence as reported by Kalmer *et al.* (8). These changes were confirmed in two independent rounds of PCR amplification. The first change involves the substitution of guanine for adenosine at nucleotide 271 and results in glycine replacing serine at amino acid position

91, while the second change constitutes substituting adenosine for thymidine at nucleotide 340 subsequently replacing cysteine 114 with serine. The base substitution at nucleotide 340 also introduces an additional *Pst*I restriction site into the sequence. Both changes reported here increase the homology between rat liver and yeast cytidyltransferase (1). The differences between the previously reported cytidyltransferase sequence (8) and that obtained by us are probably due to PCR error in the previous sequence, although the possibility of isozymes or differences between the strains of rats used in the two studies has not been ruled out.

We attempted to express rat liver cytidyltransferase in *E. coli* using the T7 polymerase pET11 vector and the pGEX-KG vector in which the target protein is synthesized as a fusion protein with glutathione-*S*-transferase (18). With pET11, however, expression of cytidyltransferase was extremely low and with pGEX-KG most of the synthesized fusion protein was proteolyzed. Attempts to alleviate proteolysis of the fusion protein were made without success using a number of different strategies such as expression at low temperatures (15–25°C) in protease-deficient strains of *E. coli*, coexpression with a chaperone protein (GRO-ESL; 19,20), and addition of sequences to the 3' end of the cytidyltransferase cDNA, which had previously been shown to enhance stability of proteins expressed in *E. coli* (21,22). In light of these results we decided to explore alternative systems in which to express cytidyltransferase.

The cDNA encoding the full-length coding sequence of rat liver CTP:phosphocholine cytidyltransferase was cloned into the *Nhe*I site of the cloning vector pBlueBac and Sf-9 insect cells were then cotransfected with the cytidyltransferase-containing construct and wild-type baculovirus DNA. To verify the correct insertion of cytidyltransferase in the viral genome, recombinant viral DNA was isolated following clonal amplification and digested with *Kpn*I, separated on a 1% agarose gel, transferred to a nylon membrane, and probed with a <sup>32</sup>P-labeled cDNA encoding about 1000 bases from the 3' end of cytidyltransferase. Digestion of pBBCT with *Kpn*I gives a fragment of about 1.6 kb containing all but the first 55 bases of cytidyltransferase plus 459 bases between the *Bam*HI and *Kpn*I restriction sites of the polyhedrin gene (Fig. 1). Cytidyltransferase cDNA labeled with <sup>32</sup>P hybridized strongly to this 1.6-kb band in both the plasmid and the recombinant viral DNA digests while no such band was observed in *Kpn*I digests of wild-type viral DNA (data not shown). The 1.6-kb fragment was also observed in *Kpn*I digests of total DNA isolated from cells infected with recombinant virus (data not shown).

Once the insertion of cytidyltransferase within the viral genome was confirmed we were then able to express the enzyme. Virus was added to Sf-9 cells in suspension culture and infection was allowed to proceed for

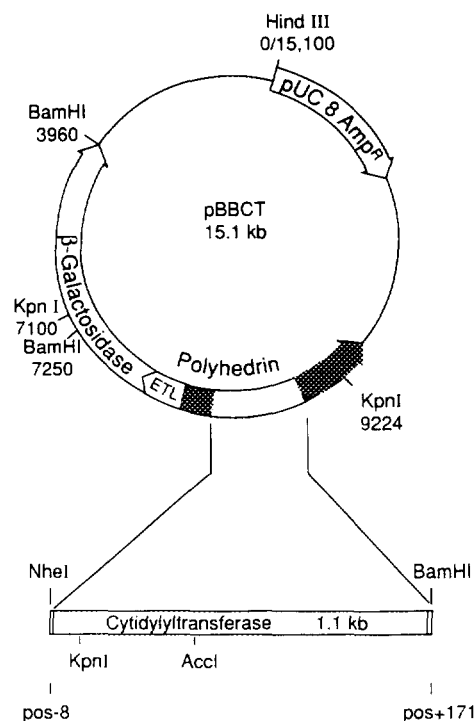
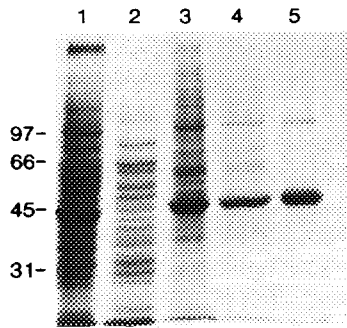


