Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3015–3019, May 1985
Microbiology

Cloning and expression of the *Rickettsia prowazekii* ADP/ATP translocator in *Escherichia coli*

(obligate intracellular parasite/carrier-mediated exchange/recombinant DNA)

DUNCAN C. KRAUSE, HERBERT H. WINKLER, AND DAVID O. WOOD*

Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, AL 36688

Communicated by Saul Roseman, December 17, 1984

ABSTRACT **Cosmid clone banks of *Rickettsia prowazekii* genomic DNA were established in *Escherichia coli* and screened for expression of the rickettsial carrier-mediated ADP/ATP translocator. Out of 2700 clones screened, a single clone, designated MOB286, accumulated radioactivity when incubated with [α-^{32}P]ATP in 100 mM sodium phosphate buffer. This clone carried a plasmid, pMW286, containing a 9-kilobase-pair insert of rickettsial DNA, as established by DNA·DNA hybridizations. Transformation studies with purified pMW286 established that the ability of *E. coli* cells to accumulate radioactivity was mediated by the recombinant plasmid. Results from experiments in which [^{3}H]ATP was substituted for [α-^{32}P]ATP strongly suggested that the radiolabeled ATP was transported intact. Furthermore, [^{3}H]ATP was incorporated into 10% (wt/vol) trichloroacetic acid-precipitable material in a time-dependent manner. Uptake of ATP was also temperature-dependent, insensitive to atractyloside, *N*-ethylmaleimide, and dinitrophenol, and specific for ADP and ATP. Efflux of radiolabeled nucleotide was observed in the presence of extracellular ADP or ATP but not AMP and was not observed in the absence of extracellular adenine nucleotides. The successful cloning and expression of the rickettsial ADP/ATP translocator in *E. coli* will permit better characterization of rickettsial bioenergetics and of the metabolic regulation of obligate intracellular parasitism.**

Rickettsia prowazekii, the etiologic agent of epidemic typhus, is an obligate intracellular parasite. Whereas other intracellular bacteria are generally found surrounded by a membrane of host origin, rickettsiae escape quickly from phagocytic vacuoles and grow directly within the cytoplasm (and occasionally the nucleus) of their host cell. Rickettsiae are not leaky, as had been postulated, but possess both usual and unusual transport systems (1–4), most notably, a carrier-mediated ADP/ATP translocator (1). This activity, similar to that present in mitochondria, permits the exchange of ADP from the rickettsia for ATP present in the host cell's cytoplasm. The carrier is specific for ADP and ATP, operates equally well in either direction, has no energy requirement, and is of an obligatory exchange type—i.e., for every molecule of ATP transported into a rickettsia, a molecule of ADP must be transported from the rickettsia, or vice versa. ADP/ATP exchange is regulated at least in part by the concentration of inorganic phosphate (5).

Characterization of the ADP/ATP translocator is fundamental to gaining an understanding of rickettsial physiology and the regulation thereof. This task is complicated enormously by the difficulty in obtaining large quantities of highly purified rickettsiae due to the required intracellular propagation of these bacteria, usually in embryonated hen eggs. Furthermore, attempts to form membrane vesicles from *R. prowazekii*, which would be very important in this characterization, have been unsuccessful (unpublished observation). The cloning and expression in *Escherichia coli* of the gene(s) responsible for ADP/ATP exchange would permit circumvention of these problems. Purification of the translocator would be facilitated immensely, modulation of this activity by various effectors could be studied against a well-defined *E. coli* physiological background, membrane vesicles could be formed, and gene organization and regulation could be assessed. We report here the cloning in *E. coli* of a *R. prowazekii* genetic locus that directs the expression in *E. coli* of the rickettsial ADP/ATP translocator.

