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Antisense oligonucleotides are effective inhibitors of hepatitis B virus replication in vitro

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Abstract

Antisense oligonucleotides are currently being used in numerous laboratories as potential anticancer and antiviral agents. The unique replication cycle of hepatitis B virus (HBV) contains several different steps which are potentially amenable to modulation by these molecules. We have examined the ability of 56 different single-stranded, oligodeoxyribonucleotides (14–23 nucleotides in length), which target several HBV-specific functions, to inhibit HBV replication in the human hepatoblastoma cell line, 2.2.15. None of the oligonucleotides examined were toxic at concentrations up to 500 μM. Oligonucleotides directed against the HBV surface antigen (HBsAg) gene (S gene), the preS1 open reading frame, and the HBV core antigen (HBcAg) gene (C gene) were effective at depressing virus production, while molecules targeting the HBV e antigen (HBeAg) open reading frame and the HBV polymerase (POL) gene were ineffective. Oligonucleotides directed against the HBV encapsidation signal/structure (ϵ) comprised some of the most effective antiviral molecules against HBV. None of 5 oligonucleotides complementary (i.e., 'sense' orientation) to the antisense oligonucleotides targeting HBsAg, HBcAg, HBeAg, preS1 and POL had any measurable effect on HBV production. The relative effectiveness of oligonucleotides targeting the S and C genes on HBV replication was highly correlated with an effect on HBsAg or HBcAg levels, respectively. None of the antisense oligonucleotides examined affected either the levels or the sizes of HBV-specific RNA transcripts. Since antisense oligonucleotides can exert biologic effects on HBV in 2.2.15 cell cultures in a sequence-specific manner which are consistent with predicted modes of action, such molecules may have practical applications in the therapy of chronic HBV infection.

Keywords: Hepatitis B virus; Antisense oligonucleotide; S gene; C gene; Replication inhibitor

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1. Introduction

At present, no satisfactory antiviral therapy has been established for individuals with chronic hepatitis B virus (HBV) infection. The only licensed agent for the treatment of chronic HBV infection is α-interferon. However, this drug is not an effective treatment for most HBV chronic carriers and produces substantial side effects in some individuals during prolonged treatment. The majority of antiviral approaches against HBV are focused on the use of nucleoside analogues to inhibit viral polymerase activities (see Hoofnagle (1990) for a review). However, the organization of the HBV replication cycle (Summers and Mason, 1982; Tiollais et al., 1988) indicates that several other steps in the viral replication pathway are suitable targets for effective antiviral therapy. Antisense oligonucleotides, which represent a novel class of antiviral agents (see Cohen (1991), and Stein and Cheng (1993) for reviews), have the potential to target several different parts of the viral replication pathway by annealing to HBV-specific RNA.

Since the replication cycle of HBV is generally well understood, the successful targeting of specific viral proteins and functions can be confirmed by established assays for HBV-specific functions. An examination of the replication pathway of HBV predicts virologic responses to different antisense oligonucleotides which can be used to confirm probable modes of action. For example, oligonucleotides interfering with the production of HBsAg would not be expected to directly interfere with intracellular HBV DNA replication since HBsAg is involved with steps in the HBV replication cycle which follow viral DNA synthesis (Summers and Mason, 1982; Tiollais et al., 1988). By contrast, oligonucleotides inhibiting HBcAg production would be expected to inhibit both intracellular virus replication and virion release by interrupting genomic RNA packaging, which occurs prior to HBV DNA synthesis (Summers and Mason, 1982; Tiollais et al., 1988; Pollack and Ganem, 1993).

Mutational analyses of the HBV genome in numerous laboratories have identified several critical viral functions which, if suppressed, could disrupt HBV production at specific points in the replication cycle. Due to technical considerations, such studies have been conducted by transient transfection of cultured cells. As such, the effect of modulating many of the critical HBV functions under conditions of a pre-existing chronic virus replication have not been studied. Antisense oligonucleotides provide a means to test if the selective disruption of specific HBV proteins or functions can induce predicted biologic consequences under conditions of chronic virus replication.

The current study was designed to address several fundamental issues related to the control of HBV replication. Can specific HBV functions be selectively disrupted under conditions of a pre-existing chronic virus replication? Will such disruptions produce patterns of biologic effects which are consistent with current models of HBV replication? Can antisense molecules exert predicable patterns of biological effects on HBV replication? Which sequences on the HBV genome have the most potential as targets for the antiviral activity of antisense oligonucleotides? Some of the HBV targets identified in this study may be accessible by means other than antisense oligonucleotides, while others may be best modulated by such molecules. For example, the HBV encapsidation signal/sequence (ϵ), due to its fundamental role in the initiation of HBV DNA synthesis, has been previously suggested to be a key potential antiviral target (Wang and

Seeger, 1992, 1993). As shown in the current study, antisense oligonucleotides can be used to effectively target ϵ, and other HBV genetic elements, and inhibit HBV replication.

2. Materials and methods

2.1. Cell culture and antiviral treatments

Antiviral activity and toxicity of oligonucleotides were assessed using a standardized culture assay (Korba and Gerin, 1992), which uses confluent cultures of the HBV-producing, human hepatoblastoma cell line, 2.2.15 (Sells et al., 1988). This cell line, which chronically produces infectious HBV (Acs et al., 1987), has been shown to be an accurate and predictive model for all measured aspects of cellular HBV replication and for the response of HBV to several antiviral agents which have been used in vivo (Sells et al., 1988; Korba and Milman, 1991; Korba and Gerin, 1992). HBV DNA replication and gene expression in this cell line is at stable, maximal levels only in confluent cultures (Sells et al., 1988; Korba and Gerin, 1992). Oligonucleotides (usually 0.3–10 μM) were added to confluent cultures of 2.2.15 cells every 24 h for 9 consecutive days in fresh culture medium (RPMI-1640 with 2% fetal bovine serum). Analyses of HBV nucleic acids and proteins were performed 24 h following the last treatment of oligonucleotides or 2′,3′-dideoxycytidine (2′,3′-ddC). These assays therefore reflect the production of virus products during the final day of treatment. In these studies, 2′,3′-ddC, a selective and effective antiviral agent in 2.2.15 cells (Korba and Gerin, 1992), was used as a positive assay standard.

