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The repeated 36 amino acid motif of *Chlamydia trachomatis* Hc2 protein binds to the major groove of DNA

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1                   **a) The repeated 36 amino acid motif of *Chlamydia***  
2                   ***trachomatis* Hc2 protein binds to the major groove of**  
3                   **DNA**

4  
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26 **b) Abstract**

27

28 The gram-negative, obligate intracellular human pathogen, *Chlamydia*  
29 *trachomatis* has a bi-phasic developmental cycle. The histone H1-like *C. trachomatis* DNA  
30 binding protein, Hc2, is produced late during the developmental cycle when the dividing  
31 reticulate body transforms into the smaller, metabolically inactive elementary body. Together  
32 with Hc1, the two proteins compact the chlamydial chromosome and arrest replication and  
33 transcription. Hc2 is heterogeneous in length due to variation in the number of lysine rich  
34 pentamers. Six pentamers and one hexamer constitute a 36 amino acid long repetitive unit that,  
35 in spite of variations, is unique for *Chlamydiaceae*.

36 Using synthetic peptides, the DNA-binding capacity of the 36 amino acid  
37 peptide and that of a randomized peptide was analyzed. Both peptides bound and compacted  
38 plasmid DNA, however, electron microscopy of peptide/DNA complexes showed major  
39 differences in the resulting aggregated structures. Fluorescence spectroscopy was used to  
40 analyze the binding. After complexing plasmid DNA with each of three different intercalating  
41 dyes, increasing amounts of peptides were added and fluorescence spectroscopy performed.  
42 The major groove binder, methyl green, was displaced by both peptides at low concentrations,  
43 while the minor groove binder, Hoechts, and the intercalating dye, Ethidium Bromide, were  
44 displaced only at high concentrations of peptides.

45

46 Keywords: *Chlamydia trachomatis*; Histone H1-like protein; Hc2; DNA packing;  
47 fluorescence spectroscopy; methyl green

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50

## 51 Abbreviations

52 AA: amino acid; EMBOSS: the European Molecular Biology Open Software Suite; Hc2:

53 *Chlamydia* histone H1-like protein 2; MALDI-TOF: matrix-assisted laser desorption

54 ionization-time of flight instrument;

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### 55 c) Introduction

56 *Chlamydia trachomatis* is an obligate intracellular gram-negative human  
57 pathogen with a unique biphasic developmental cycle in which the small infectious  
58 extracellular form with limited metabolic activity, the elementary body (EB) of 0.3  $\mu\text{m}$ ,  
59 alternates with the larger dividing, intracellular form, the reticular body (RB) of 1  $\mu\text{m}$  [1]. EB  
60 can infect genital tract and conjunctival epithelial cells. They attach to the surface of the cells  
61 at which point they secrete the translocated actin-recruiting phosphoprotein, TARP, which  
62 mediates phagocytosis and recruits actin [2,3]. Upon uptake in an intracellular vesicle, the  
63 chlamydial inclusion, EB transforms into the metabolically more active RB, protein synthesis  
64 is initiated and the inclusions are transported to the perinuclear space [4]. Early after uptake,  
65 chlamydial synthesized proteins of the inclusion membrane protein (Inc) family are secreted  
66 by the type 3 secretion system (T3SS) and inserted into the inclusion membrane where they  
67 promote inclusion fusion [5]. After multiple rounds of replication, RB are converted into EB.  
68 This transition is accompanied by synthesis of late cycle proteins, of which two are cystein-  
69 rich outer membrane proteins (Omp2 and Omp3) [6] and two are histone H1-like DNA and  
70 RNA binding proteins [7]. During the transition, the outer membrane is cross-linked by  
71 disulfide bounds, the diameter of the chlamydiae is reduced from 1  $\mu\text{m}$  to 0.3  $\mu\text{m}$ , the  
72 nucleoid is condensed, transcription and replication are arrested and the infectious EB are  
73 released through inclusion burst [8].

