



Involvement of PUF60 in transcriptional and post-transcriptional regulation of hepatitis B virus pregenomic RNA expression

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	作成者: 孫, 鎖鋒	
	メールアドレス:	
	所属:	
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2	Regulation of Hepatitis B Virus Pregenomic RNA Expression
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4	Suofeng Sun, ¹ Kenji Nakashima, ¹ Masahiko Ito, ¹ Yuan Li, ¹ Takeshi Chida, ¹
5	Hirotaka Takahashi, ² Koichi Watashi, ³ Tatsuya Sawasaki, ² Takaji Wakita, ³
6	Tetsuro Suzuki ^{1*}
7	
8	¹ Department of Virology and Parasitology, Hamamatsu University School of
9	Medicine, Shizuoka 431-3192, Japan
10	² Proteo-Science Center, Ehime University, Ehime, 790-8577, Japan
11	³ Department of Virology II, National Institute of Infectious Diseases, Tokyo
12	162-8640, Japan
13	
14	
15	Address correspondence to:
16	Tetsuro Suzuki, E-mail: tesuzuki@hama-med.ac.jp
17	Department of Virology and Parasitology, Hamamatsu University School of
18	Medicine,
19	1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan
20	Tel: +81-53-435-2336
21	Fax: +81-53-435-2338
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25 Abstract

26	Here we identified PUF60, a splicing factor and a U2 small nuclear
27	ribonucleoprotein auxiliary factor, as a versatile regulator of transcriptional and
28	post-transcriptional steps in expression of hepatitis B virus (HBV) 3.5 kb,
29	precore plus pregenomic RNA. We demonstrate that PUF60 is involved in: 1)
30	up-regulation of core promoter activity through its interaction with transcription
31	factor TCF7L2, 2) promotion of 3.5 kb RNA degradation and 3) suppression of
32	3.5 kb RNA splicing. When the 1.24-fold HBV genome was introduced into
33	cells with the PUF60-expression plasmid, the 3.5 kb RNA level was higher at
34	days 1–2 post-transfection but declined thereafter in PUF60-expressing cells
35	compared to viral replication control cells. Deletion analyses showed that the
36	second and first RNA recognition motifs (RRMs) within PUF60 are responsible
37	for core promoter activation and RNA degradation, respectively. Expression of
38	PUF60 mutant deleting the first RRM led to higher HBV production.
39	To our knowledge, this is the first to identify a host factor involved in not only
40	positively regulating viral gene expression but also negative regulation of the
41	same viral life cycle. Functional linkage between transcriptional and
42	post-transcriptional controls during viral replication might be involved in
43	mechanisms for intracellular antiviral defense and viral persistence.
44	(199 words)
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48 Introduction

Hepatitis B virus (HBV) is a hepatotropic, enveloped virus of the 49 Hepadnaviridae family with a partial double-stranded relaxed circular DNA 50 genome. Approximately 240 million people worldwide are chronically infected 51 52 with HBV. It is estimated that one million deaths occur annually due to HBV-related severe liver diseases such as liver cirrhosis, liver failure and 53 hepatocellular carcinoma¹. Although nucleoside analogues and interferons 54 55 are the major chemotherapies for HBV-positive patients to date, they do not 56 achieve HBV clearance or eliminate the viral genome when in the covalently 57 closed circular (ccc) DNA form, which resides in the nucleus of infected cells. 58 Long-term treatment with these antivirals may also have drawbacks such as development of drug-resistant variants and adverse side effects ^{2,3}. Thus, 59 60 understanding of molecular mechanisms that determine viral replication, 61 persistence and latency is urgently needed to develop novel treatments to achieve virological cure. 62 Upon infection, the uncoated viral genome is transported to the nucleus 63

64 and converted into cccDNA, which serves as the template for synthesis of viral transcripts. Four unspliced viral RNAs, 3.5, 2.4, 2.1 and 0.7 kb, are transcribed 65 66 from their respective promoters and two enhancer regions (ENI and ENII). The 3.5 kb RNA includes precore and pregenomic RNA species. Precore mRNA 67 encodes precore antigen (HBeAq), and pregenomic RNA directs translation of 68 69 core antigen (HBcAg) and polymerase. Pregenomic RNA also serves as a 70 reverse transcription template after encapsidation. A variety of liver-enriched and ubiquitous transcription factors target the promoter and enhancer regions 71

72	to regulate viral transcription and replication (as reviewed in ^{4,5}). In addition,
73	several forms of spliced RNAs are generated from 3.5 kb RNA. These spliced
74	forms have been observed in sera and livers of hepatitis B patients as well as
75	in cultured cells transfected with the viral genome ⁶⁻⁸ . However, their
76	significance and regulatory mechanisms underlying post-transcriptional
77	processing events in the HBV life cycle are essentially unclear.
78	In this study, we aimed to clarify molecular mechanisms controlling
79	transcriptional and post-transcriptional processes during HBV replication, in
80	particular mechanistic coupling between transcriptional regulation and
81	post-transcriptional mRNA processing. During the course of investigating
82	involvement of host cell factors with dual DNA- and RNA-binding capacities in
83	HBV replication in siRNA-mediated gene knockdown and over-expression
84	experiments, we identified PUF60 as a versatile regulator of both
85	transcriptional and post-transcriptional steps in expression of HBV 3.5 kb RNA.
86	PUF60 was first discovered as a poly-U binding, 60-kDa splicing factor that is
87	important for efficient splicing of multiple introns ⁹ . In addition, PUF60 forms a
88	complex with far upstream element (FUSE) and FUSE-binding protein (FBP),
89	acting as an FBP-interacting repressor (FIR), and is a transcriptional repressor
90	of human c-myc gene ¹⁰ . Here, we found that PUF60 up-regulated core
91	promoter activity through its interaction with transcription factor 7-like 2
92	(TCF7L2), which is necessary for direct binding with the ENII region. PUF60
93	also contributed to 3.5 kb RNA degradation and suppression of 3.5 kb RNA
94	splicing.

97 **Results**

98

99 Involvement of PUF60 in positive and negative regulation on HBV

100 replication

101 First, to address how PUF60 is involved in gene expression and HBV 102 replication, viral RNAs in cells co-transfected with pUC-HB-Ce carrying the 103 1.24-fold HBV genome derived from genotype C and the FLAG-tagged 104 PUF60-expressing plasmid (pcDNA-F-PUF60) were analyzed by northern 105 blotting. At day 2 post-transcription (pt), the level of 3.5 kb RNA, but not 3.5 kb 106 RNA -derived, 2.2-kb spliced (Sp1) RNA lacking intron nt 2447/489, was higher 107 in PUF60-expressing cells compared to control cells. In contrast, at day 4 pt, 108 both 3.5 kb RNA and Sp1 RNA levels were severely diminished in 109 PUF60-expressing (Fig. 1a, probe PG). Influence of PUF60 expression on the HBs RNA level appeared limited compared to that on 3.5 kb RNA and Sp1 110 RNA levels (Fig. 1a, probe S). We confirmed that no cytotoxic effect was 111 observed by over-expressing PUF60, as judged by ribosomal RNA levels (Fig. 112 1a) and quantification of cellular RNAs (data not shown). In semi-quantitative 113 114 (Fig. 1b) and quantitative (Fig. 1c, Supplementary Fig. S1) RT-PCR analyses, both a marked increase and decrease in the 3.5 kb RNA level were observed 115 in PUF60-expressing cells at days 2 and 4 pt, respectively, compared to data 116 obtained from northern blotting. No contamination of the transfected HBV 117 118 plasmids in our RNA preparation was confirmed by no detection of amplified DNA without reverse transcription (Fig. 1b). At both time points, Sp1 RNA 119

