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In vivo inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides

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Antisense oligodeoxynucleotide strategies have been employed in a variety of eukaryotic systems both to understand normal gene function and to block gene expression. Pharmacologically, 'code blockers' are ideal agents for antitumour and antimicrobial treatments because of their specific mode of action. Here we report the inhibition of duck hepatitis B virus (DHBV) by antisense oligodeoxynucleotides in primary duck hepatocyte cultures *in vitro* as well as in DHBV-infected Pekin ducks *in vivo*. The most effective antisense oligodeoxynucleotide was directed against the 5′ region of the pre-S gene and resulted in a complete inhibition of viral replication and gene expression *in vitro* and *in vivo*. These results demonstrate the application of antisense oligodeoxynucleotides *in vivo* and exemplify their potential as human antiviral therapeutics.

Key words: antisense oligodeoxynucleotides/antivirals/duck hepatitis B virus/hepadnaviruses

Introduction

Hepatitis B virus (HBV) infection is a major cause of chronic liver disease and hepatocellular carcinoma (Hoofnagle, 1990). In the past few years, several antiviral strategies designed to terminate chronic HBV infection have been studied. Among them, α-interferon has been shown to be useful with a clinical, biochemical and serological remission in 30–40% of highly selected patients (Perrillo *et al.*, 1990). To date, no satisfactory medical treatment of chronic HBV infection is available, however.

Two major achievements facilitated the evaluation of therapeutic strategies to terminate HBV infection: (i) the discovery of animal viruses closely related to HBV, e.g. duck hepatitis B virus (DHBV), woodchuck hepatitis virus, ground squirrel hepatitis virus and others (Tiollais *et al.*, 1985); (ii) the development of *in vitro* systems permissive for viral replication, e.g. primary duck hepatocyte cultures which can be infected by DHBV (Tuttleman *et al.*, 1986). Antisense oligonucleotide strategies have been employed in a variety of eukaryotic systems both to understand normal gene function and to block gene expression therapeutically *in vitro* (Stein and Cohen, 1988; Calabretta, 1991). This concept has been successfully applied *in vitro* to inhibit influenza virus (Kabanov *et al.*, 1990), Rous sarcoma virus (Zamecnik and Stephenson, 1978; Chang and Stoltzfus, 1987), human T cell leukaemia virus type I (Rueden and Gilboa, 1989), human immunodeficiency virus (Zamecnik *et al.*, 1986; Matsukara *et al.*, 1987; Agrawal *et al.*, 1989), vesicular stomatitis virus (Lemaitre *et al.*, 1987), herpes simplex virus (Smith *et al.*, 1986), bovine papilloma virus type I (Bergman *et al.*, 1986), Sendai virus (Gupta, 1987) and hepatitis B virus (Goodarzi *et al.*, 1990; Blum *et al.*, 1991; Wu and Wu, 1992). Therapeutically, 'code blockers' are ideal antitumour or antimicrobial agents because of their specific mode of action (Riordan and Martin, 1991). Here we report the inhibition of DHBV replication and gene expression by antisense oligodeoxynucleotides *in vitro* as well as *in vivo*.

