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Journal of Chromatography A, 781 (1997) 307–314

JOURNAL OF CHROMATOGRAPHY A

Method for detection of epsilon-secondary structure in the precore region of human hepatitis B virus DNA using a fluorescence-based polymerase chain reaction–single-strand-conformation polymorphism technique with capillary electrophoresis

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Abstract

A portion of the precore region of the human hepatitis B virus (HBV) genome is the signal sequence with an epsilon secondary structure, which plays a role in the encapsidation of HBV pregenome RNA. To determine the genetic mutations which occur in the precore region of HBV, we have devised a typing method using a fluorescence-based polymerase-chain-reaction–single-strand conformation polymorphism technique with automated capillary electrophoresis (CE–FSSCP). Using the cloning sequencing method, we analyzed serum samples from 10 patients with hepatitis B, and detected three types of HBV-DNA including two mutants which are crucial to the function of the encapsidation sequence: position 1896 G (guanine) to A (adenine, stop codon), position 1899 G to A, and wild-type. We performed CE–FSSCP analysis of these three types of HBV-DNA and described conditions for determination of the mutations which play roles in the encapsidation of the HBV pregenome. The two types of epsilon mutants and wild-type DNA were identified as separate individual peaks respectively. The observed migration times of the three types of DNAs agreed fairly well with estimates obtained from total RNA secondary structure energy. © 1997 Elsevier Science B.V.

Keywords: Hepatitis B virus; Single-strand conformation polymorphism; Detection, electrophoresis; DNA; RNA

1. Introduction

Single-strand conformation polymorhism (SSCP) analysis is a rapid and efficient method for the detection of mutations and polymorphisms in genomic and cDNA sequences [1]. We have reported a fluorescence-based SSCP analysis following polymerase chain reaction (PCR) amplification (PCR–FSSCP) using fluorescence-labeled primers and an autosequencer for the detection of mutations in the p53 tumor suppressor gene [2]. Using an automated DNA sequencer, the results can be interpreted quantitatively, since DNA zones are detected as peaks in the fluorogram and peak height areas are proportional to the amounts of DNA of interest in a wide dynamic range. Inputting of data to a personal computer permits objective interpretation of results; moreover, data from multiple samples can be processed to decrease the possibility of analytical errors

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0021-9673/97/$17.00
PII S0021-9673(97)00593-1

[3]. However, it is probably impossible to develop a completely automatic SSCP system based on an autosequencer since the preparation and replacement of slab gels for the autosequencer must be performed manually and requires meticulous care.

Recent capillary electrophoresis (CE) studies have shown that this technique is not only rapid and convenient for the analysis of PCR products, but also applicable to a completely automatic SSCP system. In the CE format, shrinkage of the gels results in formation of bubbles saturated with water vapor, particularly at high field strengths. These drawbacks preclude the repeated use of a gel-filled capillary and thereby prevent automation of runs. Molecular-sieving polymer solutions have correspondingly become very popular as substitutes for gels. They are replaceable, i.e., the same capillary can be used in a large number of experiments, and after each run, the used polymer solution is sucked or pressed out of the capillary and replaced by a fresh one. These features guarantee easy operation and reproducible results.

We have devised a completely automatic CE–FSSCP system for the differential detection of point mutations that does not require SSCP with radioisotopes and polyacrylamide gels and that makes use of using an automated CE technique with molecular-sieving polymer solution [4]. This automatic CE–FSSCP system was developed for reproducible operation in the denaturation of double-stranded DNA and electrophoresis of single-stranded DNA. In clinical testing, this system will be the most rapid and versatile one available for the differential detection of point mutations in a large number of DNA samples.

Here we describe a simple and rapid method for the determination of epsilon-secondary structure in the precore region of the hepatitis B virus (HBV)-genome using CE–FSSCP analysis. HBV comprises a subcategory of hepadnaviruses containing a double-stranded circular DNA genome with a characteristic DNA of 3200 bp. Infection with HBV in humans can be identified by serum positivity for HBs antigen, HBe antigen or DNA polymerase activity. A precore region, which has 87 bases in the same coding frame as the core gene, encompasses the region upstream of the codon for HBc antigen in the HBV-DNA genome. Some HBV are unable to produce the HBe antigen following the introduction of a point mutation in the precore region. Specifically, if a point mutation from G guanine to A (adenine) is introduced into HBV-DNA at nucleotide position 1896 in the precore region, the resulting 28th triplet codon for transcription is changed from TGG (Trp) to TAG (stop) and HBV is unable to produce HBe antigen [5,6]. These findings suggested that HBV-DNA in serum which is negative for HBe antigen may have this point mutation in the precore region.