2.2. Oligonucleotides

Phosphorothioate-substituted (Cohen, 1991; Stein and Cheng, 1993) oligonucleotides were produced using an Applied Biosystems DNA Synthesizer (Model 384B). The base sequence used to synthesize the oligonucleotides for these studies is a consensus sequence for HBV/ayw (Galibert, 1979). The HBV genome in 2.2.15 cells has been previously identified to be subtype HBV/ayw (Acs et al., 1987). Following deprotection and lyophilization, oligonucleotides were resuspended in sterile deionized water. Oligonucleotide concentrations were determined by measuring absorbance at 260 nm (A_{260}) using a conversion of 1.0 A_{260} unit to 35 μg DNA/ml (Applied Biosystems, Inc.).

2.3. Analysis of HBV nucleic acids and proteins

HBV virion DNA in culture medium, and intracellular HBV RI and HBV RNA levels were determined by quantitative blot hybridization analyses (dot, Southern, and Northern blot, respectively) (Korba and Milman, 1991; Korba and Gerin, 1992). Nucleic acids were prepared by previously described procedures (Korba and Milman, 1991; Korba and Gerin, 1992). Integrated HBV DNA, which remains at a stable level per cell during the

treatment period, was used to quantitate the amount of cellular DNA transferred in each Southern gel lane (Korba and Milman, 1991; Korba and Gerin, 1992). For the HBV RNA analyses, the levels of ß-actin RNA were used to quantitate the amount of cellular RNA transferred in each Northern gel lane. Previous examinations of ß-actin-specific RNA in confluent cultures of 2.2.15 cells demonstrated a steady state level of approximately 1.0 pg ß-actin RNA/μg unfractionated cellular RNA (Korba and Milman, 1991). EC_{90} values (10-fold depression of HBV DNA levels relative to untreated (control) cultures were determined by linear regression (Korba and Gerin, 1992). EC_{90} values are used for comparison since, in this culture system, DNA levels within 3-fold of control values are not generally statistically significant (Korba and Gerin, 1992).

Values for HBV proteins were determined by semi-quantitative EIA performed as previously described (Muller et al., 1992). For the EIA analyses, test samples were diluted (2- to 10-fold) so that the assay values produced were within the linear dynamic range of the EIA assays. Standard curves using serial dilutions of positive assay controls were included in each set of EIA analyses. HBV surface antigen (HBsAg), preS1 protein, and HBe antigen (HBeAg) are released as extracellular products and were therefore analyzed in culture medium obtained 24 h following the last treatment dose of oligonucleotides or 2',3'-ddC. HBV core antigen (HBcAg) is an intracellular viral protein and was assayed in cell extracts produced by Triton-X-100 lysis (Muller et al., 1992).

Cultures for HBV RNA were maintained on 6-well plates, cultures for HBV virion DNA analyses were maintained on either 96- or 24-well plates, and cultures for all other HBV parameters were maintained on 24-well plates.

2.4. Toxicity analyses

Cytotoxicity in 2.2.15 cells was determined by uptake of Neutral red dye using previously described procedures (Korba and Gerin, 1992). Cultures for cytotoxicity analyses were maintained on 96-well flat-bottomed plates. Cultures were seeded, maintained, and treated with test compounds under conditions which were identical to those used for the antiviral analyses.

3. Results

3.1. Antiviral effects of antisense oligonucleotides

The relative activity of antisense oligonucleotides directed against a specific gene is generally inversely proportional to its location relative to the AUG start codon of the target gene (Cohen, 1991; Stein and Cheng, 1993). In a previous report, antisense oligonucleotides targeting sequences more than 60 bases downstream from the AUG start codon of integrated HBV surface antigen (HBsAg) gene (S gene) sequences in HBsAg-producing PLC/PRF/5 cells were ineffective in inhibiting HBsAg production, while oligonucleotides placed within 20 bases of the AUG inhibited HBsAg production by 50–90% (Goodarzi et al., 1990). Accordingly, the S gene was chosen as a model to

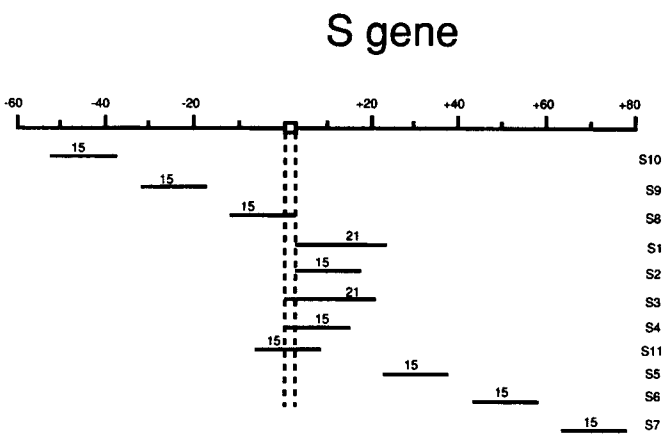


Fig. 1. Effect of antisense oligonucleotides targeting the S gene on HBV virion production. Confluent cultures of 2.2.15 cells, maintained in RPMI-1640 (with 4% fetal bovine serum) on 96-well flat-bottomed plates, were treated with oligonucleotides (0.3–20 μM, 3–6 cultures each) daily for 9 consecutive days. The open box denotes the AUG start codon of the S gene. Coordinates are nucleotides relative to the 'A' (nucleotide '+1') of the AUG start codon. Lines indicate the relative position of the different oligonucleotides; numbers above each line indicate the length (in nucleotides) of each molecule. The nucleotide sequences of each oligonucleotide are those contained in a previously published sequence of HBV/ayw (Galibert, et al., 1979). In this numbering scheme, nucleotide '1' is the first base following the single *Eco*RI cleavage site. Using this numbering scheme as a reference, the coordinates of the first nucleotide in each antisense oligonucleotide are as follows: S1, 160; S2, 160; S3, 157; S4, 157; S5, 177; S6, 197; S7, 217; S8, 145; S9, 125; S10, 105; S11, 151.

test these principles in confluent cultures of 2.2.15 cells where over 95% of intracellular HBV DNA is episomal (Sells et al., 1988; Korba and Milman, 1991; Korba and Gerin, 1992). In the current study, the relative activities of 11 antisense oligonucleotides which targeted the S gene against HBV virion DNA and HBsAg production by 2.2.15 cells were correlated with their proximity to the AUG start codon (Fig. 1 and Table 1). Oligonucleotides targeting sequences more than 20 nucleotides upstream or downstream of the AUG were essentially inactive in these analyses (Table 1).

