*Journal of Bioenergetics and Biomembranes, Vol. 28, No. 1, 1996*

# High-Level Bacterial Expression of Mitochondrial Transport Proteins

**Ronald S. Kaplan**[1]

*Received October 16, 1995*

The inability to obtain abundant quantities of purified, functional membrane proteins has represented a significant impediment to the goal of obtaining high-resolution structural information. In this review, procedures are described which have been developed in this laboratory and enable the high-level bacterial expression and subsequent purification of functional mitochondrial citrate transport proteins from yeast and rat liver. The data that we have obtained using these procedures and related results from other laboratories are discussed. Additionally, the general applicability of this approach to most mitochondrial transport proteins as well as to other types of membrane proteins is considered. Finally, relevant considerations when contemplating the use of this methodology and the likely value of this approach in future areas of research are explored.

**KEY WORDS:** Mitochondria; transporter; overexpression; tricarboxylate; citrate; membrane proteins.

## INTRODUCTION

Until recently, a major obstacle that has hindered progress toward an understanding of the structure and function of the mitochondrial inner membrane transporters at the molecular level has been an inability to obtain abundant quantities of either wild-type or site-specifically mutated transport proteins. This impediment has extended to other eukaryotic integral membrane proteins as well (for a review see Schertler, 1992). A breakthrough was achieved when Barnes *et al.* (1991) demonstrated high-level heterologous expression of the microsomal integral membrane protein 17α-hydroxylase cytochrome P450 in *E. coli.* They noted that an essential requirement for this expression was modification of the first several codons of the heterologous cDNA to codons more compatible with expression in *E. coli.* Fiermonte *et al.* (1993) subsequently demonstrated abundant expression of the mitochondrial inner membrane α-ketoglutarate carrier in a bacterial system. Most importantly, they showed that the anionic detergent sarkosyl enabled the extraction of the expressed transporter from inclusion bodies in a form that could be functionally reconstituted in liposomes. This strategy also enabled the high-level expression and functional reconstitution of the mitochondrial phosphate carrier (Wohlrab and Briggs, 1994).

Recently, utilizing variations and extensions of this approach, we have developed procedures enabling the expression and purification of high levels of functional tricarboxylate transporter (i.e., citrate transport protein) (CTP)[2] from both yeast (Kaplan *et al.*, 1995) and rat liver (Xu *et al.*, 1995) mitochondria. This review is intended to: (i) outline in detail the methodology that we have utilized to obtain overexpression; (ii) summarize our findings; (iii) discuss the general applicability of this approach for the high-level expression of a variety of other eukaryotic membrane proteins; and (iv) examine relevant considerations when

[1] Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688.

[2] Abbreviations used: BTC, 1,2,3-benzenetricarboxylate; CTP, citrate transport protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.



0145-479X/96/0200-0041$09.50/0 

contemplating the use of this approach and the likely value of this methodology in areas of future research.

## PROCEDURE FOR HIGH-LEVEL EXPRESSION OF MITOCHONDRIAL CITRATE TRANSPORT PROTEINS

We now describe the procedures that we have used to overexpress the citrate transport protein from yeast (Kaplan *et al.*, 1995) and rat liver (Xu *et al.*, 1995) mitochondria. It is important to note that, at the amino acid level, these two transporter sequences are substantially different (i.e., 37.7% identity) (Kaplan *et al.*, 1993; Holmstrom *et al.*, 1994; Kaplan *et al.*, 1995), and thus we suggest that the procedures outlined below are likely to prove successful with many other mitochondrial transporters.