74 The two histone H1-like proteins, Hc1 and Hc2, are encoded by *hctA* and *hctB*,  
75 respectively [7,9]. Both proteins are capable of condensing both DNA and RNA into tightly  
76 packed spheres and arrest replication and transcription [10–12]. Both proteins are abundant in  
77 the EB, where Hc1 constitute 6% of the total protein equal to one molecule of Hc1 per 37 bp  
78 of the genome [13]. Based on genome analysis, Hc2 is found in all members of  
79 *Chlamydiaceae*, whereas in other genera, though proteins with similar amino acid

80 composition were found, the very regular presence of repeats was not observed. Thus, Hc2 is  
81 believed to be ubiquitous in *Chlamydiaceae* [14] . While Hc1 is genetically stable in all *C.*  
82 *trachomatis* serovars, Hc2 varies in size between serovars [9] and the size variation is caused  
83 by variation in the number of repeated elements within the *hctB* gene [14]. The repeated  
84 elements consist of 36 amino acids (AA) of which many are positively charged residues. Each  
85 element is composed of six pentamers and one hexamer in which both AA substitutions and  
86 deletions result in a high number of variants among *C. trachomatis* isolates. The sequence  
87 variation makes Hc2 suitable for phylogenetic analysis [14] and is included in a multilocus  
88 sequence typing scheme for genotyping of *C. trachomatis* [15] but so far it has not been  
89 possible to link variants to clinical disease [12].

90           Within the repeat [14] the positively charged residues are evenly distributed  
91 with two positively charged residues: lysine (K) and arginine (R) separated by three amino  
92 acids of which many are either polar uncharged or hydrophobic residues alanine (A),  
93 threonine (T) and valine (V). In addition, two prolines (P) are present. Prolines provide  
94 conformational rigidity to a secondary helical structure with a kink of the  $\alpha$ -helix [16].  
95 To analyze the importance for DNA binding of conserved primary sequence of the repeated  
96 36 AA element of Hc2 we synthesized by peptide synthesis the 36 AA peptide (Hc2rep) and a  
97 36 AA peptide in which the sequence was randomized (Hc2scrbled). The two peptides were  
98 analyzed for their DNA-binding capacity by a gel shift assay and electron microscopy.  
99 Fluorescence spectroscopy was used to determine where on the DNA helix the peptide bound.

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## 107 **d) Materials and Methods**

### 108 *2.1 Peptide synthesis*

109 The Hc2-peptide of 36 AA residues, Hc2rep, Accession number GeneBank:  
110 ADD14374 aa 61-96 [14] is shown in Fig. 1A. The Hc2rep sequence was pseudorandomized  
111 using EMBOSS software to create the Hc2scrambled peptide sequence (Fig 1B). The peptides  
112 were synthesized using Fmoc solid-phase-peptide synthesis on an automatic ABI 433  
113 synthesizer (Applied Biosystems, Waltham, MA, USA) according to Holm et al. [17]. The  
114 peptides were made with a terminal amide, mimicking an internal peptide bond. The mass of  
115 each synthesized peptide was verified by mass spectrometry using an Autoflex matrix-  
116 assisted laser desorption ionization-time of flight instrument (MALDI-TOF) (Bruker  
117 Daltonics, Bremen, Germany).

118

### 119 *2.2 Bioinformatic*

120 Secondary structure predictions Garnier, Osguthorpe and Robson [18] and  
121 Helical wheels for the peptides Hc2rep and Hc2scrambled were performed using "the  
122 European Molecular Biology Open Software Suite" EMBOSS [19].

123

### 124 *2.3 Gel shift assay*

125 pBluscript SK+ (Stratagene, La Jolla, CA, USA) plasmid DNA was purified  
126 from *Escherichia coli* XL1-blue (Stratagene) using cesium chloride gradient centrifugation.  
127 DNA concentration and purity were determined by UV scan 220-300 nm (Hitachi, Tokyo,  
128 Japan) [20]. Plasmid DNA at a concentration of 33.3 µg/ml was mixed with the Hc2rep  
129 peptide and with Hc2scrambled peptide, respectively, in order to obtain final concentrations  
130 of peptide of 0, 3.1, 6.2, 12.5, 25 and 50 µg/ml (peptide/DNA ratios of 0, 0.09, 0.19, 0.38,  
131 0.75, 1.5, respectively) in PBS, incubated at 37 °C for 5 min and subjected for electrophoresis

132 in a 0.7% agarose gel [12]. The experiments were repeated twice. The gels were scanned and  
133 analyzed by ImageJ [21].