Numerous investigators from various parts of the world have reported the same mutation: a G to A change at nucleotide position 1896, creating a stop codon at codon 28 [7–14]. Moreover, some patients have an additional mutation: G to A, at nucleotide position 1899 [5,7,9,11,14,15]. The significance of this mutation is unknown, since it is downstream of the stop codon mutation. Junker-Niepmann et al. demonstrated that packaging of core mRNA is governed by a short, *cis*-acting element encompassing the last two-thirds of the precore region and the beginning of the core gene [16], a highly conserved sequence segment homologous to the US sequence of type C retrovirses [17]. This element, termed pregenome encapsidation signal epsilon, is predicted to form a hairpin structure composed of two stems, one loop and one bulge. Ogata et al. showed that a G to A change at nucleotide position 1896 and a G to A change at nucleotide position 1899 serves to stabilize the stem [18].

In this study, we devised a procedure for the determination of nucleotide position 1896 G to A type mutation, 1899 G to A type mutation, and wild-type in the precore region of HBV–DNA using an automated method of CE–FSSCP analysis. The observed migration times of three-type DNAs supported the stability of the pre-genome RNA encapsidation sequence secondary structure.

2. Experimental

2.1. Materials

Serum samples studied were obtained from patients with HBV seen at the Department of Gastroenterology, Toranomon Hospital (Kanagawa, Japan). Serum samples from patients with HBV were each

denatured with an equal volume of 0.2 *M* NaOH and incubated for 15 min at 37°C. The mixture was then neutralized with 0.2 *M* HCl, and a 5.0-μl aliquot of the resulting mixture was subjected to PCR [19].

2.2. Primers

The primer sequences used for this study are shown in Table 1. The primers were obtained with a DNA Synthesizer LKB Gene Assembler Plus (Pharmacia Biotech, Uppsara, Sweden) using a protocol previously described [20]. The sense primers, HBV-PC3 and HBV-PC5, subjected to examination by CE–FSSCP analysis, were labeled at their 5′ ends with fluorescence in derivative by the Fluore method (Pharmacia Biotech).

2.3. Sequence analysis

The precore and core regions between positions 1786 and 2097, which were present in the genome of HBV in serum obtained from ten patients with HBV was amplified by PCR in the presence of OAL-47 and OAL-46 primers at final concentrations of 1 μ*M* each. The amplified fragments were cloned in the TA cloning site of pCRII cloning vector (Invitrogen, San Diego, CA, USA) using T4 DNA ligase. The sequence analysis of these PCR-amplified fragments in the cloning vector was performed using the ALF II automatic sequence analyzer (Pharmacia Biotech) with M13 primers.

2.4. Instrumentation

The automatic CE–FSSCP system was developed for reproducible operation in the denaturation of double-stranded DNA and capillary electrophoresis of single-stranded DNA. Details of the instrumental setup are described elsewhere [4]. For electrophoresis, a high-voltage electric field in the range of −30 kV to +30 kV at up to 300 μA is applied with a high-voltage power supply (HCZ30PN-OW, Matsusada Precision, Shiga, Japan), while the capillary cassette is thermostated by forced air controlled between 100°C and 45°C within an accuracy of ±0.1°C. The capillary cassette used was fitted with a 75-μm I.D. synthetic silica capillary (Otsuka Electronics, Osaka, Japan) 300 mm in length and 225 mm to detector. The inner surface of the capillary was coated with polyacrylamide using the method of Hjertén [21] in order to suppress electroosmotic flow.