**Step 1: Construction of the Expression Plasmid.** The open reading frame (ORF) encoding the CTP plus additional 3′ untranslated sequence is amplified by PCR. In these amplification reactions, a forward primer is utilized which contains an *Nde*I site plus additional 5′ sequence (we typically add 10 bases) in order to allow efficient restriction digestion. If necessary, modifications are introduced into the forward primer in order to eliminate existing rare codons at the beginning of the ORF which might minimize the level of expression in *E. coli.* For example, with the rat liver CTP we substituted CCG for CCC in the second codon. However, care must be taken to ensure that sequence modifications are not introduced near the 3′ end of the primer, in order for efficient amplification to proceed unperturbed. If necessary, the primer can be extended in the 3′ direction. A reverse primer is designed which corresponds to sequence following the end of the ORF. The location of this primer is chosen to correspond to a region of the template that is both near the end of the ORF and will allow specific priming. The reverse primer contains a *Bam*HI restriction site plus additional 5′ bases (we typically add 10 bases). Amplifications are performed using standard conditions that have been previously described in detail (Kaplan *et al.*, 1995). We typically obtain a single predominant amplification product of the expected size which is then purified using the Geneclean II Kit (Bio 101, Inc.). The purified product is sequentially digested with *Nde*I and *Bam*HI. Because of its limited stability, *Nde*I is added in two separate aliquots (10 units/aliquot) at 30-min intervals. The resulting digest is extracted and purified with the Geneclean II Kit. We then directionally clone the resulting CTPs into either the pET-21a(+) plasmid or the pET-28a(+) plasmid. These plasmids are first sequentially digested with *Nde*I and *Bam*HI and purified by "genecleaning." (The plasmids are obtained from Novagen and their properties are described in a later section.) The ligation and subsequent transformation of NovaBlue Competent Cells (Novagen) are performed essentially according to the manufacturer's instructions (Novagen pET System Manual, 1995). Transformants are screened for the presence of the CTP insert via: (i) direct colony PCR (using the same primer combination as above); and (ii) restriction analysis of purified plasmid DNA using *Nde*I and *Bam*HI. BL21(DE3) competent cells (Novagen), the expression host, are then transformed with 1 μl of a 50-fold dilution of plasmid DNA, purified (via the Wizard Minipreps DNA Purification System (Promega)) from NovaBlue cells, the storage host. Transformants are screened for inserts as described above. The cloned CTP fragment is then sequenced in its entirety, using plasmid DNA purified from BL21(DE3) cells as the template, in order to ensure that no mutations have occurred.

**Step 2: Induction of High-Level Expression of the CTP.** A single colony of *E. coli* BL21(DE3) cells containing the target plasmid was used to inoculate LB media (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) plus antibiotic (i.e., pET 21a(+) plasmid, 50 μg of carbenicillin/ml; pET 28a(+) plasmid, 30 μg of kanamycin/ml) (Novagen pET System Manual, 1995). The culture is incubated with shaking (350 rpm) at 37°C until an absorbance at 600 nm of 0.6–0.8 is obtained. A 200-ml aliquot is then removed, placed on ice for 5 min, and harvested as described below. Then 1.0 mM IPTG is added to the remainder of the culture in order to induce CTP expression and the incubation is continued for an additional 2 h. At this time 200-ml aliquots are removed and placed on ice for 5 min.

**Step 3: Purification of the Overexpressed CTP.** Cells are harvested by centrifugation at 5,000 × *g* for 5 min at 4°C. The supernatants are discarded and the sample pellets are stored on ice until all tubes are processed. The pellets are then resuspended in 20 ml of Buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8.0). Cell lysis is accomplished by incubation of the suspension with 100 μg of lysozyme/ml (freshly prepared in Buffer A) and 0.1% (v/v) Triton X-100 for 15 min at 30°C with occasional mixing (Novagen pET System Manual, 1995). The cell lysate is then sonicated, in order to shear the DNA, until there is a marked decrease in viscosity (i.e., typically 3–7 cycles of 30 sec sonication on ice followed by 30 sec of cooling/

cycle; 70% duty cycle; output control = 1.6; Branson sonifier 250; large tip). The suspension is centrifuged at 12,000 × *g* for 15 min, and the pellet is resuspended in 2 ml of Buffer B (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.0; 1.0 mM dithioerythritol) and recentrifuged. The resulting pellet is resuspended in 2 ml of Buffer B and the inclusion body fraction isolated by centrifugation at 131,000 × *g* for 4.5 h through a step gradient consisting of 12.4 ml of 40% (w/v) sucrose and 18.6 ml of 53% sucrose (Fiermonte *et al.*, 1993) (the sucrose solutions are prepared in Buffer B). The inclusion body pellet is resuspended in 30 ml of Buffer B and centrifuged at 12,000 × *g* for 15 min. The CTP is then extracted by resuspension of the inclusion body pellet in 2 ml of 1.2% (w/v) sarkosyl dissolved in Buffer B (Fiermonte *et al.*, 1993). Following centrifugation at 314,000 × *g* for 30 min, the resulting supernatant contains the solubilized CTP.

