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ORIGINAL ARTICLE

Mutations in Core Gene Region of Hepatitis B Virus in Patients with Chronic Hepatitis B

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SUMMARY

Background: **The HBV core protein plays a major role in host immune response. Mutations occurring in the HBV core gene may cause alterations in the major epitopes being effective in the host immune response. Until now, the persistent effects of core gene mutations on HBV infections have not been fully understood. The aim of this study is to analyze the core gene mutations for epitopes in the T lymphocytes [T helper (Th) and cytotoxic (CTL)] and B cell and C terminal region in patients with chronic hepatitis using ultra-deep pyrosequencing (UDPS) method.**
Methods: **Eleven patients with chronic hepatitis B infection were included in the study. Amplification of the core gene was performed by a conventional PCR method. Mutations in the epitopes for T lymphocytes (Th and CTL) and B cell and in the C terminal region of HBV core gene were screened by UDPS. These mutations were analyzed in HBeAg positive and negative patients.**
Results: **The minimum percentages of amino acid substitutions were found with 0.9% in HBeAg positive patients and 1.2% in negative patients. The number of missense mutation was higher in patients with HBeAg positive than negative patients ($p < 0.005$). The number of amino acid substitutions in the region of aa49 - 69 in the Th epitopes was found to be the highest in both HBeAg positive and negative patients. The mutation frequency was higher in the C-terminal region of the core protein compared to the Th, CTL, and B cell regions and these were more common in subjects with high-grade fibrosis. Some types of mutations (V27I, R47H, Y132I, R174STOP, S181P, Q182K) were only detected in subjects with liver cirrhosis.**
Conclusions: **Unlike literature, our results show that there is no significant increase in number of mutations in the core gene of the virus during the anti-HBe positive period. The role of low abundance variants and mutations in the immune system can be understood using methods such as UDPS in the near future.**
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KEY WORDS

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INTRODUCTION

Hepatitis B virus (HBV) infection with more than 350 million chronic carriers is a major health problem worldwide. HBV, besides the chronicity, leads to serious consequences such as cirrhosis and hepatocellular carcinoma [1]. Host immune response is the most important factor determining the progression of HBV in-

fection, but mutations in HBV core gene are also related to the progression of the disease [2].

The core protein plays an important role at different stages of the virus life cycle. Besides interacting with other host and viral proteins, it can protect viral RNA and DNA by forming a stable capsid. Moreover, it can release viral DNA at the right time in the viral life cycle. Therefore, core protein is an important goal for the development of safe and effective virus-selective antiviral agents to improve the treatment options of HBV infection [3,4]. For this reason, mutations in the HBV core protein should be described in detail in patients with hepatitis B infection.

Previous studies have shown that HBc particles have a high immunogenicity that strongly stimulates the immune response of T and B lymphocytes [5-7]. Different epitopes have been identified in the HBV core gene associated with immunity. In particular, two epitopes play an important role for B and CD4+ T helper (Th) lymphocytes, the sequence from amino acid 74 to 84 and the sequence from amino acid 49 to 69, respectively [8, 9]. On the other hand, HBcAg is an important target of CTL attack for viral clearance, HLA class-I restricted CTL epitopes are being strongly investigated due to the detection of a strong T cell response against 18 - 27 residues [10,11]. Yet the immunological determinants of successful HBV elimination are still not fully understood.

Researchers reported that the mutations in the core gene for HBV could easily escape from the immune system with an alteration of the recognition sites of HBcAg. Thus, an increase in the number of mutations has been identified in the core gene of hepatitis B infected patients after the immunological tolerance phase [12]. Previous studies have shown that the core gene mutations are commonly seen in patients with chronic active hepatitis or fulminant hepatitis [13,14].

The development of core gene low-abundance viral quasi species occurring in response to host immune suppression may also be important for chronic hepatitis B patients. Therefore, high-sensitive methods are needed to detect changes in this gene. The method of ultra-deep pyrosequencing (UDPS) enables detection of additional changes in frequency of minor variants and quasi species. This method is highly sensitive compared to the conventional population-based sequencing method. Thanks to this method, Holm et al. have reported that some immune-stimulating activity of the minor Th28-47 epitope might be associated with nucleoside/nucleotide analogues [15].