134

#### 135 *2.4 Electron microscopy*

136 Samples with pBluescript SK+ plasmid DNA (33.3  $\mu\text{g/ml}$ ) and  
137 Hc2rep/Hc2scrambled (0, 3.1, 6.2, 12.5, 25 and 50  $\mu\text{g/ml}$ ) in PBS were prepared and  
138 incubated at 37 °C for 5 min in order to obtain peptide/DNA ratios of 0, 0.09, 0.19, 0.38, 0.75,  
139 1.5, respectively. Aliquots of the samples were mixed with spermidine buffer [22] and  
140 mounted for 5 min onto 400 mesh copper grids coated with a glow discharged carbon film.  
141 The grids were then rinsed in double distilled water and dehydrated in increasing  
142 concentrations of ethanol at 25, 50, 75 and 96%, blotted dry and rotary shadowed with  
143 tungsten wire at vacuum. Electron microscopy was carried out at 60 keV using a JEM 1010  
144 electron microscope (JEOL, Echting, Germany). Images were obtained using a KeenView  
145 digital camera (Olympus, Center Valley, PA, USA). For size determination a carbon replica  
146 grid (2160 lines/mm) was used. The experiments were repeated three times.

147

#### 148 *2.5 Fluorescence spectroscopy*

149 Fluorescence spectroscopy was performed twice without stirring and once with  
150 magnetic stirring using a QuantaMaster 400, PTI (Photon Technology International Canada,  
151 Ontario, Canada) equipped with a xenon lamp using a 1.0 cm light pathway quartz cell. Three  
152 different dyes were used for the steady state fluorescence experiments: Hoechst 33342  
153 (Thermo Fisher Scientific, Waltham, MA, USA), Methyl Green (Sigma-Aldrich, St. Louis,  
154 MO, USA) and Ethidium Bromide (Merck Millipore, Billerica, MA). Methyl green (1 mg/ml  
155 in 0.1% acetic acid) was extracted 3 times with chloroform to remove impurities before the  
156 steady state measurements [23].

157                   Excitation wavelength of 343 nm was used for Hoechst 33342 (Thermo Fisher  
158 Scientific) and emission spectra were obtained from 375-600 nm. pBluscript SK+ plasmid  
159 DNA was added to obtain a concentration of 0.32 µg/ml in a final volume of 2.8 mL and  
160 scanned 6 minutes after addition. Subsequently, 2 µL aliquots of Hc2rep or Hc2scrambled  
161 (stock solution at 0.8 mg/mL) were added with regular intervals of 6 min in order to achieve  
162 the Peptide/DNA ratios (table 1), after which the emission spectra were recorded with a  
163 scanning rate of 1 nm/s.

164                   The same experimental procedure was applied for Methyl green (1.4 µg/ml) and  
165 Ethidium Bromide (0.5 µg/ml), using an excitation wavelength of 633 nm (emission: 640-800  
166 nm) [24] and 471nm (emission: 500-700 nm) [25], respectively. As a dilution control, 2 µL of  
167 PBS 1x were added successively instead of Hc2rep or Hc2scrambled with regular intervals of  
168 6 min.

169 The relative fluorescence intensity at the maximum peak was then calculated for each  
170 protein/DNA ratio tested and plotted for each of the dyes tested.

