The inhibitory effects of antisense RNA on hepatitis B virus surface antigen synthesis

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Antisense RNA-mediated inhibition of gene expression has the potential for gene therapy of virus infections. We studied the inhibitory effect of antisense RNA directed against the hepatitis B virus (HBV) genome on the expression of the HBV surface antigen (HBsAg). Three prokaryotic antisense RNA expression constructs were produced which expressed antisense RNA complementary to the entire coding region (1–4 kb) and to 1.0 kb and 582 bp of the 5' region of HBsAg mRNA, respectively. In an in vitro translation system, all three antisense RNAs showed concentration-dependent inhibitory effects on translation of HBsAg mRNA. In a coupled in vitro transcription and translation system, concentration-dependent inhibition of HBsAg synthesis was observed for all above-mentioned antisense RNAs. Three mammalian antisense RNA expression vectors were then constructed, expressing the same antisense RNAs as used above. Transfection of the vectors into Hep3B cells (an HBsAg secreting cell line) resulted in almost complete blockage of HBsAg production, whereas control vector transfected cells secreted high levels of HBsAg. The inhibitory effect lasted for more than 10 months post-transfection. To examine the possible mechanism of the antisense RNA effect in the cell line, we measured HBV mRNA levels in the transfected cells and found that the mRNA levels in the antisense RNA expressing cells were much lower than those in the control cells. Therefore, in Hep3B cells, the antisense RNAs inhibited HBsAg synthesis, at least partially, through the reduction of HBV mRNA levels.

Introduction

Antisense RNA-mediated gene inhibition is a widely used strategy not only for studying gene regulation and revealing gene function, but also for targeting disease states, especially cancer and viral infection. Since Chang & Stoltzfus (1985) demonstrated the inhibitory effect of antisense RNA on Rous sarcoma virus gene expression, the effects have been investigated on several other viruses, including human immunodeficiency virus 1 (HIV-1; Chatterjee et al., 1992), adenovirus (Miroshnichenko et al., 1989), herpes simplex virus (Cantin et al., 1992), human cytomegalovirus (CMV; Bryant & Sinclair, 1993), human T cell leukaemia virus type I (von Ruden & Gilboa, 1989), Moloney murine leukaemia virus (MoMLV; Sullenger et al., 1990) and polyomavirus (Ottanvio et al., 1992). Successful inhibition of gene expression has been demonstrated for these viruses, especially for HIV-1. These studies suggest that antisense RNA may be useful in future gene therapy strategies for chronic viral infections.

Hepatitis B virus (HBV) is an infectious agent with about 200 million carriers worldwide. Many patients with HBV infection develop chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Unfortunately, treatment for chronic infection by this virus is far from satisfactory. The most successful therapeutic agent so far available is interferon-alpha, which shows a 40% response rate with a 40% relapse rate for patients after completion of therapy. Since several viruses have become successful targets of the antisense RNA approach, this strategy may be promising in targeting chronic HBV infection.

So far, the effects of antisense RNA on HBV gene expression have not been investigated. Goodarzi et al. (1990) and Wu & Wu (1992) have successfully demonstrated the inhibitory effects of antisense oligodeoxynucleotides on HBV gene expression. However, oligodeoxynucleotides can only temporarily suppress the pathogenic effect of integrated virus
unless given continually, whereas antisense RNA can be constitutively expressed in a stably transfected cell line, and thus, potentially provide long term protection against the pathogenic effect of integrated virus.

HBV surface antigen (HBsAg) is a group of HBV envelope proteins involved in HBV virion formation, budding and entry into hepatocytes. In addition, HBsAg may play an important role in HBV related hepatocarcinogenesis, as shown by the observations that (i) overproduction of HBsAg leads to the development of HCC in transgenic mice (Chisari et al., 1989), and (ii) 3′-truncated HBsAg is capable of transactivating some cellular oncogenes, e.g. c-myc and c-fos (Kekule et al., 1990; Schluter et al., 1994). In patients with chronic HBV infection, circulating HBsAg can bind to its antibody and form immune-complexes, which may deposit in small blood vessels of different organs, resulting in vasculitis (Brzosko et al., 1974; Gocke, 1975).

In an attempt to explore the therapeutic potential of antisense RNA for chronic HBV infection, we studied the effects of antisense RNA on HBsAg expression. We demonstrated that antisense RNAs targeted to the HBsAg coding region can specifically inhibit envelope protein synthesis both in an in vitro translation system, and more importantly, in an HBsAg secreting cell line. The effects were long lasting, and remained demonstrable for more than 10 months post-transfection.

