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The Chlamydial EUO Gene Encodes a Histone H1-Specific Protease

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Chlamydia trachomatis **is an obligate intracellular pathogen, long recognized as an agent of blinding eye disease and more recently as a common sexually transmitted infection. Recently, two eukaryotic histone H1-like proteins, designated Hc1 and Hc2, have been identified in *Chlamydia*. Expression of Hc1 in recombinant *Escherichia coli* produces chromatin condensation similar to nucleoid condensation observed late in the parasite's own life cycle. In contrast, chromatin decondensation, observed during the early life cycle, accompanies down-regulation and nondetection of Hc1 and Hc2 among internalized organisms. We reasoned that the early upstream open reading frame (EUO) gene product might play a role in Hc1 degradation and nucleoid decondensation since it is expressed very early in the chlamydial life cycle. To explore this possibility, we fused the EUO coding region between amino acids 4 and 177 from *C. trachomatis* serovar L_2 with glutathione *S*-transferase (GST) and examined the effects of fusion protein on Hc1 in vitro. The purified fusion protein was able to digest Hc1 completely within 1 h at 37°C. However, GST alone exhibited no Hc1-specific proteolytic activity. The chlamydial EUO-GST gene product also cleaves very-lysine-rich calf thymus histone H1 and chicken erythrocyte histone H5 but displays no measurable activity towards core histones H2A, H2B, H3, and H4 or chlamydial RNA polymerase α-subunit. This proteolytic activity appears sensitive to the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) and aspartic protease inhibitor pepstatin but resistant to high temperature and other broad-spectrum protease inhibitors. The proteolytic activity specified by the EUO-GST fusion product selectively digested the C-terminal portion of chlamydial Hc1, the domain involved in DNA binding, while leaving the N terminus intact. At a molar equivalent ratio of 1:1 between Hc1 and DNA, the EUO gene product cleaves Hc1 complexed to DNA and this cleavage appears sufficient to initiate dissociation of DNA-Hc1 complexes. However, at a higher molar equivalent ratio of Hc1/DNA (10:1), there is partial protection conferred upon Hc1 to an extent that prevents dissociation of DNA-Hc1 complexes.**

Chlamydia trachomatis is an obligate intracellular bacterium with a complex life cycle that involves two developmental forms, the extracellular, small (0.2- to 0.3-μm) elementary bodies (EBs) and the intracellular, large (1-μm) fragile reticulate bodies (RBs) (10, 14). The EB is metabolically inactive and resistant to osmotic lysis due to its rigid outer membrane. Proliferation of the organism is initiated by EB uptake and enlargement in the host cell, circumventing phagosomal fusion to form noninfectious, metabolically active RBs which divide within the cytoplasmic inclusions by binary fission (19). The life cycle is completed with the reorganization of RBs into EBs, which subsequently leave the disrupted host cell ready to infect new cells (29). However, little is known about the signals that trigger these events during the chlamydial life cycle.

Among the most notable features of the chlamydial EB is the presence of a condensed, discrete, and electron-dense nucleoid which is unique among eubacteria (8). In contrast, the RB developmental form contains a more relaxed chromosome with fine fibrils diffused throughout the cell. Recently, several groups have cloned and sequenced genes for chlamydial histone H1-like proteins whose expression is closely associated with dedifferentiation of RBs into EBs (5, 13, 22, 28). These histone H1-like protein homologs appear to play a major role in controlling the chlamydial developmental cycle through their ability to modify DNA structure (2, 3, 7, 20). The chlamydial histone H1-like proteins are two lysine-rich proteins of 18 and 32 kDa similar to eukaryotic histone H1. The common 18-kDa protein, designated Hc1, is conserved among all *C. trachomatis* serovars, whereas the second protein, designated Hc2, exhibits variable molecular sizes of 25 to 32 kDa depending upon the serovar (13, 16). Both proteins are expressed late in the life cycle at a time when chlamydial DNA is undergoing compaction into its dense nucleoid form, accompanied by down-regulation of transcription and metabolic processes. Several groups including ours have shown that expression of chlamydial histone H1-like protein in *Escherichia coli* is sufficient to induce nucleoid compaction, an observation which lends support to its role in DNA condensation (2, 7, 23). These results are supported by in vitro studies showing that purified Hc1 forms complexes with double-stranded DNA, leading to its coiling, shrinking, aggregation, and formation of condensed spherical bodies (7, 17, 20).

Within a few hours of entering the host cell, EBs lose their prominent electron-dense DNA core, Hc1- and Hc2-specific proteins become nondetectable (12, 16), the cell envelope loses its rigidity, and the cell increases in size from 0.3 to 1.0 μm and begins to transcribe early-stage-specific genes. It is clear, however, that the transcriptional initiation and cellular growth of *Chlamydia* is closely associated with DNA decondensation and down-regulation of Hc1 and Hc2. In this paper, we provide unequivocal evidence that the EUO gene product is an Hc1-specific protease that specifically cleaves the carboxyl-terminal portion of Hc1, which in turn initiates dissociation of DNA-Hc1 complexes.