A series of 15 different antisense oligonucleotides were then targeted to: (1) the region upstream of the S gene which encodes for preS1; (2) the region upstream of the HBV core antigen (HBcAg) gene (C gene) which encodes for the HBV e antigen (HBeAg); and (3) the HBV polymerase (POL) gene. Based on the results of oligonucleotides directed against the S gene, these series of oligonucleotides were clustered around the AUG start codons of the different HBV coding regions (Fig. 2).

Oligonucleotides targeting the preS1 coding region were also effective at inhibiting HBV virion production as oligonucleotides targeting analogous regions of the S gene (Table 2). Oligonucleotides targeting the HBeAg coding region or the HBV POL gene did not inhibit HBV virion production in 2.2.15 cells (Table 2). Oligonucleotides directed against HBeAg were, however, effective in inhibiting HBeAg production by 2.2.15 cells (Table 2). Oligonucleotides A–C (but not D and E) directed against the HBeAg coding region (Fig. 2) also encompassed the beginning of DR1, the initiation

Table 1
Effect of anti-S oligonucleotides on HBV production

Oligonucleotide	Virion DNA	EC_{90} (μM, ±S.D.)		
		HBsAg	HBeAg	HBcAg
S1	3.0±0.4	2.0±0.2	> 20	> 20
S2	2.8±0.3	1.8±0.2	> 20	> 20
S3	3.1±0.3	1.5±0.1	> 20	> 20
S4	2.8±0.2	1.7±0.2	> 20	> 20
S5	9.8±1.0	11.0±1.3	> 20	> 20
S6	> 20	> 20	> 20	> 20
S7	> 20	> 20	> 20	> 20
S8	6.9±0.8	3.2±0.3	> 20	> 20
S9	14.4±1.6	> 20	> 20	> 20
S10	> 20	> 20	> 20	> 20
S11	5.1±0.5	2.6±0.3	> 20	> 20

Confluent cultures of 2.2.15 cells were treated with oligonucleotides, and the levels of HBV virion DNA, HBsAg, HBeAg, and HBcAg were measured as described in Materials and methods. See Fig. 1 for sizes and locations of the oligonucleotides relative to the S gene sequence. EC_{90} values are expressed as micromolar concentration (μM). Standard deviations (S.D.) for the EC_{90} values are also presented. The designation '> 20' indicates that no inhibition of HBV virion DNA or HBV proteins (relative to control cultures) was observed for the indicated oligonucleotide at concentrations up to 20 μM.

site for the first strand of HBV DNA synthesis which is located only 8 bases inside the HBeAg coding region (Galibert et al., 1979; Tiollais et al., 1988).

The effect of oligonucleotides directed against the HBV C gene on HBV replication was also examined. Due to the proximity of the single polyadenylation signal/sequence (polyA signal) on the HBV genome (Galibert et al., 1979; Tiollais et al., 1988) (open box in Fig. 3), a series of 7 oligonucleotides was constructed to test the relative contribution of sequences near the beginning of the C gene and those associated with the polyA signal in inhibiting HBV. Oligonucleotides directed against regions close to, or overlapping, the AUG start codon of the C gene (filled box in Fig. 3), and which also encompassed all, or part of, the single polyA signal in the HBV genome (C1, C3, C4, C7), were the most effective at inhibiting HBV virion DNA and HBcAg production (Table 3). Oligonucleotides directed primarily at the polyA signal were inactive (C6), or

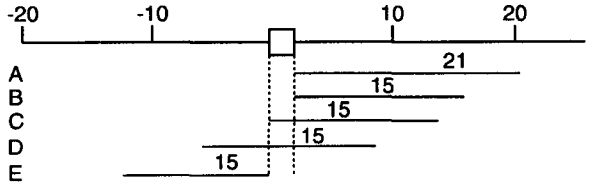


Fig. 2. Effect of antisense oligonucleotides on HBV virion production. The open box denotes the AUG start codon of the HBV genes or coding regions: preS1 (preS1), e (HBeAg), POL (polymerase). See legend to Fig. 1 for other details. The coordinates of the first nucleotide in each antisense oligonucleotide are as follows: preS1, (A) 2853, (B) 2853, (C) 2850, (D) 2844, (E) 2838; HBe, (A) 1818, (B) 1818, (C) 1815, (D) 1809, (E) 1803; POL, (A) 2311, (B) 2311, (C) 2308, (D) 2302, (E) 2296.

Table 2
Effect of antisense oligonucleotides on HBV production

HBV target	Oligonucleotide	Virion DNA	EC_{90} (μM, $\pm$S.D.)		
			HBsAg	HBeAg	HBcAg
preS1	A	6.6±0.6	>20	>20	>20
	B	6.0±0.7	>20	>20	>20
	C	6.2±0.6	>20	>20	>20
	D	8.7±0.7	>20	>20	>20
	E	12.0±1.5	>20	>20	>20
HBeAg	A	>20	>20	6.8±0.7	>20
	B	>20	>20	5.4±0.5	>20
	C	>20	>20	5.0±0.6	>20
	D	>20	>20	9.8±0.9	>20
	E	>20	>20	11.3±1.4	>20
POL	A	>20	>20	>20	>20
	B	>20	>20	>20	>20
	C	>20	>20	>20	>20
	D	>20	>20	>20	>20
	E	>20	>20	>20	>20

See Fig. 2 for sizes and locations of the oligonucleotides relative to the target HBV sequences. See Table 1 for other experimental details.

less active (C2, C5) than those molecules which were directed at the beginning of the C gene (Table 3).

