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EUKARYOTIC-LIKE HISTONES IN *CHLAMYDIA*.

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1. ABSTRACT

A fundamental process in all organisms is their ability to regulate gene expression in response to developmental and environmental signals. In *Chlamydia*, changes in gene expression are closely linked to the presence or to undetectability of eukaryotic-like histones observed late in the parasites life cycle. It is becoming increasingly clear that these histone-like proteins are involved in macromolecular confirmation of DNA. However, their functional role(s) in chlamydial development and the underlying mechanism(s) involved in their degradation and dissociation are largely unknown. It is not surprising therefore that eukaryotic-like histones are a focus of intense research in several laboratories around the world. Recent studies on the interaction of eukaryotic- like histones with DNA, the role of phosphorylation and identification of a histone specific protease are beginning to unravel the mechanism of stage specific differentiation and gene expression in *Chlamydia*. In this article we review recent advances on the eukaryotic-like histones that have set the stage for elucidation of the chlamydial developmental cycle.

2. INTRODUCTION

Chlamydiae are obligate intracellular parasites which have evolved within host eukaryotic cells. Once classified as viruses, they are now considered specialized bacteria that possess discrete cell walls and contain both DNA and RNA. Chlamydiae differ from conventional bacteria in their intracellular nature, small size, longer generation time and a requirement for an exogenous energy supply (1). The order Chlamydiales consists of one family, Chlamydiaceae, and one genus *Chlamydia,* containing three species - *Chlamydia trachomatis, C. psittaci* and *C. pneumoniae. C. trachomatis* has been recognized for centuries as the agent of trachoma which remains the leading preventable cause of blindness worldwide (2). However, in both developing and industralized countries *C. trachomatis* is a major agent of sexually transmitted disease, estimated to cause more than 4 million new cases each year in North America (3, 4). The major sequelae of chlamydial genital tract infections occur in women, and include salpingitis, ectopic pregnancy and infertility (5). Maternal carriage rates have a profound effect on neonatal health since conjunctivitis and pneumonia are seen in up to 50% and 20% respectively of infants born to infected mothers (6). *C. psittaci* is a heterogeneous species and primarily an animal pathogen that rarely causes human disease. *C. pneumoniae* is a relatively new species containing the TWAR strains, and is thought to be an important cause of community acquired pneumonia (7).

3. LIFE CYCLE

Chlamydiae have evolved a complex and unique developmental cycle alternating between two morphologically distinct forms: small (0.2-0.3 μm), extracellular, elementary bodies (EB) and large (1 μm) intracellular, metabolically active reticulate bodies (RB). Infection is initiated by attachment of infectious and metabolically inactive EB to susceptible host cells (8, 9). Attachment is followed by uptake and enlargement within the host cell by circumventing phagosomal fusion to form non-infectious RB which divide within the cytoplasmic inclusions by binary fission (10). The life cycle is completed with the reorganization of RB into EB which subsequently leave the disrupted host cell ready to infect new cells (11). The whole developmental cycle proceeds over 48-72 h depending upon the strain, culminating in completely deranged host cellular and sub-cellular structure. During the morphological transformation of RB to EB, the bacterial chromosome becomes highly condensed and its outer membrane becomes extensively disulfide cross-linked, rendering the EB membrane resistant to osmotic lysis (12, 13). As a result of these changes, the EB nucleoid appears electron dense, localized at the center of the cell, whereas RB chromatin is more relaxed with diffused fine fibrils extending throughout the cell (12). These characteristics are believed to render EB incapable of participating in the transcription and replication processes, as compared to RB whose nucleoid structure is quite relaxed and compatible with replication (14). Wager and Stephens (15) reported the presence of three DNA binding proteins in *Chlamydia* with molecular masses of 17, 25 and 58 kDa. They suggested

that these proteins may be involved in DNA condensation during the transition from RB to EB form. The molecular mass of two of the three DNA binding proteins correspond to previously described HeLa cell binding proteins (16, 17). It is now believed that the unusual composition of DNA binding proteins and their charge are responsible for many of the properties that lead to their affinity for eukaryotic cell surface components.