171

## 172                   2.6 Data treatment

173                   The fluorescence emission spectra of each dye, without DNA, were subtracted  
174 from the fluorescence emission spectra obtained for the conditions stated in Table 1. The  
175 emission signal was then smoothed (10 points smooth) in Origin 8.1 (OriginLab Corporation,  
176 Northampton, MA, USA) and the relative fluorescence of all the emission spectra calculated  
177 in Microsoft Excel 2010 (Microsoft Corporation) considering the fluorescence emission  
178 maximum of the dyes with DNA but no protein the highest emission (100%). The relative  
179 fluorescence of each aliquot at the maximum intensity peak was then plotted for all the dyes  
180 in Origin Pro 8 and fitted according to a linear model ( $f(x) = a + b*x$ ), where  $a$  is the value of  
181 y-intercept of the line and  $b$  is the slope.  $a$  was fixed at 100, the root mean square error was

182 calculated as well as the parameter values and corresponding errors. The parameters obtained  
183 for the linear fit and the corresponding uncertainties are displayed in Table 2.  
184

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185 e) **Results**

186 3.1 *Bioinformatics determination of the structure of the C. trachomatis Hc2rep*

187 The 36-mer Hc2rep peptide is composed of only six different AA (11 K, 10 A, 6  
188 V, 4 T, 3 R and 2 P). Its secondary structure was analyzed by Garnier, Osguthorpe and  
189 Robson prediction [18]. The structure of the Hc2rep 36-mer peptide was predicted to form an  
190 uninterrupted  $\alpha$ -helix (Fig. 1A). Using the pepwheel program on the Hc2rep (Fig. 1C) it was  
191 seen that the positively charged AA, R and K, are evenly distributed around the  $\alpha$ -helix,  
192 which also is the case for the remaining AA, A, V, T and P. The Hc2scrambled peptide has  
193 the same AA composition as the Hc2rep peptide but with a pseudorandomized sequence. The  
194 analysis of the secondary structure by Garnier, Osguthorpe and Robson prediction (Fig 1B)  
195 showed that the Hc2scrambled peptide was predicted to form an  $\alpha$ -helix, interrupted with  
196 turns and coils. Using the pepwheel program on Hc2scrambled (Fig 1D), it was observed that  
197 there was a different distribution of the positively charged AA, R and K, and that there was no  
198 repeated pentamers.

199

200 3.2 *Gel shift assay*

201 To determine whether the Hc2rep peptide and the Hc2scrambled peptide could  
202 bind to DNA, plasmid DNA (33.3  $\mu$ g/ml) was mixed with decreasing amounts of each of the  
203 peptides and complex formation was analyzed by a gel shift assay. Results are shown in Fig.  
204 2. In lanes 0 no peptides were added to the DNA, and three DNA bands are seen: supercoiled,  
205 covalently closed circular (CCC, lower band), nicked open circular (OC, upper band) and  
206 linear (L) DNA (middle band) (Fig. 2, inserted at the right side). For both peptides, at 1.5 and  
207 0.75 weight ratio peptide/DNA, no DNA entered the gel, but was retained in the slots,  
208 indicating that all DNA was complexed with the peptides. The DNA bands appeared bright in  
209 the gel slots when complexed with Hc2rep but with a fainter intensity when complexed with

210 Hc2scrambled. At a ratio of 0.38 Hc2rep/DNA, three bands of DNA are entering the gel as  
211 supercoiled (CCC, lower band), nicked (OC, upper band) and linear (L) DNA (middle band).  
212 However, the bands are fuzzy indicating that the DNA molecules were complexed with  
213 various amounts of peptide (Fig. 2, lanes 0.38). This indication was also supported by a faint  
214 band observed at the gel slot where part of the DNA appeared to be retained. Similar  
215 observations were made for Hc2scrambled/DNA at these ratios. At ratio of 0.19 and 0.09, the  
216 DNA bands are increasingly distinct, more defined and no DNA was retained in the slots of  
217 the gel. However, it is seen that the lower bands on the gel migrated to a higher position than  
218 seen when no peptides were added (Fig. 2, lanes 0). By gel scan there was no indication of a  
219 preferential binding of peptides to any of the plasmid forms (CCC, OC or L). The gel scan  
220 indicated, however, that the Hc2scrambled peptide was able to complex with DNA (all three  
221 forms) better than the Hc2rep peptide (1.5 times better at ratio 0.38; and 1.9 times better at  
222 ratio 0.19) as also indicated by visual inspection of the gel (Fig. 2).