MATERIALS AND METHODS

Enzymes and Reagents. Ampicillin, Tris, adenine, adenosine, AMP (sodium salt, type II), ADP (sodium salt, grade IX), ATP (disodium salt), atractyloside (disodium salt), thiamine, EDTA, and boric acid were purchased from Sigma. *N*-ethylmaleimide, 2,4-dinitrophenol, and trichloroacetic acid were purchased from Fisher. Agarose, restriction endonucleases, dithiothreitol, and T4 DNA ligase were obtained from Bethesda Research Laboratories. Nitrocellulose filters were purchased from Schleicher & Schuell. Membrane filters (HAWP025, 0.45-μm pore size) were purchased from Millipore. [2,8,5′-^{3}H]ATP was purchased from ICN. [α-^{32}P]ATP was purchased from ICN and New England Nuclear.

Bacterial Strains and Culture Conditions. *R. prowazekii* strain Madrid E was cultivated in yolk sacs of antibiotic-free embryonated chicken eggs and purified as described elsewhere (1). The purification protocol included Renografin density gradient centrifugation to remove contaminating yolk sac mitochondria (6). The *E. coli* strains used in this study were DH1 (*gyrA96 recA1 relA1*(?) *endA1 thi-1 hsdR17 glnV44* λ^- F^-) and HB101 (7) (*hsdS20* r_B^- m_B^- *recA13 xyl-5 mtl-1 ara-14 proA2 lacY1 galK2 leuB6 rpsL20 thi-1 glnV44* λ^- F^-). The former was obtained from B. Bachmann (*E. coli* Genetic Stock Center, New Haven, CT); the latter strain was supplied by D. Kopecko (Walter Reed Army Institute of Research, Washington, D.C.). *E. coli* strains were cultured at 37°C in L broth or on L agar plates (8), except where indicated.

Cosmid Clone Bank Formation. The protocol for formation of cosmid clone banks of the *R. prowazekii* genome has been published in detail (9). Briefly, rickettsial DNA was extracted according to Myers and Wisseman (10) and purified by CsCl density gradient equilibrium centrifugation. The cosmid vector pHC79 (11) was purified according to Clewell and Helinski (12). *Sau*3A-digested rickettsial DNA was inserted into the *Bam*HI site of pHC79. The ligated DNA was packaged into capsids by using packaging extracts prepared from *E. coli* strains BHB2688 and BHB2690 (13, 14) as described by Davis *et al.* (15). *E. coli* DH1 was cultured in L broth

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: kb, kilobase pair(s).

*To whom reprint requests should be addressed.

containing maltose rather than glucose, infected with the packaged recombinant cosmids, and cultured on L agar containing ampicillin at 15 μg/ml. Ampicillin-resistant colonies were stocked individually in 96-well Microtiter dishes in 200 μl of L broth containing ampicillin and approximately 10% glycerol.

***In Situ* Screening of Clone Banks for ATP Transport.** Cosmid clones were replica plated to 96-well Microtiter dishes containing 200 μl of L broth with ampicillin (15 μg/ml) per well and incubated overnight at 37°C. The bacterial suspensions were transferred to a 96-well vacuum manifold, in which the cells were collected on a nitrocellulose filter (pore size 0.45 μm). After two washes with 100 mM sodium phosphate buffer, pH 7.2 (PB), suction was removed and 200 μl of PB containing [α-^{32}P]ATP (1 μCi/ml, 5–25 Ci/mmol; 1 Ci = 37 GBq) was added per well. The cells were incubated in the presence of radiolabeled ATP for 20 min at room temperature, after which the vacuum was restored and the filters were washed four times with PB, using approximately 0.5 ml per wash. The filters were then removed from the manifold and placed on blotting paper saturated with PB for an additional 15–20 min to permit removal of radioactivity that might have seeped between the wells. After air-drying, the filters were heated to 80°C for 45 min and exposed to x-ray film (Kodak X-Omat RP) overnight. Autoradiography was enhanced with an intensifier screen. Transport of ATP and exchange of ADP and ATP by *E. coli* clones or *R. prowazekii* were assayed in uptake experiments as detailed elsewhere (1). Briefly, *E. coli* cells were cultured to late logarithmic phase (optical density at 600 nm of 0.6–0.7) in L broth with ampicillin at 15 μg/ml, washed once in PB, and resuspended in 0.1 volume of PB. Assays were initiated with the addition of 0.1 ml of cell suspension to 0.9 ml of PB containing 1 μCi of [α-^{32}P]ATP or [^{3}H]ATP. With the latter isotope, adenine was included at a final concentration of 1 mM to competitively inhibit uptake of any radioactively labeled hydrolytic product by normal *E. coli* purine-transport mechanisms. Except where indicated, uptake assays were performed at room temperature.

