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Protein Synthesis Early in the Developmental Cycle of *Chlamydia psittaci*

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The incorporation of [^{35}S]methionine into protein by intracellular and host-free *Chlamydia psittaci* 6BC was analyzed at intervals between 15 min and 28 h postinfection by autoradiography of sodium dodecyl sulfate-polyacrylamide gels. The profiles of proteins synthesized in the two systems were similar at all times, indicating that the host-free system can be used to monitor the temporal expression of genes in chlamydiae. The host-free system permitted detection of synthesis of chlamydial proteins as early as 15 min postinfection. Some of the proteins synthesized during the initial phases of reorganization of elementary bodies to reticulate bodies either were not synthesized or were synthesized in greatly reduced amounts during the other phases of the developmental cycle. The effects of rifampin and actinomycin D indicated that host-free protein synthesis was at least partially dependent on the initiation and continuation of RNA synthesis in the isolated organisms.

Chlamydia psittaci and *Chlamydia trachomatis* are obligate parasitic bacteria characterized by a unique developmental cycle that takes place entirely within a membrane-bound vacuole in the cytoplasm of eucaryotic host cells. The cycle is initiated by the endocytosis of osmotically stable, metabolically inactive elementary bodies (EBs). Once the bacteria are within host cells, chlamydial metabolic activity is activated and EBs reorganize through a continuous progression of intermediate forms to osmotically fragile reticulate bodies (RBs), a process which is completed between 8 and 10 h postinfection by *C. psittaci* 6BC in L cells. RBs of *C. psittaci* divide logarithmically until about 18 h postinfection, at which time some RBs commence reorganization to the EB form while others continue the process of cell division. RB multiplication and reorganization to EBs cease between 40 and 48 h postinfection, when the host cell lyses and infectious EBs and poorly defined, noninfectious intermediate forms are released (for reviews see references 3 and 25).

Although chlamydiae appear to possess all of the cellular components for independent RNA (28), DNA (2, 26), and protein (1) synthesis, they carry out macromolecular syntheses inefficiently and for only brief periods when isolated from host cells (9, 11, 13). The reasons for the lack of sustained metabolic activity by host-free chlamydiae is unknown, although the failure to reproduce the precise ionic and nutritional environment of the intracellular vacuole within which chlamydiae reside is undoubtedly a contributory factor. Signals which trigger the intracellular interconversion of the morphologically distinct life-cycle forms are also unknown, and very few differences between EBs and RBs, other than functional and morphological differences, have been well characterized. Several outer membrane proteins present in EBs are known to be synthesized only late in the developmental cycle (7, 10, 12, 20, 24, 30), and the RNA-to-DNA ratio is higher in RBs than in EBs (18, 29). Also, outer membrane proteins in EBs are disulfide cross-linked, while those of RBs are not (8, 10, 12, 20, 21), and the lipopolysaccharide appears to possess an additional epitope and to be more closely associated with the major outer membrane protein (MOMP) (5, 14, 23) in EBs relative to RBs (4).

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This study was undertaken to characterize protein synthesis by *C. psittaci* during the critical period of reorganization of EBs to RBs. Early protein synthesis has not been characterized previously, primarily because of the low synthetic activity of early developmental forms, the cytotoxic activity associated with high multiplicities of infection (19), and the dilution of radioactive tracer amino acids by unlabeled host cell amino acid pools. We compensated for these problems by examining the incorporation of [^{35}S]methionine of high specific activity into protein by chlamydiae isolated from a large number of host cells. Using this host-free system, we detected the synthesis of temporally regulated proteins as early as 15 min postinfection.

MATERIALS AND METHODS

Growth of *C. psittaci*. EBs of *C. psittaci* 6BC were harvested by Nonidet P-40 lysis of 48-h-infected 929 L cells, as described by Hatch et al. (14).

