



H-NS蛋白对副溶血弧菌*hcp1*的转录调控

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摘要: 【目的】研究调控子H-NS对副溶血弧菌T6SS1结构蛋白基因*hcp1*的转录调控机制。【方法】利用Western blot检测Hcp1蛋白在野生株(WT)和*hns*基因敲除株(Δhns)中表达水平的差异。提取WT和 Δhns 的总RNA, 采用实时定量RT-PCR的方法验证H-NS对*hcp1*的转录调控关系。进而采用引物延伸实验研究*hcp1*的转录起始位点, 并根据产物的丰度判断H-NS对*hcp1*的调控关系。PCR扩增*hcp1*的整个启动子区DNA序列, 并纯化His-H-NS蛋白, 通过凝胶阻滞实验(EMSA)验证His-H-NS对*hcp1*启动子区是否具有直接的结合作用。【结果】Western blot和实时定量RT-PCR结果显示H-NS能抑制*hcp1*的表达; 引物延伸结果显示*hcp1*只有一个转录起始位点T (-62)(翻译起始位点为+1), 且其转录活性是H-NS和 σ^{54} 依赖性的; EMSA实验表明H-NS对*hcp1*的启动子区具有直接的结合作用。【结论】H-NS能直接结合到*hcp1*启动子区而抑制其转录表达。

关键词: 副溶血弧菌, H-NS, *hcp1*, 转录调控

副溶血弧菌(*Vibrio parahaemolyticus*)是广泛分布于海水、海河交界处及海产品中的一种革兰氏阴性弧菌, 也是一种食源性致病菌。当人们食用被其污染的海产品时, 可能会被感染, 引起以呕吐、腹泻、发热、头疼等为主要症状的急性胃肠炎, 偶见由海水污染伤口而引起的败血症或组织感染, 表明副溶血弧菌也有一定的组织入侵

能力^[1]。

副溶血弧菌能表达多种致病性毒力因子, 如耐热性直接溶血素(TDH)、TDH 相关溶血素(TRH)、III型分泌系统(T3SS)、VI 型分泌系统(T6SS)等^[1-2]。其中, T6SS是一种由多组分蛋白复合体构成的跨膜“针状”注射装置, 其跨膜结构通常由VipA/VipB构成、菌体表面的“针状”结构通常

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由Hcp构成,而在Hcp顶端有一个由VgrG蛋白构成的3聚体结构^[3-4]。T6SS能将效应蛋白注入动物细胞或其他细菌胞内,引起细胞凋亡或杀灭细菌^[3-4]。副溶血弧菌的大小染色体上分别含有一套T6SS基因位点,分别称为T6SS1和T6SS2。T6SS1常见于临床分离株,在模拟海洋环境条件(2% NaCl和23–30 °C)下表达水平高,主要与副溶血弧菌对宿主细胞粘附活性和对其它种类细菌的杀菌活性有关^[5]。T6SS2存在于所有副溶血菌株中,在模拟宿主体内环境条件(0.5% NaCl和37 °C)下表达水平高,主要与细胞粘附活性相关^[5-6]。

H-NS是一种普遍存在于革兰氏阴性菌中的拟核相关DNA结合蛋白,也是一个整体负调控因子。H-NS对富含AT的DNA序列具有高亲和力,因而其靶基因通常是通过水平转移获得的“外源基因”^[7-8]。H-NS选择性的结合到“外源基因”启动子区序列上负调控基因转录,从而有效避免外源基因的有害表达,并为这些外源性遗传物质整合到细菌基因组中提供先决条件^[8]。在副溶血弧菌中,已有研究表明:H-NS能直接负调控关键毒力因子T3SS、TDH和T6SS2相关基因的转录,这些基因都可能是通过水平转移获得的^[9-10]。另外,Dor Salomon等利用Western blot实验证明H-NS对副溶血弧菌T6SS1结构蛋白Hcp1(VP1393)的表达也具有负调控作用,但是并未阐明这种负调控机制是否发生在转录水平上,以及其具体的分子机制^[11]。本文中,我们利用Western blot、实时定量RT-PCR、引物延伸及EMSA等实验技术证明了H-NS能直接结合到的启动子区,进而抑制其转录表达。