The effects of antisense oligonucleotides on the HBV encapsidation signal/sequence (ϵ), which is essential for HBV replication (Nassal et al., 1990; Pollack and Ganem,

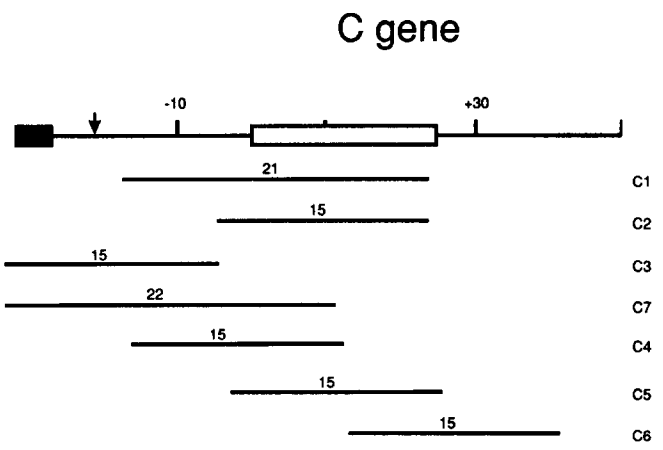


Fig. 3. Effect of oligonucleotides targeting the C gene. The filled box denotes the AUG start codon for the C gene, the open box denotes the location of the single HBV polyadenylation signal/sequence, and the arrow indicates the location of the last base in the ϵ stem-and-loop structure. See legend to Fig. 1 for other details. Coordinates of the first nucleotides in the oligonucleotides displayed in this figure are: C1 and C4, 1910; C2, 1916; C3 and C7, 1903; C5, 1917; C6, 1925.

Table 3
Effect of anti-C oligonucleotides on HBV production

Oligonucleotide	Virion DNA	EC_{90} (μM, $\pm$S.D.)		
		HBsAg	HBeAg	HBcAg
C1	2.8 $\pm$ 0.3	> 20	> 20	3.9 $\pm$ 0.5
C2	16.6 $\pm$ 1.3	> 20	> 20	> 20
C3	3.4 $\pm$ 0.3	> 20	> 20	4.1 $\pm$ 0.5
C4	5.5 $\pm$ 0.4	> 20	> 20	7.2 $\pm$ 0.8
C5	15.5 $\pm$ 1.7	> 20	> 20	> 20
C6	> 20	> 20	> 20	> 20
C7	4.8 $\pm$ 0.4	> 20	> 20	4.5 $\pm$ 0.6

See Fig. 3 for sizes and locations of the oligonucleotides relative to the C gene sequence. See Table 1 for other experimental details.

1993; Wang and Seeger, 1993), were investigated using a series of 18 separate molecules which covered different portions of the ϵ stem-and-loop structure. Some of the oligonucleotides directed at ϵ were the most highly effective molecules at inhibiting HBV production by 2.2.15 cells in this study (Fig. 4 and Table 4). The most active oligonucleotides among all the molecules tested in this study (L2c, L3e) were directed at the upper unpaired loop and the upper stem. An analogous molecule to L2c (L2b) which extended 5 nucleotides further into the lower stem was 10-fold less active. Other oligonucleotides targeting the upper stem (L2d, L2e, L3d) demonstrated the importance of the inclusion of the nucleotides associated with the upper unpaired loop in the active L2c and L3e sequences (Fig. 4). One of the oligonucleotides directed at the lower stem-and-loop structure (L1b) was also effective at inhibiting HBV virion production.

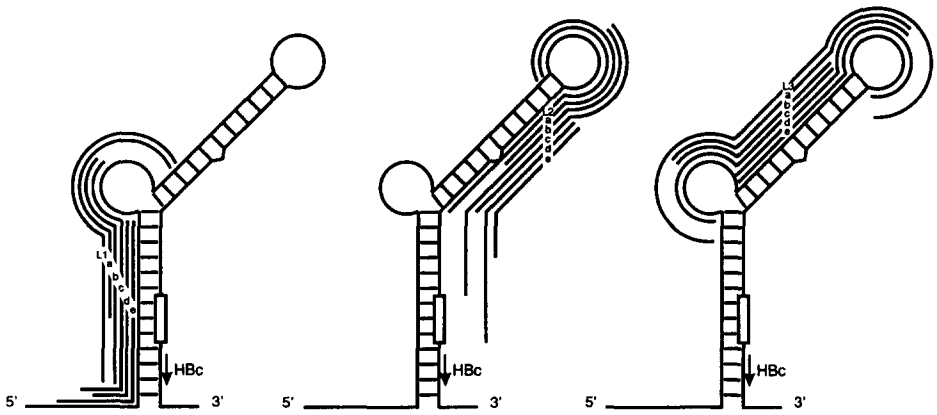


Fig. 4. Effect of oligonucleotides targeting the ϵ packaging signal. The open box denotes the AUG start codon for the C gene. The sizes of each oligonucleotide are indicated in parentheses. In the numbering scheme used in this study, the nucleotide located at the bottom of the 5'-side of the lower stem is nucleotide 1849 and is paired with nucleotide 1909. Coordinates of the first nucleotides in the oligonucleotides displayed in this figure are: L1, 1849; L1a, 1854; L1b, 1849; L1c, 1844; L1d, 1846; L1e, 1841; L2, 1879; L2a, 1879; L2b, 1884; L2c, 1884; L2d, 1887; L2e, 1887; L3, 1862; L3a, 1865; L3b, 1865; L3c, 1862; L3d, 1868; L3e, 1868.

Table 4
Effect of anti-ϵ oligonucleotides on HBV production

Oligonucleotide	Virion DNA	EC_{90} (μM, ±S.D.)		
		HBsAg	HBeAg	HBcAg
L1 (21)	8.8±0.9	>20	>20	>20
L1a (16)	8.7±0.8	>20	>20	>20
L1b (16)	5.1±0.5	>20	>20	>20
L1c (20)	12.2±1.4	>20	>20	>20
L1d (16)	9.7±1.1	>20	>20	>20
L1e (21)	11.0±1.2	>20	>20	>20
L2 (21)	15.9±1.7	>20	>20	>20
L2a (16)	14.4±1.6	>20	>20	>20
L2b (21)	11.8±1.3	>20	>20	18.8±1.5
L2c (16)	1.6±0.1	>20	>20	18.5±1.8
L2d (21)	12.9±1.7	>20	>20	19.1±2.3
L2e (16)	9.0±1.2	>20	>20	17.3±2.0
L3 (23)	5.4±0.6	>20	>20	>20
L3a (17)	5.2±0.5	>20	>20	>20
L3b (14)	4.5±0.5	>20	>20	>20
L3c (17)	3.0±0.4	>20	>20	>20
L3d (14)	12.2±1.5	>20	>20	>20
L3e (17)	1.3±0.1	>20	>20	>20

See Fig. 4 for sizes and locations of the oligonucleotides relative to the ϵ packaging signal/structure. The lengths of the oligonucleotides are listed in parentheses. See Table 1 for other experimental details.