**Step 4: Functional Reconstitution of the Expressed CTP.** The purified CTP is reconstituted into preformed asolectin vesicles via the freeze-thaw-sonication technique. This established technique, which has been utilized to reconstitute the function of the CTP [from a variety of sources (Kaplan *et al.*, 1990, 1995; Xu *et al.*, 1995)], as well as the function of other mitochondrial anion transporters (Kramer and Klingenberg, 1979; Kaplan and Pedersen, 1985; Kaplan *et al.*, 1986; Nalecz *et al.*, 1986), is carried out as follows. Asolectin vesicles are prepared by bath sonication of dried asolectin (233.2 mg, Associated Concentrates) in 2.1 ml of Buffer C (120 mM Hepes, 50 mM NaCl, 1 mM EDTA, pH 7.4). Purified CTP (5 μl, 12–15 μg, yeast CTP; 50 μl, 408–486 μg, rat liver CTP) is added to a mix consisting of 525 μl of asolectin, 60 μl of Buffer C, Buffer B (200 μl, yeast CTP; 150 μl rat liver CTP), 120 μl of 400 mM citrate, 40 μl of 10% Triton X-114, and 1.2% sarkosyl in Buffer B (45 μl, yeast CTP; 50 μl, rat liver CTP). This mixture is briefly vortexed and frozen in liquid nitrogen, at which point the CTP is stable. (The rat liver CTP is incubated on ice for 90 min immediately prior to freezing in liquid nitrogen.) We routinely quantify CTP function by measuring 1,2,3-benzenetricarboxylate (i.e., BTC)-sensitive [$^{14}$C]citrate/citrate exchange using methodology that we have previously described in detail (Kaplan *et al.*, 1990).

## COMMENTS REGARDING THE pET VECTORS

Our expression studies utilized two of the pET plasmids [i.e., pET-21a(+) and pET-28a(+)]. Both plasmids contain strong bacteriophage T7 transcription and translation signals including a T7/*lac* promoter which consists of the lac operator sequence downstream from the T7 promoter sequence (for additional detail see Novagen pET System Manual, 1995). In addition, they encode the *lac* repressor (*lacI*) which results in a reduction of the basal expression (i.e., expression in the absence of IPTG) of the target gene, so that even toxic gene products are usually not produced in sufficient quantities to prevent stable establishment of the plasmid. For CTP expression, the recombinant plasmid is transformed into BL21(DE3) cells which contain a chromosomal copy of the T7 RNA polymerase gene under the control of the inducible *lacUV5* promoter. Thus, addition of IPTG to a growing culture induces the polymerase which then transcribes the CTP DNA.

It is also important to note that we have used the pET-21a(+) plasmid to direct expression of the native yeast CTP beginning with its amino terminal methionine without the addition of extra residues. In contrast, we have employed the pET-28a(+) vector to express the mature form of the rat liver CTP plus a 21 amino acid N-terminal fusion sequence which contains a stretch of 6 His residues that would enable rapid affinity purification (if needed) of the recombinant CTP.

## QUANTITY AND PURITY OF OVEREXPRESSED CITRATE TRANSPORT PROTEINS

We have overexpressed the yeast mitochondrial CTP, after directionally cloning the yeast CTP gene into the pET-21a(+) plasmid, as described above. Data on the amount and purity of the overexpressed CTP are depicted in Table I and Fig. 1. Thus, we typically obtain 26 mg of purified CTP per liter of *E. coli* culture. Based on scanning densitometric analysis, the purity

**Table I.** Quantity and Purity of Overexpressed Citrate Transport Proteins

| CTP source/pET vector | Quantity of purified CTP (mg protein)[a] | CTP purity (percent)[b] |
|---|---|---|
| Yeast CTP/pET-21a(+) | 26 | 80 |
| Rat Liver CTP/pET-28a(+) | 91 | 95 |

[a] Denotes mg of purified CTP obtained per liter of starting *E. coli* culture.

[b] Determined by scanning densitometric analysis.

