Research Article

Activation of CXCL-8 Transcription by Hepatitis E Virus ORF-1 via AP-1

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Hepatitis E virus (HEV) is a small nonenveloped single-stranded positive-sense RNA virus and is one of the major causes for acute hepatitis worldwide. CXCL-8 is a small multifunctional proinflammatory chemokine. It was reported recently that HEV infection significantly upregulates CXCL-8 gene expression. In this study, we investigated the mechanism of HEV-induced CXCL-8 transcriptional activation. Using CXCL-8 promoter reporters of different lengths ranging from −1400 to −173, we showed that −173 promoter has the highest promoter activity in the presence of HEV genomic RNA, indicating that the −173 promoter contains sequences responsible for CXCL-8 activation by HEV. Ectopic expression of the ORF-1 protein can upregulate the −173 CXCL-8 promoter activity. In contrast, expression of the ORF-2 protein suppresses the CXCL-8 promoter activity and expression of the ORF-3 protein has no effect on the CXCL-8 promoter activity. We further showed that AP-1 is required for CXCL-8 activation because neither HEV genomic RNA nor the ORF-1 protein can upregulate the −173 CXCL-8 promoter in the absence of the AP-1 binding sequence. Taken together, our results showed that HEV and HEV ORF-1 protein activate the CXCL-8 promoter via AP-1. This novel function of HEV ORF-1 protein should contribute to our understanding of HEV-host interactions and HEV-associated pathogenesis.

1. Introduction

Hepatitis E virus (HEV), first identified in 1983, is a small nonenveloped single-stranded positive-sense RNA virus. It belongs to the genus *Hepevirus* in the Hepeviridae family and is classified into four genotypes [1, 2]. According to WHO, about two billion people are estimated to be infected with HEV with 14 million people showing clinical symptoms [3, 4]. HEV mainly causes self-limiting acute hepatitis, but the mortality rate is as high as 15–25% among pregnant women. Chronic hepatitis develops exclusively in immunocompromised patients infected with genotype 3 or 4 HEV [5–7]. HEV genome is approximately 7.2 kb and consists of three partially overlapping open reading frames (ORFs) flanked by short 5′ and 3′ untranslated regions [8, 9].

ORF-1 encodes a nonstructural polyprotein that can be cleaved into methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase, which are essential for virus replication. ORF-2 encodes the viral capsid protein which contains neutralizing epitopes and ORF-3 encodes a small phosphoprotein that functions in virion morphogenesis, release, and pathogenesis [10–12].

CXCL-8, also known as interleukin-8, is a small multifunctional proinflammatory chemokine in the CXC chemokine family, which plays an important role in immune defence processes [13–15]. CXCL-8 is secreted at the inflammatory sites by different cells, such as neutrophils, macrophages, monocytes, and endothelial cells. CXCL-8 primarily targets the polymorphonuclear leukocytes which act as the first line of immune defence [16, 17]. Furthermore, CXCL-8 can also

induce tumorigenesis, metastasis, and angiogenesis [17, 18]. CXCL-8 gene transcription can be regulated by NF-κB and activator protein-1 (AP-1) [19].

Since it was recently shown that HEV infection significantly upregulates CXCL-8 gene expression [20], we investigated how HEV mediates CXCL-8 transcriptional activation. We showed that HEV genomic RNA and the ORF-1 protein upregulated CXCL-8 promoter-mediated transcription via AP-1.

2. Material and Methods

2.1. Plasmids. A cell culture adapted HEV genotype 3 Kernow C1 strain cDNA clone was received from Dr. Emerson [21]. The coding sequences of ORF-1, ORF-2, and ORF-3 were cloned into a pEF 3xFlag vector. Human CXCL-8 promoter-luciferase reporters of different lengths, −1400, −500, −230, −193, and −173, were received from Dr. Buendia [22]. Mutant −173 CXCL-8 promoters with mutations for the AP-1 binding sequence ($^{-126}$TGACTCA^{-120} to $^{-126}$TatCTCA^{-120}, mAP-1) [23] or the negative regulatory *cis*-element sequence (NRE) ($^{-76}$ATTTCCTCTGA^{-66} to $^{-76}$ATTTCCcCcGA^{-66}, mNRE) [24] were generated by site-directed mutagenesis. The AP-1-luciferase reporter was generated by inserting seven tandem repeats of the AP-1 binding sequence into the pGL4.27 vector (Promega). All plasmids were verified by DNA sequencing.