The relative activities of oligonucleotides L3b and L3c, as well as L1d and L1e, demonstrate that the unpaired nucleotides in the lower loop/bulge of ϵ are also effective antiviral targets. Although the anti-ϵ oligonucleotides were located immediately upstream of the C gene, these molecules were relatively ineffective at lowering the intracellular levels of HBcAg (Table 5).

3.2. Target specificity

The specific patterns of effects on HBV proteins and HBV nucleic acid molecules for several different antisense oligonucleotides was used to confirm predicted probable modes of action. The S1 oligonucleotide (see Fig. 1) inhibited both extracellular HBV virion and HBsAg production, but had no effect on the levels of intracellular HBV replication intermediates (HBV RI), extracellular HBeAg levels, or intracellular HBcAg levels, consistent with its expected mode of action (Tables 1 and 5). Similarly, the other anti-S oligonucleotides were inactive against the production of HBeAg and HBcAg (Table 1). The E1 oligonucleotide ('A' for the 'e' coding region in Fig. 2 and Table 2) inhibited the release of HBeAg, but did not affect the levels of HBsAg, HBcAg, HBV virion DNA, or HBV RI, again, consistent with expected modes of action (Tables 2 and 5). The other anti-ϵ oligonucleotides were also inactive against HBsAg and HBcAg production (Table 2). The C1 oligonucleotide (see Fig. 3) lowered the levels of both HBV virion DNA and HBV RI, and interfered with HBcAg production (Tables 1 and 5). The C1 oligonucleotide, which extends over the single polyA signal on the HBV

Table 5
Levels of HBV proteins and nucleic acids in 2.2.15 cells treated with antisense oligonucleotides

Antiviral agent	HBV target	HBV proteins and nucleic acids (% control)						
		Virion DNA	HBV RI	HBV RNA transcripts		HBsAg	HBeAg	HBcAg
				3.6 kb	2.1 kb			
S1	HBsAg	4 [a]	101	98	95	2 [a]	91	90
PS1	preS1	6 [a]	89	88	106	41	95	89
E1	HBeAg/DR1	91	90	96	92	101	6 [a]	106
C1	HBcAg/polyA	2 [a]	14 [a]	95	91	53	48	4 [a]
L1b	ϵ	5 [a]	11 [a]	90	94	89	98	48
L2c	ϵ	1 [a]	1 [a]	88	95	103	90	15 [a]
L3e	ϵ	1 [a]	1 [a]	91	102	93	89	48
P1	HBV POL	102	96	100	92	94	99	97
'Sense' S1	HBsAg	98	102	ND [b]	ND	93	103	99
'Sense' PS1	preS1	90	92	ND	ND	109	94	88
'Sense' C1	HBcAg/polyA	101	87	ND	ND	97	95	105
'Sense' E1	HBeAg/DR1	91	97	ND	ND	107	91	96
'Sense' P1	HBV POL	88	99	ND	ND	90	89	90
2′,3′-ddC	HBV POL	1 [a]	6 [a]	92	105	96	95	102

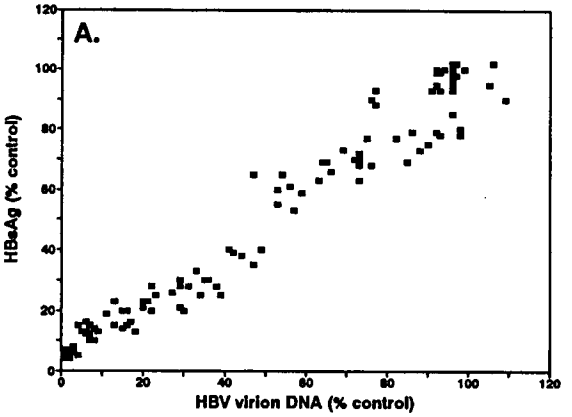
See text and legends to Figs. 1–4 for specific locations of oligonucleotide target sequences. Cultures were treated daily with 10 μM concentrations of the indicated agents for 9 days (Korba and Gerin, 1992). The different antisense oligonucleotides listed in this table have different EC_{90} values (see Tables 1–4). For purposes of direct comparison, values presented are a percentage of the average values observed in untreated (control) cultures maintained in parallel with the treated cultures. Each value represents the average of observations obtained from 8–12 separate cultures in 2 or 3 experiments. See legend to Fig. 1 for other experimental details. The two major classes of HBV RNA transcripts (2.1 kb and 3.6 kb) are listed separately. The 2.1-kb transcripts primarily encode for HBsAg, while the 3.6-kb transcripts encode for all other HBV proteins and serve as the template for the first strand of HBV replication (Summers and Mason, 1982; Tiollais et al., 1988). The levels of HBV nucleic acids and proteins following treatment with 2′,3′-dideoxycytidine (2′,3′-ddC) are presented for reference.

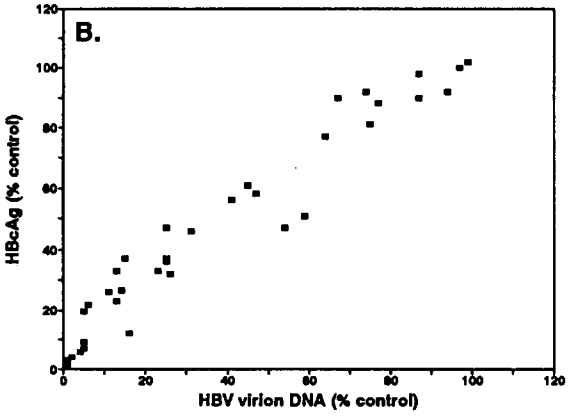
[a] Significantly different from control values ($P < 0.05$), using a one-tailed t-test with corrections for small numbers to assign Fisher's probability values.