RESULTS

Identification of an ATP-Transporting Clone. Approximately 2700 cosmid clones were screened for the ability to transport [α-^{32}P]ATP. A single clone, 9B, showed significant accumulation of radioactivity compared to controls (Fig. 1).

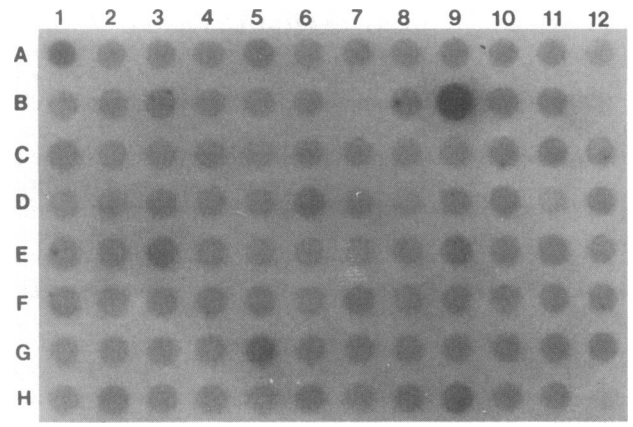

FIG. 1. Autoradiograph of *E. coli* cosmid clones incubated with [α-^{32}P]ATP for 20 min at room temperature and then washed extensively. A single clone (9B, designated hereafter as MOB286) accumulated a significant level of radioactivity. *R. prowazekii* (1A) and *E. coli* DH1 (12G) were included as positive and negative controls, respectively. The film was exposed for 18 hr in the presence of an intensifier screen.

R. prowazekii was included as a positive control in well 1A, while *E. coli* DH1 was present in 12G. The intensity of 9B in this autoradiograph was significantly greater than that of the other clones present, the *E. coli* negative control, and the rickettsial positive control. Variability in the intensity of the positive control from experiment to experiment was a function of total rickettsial cell number and viability as indicated by hemolytic activity (16).

Physical Characterization of the Recombinant Plasmid. Clone 9B has been designated MOB286, and the recombinant plasmid associated with this clone, pMW286. Plasmid DNA was extracted from MOB286 according to Hansen and Olsen (17) and purified by two successive cesium chloride/ethidium bromide density gradient equilibrium centrifugations. Plasmid pMW286 was digested to completion with restriction endonuclease *Hin*dIII and analyzed by agarose gel electrophoresis (Fig. 2*A*). *Hin*dIII restriction fragments totaling approximately 15.4 kilobase pairs (kb) were observed (Fig. 2*A*, lane d). *Hin*dIII digestions of *R. prowazekii* genomic DNA, *E. coli* DNA, and chicken egg yolk sac DNA were included for comparison. The DNA shown in Fig. 2*A* was transferred to nitrocellulose paper according to Southern (18) and probed with pMW286 biotinylated by nick-translation (19), using a kit obtained from Bethesda Research Laboratories. Hybridization was carried out under conditions requiring greater than 90% DNA sequence homology. No hybridization was observed to *E. coli* or yolk sac DNA. Some hybridization to bacteriophage λ DNA was observed and probably can be attributed to the λ *cos* site in the cosmid vector pHC79. The probe hybridized to five restriction fragments of *R. prowazekii* genomic DNA (Fig. 2*B*, lane a, arrowheads), demonstrating the rickettsial origin of the cloned DNA. The rickettsial *Hin*dIII fragments were of approximately 3.5, 2.8, 1.8, 1.1, and 0.7 kb. No hybridization to rickettsial DNA was observed when vector pHC79 lacking an insert was substituted for pMW286 (data not shown).