223

### 224 *3.3 Electron microscopy*

225 To determine how the complex formation between the Hc2rep peptide and the  
226 plasmid DNA appeared, electron microscopy was performed (Fig. 3). Decreasing amounts of  
227 Hc2rep peptide were added to plasmid DNA, and the complexes were visualized following  
228 dehydration and rotary shadowing [22]. At ratio of 1.5 and 0.75 of Hc2rep/DNA, complexes  
229 were found in large aggregates seen as tight centers of various sizes with an average of 195  
230 nm in diameter (from 50 to 340 nm in diameter ) (Fig. 3A, white arrow), from which loops of  
231 DNA were seen (white arrowhead), (Fig. 3A). Other central structures were more elongated  
232 (100 x 300 nm) with indication of being built up of twisted DNA strands (black arrow, Fig.  
233 3B). DNA strands (white arrowhead) were seen to protrude from the elongated center. No  
234 DNA molecules were seen in the background indicating that at this peptide/DNA ratio all

235 DNA was complexed with peptides in agreement with Fig. 2 where at this peptide/DNA ratios  
236 no DNA was entering the gel.. There was no clear structure in the aggregated centers but  
237 clearly each center had been complexed with a high number of plasmid DNA molecules. At  
238 ratio of 0.38 the DNA was complexed with the peptide, forming coiled, elongated central  
239 structures (from 200-50 nm) from which loops of DNA could be seen (black arrow, Fig. 3C).  
240 In these elongated central structures, it appeared that there was a macrolevel coiling of  
241 various numbers of DNA strands. A high number of DNA loops were seen protruding from  
242 the elongated centers (Fig. 3C white arrowhead). Similar elongated, coiled central structures  
243 (black arrow) appeared at ratio of 0.19 of peptide/DNA (110 – 70 nm), from which loops of  
244 DNA could be seen. In addition, supercoiled DNA molecules were seen (Fig. 3D, black  
245 arrowhead). At ratio of 0.09 the central structures were shorter (up to 36 nm) and clearly  
246 twisted (Fig. 3E, black arrow), forming more loosely organized structures from which loops  
247 of both supercoiled (black arrowhead) and linear DNA (L) could be seen protruding. In  
248 addition, uncomplexed plasmid DNA molecules could be seen (black arrowhead, Fig. 3E). In  
249 Fig. 3F, in which no Hc2rep peptide was added to the plasmid DNA, only uncomplexed  
250 plasmid DNA was seen both as supercoiled (Fig. 3F, black arrowhead), open circles and as  
251 linear molecules (Fig. 3F), in agreement with the three bands seen by gel electrophoresis (Fig.  
252 2A lane 0).

253 In Fig. 4 are depicted two electron microscopy images obtained after complex  
254 formation between the Hc2scrambled peptide and plasmid DNA at ratios 1.5 (Fig. 4A) and  
255 0.38 (Fig.4B). At ratio 1.5 a smooth compact structure of 1400 x 75 nm (black arrow) is  
256 shown, from which a few DNA loops are protruding (black arrowhead) (Fig. 4A, insert). A  
257 macrolevel coiling of DNA strands appeared when Hc2scrambled peptide was added to  
258 plasmid DNA (Fig. 4B, black arrow) and at this ratio a number of DNA molecules are seen to  
259 protrude from the dense central structure (black arrowhead, Fig. 4B) .



260

261 *3.4 Fluorescence spectroscopy*

262 Since both the gel shift assay and electron microscopy showed that the Hc2rep  
263 peptide was able to form complexes with the plasmid DNA, the next step was to determine  
264 how the interaction between plasmid DNA and the peptide was established. Hc2scrambled  
265 was used for comparison in these experiments. To determine to which groove the peptide  
266 binds, we used a competition assay with three fluorescent DNA binding: the major groove  
267 binder, methyl green [23,24]; the minor groove binder, Hoechst 33342 [26,27]; and the  
268 intercalator probe, ethidium bromide [28]. Plasmid DNA and the respective fluorescent dye  
269 were mixed in a cuvette with magnetic stirring and scanned prior to addition of peptide.  
270 Aliquots of peptide were added and after each addition of peptide a new scanning was  
271 performed.