1 材料和方法

1.1 材料

1.1.1 菌株和抗体:实验所用的副溶血弧菌RIMD2210633株(野生型,WT;1996年从日本旅

游腹泻病人的粪便中分离而得,属O3:K6型大流行菌株,KP⁺,tdh⁺,trh⁻)及其hns非极性突变株(Δ hns)^[10]、His-H-NS重组蛋白表达菌、兔抗Hcp1多克隆抗体等均由北京微生物流行病学研究所病原微生物国家重点实验室保存。

1.1.2 主要试剂:HI肉汤培养基(2.5% Bacto™ heart infusion)购自BD Bioscience; TRIzol Reagent购自Invitrogen公司; Primer Extension System和fmol® DNA Cycle Sequencing System为Promega产品; Micro BCA™ Protein Assay Kit购至Thermo Scientific公司; Taq DNA聚合酶和dNTPs购自Fermentas公司; PCR产物纯化试剂盒购自QIAGEN公司; DNA-free™ Kit购自Amibion公司; LightCycler® 480 SYBR Green Master购自Roche公司; 酶标羊抗兔抗体购自SIGMA公司。

1.2 细菌培养

取15 μL甘油菌种接种于15 mL的HI肉汤中(50 mL的三角烧瓶,下同),37 °C下200 r/min培养12–14 h,按1:50稀释接种至15 mL新鲜的HI肉汤中,37 °C下200 r/min培养至OD₆₂₀≈1.0,再按1:1000稀释接种至15 mL新鲜的HI肉汤中,37 °C、200 r/min培养至OD₆₂₀≈1.0,收集菌体。

1.3 Western blot检测

细菌按1.2的方法培养后,收集菌体,用PBS缓冲液悬起菌体并超声碎菌,4 °C、12000 r/min离心10 min后,弃沉淀取上清,再用Micro BCA™ Protein Assay Kit测定上清中的总蛋白含量。取等总蛋白量的WT和 Δ hns的裂解上清进行12% SDS-PAGE电泳,而后转移至PVDF膜上,以免抗Hcp1多克隆抗体做一抗,荧光标记的羊抗兔抗体做第二抗体,进行免疫印记实验分析^[12],通过比较特异性条带的差异来判定H-NS对的调控关系。

1.4 实时定量RT-PCR

提取WT和 Δ hns的总RNA,用DNA-free™ Kit

(Amibion)消化去除其中可能污染的基因组DNA, 并用N6随机引物将其逆转录成cDNA, 最后用Roche的LightCycler system作实时定量RT-PCR分析。以16S rRNA的表达量为内参, 利用经典的 $2^{-\Delta\Delta C_t}$ 的方法对靶基因的表达进行相对定量^[13]。

1.5 引物延伸实验

采用TRIzol法提取WT和 Δhns 的总RNA, 并通过琼脂糖凝胶电泳监测RNA质量。将与*hcp1*的mRNA互补的特异性引物(见表1) 5'-末端用 $[\gamma\text{-}^{32}\text{P}]$ ATP (5000 Ci/mmol)进行放射性标记^[14]。分别以WT和 Δhns 的总RNA为模板(总RNA的量一致), 利用AMV酶将*hcp1*的mRNA逆转录成cDNA。逆转录产物配伍测序条带[fmol[®] DNA Cycle Sequencing System (Promega)]进行6%聚丙烯酰胺变性胶电泳, 放射自显影后分析结果。

1.6 EMSA实验

PCR扩增16S rRNA编码区和*hcp1* (引物见表1)的启动子区序列并对产物纯化回收, 用T4多聚核苷酸激酶(T4 PNK)对DNA片段5'-末端进行标记, 以制备DNA探针^[14]。利用Ni-NTA柱法纯化His-H-NS蛋白^[14]。将不同浓度的His-H-NS蛋白与DNA探针在10 μ L结合反应体系中, 室温共同孵育20 min后, 进行4%非变性聚丙烯酰胺凝胶中进行电泳, $-20\text{ }^{\circ}\text{C}$ 放射自显影后分析结果。

2 结果和分析

2.1 H-NS负调控*hcp1*的表达

利用Western blot实验研究H-NS对*hcp1*的表达调控, 结果如图1-A, 可以看出: 在 Δhns 中检测出的Hcp1条带明显粗于WT中的, 表明H-NS能抑制