That a rickettsial gene on pMW286 was responsible for directing the uptake of radioactivity, putatively ATP, was determined in a coinheritance experiment. $CaCl_2$-treated *E. coli* DH1 cells were transformed with the recombinant plas-

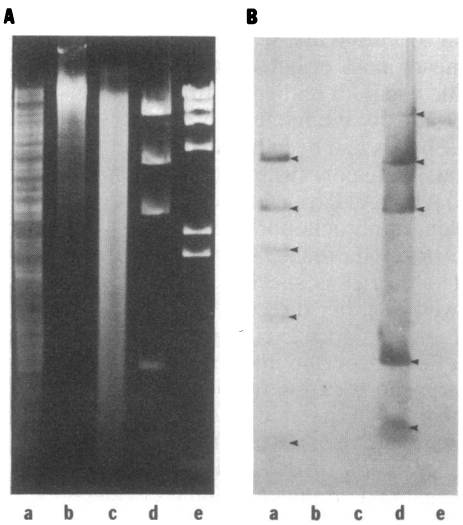

FIG. 2. (*A*) Agarose gel electrophoretic analysis of restriction endonuclease *Hin*dIII digests of *R. prowazekii* DNA (lane a), *E. coli* DNA (lane b), chicken egg yolk sac DNA (lane c), and pMW286 (lane d). Bacteriophage λ DNA digested with *Hin*dIII was included (lane e) for size determinations of linear DNA. The λ DNA bands are, from top to bottom, at 23.3, 9.3, 6.5, 4.3, 2.2, 1.9, and 0.5 kb. Bands were visualized with ethidium bromide. (*B*) Hybridization of pMW286, biotin-labeled by nick-translation, to a Southern blot (18) of the DNAs shown in *A*. Bands of interest are indicated with arrowheads.

mid pMW286 or with the vector pHC79 according to Davis *et al.* (15). Transformants were selected on L agar containing ampicillin at 15 μg/ml. Ampicillin-resistant colonies were inoculated into individual wells of a 96-well Microtiter dish in L broth containing ampicillin, incubated for approximately 6 hr at 37°C, and screened for the ability to accumulate radioactive ATP. *R. prowazekii* transported a significant amount of [^{32}P]ATP, as indicated by the intensity of the spots corresponding to this positive control (Fig. 3*A*). In contrast, *E. coli* DH1 containing only the vector pHC79 accumulated very little radioactivity (Fig. 3*B*). All of the ampicillin-resistant transformants acquiring pMW286 were also able to transport [^{32}P]ATP (Fig. 3*C*), indicating coinheritance of ampicillin resistance and ATP transport and thus localizing the latter activity to the recombinant plasmid. Similar results were obtained when *E. coli* strain HB101 was substituted for strain DH1, indicating that expression of this trait was not limited to one strain of *E. coli* (data not shown).

Incorporation of Transported Nucleotide. The fate of transported ATP was evaluated as follows. MOB286 or control *E. coli* were grown to an OD_{600} of 0.6 in minimal medium (20) containing 0.5% glucose, 0.5% Casamino acids, thiamine (2 μg/ml), and ampicillin (15 μg/ml). Adenine was added to 1 mM and [^{3}H]ATP (46.2 Ci/mmol) was added to 1 μCi/ml. Samples were taken at 15 and 30 min and either processed to determine total [^{3}H]ATP uptake or treated with 10% (wt/vol) Cl_3CCOOH to determine the amount of label incorporated into Cl_3CCOOH-precipitable material. After 1 hr on ice, precipitated material was collected on filters. The filters were washed with 5% Cl_3CCOOH and air-dried, and radioactivity was determined by liquid scintillation counting. Results from a representative experiment are shown in Fig. 4. Negligible radioactivity, either total cell-associated or Cl_3CCOOH-precipitable, was detected with the *E. coli* negative control. In contrast, [^{3}H]ATP was transported by MOB286 in a time-dependent fashion. Hence, MOB286 will accumulate radioactivity when incubated with radiolabeled ATP whether the radioactivity is in the phosphorous (as in the screening assays) or in the adenosine portion of the ATP, strongly suggesting that the ATP molecule is transported intact. By 30 min, up to 30% of the cell-associated radioactivity was precipitable with Cl_3CCOOH, verifying that the ATP