2.5. Automated CE–FSSCP analysis

PCR using the HBV-PC3/PC4 or HBV-PC5/PC6 primer pairs, with the sense primer labeled at the 5′ end with fluoresce in derivative, was performed as described above. Then 10^8 copies of recombinant plasmids were amplified in a total volume of 50 μl in the buffer recommended by Perkin–Elmer Roche (Branchburg, NJ, USA). The PCR products which were placed in the autosampler of the CE–FSSCP system were diluted 2-fold with a formamide buffer (Pharmacia Biotech), heated at 95°C for 5 min, placed on 0°C for 5 min, and then injected at low pressure (0.5 p.s.i.) for 30 s (1 p.s.i.=6894.76 Pa). Separation was carried out at 10 kV (negative to positive) with a system current of 15 μA and the capillary maintained at 25°C. The molecular-sieving polymer was 1×TBE (90 m*M* Tris, 90 m*M* boric acid and 2 m*M* EDTA, pH 8.5) containing 2% polyacrylamide polymer with 5% glycerol or 2% polyacrylamide polymer with 2% dextran (M_r=

Table 1
Sequences of oligonucleotide primers and their locations in the HBV genome

Primer	Sequence (5′ to 3′)	Length (mer)	Sense	Nucleotide positions
OAL 47	5′-GGCATAAATTGGTCTGTTCA-3′	20	+	1786–1805
OAL 46	5′-ATCAACTCACCCCAACACAG-3′	20	−	2078–2097
HBV-PC3	F-5′-TTTCACCTCTGCCTAATCATC-3′	21	+	1823–1843
HBV-PC4	5′-AGCTCCAAATTCTTTATACGGG-3′	22	−	1912–1933
HBV-PCS	F-5′-ACCTCTGCCTAATCATCTCATG-3′	22	+	1827–1848
HBV-PC6	5′-TTATACGGGTCAATGTCCAT-3′	20	−	1901–1920

F indicates carboxyfluorescein linked to the 5′-nucleotide via a linker and phosphate.

2 000 000, Sigma, St. Louis, MO, USA). The polyacrylamide (M_r=700 000–1 000 000) solution which was 10% in water was purchased from Tokyo Kasei (Tokyo, Japan).

2.6. Prediction of the total RNA secondary structure energy

The total RNA secondary structure energy in the HBV encapsidation signal (1827 to 1920) was calculated with DNASIS-Mac software (Hitachi Software Engineering, Tokyo, Japan).

3. Results and discussion

3.1. Cloning and sequence analysis

We analyzed serum samples from 10 patients with HBV by the cloning and sequencing method. The results of the sequence analysis are shown in Fig. 1. These HBV-DNAs were assigned to 4 types of HBV including 3 mutants: clone 27-2 (wild-type), clone 34-2 (nucleotide position 1846 A to T, 1896 G to A, and 1908 T to C changes), clone 28-4 (nucleotide position 1846 A to C change), and clone 8-3 (nucleotide position 1846 A to C and 1899 G to A changes) in the precore and core region.

3.2. CE–FSSCP analysis using a glycerol buffer system

Polyacrylamide gel electrophoresis containing glycerol is widely used for PCR–SSCP analysis. The conformation of single-stranded DNA is presumably determined by the balance between thermal fluctuation and weak local stabilizing forces such as short intra-strand base pairings and base stacking. Therefore, changes in environmental conditions such as temperature and the presence of denaturant are likely to cause changes in conformation, which can be detected in SSCP analysis as alterations in mobility [1]. We previously found the glycerol buffer system to be ideal for CE–FSSCP of the mutation detection