表1. 本文所用引物

Table 1. Oligonucleotide primers used in this study

Target	Primers (5'→3', forward/reverse)
RT-PCR	
<i>hcp1</i>	TCAGTAGGTAACACATGGCAAG/TGGAGAAGAACGGTCTTGAAC
16S rRNA	GACACGGTCCAGACTCCTAC/GTGCTTCTTCTGTCGCTAAC
Primer extension	
<i>hcp1</i>	GAGTTTCACCGTTGATAGAC
EMSA	
<i>hcp1</i>	GCTATCGGGTGTAGACGCTG/GAGTTTCACCGTTGATAGAC
16S rDNA	GACACGGTCCAGACTCCTAC/GTGCTTCTTCTGTCGCTAAC

Hcp1的表达, 这也与Dor Salomon等的结果一致^[11]。进一步利用实时定量RT-PCR的方法研究H-NS对*hcp1*的转录调控关系, 其结果如图1-B所示: 在 Δhns 中检测到的*hcp1* mRNA相对表达丰度明显高于WT中, 且二者具有显著性差异, 这说明H-NS能抑制*hcp1*的转录。总之, H-NS负调控*hcp1*的表达。

2.2 H-NS抑制*hcp1*的启动子区活性

引物延伸实验结果(图2-A)显示*hcp1*有一个位于-62位的转录起始位点T(翻译起始位点为+1),

说明该基因有一个以T(-62)为转录起始位点的启动子区; 另外, 我们在 Δhns 中检测到的引物延伸条带明显浓于WT中的, 这说明H-NS能抑制T(-62)启动子区活性。T(-62)上游含有-12(TTGA)和-24(TGGCACG)元件组成的核心启动子区(图2-B), 说明T(-62)的转录受 σ^{54} 的调节^[15]。总之, T(-62)的转录是H-NS和 σ^{54} 依赖性的。

2.3 H-NS能直接结合到*hcp1*的启动子区

H-NS没有保守的识别基序, 但是对富含AT的DNA序列具有较强的亲和力。*hcp1*启动子区

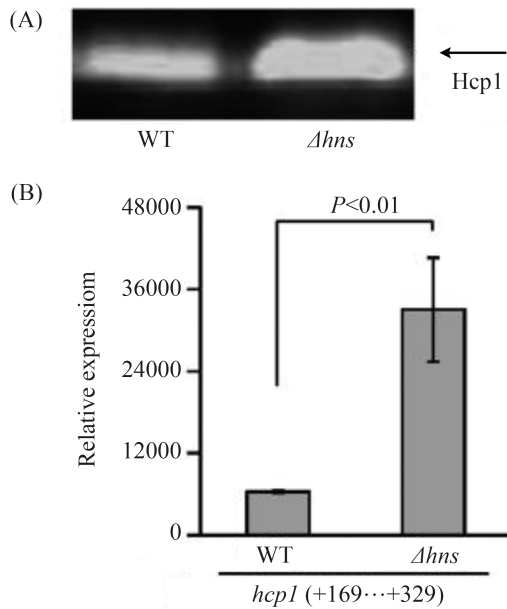


图1. H-NS抑制*hcp1*的表达

Figure 1. Expression of *hcp1* was repressed by H-NS. A: Western blot. The Hcp1 production was detected in WT or Δhns using the anti-Hcp1 polyclonal antibody. B: RT-PCR. The mRNA levels of *hcp1* were compared between Δhns and WT. On the basis of the standard curves of 16S rRNA expression, the relative mRNA level was determined by calculating the threshold cycle (ΔC_t) of the gene using the classic ΔC_t method. The positive numbers indicated the nucleotide position downstream of translation start.

DNA序列的AT含量高达72%，这提示H-NS可能对*hcp1*启动子区具有结合作用。EMSA结果(图3)显示：当His-H-NS浓度达到25.6 pmol时，即可出现阻滞条带，且随着蛋白浓度的增高，阻滞条带也逐渐增强，这说明His-H-NS对*hcp1*启动子区DNA序列的结合是浓度梯度依赖性的；而在所有的蛋白浓度下，阴性对照16S rDNA均未出现阻滞条带，这说明了实验结果的可靠性。总之，H-NS能直接作用于*hcp1*的启动子区。