levels in PUF60-expressing cells were lower than those in control cells. Levels 120 of HBV proteins such as HBs and HBc were also lower in the culture of 121 122 PUF60-expressing cells (Supplementary Fig. S2). Immunoblotting showed that 123 similar levels of PUF60, detectable as monomer and SDS-resistant dimer forms¹¹, were expressed at days 2 and 4 pt in cells (Fig. 1b). Nuclear and 124 125 cytoplasmic fractions of cells transfected with pUC-HB-Ce with or without 126 pcDNA-F-PUF60 were isolated and 3.5 kb RNA levels in each fraction were 127 determined.. At day 1 pt, PUF60 expression resulted in a marked increase in 128 the nuclear 3.5 kb RNA level (Fig. 1d, left). In contrast, at day 4 pt, PUF60 129 expression led to significantly (p<0.01) low 3.5 kb RNA levels in both the 130 nucleus and cytoplasm (Fig. 1d, right). Isolation of the nuclear and cytoplasmic 131 fractions was confirmed by immunoblotting to detect each marker protein 132 (Supplementary Fig. S3). A dose-dependent increase and decrease in the 3.5 133 kb RNA level by PUF60 expression from various concentrations of plasmids transfected were also observed (Fig. 1e). Impact of PUF60 on the 3.5 kb RNA 134 expression was further assessed in other HBV genotypes (Fig. 1f). Increased 135 3.5 kb RNA levels at day 2 pt and subsequent decreased levels at day 4 pt in 136 HBV-replicating cells with PUF60 expression were detected not only in HBV 137 138 genotype C, but also HBV genotypes A and B. PUF60 is known as a member of the U2 small nuclear ribonucleoprotein 139 auxiliary factor (U2AF) family and contains two canonical RNA recognition 140

141 motifs (RRMs) at its N-terminal (aa 129-207) and central (aa 226-304) regions.

142 An additional unusual RRM called the U2AF homology motif (UHM) ¹² is

located at the C-terminus (aa 462-549) of PUF60. To identify the regions in

PUF60 responsible for its effects on increased and decreased 3.5 kb RNA 144 expression, PUF60 deletion mutants, pcDNA-F-PUF60-D1, -D2 and -D3, 145 which encode PUF60 lacking one of the motifs (PUF60-D1, -D2 and -D3) 146 147 indicated above, respectively, with a FLAG tag were constructed (Fig. 2a). The 148 subcellular localization of wild-type and mutant PUF60 strains was determined 149 by immunostaining with anti-PUF60 antibody (Fig. 2b). Wild-type PUF60 150 mainly localized to the nucleus and was partly present in the cytoplasm. The 151 mutants PUF60-D1 and -D3 also mainly localized to the nucleus. In contrast, 152 PUF60-D2 expression showed a homogeneous cytoplasmic distribution. It is 153 thus likely that the second RRM, but not the first RRM and UHM within PUF60, 154 is critical for its nuclear localization. 155 Effects of over-expression of each PUF60 deletion mutant on 3.5 kb RNA 156 expression at days 2 and 4 pt were tested in cells co-transfected with 157 pUC-HB-Ce (Fig. 2c). At day 2 pt, 3.5 kb RNA levels in cells expressing wild-type PUF60, PUF60-D1 or -D3 were 5- to 6-fold higher than that in control 158 159 cells. In contrast, the 3.5 kb RNA level in cells expressing PUF60-D2 was comparable to that of control cells. At day 4 pt, although expression of 160 wild-type PUF60, PUF60-D2 or -D3 led to decreases in the 3.5 kb RNA level, 161 162 increased 3.5 kb RNA levels (e.g., 3-fold higher compared to that of the control) was maintained in cells expressing PUF60-D1. These results strongly suggest 163 that the central (second) and N-terminal (first) RRMs, respectively, are 164 165 important for up-regulation of 3.5 kb RNA expression at early time points after 166 introduction of the HBV genome (day 2 pt) and its subsequently decreased

effect on 3.5 kb RNA observed at a later time point (day 4 pt).

168 Next, to determine the effect of PUF60 on HBV production,

169	particle-associated HBV DNA in culture supernatants of cells transfected with
170	pUC-HB-Ce with or without pcDNA-F-PUF60 was quantitatively measured (Fig.
171	3a). The results were comparable to those of 3.5 kb RNA in cells (Fig. 1a, b
172	and c). Although the viral DNA level at day 2 pt was 2.5-fold higher in the
173	culture with PUF60 expression compared to that of the control culture, the
174	DNA level in the supernatant of PUF60-expressing cells at day 5 pt was 5-fold
175	lower than that of control cells. Collectively, these findings suggest
176	involvement of PUF60 in both positive and negative regulation of HBV
177	replication.
178	We next used the HBV infection system with NTCP-expressing HepG2
179	cells, HepG2-hNTCP-C4 cells ¹³ , to assess the influence of PUF60 on viral
180	infection (Fig. 3b). After 12 h of transfection with pcDNA-F-PUF60 or an empty
181	vector, HepG2-hNTCP-C4 cells were inoculated with HBV prepared from the
182	culture supernatant of HepG38.7-Tet ¹⁴ and cultured for 5 days. Total cellular
183	RNA levels were then determined by reverse-transcription quantitative PCR
184	(RT-qPCR). As expected, both 3.5 kb RNA and Sp1 RNA levels in infected
185	cells with PUF60 expression were significantly lower than those in control
186	infected cells. At earlier time points, such as day 2 post-infection, it was difficult
187	to assess the influence of PUF60 expression since basal levels of HBV RNAs
188	were quite low in this setting.
189	

190 PUF60 as a positive regulator of HBV core promoter activity

Based on the positive effect of PUF60 on 3.5 kb RNA, in particular at the

192	nuclear level, at the early phase pt (Fig. 1a, b, c and d), we next investigated
193	whether PUF60 plays a role in transcriptional regulation of 3.5 kb RNA. Effect
194	of PUF60 on HBV promoter activities was analyzed by transfection of HuH-7
195	cells with a luciferase reporter carrying either the entire core promoter (nt
196	900-1817), ENII/basal core promoter (BCP) (nt 1627-1817), preS1 promoter
197	(nt 2707-2847) or preS2/S promoter (nt 2937-3204) with or without
198	pcDNA-F-PUF60. Reporter activities in the cells were measured at 24 h pt (Fig.
199	4a). The activities of both the entire core promoter and ENII/BCP were
200	significantly higher in cells over-expressing PUF60. In contrast, preS1 and
201	preS2/S promoter activities were not affected by PUF60 expression. PUF60
202	also had little or no influence on human ubiquitin C promoter and human
203	elongation factor 1α promoter activities. At day 4 pt, no significant effect of
204	PUF60 expression on core promoter activity was observed (Supplementary
205	Fig. S4). Effects of PUF60 knockdown on core promoter activity and 3.5 kb
206	RNA expression were further assessed (Fig. 4b). As expected, at day 2 pt,
207	siRNA-based silencing of PUF60 reduced both promoter activity and the 3.5 kb
208	RNA level. In contrast, at day 4 pt, PUF60 knockdown led to a marginal effect
209	on the core promoter activity but, somewhat unexpectedly, reduced the 3.5 kb
210	RNA level (Supplementary Fig. S5). Knockdown efficiency of PUF60 gene was
211	confirmed by immunoblotting (Supplementary Fig. S6).
212	PUF60 deletion experiments (Fig. 2) identified protein regions critical for
213	positive and negative regulation of 3.5 kb RNA expression by PUF60. Effect of
214	PUF60 deletions on activation of the core promoter was further assessed (Fig.
215	4c). Consistent with the result shown in Fig. 2c, the increase in core promoter

activity induced by PUF60 expression was cancelled with expression of
PUF60-D2. Thus, it appears that up-regulation of core promoter activity
mediated by the central RRM within PUF60 led to an increase in the 3.5 kb
RNA level seen at day 2 pt with the PUF60-expressing plasmid.