4. IDENTIFICATION OF EUKARYOTIC-LIKE HISTONE GENES

The application of molecular cloning techniques over the past decade has provided insight into the regulation of a few gene products including histone H1-like proteins that appear to play a major role in controlling the chlamydial developmental cycle through their ability to modify DNA structure. In 1991, two groups working independently reported the cloning and characterization of a very basic protein with an estimated pI of 10.71 (18, 19). The highly basic nature of this polypeptide probably explained the discrepancy between its estimated (18,000 daltons) and calculated (13,689 daltons) molecular weights. Protein data base searches identified significant homology between the cloned gene product and eukaryotic histone H1. This protein is now commonly termed Hc1. Monoclonal antibody generated against chlamydial Hc1 displayed immunoblot cross reactivity to Hc1 and a related EB specific protein with an apparent molecular mass of 32kDa. These antibodies also revealed antinuclear specificity suggestive of cross reactivity to histone H1 (19). Late stage specific expression of Hc1 was confirmed by Northern blot analysis as well as by immunoblot analysis. Hc1 specific transcript was detected 12h post infection among *Chlamydia* infected host cells. All *C. trachomatis* serovars revealed complete conservation of the Hc1 gene product. However, interspecies structural diversity resulted in a smaller protein with 117 amino acids for *C. psittaci* as compared to a product of 125 amino acids for *the C. trachomatis* serovars. Greater preservation of amino acid residues was observed among 66 amino terminal residues (87% identity; 20). In contrast, Hc2 exhibited variable molecular weights of 25-32 kDa depending upon the serovar. Perara *et al* (21) identified the gene encoding the 26-kDa Hc2 from *C. trachomatis* serovar MoPn. Examination of the derived amino acid sequence identified pentapeptide motifs containing three aliphatic residues (usually valine or alanine) and two basic residues (lysine and arginine) which appear crucial for DNA binding. Recently, the Hc2 gene encoding the 32 kDa protein from *C. trachomatis* serovar L_2 has been cloned and expressed by Brickman *et al* (22). This Hc2 polypeptide chain also contains numerous pentapeptide repeats of three aliphatic and two basic residues. It has been suggested that the range of molecular weights observed in different serovars is due to truncated forms of a common protein, since they all share amino acid sequence in the amino terminus. Considering the sequence variability of Hc2 from different *C. trachomatis* serovars and its absence in *C. psittaci* strain Meningopneumonitis (Mn), the functional importance of Hc2 *in vivo* remains to be elucidated (23). It must be emphasized that in general all prokaryotic cells synthesize a set of small, usually basic proteins collectively called histone-like proteins (because their biochemical properties resemble eukaryotic histones) that bind and compact DNA (24). However, unlike chlamydial histone H1-like protein Hc1, which shows nearly 74% similarity (considering 38 perfectly matched residues and 45 conservative substitutions) to eukaryotic histone H1, these prokaryotic nucleoproteins exhibit very little sequence homology. Eukaryotic-like histones have been described in two other prokaryotes, *Pseudomonas aeruginosa* (25), and *Bordetella pertussis* (26). In *P. aeruginosa* histone H1-like protein AlgR3 binds directly to a specific DNA sequence leading to positive regulation of exopolysaccharide alginate biosynthesis (27) while in *Bordetella pertusis* histone-like protein BpH1 plays a role in chromatin formation and condensation (26).