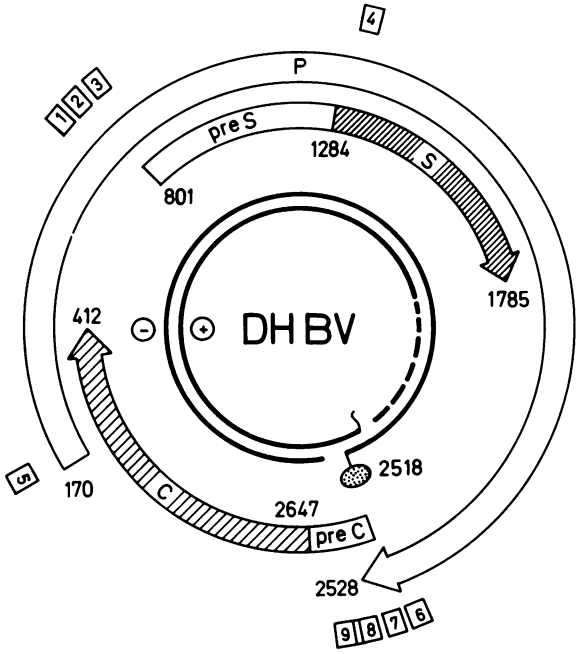
Results

Effect of antisense oligodeoxynucleotides in vitro

DHBV-infected primary duck hepatocyte cultures were used to assess the antiviral activity of antisense oligodeoxynucleotides *in vitro*. Antisense oligodeoxynucleotides were synthesized as phosphorothioate analogues (see Materials and methods). These are more resistant to nucleases and are taken up by the cells more efficiently than unmodified oligodeoxynucleotides (Eckstein, 1985; Matsukara *et al.*, 1987; Agrawal *et al.*, 1988). The genetic organization of the DHBV genome and the position of the nine antisense oligodeoxynucleotides evaluated in this study are shown in Figure 1: four antisense oligodeoxynucleotides (AS 1–4) are located in the pre-S/S-region, one oligodeoxynucleotide (AS 5) at the start of the polymerase region and four oligodeoxynucleotides (AS 6–9) in the pre-C/C-region.

Primary duck hepatocytes were isolated from DHBV-infected ducklings and cultured as described by Offensperger *et al.* (1991a,b). The infected hepatocytes were kept in culture with daily change of medium containing the respective antisense oligodeoxynucleotides at a final concentration of 1.5 μM. After 10 days in culture the cells were harvested; DNA was isolated and analysed by Southern blot hybridization. As shown in Figure 2a, incubation with the nine antisense oligodeoxynucleotides for a period of 10 days led to a decrease of intracellular viral replicative intermediates for all oligodeoxynucleotides tested. Two antisense oligodeoxynucleotides, however, showed a particularly high efficiency: AS 2, directed against the start of the pre-S-region, resulted in a very strong inhibition of viral replication with only residual covalently closed circular DNA (ccc DNA) molecules left. Also AS 6, directed against the direct repeat II (DR II) region, resulted in a strong inhibition of viral replication with only residual single-stranded, relaxed circular and ccc DNA species left.

These inhibitory effects were quantified by liquid scintillation counting of the radiolabelled spots after dot blot hybridization of DNA from primary duck hepatocytes with a DHBV-specific probe. As compared with the control



AS 1: nucl. 763 - 778
AS 2: 795 - 812
AS 3: 829 - 844
AS 4: 1280 - 1295
AS 5: 170 - 186
AS 6: 2469 - 2488
AS 7: 2491 - 2508
AS 8: 2518 - 2533
AS 9: 2529 - 2546

Fig. 1. Genetic organization of DHBV genome and location of the nine antisense oligodeoxynucleotides, AS 1–9.

culture (100%), the hybridization signal was 3.7% after incubation of the cells with AS 2 and 9.4% after incubation with AS 6. These data indicate that viral replication was inhibited by >90%.

The following observations exclude the possibility that a direct toxic effect of the antisense oligodeoxynucleotides is responsible for the inhibition of the DHBV replication: (i) trypan blue exclusion demonstrated identical viability of cells kept in culture media without or with the antisense oligos; (ii) the total RNA content of hepatocytes cultured in the absence or presence of the antisense oligos was identical.

In addition to the DHBV-specific antisense oligodeoxynucleotides mentioned above, we tested the effect of the 18mer homooligomers of deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine on DHBV replication *in vitro* under the same experimental conditions as described above. These homooligodeoxynucleotides have previously been shown to strongly inhibit human immunodeficiency virus (HIV) replication (Matsukara *et al.*, 1987; Agrawal *et al.*, 1988), possibly mediated through a direct action on viral RNA polymerase/reverse transcriptase and an additional translational arrest (Boiziau *et al.*, 1992). In contrast to these findings in the HIV system, DHBV replication was not significantly reduced by these homooligodeoxynucleotides as compared with the AS 2 and 6 (data not illustrated).