### Methods

#### Construction of antisense HBsAg RNA expression vectors

Prokaryotic antisense RNA expression vectors. The entire HBsAg coding region was isolated from pHBV-1 (Hirschman et al., 1989), and subcloned into pBluescript SK (Stratagene), resulting in vector pBSk/HBs1-4kb, as shown in Fig. 1. When transcribed from the T7 promoter, pBSk/HBs1-4kb produced 1.4 kb antisense RNA complementary to the full-length HBsAg gene, and if transcribed with T3 RNA polymerase, it generated 1.4 kb sense RNA. This sense RNA was used for the translation of HBsAg in an in vitro translation system.

Next, 849 bp of the 3′ end of the S coding region was removed from pBSk/HBs1-4kb with HindIII digestion, and the resulting plasmid was self-ligated (pBSk/HBs582bp; Fig. 1). When transcribed from the T7 promoter, pBSk/HBs582bp generated 582 bp of antisense RNA covering 582 bp of the 5′ end of the HBsAg coding region. Lastly, a 1.0 kb HBV DNA fragment with a partial deletion of the 3′ end of the S coding region was isolated from pBSk/HBs1-4kb with SpeI digestion and subcloned into pBluescript KS (Stratagene), generating pBSk/HBs1-0 kb (Fig. 1) which, when transcribed from the T3 promoter, produced 1.0 kb of antisense RNA targeted to the 1.0 kb 5′ end of the HBsAg coding region.

Mammalian antisense RNA expression vectors. An episomally replicating vector, pCPE4 (Invitrogen), was used for constructing the mammalian antisense RNA expression vectors. The respective HBsAg or 3′ end truncated HBsAg inserts were isolated from the respective prokaryotic antisense RNA expression plasmid (Fig. 1), and subcloned into pCPE4 in a reverse orientation to the CMV promoter. The resulting vectors, pCPE4/HBs582bp, pCPE4/HBs1-0 kb and pCPE4/HBs1-4kb, could express 582 bp, 1.0 and 1.4 kb antisense HBsAg RNAs, respectively.

- **In vitro transcription.** To make run-off sense and antisense RNA, the appropriate plasmid was linearized downstream of the insert with an appropriate restriction enzyme, and transcribed with T3 or T7 RNA polymerase (Promega). The in vitro transcribed RNA was purified by phenol-chloroform extraction followed by ethanol precipitation.

- **In vitro translation.** In vitro translation was performed by using Ambion’s retic lysate IVT kit according to the manufacturer’s protocol. For this purpose, 0.5 µg of 1.4 kb run-off sense RNA was translated in the absence or presence of different amounts of run-off antisense RNA in a total of 25 µl reaction volume containing [35S]methionine (approximately 1200 Ci/mmole, Amersham). The in vitro translated products were analysed on SDS–PAGE.

- **Coupled in vitro transcription and translation.** The single tube protein (STP) system (Novagen) was used for coupled in vitro transcription and translation according to the manufacturer’s protocol. In this system, HindIII linearized pBSk/HBs1-4kb was transcribed and translated in the absence or presence of different amounts of antisense RNAs in the rabbit reticulocyte lysate containing T3 RNA polymerase. The in vitro translated products were analysed by SDS–PAGE.

- **Cell culture.** Hep3B, a human hepatoma cell line secreting HBsAg (Aden et al., 1979), was routinely cultured in a minimal essential medium (Gibco-BRL) supplemented with 10% foetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ 95% air at 37 °C.

- **Lipofectin mediated DNA transfection.** Cells (10⁵) were seeded in 60 mm culture dishes 1 day before transfection. DNA transfection was performed with lipofectin (Gibco-BRL) according to the manufacturer’s protocol. Seventy-two hours later, the transfected cells were subcultured in selection medium containing 100 µg/ml hygromycin B (Calbiochem). Hygromycin B resistant cells were used for further characterization.

- **HBsAg detection.** Cell culture media were centrifuged at 1000 r.p.m. for 10 min to remove any cells prior to HBsAg detection. Cell lysates were prepared by the freeze-thaw method. HBsAg was detected by ELISA (Bionike) according to the manufacturer’s protocol.
Metabolic labelling. Cells were incubated with methionine-deficient Dulbecco’s modification of Eagle’s media (ICN) containing l-[35S]methionine and l-[35S]cysteine (Trans35S-Label, ICN). After a 4 h incubation period cells were lysed on ice in NP40 lysis buffer, followed by centrifugation at 4 °C to remove cell debris. The supernatant was used to measure the amount of incorporation of radiolabelled precursor into protein using the trichloroacetic acid precipitation method.