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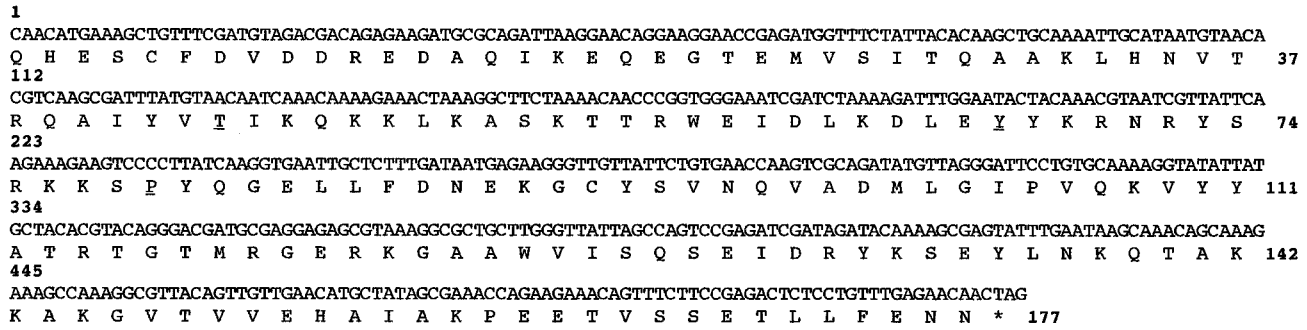

FIG. 1. Complete nucleotide sequence of a 543-bp fragment encoding the *C. trachomatis* EUO protein. The deduced amino acid sequence is given in the single-letter code below the nucleotide sequence. The numbers above the sequence refer to nucleotide positions, while the numbers on the right refer to amino acids. The amino acids that differ from the *C. psittaci* sequence are underlined.

MATERIALS AND METHODS

C. trachomatis L_2/434/Bu was grown in HeLa cells, and EBs were purified as described previously (30). *E. coli* BL21 (25) was used as a host for the expression of recombinant proteins under the control of T7 RNA polymerase (26), while *E. coli* DH5αF′ served as a host for plasmid pGEX-3X and its recombinant derivatives (24). Plasmid DNA was isolated by an alkaline lysis method with Qiagen (Chatsworth, Calif.) purification kits. Restriction endonuclease-generated DNA fragments were extracted from agarose gels with a Bio 101 (La Jolla, Calif.) GENECLEAN kit. All proteinase inhibitors used were purchased from Boehringer Mannheim. Core histones and histone H1 were extracted from freshly frozen calf thymus glands (15). Histone H5 was extracted from chicken erythrocyte nuclei with 5% perchloric acid and further purified by high-performance liquid chromatography (HPLC) on a Vydac C_4 reverse-phase column with a 0 to 50% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing H5 were lyophilized.

Cloning and sequence analysis of the EUO gene. To explore the role of the EUO protein, we cloned the EUO gene from *C. trachomatis* serovar L_2 into plasmid pGEX-3X. The resulting plasmid pGST-EUO produced a fusion protein comprising glutathione *S*-transferase (GST) and the EUO coding sequence between amino acids 4 and 177. Briefly, PCR amplification of the EUO coding region was carried out following construction of two complementary oligonucleotide primers with a forward primer (5′-CGTATGGAATGGATCCAACATG-3′) comprising the start codon and a *Bam*HI restriction site and a reverse primer (5′-GAAGAAGACGAATTCTGAACTAG-3′) comprising the stop codon and *Eco*RI site based on a published sequence (31). The amplified gene sequence was subsequently digested with restriction endonucleases *Bam*HI and *Eco*RI and ligated into plasmid pGEX-3X, which had been cleaved with the same enzymes. Ligated DNA was transformed into *E. coli* DH5αF′, and ampicillin-resistant clones were characterized by restriction endonuclease digestion. One positive clone, designated pGST-EUO, containing the EUO gene fused to GST, was expressed in *E. coli* after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). A fusion product of approximately 48 kDa was identified among cells harboring pGST-EUO as compared to the GST product of 28-kDa among controls harboring pGEX-3X alone. The authenticity of this clone was further confirmed by DNA sequencing with the dideoxy-chain termination method.

Purification of the EUO-GST fusion protein. The EUO-GST fusion protein was purified from insoluble inclusions by solubilization in 4 M guanidine followed by affinity purification over glutathione-Sepharose. Essentially, cells harboring plasmid pGST-EUO were grown at 37°C to a cell optical density at 600 nm of approximately 1.0 and induced with 0.1 mM IPTG for 2 h with vigorous shaking. Induced cells were harvested and suspended in HEMGN buffer (100 mM KCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol) containing 500 μg of lysozyme per ml. Cells were incubated on ice for 30 min and sonicated, and cell lysates were spun at 27,000 × *g* for 15 min. The cell pellet was suspended in HEMGN buffer containing 4 M guanidine, incubated on ice for 30 min, and centrifuged at 87,000 × *g* for 30 min. The guanidine-soluble fraction was passed through Bio-Gel P6 desalting columns (Bio-Rad Laboratories, Hercules, Calif.), and the eluate comprising the soluble fusion protein was incubated with glutathione-Sepharose. The fusion protein-bound glutathione-Sepharose beads were washed with phosphate-buffered saline (PBS) three times to remove unbound proteins. Washed beads were incubated with 10 mM reduced glutathione for 10 min, and dissociated protein was recovered by centrifugation. Approximately 5 μg of affinity-purified fusion protein was recovered from 100 ml of culture. The purity and recovery of the fusion protein were tested by resolving 10 μg of whole-cell extracts from uninduced and induced cells and 1 μg of affinity-purified proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fusion protein thus purified was used in further studies.