Host-free and in vivo protein synthesis. L cells in suspension culture were infected at zero time with 25 times the dose required to infect 50% of the cells; unabsorbed EBs were not removed during incubation with host cells at 37°C. At various times postinfection, duplicate samples of 2×10^7 L cells were removed and pelleted at $1,000 \times g$ for 10 min. For in vivo protein synthesis studies, the cells were suspended in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.)–10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.2) containing 100 μg of cycloheximide per ml. Infected cells were incubated for 1 to 2.5 h at 37°C with 50 μCi of [^{35}S]methionine (1,115 Ci/mmol) in a final volume of 200 μl. The reaction was stopped by the addition of 1 ml of cold (4°C) Dulbecco phosphate-buffered saline (pH 7.4), and chlamydiae were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by lysis of the infected cells in a Mettler ultrasonic bath (14), pelleting the chlamydiae at $10,000 \times g$ for 10 min, and suspension of the pellet in 100 μl of gel solubilization mixture (15). In some in vivo experiments, infected cells were lysed by the addition of Nonidet P-40 to 0.5%, and host cell nuclei were removed by centrifugation at $1,000 \times g$ for 5 min before the chlamydiae were pelleted at $10,000 \times g$ for 20 min. For studies of the host-free chlamydiae, the infected cells were suspended in phosphate-

buffered saline and lysed in a sonic bath. The chlamydiae were concentrated by centrifugation for 20 min at 10,000 × *g*, suspended in TK buffer (50 mM Tris hydrochloride, 100 mM KCl [pH 7.0]), and incubated at 37°C in 200 μl of host-free protein synthesis reaction mixture, as previously described (11). The reaction mixture consisted of TK buffer containing 5 mM $MgCl_2$, 7.8 mM creatine phosphate, 1.5 mg of phosphocreatine kinase per ml, 1 mM ATP, 19 unlabeled amino acids (each present at a final concentration of 50 mM), 10 or 50 μCi of [^{35}S]methionine, as noted, and 100 μg of cycloheximide per ml. The reaction was stopped after 1 to 2 h of incubation by the addition of 1 ml of cold phosphate-buffered saline, and chlamydiae were prepared for electrophoresis by pelleting at 10,000 × *g* for 20 min and suspending the pellet in 100 μl of gel solubilization mixture. In the RNA inhibition experiment, chlamydiae were purified by Renografin density centrifugation prior to incubation with the reaction mixture (14).

SDS-PAGE. Preparations were heated to 100°C for 3 min in the presence of 5% (vol/vol) 2-mercaptoethanol, and samples (5 to 20 μl) were subjected to electrophoresis in one dimension on a 7.5 to 15% gradient polyacrylamide gel with the Tris-glycine buffer system of Laemmli (15). Coomassie brilliant blue R-stained gels were dried, and radiolabeled bands were visualized by autoradiography with Kodak X-Omat AR film. In one experiment, protein bands were excised from the dried gel, rehydrated in 100 μl of water, and incubated at 45°C for 4 h in 10 ml of Biofluor containing 10% Protosol (Du Pont, NEN Research Products, Boston, Mass.), and the radioactivity incorporated into the bands was quantitated by scintillation spectrometry.

Materials. [^{35}S]methionine was purchased from Du Pont, NEN. Rifampin, amino acids, and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. Actinomycin D was obtained from Calbiochem-Behring, La Jolla, Calif.

RESULTS

Host-free and in vivo protein synthesis by *C. psittaci* during the developmental cycle. The incorporation of [^{35}S]methionine into protein by *C. psittaci* 6BC within host L cells (in vivo) and in partially purified lysates of infected cells (host-free) at 1 to 2, 5 to 6, 11 to 12, 18 to 19, and 28 to 29 h postinfection is shown in Fig. 1A. The host-free pattern was similar but not identical to the in vivo pattern at each time postinfection. For example, a number of bands which were well labeled relative to the MOMP band in the host-free system at 28 h were also present in the in vivo-labeled sample, but the intensity of these bands relative to the MOMP band was low. Another significant difference between the two systems was the better overall incorporation of radioactive label in the host-free system at early times postinfection. The difference in efficiency of labeling probably was due to the direct incorporation of high-specific-activity [^{35}S]methionine by host-free chlamydiae, compared with the incorporation of label diluted with unlabeled host cell methionine pools by intracellular chlamydiae.