3 讨论

H-NS是一个小而丰富的拟核相关DNA结合蛋

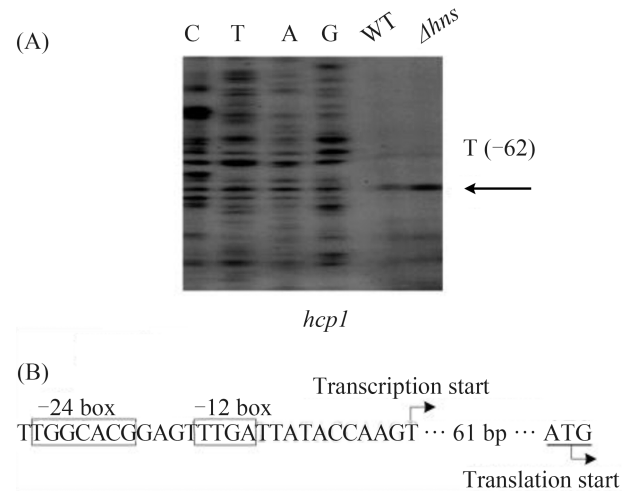


图2. H-NS抑制*hcp1*启动子区活性

Figure 2. H-NS represses promoter activity of *hcp1*. A: Primer extension. An oligonucleotide primer was designed to be complementary to the RNA transcript of *hcp1*. The primer extension products were analyzed with 8 mol/L urea-6% acrylamide sequencing gel. The transcription start site of *hcp1* was indicated by arrow with nucleotide and position. Lanes C, T, A, and G represent the Sanger sequencing reactions. The negative number indicated the nucleotide site upstream of the translation start (+1). B: Structure of *hcp1* core promoter. Based on the primer extension result, organization core promoter of *hcp1* was depicted, in which shown were translation and transcription starts, -24 and -12 core promoter elements for σ^{54} (RpoN) recognition.

白，主要参与细菌拟核基因组装配和调控基因转录。作为转录调控因子，H-NS通常有选择地结合到基因启动子区富含AT的碱基序列上并形成高级的“桥链”结构，从而抑制基因转录^[8]。H-NS是革兰氏阴性菌的整体负调控因子，如在大肠杆菌K-12菌株中，至少有4%的基因转录是受H-NS抑制的，包括许多应激适应性相关基因^[16]。目前的研究表明：H-NS是致病性弧菌毒力因子表达的主要抑制因子。在霍乱弧菌(*V. cholerae*)中，H-NS对*hapA* (编码血凝素/蛋白酶)基因、*tcp* (编码菌毛粘附因子)和*ctx* (编码霍乱毒素)操纵子的转录均具有

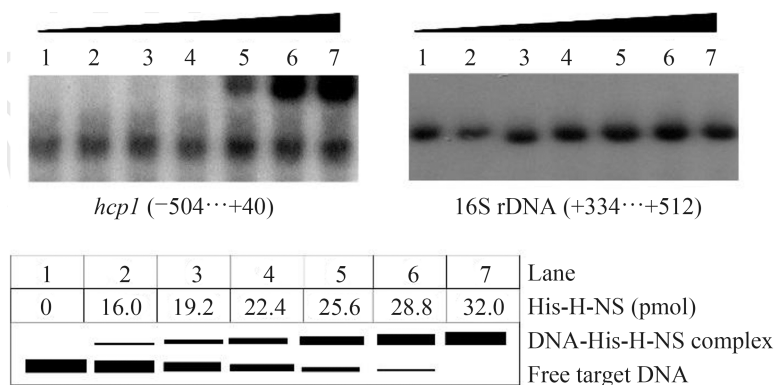


图3. H-NS能直接结合到*hcp1*启动子区

Figure 3. His-H-NS bound to the promoter-proximal DNA region of *hcp1*. The positive and minus numbers in the brackets indicated the nucleotide sites upstream and downstream of the translation start (+1), respectively. Shown below was the schematic representation of the EMSA design.