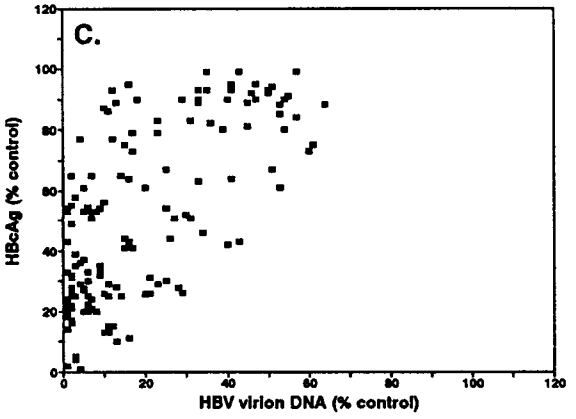
[b] ND, not determined.

genome, also induced a slight depression in the levels of HBsAg and HBeAg at a concentration of 10 μM (Table 5). Despite this modest effect on HBsAg and HBeAg levels by one anti-C oligonucleotide, the anti-C oligonucleotides were found to be inactive against the production of HBeAg and HBsAg (Table 3). Oligonucleotides L1b, L2c, and L3e, the most active antiviral anti-ϵ molecules (see Fig. 4 and Table 4), inhibited the levels of both HBV virion DNA and HBV RI, but, of these 3 molecules,

Fig. 5. Comparison of HBV virion and HBV protein levels for cultures treated with antisense oligonucleotides. The relative levels (as compared to control (untreated) cultures) of HBV virion DNA and either HBsAg (A) or HBcAg (B and C) in cultures treated with various concentrations of different antisense oligonucleotides were directly compared. Each plotted point represents an average of values obtained from 3 to 4 separate cultures. A: cultures treated with anti-S oligonucleotides. B: cultures treated with anti-C oligonucleotides. C: cultures treated with anti-ϵ oligonucleotides.


A.
HBeAg (% control)
HBV virion DNA (% control)
0
20
40
60
80
100
120


B.
HBcAg (% control)
HBV virion DNA (% control)
0
20
40
60
80
100
120


C.
HBcAg (% control)
HBV virion DNA (% control)
0
20
40
60
80
100
120

only L2c had a significant effect on HBcAg levels at 10 μM (Table 5). In general, the anti-ϵ oligonucleotides were inactive against HBcAg production (Table 4). None of the anti-ϵ oligonucleotides affected the levels of HBsAg or HBeAg (Tables 4 and 5).

In addition to directly interfering with the translation of specific RNA molecules, phosphorothioate-substituted (PS) antisense oligonucleotides have been associated with the destruction of targeted and, at high concentrations, non-targeted RNA transcripts (Cohen, 1991; Stein and Cheng, 1993). A subset of antisense oligonucleotides, which included some of the most active molecules in this study, were chosen to test for such activity which would be manifested as a loss of specific HBV RNA transcripts. Northern blot hybridization analysis of HBV-specific RNA from treated 2.2.15 cells demonstrated that the S1, C1, E1, L1b, L2c, and L3e oligonucleotides had no measurable effect on either the overall levels of HBV RNA or the relative levels of either of the two major classes of HBV RNA transcripts in 2.2.15 cells (Table 5).

In order to determine if the effects on HBV protein levels were correlated with the effects on HBV production, the relative levels of HBV virion DNA and HBV protein levels were directly compared in cultures treated with the different anti-S or anti-C oligonucleotides. A strong correlation was observed between virion DNA and either HBsAg or HBcAg levels in cultures treated with either the anti-S or the anti-C oligonucleotides (linear regression coefficients (r) were 0.95 and 0.96, respectively) (Fig. 5A, B). A similar analysis was performed for the anti-ϵ oligonucleotides. Because of the close proximity of ϵ to the beginning of the C gene sequence, the levels of HBcAg in cultures treated with the anti-ϵ oligonucleotides were compared to the levels of HBV virion DNA. Little correlation was observed (r value of 0.49) between HBcAg

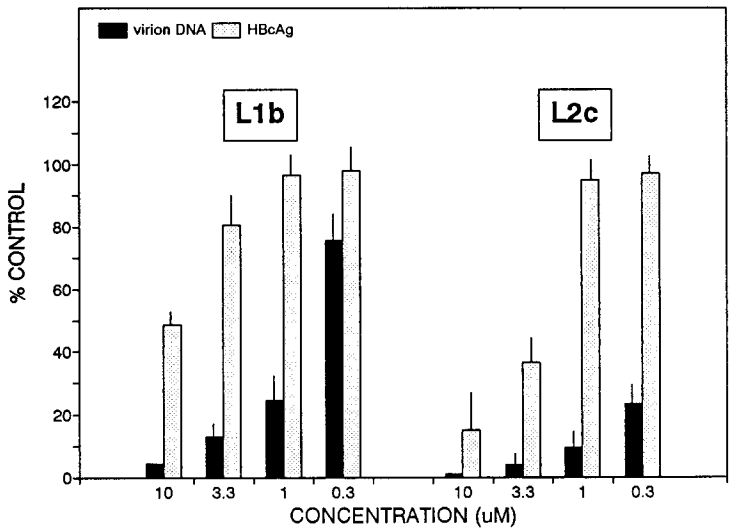


Fig. 6. Effect of anti-ϵ antisense oligonucleotide concentration on HBV virion DNA and HBcAg levels. The relative levels (as compared to control (untreated) cultures) of HBV virion DNA and HBcAg following treatment with different cultures of oligonucleotides L1b or L2c are displayed. Bars represent an average of values obtained from 4 to 12 separate cultures from 2 to 3 experiments. Lines indicate standard deviations.

levels and HBV virion DNA in these cultures (Fig. 5C). As shown for oligonucleotides L1b and L2c, high concentrations (e.g. greater than 3-fold above the EC_{90} values for virion DNA) of some anti-ϵ oligonucleotides were able to effectively inhibit HBcAg levels (Fig. 6 and Table 4). However, at the EC_{90} for virion DNA, little or no inhibition of HBcAg was observed (Fig. 6).