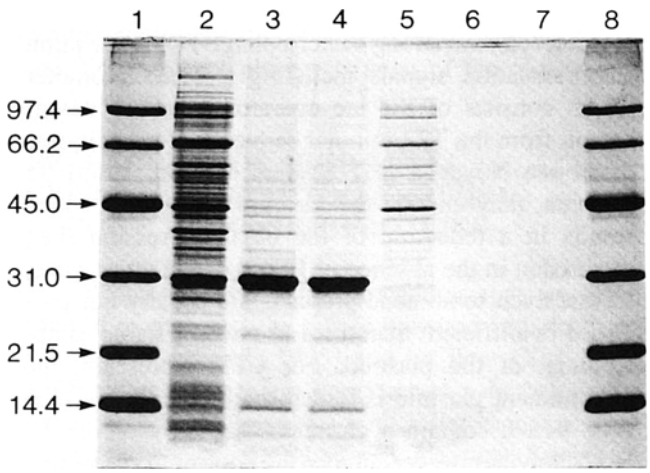


**Fig. 1.** Coomassie-stained SDS-polyacrylamide gradient gel depicting expression of the yeast mitochondrial citrate transport protein in *E. coli.* Proteins were separated in a 4.5% polyacrylamide stacking gel followed by a 14–20% linear gradient gel employing the buffer system of Laemmli (1970). Lanes 1 and 8, 1 μl of Bio-Rad SDS-PAGE low-range molecular weight standards: phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). Lanes 2 and 5, 9.5 μl of *E. coli* cells harvested either 2 h following induction with IPTG (lane 2) or immediately prior to the induction (lane 5). Lanes 3 and 6, 4 μl of the inclusion body fraction originating from cells harvested either 2 following induction (lane 3) or immediately prior to induction (lane 6). Lanes 4 and 7, 4 μl of the sarkosyl-solubilized inclusion body fraction originating from cells harvested either 2 h following induction (lane 4) or immediately prior to induction (lane 7). Reproduced with permission from *J. Biol. Chem.* (Kaplan *et al.*, 1995).

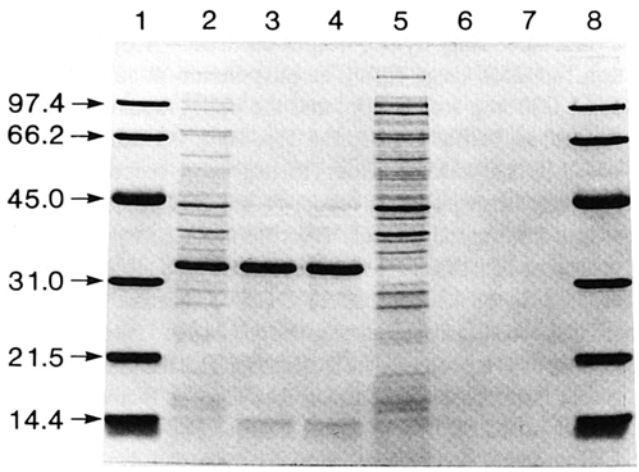


**Fig. 2.** Coomassie-stained SDS-polyacrylamide gradient gel depicting expression of the rat liver mitochondrial citrate transport protein in *E. coli.* SDS-PAGE was carried out as described in the legend to Fig. 1. Lanes 1 and 8, 1 μl of Bio-Rad SDS-PAGE low-range molecular weight standards. Lanes 2 and 5, 18 μg of *E. coli* cells harvested either 2 h following induction with IPTG (lane 2) or immediately prior to the induction (lane 5). Lanes 3 and 6, 8 μl of a 1/10 dilution of the inclusion body fraction originating from cells harvested either 2 h following induction (lane 3) or immediately prior to induction (lane 6). Lanes 4 and 7, 8 μl of a 1/10 dilution of the sarkosyl-solubilized inclusion body fraction originating from cells harvested either 2 h following induction (lane 4) or immediately prior to induction (lane 7). Reproduced with permission from *Biochem. Biophys. Res. Commun.* (Xu *et al.*, 1995).

of this material (i.e., Fig. 1, lane 4) is approximately 80%. Figure 1 depicts the SDS-PAGE profile of the overexpressed CTP. Lane 2 shows that 2 h following the induction of expression with IPTG, there is a high-level expression of a protein with an apparent molecular mass of 30–31 kDa (i.e., the yeast CTP) in the whole cell fraction. Scanning densitometry indicates that the CTP represents approximately 18% of total cellular protein. Lane 3 indicates that this protein is highly abundant in the inclusion body fraction isolated from the induced cells and lane 4 demonstrates that the CTP is effectively extracted from the inclusion bodies with the detergent sarkosyl. Importantly, prior to induction with IPTG, negligible amounts of CTP are present in the whole cell (lane 5), the inclusion body (lane 6), and the sarkosyl-extracted inclusion body (lane 7) fractions.