272 To compare the changes in fluorescence between the dyes at increasing amounts  
273 of Hc2rep or Hc2scrambled peptide, the maximal emissions were set to 100% without peptide  
274 and plotted against peptide/DNA ratio for each dye. The plotted values were then fitted  
275 according to a linear model  $f(x) = a + b \cdot x$  (Fig. 5). The dynamics of peptide/DNA binding at  
276 the tested ratios followed a linear trend with good adjusted root-mean-square ( $R^2$ ) values  
277 (Table 2).

278 Addition of Hc2rep or Hc2scrambled peptides to the DNA/methyl green (major  
279 groove binder), led to a decrease in the relative fluorescence intensity with increase of  
280 peptide/DNA ratio, indicating that the peptide had displaced the fluorescent dye from the  
281 major groove of DNA. In Fig.5A, it was observed that for the highest peptide/DNA ratio  
282 (10.7), the addition of Hc2rep led to a decrease in relative fluorescence intensity of 27%  
283 whereas the addition of Hc2scrambled led to a decrease of 35% when compared to the  
284 fluorescence decrease of the PBS control with no peptide. Thus, both peptides displaced

285 methyl green from the major groove in a similar manner. The linear fittings for both Hc2rep  
286 and Hc2scrambled showed adjusted  $R^2$  of 0.999 and 0.997, respectively (Table 2), and the  
287 slope obtained for the fitted lines of Hc2rep (-4.2) and Hc2scrambled (-5.0) were statistically  
288 different from the slope of the control with PBS (-1.8), determined by the confidence intervals  
289 (Table 2).

290 Similarly, the minor groove binder Hoechst 33342 was analyzed (Fig. 5B).  
291 Addition of Hc2rep or Hc2scrambled peptides to the DNA- Hoechst 33342 solution decreased  
292 the relative fluorescence intensity by 6% and 11%, respectively. This decrease was lower than  
293 what was seen for methyl green, indicating that the displacement of Hoechst 33342 from the  
294 minor groove of DNA by Hc2rep and Hc2scrambled peptides was less than what was  
295 observed for the displacement from the major groove with methyl green. The slopes obtained  
296 from the linear model fitting corroborate these observations, as the slope for Hc2scrambled  
297 was more negative than the slope value obtained for Hc2rep (Table 2). Thus, at the highest  
298 peptide/DNA ratio a decrease in fluorescence of 8% for Hoechst 33342 compared to  
299 compared to 27% with methyl green.

300 The intercalating dye ethidium bromide (0.5  $\mu\text{g/ml}$ ) was excited at 471 nm and  
301 scanned from 500 – 700 nm (Fig. 5C). Addition of Hc2rep or Hc2scrambled showed a  
302 decrease in relative fluorescence intensity at 600nm, with a higher decrease observed for  
303 Hc2scrambled. At the highest peptide/DNA ratio (10.7) a decrease of 1% and 9% was  
304 registered for Hc2rep and Hc2scrambled/DNA complexes, respectively. The slopes obtained  
305 from the linear model showed a statistical significant difference between PBS/Hc2rep and  
306 HC2scrambled (Table 2).

307                   The fluorescence spectroscopy thus showed that methyl green showed the  
308 highest displacement with both Hc2rep and Hc2scrambled, while the largest difference  
309 between Hc2rep and Hc2scrambled displacement was observed for ethidium bromide, with  
310 the highest displacement shown for Hc2scrambled (Fig. 5, Table 2).