In this study, we aimed to analyze the core gene mutation in terms of CTL, Th, and B cell epitopes and the C-terminal region in patients with HBeAg positive and negative chronic hepatitis B using the UDPS method.

MATERIALS AND METHODS

Study population

The study included the 11 chronic infected HBV patients, 7 of whom are hepatitis B e antigen (HBeAg) positive.

The inclusion criteria for the study were hepatitis B surface antigen (HBsAg) positivity and HBV DNA positivity for more than 6 months. Exclusion criteria included hepatitis C virus (HCV), hepatitis D virus (HDV) and/or human immunodeficiency virus (HIV) co-infection and autoimmune liver disease. HBV DNA positivity was determined by using the TaqMan real-time PCR method (COBAS AmpliPrep/COBAS TaqMan; Roche). Serum samples collected from all patients stored at -70°C until analysis.

Amplification of core gene region

Amplification of the core gene was performed by a conventional PCR method. HBV genomic sequences from the NBCI database (http: //www.ncbi.nlm.nih.go/) were selected to design 10 pairs of overlapping amplicons to detect the C gene using Polymerase Chain Reaction (PCR) primers (Table 1) (IDT, Bradford, CT, USA).

Amplicon primers were designed according to manufacturer's amplicon sequencing protocol (http://www.454.com). All fragments were amplified by using a proofreading enzyme (Fast Start high-fidelity enzyme; 125 Roche, Manheim, Germany). PCR reaction mix contained: 50 µmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 10 pmol primers, and 0.15 U Taq polymerase. After addition of 100 ng of HBV plasmid DNA to PCR mix, amplification was performed as follows: 1 cycle at 95°C for 3 minutes, and 35 cycles comparing; 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, and 1 final cycle at 72°C for 5 minutes.

Ultra-deep pyrosequencing (UDPS)

QIAspin mini-elute spin kit (QIAgen Co. USA) was used for purification of the PCR products (~400 base-pairs [bp] long). PCR amplified DNA was used to generate the amplicon library according to the manufacturer's protocols. (Roche Diagnostics Co., Indianapolis, IN, USA). DNA fragments were cleaned by using the Mini-Elute Kit (Qiagen 137 Inc, Valencia, CA, USA), and size and quality of both fragments were verified by Agilent Bioanalyzer 2100 (Agilent Technologies, Anaheim, CA, USA). The sequencing of fragments was performed on a Genome Sequencer FLX using the GS FLX kits according to the manufacturer's protocols (Roche Diagnostics Co.). Sequencer (Genes Codes Corporation, Ann Arbor, MI, USA) software was used for the analysis of the UDPS results. AVA software was set to a cut-off value of 1% for estimation of the mutation frequency in the samples. Cloned HBV plasmid was absent in this study. Different particle lengths of A, T, C, and G bases were used as internal controls in our study. Moreover mathematical and statistical "error correction" was performed for the definition of the minimum threshold

Table 1. Amplification primers used in the PCR for UDPS of HBV-Core gene.

Primer No.	Primer Sequences (5′→3′)
1	GCCTCCCTCGCGCCATCAGCTCCACCACGTTCCACCAAACTC
2	GCCTTGCCAGCCCGCTCAGAAGAAGATGAGGCATAGCAGCAGG
3	GCCTCCCTCGCGCCATCAGCCAAATCTCCAGTCACTCACC
4	GCCTTGCCAGCCCGCTCAGGAATACAGGTGCAGTTTCCGTCC
5	GCCTCCCTCGCGCCATCAGAGAACCTGCACGACTCCTGCTCA
6	GCCTTGCCAGCCCGCTCAGGACATACCTTCCAATCAATAGG
7	GCCTCCCTCGCGCCATCAGCTTGAGTCCCTTTATGCCGCTG
8	GCCTTGCCAGCCCGCTCAGCGCAGTATGGATCGGCAGAGG
9	GCCTCCCTCGCGCCATCAGGTGCCAAGTGTTTGCTGACGC
10	GCCTTGCCAGCCCGCTCAGGAAGTATGCCTCAAGGTCGGTC

Table 2. Amino acid substitutions in three immune epitopes and C- terminal region of HBV core gene.