The data we have obtained following overexpression of the rat liver CTP in the pET-28a(+) plasmid are depicted in Table I and Fig. 2. As indicated, we are able to prepare 91 mg of purified CTP per liter of *E. coli* culture. Scanning densitometric analysis indicates that this material (i.e., Fig. 2, lane 4) is 95% pure. Figure 2 depicts the SDS-PAGE profile of this material. Lane 2 demonstrates that 2 h following the addition of IPTG, there is a high-level expression of a protein with an apparent molecular mass of approximately 34 kDa (i.e., the rat liver CTP) in the whole cell fraction. Scanning densitometry indicates that the CTP represents approximately 48% of total cellular protein. Lane 3 demonstrates that the CTP is highly abundant in the isolated inclusion body fraction and lane 4 shows that the CTP is effectively extracted from the inclusion bodies with sarkosyl. Prior to induction with IPTG, negligible CTP is present in the whole cell (lane 5), the inclusion body (lane 6), and the sarkosyl-extracted inclusion body (lane 7) fractions.

## FUNCTIONAL PROPERTIES OF RECONSTITUTED OVEREXPRESSED CITRATE TRANSPORT PROTEINS

The functional properties of the overexpressed CTPs have been determined by incorporation of the

sarkosyl-solubilized inclusion body fraction (lane 4 of Figs. 1 and 2) into phospholipid vesicles and subsequent measurement of BTC-sensitive [$^{14}$C]citrate/citrate exchange. As depicted in Table II, the overexpressed yeast CTP displays a $K_m$ of 0.36 mM which is similar to the value reported for this transporter in isolated yeast mitochondria (i.e., 0.24 mM) (Sandor *et al.*, 1994). Importantly, the overexpressed CTP displays a $V_{max}$ value of 2.5 μmol/min/mg protein which compares favorably to published values (i.e., 0.4–2.0 μmol/min/mg protein) (Claeys and Azzi, 1989; Bisaccia *et al.*, 1990; Kaplan and Mayor, 1993) for CTPs that were isolated from a variety of other sources and were assayed under similar conditions. It should be noted that since the native CTP has not been purified from isolated yeast mitochondria, a direct comparison of the $V_{max}$ of the purified native versus overexpressed yeast CTP is not possible. Importantly, reconstitution of the sarkosyl-solubilized inclusion body fraction obtained from cells harvested prior to induction with IPTG did not yield detectable BTC-sensitive citrate transport. Finally, as we have previously reported (Kaplan *et al.*, 1995), the overexpressed CTP: (i) maintains a strict requirement for intraliposomal substrate and thus catalyzes an obligatory exchange reaction; and (ii) displays a generally similar substrate specificity to that observed for this transporter in intact yeast mitochondria. In summary, the overexpressed yeast CTP exhibits a high degree of functional competence and displays characteristics which are quite similar to the native transporter.

The kinetic properties of the overexpressed rat liver CTP are also depicted in Table II. Thus, the rat liver CTP displays a $K_m$ of 0.37 mM which is generally similar to values reported for the native transporter in both isolated mitochondria (i.e., 0.12–0.25 mM) (Robinson *et al.*, 1971; Palmieri *et al.*, 1972) as well as after purification (i.e., 0.13 mM) (Bisaccia *et al.*, 1990; Kaplan and Mayor, 1993). The $V_{max}$ for the overexpressed transporter is 0.1 μmol/min/mg protein. While this value is higher than the values obtained for the native transporter in isolated mitochondria (i.e., 4–23 nmol/min/mg protein) (Robinson *et al.*, 1971; Palmieri *et al.*, 1972), it is substantially reduced compared with values reported for the purified native rat liver CTP (i.e., 1.6–2.0 μmol/min/mg protein) (Bisaccia *et al.*, 1990; Glerum *et al.*, 1990; Kaplan and Mayor, 1993). The reduced $V_{max}$ value is unlikely to be due to the presence of the amino terminal fusion sequence on the recombinant transporter, since our studies with rat liver CTP where expression was directed by the pET-21a(+) plasmid (which adds only a single methionine residue to the amino terminus of the native transporter) also indicate a substantially reduced $V_{max}$ value (R.S. Kaplan, unpublished results). Instead, we suggest that the reduced $V_{max}$ is most likely a consequence of a reduction in the proportion of reconstituted transporter that is in fact active due to incomplete renaturation following disaggregation of the inclusion bodies with sarkosyl. Finally, as previously noted (Xu *et al.*, 1995), the overexpressed rat liver CTP: (i) maintains a requirement for intraliposomal substrate and thus catalyzes an obligatory exchange reaction; and (ii) displays a substrate specificity that is virtually identical to that observed with the native purified transporter. Moreover, reconstitution of the sarkosyl-solubilized inclusion body protein obtained from cells harvested prior to induction with IPTG did not yield detectable BTC-sensitive citrate transport. In combination, the above findings establish the functional competence (albeit with a reduced $V_{max}$) of the overexpressed rat liver CTP.