Total cellular RNA and poly(A) RNA isolations. Total cellular RNA was isolated using the ultraspec RNA isolation system (Biotecx). Poly(A) RNA was purified from total cellular RNA on oligo(dT) cellulose columns (Gibco-BRL).

Ribonuclease protection assay (RPA). RPA was performed using the RPAII kit (Ambion) according to the manufacturer’s protocol. The 361 bp sense RNA probe was made with T3 RNA polymerase from EcoRI linearized pBsk/HBs1-4kb containing the 5’ 361 bp of the HBsAg reading frame. The probe was gel-purified.

RT–PCR. Ten µg of cellular RNA was digested with 50 U DNase (Promega) in 10 mM Tris, 7 mM MgCl2 at room temperature for 30 min to remove contaminating vector DNA, followed by phenol–chloroform extraction and ethanol precipitation. One µg of purified RNA was subjected to RT–PCR using S3 and G2 primers. S3 primer was 5’ GTT CTT CTC GAC TAC CAA GG 3’ (complementary to the HBsAg coding region at position 442–461 bp) and G2 primer was 5’ TTC CTG CAG CCC GGG GGA T 3’ (complementary to the region between the CMV promoter and HBsAg coding region).

Northern blot analysis. RNA was fractionated by agarose gel electrophoresis and then blotted onto a zeta-probe membrane (Bio-Rad). For the RNA probe, prehybridization and hybridization were performed at 60 °C in 50% formamide, 0.25 M Na2HPO4, 1.0 mM EDTA, 0.25 M NaCl and 7% SDS. For the DNA probe, prehybridization and hybridization were carried out at 65 °C in the buffer containing 0.25 M Na2HPO4 (pH 7.2), 7% SDS, 1.0 mM EDTA. The cDNA probe was made with a nick translation kit (Promega) according to the manufacturer’s protocol.

Southern blot analysis. DNA was fractionated by agarose gel electrophoresis at 100 V, and transferred to a zeta-probe membrane (Bio-Rad). Prehybridization and hybridization were performed at 42 °C in 50% formamide, 0.12 M Na2HPO4 (pH 7.2), 0.25 M NaCl, 7% SDS and 1.0 mM EDTA.

Statistical analysis. Statistical analysis were done with Dunnett’s t-test for comparing multiple experimental conditions to a single control.

Results

The effects of antisense RNAs on HBsAg translation in an in vitro translation system

The effects of antisense RNAs on HBsAg synthesis were first evaluated in an in vitro translation system (data not shown). Each antisense RNA was mixed with the full-length sense RNA (1:4 kb) at molar ratios of 1:1 and 10:1, and was translated in vitro. Results indicated that each antisense RNA inhibited HBsAg synthesis, especially at 10-fold higher concentration than sense RNA, and the 1:0 and 1:4 kb antisense RNAs showed greater inhibition than the 582 bp antisense RNA. All three antisense RNAs failed to inhibit the translation of Xenopus elongation factor-I (Xef-I) RNA. Therefore, the effects of the antisense RNAs were specific for the HBsAg sense RNA.

The effects of antisense RNAs on HBsAg translation in a coupled in vitro transcription and translation system

The effects of antisense RNA were also tested in a coupled in vitro transcription and translation system, a more efficient in
Fig. 3. Effect of antisense RNA on HBsAg production by Hep3B cells transfected with antisense RNA expression vector. At the indicated days after cell seeding, HBsAg levels in the media of transfected cells were measured. HBsAg levels are normalized for cell number and shown as percentage HBsAg level in control vector transfected cells (Hep3B/pCEP4). Standard deviations for some data are too small to be shown on the graphs.

vitro protein synthesis system. HindIII linearized pBsk/HBs1-4kb was incubated with different amounts of antisense RNA (0, 1.5, 15 pmol, respectively) in the presence of T3 RNA polymerase, followed by SDS–PAGE and autoradiography. As shown in Fig. 2, all three antisense RNAs inhibited HBsAg synthesis, especially at 15 pmol. In the uncoupled system, 1.0 and 1.4 kb antisense RNAs showed greater inhibition than the 582 bp antisense RNA. None of the three antisense RNAs inhibited the protein synthesis from pXef-I (Fig. 2). Therefore, the inhibitory effect of the antisense RNAs was specific for HBsAg.