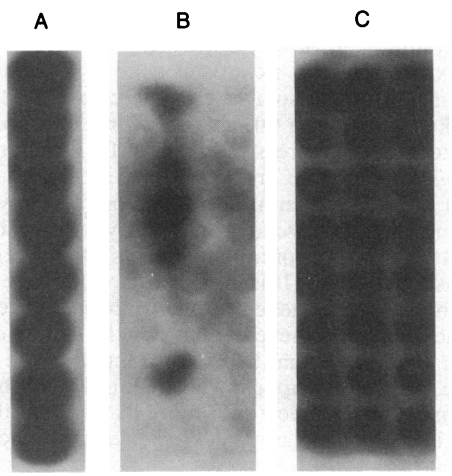

FIG. 3. Autoradiograph of *R. prowazekii* (*A*) or ampicillin-resistant transformants of *E. coli* DH1 receiving only vector pHC79 (*B*) or the recombinant cosmid pMW286 (*C*) incubated with [α-^{32}P]ATP for 20 min at room temperature, washed extensively, and exposed to film for 18 hr in the presence of an intensifier screen. The dark areas between some wells in *B* do not correspond to cell-associated radioactivity but are the result of inadequate removal of free [^{32}P]ATP that had seeped between some wells in the manifold.

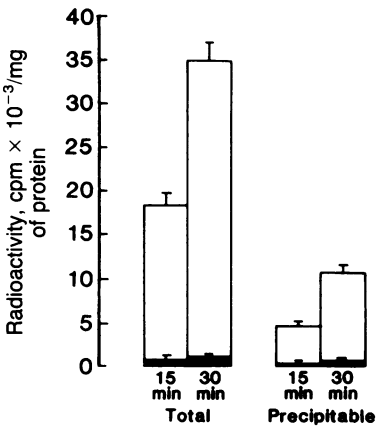

FIG. 4. Uptake of [^{3}H]ATP by *E. coli* DH1 containing vector pHC79 (solid bars) or pMW286 (open bars) and incorporation of radioactivity into 10% Cl_3CCOOH-precipitable material. *E. coli* cells were grown and labeled as described in the text and sampled at the indicated times. Samples were either collected on filters for total ATP uptake or treated with Cl_3CCOOH before filtering. The data were adjusted for background radioactivity observed when parallel samples were processed at 0°C. Each bar represents the mean of duplicate samples, with positive standard deviations indicated.

is transported to the intracellular nucleotide pool and accessible for incorporation into nucleic acids.

Specificity of Nucleotide Uptake. The specificity of ATP transport was assessed by testing the ability of various nonradioactive effectors to inhibit uptake of [α-^{32}P]ATP. Putative effectors were added to the phosphate buffer to a final concentration of 1 mM (25,000-fold excess) prior to the addition of cells. As shown in Fig. 5, adenine, adenosine, and AMP had little effect on the uptake of ATP. Again, this suggests that intact ATP rather than a product of hydrolysis was transported. However, both ADP and ATP effectively inhib-