A number of chlamydial proteins, including those marked a, c [a doublet], e, f, h, and i, could be detected by a 1-h pulse of methionine added at 1 h postinfection to the host-free system (Fig. 1A). Most of these bands could be detected in the in vivo sample at 1 h, although the total incorporation of label was lower in vivo and the relative intensities of the bands were different in the two systems. A closer correlation between the host-free and in vivo patterns was evident in the 5- and 11-h samples, when the total

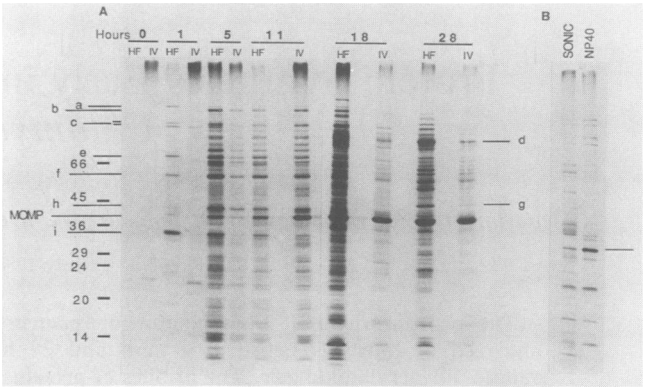

FIG. 1. (A) In vivo (IV) and host-free (HF) synthesis of proteins by *C. psittaci* during the developmental cycle. Infected cells (IV) or chlamydiae isolated from sonic lysates of infected cells (HF) were incubated for 1 h in the presence of 50 μCi of [^{35}S]methionine at the hour postinfection indicated, and the incorporation of radioactivity into chlamydial proteins was analyzed by autoradiography of an SDS-polyacrylamide gel. Samples labeled at 0 (uninfected cells), 1, 5, and 11 h were exposed to film for 14 days; samples labeled at 18 and 28 h postinfection were exposed for 2 days. Cycloheximide-resistant incorporation of label into a number of host cell peptides (with molecular masses below 24 kilodaltons) can be detected in the early IV time samples. The numbers on the left refer to molecular mass standards in kilodaltons; the letters note protein bands referred to in the text. (B) Differential recovery of labeled proteins depending upon the method of lysis of host cells. Infected L cells were labeled with 50 μCi of [^{35}S]methionine between 1 and 2.5 h postinfection, and chlamydiae were isolated after sonic or Nonidet P-40 lysis of host cells. Chlamydial protein synthesis was analyzed by SDS-PAGE and autoradiography.

amount of label incorporated in the two systems was similar. One protein (b) was barely detected in the 1-h host-free sample but was prominent in the host-free and in vivo samples at 5 and 11 h. At later times during the cycle, when some RBs were still undergoing cell division while others were reorganizing to the EB form (18 and 28 h), a number of chlamydial proteins were synthesized (including those marked d and g) which were not evident at earlier times. Because of the large number of proteins synthesized by 18 h, it is difficult to determine whether the early gene products continued to be synthesized at later times. The temporal synthesis of chlamydial proteins is more evident in the results shown in Fig. 2. The pair of early proteins (marked c) appeared to be no longer synthesized at 9 h postinfection. Other proteins (such as those marked a and i) may have been synthesized at 9 h but were produced in distinctly greater amounts during the first few hours postinfection. A protein with the same relative molecular weight as that of the MOMP appeared to be synthesized as early a 1 h postinfection and continued to be synthesized in increasing amounts relative to other proteins through 24 to 28 h (Fig. 1A and 2).

The host-free labeling experiments reported in Fig. 1 and 2 were carried out with the 10,000-×-*g* pellet of sonic lysates of infected cells suspended in the host-free reaction system. However, a similar pattern of protein synthesis (albeit at lower levels) was noted when host-free chlamydiae were purified by Renografin density centrifugation (data not shown). This observation suggests that the temporal pattern of protein synthesis by host-free chlamydiae was an inherent property of the chlamydiae at the time of their isolation rather than a reflection of host cell regulatory factors present in the reaction mixture.

