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SITE-DIRECTED MUTAGENESIS OF HEPATITIS B SURFACE ANTIGEN SEQUENCE AT CODON 160 FROM ARGININE TO LYSINE FOR CONVERSION OF SUBTYPIC DETERMINANT FROM r TO w

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SUMMARY Site-directed mutagenesis from G to A was induced at nucleotide 479 in the S gene of hepatitis B virus DNA, cloned from an individual carrying the surface antigen of subtype ayr. HepG2 cells were transfected with the plasmid DNA containing the mutant. They produced surface antigen of subtype ayw, unlike HepG2 cells harboring the parent viral DNA that produced surface antigen of subtype ayr. These results indicate that a point mutation from G to A at nucleotide 479 in the S gene, changing codon 160 for arginine to that for lysine, can convert the subtypic determinant of hepatitis B surface antigen from r to its allelic determinant w.

The envelope protein of hepatitis B virus (HBV), known as hepatitis B surface antigen (HBsAg), bears the group-specific determinant called a. In addition, it carries one or the other from each of the two pairs of mutually-exclusive, subtypic determinants named d and y (1), as well as w and r (2). These seemingly allelic determinants have been deduced to arise from substitutions of amino acid residues in the translation product of the S gene (3, 4).

We have reported the single amino acid substitution responsible for the d/y allelic determinants in the S gene product, Lys 122 for d and Arg 122 for y (5). The amino acid substitution controlling the w/r allelic determinants has not been identified. It is not clear which of Val 159 or Lys 160 specifies w, nor is it certain which of Ala 159 or Arg 160 determines r (5); comparison of the S gene sequences of previously reported HBV DNA clones strongly suggested that the amino acid residue 160 of lysine or arginine would specify w or r.

With the advent of site-directed mutagenesis (6, 7), we converted codon 160 of the S gene sequence from arginine to lysine in an HBV DNA clone of subtype ayr. The mutant HBV DNA clone showed the capacity to code for HBsAg of subtype ayw in HepG2 cells.

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Abbreviations used: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

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MATERIALS AND METHODS

Enzymes and Radioisotopes. Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs (Boston, MA) or Takara Biochemicals (Kyoto, Japan). [α-^{32}P]dCTP (400 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham (Buckinghamshire, England).

In vitro Mutagenesis. An HBV DNA clone (pYRB259) was obtained from an individual who carried HBsAg of subtype ayr (8). The 1,154-base-pair XbaI - BamHI fragment of the HBV genome, cut out from pYRB259, was cloned into the phage vector M13mp10 (Amersham). The single-stranded DNA generated from it was subjected to the site-specific mutagenesis with a synthetic oligonucleotide, 5' TCGCAAAATTCCTATGG 3' (17-mer, mismatch at nucleotide 479 underlined), for the purpose of converting codon 160 of the S gene from AGA (arginine) to AAA (lysine). The mutagenesis was carried out by the method of Taylor et al. (6, 7), with the oligonucleotide-directed in vitro mutagenesis system (Amersham). Clones that had undergone the required mutation were selected by radiolabeled probe hybridization using the mutagenic oligonucleotide.

The XbaI - BamHI fragment was excised from the M13mp10 harboring HBV DNA in a replicative, double-stranded form. It was religated into the XbaI - BamHI site of the parent HBV DNA (pYRB259). The plasmid thus obtained (pYRB-w160) was subjected to sequencing of the carried HBV DNA by the dideoxy-chain-termination method (9).

Transfection of HepG2 Cells. Two copies of the entire HBV genome of the mutant plasmid (pYRB-w160) in a head-to-tail arrangement were inserted into the BamHI site of pSP65 (Promega Biotec, Madison, WI). The recombinant plasmid thus obtained (p2YRB-w160) was introduced into HepG2 cells (10) by the calcium phosphate transfection method (11). Cells were grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C in an atmosphere containing 5% CO_2. HepG2 cells were transfected also with a tandem dimer of the parent HBV DNA (p2YRB259).

Serological Tests. HBsAg was determined by reversed passive hemagglutination (12), and the results were expressed by the highest twofold dilution of the sample that induced hemagglutination. Subtypes of HBsAg were determined by the solid-phase enzyme immunoassay with monoclonal antibodies (Institute of Immunology, Co., Ltd., Tokyo, Japan), by sandwiching HBsAg between monoclonal antibody against the common determinant a (monoclonal No. 3207), fixed on a solid support, and one or other of monoclonal anti-d (No. 3423), anti-y (No. 3457), anti-w (No. 4111) and anti-r (No. 313) which had been labeled with horseradish peroxidase.

RESULTS

Fig. 1 depicts the nucleotide sequence and translation product of the S gene of an HBV DNA clone of subtype ayr (pYRB259), along with those of its artificial mutant (pYRB-w160) that was expected to code for HBsAg of subtype ayw. They were different in the single nucleotide at position 479. The parent clone had G, contributing to codon 160 for arginine as the second letter, while the mutant clone had A, contributing to that for lysine.