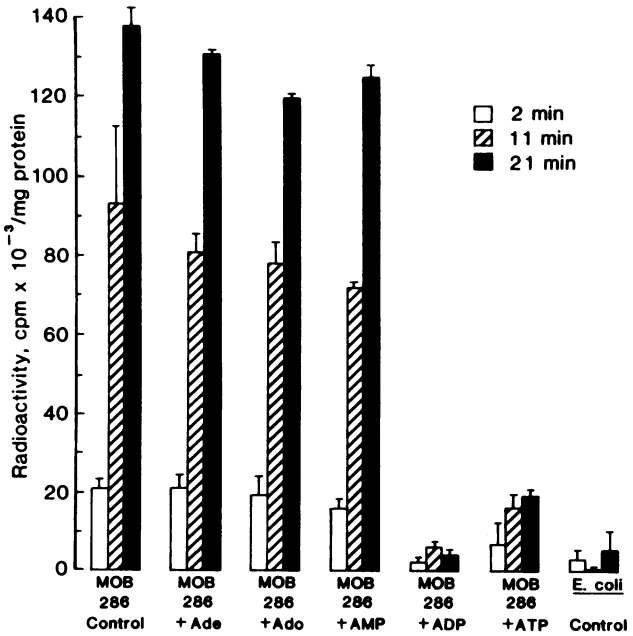

FIG. 5. Competitive inhibition by adenine compounds of uptake of [α-^{32}P]ATP by MOB286. *E. coli* cells were added to phosphate buffer containing 40 nM [^{32}P]ATP (1 μCi/ml) and the indicated compound (1 mM). Control, no inhibitor; Ade, adenine; Ado, adenosine. Samples were taken at 2 min (open bars), 11 min (hatched bars), and 21 min (solid bars). Each bar represents the mean of duplicate samples, with positive standard deviations indicated.

ited transport of the radioactive label. GTP, CTP, or UTP at a 25,000-fold excess competitively inhibited ATP uptake by only ≈20% (data not shown). Thus, if these compounds can serve as substrates for the cloned translocator, they do so with a much lower affinity than do ADP or ATP.

Examination of the Efflux Component of the Translocator. An important characteristic of the *R. prowazekii* translocator is its reciprocal exchange nature. For every molecule of ATP transported into a cell a molecule of ADP is transported out of the cell, and vice versa. Rickettsiae are able to maintain the intracellular concentration of substrate when there are extreme changes in the extracellular concentrations of substrate (1). In the absence of exchangeable nucleotide there is no efflux, as is typical for carrier-mediated exchange. The efflux component of the cloned translocator in *E. coli* was evaluated by loading the cells with [α-^{32}P]ATP and examining the dependence of efflux on the presence of exchangeable nucleotide in the extracellular medium. As shown in Fig. 6, a reduction of substrate concentration by a factor of 250 in the absence of any nucleotide or in the presence of 1 mM AMP resulted in only a slight reduction of intracellular radioactivity. In contrast, there was substantial exchange of unlabeled ADP or ATP for the radioactive ATP when either of these effectors was included in the diluent.

To further characterize the cloned translocator, uptake experiments were carried out in the presence of 1 mM 2,4-dinitrophenol or *N*-ethylmaleimide or 25 μM atractyloside. Mitochondrial ADP/ATP translocators, including that of yolk sac mitochondria, are sensitive to atractyloside, whereas the rickettsial translocator is not (1). Similarly, the rickettsial translocator is not inhibited by protonophores such as dinitrophenol or the thiol reagent *N*-ethylmaleimide (1). Uptake of [^{32}P]ATP in *E. coli* expressing the cloned rickettsial translocator was not inhibited by any of these agents (data not shown). In contrast, for example, adenine uptake by *E. coli* DH1 was reduced significantly in the presence of dinitrophenol.

DISCUSSION

The ability to exploit host-cell ATP pools as an energy source and sense them as a regulatory signal (21) represents an important aspect of successful parasitism by *R. prowazekii*. Characterization of the rickettsial ADP/ATP translocator is fundamental to gaining an understanding of rickettsial bioenergetics and the metabolic regulation of obligate intracellular parasitism. The successful cloning and expression of the rickettsial ADP/ATP translocator in *E. coli* should prove to be a significant step towards this goal.