2.2. In Vitro Transcription. HEV genomic RNA was produced by *in vitro* transcription from linearized HEV cDNA using the mMESSAGE mMACHINE T7 kit (Ambion).

2.3. Cell Lines and Transfection. Human hepatoma HuH-7 and human embryonic kidney HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) with 10% (v/v) fetal bovine serum (FBS, Life Technologies) at 37°C and 5% CO_2. For luciferase assays, cells in 24-well plates were cotransfected with 1 μg of HEV RNA and 1 μg of luciferase reporter DNA using the jetPEI reagent (Polyplus), or with 0.5 μg of plasmid DNA expressing HEV ORF proteins and 0.5 μg of luciferase reporter DNA using the calcium phosphate precipitation method [25]. In the immunoprecipitation experiments, cells in 6-well plates were transfected with 2 μg of plasmid DNA expressing HEV ORF proteins.

2.4. Luciferase Assay. Cells were lysed in Passive Lysis Buffer (Promega) and the luciferase activity was measured using a Luciferase Assay System (Promega). Luciferase activity was normalized to the protein concentration quantified using the Bradford assay reagent (Bio-Rad).

2.5. Immunoprecipitation, Western Blotting, and Antibodies. Cell lysates in RIPA buffer were immunoprecipitated with an anti-Flag antibody (Sigma-Aldrich) using the SureBeads magnetic beads system (Bio-Rad). Precipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in 5% skim milk in PBS for 1 hour and incubated with the anti-Flag antibody
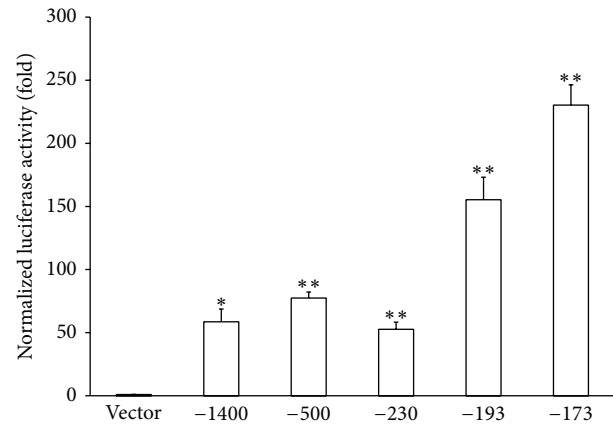

FIGURE 1: CXCL-8 promoter activities in the presence of HEV genomic RNA. HuH-7 cells were cotransfected with HEV genomic RNA and CXCL-8 promoter-luciferase reporters of different lengths or a luciferase reporter vector without the CXC-8 promoter. Luciferase assay was performed 48 hours after transfection. Luciferase activity was normalized against the protein concentration of the same samples. ∗: $p \leq 0.05$; ∗∗: $p \leq 0.01$ by Student's t-test.

overnight at 4°C. After washing with PBS, the blots were incubated with an infrared dye-labeled secondary antibody (Li-Cor Biosciences) for 1 hour at room temperature and then washed again. The blot was scanned using Odyssey Infrared Imaging System (Li-Cor Biosciences).

2.6. Statistical Analysis. All experiments were performed three times in triplicate and differences between samples were assessed by Student's t-test. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Determination of CXCL-8 Promoter Activities in the Presence of HEV Genomic RNA. A recent study showed that HEV infection can induce inflammatory cytokines and chemokines, including CXCL-8 [20]. To determine the minimum region of CXCL-8 promoter required for HEV enhanced CXCL-8 transcription, HuH-7 cells were cotransfected with HEV genomic RNA and CXCL-8 promoter-luciferase reporters of different lengths. The HEV genomic RNA can replicate and generate infectious HEV in cell culture [21]. Luciferase activity, as an indicator of CXCL-8 promoter activity, was measured 48 hours after transfection. As shown in Figure 1, we observed substantial luciferase activities when transcribed from the −1400, −500, −230, −193, and −173 CXCL-8 promoters. The −173 bp promoter displayed the highest promoter activity, suggesting that the −173 promoter contains the sequences necessary for CXCL-8 promoter activation by HEV.