T helper epitope (n)	CTL epitope (n)	B cell epitope (n)	C- terminal region (n)
T12S (3)	S21T (2)	E77D (1)	T147S (4) T147C (1)
V13L (1)	V27I (1)	I80T (7)	R151Q (5)
V14E (1) V14L (1)	S87N (1)	D83G (1) E83D (1)	G153C (3)
S49T (2)	H88Y (7)	S87N (1)	R154G (1)
I59T (1)	F91I (1)	H88Y (7)	S155T (6)
W62L (1) W62G (1)	T147S (4) T147C (1)	I116L (1) I116V (1)	S162P (2)
D64E (3) D64N (1) D64K (1)	R151Q (5)	P130L (1) P130R (1)	R165S (1)
T67N (2)		Y132I(1)	R167K (1)
A69G (1)		R151Q (5)	S168P (1)
P130L (1) P130R (1)		G153C (3)	Q169P (1)
		R154G (1)	P171R (1)
		S155T (6)	R172H (1)
			R174STOP (1)
			V175K (1)
			Q177K (1)
			S178Y (1)
			E180G (1)
			S181P (2) S181A (1)
			Q182K (1) Q182E (1)
			C183Q (1)

level. The HBV region analyzed covered amino acids from 1 to 183 of the core gene. The raw sequences obtained and each individual sequence read was determined. Detection of single nucleotide polymorphisms and deletion-insertion polymorphisms in each patient was performed according to the following criteria; (i) variables must be determined in at least three unique (non-duplicate) sequencing reads with both forward and re-

Table 3. Frequency distribution of mutations in different epitopes within core protein of HBeAg positive and negative patients.

	T helper (n:15)			CTL (n:8)			B cell (n:15)			
	1 - 20	49 - 69	117 - 131	18 - 27	84 - 101	141 - 151	74 - 89	107 - 118	130 - 138	148 160
HBeAg pos. (n:7)	4	7	2	2	3	3	4	2	3	4
HBeAg neg. (n:4)	1	2	0	1	1	2	4	0	0	2

Table 4. Emerging HBV core gene amino acid substitutions detected by UDPS.

Pt. no.	Age (years)	Gender (M/F)	ALT values (IU/mL)	HBeAg status	Fibrosis stage	Mutations in core gene
1	25	F	60	+	1	W62L, D64E, G73S, G73C, I80T, D83G, H88Y, I105T, R151Q, R153C, S155T, S168P, Q169P, P171R, R172H
2	49	M	285	+	4	V14L, S87T, P130R, P130L, R165S, R167K, E180G, S181A
3	40	M	45	+	4	I80T, H88Y, *Y132I*, T147S, S162P, *R174STOP*
4	21	F	207	+	1	I80T, H88Y, F91I, T147C, Q177K, S178Y
5	34	M	50	+	2	V13L, D29H, D64K, T67N, A69G, R153C, R154G, S155T, S162P, C189G
6	47	F	58	+	2	V14E, D29H, I59T, D64N, D64E, T147S, R151Q, R153C, S155T
7	51	M	51	+	4	T12S, S21T, *V27I, R47H*, D64E, T67N, G73S, R151Q, S155T, S181P, *Q182K*
8	32	M	347	-	3	T12S, S21T, S49T, I80T, H88Y, I116L, R151Q, S155T, V175K, S181P
9	33	F	52	-	1	T12S, E40D, S49T, E77D, I80T, H88Y, C105V, I116V, Q182E
10	34	M	96	-	1	I80T, H88Y, T147S, R151Q, S155T
11	20	F	164	-	1	E43T, W62G, I80T, E83D, H88Y, T147S

Pt - patient, F - female, M - male, ALT - alanine aminotransferase, HBeAg - hepatitis B antigen.
*** The mutations in only liver cirrhotic patients are italicized while similar mutations in stage-3 liver fibrosis and liver cirrhotic patients are underlined.**

verse reads; (ii) having a frequency of at least 10% between the total unique sequencing reads in that region, and (iii) each polymorphism must have high-quality scores (Q20 for variants and Q15 for three nucleotides on each side of the variant). Consensus sequences were generated from reference sequences from Gen Bank (accession no. AY721608, AY796030, AY661792, and Q486022).