Expression of recombinant Hc1. *E. coli* BL21 (DE3) harboring recombinant plasmid pH1W, which encodes the first six amino acids of plasmid pT7-7 polylinker along with amino acids 2 to 125 of Hc1, pH1N, which encodes amino acids 2 to 65, and pH1C, encoding amino acids 68 to 125, were grown in Luria broth medium containing ampicillin. All three gene products were overexpressed in *E. coli* following induction with 1 mM IPTG as described earlier (23). We propose to use the term Hc1 instead of H1W throughout this paper to avoid confusion. Spheroplasts isolated from *E. coli* were lysed in a French pressure cell at 10,000 lb/in^2, and the cellular extracts were suspended in a final concentration of 5% perchloric acid followed by centrifugation at 3,000 × *g*. Individual supernatants containing perchloric acid-soluble proteins were neutralized immediately with 1 M triethanolamine and precipitated overnight with 10 volumes of chilled ethanol. Each pellet was washed twice with acetone and dessicated in the cold. The dry pellet was subsequently dissolved in 5% acetonitrile and loaded on a C_4 reverse-phase HPLC (RPHPLC) column, and the pure Hc1, H1N, and H1C were fractionated over a 0 to 60% acetonitrile gradient. The column was run at a speed of 1 ml/min, and pure Hc1 eluted between fractions 39 and 43. The purity of individual peptides was checked by SDS-PAGE and Western blot (immunoblot) analysis with anti-amino-terminal (H1N) and anti-carboxyl-terminal (H1C) antibodies. The protein concentration was determined colorimetrically with Bio-Rad protein assay kits.

EUO activity. Histone H1-specific proteolytic activity associated with the EUO-GST fusion protein was monitored by incubating purified calf thymus histone H1 and chicken erythrocyte histone H5 and/or chlamydial histone H1-like protein (Hc1) for 60 min at 37°C in PBS. The digested Hc1 and H1 were subsequently resolved by SDS-PAGE. The role of a proteinase inhibitor(s) was examined by preincubating the EUO-GST fusion protein with individual inhibitors for 15 min before the addition of substrate Hc1 or H1. The thermostability of the fusion protein was examined by heating the enzyme at 100°C for 3 min. The ability of the EUO-GST fusion protein to cleave DNA-Hc1 complexes was examined by either (i) digesting preformed DNA-Hc1 complexes formed by incubating 100 ng of DNA with either equimolar amounts (10 ng) or a 10-fold molar excess (100 ng) of purified Hc1 for 30 min at room temperature followed by incubations with fusion protein or (ii) predigesting 10 or 100 ng of Hc1 with fusion protein prior to its interaction with 100 ng of DNA. A 520-bp *Bam*HI-*Xho*I fragment of chlamydial plasmid comprising a high-affinity Hc1 binding site (17) was used for DNA-Hc1 interactions. Complexes were subsequently electrophoresed on a 1% agarose gel to examine the shift in DNA mobility or, alternatively, resolved on a SDS–14% PAGE gel to confirm Hc1 cleavage.

To identify the sites on Hc1 that are sensitive to cleavage by the EUO-GST fusion protein, we immobilized the affinity-purified fusion protein on nitrocellulose discs for 1 h at room temperature. The discs were subsequently washed with PBS containing 0.2% Tween 20. Enzyme-immobilized discs were then immersed in the reaction solution containing Hc1 and incubated for different time intervals until complete digestion was obtained as monitored by SDS-PAGE analysis. The digested sample was then loaded onto a Vydac C_{18} RPHPLC column and fractionated over a 0 to 60% acetonitrile gradient. The column was run at a speed of 1 ml/min, and a major cleavable peptide that eluted between fractions 40 and 42 was recovered, lyophilized, and amino acid analyzed. Amino acid analysis of this peptide was performed at the Protein Structure Laboratory, University of California, Davis. Amino acid composition was calculated by adding the total number of nanomoles of the individual amino acids (excluding NH_3 and internal standards) and dividing the amount of each amino acid by the total. Additional characterization of this purified peptide was carried out by both immunoblotting and Southwestern blotting techniques as described previously (23).