5. FUNCTIONAL ROLE(S) OF EUKARYOTIC-LIKE HISTONES AND THEIR TERMINI

Condensation of chlamydial nucleoid occurs late in its life cycle, concomitant with the expression of Hc1 and Hc2 and accompanied by down regulation of transcription and metabolic processes. In order to examine the role of eukaryotic-like histones in macromolecular confirmation of DNA Barry *et al* (28) expressed *C. trachomatis* Hc1 in *E. coli* . *E. coli* expressing recombinant Hc1 revealed a condensed nucleoid structure similar to that of chlamydiae when examined by light and electron microscopy. Further, Hc1 was shown to co-sediment with purified recombinant *E. coli* nucleoid. These results strongly support a role for Hc1 in condensation of the chlamydial nucleoid. Additional evidence invoking the role of Hc1 in DNA condensation was derived from *in vitro* studies showing purified Hc1 complexed to double stranded DNA, leading to the formation of large aggregates, often in the form of condensed spherical bodies (29, 30). Expression of Hc1 in *E. coli* was shown to down regulate translation, transcription and replication at concentrations similar to those observed in chlamydial EB (31). These authors also demonstrated that low level expression of Hc1 in *E. coli* results in net relaxation of chromosomal DNA - suggesting a bifunctional role for Hc1 depending upon its concentration. These observations agreed with the earlier findings demonstrating that highly supercoiled DNA is associated with the EB stage of the developmental cycle (32). Pedersen *et al* (33) have shown that purified Hc1 inhibits transcription and translation *in vitro* by interacting directly with DNA and RNA. The tight coupling of Hc1 gene expression with DNA replication accounts for its role in cell growth and division. Interestingly, expression of Hc2 in *E. coli* induces compaction of bacterial chromatin distinct from that mediated by Hc1, suggesting differential DNA-binding modes for Hc1 and Hc2 (31). Pedersen *et al* (33), on the other hand, concluded that that DNA-condensation is not the principal function of Hc2 because of their failure to observe nucleoid condensation among *E. coli* expressing Hc2. Further, Hc2 immobilized on nitrocellulose displayed a higher affinity for single stranded DNA and RNA than for double stranded DNA (30).

A first step towards understanding the role of chlamydial Hc1 is to delineate the regions responsible for its characteristics. We have recently subcloned segments of the Hc1 gene corresponding to its amino terminal portion (amino acids 2-65, designated H1N) and carboxyl terminal portion (amino acids 68-125, designated H1C) (34). Expression of these subunits in *E. coli* has identified peptides with estimated molecular weights of 14,000 for the carboxyl and 8,000 for the amino terminal portion of

histone H1. Again, the highly basic nature of the carboxyl terminal domain (pI 13.2) may explain the discrepancy between its estimated (14,000 daltons) and calculated (7,100 daltons) molecular weights. Polyclonal antibodies raised against either amino or carboxyl termini of Hc1 react with recombinant *E. coli* as well as to *C. trachomatis* EB, suggestive of antigenic conservation. Subsequently, we examined the binding of double stranded DNA to Hc1 and its two terminal portions using Southwestern blotting. Hc1 and its carboxyl terminal portion were found to bind DNA; however, no binding was observed between the amino terminus and DNA. *E. coli* expressing either whole Hc1 or its carboxyl terminal peptide was observed to condense its own DNA. The condensation ranged from highly electron dense particles in cells expressing whole Hc1 to loose condensation in the case of carboxyl terminal expression. No condensation was observed in cells expressing the amino terminal portion. Structurally, cells expressing the amino terminus appeared similar to controls. These results clearly imply a role for the carboxyl terminal portion in DNA:protein interaction, a function similar to its eukaryotic counterpart. However, this interaction alone does not appear sufficient to condense DNA tightly; intramolecular protein:protein interactions seem necessary to mediate that effect. Given the sequence conservation at its amino terminus it is tempting to speculate its role in protein-protein interaction. Recently Pedersen *et al* (35) presented experimental evidence to show a potential dimerization site at the N-terminal domain of Hc1. Based on these results, the authors concluded that the amino terminus is functionally important in protein-protein interactions despite the fact that it is not involved in any appreciable interaction with DNA.These results may also help to explain why Hc1 forms higher order complexes with DNA.