FIG. 1. Structure of the baculovirus transfer vector pBBCT. The plasmid contains the ampicillin resistance gene (*amp*<sup>r</sup>) for propagation in *E. coli*. A  $\beta$ -galactosidase gene controlled by the viral ETL promoter is present in pBBCT. Target protein expression is controlled by the polyhedrin promoter. Clonal insertion relative to the polyhedrin ATG codon is shown.

3–4 days. The cells were then sedimented, washed, resuspended in lysis buffer, and fractionated into the cytosolic and particulate components by ultracentrifugation. Subsequent purification was essentially per Weinholt *et al.* (6,7) with some modifications.

The initial step of the purification involves an acid precipitation of cytidyltransferase subsequent to the addition of sonicated phosphatidylcholine:oleic acid vesicles (1:2, mol/mol). Purification of cytidyltransferase from rat liver calls for the addition of 80 nmol phosphatidylcholine/mg protein (7); however, for baculovirus expression, addition of only 80 nmol of phosphatidylcholine/mg protein resulted in a considerable amount of cytidyltransferase remaining in the supernatant liquid following centrifugation of the acid precipitate. Cytidyltransferase was completely precipitated by increasing the amount of lipid by a factor of five as evidenced by the complete lack of the 42-kDa protein in the acid supernatant liquid (Fig. 2, lanes 2 and 3).

Cytidyltransferase was solubilized from the acid precipitate by the addition of octylglucoside and was loaded onto a DEAE-Sepharose column after the addition of one-tenth volume of freshly sonicated phosphatidylcholine:oleate vesicles (1:2 molar ratio). We did not find it necessary to run a gradient of 150 to 300 mM



**FIG. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractions from Table 1 detailing the steps in the purification of cytidylyltransferase from infected insect cells (see Materials and Methods). Lane 1, cytosol; lane 2, acid precipitate of the cytosolic portion, supernatant; lane 3, acid precipitate, pellet; lane 4, cytidylyltransferase preparation from the DEAE column eluted with 400 mM NaCl and 50 mM octylglucoside; lane 5, purified cytidylyltransferase eluted with 200 mM phosphate, 0.03% Triton X-100 from hydroxylapatite.

NaCl as previously described (6,7) but rather went straight from 150 to 300 mM NaCl in a stepwise fashion. The addition of sonicated lipid vesicles to the octylglucoside-solubilized extract prevented the elution of cytidylyltransferase in the 300 mM NaCl step. Cytidylyltransferase was then recovered from the column in 400 mM NaCl, 50 mM octylglucoside and applied to a column of hydroxylapatite.

We employed a four-step procedure to recover cytidylyltransferase from hydroxylapatite, avoiding the octylglucoside gradient and the 100 mM octylglucoside wash previously described (6,7). A summary of the purification is shown in Table 1 and described in the legend to Fig. 2. The band migrating at about 90 kDa in Fig. 2, lane 5, corresponds in size to the previously reported oxidized homodimer of cytidylyltransferase (23). The amount of enzyme recovered from hydroxylapatite represented 30% of that applied to the column (Table 1) and the total recovery of cytidylyltransferase from cytosol was 20%. It should be noted that the enzyme activity peak from hydroxylapatite had an extensive tail that we could never eliminate and the yields of pure cytidylyltransferase (Table 1) are based on activity derived from pooling the peak fractions only. Thus the actual total

yield from hydroxylapatite is probably somewhat higher than that reported (Table 1). The specific activity of the purified recombinant enzyme is lower than that reported previously for purified rat liver cytidylyltransferase. Weinhold and Feldman (6) reported a specific activity of 47.5  $\mu\text{mol}/\text{min}/\text{mg}$  protein, while Sanghera and Vance reported a specific activity of 34.2  $\mu\text{mol}/\text{min}/\text{mg}$  protein (24). The reasons for the lower activity of the purified recombinant enzyme are not known.