The study was approved by the Local Ethical Committee of the Istanbul University.

Statistical analysis

Statistical analysis of the presence or absence of mutations in HBeAg positive and negative patients was performed using the Chi-square test. p-value of < 0.05 was considered to be statistically significant.

RESULTS

Patients and samples

The study group consisted of 11 patients with chronic hepatitis B. The mean age was 35 years and 54% were males [(6 males, mean age 35.1 ± 10.7 years)]. Seven patients had positive hepatitis B e antigen [HBeAg]. During core genome analysis, the average reading length of the sequences is 249 bp. All patients had genotype D and they were known in advance.

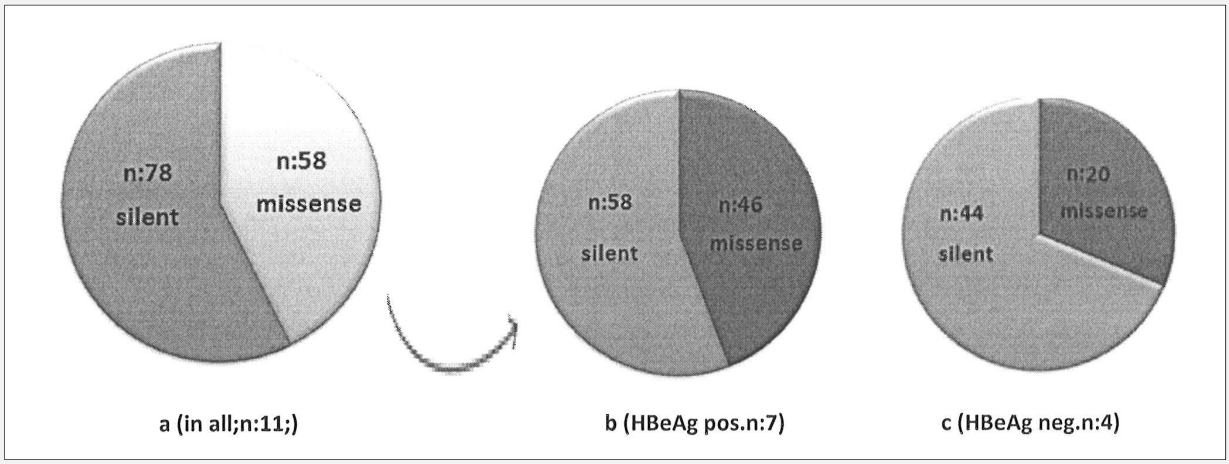

Figure 1. Distribution of missense and silent mutations in the core gene region.

Amino acid substitutions in the core region

Amino acid sequences of the HBV core protein (aa1-183) were analyzed in all serum samples by UDPS. In 11 patients a total number of 58 different missense mutations in the core gene were detected while 78 different silent mutations were found (Figure 1a). Some of the silent and missense mutations were the same as those in patients who were HBeAg positive and negative. The number of missense and silent mutations were found to be 46 and 58 in HBeAg positive patients and 20 and 44 in negative patients, respectively (Figure 1b, 1c). The number of missense mutations was higher in patients who were HBeAg positive than who were negative patients ($p < 0.005$). On the other hand, there was a significant difference between silent mutations and HBeAg negative patients compared to positive patients ($p < 0.001$). The minimum percentages of amino acid substitutions were found to be 0.9% in HBeAg positive patients and 1.2% in negative patients.

Amino acid mutations in the core protein immune epitopes

In total, 38 amino acid mutations have been detected in different immune epitopes within the core protein, 15 (~40.5%) in B cell epitopes, 15 (~40.5%) in Th cell epitopes, and 8 (19%) in CTL epitopes. Twenty additional substitutions were found outside the B, CTL, and Th cell epitopes. Amino acid changes in different epitopes within the core region of HBeAg positive and negative patients are shown in Table 2.