Specificity of antisense oligodeoxynucleotides in vitro

To demonstrate the antisense specificity of the inhibition of DHBV replication *in vitro*, we synthesized the sense oligodeoxynucleotides complementary to the antisense oligodeoxynucleotides AS 2 and AS 6. The parallel analysis of these antisense and sense oligodeoxynucleotides *in vitro* demonstrated that the inhibitory effect on DHBV replication

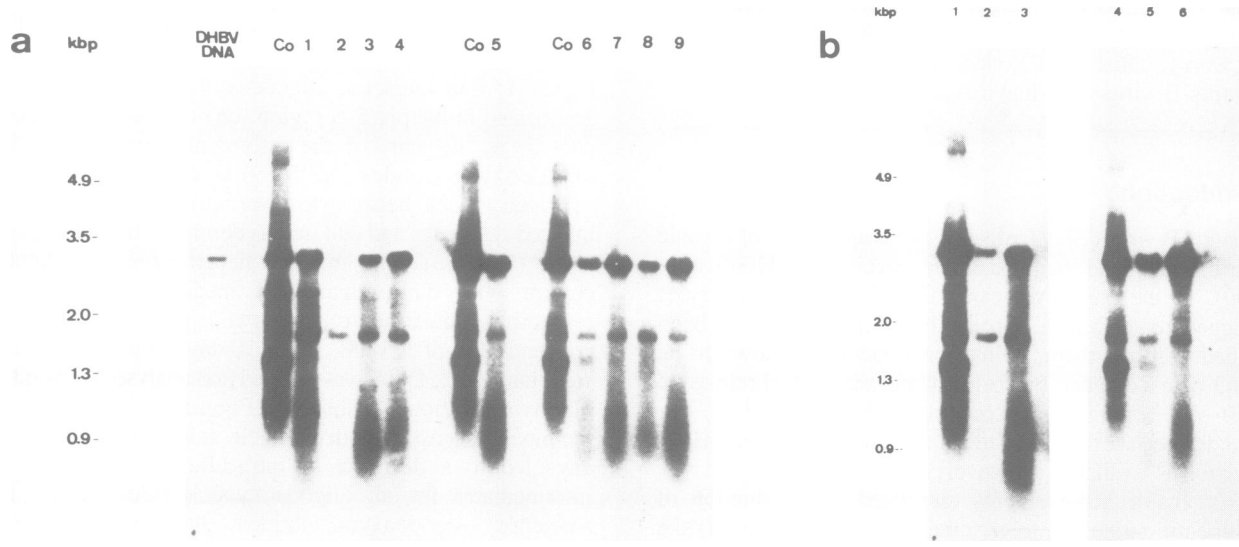


Fig. 2. (**a**) Effect of antisense oligodeoxynucleotides on DHBV DNA replication *in vitro*. Co: untreated control cells. Numbers on top indicate antisense oligodeoxynucleotides used; for their location in DHBV genome see Figure 1. (**b**) Effect of antisense oligodeoxynucleotide 2 (lane 2), sense oligodeoxynucleotide 2 (lane 3), antisense oligodeoxynucleotide 6 (lane 5) and sense oligodeoxynucleotide 6 (lane 6) on DHBV replication *in vitro*. Untreated control cultures are shown in lanes 1 and 4, respectively. Southern blot analysis was performed with 20 μg total liver DNA per lane. Size marker was *Hin*dIII-digested lambda DNA and cloned full-length DHBV DNA (10 pg) of 3.0 kbp length. The replicating DHBV DNA species in the liver are: single-stranded DNA molecules of minus-strand polarity migrating as a smear of molecules of increasing length between the 0.5 kbp and 1.5 kbp positions, the prominent band in the 1.5 kbp position representing full-length minus-strand DNA; partially double-stranded DHBV DNA species, migrating between 1.5 kbp and 3.0 kbp, and fully doubled-stranded DHBV DNA of 3.0 kbp length.

was polarity-specific (Figure 2b): antisense oligodeoxynucleotides 2 and 6 resulted in an almost complete elimination of viral replicative intermediates while the corresponding sense oligodeoxynucleotides did not significantly affect viral replication.