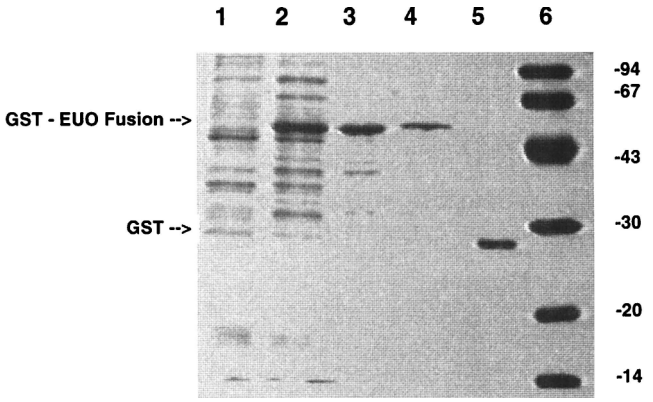

FIG. 2. Coomassie blue-stained SDS-PAGE analysis of purified recombinant EUO-GST protein. Lanes: 1 to 5, protein profiles of uninduced *E. coli* harboring pGST-EUO (lane 1), IPTG-induced *E. coli* harboring pGST-EUO (lane 2), guanidine-soluble fraction following Bio-Gel P6 extraction (lane 3), affinity-purified EUO-GST fusion product (lane 4), and affinity-purified GST alone (lane 5). Approximately 10 μg of total cellular protein (lanes 1 and 2), 2 μg of Bio-Gel-purified protein (lane 3), or 1 μg of affinity-purified protein (lanes 4 and 5) was loaded onto each lane. The mobilities of the 48-kDa EUO-GST fusion peptide and the 28-kDa GST are indicated. Lane 6 represents the mobility of low-molecular-size markers in kilodaltons.

RESULTS

Cloning, sequencing, and expression of the EUO gene. We amplified a 549-bp fragment encoding the entire open reading frame except the first four amino acids of the *C. trachomatis* serovar L_2 EUO gene into plasmid pGEX-3X. This strategy produced a fusion gene product between *C. trachomatis* EUO and GST of plasmid pGEX-3X. Subsequently, we sequenced the entire 550 bp of this cloned gene in both orientations to determine the extent of interspecies homology between *Chlamydia psittaci* and *C. trachomatis* EUO genes. Figure 1 shows the complete nucleotide sequence of the serovar L_2 EUO gene along with its predicted amino acid sequence. The deduced amino acid sequence comprises 177 amino acids with a calculated molecular size of 20,409 Da. However, when the first four amino acids that were excluded in our cloning strategy are included, the EUO protein is 181 amino acids long and has a molecular size of 20,885 Da. Comparison of gene sequences among *C. trachomatis* and *C. psittaci* revealed a total of eight nucleotide alterations, leading to a predicted product that is one amino acid shorter for *C. trachomatis*. Three other alterations lead to a switch in amino acids 49, 72, and 84 from Ala to Thr, Asp to Tyr, and Leu to Pro, respectively, whereas two other nucleotide alterations have no effect on the amino acid makeup. Overall, the *C. trachomatis* EUO protein is basic in nature, with an estimated pI of 8.77 and acidic/basic ratio of 9:11.

Purification of EUO-GST fusion protein. The recombinant pGST-EUO containing the EUO gene fused to GST gene was expressed in *E. coli* following induction with IPTG. A fusion product of approximately 48 kDa was identified among cells harboring pGST-EUO as compared to the GST product of 28 kDa among controls harboring pGEX-3X alone. The size of this fusion protein agreed closely with its expected molecular weight. The 48-kDa fusion product was subsequently recovered from insoluble inclusions following solubilization in 4 M guanidine and affinity purification over glutathione-Sepharose (Fig. 2). The few minor low-molecular-weight bands present in some preparations of the affinity-purified protein simply represent degradation products generated during the purification process. We avoided the use of protease inhibitors during the purification process because they may inhibit the enzyme activity associated with EUO. Additionally, the susceptibility of the EUO-GST fusion protein to factor Xa cleavage was investigated. While the fusion product was susceptible to factor Xa, factor Xa itself cleaves Hc1, H1, and H5 (data not shown). This observation explains why we chose to use the EUO-GST fusion product rather than purified EUO by itself.

Histone-specific proteolytic activity. We studied the role of the EUO gene product by analyzing its functional activity. The purified 48-kDa fusion protein was able to digest Hc1 in a time-dependent fashion. While complete digestion of Hc1 protein was achieved within 1 h at 37°C in PBS (Fig. 3, lanes 2 to 5), no proteolytic activity was observed among GST-treated samples (Fig. 3, lanes 6 to 8). To address whether our failure to digest Hc1 with GST was due to some aberrant stabilizing effect mediated through protein-protein interactions, we incubated a mixture of GST and Hc1 with the EUO-GST fusion protein. The fusion protein selectively digested Hc1 (Fig. 3, lane 9). The requirement for substrate specificity of the EUO gene product was assessed by incubating the EUO-GST fusion protein with the following: calf thymus histone H1, calf thymus core histones (comprising H2A, H2B, H3, and H4), chicken erythrocyte histone H5, a mixture of low-molecular-weight markers (comprising phosphorylase β, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin) and chlamydial RNA polymerase α-subunit (11) (Fig. 4). The enzyme cleaved calf thymus histone H1 (Fig. 4, lane 8) and chicken erythrocyte histone H5 (Fig. 4, lane 6) completely within 1 h under the experimental conditions described above, while at the same time no measurable activity was observed towards calf thymus core histones (Fig. 4, lane 10) and chlamydial RNA polymerase α-subunit (Fig. 4, lane 4). EUO also failed to cleave a mixture of low-molecular-weight markers (Fig. 4, lane 2), supporting a proposed role for the EUO gene product as a histone H1-specific protease.