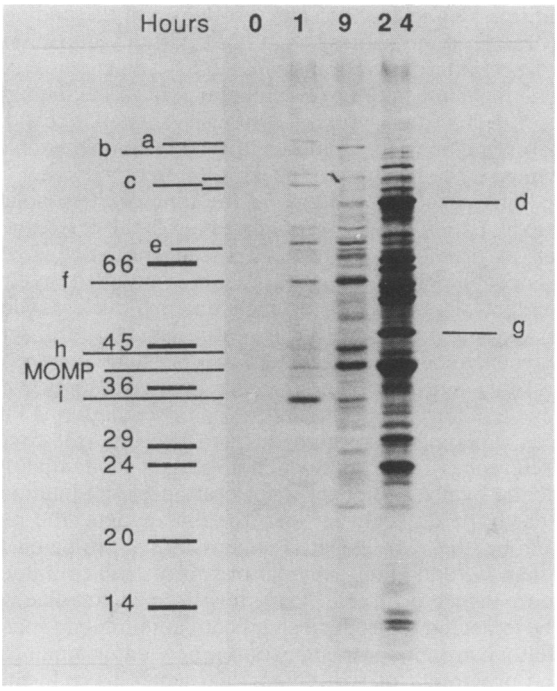

FIG. 2. Autoradiography of SDS-PAGE profiles of proteins synthesized by early- and midcycle host-free chlamydiae. Host-free chlamydiae, isolated at the hour postinfection indicated, were pulsed for 2 h with 50 μCi of [^{35}S]methionine. Letters indicate reference proteins mentioned in the text. The numbers on the left are molecular mass standards in kilodaltons.

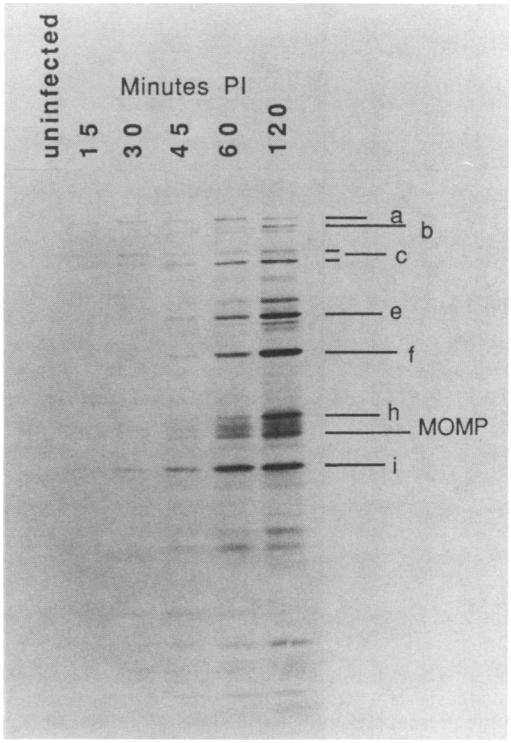

FIG. 3. Early protein synthesis by host-free chlamydiae. Host-free chlamydiae, isolated at the minutes postinfection (PI) indicated, were incubated for 1 h in the presence of 50 μCi of [^{35}S]methionine. Letters indicate reference proteins mentioned in the text.

The recovery of some proteins labeled in vivo at early times postinfection was dependent on the method of lysis of host cells. For example, one protein with a relative molecular weight of about 35,000 (marked i) was clearly the most prominently labeled protein in samples prepared by lysis with the nonionic detergent Nonidet P-40 but was no better labeled than several other proteins in samples prepared by sonic lysis (Fig. 1B). Nonidet P-40 is known to lyse osmotically fragile forms of chlamydiae which develop from stable EBs at some undetermined time shortly after infection. The results shown in Fig. 1B suggest that the early, osmotically stable forms may preferentially synthesize the 35-kilodalton protein, while the later, more fragile forms may synthesize this and other proteins equally. The 35-kilodalton protein could not be distinguished from the background in Coomassie brilliant blue-stained gels of either 1-h-postinfection chlamydiae or EBs (data not shown).