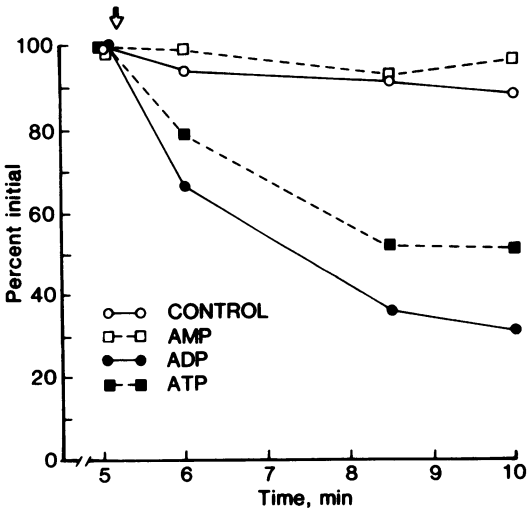

FIG. 6. Efflux of intracellular ATP from MOB286. *E. coli* DH1 containing pMW286 were loaded with [α-^{32}P]ATP (80 nM, 1 μCi/ml) for 5 min at room temperature and then diluted 250-fold into phosphate buffer. AMP, ADP, or ATP were added to a final concentration of 1 mM immediately after dilution (arrow). Samples were taken at the indicated times.

Approximately 2700 cosmid clones were screened for ATP transport before a positive clone was identified. The restriction nuclease *Sau*3A was chosen to digest rickettsial genomic DNA into fragments suitable for cloning. This enzyme recognizes a 4-base-pair sequence and thus is regarded as a "frequent-cutter" that can generate practically random restriction fragments in partial digestions of rickettsial DNA. Insertion of *Sau*3A-digested DNA into the *Bam*HI site generally does not regenerate *Bam*HI restriction sites at the points of insertion, therefore it was not possible to excise intact the cloned rickettsial DNA. For this reason, there was not a 1:1 correspondence between all of the DNA bands generated when *R. prowazekii* DNA and pMW286 were digested with *Hin*dIII and probed with biotin-labeled pMW286 (Fig. 3*B*, lanes a and d, arrowheads). The rickettsial origin of the cloned insert was confirmed by DNA·DNA hybridizations. The absence of hybridization to yolk sac DNA and the insensitivity of uptake of ATP to atractyloside establish that a eukaryotic gene for mitochondrial ADP/ATP transport had not been cloned. That the hybrid plasmid responsible for directing this activity was only 15.4 kb long was somewhat surprising, in view of the fact that bacteriophage λ packaging preparations generally package cosmids of 35–50 kb. The instability of large recombinant cosmids has been noted by us (9) as well as by others (22), and the 15.4-kb pMW286 is probably a deletion derivative of a larger parent plasmid.

The cloned rickettsial ADP/ATP translocator exhibited the same characteristics as the translocator in *R. prowazekii*. Both are time- and temperature-dependent (data for the latter not presented); both are saturable and, hence, can be competitively inhibited; both are specific for ADP and ATP; and both possess influx and efflux components. It is noteworthy that with *R. prowazekii*, ADP can exchange for >90% of the radioactive ATP (1). In contrast, ADP could exchange for only about 70% of the intracellular radioactive material of the *E. coli* clone MOB286 (Fig. 6). This lower exchange may be due to the incorporation of accumulating radioactive ATP into nonexchangeable macromolecules with the cloned translocator in *E. coli* (Fig. 4), an incorporation that does not occur in *R. prowazekii*.

The expression of a rickettsial gene in *E. coli* has been accomplished for the soluble enzyme citrate synthase (23), for a rickettsial antigen (9), and now for a transport system. Undoubtedly, the expression of the ADP/ATP translocase in *E. coli* required the proper processing of the protein(s) involved into the bacterial inner membrane. However, this processing may not have to be especially rigorous because of the greater symmetry of an exchange system versus an active transport system. It is conceivable that there was a fusion of the rickettsial gene product to the amino terminus of the tetracycline-resistance gene product to allow membrane insertion. The rickettsial DNA was cloned in the middle of this gene, which does appear to code for a hydrophobic leader sequence (24). We feel that a more likely alternative is that the translocator gene has a leader sequence of its own that is functional in *E. coli*.

Rickettsiae are not strict energy parasites. They possess a functional tricarboxylic acid cycle and thus can generate their own ATP. Rickettsiae do not find themselves in a static environment; as host-cell ATP is depleted or during transit between cells, the ADP/ATP translocator becomes less useful as the rickettsiae are required to synthesize their own ATP. We anticipate that as a result of cloning the rickettsial genes for citrate synthase and the ADP/ATP translocase, we

will soon be able to address to a greater extent the regulation of these genes and their products during changes in the environment of the host cell's cytoplasm.