To define the site(s) of fragmentation on Hc1, we created proteolytic probes by immobilizing the EUO-GST fusion protein on nitrocellulose discs. The nitrocellulose discs were sub-

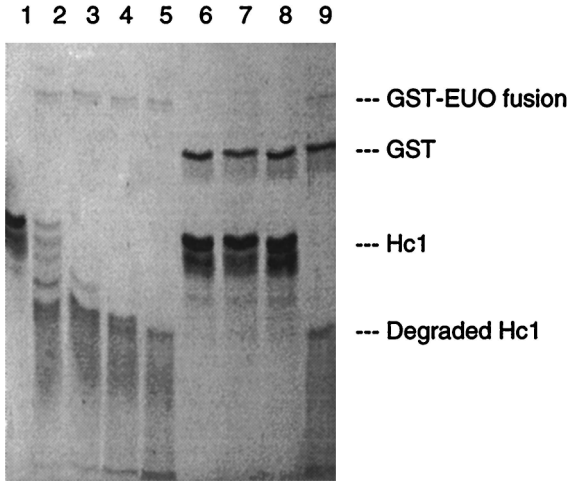

FIG. 3. SDS-PAGE analysis of purified Hc1 cleaved with the EUO-GST fusion product. A 1.5-μg amount of Hc1 was incubated with 0.10 μg of affinity-purified EUO-GST fusion protein (lanes 2 to 5) or 0.25 μg of GST (lanes 6 to 8) for 15 min (lane 2), 30 min (lanes 3 and 6), 45 min (lanes 4 and 7), or 60 min (lanes 5 and 8). The codigestion of affinity-purified Hc1 and GST with EUO-GST (lane 9) and the mobility of untreated Hc1 (lane 1) are also shown. Samples were resolved on 15% gels and stained with Coomassie blue. It is important to note the complete degradation of Hc1 in lanes 5 and 9.

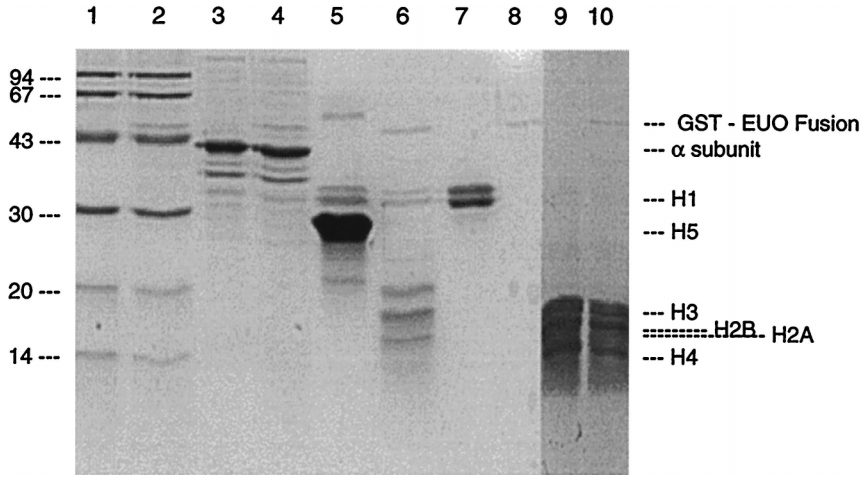

FIG. 4. Proteolytic cleavage of low-molecular-size markers (lanes 1 and 2), affinity-purified chlamydial RNA polymerase α-subunit (lanes 3 and 4), chicken erythrocyte histone H5 (lanes 5 and 6), calf thymus histone H1 (lanes 7 and 8), and calf thymus core histones (lanes 9 and 10). Proteolytic cleavage in the presence (lanes 2, 4, 6, 8, and 10) and absence (lanes 1, 3, 5, 7, and 9) of the EUO-GST fusion protein is shown. It is important to note the complete degradation of histone H5 and H1 in lanes 6 and 8 compared to that of corresponding controls. The samples were resolved on an SDS–15% PAGE and stained with Coomassie blue. The numbers on the left indicate the sizes of the markers in kilodaltons.

sequently incubated with Hc1 in solution, and aliquots of digested Hc1 were removed at various time points. Figure 5A shows the Coomassie blue-stained SDS-PAGE pattern of Hc1 digested with EUO. While the majority of Hc1 was cleaved within 2 h by this experimental protocol, complete digestion was observed by 8 h as revealed by SDS-PAGE analysis. The digested samples were examined by immunoblotting with anti-H1N antibodies (Fig. 5B). A strong immunoreactive band migrating around 10 kDa corresponding to a major Coomassie blue-stained band was identified among all aliquoted samples. As expected, two light immunoreactive bands with intermediate mobility between intact Hc1 and the digested fragment were also visualized in lanes 2 and 3 and probably represent incomplete digests. Further characterization of digested Hc1 (8 h of incubation with immobilized EUO fusion protein) was achieved through fractionation on RPHPLC, and the major eluted fraction was analyzed for amino acid composition. The actual amino acid composition of the eluted peptide was in agreement with the amino-terminal proteolytic fragment of Hc1 comprising amino acids 3 to 72. Further proof that the purified proteolytic fragment represents the amino terminus of Hc1 was obtained by Southwestern blotting (Fig. 5C). While ^{32}P-labeled DNA was able to bind purified whole Hc1 and C-terminal H1C peptides (lanes 1 and 4), no binding signals were observed with either purified N-terminal H1N peptide (lane 2) or proteolytic fragment (lane 3). We also examined the effect of the GST-EUO fusion protein on H1C and H1N peptides. While the fusion protein was able to digest intact Hc1 (Fig. 6, lane 3) and its carboxyl-terminal peptide H1C (Fig. 6, lane 7), no significant cleavage of N-terminal peptide H1N was observed under similar conditions (Fig. 6, lane 5).