Biochemical characterization of Hep3B cells expressing antisense RNAs

To test the effects of antisense RNAs on HBsAg production intracellularly, the Hep3B cells (HBsAg secreting cells) were transfected with the mammalian antisense RNA expression vectors (pCEP4/HBs582bp, pCEP4/HBs1-0kb and pCEP4/HBs1-4kb, respectively), and the parent vector pCEP4 (as a control). Stably transfected cells were subjected to the following studies:

HBsAg levels in media and cell lysate. HBsAg levels produced by transfected Hep3B cells were determined using ELISA at 12, 16, 20 and 24 days after cell seeding. At the same time, cells were counted by the trypan blue exclusion method. HBsAg levels were determined by ELISA. In both media (Fig. 3) and cell lysates (data not shown), HBsAg production was almost completely blocked in antisense RNA expression vector transfected cell lines (HBsAg was negative according to the ELISA kit standard) whereas, in control vector transfected cells, high levels of HBsAg persisted. The inhibitory effect remained for the entire 10 month observation period. To determine whether the decreased HBsAg levels in antisense RNA transfected cells were simply due to non-specific inhibitory effects of the antisense RNAs on cellular total protein synthesis, the cells were also monitored for total protein synthesis using the [35S]methionine-labelling method. There was little decrease in total protein synthesis in antisense RNA transfected cells (data not shown).

Detection of antisense RNA expression. To determine whether the antisense RNAs were expressed in antisense RNA transfected cells, total cellular RNA was subjected to RPA. As shown in Fig. 4, antisense RNA expression was detectable in pCEP4/HBs582bp and pCEP4/HBs1-4kb transfected cells, but repeatedly undetectable in pCEP4/HBs1-0kb transfected cells, probably due to the low level of expression. To demonstrate the expression of antisense RNA in pCEP4/HBs1-0kb transfected cells, RT–PCR was performed on DNase pre-treated cellular RNA from pCEP4/HBs1-0kb transfected cells using primers complementary to the HBsAg coding region (S3 primer) and the sequence flanking the HBsAg coding region (G2 primer), followed by Southern blot analysis using HBV cDNA as a probe. Results showed the presence of 1.0 kb antisense RNA (data not shown).

Detection of HBV mRNA levels. To determine whether HBV mRNA levels in the antisense RNA expressing cells were reduced, poly(A) RNA was isolated from the cells 20 days after
In this study, we demonstrated that antisense RNA inhibited the synthesis and production of HBsAg in cell free and cellular systems in vitro. Our initial hypothesis concerning the mechanism of the antisense RNA effect was that antisense RNA would bind to the corresponding mRNA and prevent translation from the mRNA. To directly confirm this hypothesis, we investigated the effect of antisense RNA on the mechanism of the antisense RNA effect was that antisense RNA in the presence of dsRNA (data not shown), probably because the amount of dsRNA in our system is much higher than that activating DAI. Therefore, the antisense RNAs were capable of inhibiting the translation of mRNA, which may be one of the major mechanisms of the antisense RNA effect.

If inhibition of translation is one of the major mechanisms of antisense RNA action, it is probable that the 5' end of the mRNA is more likely to be an effective target, because inhibition of initiation seems more effective than inhibition of elongation. This view is supported by the experiments involving injection of antisense RNA into the cytoplasm of Xenopus oocytes. In Xenopus oocytes, the RNAs that did not hybridize to the 5' end of the target message were either ineffective (Melton, 1985) or less efficient (Harland & Weintraub, 1985) than those covering the initiation region. Based on the above experiments, we chose to target the 5' end of the HBsAg coding region by constructing the expression vectors expressing three different lengths of antisense RNAs (582 bp, 1-0 and 1-4 kb) covering all the initiation codons in the HBsAg message. We first tested the effects of the antisense RNAs on HBsAg synthesis in a cell free system. In both coupled and uncoupled in vitro transcription and translation systems, all three antisense RNAs specifically inhibited HBsAg translation. The inhibition was concentration-dependent.