Effect of antisense oligodeoxynucleotides in DHBV-infected Pekin ducklings

In an extension of the *in vitro* studies described above, the effect of AS 2 on DHBV replication was analysed *in vivo*. One day-old ducklings were intravenously injected with DHBV DNA positive duck serum, resulting in DHBV infection in all animals. Two weeks later, daily intravenous injection of AS 2 was started. Ten days later liver DNA was analysed for the presence of DHBV DNA replicative intermediates by Southern blot hybridization. As shown in Figure 3, the *in vivo* administration of AS 2 resulted in a dose-dependent inhibition of viral replication with a nearly complete elimination of viral DNA from liver cells at a daily dose of 20 μg per gram body weight, applied for a total of 10 days.

Summarizing five consecutive series of *in vivo* therapy of DHBV-infected ducklings, a total number of 14 animals was treated with AS 2. Without exception all ducklings showed a nearly complete inhibition of viral replication after treatment with this antisense oligodeoxynucleotide, indicating that the antisense effect observed is highly reproducible.

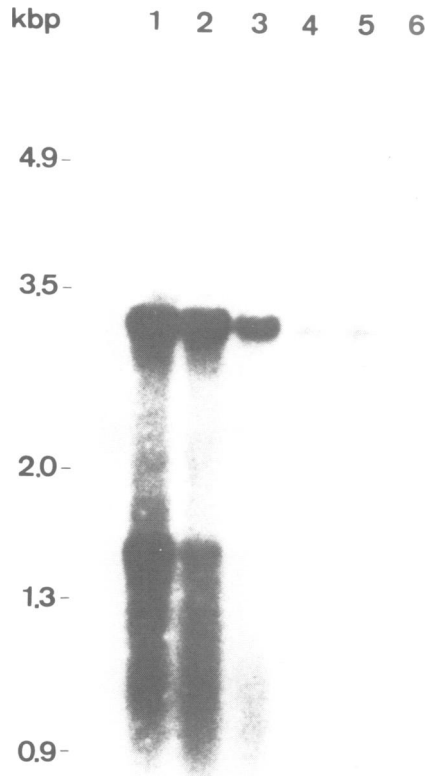


Fig. 3. Effect of AS 2 on DHBV DNA replication *in vivo* in DHBV-infected ducks. Lane 1, control duck; lanes 2–6, ducks treated for 10 days with daily intravenous injection of AS 2 at a concentration of 5 μg (lane 2), 10 μg (lane 3) and 20 μg per gram body weight (lanes 4–6). For further experimental details see legend to Figure 2.

The effect of AS 2 on viral gene expression *in vivo* was assessed by the detection of viral surface antigen (DHBsAg) in serum and of viral core antigen (DHBcAg) in liver by Western blot analysis. As demonstrated in Figure 4a for serum and Figure 4b for liver, the administration of AS 2 *in vivo* resulted in a block of DHBV gene expression with disappearance of viral pre-S and S antigens from serum and of viral pre-C and C antigens from liver.

To test possible toxic side effects of AS 2 on the animals, several clinico-chemical parameters were measured: the levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, cholinesterase, total protein and albumin in serum. The results were identical in AS 2-treated and untreated ducks, suggesting that AS 2 had no direct hepatotoxic effect.

Effect of antisense oligodeoxynucleotides on virus infectivity in vivo

Two DHBV-negative Pekin ducklings were treated with AS 2 at a concentration of 20 μg per gram body weight starting on day 3 after hatching. Twelve hours later they were infected by intravenous injection of 100 μl DHBV DNA positive serum (about 10^9 virions/ml). The daily intravenous AS 2 treatment was continued until day 12 at which time the ducklings were sacrificed. As shown in Figure 5a and b, the absence of viral DNA and pre-S/S proteins demonstrates the successful prevention of the infection by pretreatment of ducklings with AS 2, as compared with a productive DHBV infection in untreated ducklings.