The relatively efficient labeling afforded by the host-free system permitted the detection of protein synthesis in chlamydiae isolated as early as 15 min postinfection (Fig. 3). Total incorporation increased at each succeeding time of isolation, while the pattern of major proteins synthesized appeared to remain constant. No protein synthesis could be detected in host-free EBs, even when up to 10^9 infectious units were incubated in the presence of 500 μCi of [^{35}S] methionine (data not shown).

Transcription by host-free *C. psittaci*. Host-free protein synthesis may be the result of the translation of transcripts synthesized either in vivo or in the host-free system. To distinguish between these possibilities, the effects of rifampin, an inhibitor of transcription initiation, and actinomycin D, a general inhibitor of RNA synthesis, on protein synthesis by host-free chlamydiae isolated at 2 and 22 h postinfection were determined (Fig. 4). Actinomycin D almost completely inhibited incorporation of [^{35}S]methionine into most proteins at both times and reduced incorporation into the MOMP by 69% at 22 to 24 h and by 82% at 2 to 4 h, indicating that synthesis of proteins was coupled to host-free transcription. The higher level of incorporation of [^{35}S]methionine into the MOMP in the presence of actinomycin D relative to most other proteins suggests that some MOMP synthesis may reflect translation of messages synthesized in vivo. This was particularly surprising in the case of the 22- to 24-h samples because the host-free chlamydiae were purified, a process that includes a 30-min incubation with DNase at 37°C and several centrifugation steps (4°C) that take approximately 1.5 h to complete. The inhibitory effect of rifampin was less complete, particularly at 2 to 4 h, but nonetheless suggested that host-free protein synthesis was also partially dependent on the initiation of transcripts by host-free chlamydiae.

DISCUSSION

The synthesis of *C. psittaci* proteins as early as 15 min postinfection is reported here for the first time. The profile of proteins synthesized through 2 h postinfection remained relatively constant, with increased intensity of labeling of all major bands at each succeeding time examined. The increase in incorporation of label over time probably was a reflection of continued entry of chlamydiae into host cells, as well as of continued activation of gene expression of intracellular chlamydiae. The mechanism by which early chlamydial protein synthesis is activated is unknown. Although protein synthesis could not be detected in extracellular EBs, it is possible that initial translation is directed from stable transcripts present in the infecting EB forms.

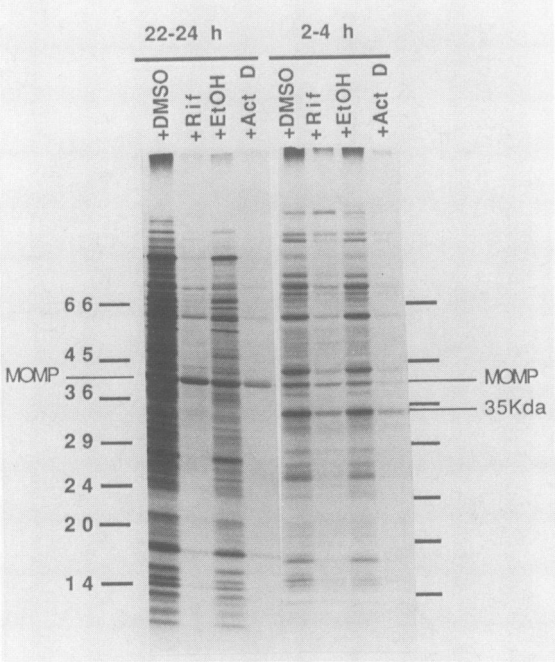

FIG. 4. Effects of rifampin and actinomycin D on host-free protein synthesis. Host-free samples were preincubated at 37°C for 5 min in the presence of inhibitors (50 μg/ml) or the solvents (final concentration of 5%) in which the inhibitors were dissolved and then pulsed for 2 h in the continued presence of inhibitors with 10 μCi of [^{35}S]methionine plus unlabeled carrier methionine. The final concentrations of methionine (0.6 μM at 2 h and 4 μM at 22 h) were sufficient to allow linear incorporation of radioactivity over the 2-h incubation period. Chlamydiae isolated at 22 h were purified by Renografin density centrifugation. Rifampin (Rif) was dissolved in dimethyl sulfoxide (DMSO), and actinomycin D (Act D) was dissolved in ethanol (EtOH). Numbers refer to molecular mass standards in kilodaltons.