220

Involvement of TCF7L2 in up-regulation of core promoter activity

222 potentially via interaction with the ENII region and PUF60

223 To address the molecular mechanism underlying PUF60 regulation of the 224 core promoter, a series of reporter constructs with partial ENII/BCP deletions 225 were first generated (Fig. 5, left) to identify the element(s) responsible within 226 the ENII/BCP sequence for transcriptional regulation by PUF60. Luciferase 227 activities were determined by co-transfection of HuH-7 cells with or without 228 pcDNA-F-PUF60 (Fig. 5, right). Although most deletions tested maintained the 229 increase in reporter activity by PUF60 expression, HBenIIcp-del-5 (deletion of nt 1689-1726) and -6 (deletion of nt 1710-1742) cancelled the effect by PUF60. 230 This result indicates that the nt 1689-1742 region, located in ENII, is important 231 for transcriptional regulation of 3.5 kb RNA mediated by PUF60. 232 233 From our transcription factor database search, six transcription-related 234 proteins, HNF1 α , SRY, TCF7L2, SP1, FOXM1 and KLF5, were predicted to be possible binding factors within the nt 1689-1742 region of ENII. Thus, whether 235 these proteins and PUF60 are able to bind directly to the sequence was 236 237 assessed by the gel shift assay using *in vitro* synthesized proteins and end-labeled oligonucleotide probes spanning the nt 1689-1726 and nt 238 1710-1742 regions. Among the proteins tested, TCF7L2¹⁵, a member of the 239

TCF family of transcription factors that is predicted to bind to nt 1708-1713
(TTCAAAG) from the search program, was found to bind to the nt 1689-1726
sequence (Fig. 6a) but not to the nt 1710-1742 sequence (data not shown).
Other proteins including PUF60 did not bind to these sequences. From these
results, we hypothesized that PUF60 possibly accesses ENII of the HBV core
promoter via interaction with ENII-binding partner, TCF7L2, leading to
up-regulation of 3.5 kb RNA transcription.

247 To address this hypothesis, interaction between PUF60 and TCF7L2 in 248 cells was tested (Fig. 6b). PUF60 co-precipitated with TCF7L2 but not with 249 HNF4α. PUF60-TCF7L2 interaction was largely cancelled in 250 PUF60-D2-expressing cells (Fig. 2a), which cannot increase the 3.5 kb RNA 251 level (Fig. 2c) or core promoter activity (Fig. 4c). The Chromatin 252 immunoprecipitation (ChIP) assay with or without knockdown of TCF7L2 was 253 further performed to determine PUF60 recruitment to the ENII region (Fig. 6c). Amplified DNA covering the nt 1589-1828 region was detectable after 254 255 immunoprecipitation of cell lysates with or without expressing FLAG-tagged PUF60 with an anti-FLAG antibody. Additionally, the DNA level was clearly 256 257 lower in TCF7L2-knockdown cells, indicating involvement of TCF7L2 in PUF60 258 recruitment to the ENII region, which is important for 3.5 kb RNA expression. Involvement of TCF7L2 in ENII/BCP activity was examined by the reporter 259 assay in which luciferase activities of the cells were measured at 24 h pt (Fig. 260 261 6d). Over-expression of either TCF7L2 or PUF60 led to a comparable increase 262 in ENII/BCP activity, which was further increased by co-expression of both 263 TCF7L2 and PUF60. Effects of TCF7L2 over-expression with or without

PUF60 on 3.5 kb RNA expression at days 2 and 4 pt were assessed in cells 264 co-transfected with pUC-HB-Ce (Fig. 6e). As expected, at day 2 pt, the 3.5 kb 265 266 RNA level in cells co-expressing both TCF7L2 and PUF60 were markedly 267 higher than those in cells expressing either TCF7L2 or PUF60, in which 268 significantly increased 3.5 kb RNA levels were observed compared to control 269 cells. At day 4 pt, in contrast to the effect of PUF60 shown in Fig. 1c, the 270 increase in 3.5 kb RNA levels induced by TCF7L2 expression alone was 271 maintained. 272 These results strongly suggest that PUF60 acts as a positive regulator of 273 ENII/BCP activity during 3.5 kb RNA transcription cooperatively with TCF7L2.

It has been shown that PUF60, also known as FIR, plays a role in c-myc

transcription via interaction with FBP, which targets FUSE located upstream of

the c-myc promoter ¹⁰. Although no typical FUSE-like sequence was detected

within the HBV genome, we further examined whether FBP is involved in 3.5

kb RNA expression (Fig. 6f). No significant impact on core promoter and

279 ENII/BCP activities by over-expression of FBP was found. A proper expression

280 of HA-tagged FBP from the expression plasmid used was confirmed

(Supplementary Fig. S7). The results indicated that FBP does not participate in

the PUF60-dependent mechanism on ENII/BCP regulation.

283

284 Role of PUF60 on HBV 3.5 kb RNA degradation

In addition to the positive effect on 3.5 kb RNA expression, the findings
described above demonstrate that PUF60 potentially has a negative role on
the steady state level of 3.5 kb RNA during the HBV life cycle. To address the

288	mechanism underlying this negative regulation, the effect of PUF60 expression
289	on 3.5 kb RNA decay or degradation was determined (Fig. 7a). Cells
290	replicating the HBV genome with or without PUF60 expression were treated
291	with actinomycin D to arrest de novo RNA synthesis at day 2 pt, followed by
292	RNA isolation at 0, 6 and 12 h after addition of actinomycin D. PUF60
293	expression resulted in faster degradation of 3.5 kb RNA (Fig. 7a, left) but not of
294	cellular mRNA of constitutively expressed heat shock protein family A member
295	1B (HSPA1B) (Fig. 7a, right). At day 4 pt, it appeared difficult to evaluate effect
296	of PUF60 expression on decay of the 3.5 kb RNA since the RNA level in
297	PUF60-expressing cells was quite low even at 0 h (Supplementary Fig. S8). As
298	indicated in Fig. 2, the N-terminal RRM region within PUF60 is important for its
299	inhibitory effect on 3.5 kb RNA expression. The effect on 3.5 kb RNA
300	degradation was cancelled by deleting the RRM region (PUF60-D1; Fig. 7a,
301	left). Interaction of PUF60 with 3.5 kb RNA was detectable in HBV
302	genome-replicating cells that expressed the full-length PUF60 but not
303	PUF60-D1 by immunoprecipitation and RT-qPCR analyses (Fig. 7b). Time
304	course changes in the 3.5 kb RNA level in cells replicating the viral genome in
305	the presence of PUF60-D1 was compared with that in the presence of
306	full-length PUF60 (Fig. 7c, left). At day 2 pt, the 3.5 kb RNA level in cells
307	expressing PUF60-D1 was comparable to that in cells expressing full-length
308	PUF60 and markedly higher than control cells without PUF60 expression.
309	Interestingly, at day 4 pt, in contrast to the decreased level of 3.5 kb RNA in
310	cells expressing full-length PUF60 compared to control cells, PUF60-D1
311	expression maintained the increased level of 3.5 kb RNA seen at day 2 pt.

These findings demonstrate a critical role of the N-terminal RRM region within PUF60 in HBV 3.5 kb RNA degradation.