Inhibition of EUO activity by proteinase inhibitors. Proteinase inhibitors serve as important tools for determining the site specificity of enzymes toward their substrates. We examined the activity of 13 different proteinase inhibitors on EUO activity. Our results show that EUO-mediated Hc1 degradation is inhibited completely by 10 mM serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; pefabloc) (Fig. 7, lane 8) and to a lesser extent by aspartic protease inhibitor pepstatin (Fig. 7, lane 6). The enzyme activity is resistant towards a host of other broad-spectrum proteinase inhibitors, including another serine protease inhibitor, phenylmethylsulfonyl fluoride (Fig. 7, lane 13). Similar results were obtained when calf thymus histone H1 was used as a substrate instead of chlamydial Hc1 (data not shown). We tested the heat dependence of EUO protease. The proteolytic activity associated with the EUO gene product appeared heat resistant and remained stable even after the enzyme was boiled (Fig. 7, lane 14). We also tested the influence of various metal ions on EUO activity. Metal ions exhibited no influence on EUO activity (data not shown).

Effect of EUO on DNA-Hc1 complexes. DNA condensation in chlamydial EB is believed to be mediated through DNA-Hc1 interaction. Whether EUO can cleave Hc1 in DNA complexes, leading to relaxation of condensed DNA, was addressed by digesting preformed in vitro DNA-Hc1 complexes with the EUO-GST fusion protein. At molar equivalent ratios of 1:1 and 1:10 between HPLC-purified Hc1 and the 550-bp *Bam*HI-*Xho*I fragment of chlamydial plasmid, the majority of the complexed Hc1 was cleaved by the EUO-GST fusion protein (Fig. 8A, lanes 5 and 9). While a significant proportion of complexed Hc1 was digested, a small amount remained undigested even after 2 h of incubation with the fusion protein. The proportional amount of undigested Hc1 was greater among complexes formed at a molar equivalent ratio of 10:1 (Hc1/

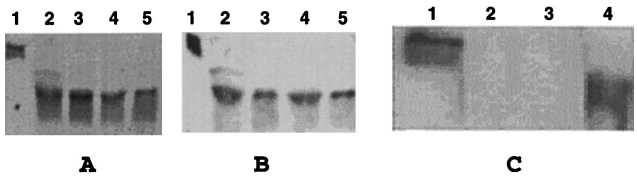

FIG. 5. Identification of the EUO-specified cleavage site on substrate Hc1. (A) HPLC-purified Hc1 (lane 1) was incubated with EUO immobilized on nitrocellulose discs. Samples were removed at 2 (lane 2), 4 (lane 3), 6 (lane 4), and 8 (lane 5) h, resolved by SDS-PAGE, and stained with Coomassie blue. (B) Immunoblot corresponding to gel shown in panel A probed with preabsorbed polyclonal antibodies to anti-H1N antibodies. (C) Southwestern blot analysis of purified Hc1 (lane 1), H1N (lane 2), major cleavable Hc1 peptide following HPLC purification (lane 3), and H1C (lane 4). The blot was probed with a ^{32}P-labeled 500-bp *Bam*HI-*Xho*I fragment of the chlamydial plasmid.

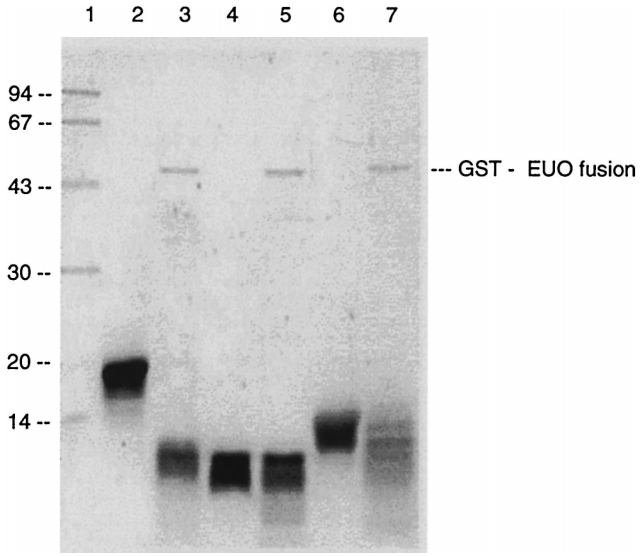

FIG. 6. SDS-PAGE analysis of 1.5 μg of purified Hc1 (lanes 2 and 3), amino-terminal peptide H1N (lanes 4 and 5), and carboxyl-terminal peptide (lanes 6 and 7) digested with 0.1 μg of the EUO-GST fusion protein. Purified peptides were incubated with (lanes 3, 5, and 7) or without (lanes 2, 4, and 6) the EUO-GST fusion protein. Lane 1 shows the mobility of standard small-molecular-size markers (numbers on the left are sizes of markers in kilodaltons).