Evidence is mounting that Hc1 serves as a non specific yet carefully regulated transcriptional repressor in a manner similar to eukaryotic histone H1 and prokaryotic histone H1-like protein H-NS (30-31, 36-38). In eukaryotes, selective removal of histone H1 renders some genes transcriptionally active, while addition of H1 protein has been shown to repress *in vitro* transcription (39). *In vivo* histone H1 is more prevalent on chromatin of transcriptionally inactive genes than on transcriptionally active genes (40). Although it seems certain that all kinds of DNA will bind histone H1, there are reports that some sequences bind H1 better than others (41-42). However, the association of Hc1 with transcriptionally active genes during the chlamydial growth cycle has not been examined. To identify sequence specific and sequence independent interactions between Hc1 and chlamydial DNA we developed a cross-linking immunoprecipitation protocol to immune precipitate chlamydial Hc1 cross linked to DNA (43). The DNA thus purified was used to probe Southern blots. Our data clearly indicate the presence of sequence specific binding sites on the chlamydial plasmid and Hc1 gene upstream of its open reading frame, in addition to other sequence-independent sites. The sequence-specific high affinity binding sites on the chlamydial plasmid and Hc1 gene were mapped to 24-bp regions that were 70% identical. No intrinsic curvature(s) was detected within these high affinity sites arguing against the role of DNA bends in Hc1 binding. We also ruled out the possibility that such interaction was mediated through a high A+T ratio of the 24-bp fragment. Control fragments with similar A+T ratio failed to associate under similar experimental conditions. More experiments are required to address what mediates sequence specific binding. The observation that Hc1 preferentially binds to only one strand of plasmid DNA is intriguing. Mathews and Sriprakash (44) reported earlier a strand specific endonucleolytic activity in high salt extracts of *C. trachomatis*. Coincidentally, this activity was specific for a region that lies adjacent to the primary Hc1 binding site on chlamydial plasmid. Whether an interplay between endonucleolytic activity and Hc1 induced superhelicity exists remains to be explored. The site specific affinity of Hc1 was further demonstrated by atomic force microscope (AFM) data images. Hc1 binding was always followed by coiling, shrinking and aggregation of the affected DNA (43).

6. Histone H1 specific protease

Within a few hours of entering the host cell EB lose their prominent electron-dense DNA core, the two histone H1-like proteins are undetectable, the cell envelope loses its rigidity, the cell increases in size from 0.3 to 1.0 m and begins to transcribe early stage-specific genes. It is clear, however, that the transcriptional initiation and cellular growth of *Chlamydia* is contingent upon DNA decondensation, which is closely associated with the lack of detection of two histone H1-like molecules. We reasoned that an early upstream open reading frame (EUO) gene product described by Wichlam and Hatch (45) might play an important role in Hc1 degradation and nucleoid decondensation since i) it is expressed very early in the chlamydial life cycle and ii) is highly transcribed compared with MOMP and ^{66} signals. In order to explore this possibility we fused the EUO coding region with glutathione S-transferase (GST) and examined the effect of the fusion protein on Hc1 *in vitro*. The purified fusion protein was able to digest Hc1 completely within 1 h at 37^0C. Also digested were very lysine rich calf thymus histone H1 and chicken erythrocyte histone H5. No measurable activity was observed towards core histones H2A, H2B, H3 and H4 (46). The proteolytic activity specified by the fusion protein preferentially cleaved the C-terminal portion of Hc1, the domain involved in DNA binding, while leaving the N-terminus intact. In addition to cleaving free Hc1, the fusion protein was able to cleave DNA:Hc1 complexes at a molar equivalent ratio of 1:1 between Hc1 and DNA. However, at a higher molar equivalent ratio of Hc1:DNA (10:1), partial protection was conferred upon Hc1 to an extent that prevented complete dissociation of DNA-Hc1 complexes. It is not clear whether partial dissociation of DNA:Hc1 complexes can facilitate DNA decondensation *in vivo*. Also, whether failure to dissociate the complex completely is due to inaccessibility of histone protease remains to be determined. In any event, our results support the nomination of EUO as a histone specific protease. The expression of histone protease during the early life cycle represents an important component of the process leading to chromatin decondensation.