Antisense specificity of oligodeoxynucleotides in vivo

Two 'control' phosphorothioate oligodeoxynucleotides were tested: the sense oligodeoxynucleotide complementary to AS 2, and the random oligodeoxynucleotide 5′-GCAGGCAA-ACCATTTGAATG-3′. As illustrated in Figure 6, the administration of the two oligodeoxynucleotides to DHBV-infected Pekin ducks did not cause a reduction in viral replication, demonstrating the specificity of action of the antisense oligodeoxynucleotides *in vivo*. Also no major inhibition of virus infectivity could be observed *in vivo* with these two 'control' oligodeoxynucleotides (Figure 5a and b).

Discussion

Hepadnaviruses, including DHBV, utilize the unique pathway of reverse transcription of a RNA pregenome for viral DNA replication (Summers and Mason, 1982). Because viral RNAs, including the viral pregenome, are intermediates in the replicative and the translational pathways (Tiollais *et al.*, 1985), it follows that antisense oligodeoxynucleotides, acting via translational arrest by interfering with a number of post-transcriptional steps, e.g. RNA processing, stability and translation (Riordan and Martin, 1991), effectively block viral replication as well as viral gene expression.

The reasons why the nine DHBV-specific antisense sequences tested show different antiviral activity *in vitro* are not completely understood. Possible factors are the

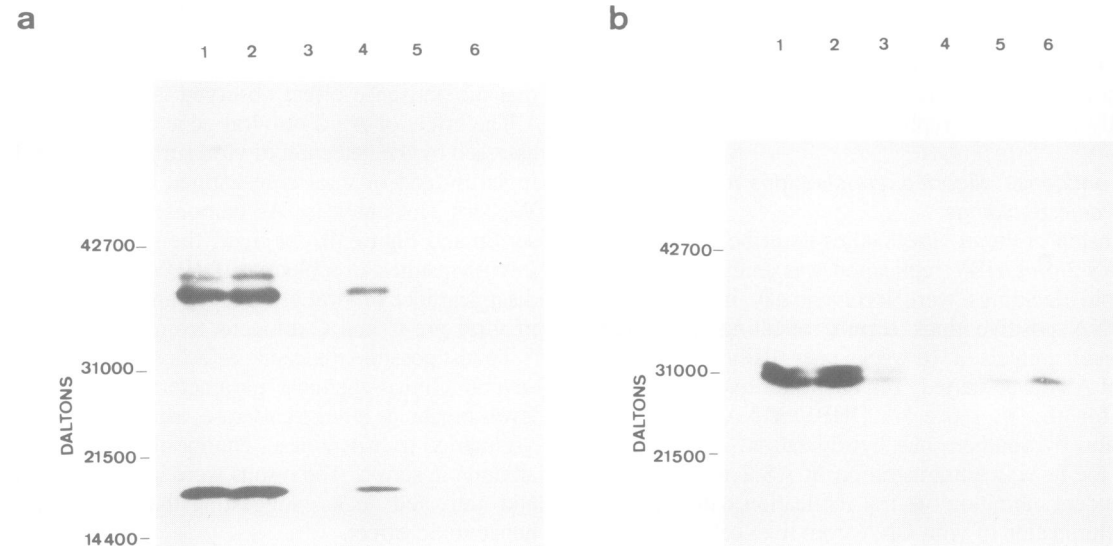


Fig. 4. (**a**) Western blot analysis of sera obtained from DHBV-infected ducks treated with AS 2 *in vivo* using a polyclonal antibody against native DHBsAg. The experimental design corresponds to Figure 3. Lane 1, control duck; lanes 2–6, ducks treated for 10 days with daily intravenous injection of AS 2 at a concentration of 5 μg (lane 2), 10 μg (lane 3) and 20 μg per gram body weight (lanes 4–6). (**b**) Western blot analysis of liver extracts obtained from DHBV-infected ducks treated with AS 2 *in vivo* using a polyclonal antibody against DHBcAg. The numbers on top of the lanes correspond to those in panel a.