The recombinant cytidylyltransferase compares favorably with cytidylyltransferase isolated from rat liver. Both the recombinant enzyme and cytidylyltransferase purified from rat liver reacted identically when probed on Western blots with antibodies generated respectively against peptides corresponding to the N and C termini of cytidylyltransferase (Fig. 3). In addition, the recombinant enzyme is activated by lipids in a manner which is identical with cytidylyltransferase purified from rat liver (Table 2; Refs. 7,25,26).

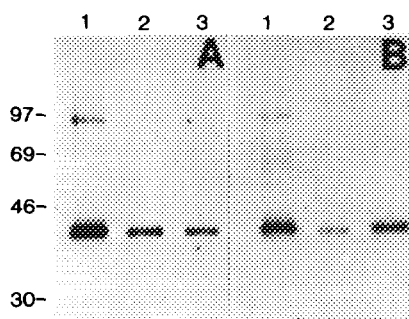
## DISCUSSION

In this study we describe the expression and purification of rat liver CTP:phosphocholine cytidylyltransferase using recombinant baculovirus technology. Baculovirus enjoys a certain advantage over bacteria in the expression of eukaryotic proteins in that the host cells themselves are eukaryotic and therefore protein stability is more likely to be maintained among proteins expressed in these cells as opposed to *E. coli*. Indeed we found this to be the case when we attempted to express cytidylyltransferase in *E. coli* and found the degree of proteolysis to be prohibitive. Interestingly, cytidylyltransferase from *Saccharomyces cerevisiae* has been expressed in *E. coli* (27).

The purification procedure employed for recombinant cytidylyltransferase is based on the procedure previously published by Weinhold *et al.* (6,7). We initially attempted a major modification in the established procedure by performing ion-exchange chromatography directly on the cytosolic fraction rather than on the octylglucoside extract of the acid precipitate. The results of this strategy were unsuccessful in that cytidylyltransferase activity was spread over three distinct and very broad peaks. We therefore decided to follow the

**TABLE 1**  
Purification of Recombinant Cytidylyltransferase

Fraction	Protein (mg)	Activity ( $\mu\text{mol}/\text{min}$ )	Specific activity (nmol/min/mg protein)	Yield (%)
Cytosol	246	152	620	100
Octylglucoside extract	26	121	4,600	79
DEAE-Sepharose	13	98	9,800	64
Hydroxylapatite	2.1	29.7	21,644	20



**FIG. 3.** Western blot comparison of purified recombinant cytidylyltransferase and cytidylyltransferase purified from rat liver. Cytidylyltransferase preparations were electrophoresed, transferred to Immobilon polyvinylidene difluoride, and probed with two separate antibodies, one generated against an amino-terminal peptide of cytidylyltransferase ("N-antibody") and the other generated against a carboxy-terminal peptide of cytidylyltransferase ("C-antibody"; see Materials and Methods). Immunoblot with the N-antibody is represented in A, while that with the C-antibody is shown in B. Lanes 1, purified recombinant cytidylyltransferase; lanes 2, purified cytidylyltransferase from rat liver; lanes 3, partially purified cytidylyltransferase from rat liver, DEAE fraction.