3.2. CXCL-8 Promoter Activation by HEV ORF-1 via AP-1. HEV encodes three proteins. To determine which HEV protein is responsible for activating the CXCL-8 promoter, we
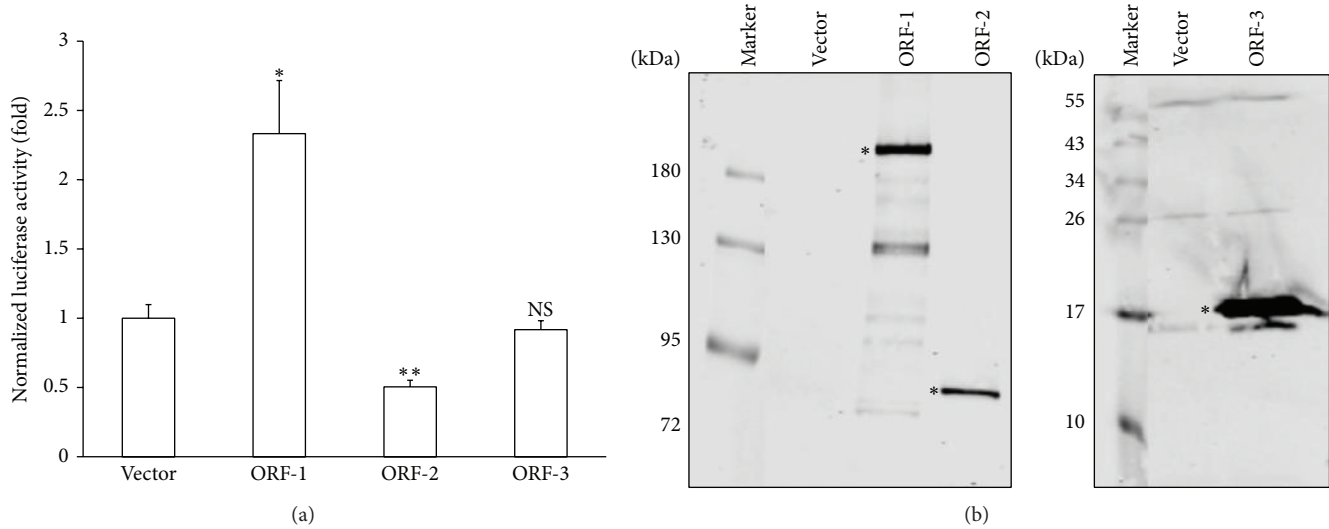
FIGURE 2: CXCL-8 transcriptional activation by HEV ORF-1. (a) HuH-7 cells were cotransfected with plasmid vector, or plasmids expressing HEV ORF-1, ORF-2, or ORF-3 proteins and the −173 CXCL-8 promoter-luciferase reporter. Luciferase assay was performed 48 hours after transfection. Luciferase activity was normalized against the protein concentration of the same samples. ∗: $p \leq 0.05$, ∗∗: $p \leq 0.01$, and NS: not significant by Student's t-test. (b) The expression of HEV ORF-1, ORF-2, or ORF-3 proteins with a N-terminal Flag-tag after transfection into HEK293 cells was analyzed by immunoprecipitation followed by Western blotting with an anti-Flag antibody. The location of HEV proteins was indicated by ∗ in the blot.

cotransfected HuH-7 cells with plasmids expressing the ORF-1, ORF-2, or ORF-3 proteins, or the vector control, together with the −173 CXCL-8 promoter reporter. As shown in Figure 2(a), the expression of the ORF-1 protein significantly increased CXCL-8 promoter activity in comparison to vector control, whereas the ORF-2 protein inhibited and the ORF-3 protein had no effect on CXCL-8 promoter activity. The expression of HEV proteins was confirmed by immunoprecipitation followed by Western blotting (Figure 2(b)). These results demonstrate that HEV ORF-1 protein can activate the CXCL-8 promoter.

The −173 CXCL-8 promoter contains the binding sequence for AP-1 [23]. To investigate the role of AP-1, the AP-1 binding sequence was removed from the CXCL-8 promoter by mutagenesis. As shown in Figure 3(a), the ORF-1 protein could no longer activate the mAP-1 CXCL-8 promoter. As a control, we used another mutant CXCL-8 promoter with the NRE sequence removed. HEV ORF-1 protein could activate the mNRE CXCL-8 promoter to a comparable level as to the wild-type CXCL-8 promoter (Figures 3(a) and 2(a)). These results demonstrate that AP-1 plays a role in CXCL-8 promoter activation by HEV ORF-1 protein.