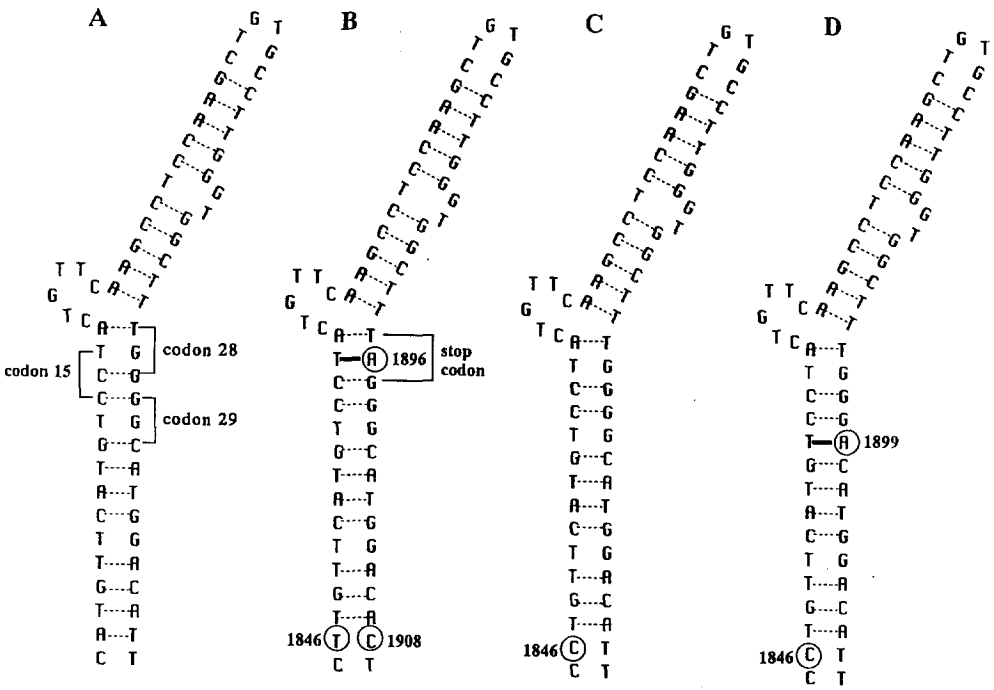

Fig. 1. Location and type of mutations detected in relation to the nucleotide sequence. Proposed secondary structures of the HBV pre-genome encapsidation sequence [16], clone 27-2 (A; wild-type), clone 34-2 (B), clone 28-4 (C) and clone 8-3 (D). Substituted nucleotides are shown in open circles.

in exon 7 of p53 gene [4], as regarded by other investigators as well [22].

Three mutated DNA samples (clone 34-2, 28-4, 8-3) and wild-type DNA sample (clone 27-2) were analyzed using the automatic CE–FSSCP system. The PCR products amplified with the HBV–PC3/PC4 primer set were mixed and then subjected to CE–FSSCP system analysis. Fig. 2 shows the CE–FSSCP electropherograms of the PCR-amplified mixture in a capillary equilibrated with 2% polyacrylamide polymer solution containing 5% glycerol. Clone 27-2 and clone 34-2 each had a peak for non-reacted primer (n-primer), a peak for double-stranded DNA molecules (ds-DNA), and two peaks for single-stranded DNA molecules (SS-1 and SS-2), as shown in Fig. 2A,B. The migration times of ds-DNA and ss-1 exhibited good agreements in all measurements of this series. In contrast, a mixture of clone 27-2 and clone 34-2 had three peaks for single-stranded DNA molecules. Two of these peaks, indicated by W-1 and 34-2 (Fig. 2C), migrated to the same positions as SS-2 of clone 27-2 and clone 34-2 in Fig. 2A,B. For mixtures of clone 27-2 and other clones (clones 28-4, 8-3), mutated clones were separated from the wild-type clone 27-2 (data not shown). However, for mixtures of clone 28-4 and clone 34-2 or 8-3, we detected no mobility shifts of peaks corresponding to two clones (Fig. 2D). These findings suggest that the differences in mobility shifts between these clones resulted from mutations at nucleotide positions 1846 and 1908.