Ratio of spliced RNAs/unspliced 3.5 kb RNA, was also compared in cells 314 expressing full-length PUF60 versus PUF60-D1 (Fig. 7c, middle). The ratio in 315 316 cells expressing PUF60-D1 was significantly higher (p<0.05) than that of cells 317 expressing full-length PUF 60 but was still lower than that of the control. By 318 semi-guantitative RT-PCR, not only a change in the ratio of spliced/unspliced 319 3.5 kb RNA but variation of alternative splicing induced by PUF60- or 320 PUF60-D1 expression were observed (Fig. 7c, right). Expression of PUF60 or 321 PUF60-D1 resulted in decreasing Sp1 RNA, which was dominant in the control 322 cells, but in increasing other spliced RNAs, indicating involvement of PUF60 323 on splicing events of HBV pregenomic RNA. In contrast, PUF60 knockdown 324 resulted in increase of the spliced/unspliced 3.5 kb RNA ratio (Fig. 7d). These 325 findings suggest the N-terminal RRM region is important for regulation of 3.5 kb RNA splicing mediated by PUF60 but additional region(s) in PUF60 may 326 also be involved in this regulation. It may be likely that PUF60-D1 expression 327 contributes to an increased level of HBV production. To address this issue, we 328 evaluated HuH-7 cells replicating the HBV genome transfected with the 329 330 PUF60-D1 expression plasmid. Approximately 2-fold higher levels of particle-associated HBV DNA in the culture supernatant as well as 3.5 kb RNA 331 in these cells compared to cells without PUF60-D1 expression was observed 332 333 at day 5 pt (Fig. 7e).

Collectively, these results suggest that human hepatoma cells with ectopic expression of cell-derived proteins such as PUF60-D1, which contribute to

³³⁶ up-regulation of 3.5 kb RNA but have no effect on its degradation, are

337 potentially useful to increase HBV production.

338

339

340 **Discussion**

341 In general, nucleic acid binding proteins play roles in a variety of cellular 342 processes, including transcriptional regulation, pre-mRNA splicing and nucleic 343 acid transport. Although proteins that bind both mRNAs and their encoding 344 promoters are considered to have functional advantages or flexibility in 345 generating cellular responses, DNA- and RNA-binding proteins have been 346 largely studied independently in modulating gene expression. In the course of 347 study to determine roles of host proteins that have DNA- and RNA-binding 348 properties in HBV replication, we found that PUF60 potentially functions as a 349 versatile regulator of both transcriptional and post-transcriptional steps of HBV 350 pregenome expression.

In this study, we demonstrated that PUF60 expression leads to: 1)

352 up-regulation of core promoter activity, 2) suppression of pregenome-derived 353 RNA splicing and 3) promotion of pregenome degradation. When the 1.24-fold 354 HBV genome plasmid was introduced into cells with co-transfection of the 355 PUF60-expression vector, the 3.5 kb RNA level increased at an early time point, such as days 1-2 pt, but subsequently decreased compared to control 356 357 HBV-replicating cells. To our knowledge, this is the first study to demonstrate 358 involvement of a host factor in not only positively regulating gene expression 359 and replication of virus, but also the negative regulation of the same viral life

cycle. While why both decreased and increased expression of PUF60 lowered
the 3.5 kb RNA at day 4 pt (Fig. 1, Supplementary Fig. S5) is unclear to date,
critical contributions of PUF60 to diverse biological pathways in transcriptionaland post-transcriptional processes during the viral replication potentially in a
time-dependent manner might lead to such an unusual regulatory paradigm.

365 PUF60 is a splicing factor that associates with splicing factors involved in 366 early spliceosome assembly and plays a role in the recognition of the 3' splice 367 site during recruitment of small nuclear ribonucleoproteins (snRNPs) to the intron for splicing ^{16,17}. PUF60 is classified as a member of the U2AF protein 368 369 family, where canonical RRMs with distinct features of protein recognition are conserved ¹². U2AF-related proteins are potentially involved in changes in 370 371 available splice sites by preventing initial binding of U1 snRNP and U2AF 372 during spliceosome assembly.

373 Although mRNA turnover is critical for gene expression in eukaryotic cells, contribution of decay factors to mRNA degradation machineries remains poorly 374 375 understood because of their complexity. In particular, evidence for roles of 376 U2AF-related proteins in mRNA degradation is guite limited. T-cell intracellular 377 antigen 1 (TIA1), known to possess U2AF homology motifs, has been shown 378 to contribute to modulation of the mRNA level of programmed cell death 4 (PDCD4) through binding to PDCD4 3' UTR mRNA¹⁸. Competition between 379 TIA1 and another RNA-binding factor, HuR, for binding on PDCD4 mRNA is 380 381 thought to be important for fine-tuning PDCD4 expression in cells. Additionally, 382 TIA1 has been shown to contribute to HBs expression possibly via interaction 383 with a particular HBV RNA sequence, post-transcriptional regulatory element

384 (PRE) ¹⁹.

385	RNA decay mechanisms such as innate immune recognition,
386	nonsense-mediated decay, RNA exosome and canonical RNA decay
387	machinery are now recognized to play an important role in antiviral defense in
388	mammalian cells. For example, as anti-HBV defense mechanisms, the zinc
389	finger antiviral protein ZAP has been shown to target HBV 3.5 kb RNA,
390	resulting in RNA decay ²⁰ , as seen in retroviruses, alphaviruses and filoviruses
391	²¹⁻²³ . Cytidine deaminase possibly triggers HBV RNA degradation by tethering
392	the RNA exosome to the viral protein/RNA complex ²⁴ . Non-stop-mediated
393	RNA quality control is potentially involved in degradation of the viral X mRNA at
394	the RNA exosome complex 25 . To our knowledge, this study is the first to reveal
395	the role of PUF60 in mRNA degradation, and PUF60-mediated degradation of
396	viral RNAs might be a novel type of antiviral defense mechanism. PUF60 was
397	first identified as a 60-kDa protein that efficiently binds to the poly-U tract.
398	However, no typical or consensus motif for PUF60 binding is observed in HBV
399	RNAs. To determine the HBV RNA degradation mechanism mediated by
400	PUF60, we found direct binding of PUF60 to PRE within HBV RNA but not with
401	its reverse sequence in the in vitro assay (Supplementary Fig. S9). Although
402	HBV PRE has been reported to be involved in viral mRNA regulation such as
403	nuclear export, mRNA stability and splicing ²⁶⁻²⁹ , little is understood about the
404	underlying molecular mechanisms. Further study to elucidate the significance
405	of PUF60-PRE interaction on stability of viral RNAs and PUF60-dependent
406	pathway of RNA degradation is currently underway.

407 In addition to roles as an RNA-binding factor, it has been shown that

PUF60 potentially controls the expression of c-myc at the transcriptional step by inhibiting the transcription factor, FBP ¹⁰. PUF60 is thus termed FIR. It is thought that the interplay between FUSE, FBP and FIR/PUF60 influences the timing and level of c-myc expression ³⁰.