DNA). Whether a higher amount of substrate Hc1 in the complex was responsible for partial cleavage was analyzed by digesting an equal amount of uncomplexed Hc1 with the fusion protein. Complete digestion of Hc1 was observed under those conditions (Fig. 8A, lanes 2 and 7). In contrast, DNA by itself had no effect on either Hc1 (Fig. 8A, lanes 1 and 6) or EUO-GST fusion protein (Fig. 8A, lane 3) proteolysis. We then examined whether Hc1 cleavage mediated by the fusion protein was sufficient to dissociate DNA-Hc1 complexes by comparing the mobilities of cleaved DNA-Hc1 complexes to those of free uncomplexed DNA by gel shift assays. Whereas undigested DNA-Hc1 aggregates failed to migrate and were retained in gel wells (Fig. 8B, lane 2), precleaved Hc1 incubated with DNA or DNA-Hc1 complexes formed at 1:1 molar equivalent ratios and digested with the fusion protein migrated through the agarose gel (Fig. 8B, lanes 4 and 5). However, only a small portion of DNA from preformed DNA-Hc1 complexes digested with the fusion protein migrated through the gel compared to free uncomplexed DNA as well as precleaved Hc1 complexed to DNA (Fig. 8B, compare lanes 1, 4, and 5). The remainder of complexed DNA was either retained in the gel well or migrated more slowly than uncomplexed DNA (Fig. 8B, lane 5). Preformed DNA-Hcl complexes formed at molar equivalent ratios of 10:1 and subsequently digested with the fusion protein or precleaved Hc1 incubated with DNA at a 10:1 ratio failed to migrate through the gel and were retained in wells, similar to uncleaved DNA-Hc1 complexes (Fig. 8B, lanes 6 to 8). It is important to note that dissociation of Hc1 is a prerequisite to mobility shift. As a control, the ability of the EUO-GST fusion protein to associate with DNA was tested. No shift in DNA mobility was observed under those conditions despite the basic nature of the EUO gene product (Fig. 8B, lane 3).

DISCUSSION

The process of chromatin decondensation in *Chlamydia* during its early life cycle appears to coincide with the inability to detect histone-like proteins from the internalized EB. The mechanism of apparent disappearance of Hc1 and Hc2 following invasion of host cells remains a puzzle. We have found that the product of the EUO gene, expressed very early in the chlamydial life cycle, is a protease capable of cleaving Hc1 and is therefore a candidate for an important component of the process leading to chromatin decondensation. Interestingly, the proteolytic activity specified by the EUO gene product preferentially cleaves the C-terminal portion of Hc1, leaving the N terminus intact. Previous studies from our laboratory have invoked the C terminus in DNA-protein interaction, leading to initiation of DNA condensation in the absence of the N terminus (28). The finding that preformed DNA-Hc1 complexes are susceptible to cleavage by the EUO-GST fusion protein (although the extent of cleavage is dependent upon the molar equivalent ratios of DNA/Hc1) is intriguing. However, it is not clear whether such partial dissociation of DNA-Hc1 complexes can facilitate DNA decondensation in vivo. This is important considering physiological ratios of histone/DNA of 1.1:1.3 (4). Whether failure to dissociate the complex completely is due essentially to inaccessibility of the enzyme or to protection conferred upon Hc1 following its interaction with DNA remains to be determined. Recent studies from our laboratory have ruled out the possibility that DNA-Hc1 interaction blocks accessibility of DNA ligase at low protein/DNA ratios (17). The fact that we observe Hc1 cleavage irrespective

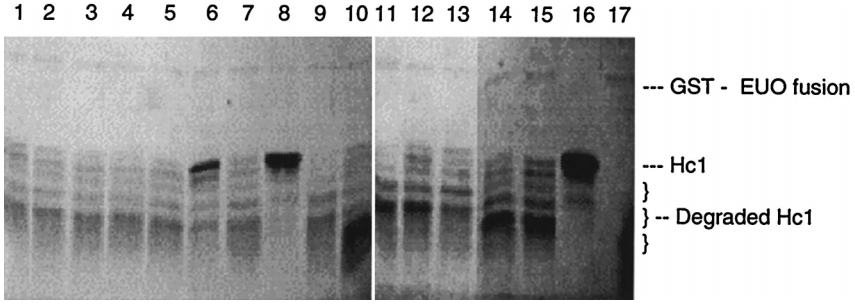

FIG. 7. Effect of proteinase inhibitors, temperature, and pH on the EUO-GST-mediated degradation of Hc1. Coomassie blue-stained SDS–15% PAGE profile of 1.5 μg of Hc1 incubated with 0.1 μg of fusion protein in the presence of 10 mM antipain dihydrochloride (lane 1), bestatin (lane 2), chymostatin (lane 3), E-64 (lane 4), leupeptin (lane 5), pepstatin (lane 6), phosphoramidon (lane 7), pefabloc AEBSF (lane 8), disodium EDTA (lane 9), aprotinin (lane 10), TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) (lane 11), TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) (lane 12), and phenylmethylsulfonyl fluoride (lane 13) is shown. The EUO-GST fusion protein was boiled for 3 min prior to incubation with Hc1 (lane 14). Control Hc1 incubated with (lane 15) or without (lane 16) fusion protein is also shown. All incubations were carried out in PBS (pH 7.4) for 60 min. Lane 17 shows the mobility of the EUO-GST fusion protein alone. The mobilities of the EUO fusion protein, Hc1, and cleaved Hc1 are indicated.