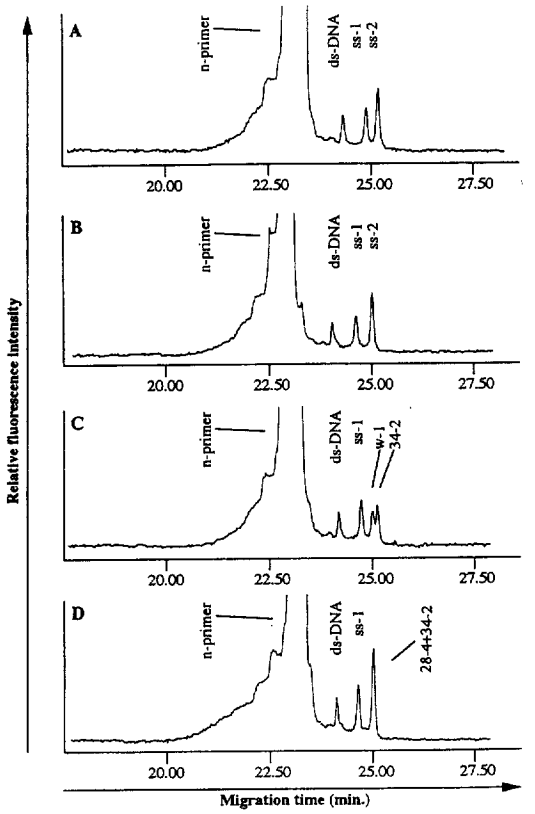

Fig. 2. CE–FSSCP analysis using a 5% glycerol polymer system and HBV PC3/PC4 primer pair. DNA samples examined were from clone 27-2 (A) and clone 34-2 (B). The mixture of clone 27-2 and 34-2 was separated at peaks indicated by w-1 and 34-2 corresponding to clone 27-2 and clone 34-2 (C). The mixture of clone 28-4 and clone 34-2 was not separated (D). The peak indicated by 28-4+34-2 corresponds to ss-2. Positions of non-reacted primer, double-stranded DNA and single-stranded DNA are indicated by n-primer, ds-DNA, ss-1 and ss-2.

To detected mutations in positions 1896 and 1899, we used the HBV-PC5 and HBV-PC6 primer set and performed CE–FSSCP analysis. Fig. 3 shows CE–FSSCP electropherograms of a PCR-amplified mix-

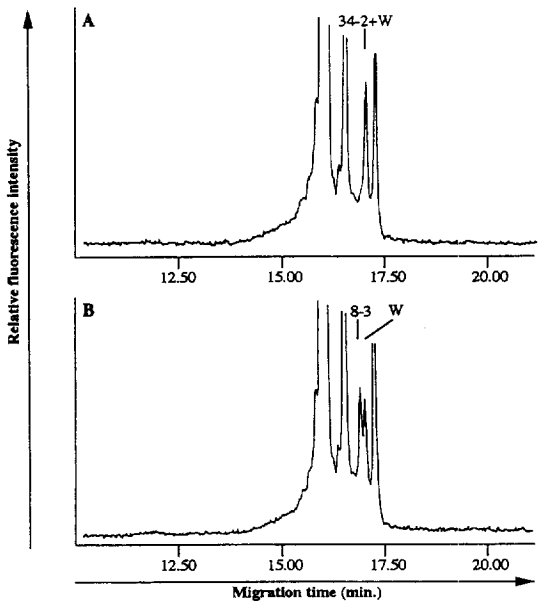

Fig. 3. CE–FSSCP analysis using a 5% glycerol polymer system and HBV PCS/PC6 primer pair. The mixture of clones 27-2 and 34-2 was not separated (A), but the mixture of clone 27-2 and 8-3 was separated at peaks indicated by 8-3 and w. The peaks indicated by 34-2+w, 8-3 and w corresponded to clone 34-2 and 27-2 (wild-type), 8-3 and 27-2.

ture in a capillary equilibrated with 2% polyacrylamide polymer solution with 5% glycerol. In contrast to that shown in Fig. 2A–D, the migration time of ss-2 was constant, though that of ss-1 varied. In the mixture of clone 27-2 and clone 8-3, the mutated clone was separated from the wild-type clone (Fig. 3B). However, this polymer solution did not adequately separate clone 34-2 from clone 27-2 (Fig. 3A).