3.3. AP-1 Upregulation by HEV ORF-1. Since the AP-1 binding sequence within the CXCL-8 promoter was shown to be required for activation by HEV ORF-1 protein, we hypothesized that HEV ORF-1 protein can directly increase transcription mediated by AP-1. We therefore cotransfected HuH-7 cells with a HEV ORF-1 protein expressing plasmid and a luciferase reporter plasmid with seven tandem repeats of the AP-1 binding sequence. Figure 3(b) shows that the expression of HEV ORF-1 protein resulted in greater than threefold

increase in the luciferase activity expressed downstream from the tandem AP-1 motifs when compared with basal promoter control. These results suggest that HEV ORF-1 protein can indeed upregulate AP-1-mediated transcription.

3.4. CXCL-8 Promoter Activation by HEV Genomic RNA Requires AP-1. Although we showed that AP-1 is involved in CXCL-8 promoter activation by HEV ORF-1, it was important to demonstrate that transcriptional activation of the CXCL-8 promoter by HEV also requires AP-1. To address this question, we cotransfected HuH-7 cells with the HEV genomic RNA, together with wild-type or mAP-1 −173 CXCL-8 promoter-luciferase reporters. Results showed that elimination of the AP-1 binding sequence resulted in a significantly lower luciferase activity in comparison to the wild-type promoter (Figure 4). This finding demonstrates the importance of AP-1 in CXCL-8 promoter activation by HEV.

4. Discussion

Inflammatory cytokines and chemokines play a critical role in the pathology associated with viral infections. Although elevated inflammatory cytokine/chemokine levels are detected in HEV-associated liver failure patients [26], there is very limited information on how HEV regulates these genes at the molecular level. Devhare et al. showed upregulation of several inflammatory cytokine/chemokine genes and downregulation of interferons after HEV infection in a cell culture model [20]. Consistently, we showed that transfection of a HEV genomic RNA is associated with substantial CXCL-8 transcription in a promoter-luciferase reporter assay. We further demonstrated that the ORF-1 protein, but not the
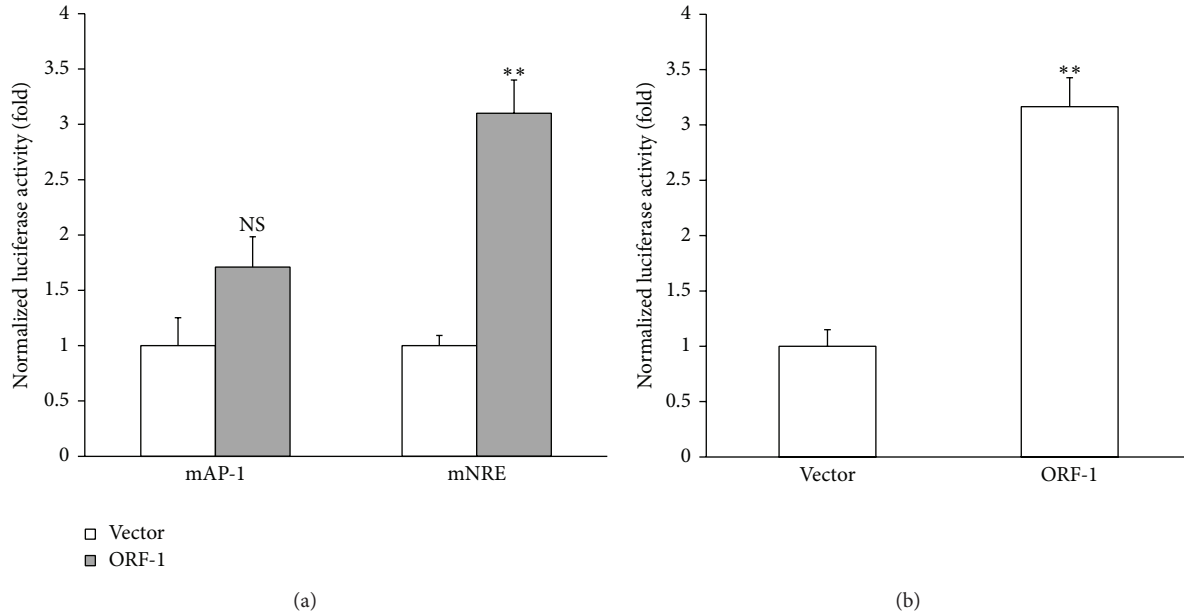
(a)