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313 **f) Discussion**

314 In the present study we analyzed how the 36-mer repeated part of the *C.*  
315 *trachomatis* histone H1-like protein, Hc2rep, could form large aggregates with plasmid DNA,  
316 and found that the peptide preferentially was bound in the major groove of DNA. The 36-mer  
317 peptide has a predicted  $\alpha$ -helix structure. As one  $\alpha$ -helical turn is made up of 3.6 AA the 36-  
318 mer peptide has 10 turns. The peptide is rich in the positively charged AA, R and K, which  
319 can interact electrostatically with the negatively charged phosphates of the DNA backbone,  
320 and the hydrophobic AA, A and V, known to stabilize the  $\alpha$ -helix in short peptides in aqueous  
321 solutions [29], similarly distributed over the  $\alpha$ -helix. Within the 36-mer peptide Hc2rep (Fig.  
322 1A), a pronounced symmetry is seen: two positively charged AA are separated by three  
323 uncharged or hydrophobic AA so that each 36-mer peptide is composed of six pentamers and  
324 one hexamer [14]. In contrast to the helical wheel analysis of Hc2rep, that showed the  
325 positively charged AA to be evenly distributed around the  $\alpha$ -helix, the Hc2scrambled peptide  
326 had an uneven distribution of positively charged AA and disruption of the predicted  $\alpha$ -helix  
327 (Fig. 1 B and D)

328 Large aggregates were formed when plasmid DNA was mixed with high peptide  
329 concentrations (Fig. 2-4), and since interactions between the positively charged AA and the  
330 sugar-phosphate backbone of the DNA is largely independent of the base sequence [30], a  
331 single 36-mer peptide molecule must cross-link several DNA molecules.

332 At high peptide concentrations, large aggregates of peptides/DNA were formed  
333 by both peptides (Fig. 2 - 4). It is clear that at these concentrations the peptides are not  
334 binding uniformly along a DNA strand (Fig. 3) but rather aggregating many DNA molecules  
335 similarly to what is seen when recombinant *C. trachomatis* Hc2 is complexed with plasmid  
336 DNA [12]. Also at the lower concentrations of Hc2rep peptide the complexes formed with  
337 plasmid DNA are similar in structure to what was seen with the complete recombinant Hc2,

338 where cores of coiled DNA molecules were seen [12]. It thus seems that binding of Hc2rep  
339 peptides to plasmid DNA results in coiling of several plasmid DNA molecules forming less  
340 tightly wound up aggregates when the Hc2rep peptide concentration was reduced (Fig. 3 C-E).  
341 Coil-like structures were also observed when recombinant Hc2 was expressed in *E. coli*, and  
342 cells expressing Hc2 were analyzed by electron microscopy [9]. Purified nucleoids from such  
343 cells were resistant to DNase I degradation, indicating the intimate binding of Hc2 to the *E.*  
344 *coli* chromosome [9]. The Hc2rep AA sequence is highly similar to part of the AA sequence  
345 of the 26 kDa lysine and alanine rich protein of *Chlamydia muridarum* [31]. In their paper  
346 Perara et al. [31] suggested that the regular spacing of prolines within the penta-peptide repeat  
347 region would result in a kinked helical structure that would assist the fit into the major groove  
348 of DNA, and that this would allow K and R residues to form electrostatic and hydrogen-bonds  
349 with the phosphate backbone of DNA [9,31]. This is in agreement with our findings, that  
350 Hc2rep predominantly binds to the major groove of DNA.

351           The results obtained with steady state fluorescence with 3 different DNA-  
352 binding dyes, showed distinct degrees of binding according to the type of binding dye used  
353 (intercalating, binding to the major groove or binding to the minor groove). Binding of the  
354 peptides to DNA led to the displacement of the dye which was translated into a decrease in  
355 relative fluorescence intensity. The highest decrease was registered for methyl green  
356 indicating that the peptides bound primarily to the major groove of the DNA. Even though  
357 dilution plays a role in fluorescence intensity decrease, the controls performed with PBS  
358 showed that the registered fluorescence intensity decrease for the both Hc2 rep and  
359 Hc2scrambled peptides were due to binding and not just due to dilution effect. The acquired  
360 data for the tested ratios could be approximated with a linear trend. A good fitting was  
361 obtained for all data in the range.