established procedure with only the modifications outlined under Materials and Methods. The most important modifications are the use of larger amounts of lipids in the acid precipitation and certain changes in the ion-exchange chromatographic steps. Initially we increased the amount of lipid only to precipitate all the cytidylyltransferase from the cytosol but despite this we still found that about half of the cytidylyltransferase was eluted from the DEAE column in the 300 mM NaCl wash. The addition of one-tenth volume of sonicated lipid vesicles to the octylglucoside extract prior to DEAE chromatography alleviated the problem of cytidylyltransferase elution in the 300 mM NaCl step. It should be noted that a significant amount of enzyme was always recovered from the DEAE column flow-through. In most instances this material was discarded but in a separate experiment the flow-through fractions were pooled and rechromatographed on DEAE and hydroxylapatite with a total recovery of about 200  $\mu$ g of pure cytidylyltransferase. Thus the DEAE flow-through fractions identify a significant point of loss of cytidylyltransferase. No cytidylyltransferase activity was detected in the hydroxylapatite column flow-through fractions.

We also generated a recombinant baculovirus clone in which cytidylyltransferase is expressed as a fusion protein with glutathione-S-transferase. The glutathione-S-transferase gene used in this construct was from pGEX-KG (18) and contains a thrombin cleavage site in the linker between the two proteins. In expressing cytidylyltransferase as a fusion protein with glutathione-S-transferase we could reduce the purification of the enzyme to three simple steps involving first the purifica-

tion of the fusion protein by affinity chromatography over glutathione-agarose followed by thrombin cleavage to liberate cytidylyltransferase and separation of the two proteins by a second passage over glutathione-agarose. The results of this effort were disappointing as the efficiency of thrombin digestion was extremely low. Furthermore, upon prolonged incubation with elevated levels of thrombin, cytidylyltransferase itself was in fact partially proteolyzed, despite the fact that purified rat liver cytidylyltransferase was insensitive to thrombin.

In many cell types activation of phosphatidylcholine synthesis *in vivo* is accompanied by a net movement of cytidylyltransferase from the cytosol to the membranes (1). In Chinese hamster ovary (CHO) cells the target membrane appears to be the nuclear envelope (15). Cytidylyltransferase is a phosphoprotein and recent evidence suggests that translocation of the enzyme in CHO cells is accompanied by changes in its overall phosphorylation state (4). The nature of these phosphorylation-dephosphorylation events is not known and an initial step in understanding how cytidylyltransferase is regulated by phosphorylation would be to map the phosphorylation sites within the enzyme. In addition, it is possible to identify the kinases and phosphatases involved in the regulation of cytidylyltransferase by using recombinant enzyme as a substrate for phosphorylation. Cytidylyltransferase expressed in baculovirus-infected insect cells should be useful in these studies.

The baculovirus-expressed protein will be important for enzymological studies, especially those regarding the interaction of cytidylyltransferase and lipids. Activation of cytidylyltransferase by lipids relates to the amphitropic nature of the enzyme and involves a high degree of selectivity with respect to the nature of the lipid (7,25,26). Lipid activation appears to be a function of a number of different factors such as acyl chain length, lipid phase, and overall charge (25,26) but little is known about how various lipids interact with cytidylyltransferase. One significant structural feature of cy-

**TABLE 2**  
Activation of Purified Cytidylyltransferase by Lipids<sup>a</sup>

	Activity (nmol CDP-choline/min)	Relative activity (%)
100 $\mu$ M PC:oleate	0.81	100
50 $\mu$ M PC:oleate	0.64	79
10 $\mu$ M PC:oleate	0.59	72
10 $\mu$ M PG	0.73	90
50 $\mu$ M PG	0.64	79
No lipid	0.21	26

<sup>a</sup> PC:oleate denotes 1:1 molar ratio of sonicated phosphatidylcholine:oleic acid vesicles. PG denotes sonicated phosphatidylglycerol vesicles.

tidyltransferase is a prominent amphipathic helix which may play a role in lipid binding (8). It would be interesting to construct mutants in this helical domain, express the mutant proteins in insect cells, and examine changes in lipid binding and activation.

#### ACKNOWLEDGMENTS

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