412 Our findings suggest that PUF60 positively regulates ENII/BCP activity via 413 interaction with TCF7L2, which can bind directly to the ENII sequence. 414 Interaction of PUF60 with TCF7L2 was cancelled by deleting the aa 210-281 415 region of PUF60 (Fig. 5), which is essential for the positive regulation of 3.5 kb 416 RNA expression (Fig. 2c). Despite the lack of direct binding between PUF60 417 and the ENII sequence (Fig. 6a), results of the ChIP assay indicated that 418 PUF60 can be recruited to the ENII region of the HBV genome, and this 419 recruitment is impaired by knockdown of TCF7L2 (Fig. 6c). The highest 420 positive impact on ENII/BCP activity and 3.5 kb RNA expression was found in 421 cells co-expressing PUF60 and TCF7L2 compared to cells over-expressing 422 either PUF60 or TCF7L2 alone (Fig. 6d and e). It is also noted that the 423 consensus DNA sequence for TCF7L2 binding is well conserved within the 424 ENII region among HBV isolates including HBV genotypes A to D. 425 TCF7L2 is a key member of the TCF family of transcription factors, which are known as downstream transcriptional effectors of Wnt signaling ³¹ and 426 427 have been shown to bind DNA directly and recruit multiple transcriptional factors such as GATA3. β -catenin. HNF4 α and FOXO1 ³²⁻³⁴. Genetic variants 428 429 of TCF7L2 showed the strongest association with type 2 diabetes/gestational 430 diabetes mellitus to date. Several studies have demonstrated that, in the liver,

431 TCF7L2 potentially serves as an important regulator of glucose homeostasis

432	by regulating proinsulin production and processing ^{35,36} . It is further suggested
433	that TCF7L2 also plays metabolic roles in lipid and amino acid metabolism,
434	and such diverse roles are possibly accomplished via its interactions with
435	various transcriptional factors as shown above. Here, we found that TCF7L2
436	also plays a role in viral transcription. TCF7L2 functions as a positive regulator
437	of ENII/BCP activity in HBV via binding to the ENII region. Moreover, its
438	interaction with PUF60 leads to further acceleration of ENII/BCP activity and
439	3.5 kb RNA expression. It will be of interest to determine if 3.5 kb RNA
440	expression regulated by TCF7L2 has an influence on metabolic gene
441	expression mediated by TCF7L2 and if the interaction with PUF60 is also
442	involved in TCF7L2-dependent regulation of cellular gene expression. Given
443	the role as a host restriction factor that limits HBV replication, the positive
444	regulation of ENII/BCP activity induced by PUF60, coupled with TCF7L2,
445	might be an evolutionally acquired strategy to avoid or reduce antiviral effects
446	via the RNA decay pathway.
447	In conclusion, we identified PUF60 as a versatile regulator of the HBV life
448	cycle, capable not only of transcriptional up-regulation of 3.5 kb RNA
449	expression, but also post-transcriptional involvement including accelerating 3.5
450	kb RNA decay and suppressing 3.5 kb RNA splicing. It appears that PUF60
451	potentially changes the balance of RNA generation and decay in a
452	time-dependent manner. Although further detailed analyses to understand the
453	regulatory mechanisms of HBV life cycle mediated by PUF60 are required,
454	these findings lead to insight on the functional linkage between transcriptional
455	and post-transcriptional regulations on viral replication and a potential

456 mechanism(s) to control antiviral host defense and viral persistence.

457

458

459 Methods

460

461 Plasmids

462 Plasmids containing the 1.24-fold HBV genomes derived from HBV

463 genotypes Ae and Bj, pUC-HB-Ae and pUC-HB-Bj ^{37,38}, respectively, were gifts

464 from Dr. Mizokami (National Center for Global Health and Medicine, Japan).

⁴⁶⁵ pUC-HB-Ce, which contains the 1.24-fold HBV genome derived from a

466 consensus sequence of HBV genotype Ce, was designed in accordance with

the most common nucleotide observed among HBV genotype Ce clones

468 (AB014381, AB205124, AB033551, AB198081, AY596108, AB198080,

AB222714 and AY066028) at each position and was artificially synthesized by

470 Eurofins Genomics (Ebersberg, Germany). DNA fragments of HBV core

471 promoter derived from HBV genotypes B and C were designed in accordance

472 with the most common nucleotide among HBV genotypes Bj and Ce,

respectively, via searching the database of HBV sequences, and were then

474 synthesized by Eurofins Genomics. To construct pGL4.74-HBpg-Ce,

475 synthesized fragments corresponding to the nt 900-1817 region of the HBV

476 genome digested by KpnI and HindIII were inserted upstream of the luciferase

reporter gene of pGL4.74 (Promega, Madison, WI, USA). To construct

478 pGL4.74-HBenIIcp-Bj/Ce, synthesized fragments corresponding to the nt

479 1627-1817 region were amplified by PCR and cloned into pGL4.74 as

480	described above. The sequences of preS1 and preS2/S promoters were
481	obtained from nt 2707-2847 and nt 2937-3204 regions, respectively, from a
482	consensus sequence of genotype Ce. Promoter sequences of human ubiquitin
483	C and human elongation factor 1α subunit were obtained from pUB6 and pEF6
484	(Thermo Fisher Scientific, Waltham, MA, USA), respectively, and subsequently
485	inserted into pGL4.74. A series of deletion mutants, pGL4.74-HBenIIcp-Bj-D1-
486	D10 (Fig. 5a), were generated based on pGL4.74-HBenIIcp-Bj. To create the
487	PUF60 expression plasmid pcDNA-F-PUF60, the cDNA sequence of human
488	PUF60 (Gene ID: 22827) was amplified by PCR using HuH-7 cells as the
489	template, followed by digestion with HindIII and Xbal and subsequent insertion
490	into pcDNA3.1 (Thermo Fisher Scientific). Plasmids expressing PUF60
491	deletion mutants were generated via several PCRs using pcDNA-F-PUF60 as
492	the template, resulting in pcDNA-F-PUF60-D1, -D2 and -D3 (Fig. 2a). To
493	create the FBP expression plasmid pcDNA-HA-FBP, the cDNA sequence of
494	human FBP (Gene ID: 8880) was amplified by PCR as described above,
495	followed by digestion with HindIII and Xbal and subsequent insertion into
496	pcDNA3.1. pcDNA-F-TCF7L2 is kindly gift from Prof. Peggy Farnham
497	(University of Southern California). Expression plasmid for HNF4 $lpha$ was
498	generated previously ³⁹ .

500 Cell culture, transfection and RNA interference

Human hepatoma derived cells [HuH-7, HepG2, HepG38.7-Tet and
 HepG-hNTCP-C4 ¹³] were maintained in Dulbecco's modified Eagle medium
 (DMEM) supplemented with 10% fetal bovine serum. Cells (1×10⁵ cells/well in

a 24-well plate) were transiently transfected with 1 µg of plasmid DNA mixed
with Lipofectamine LTX (Thermo Fisher Scientific). Synthetic siRNAs were
provided by Ambion (Thermo Fisher Scientific) and were transfected into cells
using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific).

508

509 **HBV infection**

The culture supernatant of HepG38.7-Tet cells ¹⁴ was concentrated using 510 Amicon Ultra-15 Centrifugal Filter Devices (MILLIPORE, Darmstadt, Germany) 511 and the resulting HBV sample (HBV DNA copies 2×10⁸/ml) was used as an 512 513 inoculum for infection assays. HepG2-hNTCP-C4 cells cultured in a 24-well collagen-coated plate were transfected with pcDNA-F-PUF60 and then 514 515 inoculated with the HBV sample (50 µI) in DMEM containing 4% polyethylene 516 glycol (PEG) 8000 (Promega) after 12 h of transfection. The cells were washed 517 3 times with PBS after 24 h of infection and then subjected to RT-gPCR after 96 h of further culture. 518

519

520 Quantification of HBV DNA and RNA

Quantification of HBV DNA was carried out as previously described ³⁹. To quantify particle-associated HBV DNA, culture supernatants collected from transfected cells were treated with PNE solution (8.45% PEG, 0.445 M NaCl and 13 mM EDTA) for 1 h on ice. To remove free nucleic acids, the pellets were incubated at 37°C for 1 h with DNase I (TaKaRa, Shiga, Japan) and RNase (TaKaRa). After treatment with proteinase K at 56°C overnight, HBV DNA was isolated by phenol/chloroform extraction and ethanol precipitation. HBV DNA