Two commonly observed features of cell culture treatments with antisense oligonucleotides (Cohen, 1991; Stein and Cheng, 1993) were also observed for the antisense molecules used in these studies. The linear dynamic range of antiviral activity for the oligonucleotides used in this study was found to be limited. The entire range of antiviral activity (0–99% inhibition) for most of the molecules used in this study was confined within approximately a 10-fold range of concentrations (data not shown). In addition, the maximal suppression of HBV functions for the most active antisense molecules was 1–10% of untreated cell levels, even when concentrations which were 5- to 10-fold greater than the EC_{90} values were used (data not shown).

3.3. Sequence specificity and toxicity

To test whether the antisense orientation of the oligonucleotides was the active antiviral component of these molecules, a panel of 5 oligonucleotides, which were complementary to oligonucleotides S1 (Fig. 1), C1 (Fig. 3), and the 3 'A' oligonucleotides listed in Fig. 2, were examined for anti-HBV activity. None of these 5 'sense' oligonucleotides had any measurable effect on HBV production in 2.2.15 cells at concentrations up to 20 μM, indicating that the antiviral activities of the active molecules were specific to the antisense sequences (Table 5).

Toxicity of the oligonucleotides used in this study was assessed by uptake of neutral red dye relative to untreated (control) cultures (Korba and Gerin, 1992). Toxicity measurements were made under culture conditions which were identical to those used for the antiviral analyses (Korba and Gerin, 1992). None of 8 oligonucleotides examined in this study for toxicity (S1, C1, L1c, L2b, L3e, and the 3 'A' oligonucleotides in Fig. 2) induced any significant inhibition of neutral red uptake by 2.2.15 cells at concentrations of up to 500 μM, a level more than 100-fold higher than most of the effective antiviral concentrations (data not shown). By contrast, the EC_{50} of 2′,3′-ddC in these studies was approximately 240–260 μM, consistent with previous analyses of this compound in this culture system (Korba and Gerin, 1992). The EC_{50} is defined as the concentration at which a 50% inhibition of neutral red dye uptake (relative to untreated cells) is observed and is calculated by liner regression analysis (Korba and Gerin, 1992).

4. Discussion

These experiments demonstrate that small oligodeoxyribonucleotides can exert biologic effects on HBV in 2.2.15 cell cultures at non-toxic concentrations in a sequence-specific manner which are consistent with predicted modes of action. The observations compiled in this study also demonstrate that it is possible to selectively disrupt specific HBV functions other than DNA polymerase under conditions of pre-existing chronic viral replication.

The relatively high degree of success in establishing antiviral activity for the antisense oligonucleotides used in this study was most likely related to the choice of target sequences. For the most part, the target sequences chosen were key control elements which were located in regions of the HBV genome that are highly conserved among different sequenced isolates of HBV. For example, with the exception of specific HBe-negative variants (Lok et al., 1994) the ϵ nucleotide sequence is essentially 100% conserved among the published sequences of 35 different HBV isolates. A consensus HBV DNA sequence was used to specify the antisense oligonucleotide sequences since, if oligonucleotides directed at HBV-specific functions are to have a practical therapeutic application, then these molecules will have to be effective on any unspecified HBV (subtype HBV/ayw) genome. The effect of single or multiple base mismatches on the effectiveness of the oligonucleotides used in this study was not examined. Such principles, however, are being examined as part of continuing studies.

The antisense molecules used in this study exerted antiviral effects on HBV which were limited to the HBV genes or RNA sequences which were specifically targeted. The patterns of observed biologic effects of these oligonucleotides were consistent with the role of the different viral genes and RNA sequences involved. For example, the inhibition of HBsAg, the major structural protein of the HBV envelope, would inhibit encoating of intracellular viral particles and thereby prevent the release of DNA-containing virions into the culture medium (Summers and Mason, 1982; Tiollais et al., 1988; Ueda et al., 1991). PreS1, another of the 3 virus-specific structural proteins comprising the HBV surface envelope, has been shown to be essential for HBV virion release in cell culture (Ueda et al., 1991). But, like HBsAg, preS1 is not essential for intracellular HBV replication (Ueda et al., 1991). HBcAg is essential for HBV replication since synthesis of both strands of the HBV genome occurs exclusively in cytoplasmic particles comprised of HBcAg (Summers and Mason, 1982; Tiollais et al., 1988). Thus, disruption of HBcAg production would be expected to interfere with both intracellular replication as well as virion production.

The presence of HBeAg in patient serum is a marker of active virus replication, although the actual function of HBeAg in the course of HBV infection has not been elucidated (Hoofnagle, 1981; Tiollais et al., 1988; Vyas and Ulrich, 1991; Lok et al., 1994). HBe-negative HBV mutants, however, have been isolated in vivo, and these mutants are able to replicate efficiently in cell culture, demonstrating that HBeAg production is not directly required for cellular virus replication (Vyas and Ulrich, 1991). The lack of an effect on HBV replication by the antisense oligonucleotides targeting the e coding sequences, despite a reduction in HBeAg production, is consistent with such observations.

It is not readily apparent why the antisense oligonucleotides targeting the HBV POL gene failed to disrupt HBV replication in this study. Since assays for HBV polymerase protein levels are not available for the culture system used in this study, it is not known if the anti-POL molecules used were capable of exerting any biologic effect. It is possible that the anti-POL oligonucleotides were not efficiently taken up by the cells. Although uptake of the antisense molecules was not determined, a specific lack of cellular uptake of the anti-POL molecules was probably not a contributing factor since all of the molecules used in this study were relatively small, were approximately the

same size, and had approximately the same overall G–C content. The POL gene, like the HBV X gene, contains potential alternative initiation codons downstream of the targeted POL sequences (Khudyakov and Makhov, 1989), suggesting the possibility that, once the primary translational sequences were blocked, an internal initiation of POL was sufficient to produce enough active HBV polymerase activity to support HBV replication. Alternatively, despite the conservation of HBV sequences, it is possible that there is sufficient sequence divergence in POL to prevent efficient interaction with antisense molecules based on a generalized HBV sequence. There may also be some secondary structural features in the POL gene mRNA which could have influenced interaction with the oligonucleotides. However, the ϵ structure, which has a high degree of secondary structure, is a highly sensitive target for antisense oligonucleotides.