**Table II.** Kinetic Properties of Reconstituted Overexpressed Citrate Transport Proteins[a]

| CTP source/pET vector | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) |
|---|---|---|
| Yeast CTP/pET-21a(+) | 0.36 | 2.5 |
| Rat liver CTP/pET-28a(+) | 0.37 | 0.1 |

[a] The overexpressed CTP was extracted from inclusion bodies with sarkosyl and incorporated into phospholipid vesicles in the presence of 0.05 M citrate. Transport reactions were carried out for either 10 sec (yeast) or 1 min (rat liver) at 30°C in the presence of 0.17–1.50 mM external citrate. Other experimental conditions were as previously described (Kaplan *et al.*, 1995; Xu *et al.*, 1995). Kinetic parameters were calculated from best-fit lines constructed based on linear regression analysis via the method of least squares.

## REFLECTIONS AND PROJECTIONS

With regard to the results obtained to date, several points merit comment. First, the selective packaging of both the yeast and the rat liver CTPs into inclusion bodies permits a high degree of purification of the overexpressed transporters by simply isolating the inclusion body fraction. This avoids the time-consuming step of developing complicated purification procedures which would tend to decrease yield and possibly function of the expressed transporters. Second, it is interesting to note that with respect to the rat liver CTP, although we expressed recombinant CTP containing a

His tag with the idea that this tag would enable additional purification of the transporter via nickel chelate chromatography, we in fact never needed to utilize this methodology since, upon extraction of the protein from the inclusion body fraction, the CTP was already 95% pure. Nonetheless, with proteins that are less pure the His tag is clearly likely to be advantageous. Third, it is very important to note that the extent of effectiveness of one pET vector versus another in the expression of a given protein is unpredictable and can best be determined by trial and error. For example, before overexpressing the rat liver CTP in the pET-28a(+) vector, we attempted overexpression in the pET-21a(+). For reasons which are unclear, rat liver CTP expression in this vector was considerably reduced. For example, we obtained approximately 8 mg of CTP per liter of *E. coli* culture at a purity of 55%. While this level of expression and purity are respectable, they certainly pale in comparison to the results we obtained with the pET-28a(+) vector. Thus, we conclude that for a given protein, if insufficient expression is obtained with one pET vector, it is certainly worth the investment of time to experiment with other pET vectors to determine if expression can be improved. Related to this, it has been our experience that, from start to finish, it takes approximately three weeks to determine whether a transporter sequence can be overexpressed in functional form using a given pET vector. Fourth, in addition to CTP expression described in this review, the pET (or related) vectors have also been used to overexpress the mitochondrial α-ketoglutarate (Fiermonte *et al.*, 1993) and phosphate (Wohlrab and Briggs, 1994) carriers. Therefore, we conclude that this system is excellently suited for overexpression of mitochondrial anion transporters which differ substantially in sequence. Furthermore, since a similar system has enabled the overexpression of cytochrome P450 17α-hydroxylase (Barnes *et al.*, 1991), a microsomal integral membrane protein, it is tempting to speculate that the pET vectors may permit overexpression of many different types of membrane proteins. The validity of this idea must await further experimentation.