The number of amino acid substitutions in the region of aa49 - 69 among Th epitopes was found to be highest in both of HBeAg positive and negative patients. B cell epitope showed the highest variability in two regions, aa74 - 89 and aa148 - 160, respectively (Table 3). The number of amino acid mutations in HBc protein was generally higher in HBeAg positive patients than HBeAg negatives [except the B epitope (aa74 - 89)] (Table 3). Only two patients (one HBeAg positive patient with liver cirrhosis and one HBeAg negative patient with liver fibrosis stage-3) had mutations in the aa18 - 27 region of the CTL epitope. The most polymorphic region was codon 88 in the core gene. The I80T-Y88H double mutation was detected in all HBeAg negative patients.

Mutations in the carboxy-terminal end of the core gene overlapping with the polymerase gene (codon 136 - 183) have commonly been detected. This region included 23 mutations constituting 40% of the mutations detected in the core gene, and the most frequently affected codons were 151, 153, and 155. Three mutations were detected in the arginine rich RNA binding site (codon 150 - 154). Four of seven HBeAg positive patients (57%) and two of four HBeAg negative patients (50%) showed amino acid substitutions in this region. On the other hand, codon 153 and 154 mutations were detected in only HBeAg positive patients.

Core gene mutations and prognosis of HBV infection

The mutations in the core protein in 11 patients with different clinical outcomes are summarized in Table 4. The S21T, V27I, R47H, I116L, Y132I, R174STOP, S181P, and Q182K mutations were detected in the three patients with only advanced stage liver fibrosis. The S21T and S181P mutations were detected in 2 subjects, one HBeAg positive subject with liver cirrhosis and one HBeAg negative subject with liver fibrosis stage-3. The V27I and R47H mutations were detected in only one HBeAg positive patient with liver cirrhosis while other mutations (Y132I and R174STOP) occurred in another

HBeAg positive patient with liver cirrhosis. The Q182K mutation related to HCC was found in only one individual with liver cirrhosis. The I97F/L mutation was not detected in any of the patients. The T67N, E77D, and I80T mutations were also detected in our patients who are similar to those mutations found in inactive carrier patients.

DISCUSSION

Studies on quasi species dynamics are important in understanding the pathogenesis and persistence of viruses, as well as preventing disease, and treatment strategies. In our study, the mutations in the HBV core gene region were analyzed in 11 patients with chronic HBV infections using UDPS method. The studies involving core gene sequence analysis are limited in the literature. Many studies have described only the difference between precore/core mutations among HBeAg positive and negative patients. Moreover, previous studies (except Holm et al. [15]) mostly analyze these mutations using direct sequencing. However, these currently used standard methods are not sufficient enough for the determination of mutations with low prevalence. UDPS is a very sensitive method for the detection of low frequency quasi species [16]. Therefore, these methods may contribute to a better understanding of the genetic diversity of the core gene in the near future.

Researchers have shown that the long-term interaction of the host with the virus during the anti-HBe positive period may cause the development of new HBVs with many mutations. Therefore, the number of mutations in the core gene is expected to be higher in HBeAg negative patients [17]. Several studies have reported that the mutations in the core gene were more common in patients with HBeAg-negative chronic hepatitis B (CHB) [18]. Our results are not consistent with the studies showing a significant increase in post-seroconversion core gene mutations in adults. This result may be due to the fact that the UDPS is able to detect the mutations with low frequency. Our results suggest that the number of core gene mutations after HBeAg seroconversion cannot be explained by previous approaches.

The CTL(s) are one of the most important immune cells for the control of viral clearance of HBV infection. Previous studies have shown that aa18 to aa27 of HBcAg is the major recognition region for CTLs [19]. Therefore, mutations in this region may play an important role in the persistence of the disease. In chronic patients of our study group, only two mutations were detected in this region. These mutations are S21T and V27I which were detected in one HBeAg positive and one negative patient. Both of these patients had high-grade liver fibrosis; however, Mohebbi et al. also reported these mutations in inactive HBV carriers [20]. The core region is highly conserved in the immune tolerance phase of HBV infections in adults. On the other hand, the number of mutations in the B and T cell epitopes of the core gene increases following this phase of the disease [21]. We obtained similar results in our patients except for the aa141 to aa151 region of the CTL epitope. The reason for the increased number of mutations in the aa141 to aa151 region of the CTL epitope can be explained by the partial overlap with the aa148 to aa160 region of the B cell epitope.