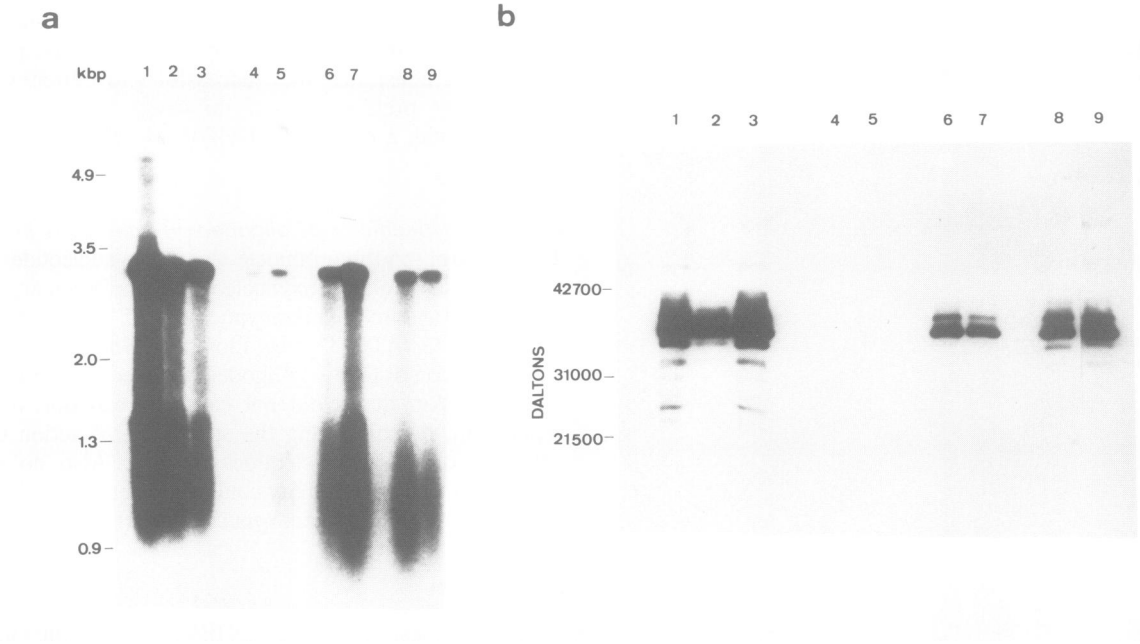


Fig. 5. *In vivo* effect of oligodeoxynucleotides on DHBV infectivity. The treatment of the ducklings with the oligodeoxynucleotides at a concentration of 20 μg per gram body weight was started 1 day before infection with DHBV positive serum. (**a**) Southern blot analysis. Lanes 1–3, three control ducklings; lanes 4 and 5, two ducklings treated for 10 days with daily intravenous injection of AS 2; lanes 6 and 7, two ducklings treated with sense oligodeoxynucleotide 2; lanes 8 and 9: two ducklings treated with the random oligodeoxynucleotide. For further experimental details see legend to Figure 2. (**b**) Western blot analysis of the sera using a polyclonal antibody against DHB-pre-SAg. The numbers above the lanes correspond to those in panel a.

significance of the targeted region for viral replication, including reverse transcription (Boiziau *et al.*, 1992), hybridization efficiency which depends on secondary or tertiary structures, abundance of RNA species complexed with protein which may prevent oligonucleotide annealing, the degree of duplex unwinding as well as the intracellular distribution of antisense oligodeoxynucleotides (Leonetti *et al.*, 1991).