- 528 copies were determined by qPCR with primers
- 529 5'-TCCCTCGCCTCGCAGACG-3' and 5'-GTTTCCCACCTTATGAGTC-3'.
- 530 Quantification of unspliced and spliced forms derived from HBV 3.5 kb
- 531 RNA and host-derived mRNAs were performed as described previously with
- some modifications ^{39,40}. Total RNA was extracted from transfected cells with
- 533 TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). After
- treatment with DNase I and RNase inhibitor, cDNA templates were
- synthesized and HBV RNAs were quantified by qPCR using the SYBR qPCR
- 536 Mix kit (Toyobo, Osaka, Japan) with the following primer sets:
- 537 5'-TCCCTCGCCTCGCAGACG-3' and 5'-GTTTCCCACCTTATGAGTC-3' for
- unspliced 3.5 kb RNA, and 5'-CCGCGTCGCAGAAGATCT-3' and
- 539 5'-CTGAGGCCCACTCCCATAGG-3' for spliced RNAs derived from 3.5 kb
- 540 RNA. 5'- TTCTACAATGAGCTGCGTGTG -3' and 5'-
- 541 GGGGTGTTGAAGGTCTCAAA-3' for β -actin mRNA,
- 542 5'-AAGGGTGTTTCGTTCCCTTT-3' and 5'- TAGTGTTTTCGCCAAGCAAA-3'
- for HSPA1B mRNA, and 5'-AGCAGCAGCTCACCAACC-3' and
- 544 5'-CATCGATTGCAAAGGTGAGA-3' for PUF60 mRNA. For semi-quantitative
- 545 RT-PCR, cDNA templates were amplified with primers
- 546 5'-AGCCTCCAAGCTGTGCCTTGGGTG-3' and
- 547 5'-AACCACTGAACAAATGGCACTAGTAAACTGAGC-3'. Unspliced and
- spliced forms of 3.5 kb RNA were analyzed by agarose gel electrophoresis.

550 Northern blot analysis

551 Total RNA was extracted from cells transfected with HBV plasmids using

552	TRI Reagent. After treatment with DNase I and RNase inhibitor, RNA samples
553	were separated on 1.2% agarose gel with 7% formaldehyde at 60 V for 3 h in
554	1x 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (20 mM MOPS, 5 mM
555	sodium acetate and 2 mM EDTA). The samples were transferred to a nylon
556	membrane (Roche Diagnostics, Tokyo, Japan) with 20x SSC transfer buffer for
557	16 h, and subsequently cross-linked to the membrane by ultraviolet light (120
558	mJ/cm ²). After washing, the blotted membrane was dried at room temperature.
559	The blot was prehybridized with DIG Easy Hybridization buffer (Roche
560	Diagnostics) in 68°C and hybridized with an appropriate DIG-labeled RNA
561	probe labeled with DIG-11-UTP at 68°C overnight using the DIG Northern
562	Starter Kit (Roche Diagnostics). To generate a DIG-labeled RNA probe with
563	specific binding to HBV pregenome and HBs RNA, PCR fragments covering
564	the nt 1998-2447 and nt 3205-488 regions were used as templates for in vitro
565	transcription for the pregenome probe and HBs probe, respectively. RNA was
566	labeled in the T7 promoter transcriptional system with DIG-11-UTP using a
567	labeling mixture from the DIG Northern Starter Kit (Roche Diagnostics).
568	Detection of the DIG-labeled probe on the blot was performed using CDP-Star
569	detection reagent (GE Healthcare, Tokyo, Japan).
570	

571 RNA degradation assay

At day 2 post-transfectin (pt) with pUC-HB-Ce and pcDNA-F-PUF60 or pcDNA-F-PUF60-D1, aliquots of the cells were harvested (designated as 0 h) and the other cells were treated with actinomycin D (5 μg/ml), followed by further culture for 6 or 12 h. At each time point, total RNA was extracted from

576	transfected cells with	n TRI Reagent and the HB	/ 3.5 kb RNA level was
		0	

577 determined by RT-qPCR using the SYBR qPCR Mix kit (Toyobo).

578

579 Luciferase reporter assay

580 Cells were transiently co-transfected with pcDNA-F-PUF60,

pcDNA-F-PUF60-D1, -D2, -D3 or empty vector and the *Renilla* luciferase
reporter which carries either of HBV promoter or host cellular promoter. At 24
or 48 h pt, luciferase activities in cell lysates were measured with the *Renilla*luciferase reporter assay kit (Promega). Total protein concentrations in cell
lysates were measured and used to normalize luciferase activities.

585 lysates were measured and used to normalize luciferase activities.

586

587 Gel mobility shift assay

588 To determine *in vitro* binding between transcriptional factors and HBV 589 DNA sequence of the ENII/BCP region, HNF1a, SRY, TCF7L2, FOXM1, SP1, KLF5 and PUF60 were synthesized in vitro. In brief, cDNAs encoding these 590 seven transcription factors were isolated from MGC clones (DNAFORM. 591 Yokohama, Japan) and individually inserted into a pEU vector ⁴¹ to express an 592 593 N-terminal FLAG-fusion protein. Each transcription factor was synthesized in a wheat cell-free system as previously described ⁴². The 3'-ends of synthesized 594 595 oligonucleotides (nt 1689-1726) were labeled by DIG-11-dUTP using the DIG 596 gel shift kit (Roche Diagnostics). The labeled oligonucleotide probe was mixed 597 with each synthesized protein, and the gel shift reaction was performed 598 according to the manufacturer's instructions. The resulting samples were 599 analyzed by native PAGE using a 6% gel. The labeled DNA-protein complexes

as well as the probe were blotted to a nylon membrane and detected using

601 CDP-Star detection reagent (GE Healthcare, Buckinghamshire, UK).

602

603 Immunoblotting and immunocytochemistry

604 Immunoblotting was performed as previously described with slight modification ⁴³. Briefly, cell lysates were separated by SDS–PAGE and 605 606 transferred onto polyvinylidene difluoride membranes. After blocking, 607 membranes were incubated with an antibody against PUF60 (GeneTex, Irvine, 608 CA), FLAG M2 (Sigma-Aldrich, Tokyo, Japan) or GAPDH (Santa Cruz 609 Biotechnology, Dallas, TX) or HA (MBL, Nagoya, Japan) for 1 h. After washing, 610 membranes were incubated with an HRP-conjugated secondary antibody (Cell 611 Signaling Technology, Danvers, MA) for 0.5–1 h. Antigen-antibody complexes 612 were detected using the ChemiDoc[™] Imaging System (BIO-RAD Laboratories, 613 Tokyo, Japan). For immunocytochemistry, cells grown on a glass bottom plate were fixed with 4% paraformaldehyde for 15 min and permeabilized in 0.5% 614 Triton X-100 in PBS, followed by blocking with 1% bovine serum albumin 615 616 (BSA). Immunocytochemistry was performed by incubation with the 617 anti-PUF60 antibody (GeneTex, Irvine, CA) for 2 h, followed by incubation with 618 Alexa Fluor 488 anti-rabbit IgG (H+L) antibody (Vector Laboratories, 619 Burlingame, CA, USA) for 2 h. Double-stranded DNA was stained with Hoechst 620 33342 (Dojin, Tokyo, Japan). Subcellular localization of PUF60 was observed 621 under a confocal microscope (Leica TCS SP8; Leica, Wetzlar, Germany). 622