3.3. CE–FSSCP using a dextran buffer system

To detect the G to A mutation at nucleotide position 1896, a polymer buffer system was chosen and optimized. On testing of several polymer buffer systems, we found that a dextran-containing buffer system was ideal for CE–FSSCP using HBV-PC5/PC6 primer set. Fig. 4 illustrates the electropherograms obtained using 2% polyacrylamide sieving medium of containing 2% dextran (M_r=2 000 000). Clone 27-2 had a peak for non-reacted primer (n-primer), a peak for primer dimmer, a peak for double-stranded DNA molecule (ds-DNA), and three peaks for single-stranded DNA molecules (ss-DNA) (Fig. 4A). The electropherographic patterns for clones 34-2 and 8-3 (Fig. 4B,C) were quite similar to that of clone 27-2. One of three single-stranded DNA peaks, that which migrated fastest and which is indicated by *, were detected at different positions for each clone. The other peaks could be shown at the same migration times with insignificant deviation. The results obtained in Fig. 4 were confirmed by analysis of clone 27-2 and clone 34-2 mixture, or clone 27-2 and clone 8-3 mixture. The electropherograms (Fig. 5) reveal the same pattern as in Fig. 4. The clone 27-2 and clone 8-3 mixture had four peaks for single-stranded DNA. The first and second of these peaks for single-stranded DNA corresponded to nucleotide position 1896 G to A type clone (34-2) and wild-type clone (w), respectively (Fig. 5A). The electropherographic patterns of clone 27-2 and clone 8-3 were quite similar, as shown in Fig. 5A (Fig. 5B). The first peak of the four derived from single-stranded DNA probably corresponded to clone 8-3, and when three clones, 27-2, 8-3 and 34-2, were mixed and then analyzed by

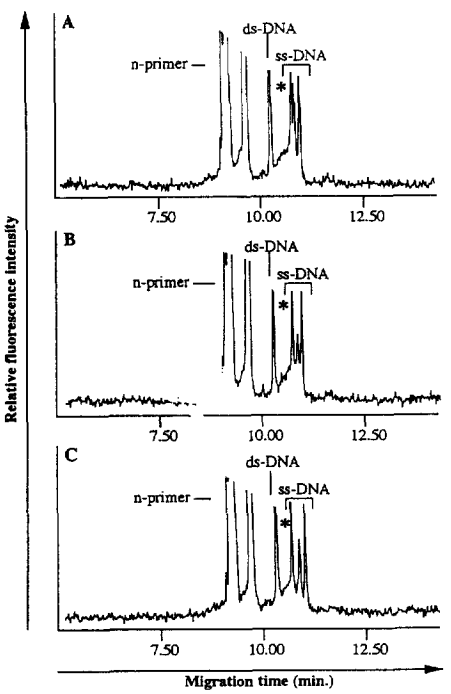

Fig. 4. CE–FSSCP analysis using a 2% dextran polymer system and HBV PCS/PC6 primer pair. DNA samples examined were from clones 27-2 (A), 34-2 (B) and 8-3 (C). One of three peaks of single-stranded DNA (ss-DNA), indicated by *, had a different migration time. Positions of non-reacted primer and double-stranded DNA are indicated by n-primer and ds-DNA.

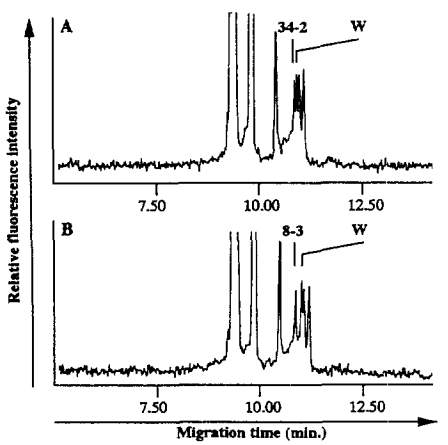

Fig. 5. Electropherograms of DNA sample mixtures. (A) The electropherogram of clone 27-2 and 34-2 mixture sample. (B) The electropherogram of clone 27-2 and 8-3 mixture sample. Single-stranded DNAs are indicated by w, 27-2 and 8-3, corresponding to clones 27-2, 8-3 and 34-2.