In contrast to the increasing evidence for antiviral (Zamecnik and Stephenson, 1978; Bergman *et al.*, 1986; Smith *et al.*, 1986; Zamecnik *et al.*, 1986; Chang and Stoltzfus, 1987; Gupta, 1987; Lemaitre *et al.*, 1987;

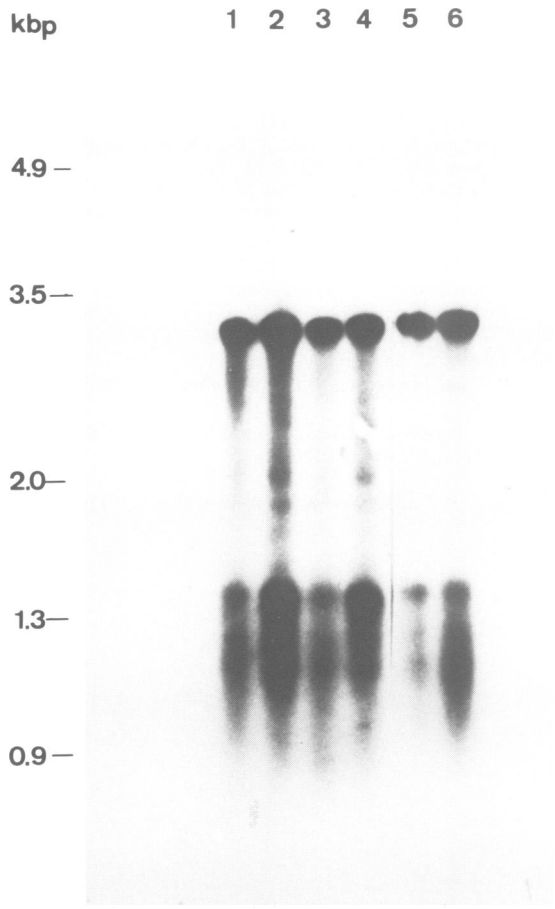


Fig. 6. Effect of sense or random oligodeoxynucleotides on DHBV DNA replication *in vivo*. Lanes 1 and 2, two control ducks; lanes 3 and 4, two ducks treated for 10 days with daily intravenous injection of the sense oligodeoxynucleotide 2 at a concentration of 20 μg per gram body weight; lanes 5 and 6: two ducks treated for 10 days with daily intravenous injection of the random oligodeoxynucleotide at a concentration of 20 μg per gram body weight. For further experimental details see legend to Figure 2.

Matsukara *et al.*, 1987; Agrawal *et al.*, 1989; Goodarzi *et al.*, 1990; Blum *et al.*, 1991; Wu and Wu, 1992) and antitumour activities of antisense oligonucleotides *in vitro* (Harel-Bellan *et al.*, 1988; Holt *et al.*, 1988; Anfossi *et al.*, 1989; Becker *et al.*, 1989; Rivera *et al.*, 1989; McManaway *et al.*, 1990; Reed *et al.*, 1990; Melani *et al.*, 1991, Saison-Behmoaras *et al.*, 1991), the application of this concept *in vivo* has been speculative to date. In our study, the feasibility of antisense oligodeoxynucleotide therapy of a viral infection is demonstrated for the first time *in vivo*. Our results show that intravenous application of antisense oligodeoxynucleotides *in vivo* is very effective. The amount of oligodeoxynucleotide required to obtain these effects *in vivo* corresponds to the requirements *in vitro* and suggests comparable uptake, metabolism and degradation of the antisense oligos *in vivo*. Additional data concerning pharmacokinetics as well as possible side effects of antisense oligonucleotides *in vivo* are needed. Basic objections against the use of antisense oligonucleotides in clinical medicine are not obvious, however.

With respect to the translation of our antisense oligodeoxynucleotide findings into clinical practice, the persistence of ccc DNA species in liver cell nuclei poses a particular problem. These DNA species are precursors of viral transcripts, comparable to a 'minichromosome', and are not completely eliminated by the 'code blocker' antiviral strategy, resulting in a very slow reactivation of replication after cessation of antisense oligodeoxynucleotide therapy (data not shown). Possibly, long-term treatment with antisense oligodeoxynucleotides will eliminate all DNA species. This concept will be tested by extended application of antisense oligodeoxynucleotides to DHBV-infected Pekin ducks *in vivo*.