With respect to the future, we believe that the ability to overexpress mitochondrial transporter sequences with this system affords several excellent opportunities. First, we concur with the suggestion of Fiermonte *et al.* (1993) that the ability to obtain abundant quantities of highly purified, functional transport proteins now permits the initiation of crystallization trials. Second, this system enables the expression of abundant quantities of site-specifically mutated tran porters (Wohlrab and Briggs, 1994; Xu *et al.*, 1996), thereby facilitating a comprehensive examination of the roles of individual amino acid residues in the translocation mechanism. Finally, as initially suggested by Fiermonte *et al.* (1993) and subsequently demonstrated by our laboratory (Kaplan *et al.*, 1995), this system will enable overexpression of the increasing number of DNA sequences that have recently appeared in GenBank and display characteristics that typify mitochondrial transporters. Thus, by overexpression of the protein encoded by a given sequence followed by its incorporation into phospholipid vesicles (utilizing established procedures that have been demonstrated to work with many different mitochondrial transporters), it should be possible to determine the transport function catalyzed by the expressed protein by merely experimenting with various substrate and inhibitor combinations in the transport assay. Once the function of a given transporter has been identified, a detailed structure–function characterization will be possible since the transport protein will already have been prepared in high abundance, and its encoding nucleotide sequence will have been identified and cloned into vectors which enable efficient mutagenesis. It is the opinion of this author that this powerful approach will result in the identification of previously undiscovered mitochondrial transporters and will enable characterization, at the molecular level, of a number of transporters that either have not yet been purified or have been purified in amounts insufficient to allow further progress.

## ACKNOWLEDGMENTS

The work described in this review was supported by grants from the National Institutes of Health (DK-44993) and the National Science Foundation (MCB-9219387). The author also wishes to thank June Mayor for critically reviewing this manuscript and the talented students, technologists, and colleagues who have participated in the work concerning this topic and whose names appear on the papers cited in the text.

## REFERENCES

Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 5597–5601.

Bisaccia, F., De Palma, A., Prezioso, G., and Palmieri, F. (1990). *Biochim. Biophys. Acta* **1019**, 250–256.

Claeys, D., and Azzi, A. (1989). *J. Biol. Chem.* **264**, 14627–14630.
Fiermonte, G., Walker, J. E., and Palmieri, F. (1993). *Biochem. J.* **294**, 293–299.
Glerum, D. M., Claeys, D., Mertens, W., and Azzi, A. (1990). *Eur. J. Biochem.* **194**, 681–684.
Holmstrom, K., Brandt, T., and Kallesoe, T. (1994). *Yeast* **10**, S47–S62.
Kaplan, R. S., and Mayor, J. A. (1993). *J. Bioenerg. Biomembr.* **25**, 503–514.
Kaplan, R. S., and Pedersen, P. L. (1985). *J. Biol. Chem.* **260**, 10293–10298.
Kaplan, R. S., Pratt, R. D., and Pedersen, P. L. (1986). *J. Biol. Chem.* **261**, 12767–12773.
Kaplan, R. S., Mayor, J. A., Johnston, N., and Oliveira, D. L. (1990). *J. Biol. Chem.* **265**, 13379–13385.
Kaplan, R. S., Mayor, J. A., and Wood, D. O. (1993). *J. Biol. Chem.* **268**, 13682–13690.
Kaplan, R. S., Mayor, J. A., Gremse, D. A., and Wood, D. O. (1995). *J. Biol. Chem.* **270**, 4108–4114.
Kramer, R., and Klingenberg, M. (1979). *Biochemistry* **18**, 4209–4215.
Laemmli, U. K. (1970). *Nature* **227**, 680–685.
Nalecz, K. A., Bolli, R., Wojtczak, L., and Azzi, A. (1986). *Biochim. Biophys. Acta* **851**, 29–37.
Novagen pET System Manual (1995), 5th edition, TB#55, Novagen Inc., Madison, Wisconsin.
Palmieri, F., Stipani, I., Quagliariello, E., and Klingenberg, M. (1972). *Eur. J. Biochem.* **26**, 587–594.
Robinson, B. H., Williams, G. R., Halperin, M. L., and Leznoff, C. C. (1971). *J. Biol. Chem.* **246**, 5280–5286.
Sandor, A., Johnson, J. H., and Srere, P. A. (1994). *J. Biol. Chem.* **269**, 29609–29612.
Schertler, G. F. X. (1992). *Curr. Opin. Struct. Biol.* **2**, 534–544.
Wohlrab, H., and Briggs, C. (1994). *Biochemistry* **33**, 9371–9375.
Xu, Y., Mayor, J. A., Gremse, D., Wood, D. O., and Kaplan, R. S. (1995). *Biochem. Biophys. Res. Commun.* **207**, 783–789.
Xu, Y., Gremse, D. A., Mayor, J. A., and Kaplan, R. S. (1996). *Biophys. J.*, in press.