The spent culture medium of HepG2 cells, transfected with the plasmid containing parent or mutant HBV DNA, was tested for HBsAg on the day 2, 5, 7, 9 and 12. The hemagglutination titer of HBsAg was the highest at 2^4 on the day 5. The two HBsAg preparations, coded for by the parent or mutant HBV DNA, were subtyped by the solid-phase enzyme immunoassay (Table 1). HBsAg produced by cells harboring the mutant HBV DNA had subtype ayw, contrasting with HBsAg of subtype ayr produced by cells harboring the parent HBV DNA.

Figure 1. Nucleotide sequence and translation product of the S gene of an HBV DNA clone of subtype ayr (pYRB259) and its mutant of subtype ayw (pYRB-w160). The site-directed mutagenesis of nucleotide 479 from G to A converted Arg 160 to Lys 160.

Table 1. Sandwich Enzyme Immunoassay for the Common and Subtypic Determinants of HBsAg Produced by HepG2 Cells Transfected with the Parent HBV DNA or its Mutant That Had Undergone the Site-Specific Mutagenesis

HBsAg produced by	Enzyme-labeled monoclonal antibody against				
	a (No. 824)	d (No. 3423)	y (No. 3457)	w (No. 4111)	r (No. 313)
HepG2 cells transfected with parent HBV DNA (p2YRB259)	> 2.00	0.06	> 2.00	0.03	> 2.00
HepG2 cells transfected with mutant HBV DNA (p2YRB-w160)	> 2.00	0.05	> 2.00	> 2.00	0.03
Control (culture medium)	0.05	0.04	0.04	0.03	0.03

HepG2 cells were transfected with the plasmid harboring a tandem dimer of the recombinant HBV DNA (p2YRB259) or its mutant (p2YRB-w160) generated by the site-specific mutagenesis from G to A at nucleotide 479 of the S gene. The spent culture media were concentrated so as to give a reversed passive hemagglutination titer for HBsAg at 2^7. Culture media containing HBsAg particles were tested for the common and subtypic determinants by sandwiching them between monoclonal antibody against the common determinant a, fixed on a solid support, and monoclonal antibody directed to common or subtypic determinant, labeled with horseradish peroxidase. Values represent the absorbance at 492 nm.

DISCUSSION

Amino acid residue 122 of the S gene product has been implicated in the d/y allelic determinants, along with residue 68, based on the comparison of HBV DNA clones of various subtypes (8). Peterson et al. observed that reductive methylation of Lys 122 decreased the reactivity with monoclonal antibody to the determinant d (4). Antoni and Peterson (13) converted Cys 121 to Ser by means of site-directed mutagenesis, and observed a decrease in the binding with monoclonal antibody directed to the common determinant a, as well as with that to a subtypic determinant y. From the plasma of a rare individual who carried HBsAg particles of a compound subtype, adyr, on which both d and y determinants occurred, two kinds of HBV DNA clones were propagated which were different in the S gene sequence at nucleotide 365 (5). NIH3T3 cells transfected with a clone with nucleotide 365 of A produced HBsAg/adr, while those transfected with a clone with that of G produced HBsAg/ayr. These observations unambiguously indicate that the substitution of nucleotide 365 of A, contributing to codon 122 for lysine, to G contributing to that for arginine, can convert the subtypic determinant d to its allelic determinant y.

A single nucleotide substitution that specifies the w/r allele has not been verified as yet. From an individual who carried HBsAg of another compound subtype, adwr, two kinds of HBV DNA clones were established which were discordant at two nucleotides in the S gene sequence (5). Clones with the capacity to code for HBsAg of subtype adw in NIH3T3 cells had T and A as nucleotides 476 and 479, respectively, contrasting with clones encoding HBsAg of subtype adr that had C and G as the respective nucleotides. On the basis of these observations alone, it is not to be decided which of Val 159 or Lys 160 is responsible for the determinant w, nor is it certain which of Ala 159 or Arg 160 specifies the determinant r. The comparison with previously reported HBV DNA clones with w or r subtype strongly indicated that Lys 160 would be responsible for the w determinant and Arg 160 for the r determinant (5).

With the advent of site-directed mutagenesis, we attempted to identify the amino acid substitution responsible for the w/r subtypic determinants. The parent HBV DNA clone of subtype ayr (pYRB259) was subjected to the mutagenesis at nucleotide 479 of the S gene to generate a mutant (pYRB-w160). A tandem dimer of parent plasmid (p2YRB259) or that of mutant plasmid (p2YRB-w160) coded for the production of HBsAg particles of subtype ayr or ayw in HepG2 cells. A single point mutation of nucleotide 479 from G to A, therefore, can convert the r determinant to its allelic determinant w, just as is the case for the d/y determinants.

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