623 ChIP assay

ChIP followed by qPCR was performed as previously described with some 624 modification ⁴⁴. Briefly, cells seeded in 100-mm dishes were transfected with 625 siTCF7L2 RNA. After 48 h, the cells were co-transfected with pUC-HB-Ce and 626 627 the pcDNA-F-PUF60 expression vector or empty vector. After 48 h, ChIP was 628 performed by the Chromatin IP kit (Cell Signaling Technology). Proteins in the 629 cells were cross-linked with DNA using 1% formaldehyde for 10 min at room 630 temperature. The cross-linking reaction was stopped by the addition of 1 ml of 631 10x glycine to each dish and incubation for 5 min at room temperature. After 632 washing two times with ice-cold PBS, the cells were scraped into PIC buffer (1 633 ml PBS and 5 µl 200x protease inhibitor cocktail) and sonicated to shear DNA 634 to lengths between 150 and 900 bp. After 5-fold dilution of the sonicated cell 635 supernatants in 100 µl 1x ChIP buffer and 0.5 µl 200x PIC, 636 immunoprecipitations were carried out overnight at 4°C with the anti-FLAG M2 637 antibody. Protein G agarose beads were added and incubated for 2 h at 4°C with rotation. DNA-protein complexes were eluted from the beads with a buffer 638 containing 1% SDS and 0.1 M NaHCO₃. The cross-links were reversed by 639 incubating the eluates with proteinase K solution (final concentration: 200 mM 640 641 NaCl and 266 µg/ml proteinase K) overnight at 65°C. DNA was recovered by 642 phenol/chloroform extraction and ethanol precipitation. ChIPped DNA was 643 analyzed for the presence of HBV gene promoter sequence by qPCR. Viral DNA covering the nt 1589-1828 region was detected by using the SYBR gPCR 644 645 Mix kit (Toyobo) with the following primer set: 646 5'-CTTCACCTCTGCACGTCGCATG-3' and

647 5'-GTGAAAAAGTTGCATGGTGCTGGTG-3'.

649 Immunoprecipitation

650	Immunoprecipitation was performed as previously described with slight
651	modification 14 . Briefly, cells were lysed with lysis buffer (0.5% NP-40 in PBS)
652	and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were
653	incubated with Protein G agarose beads, which were prewashed with lysis
654	buffer, and anti-HA antibody or anti-FLAG antibody for 60 min at 4° C. The
655	samples were then centrifuged, and the resulting pellets were washed 4 times
656	with lysis buffer and subjected to SDS-PAGE.
657	
658	Subcellular fractionation
659	Cells were suspended with hypotonic buffer (0.5% NP40,10 mM Tris-HCI
660	pH8.0, 10 mM NaCl, 3 mM MgCl2, 5mM DTT), followed by centrifugation at
661	500 × g for 5 min at 4 °C. The supernatant was collected and termed as the
662	cytoplasmic fraction. The pellet containing the nuclear fraction was
663	re-suspended with disruption buffer (1% Triton, 1% DOC, 0.1% SDS, 25 mM
664	Tris-HCI pH 7.6, 150 mM NaCl, 1 mM EDTA, 5mM DTT).
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822

823 Author Contributions Statement

- Tetsuro S., T.W., Tatsuya S., designed the study. S.S., K.N., M.I., Y.L., T.C.,
- H.T. and K.W. performed experiments and analyzed data. Tetsuro S. and S.S.
- wrote the main manuscript text. All authors reviewed the manuscript.

827

828 Competing financial interests

829 The authors declare no competing financial interests.

831 Figure legends

833	Fig. 1 Involvement of PUF60 in regulation of HBV RNA expression. (a) A
834	schematic diagram of HBV RNAs and regions used as probes for northern
835	blotting is indicated (top). pcDNA-F-PUF60 or an empty vector (EV) was
836	co-transfected with pUC-HB-Ce into HuH-7 cells. At day 2 or 4
837	post-transfection (pt), total RNA was extracted from cells and separated on an
838	agarose gel. HBV 3.5 kb RNA and spRNA (upper panels) and 3.5 kb RNA and
839	HBs RNA (lower panels) were detected by northern blotting using probe PG (nt
840	1998-2447) and probe S (nt 3205-488), respectively. Band intensities of 3.5 kb
841	RNA on the blots with PG probe were determined by Image-J software and
842	those of control samples (EV) were calculated as 1. (b) Total RNAs prepared
843	as described above were used for semi-quantitative RT-PCR with (RT(+)) or
844	without (RT(-)) reverse transcription. cDNA bands corresponding to unspliced
845	3.5 kb RNA and its spliced forms (spRNAs) were detected by agarose gel
846	electrophoresis. 18S ribosomal RNA (18S) was also detected. Immunoblotting
847	indicated expression of PUF60 and GAPDH in transfected cells. (c) RT-qPCR
848	analysis was performed to determine 3.5 kb RNA and spRNA levels in cells as
849	described above. (d) Nuclear and cytoplasmic fractions of cells transfected
850	with pUC-HB-Ce with pcDNA-F-PUF60 or EV were isolated and 3.5 kb RNA
851	levels in each fraction were determined at days 1 and 4 pt. (e)
852	Dose-dependent effect of PUF60 on 3.5 kb RNA levels was determined in cells
853	transfected with pUC-HB-Ce with various concentrations of pcDNA-F-PUF60
854	by RT-qPCR. (f) Effect of PUF60 expression on 3.5 kb RNA levels of various

HBV genotypes was determined in cells transfected with pcDNA-F-PUF60 and 855 856 a plasmid carrying the 1.24-fold HBV genome derived from HBV genotype 857 (GT) A, B or C. (c) - (f) Data are normalized to that of β -actin mRNA and values 858 of "EV" (GT-A EV in case of (f)) are set to 1. Values shown represent means ± 859 SD obtained from three independent samples. Statistical differences 860 compared with the control (EV) are shown. **p<0.01, Student's t test. 861 Full-length blots in (a) and (b) are presented in Supplementary Figures S10 862 and S11, respectively. 863

864 Fig. 2 Effect of PUF60 deletion mutants on HBV 3.5 kb RNA expression. (a) 865 Three PUF60 deletion mutants were used in this study. A schematic diagram of RNA recognition motifs (RRM1 and RRM2) and U2AF homology motif (UHM) 866 867 within PUF60 is indicated at the top. (b) Subcellular localization of wild-type 868 and mutant PUF60 strains was determined. At day 2 pt, cells were fixed and stained with Hoechst 33342, followed by immunostaining with anti-PUF60 869 870 antibody. (c) Effect of over-expression of PUF60 deletion mutants on 3.5 kb RNA expression at days 2 and 4 pt was evaluated in cells co-transfected with 871 872 pUC-HB-Ce. Data are normalized to that of β -actin mRNA and the values in 873 cells transfected with EV are set to 1. Values shown represent means ± SD 874 obtained from three independent samples. Expression of each PUF60 deletion 875 mutant was evaluated by immunoblotting. Full-length blot is presented in 876 Supplementary Figure S12. Statistical significances compared with the control (EV) were shown. *p<0.05, **p<0.01, Student's t test. 877