直接的抑制作用，它们均是霍乱弧菌最重要的毒力因子^[17-18]。在创伤弧菌(*V. vulnificus*)中，H-NS对编码RTX毒素的基因座位*rtxA1*操纵子的转录也具有直接的抑制作用^[19]；在副溶血弧菌(*V. parahaemolyticus*)中，H-NS对主要的毒力因子T3SS1、T3SS2、TDH和T6SS2相关基因的转录均具有直接的抑制作用^[9-10]。另外，Dor Salomon等利用Western blot实验证明H-NS对T6SS1相关基因的表达也具有抑制作用^[11]，但是具体的机制未被阐明。本文中，我们首先利用Western blot和实时定量RT-PCR实验(图1)证明H-NS负调控T6SS1结构蛋白基因*hcp1* (VP1393)的转录；随后的引物延伸结果(图2)表明*hcp1*有一个 σ^{54} 依赖的转录起始位点T (-62)，该位点的转录受H-NS的抑制。H-NS对富含AT的DNA序列具有较强的亲和力，而*hcp1*启动子区DNA序列的AT含量高达72%，这提示H-NS可能对*hcp1*具有直接的调控作用，EMSA结果(图3)显示H-NS确实能直接作用于*hcp1*的启动子区。

T6SS1由副溶血弧菌大染色体编码的跨膜“针状”注射装置，主要具有细胞粘附活性和对他种细菌的杀菌活性^[5]。T6SS1的表达受周围生长环境的调控，在模拟海洋环境条件(2% NaCl和23-30 °C)下表达水平最高^[5]。密度感应(Quorum sensing,

QS)系统对T6SS1也具有调控作用：当细菌处于低密度生长时，QS系统低密度下的核心调控子AphA对T6SS1相关基因的转录(我们未发表数据)具有间接的抑制作用；当细菌处于高密度生长时，QS系统高密度下的核心调控子OpaR也能间接抑制T6SS1相关基因的表达^[20]。另外，位于T6SS1基因座位内的调控子VP1391和VP1407对T6SS1相关基因的转录均具有激活作用^[11]。本文的研究结果表明H-NS对T6SS1结构蛋白基因*hcp1* (VP1393)的转录具有直接的抑制作用。可见，副溶血弧菌的T6SS1的表达是一个被多种因素紧密调控的过程。

副溶血弧菌的H-NS对靶基因启动子区的结合序列长度都在100 bp以上，并通常覆盖 σ 因子结合位点和转录起始位点，或位于转录起始位点之后，从而起负调控作用^[10]。因此，我们推测H-NS对*hcp1*启动子区的结合位点很可能覆盖-12和-24元件及转录起始位点T (-62)(图2-A)，从而抑制其转录。除VP1393-1406操纵子外，副溶血弧菌T6SS1 (VP1386-1414)至少还包含5个操纵子^[21]，即VP1386-1387、VP1388-1390、VP1392-1391、VP1409-1407和VP1410-1414，H-NS对这些操纵子基因是否具有调控作用，以及具体的调控机制值得我们后续继续深入研究。

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Transcriptional regulation of *hcp1* by H-NS in *Vibrio parahaemolyticus*

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Abstract: [Objective] To study the transcriptional regulation of the structural components Hcp1 by H-NS in *Vibrio parahaemolyticus*. [Methods] Expression of Hcp1 in the wide-type (WT) strain and *hns* mutant (Δhns) were detected by Western blot using rabbit anti-Hcp1 polyclonal antibodies. Total RNAs were extracted from WT and Δhns strains. Quantitative RT-PCR was carried out to calculate the transcriptional variation of *hcp1* between WT and Δhns strains, and then primer extension assay was used to detect the transcription start site and the promoter activity (the amount of primer extension product) of *hcp1* in WT and Δhns . The entire promoter region of *hcp1* was amplified by PCR with *ExTaq*TM DNA polymerase using WT genomic DNA as the template. The over-expressed His-H-NS was purified under native conditions with nickel loaded HiTrap Chelating Sepharose columns (Amersham). Electrophoretic mobility shift assay (EMSA) was applied to analyze the DNA-binding activity of His-H-NS to *hcp1* promoter region *in vitro*. [Results] Western blot and quantitative RT-PCR results showed that expression of Hcp1 was inhibited by H-NS. The primer extension assay detected only one transcription start site located at 62 bp upstream of *hcp1*, whose transcription was H-NS and σ^{54} -dependent. EMSA result indicated that His-H-NS was able to bind the promoter DNA region of *hcp1*. [Conclusion] The expression of *hcp1* was directly repressed by H-NS in *V. parahaemolyticus*.

Keywords: *Vibrio parahaemolyticus*, H-NS, *hcp1*, transcriptional regulation

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