Further, to deliver oligodeoxynucleotides specifically to liver cells, work is in progress to encapsidate the antisense oligonucleotides into liposomes or into liposomes conjugated with an asialoglycoprotein (ASGP). The ASGP can be targeted specifically to liver cells via a cell surface associated ASGP receptor unique to hepatocytes (Wu *et al.*, 1989; Wu and Wu, 1992). Finally, the potential of triple helix-forming oligonucleotides (Duval-Valentin *et al.*, 1992) as inhibitors of DHBV transcription will be explored as a further antiviral strategy.

Materials and methods

Oligodeoxynucleotide synthesis

Phosphorothioate-modified oligodeoxynucleotides were synthesized at the 10 μmol scale (Applied Biosystems). In view of the expense, suboptimal coupling yields and inconvenience associated with phosphorothioate synthesis via the H-phosphonate chemistry, antisense oligodeoxynucleotides were obtained with standard cyanoethylphosphoramidites using the sulfur transfer reagent ^{3}H-1,2-benzodithiol-3-one 1,1-dioxide (Iyer *et al.*, 1990) or tetraethylthiuram disulphide (Vu and Hirschbein, 1992). After synthesis, the oligodeoxynucleotides were ethanol precipitated, washed and dissolved in TE.

DHBV infection in vitro and in vivo

Primary duck hepatocytes were isolated from 10 day-old DHBV-infected Pekin ducks as described by Tuttleman *et al.* (1986) with only minor modifications (Offensperger *et al.*, 1991a,b). The hepatocytes were seeded at a density of 2×10^{5} cells/cm^{2} on Primaria tissue culture dishes using Williams' medium E supplemented with 20 mM HEPES (pH 7.4), 5 mM glutamine, 0.066 μM insulin, 10 mM dexamethasone, 100 μg/ml penicillin, 100 μg/ml streptomycin and 1.5% dimethyl sulfoxide. The cultures were maintained at 37°C and 5% CO_2 in a humidified incubator with daily medium change for the duration of the experiment. The antisense oligodeoxynucleotides were added to give a final concentration of 10 mg/ml (1.5 μM).

One day after hatching, Pekin ducklings were infected by intravenous injection of 100 μl DHBV DNA positive serum (~10^{9} virions/ml). At the age of 14 days, daily intravenous injection of AS 2 was started. Ten days later the ducks were sacrificed.

Southern blot analyses

After 2 weeks in culture, liver cells were lysed with a solution containing 1% SDS, 20 mM Tris−HCl (pH 7.4), 10 mM EDTA and 150 mM NaCl, and digested with proteinase K (0.4 mg/ml) at 37°C for 12 h. After extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), nucleic acids were precipitated with 2 vol of 100% ethanol in the presence of 0.3 M sodium acetate, pH 5.2. After lyophilization, nucleic acids were dissolved in 10 mM Tris−HCl (pH 7.4), 1 mM EDTA. Radiolabelling of the DHBV genome and Southern blot hybridization using a ^{32}P-labelled, full-length DHBV DNA probe were carried out as described earlier (Offensperger *et al.*, 1988). Autoradiographic exposure time was 1 day at −80°C; size markers were *Hin*dIII digested lambda DNA and 10 pg DHBV DNA.

Western blot analyses

For immunoblot analyses three different polyclonal antibodies were used: an antibody against native DHBsAg, an antibody against DHB-pre-SAg and an antibody against DHBcAg, all raised in rabbits. Using anti-DHBsAg,

a major band with a molecular weight of 36 kDa (pre-S/S protein), some minor bands of different molecular weights due to the different degree of glycosylation and a major band of molecular weight 18 kDa (S protein) can be detected. Using anti-DHB-pre-SAg only the pre-S/S proteins can be seen. Using anti-DHBcAg, pre-C/C proteins with molecular weights of 30 and 33 kDa can be detected.

Serum or liver samples were denatured, separated by SDS–PAGE (12%) and transferred to nitrocellulose. After blocking, the membrane was incubated with the primary antibody overnight. Using the enhanced chemiluminescence detection method the sequential steps were carried out according to the instructions of the manufacturer (Amersham Buchler, Braunschweig, Germany).

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