Some of the proteins synthesized during the first few hours postinfection appeared to have been produced only during the reorganization of EBs to RBs, while others appeared to have been synthesized at least through the late logarithmic phase of RB division (28 h) but at considerably lower rates relative to other proteins. One protein with a relative molecular weight of 35,000 is of particular interest; it was the most actively synthesized protein detected in host-free and intracellular chlamydiae (when Nonidet P-40 lysates of infected cells were analyzed by SDS-PAGE) during the first several hours of infection. Only one early event in the developmental cycle has been reported previously: Hatch et al. (12) found that the disulfide cross-linked MOMP of *C. psittaci* 6BC is reduced to monomers within 1 h postinfection by a mechanism which requires de novo chlamydial protein synthesis. Whether the 35-kilodalton protein or one of the other early proteins detected in the present investigation functions in this reductive process is unknown. Whatever their exact function may be, some of these early proteins almost certainly play an important role in the transformation of metabolically inactive EBs into RBs that are capable of multiplication.

While some of the proteins produced during the early phases of reorganization of EBs to RBs may also be synthesized by dividing RBs, the overall pattern of early synthesis is clearly different from that of mid-cycle synthesis and appears to be subject to an orderly system of developmental regulation. The observation that host-free and intracellular organisms synthesize the same proteins and the finding that host-free protein synthesis is at least partially dependent on host-free initiation of transcripts offer some clues to the general mechanism of developmental control in chlamydiae. Since the host-free protein synthesis system likely bears little resemblance to an intracellular environment, chlamydiae must be committed to the expression of a distinct set of temporally regulated proteins at the time of their isolation. One explanation for such a commitment is the existence of a series of promoters which are specifically recognized in sequence by RNA polymerase either modified by cycle-dependent sigma factors or mediated by cycle-dependent DNA-binding proteins. That is, early genes are transcribed only when an early sigma factor (or other activating protein) is present, and midcycle genes are expressed only when a midcycle factor is present. This type of regulation is known to exist in some bacteriophages and bacteria (for reviews, see references 16, 17, 22) which possess a sequential developmental cycle. Although incorporation of [^{35}S]methionine can easily be detected in host-free chlamydiae, the rate of protein synthesis by isolated chlamydiae is estimated to be less than 1% that of intracellular organisms and continues for no more than 2 to 4 h (11). Thus, host-free chlamydiae would not be expected to produce significant amounts of functional regulatory proteins in response to a new environment.

The promoters of two chlamydial genes have been analyzed to date. Stephens et al. (27) found that the MOMP gene (*omp*1) of *C. trachomatis* serovars L2, B, and C possesses two tandem promoters, one of which is active throughout the cycle and the other of which promotes synthesis of a full-length transcript only after 12 h postinfection. The authors speculated that the differential transcription may be mediated by a sigma factor or another auxiliary protein which permits chlamydial RNA polymerase after 12 h postinfection either to recognize the second promoter or to read through a putative terminator located between the tandem promoters. Stephens et al. (27) also suggested that transcription from two promoters may explain the high level of MOMP synthesis in logarithmic-phase RBs. Our finding that mRNA which encodes MOMP may be unusually stable offers an additional mechanism for high levels of MOMP production. Engel and Ganem (6) also reported finding a pair of tandem promoters in an rRNA cistron of the mouse pneumonitis strain of *C. trachomatis*. Although one was more active than the other, neither appeared to be subject to developmental control. However, the earliest transcripts detected by these investigators was at 9 h postinfection, a time when chlamydiae may have already entered the logarithmic phase of growth. It is possible, therefore, that the rRNA cistron is temporally regulated in the same manner as *omp*1; one of the promoters may be active throughout the cycle, and the other may be active only during RB cell division.

Our laboratory currently is using host-free protein synthesis and RNA radiolabeled in a host-free system to identify early genes within a chlamydial genomic library. It is hoped that analysis of regulatory sequences of these genes will provide further information on the mechanism of sequential developmental regulation of the chlamydial developmental cycle.

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