The single polyadenylation signal/sequence on the HBV genome did not appear to constitute an efficient target for antiviral therapy using non-targeted PS antisense oligonucleotides in this study. Oligonucleotides C1, C3 and C7, which cover sequences at or near the HBc gene AUG start codon, are much more potent than oligonucleotides C2, C5, and C6, which primarily cover the polyadenylation sequence region and do not involve sequences near the HBc gene AUG start codon. In addition, the anti-HBV activities of oligonucleotides targeting this region (as measured by activity against HBV DNA) are correlated with their ability to inhibit HBcAg production (see Table 4). These results are consistent with previous observations on a single oligonucleotide directed against the HBV polyA signal (Wu and Wu, 1992). Oligonucleotides specifically directed against the C gene, and which overlap the polyA signal sequence, have been shown to slightly reduce HBsAg production in this study and a previous one (Wu and Wu, 1992). Such observations illustrate the difficulty in designing antisense oligonucleotides against HBV which are confined to inducing effects on singular portions of the HBV replication pathway. Furthermore, the anti-ϵ and some of the anti-C oligonucleotides also overlap additional key control elements (DR1 and ϵ) on HBV RNA. The overlap of HBV genes and critical control elements, although sometimes a confounding feature for the discrimination of individual mechanisms related to the control of HBV replication, can be considered to be an advantage for the application of antiviral strategies, such as antisense oligonucleotides since the ultimate goal is to suppress viral replication as efficiently as possible.

Unlike most of the antisense molecules used in this study, antisense oligonucleotides targeting ϵ are postulated not to inhibit RNA translation, but to either disrupt the postulated stem-and-loop structure or directly prevent access to the unpaired bases in the loop regions. These antisense molecules used in this study were designed to test specific observations on essential parts of this sequence which include both structure and sequence-specific features (Nassal et al., 1990; Pollack and Ganem, 1993; Wang and Seeger, 1993). Disruption of ϵ by site-specific mutational analysis has been shown to inhibit packaging of HBV pregenomic RNA, an essential step in HBV replication (Nassal et al., 1990; Pollack and Ganem, 1993; Wang and Seeger, 1993).

The patterns of antiviral activity for the oligonucleotides directed against ϵ indicate that the packaging signal/sequence is an accessible target for antiviral therapy. The nucleotides located in the lower unpaired bulge constitute the template for the first 4 nucleotides of the minus strand of HBV DNA while the bases located in the upper

unpaired loop are key elements of the recognition signal to enable HBV polymerase to interact with the ϵ sequence (Nassal et al., 1990; Pollack and Ganem, 1993; Wang and Seeger, 1993). The relatively high degree of antiviral activity for oligonucleotides targeting these portions of ϵ reinforce these observations. The higher potency of oligonucleotides targeting the upper loop of ϵ indicate that interfering with the initial interaction of HBV polymerase with ϵ is more efficient than attempting to compete with polymerase for the ϵ priming sequences. Once polymerase is localized to this region it is probably tightly interacting with the RNA template. Similarly, the ineffectiveness of oligonucleotides directed against DR1 may indicate that those antisense molecules cannot efficiently compete with the HBV polymerase/primer template complex for this sequence (Wang and Seeger, 1993).

Upon initial inspection, it appears that the antiviral activity for oligonucleotides directed at the AUG start codon of the C gene (C3, C7) may have been partially related to a disruption of the ϵ stem-and-loop structure which extends 7 bases into the C gene (arrow in Fig. 3). However, further comparison of the data presented in this study demonstrates that the antiviral activities of anti-C oligonucleotides C3 and C7 (see Fig. 4) are probably not due to the fact that these molecules cover part of the lower stem of ϵ, but are due to their effect on HBcAg levels. Oligonucleotides which targeted sequences on the other side of the lower stem of ϵ, but do not cover the lower bulge (L1d and L1e) have a relatively low level of antiviral activity against HBV replication and do not effect HBcAg production. In addition, the activity of oligonucleotides C3 and C7 against HBV replication and HBcAg production is essentially the same as oligonucleotides C1 and C4 (which do not cover ϵ sequences).

As observed for antisense oligonucleotide therapies directed at other viral systems (Cohen, 1991; Stein and Cheng, 1993), it is difficult to induce suppression of targeted viral activities to levels lower than 1–10% of control values, even at very high oligonucleotide concentrations. This may involve factors such as the saturation of cellular receptors, or saturation of the cellular processes responsible for the internalization of oligonucleotides. Alternatively, it may be that the proteins involved in RNA translation and virus replication can displace oligonucleotides bound to RNA at some low level of efficiency which could vary depending on the gene sequence or the location of the oligonucleotides relative to critical control elements. It is also possible that the maximal suppression levels observed reflect an equilibrium in the rate of interaction/disassociation between each oligonucleotide and its target sequence. The lack of an effect on HBV RNA levels by the antisense oligonucleotides used in this study indicates that the primary mechanism of antiviral action of these molecules was related to an inhibition of RNA translation. It is not apparent why no evidence of RNase H activity was found in this study.

Antisense oligonucleotides constitute a class of potential antiviral agents which permits the specific targeting of selected features of HBV replication other than those accessible to agents such as nucleoside analogues. The practical application of antisense oligonucleotides to in vivo therapies is currently under aggressive development by numerous groups (see Cohen (1991), and Stein and Cheng (1993) for reviews). Because of the manufacturing costs and the size of antisense molecules, selected delivery of antisense molecules to the target disease organs or cells appears to be necessary in order

to utilize this promising antiviral approach. At least one report has provided some evidence indicating that delivery of anti-HBV antisense oligonucleotides to cultured hepatocytes via the asialoglycoprotein receptor can increase the efficacy of these molecules (Wu and Wu, 1992). However, even without the use of tissue targeting/delivery techniques, approximately 40% of phosphorothioate-substituted antisense oligonucleotides in vivo can be delivered to the liver, although it is not currently known if these molecules are effectively delivered to parenchymal cells (Stein and Cheng, 1993). Thus, antisense oligonucleotides may be especially useful for antiviral therapies against chronic HBV infection either as primary agents (with one or more antisense molecules), or in combination with other antiviral strategies.

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