This work is significant outside the field of rickettsiology as well. For example, biochemically, the ADP/ATP translocators of rickettsiae and mitochondria exhibit both a number of similarities (specificity, affinity, exchange nature, and insensitivity to metabolic inhibitors) and a number of differences (sensitivity to atractyloside and bongkrekic acid). Now that the gene for the rickettsial translocator has been cloned, it will soon be possible to compare this gene with the recently cloned and sequenced *Neurospora crassa* ADP/ATP translocator gene (25, 26) for DNA sequence homology. This information may provide new insights into the origin of mitochondria and a possible derivation from a symbiotic relationship with a prokaryote. In addition, in the field of *E. coli* bioenergetics, this activity should allow *E. coli* to transport ATP into membrane vesicles formed in the presence of adenine nucleotide, permitting studies of ATP-driven activities otherwise not possible.

We thank Dr. Barbara Bachmann and Dr. Dennis Kopecko for supplying the bacterial strains used in this study. We also thank Chee Chu for excellent technical assistance and Karen Waltman and Mary Lazzari for typing the manuscript. This work was supported by Public Health Service Research Grants AI20384 and AI15035 from the National Institute of Allergy and Infectious Diseases.

1. Winkler, H. H. (1976) *J. Biol. Chem.* **251,** 389–396.
2. Smith, D. K. & Winkler, H. H. (1977) *J. Bacteriol.* **129,** 1349–1355.
3. Winkler, H. H. & Daugherty, R. M. (1984) *J. Bacteriol.* **158,** 460–463.
4. Atkinson, W. H. & Winkler, H. H. (1981) in *Rickettsiae and Rickettsial Diseases*, eds. Burgdorfer, W. & Anacker, R. (Academic, New York), pp. 411–420.
5. Winkler, H. H. & Daugherty, R. M. (1984) *J. Bacteriol.* **160,** 76–79.
6. Weiss, E., Coolbaugh, J. C. & Williams, J. C. (1975) *Appl. Microbiol.* **30,** 456–463.
7. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–472.
8. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 433.
9. Krause, D. C., Winkler, H. H. & Wood, D. O. (1984) *Infect. Immun.* **47,** 157–165.
10. Myers, W. F. & Wisseman, C. L., Jr. (1982) *Int. J. Syst. Bacteriol.* **30,** 143–150.
11. Hohn, B. & Collins, J. (1980) *Gene* **11,** 291–298.
12. Clewell, D. B. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62,** 1159–1166.
13. Hohn, B. & Murray, K. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3259–3263.
14. Sternberg, N., Tiemeier, D. & Enquist, L. (1977) *Gene* **1,** 255–280.
15. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 130–133.
16. Walker, T. S. & Winkler, H. H. (1979) *J. Clin. Microbiol.* **9,** 645–647.
17. Hansen, J. B. & Olsen, R. H. (1978) *J. Bacteriol.* **135,** 227–238.
18. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
19. Rigby, P. W. J., Dieckmann, J., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113,** 237–251.
20. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218,** 97–106.
21. Phibbs, P. V., Jr., & Winkler, H. H. (1982) *J. Bacteriol.* **149,** 718–725.
22. Van Embden, J. D., van der Donk, H. J., van Eyk, R. V., van der Heide, H. G., de Jong, J. A., van Olderen, M. F., Osterhans, A. D. & Schouls, L. M. (1983) *Infect. Immun.* **42,** 187–196.
23. Wood, D. O., Atkinson, W. H., Sikorski, R. S. & Winkler, H. H. (1983) *J. Bacteriol.* **155,** 412–416.
24. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77–90.
25. O'Malley, K., Pratt, P., Robertson, J., Lilly, M. & Douglas, M. G. (1982) *J. Biol. Chem.* **257,** 2097–2103.
26. Arends, H. & Sebald, W. (1984) *EMBO J.* **3,** 377–382.