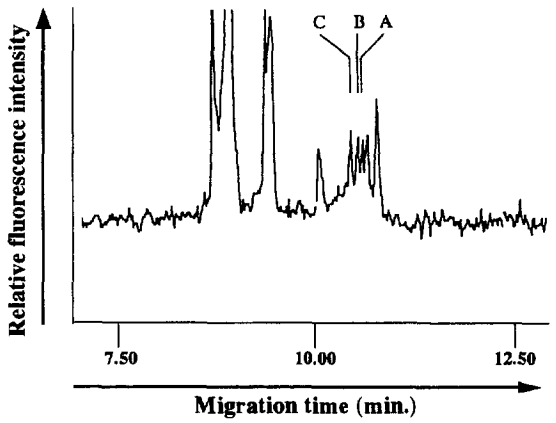

Fig. 6. Electropherogram of three-type DNA mixed samples. The single-stranded DNAs are indicated by A, B and C, corresponding to clones 34-2, 8-3 and 27-2.

CE–FSSCP, the first peak of ssDNA could be distinguished among the three, as shown in Fig. 6.

Since only the sense strand amplified with a sense primer is fluorescently labeled, one peak of single-stranded DNA is expected on fluorescence detection. However, two or three peaks of single-stranded DNA were observed (Figs. 2–4). Two possible explanations are considered. First, one of the two or three peaks of single-stranded DNA may reflect the linear shape of the strand without secondary structure. Second, these single-stranded DNA peaks may correspond to two or three different stabilized states of secondary structure.

3.4. Prediction of the total RNA secondary structure energy

To elucidate the relationship between the migration times of wild-type, nucleotide position 1896 G to A type, and nucleotide position 1899 G to A type clones and the total RNA secondary structure energy, a three-type DNA mixed sample was separated using 2% polyacrylamide polymer buffer containing 2% dextran and the total RNA secondary structure energies of three-type DNAs were predicted. Fig. 6 illustrates the electropherogram obtained using 2% polyacrylamide sieving medium containing 2% dextran. Three of five peaks for single-stranded DNAs corresponded to wild-type (peak A), nucleotide position 1896 G to A type (peak B), and nucleotide position 1899 G to A type (peak C). The values of total RNA secondary structure energy were −38.4 kcal/mol (wild-type), −39.6 kcal/mol (1896 G to A type), and −40.0 kcal/mol (1899 G to A type) (Table 2) (1 cal=4.184 J). It is possible that nucleotide sequences having lower secondary structure energy exhibited shorter migration times, since lower energy probably induces a more compact structure (Table 2).

Table 2
The relation between the migration times and total RNA secondary structure enegy

DNA type	Migration time	Secondary structure energy
Wild type	10.60 min.	−38.4 kcal/mol
1896 G to A type	10.55 min.	−39.6 kcal/mol
1899 G to A type	10.45 min.	−40.0 kcal/mol

The stem–loop structure consists of 14 nucleotides on each limb of the stem; 3 of the nucleotides are unpaired: 1858 with 1896, 1855 with 1899, and 1850 with 1904. Codon 15 and codons 28 and 29 are involved in 2 of the 3 unpaired sites (Fig. 1). Lok et al. reported that the most frequently detected mutations in patients with HBV were a T to C change at position 1858, and G to A changes at positions 1896 and 1899 [23]. The frequent occurrence of these mutations which serve to stabilize the stem structure, is intriguing. These findings suggested that the 3-nt segment (1855–1858 and 1896–1898) and perhaps the adjacent nucleotide (1855 or 1899) are required for the function of this encapsidation sequence. The occurrence in parallel of a decrease in migration time and an increase in secondary structure energy (Table 2) strongly suggested that CE–FSSCP analysis of the HBV precore region is not only useful for detection of point mutations, but also for determination of the function of the encapsidation sequence.

4. Conclusions

PCR–SSCP is a rapid and efficient method for the detection of mutations and polymorphisms in genomic or cDNA sequences. We previously reported the CE–FSSCP method for the detection of mutations in the p53 gene using a completely automatic capillary electrophoresis system [4]. In this study, we

described a simple and rapid method for detection of point mutations in the precore region of human hepatitis B virus genome using an automated CE–FSSCP system. Moreover, our findings strongly suggested that this method of CE–FSSCP analysis of the HBV precore region is useful not only for detection of point mutations, but also for determination of the function of encapsidation sequence. We expect this technique to prove useful for the diagnosis of hepatitis, determination of the therapeutic effect of interferon treatment of patients with hepatitis, and study of the molecular aspects of the mechanisms involved in the pathogenesis of hepatocellular carcinoma.

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