(b)

FIGURE 3: AP-1 is involved in CXCL-8 activation by HEV ORF-1. HuH-7 cells were cotransfected with plasmid vector or a plasmid expressing HEV ORF-1 protein, together with −173 mAP-1 or mNRE CXCL-8 promoter-luciferase reporters (a) or a luciferase reporter with seven repeats of the AP-1 binding sequences (b). Luciferase assay was performed 48 hours after transfection. Luciferase activity was normalized against the protein concentration of the same samples. $**$: $p \leq 0.01$; NS: not significant by Student's t-test.
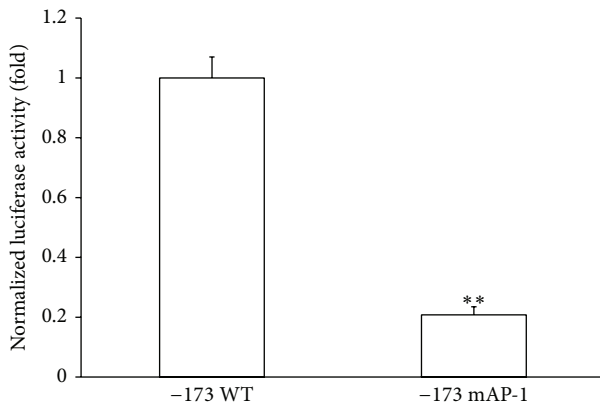

FIGURE 4: AP-1 is required for CXCL-8 activation by HEV genomic RNA. HuH-7 cells were cotransfected with HEV genomic RNA, together with the wild-type or mAP-1 −173 CXCL-8 promoter-luciferase reporters. Luciferase assay was performed 48 hours after transfection. Luciferase activity was normalized against the protein concentration of the same samples. $**$: $p \leq 0.01$ by Student's t-test.

ORF-2 or ORF-3 proteins, can activate CXCL-8 promoter. In addition, we identified the requirement of AP-1 in CXCL-8 transcription activation by both HEV genomic RNA and the ORF-1 protein.

Amongst the CXCL-8 promoters of different lengths ranging from −1400 to −173, the −193 and −173 promoters exhibit higher degree of activities in the presence of HEV RNA (Figure 1). The mechanisms of this differential activation are not fully understood. The activity of a promoter is determined by the interplay of positive and negative factors involved. The binding motifs for multiple transcriptional

factors, such as IRF-1, HNF-1, and GR, are located between the −1400 and −173 promoter region [22]. Therefore, it is possible that multiple transcription factors are involved in regulating CXCL-8 transcription by HEV. Furthermore, a few mechanisms, such as histone deacetylation and OCT-1 binding, have been shown to inhibit the CXCL-8 promoter activity [27]. Whether these mechanisms play a role in CXCL-8 promoter regulation by HEV requires further investigation.

The ORF-1 protein, essential for HEV RNA replication, consists of a few domains which can function as methyltransferase, cysteine protease, RNA helicase, and RNA-dependent RNA polymerase [6]. It would be interesting to determine which domains of ORF-1 are responsible for activating CXCL-8 transcription. The cysteine protease domain and the adjacent X domain have been shown to inhibit interferon transcription [28]. Thus, the ORF-1 protein can both upregulate inflammatory chemokine expression and downregulate interferon expression after HEV infection.

The capsid ORF-2 protein, when expressed as an intracellular protein, was found to inhibit CXCL-8 transcription (Figure 2(a)). Previous studies have shown that the UV-inactivated HEV virus, but not soluble ORF-2 protein, induces inflammatory cytokines and chemokines [20]. This indicates that the ORF-2 protein has different functional properties as a component of the viral capsid compared with an intracellular protein. Further studies are required to study the functions of the ORF-2 protein.

5. Conclusions

We showed that HEV genomic RNA and the ORF-1 protein significantly enhance CXCL-8 promoter activity which

requires the transcription factor AP-1. This newly identified function of the ORF-1 protein should help understand the molecular mechanisms of HEV-host interactions and HEV-associated pathogenesis.

Disclosure

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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