362            Electron microscopy showed a marked difference between Hc2rep and  
363 Hc2scrambled in the appearance of DNA complexes (Fig. 3 and 4). In the steady state  
364 fluorescence, it was seen that Hc2scrambled affected the intercalating ethidium bromide  
365 binding in high peptide/DNA ratios significantly, whereas Hc2rep did not (Fig. 5C, Table 2).  
366 Therefore, the different distribution of the AA in the peptide structure led to different  
367 behaviors upon binding to DNA, and thus the primary AA sequence had a specific function,  
368 explaining why the AA sequence is preserved in the chlamydial species [12].

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373 **g) Conflict of interests**

374 Svend Birkelund, Arne Holm and Gunna Christiansen are shareholders in Loke  
375 Holdingselskab, Egaa, Denmark, which provided the peptide for this study.

376

377

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477 **j) Figure legends**

478

479 **Fig. 1.** Secondary structure prediction A) Hc2rep B) Hc2scrambled. Garnier, Osguthorpe  
480 and Robson; Helical wheel C) Hc2rep and D) Hc2scrambled.

481

482 **Fig. 2.** Agarose gel electrophoresis A) Hc2rep/DNA complexes B) Hc2scrambled/DNA  
483 complexes. The weight ratio between Hc2rep/Hc2scrambled and DNA are marked  
484 above the lanes. Std. *Hind*III digested lambda  $\lambda$ -phage DNA. OC: open circular DNA; L:  
485 linear DNA; CCC: covalently closed circular DNA. At the right of the figure drawings are  
486 shown of OC, L and CCC DNA molecules.

487

488

489 **Fig. 3.** Electron micrographs of Hc2rep/DNA complexes. A-B) Hc2rep to DNA ratio 1.5.  
490 C) Hc2rep to DNA ratio 0.38. D) Hc2rep to DNA ratio 0.19. E) Hc2rep to DNA ratio 0.09.  
491 F) DNA. White arrow: aggregated central structure; white arrowhead: DNA loop; black  
492 arrow: coiled elongated central DNA structures; black arrowhead: supercoiled DNA.

493

494 **Fig. 4.** Electron micrographs of Hc2scrambled/DNA complexes. A) Hc2scrambled to  
495 DNA ratio 1.5. B) Hc2scrambled to DNA ratio 0.38. Black arrow: aggregated, compact  
496 DNA/peptide structures; black arrowhead: protruding DNA loops.

497

498 **Fig. 5.** Relative reduction in fluorescence intensity at emission maximum after addition  
499 of Hc2rep, Hc2scrambled or PBS for A) methyl green, B) Hoechts 33342 and C) ethidium  
500 bromide. Relative intensities are shown on the y-axis and peptide/DNA ratios are  
501 marked on the X-axi

502 k) Tables

503 **Table 1.** Peptide concentrations ( $\mu\text{g/mL}$ ) and peptide/DNA ratios used in fluorescence  
504 steady state measurements with Hoechst 33342, methyl green and ethidium bromide.

Peptide aliquot added	Peptide concentration ( $\mu\text{g/mL}$ )	Peptide/DNA ratio
1	0.6	1.8
2	1.1	3.6
3	1.7	5.4
4	2.3	7.1
5	2.9	8.9
6	3.4	10.7

505

506

507 **Table 2.** Linear fit parameters and respective errors using the model  $f(x) = a + b \cdot x$  ( $a$   
 508 fixed at 100) of the relative values of maximum fluorescence intensity obtained in each  
 509 condition tested with for methyl green, Hoechst 33342 and ethidium bromide.

510

Dye	Sample	Slope (b)	Adjusted R <sup>2</sup>
Methyl Green	PBS	-1.772 ± 0.032	0.999
	Hc2rep	-4.231 ± 0.052	0.999
	Hc2scrambled	-5.000 ± 0.237	0.997
Hoechst	PBS	-0.665 ± 0.048	0.999
	Hc2rep	-1.286 ± 0.041	0.999
	Hc2scrambled	-1.717 ± 0.034	0.999
Ethidium Bromide	PBS	-1.136 ± 0.120	0.999
	Hc2rep	-1.222 ± 0.027	0.999
	Hc2scrambled	-1.993 ± 0.042	0.999

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