No doubt, mutations in the aa49 - 69 region of the T helper epitope play an important role in the course of the infection. Akarca et al. reported that the highest amino acid alterations were found between aa49 to aa69 epitopes of the Th cell regions in patients with chronic hepatitis [18]. Likewise, we have found that the highest number of amino acid changes in our study were in the aa49 to aa69 region. On the other hand, the number of mutations in HBeAg-positive patients was much higher than the negative ones for this region. Carman et al. reported that the putative escape mutations occur in the aa49 to aa69 region of the Th epitopes of patients with seroconversion from HBeAg to anti-HBe positivity (who went into remission) had active hepatitis [9]. On the contrary, the mutations have been reported more frequently within the B cell epitope in patients with continuous anti-HBe ongoing disease. The sequence from amino acid 74 to 84 is an important immune-dominant region for B lymphocyte stimulation [9]. In our study, we found more frequent mutations in this region in both HBeAg positive and negative patients. Interestingly, in B cell epitopes, no mutations were detected within both aa107 - 118 region of HBeAg positive patients and the aa130 - 138 region of the HBeAg negative patients.

Researchers have noted that molecular function and biological process of the core protein may be more affected by mutations occurring in the phosphorylation region of the C-terminal domain. We found a 40% mutation rate within the C-terminal domain of the core protein in our patients. Yet Carman et al. reported that a small number of mutations were detected in the same region in patients with chronic hepatitis [9]. Moreover, in our study, all of our patients with high-grade liver fibrosis had the mutations within this site. Similar results were found in previous studies [22,23]. Mohamadkhani et al. detected an increase of liver cirrhosis in patients that had mutations in the C-terminal region [23]. Interestingly, the number of mutations in the C-terminal region of our patients was unexpectedly high in the arginine-rich region, important for RNA binding. In our study, the number of mutations in HBeAg-positive patients with slightly elevated ALT levels was slightly higher compared to HBeAg-negative subjects with quite elevated ALT levels. Akarca et al. indicated that there were no mutations in this region in the majority of patients with HBeAg seroconversion and normal ALT levels [18]. In the same study, the rate of mutations for this region was lower in patients with elevated ALT values and anti-HBe seroconversion compared to HBe seroconversion persistent patients. This result did not show similarity with our findings. Interestingly, we have detected mutations in the codon 153 and 154 in only HBeAg positive

patients. However, Akarca et al. reported that there were no mutations in codon 154 in their study [18].
A recent study has shown that some types of mutations in the core region are associated with HCC compared to chronic hepatitis and liver cirrhosis [24]. The names of these mutations are P5H/L/T, E83D, I97F/L, L100I, and Q182K/Stop. In our study, we have identified two of these mutations. The Q182K amino acid change was present in one patient with liver cirrhosis while E83D amino acid change was found in another patient with stage-1 liver fibrosis. Additionally, we have detected V27I, R47H, Y132I, R174STOP, and Q182K mutations only in the patients with liver cirrhosis. In addition to the Q182K mutation, the presence of four other mutations suggest that these mutations may contribute to the development of liver cirrhosis. However, clinical outcomes for these mutations can only be assessed by further study with more samples.

CONCLUSION

This study shows that UDPS is a quite sensitive method to study the variability of mutations and variants of epitopes in the core protein. The highest variable epitopes were found in Th49 - 69 and B74 - 84 in both HBeAg positive and negative groups. This result supports the fact that these are immune stimulator regions in the core region. According to our findings, unlike literature as in terms of the mutation frequencies in the core protein, there is no significant increase in number of mutations in the core gene of virus during the anti-HBe positive period. Contrary to the mutation frequencies in the core protein, a significant difference was found in HBeAg positive patients when compared with HBeAg negative patients. Moreover, a similar number of mutations were detected in the aa74-89 region of the B cell epitopes for HBeAg positive and negative patients. Another different result is that mutations in codon 154 in the C-terminal region were detected only in HBeAg-positive patients. The reason for these results may be due to the fact that UDPS detects low frequency mutations. Furthermore, according to our findings, some of the mutations may also be associated with the disease progression. Therefore, it is clear that mutations in the core gene should be identified with more sensitive techniques such as UDPS. However, further studies with a larger sample groups of patients are necessary to confirm our findings.

Declaration of Interest:
The authors do not declare any conflict of interest.

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