878

879	Fig. 3 Effect of PUF60 on HBV production. (a) Particle-associated HBV DNA
880	in culture supernatants of cells transfected with pUC-HB-Ce and
881	pcDNA-F-PUF60 or empty vector (EV) was quantitatively measured at days 2
882	and 5 pt. (b) After 12 h of transfection with pcDNA-F-PUF60 or EV,
883	HepG2-hNTCP-C4 cells were infected with HBV and cultured for 5 days, and
884	total cellular RNA was analyzed by RT-qPCR to determine levels of 3.5 kb
885	RNA (left) and spRNAs (right). (a) and (b) Values shown represent means \pm
886	SD obtained from three independent samples. Statistical differences
887	compared with the control (EV) are shown. *p<0.05, Student's t test.
888	
889	Fig. 4 Up-regulation of HBV core promoter activity induced by PUF60. (a)
890	Effect of PUF60 expression on HBV or cellular promoter activities was
891	analyzed by transfection of HuH-7 cells with the luciferase reporter carrying the
892	entire core promoter (nt 900-1817), ENII/BCP (nt 1627-1817), preS1 promoter
893	(nt 2707-2847), preS2/S promoter (nt 2937-3204), human ubiquitin C promoter
894	or human elongation factor 1α promoter and pcDNA-F-PUF60 or empty vector
895	(EV). Reporter activities in the cells were measured at 24 h pt. Values are
896	normalized to total protein concentrations in cell lysates. (b) Knockdown effect
897	of PUF60 on core promoter activity (left) and 3.5 kb RNA expression (middle)
898	as well as knockdown efficiency of PUF60 (right) were assessed. At 2 days
899	after introducing PUF60 siRNAs (siPUF60) or its negative control (siNC),
900	HuH-7 cells were transfected with pGLHBp900/1817 or pUC-HB-Ce and then
901	reporter activities and RNA levels, respectively, were measured after 2 days of
902	further culture. PUF60 mRNA expression was also determined. (c) Effect of

903	PUF60 deletion on activation of the core promoter was assessed. HuH-7 cells
904	were transfected with pGLHBp900/1817 and a plasmid expressing either
905	wild-type PUF60, PUF60-D1, -D2, -D3 or EV. Reporter activities were
906	measured at day 1 pt. (a) - (c) Data are normalized to that of β -actin mRNA
907	and the values in cells transfected with EV or siNC are set to 1. All assays
908	were performed in triplicate and results are presented as means \pm SD.
909	Statistical differences compared with the control (EV or siNC) are shown.
910	*p<0.05, **p<0.01, Student's t test.
911	
912	Fig. 5 Identification of the ENII/BCP element(s) responsible for transcriptional
913	regulation by PUF60. A series of reporter constructs with partial deletions

914 within ENII/BCP were generated (left). Luciferase activities were determined in

cells transfected with each reporter construct and pcDNA-F-PUF60 or empty

916 vector (EV) at day 2 pt (right). Relative changes in reporter activities induced

by PUF60 calculated as the ratios of reporter activities in cells expressing

918 PUF60 to those in control cells are shown.

919

Fig. 6 Involvement of TCF7L2 in up-regulation of core promoter activity. (a) Transcription-related proteins from database search (HNF1 α , SRY, TCF7L2, SP1, FOXM1 and KLF5) and PUF60 were synthesized *in vitro* and used for the gel shift assay with an end-labeled oligonucleotide probe (nt 1689-1726). (b) Interaction of PUF60 with TCF7L2 in cells was tested. Cells transfected with pcDNA-HA-PUF60 or -PUF60-D2 and pcDNA-F-TCF7L2 or -HNF4 α plasmids were lysed at day 2 pt and subjected to immunoprecipitation (IP) with anti-HA

927	antibody. Resulting precipitates and whole cell lysates were examined by
928	immunoblotting using anti-FLAG, anti-HA or anti-GAPDH antibody. (c) ChIP
929	assay was performed to determine recruitment of PUF60 to the core promoter
930	in cells. After 2 days with or without knockdown of TCF7L2, cells were
931	transfected with pcDNA-F-PUF60 or empty vector (EV). Further 2 days later,
932	cell lysates were immunoprecipitated with anti-FLAG antibody, and HBV DNA
933	in the precipitates was measured by qPCR. FLAG-PUF60 and GAPDH in the
934	precipitates were detected by immunoblotting. (d) Effect of TCF7L2 expression
935	on ENII/BCP activity was determined by the reporter assay. Cells were
936	transfected with pcDNA-F-PUF60 or -TCF7L2 or both and pGLHBp1627/1817.
937	At 24 h pt, Renilla luciferase activities in cells were measured. (e) Effect of
938	TCF7L2 over-expression with or without PUF60 on 3.5 kb RNA expression
939	was determined. Cells were transfected with pcDNA-F-PUF60 or -TCF7L2 or
940	both and pUC-HB-Ce. At day 2 or 4 pt, total RNA was extracted and HBV 3.5
941	kb RNA level was assessed by RT-qPCR. Results were normalized to that of
942	β -actin mRNA. (f) Effect of FBP over-expression with or without PUF60 on core
943	promoter and ENII/BCP activities was assessed. Cells were transfected with
944	pcDNA-HA-FBP or -F-PUF60 or both and pGLHBp900/1817 or
945	pGLHBp1627/1817. Luciferase activities in cell lysates were measured at day
946	2 pt. (d) - (f) The values in cells transfected with EV are set to 1. Results are
947	presented as means \pm SD from at least three independent samples. Statistical
948	differences compared with the negative control (EV only) are shown. *p<0.05,
949	**p<0.01, Student's t test. Full-length blots in (b) and (c) are presented in
950	Supplementary Figures S13 and S14, respectively.

952	Fig. 7 Involvement of PUF60 in HBV 3.5 kb RNA degradation. (a) Time course
953	changes in HBV 3.5 kb RNA and HSPA1B mRNA levels were determined. At
954	day 2 pt with pUC-HB-Ce and pcDNA-F-PUF60, -PUF60-D1 or empty vector
955	(EV), aliquots of cells were harvested (0 h) and the remaining cells were
956	treated with actinomycin D, followed by 6- or 12 h culture. At each time point,
957	total RNA was extracted and the RNA levels were assessed by RT-qPCR. The
958	values of each group at 0 h were set to 1. Expression of PUF60, PUF60-D1
959	and GAPDH was detected by immunoblotting. (b) Interaction of PUF60 with
960	3.5 kb RNA in cells was examined. HuH-7 cells were transfected with
961	pUC-HB-Ce and pcDNA-F-PUF60, -PUF60-D1 or EV, followed by
962	immunoprecipitation (IP) with anti-FLAG antibody at day 2 pt. HBV 3.5 kb RNA
963	level in the precipitates was determined by RT-qPCR (left). The precipitates
964	and whole cell lysates used in IP were examined by immunoblotting using
965	anti-FLAG or anti-GAPDH antibody. An arrowhead indicates non-specific
966	bands. (c) Effects of PUF60 and PUF60-D1 on 3.5 kb RNA and spRNA levels
967	were tested. Cells were transfected with pUC-HB-Ce and pcDNA-F-PUF60,
968	-PUF60-D1 or EV, followed by RT-qPCR for 3.5 kb RNA at days 2 and 4 pt
969	(left). At day 2 pt, spRNA levels in each transfectant were determined by
970	RT-qPCR (middle) and semi-quantitative RT-PCR (right). The values in cells
971	transfected with EV are set to 1. (d) Effect of PUF60 knockdown on the ratio of
972	spliced/unspliced 3.5 kb RNA at day 2 and 4 pt. (e) Effect of PUF60-D1
973	over-expression on HBV production was assessed. Particle-associated HBV
974	DNA in culture supernatants as well as 3.5 kb RNA in these cells transfected

- with pUC-HB-Ce and pcDNA-F-PUF60-D1 or EV were measured at day 5 pt.
- 976 (a) (e) Assays were performed in triplicate and results are presented as
- ⁹⁷⁷ means ± SD. Statistical differences compared with the control (EV) are shown.
- ⁹⁷⁸ *p<0.05, **p<0.01, one-way ANOVA followed by Tukey's test (a) or Student's t
- test (b-e). Full-length blots in (a), (b) and (c) are presented in Supplementary
- 980 Figures S15, S16 and S17.

