7. PHOSPHORYLATION OF EUKARYOTIC-LIKE HISTONE HC1

DNA decondensation of internalized EB during the early stages of chlamydial infection appears essential

for successful initiation of its growth cycle. *In vitro* studies are supportive of electrostatic interactions between DNA and histone-like proteins that play a paramount role in DNA folding and condensation. The question whether Hc1 is phosphorylated like its eukaryotic counterpart and whether phosphorylated Hc1 exhibits altered affinity for DNA leading to Hc1:DNA dissociation was recently addressed by us (47). Preliminary results are clearly indicative of Hc1 phosphorylation *in vivo*. While Hc1 phosphorylation peaked around 19 h post infection, Hc1 content increased steadily with the progression of the chlamydial life cycle. Significantly low Hc1 phosphorylation was observed late in the life cycle. Whether this represents dephosphorylation or inability to phosphorylate during later stages of developmental cycle remains to be determined. Nevertheless, these results represent the first report of Hc1 phosphorylation in *Chlamydia* during early stages of the growth cycle. Further, we were able to phosphorylate both Hc1 and its carboxyl terminus *in vitro* using protein kinase C (PKC). Recently, we localized a phosphoserine residue at the C-terminal tail of Hc1 as the potential phosphorylation site. Examination of the sequences around the phosphoserine residue identified a motif STKK - a well known PKC phosphorylation site. It remains to be determined if the same site is phosphorylated *in vivo* and what the source of PKC is. Given the availability of reagents it is possible now to ask whether phosphorylated Hc1 exhibits altered affinity for DNA and if so, whether that is sufficient to weaken the DNA:Hc1 complexes.

8. PERSPECTIVE

The presence of eukaryotic-like histones in pathogenic bacteria is intriguing and represents a heitherto unknown mechanism(s) of gene regulation. While in *P.aeruginosa* eukaryotic-like histone is involved in positive regulation of aliginate biosynthesis, chlamydial eukaryotic-like histone Hc1 condenses DNA and down regulates transcriptional and translational processes. These newly discovered basic proteins with homologies to eukaryotic histones may play a significant regulatory role in microbial pathogenesis. We have briefly summarized the functional role of Hc1 in DNA binding and condensation, along with its role in transcriptional and translational processes. However, the question why Hc1 binds preferentially to some sequences over others remains to be addressed. Structure-function study of chlamydial Hc1 has identified its carboxyl terminus as a DNA binding domain leading to chromatin compaction (as its eukaryotic counterpart) while the amino terminus is involved in protein-protein interactions. These observations may explain the ability of Hc1 to form higher order complexes with DNA that fail to migrate through agarose gels. The conservation of amino acid residues at the amino terminal domain simply supports the importance of protein-protein interactions in these processes.

Failure to detect Hc1 during early stages of the chlamydial developmental cycle has led to the suggestion that Hc1 may be lost following internalization. Identification of a novel histone protease which cleaves both free Hc1 and that associated with DNA *in vitro* may allude to Hc1 loss during the early stages of chlamydial infection. However, localization and quantitation of the amount of Hc1 associated with EB and RB forms of the developmental cycle must be determined in order to understand the sequence of events leading to Hc1 undetectability. In addition, the question how histone protease gains access to condensed chromatin *in vivo* remains to be addressed. Efforts are underway in our laboratory to examine whether relaxation of chlamydial chromatin involves Hc1 phosphorylation and subsequent degradation. Eventually, an understanding of the process of DNA condensation and decondensation, which appears fundamental to chlamydial pathogenesis, may lead to novel therapeutic strategies.

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