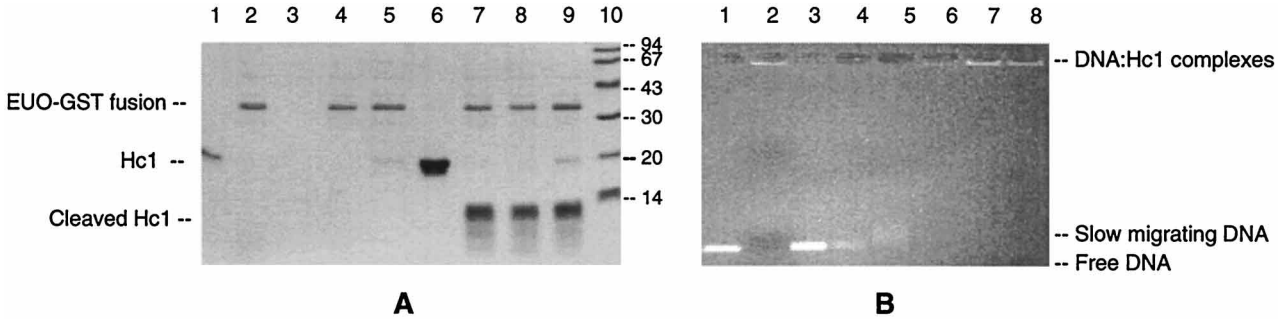

FIG. 8. Cleavage of DNA-Hc1 complexes with the EUO-GST fusion protein. DNA-Hc1 complexes were digested with the EUO-GST fusion protein and analyzed. (A) SDS-PAGE analysis of DNA complexed to Hc1 (lanes 1 and 6), Hc1 digested with fusion protein alone (lanes 2 and 7), DNA incubated with the EUO-GST fusion protein (lane 3), DNA incubated with precleaved Hc1 (lanes 4 and 8), and preformed DNA-Hc1 complexes digested with EUO-GST fusion protein (lanes 5 and 9) are shown. One hundred nanograms of a 520-bp *Bam*HI-*Xho*I DNA fragment of chlamydial plasmid comprising a high-affinity Hc1 binding site was incubated with either 10 ng (lanes 1 to 5) or 100 ng (lanes 6 to 9) of Hc1. The mobility of standard low-molecular-size markers (lane 10) is shown. (B) A gel mobility shift assay with DNA alone (lane 1), DNA complexed to Hc1 (lane 2 and 6), DNA incubated with the EUO-GST fusion protein (lane 3), DNA incubated with predigested Hc1 (lanes 4 and 7), and preformed DNA-Hc1 complexes digested with the EUO-GST fusion protein (lanes 5 and 8) is shown. DNA-Hc1 complexes were formed by incubating 100 ng of DNA with either equimolar amounts (10 ng) (lanes 1 to 5) or 10-fold molar excess amounts (100 ng) (lanes 6 to 8) of purified Hc1. It is important to note the mobilities of free, slow-migrating, and complexed DNA.

of its concentration in the complexes is also suggestive of EUO-GST accessibility. In view of our results, it is tempting to speculate at least two possible mechanisms for DNA decondensation during the early stages of the chlamydial life cycle assuming an in vivo role for EUO protease. These are (i) posttranslational phosphorylation that alters the net charge on Hc1, especially the carboxyl-terminal portion, leading to changes in its affinity for DNA followed by cleavage with EUO, and (ii) competition between resident basic proteins and EUO-degraded Hc1, which appears to affect the affinity for DNA to begin with. Failure of EUO to bind DNA despite its basic nature suggests that EUO itself does not compete with Hc1 for binding to DNA. However, this does not rule out the possibility of EUO acting as a specific or general transcriptional factor in addition to its role in Hc1 cleavage. More recently, we have been able to demonstrate the phosphorylation of Hc1 during early stages of the chlamydial life cycle (18). Nevertheless, more information is needed to define the exact mechanism of Hc1 disappearance and DNA decondensation following entry of EB into the host cell.

The ability of EUO gene product to cleave histones H1 and H5 is interesting. While histone H1 is required for the formation and stabilization of a higher-order chromatin structure (4, 9), a function similar to that of chlamydial Hc1 (2, 7, 23), histone H5 has been shown to largely replace histone H1 in avian erythrocytes (1, 27). Given the lysine-rich nature of histones H1 and H5 and chlamydial Hc1, we propose to designate the EUO gene product a lysine-rich histone-specific protease. In addition, inhibition of proteolytic activity specified by the EUO gene product is indicative of its serine protease nature; however, failure to exhibit amino acid similarities with other known serine proteases may reflect its novel nature. The novel nature of EUO is supported by the fact that its activity is also inhibited by pepstatin, an inhibitor of human immunodeficiency virus and other aspartic proteases. The only other known sequence-specific protease in bacteria that has been recently reported is germination protease GPR, which degrades small acid-soluble proteins of *Bacillus* species (6). However, unlike degradation of *Bacillus* small acid-soluble proteins, degradation of Hc1 has never been observed in vivo. Identification of an EUO gene product that exhibits Hc1-specific proteolytic activity in addition to cleaving histones H1 and H5 represents an important advance in chlamydial biology and may also help to unravel the mechanism of chromatin remodeling in eukaryotes through its ability to modulate histone H1.

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