To further investigate the effect of the antisense RNA on HBsAg production in an HBsAg secreting cell line, we constructed mammalian antisense RNA expression vectors, expressing the same lengths of antisense RNAs as used in the in vitro translation system. The vectors were then introduced into the Hep3B cell line, an HBsAg secreting HCC cell line established from a patient with HBV infection. Since this cell line is naturally infected by HBV, it should be a good model for examining the effects of antisense RNAs on HBsAg production. As shown in Fig. 3, in the cells stably transfected with antisense RNA expression vectors, HBsAg production in both culture media and cell lysates was significantly inhibited in comparison with that in control vector transfected cells ($P < 0.01$). The inhibitory effect was long lasting, and remained demonstrable for more than 10 months post-transfection. In fact, the levels of HBsAg in the antisense RNA expressing cells were negative according to the ELISA standard. To test whether the inhibitory effect of antisense RNA on HBsAg production was simply due to a non-specific inhibitory effect on cellular protein synthesis, we monitored the cellular protein synthesis levels. There was little decrease in total protein synthesis in the presence of dsRNA (data not shown), probably because the amount of dsRNA in our system is much higher than that activating DAI. Therefore, the antisense RNAs were capable of inhibiting the translation of mRNA, which may be one of the major mechanisms of the antisense RNA effect.

The mechanisms of antisense RNA action appear to be more complicated than initially thought. A typical example is the experiment conducted by Sullenger et al. (1990). They...
studied inhibition of MoMLV replication by antisense \textit{gag} RNA and demonstrated that the antisense RNA substantially reduced the synthesis of viral Gag proteins but had no effect on the cytoplasmic level of \textit{gag} mRNA. The results obtained in our \textit{in vitro} translation experiments with antisense RNA support the theory of inhibition of translation by antisense RNA. However, as the effects of antisense RNA were investigated further, other previously unidentified mechanisms have been uncovered. They include: (i) inhibition at the transcription level (Yokoyama & Imamoto, 1987); (ii) inhibition at the level of RNA splicing (Feng & Denhardt, 1992); (iii) inhibition of the transport of mRNA out of the nucleus (Kim & Wold, 1985); and (iv) modulation of dsRNA by RNA duplex unwindase which leads to the increased degradation of dsRNA or changes of the coding capacity of mRNA (Wagner \textit{et al.} 1989). To demonstrate the probable mechanism of antisense RNA action in our cellular system, we isolated poly(A) RNA from control cells and antisense RNA expressing cells, and measured HBV mRNA levels by Northern blot analysis using antisense RNA as a probe. As shown in Fig. 5, HBV mRNA levels were reduced significantly in antisense RNA expressing Hep3B cells in comparison with control vector transfected and untransfected cells, whereas there was no difference in GAPDH mRNA levels between control and antisense expressing cells. Therefore, in our cellular system, the antisense RNA exerted its inhibitory effect, at least partially, by affecting the mRNA, but the exact mechanism is unclear. Based on the mechanisms so far reported, we can exclude the possibility of inhibition of the processing of the mRNA in our system since HBV transcripts do not need the splicing process before becoming fully functional. We propose that the antisense RNA may exert its effect, either through the inhibition of transcription of HBV mRNA or through the modulation of dsRNA by unwindase activity. In fact, adenosine-to-inosine transition in the HBV mRNA may be responsible for the decreased signal in the Northern blot analysis, because the transition reduces the binding strength between mRNA and probe during hybridization. This Northern blot result further addressed the specificity of the antisense RNA action.

In general, a large excess of antisense RNA over mRNA is likely to improve the chance of successful inhibition since the antisense RNA has easy access to its target when at high concentration. However, over-expression of antisense RNA is not an absolute requirement for obtaining maximal effect of an antisense RNA. A number of reports described significant down-regulation of expression under circumstances in which the steady-state levels of mRNA substantially exceeded the steady-state levels of antisense RNA (Nishikura & Murray, 1987; Sheehy \textit{et al.}, 1988). In our cellular system, the expression of 1·0 kb antisense RNA in transfected Hep3B cells was only detectable by RT–PCR (Fig. 3). It is not known why the 1·0 kb antisense RNA still demonstrated a strong inhibitory effect on HBsAg production, at times even stronger than the other two antisense RNAs. Based on our coupled \textit{in vitro} transcription and translation experiment which showed that 1·0 kb antisense RNA seemed to exert the most potent effect, it is reasonable to propose that 1·0 kb antisense RNA also exerted a more potent effect intracellularly than the other two despite the low amount of antisense RNA. In fact, several authors have reported that antisense RNA was undetectable although a biological effect was observed (Crowley \textit{et al.}, 1985; Kasid \textit{et al.}, 1989). This could be due to the intrinsic instability of such RNAs or due to the degradation of dsRNA, which may explain why we see low 1·0 kb antisense RNA expression in transfected cells.

The research presented in this paper is the first to demonstrate an inhibitory effect of antisense RNA on HBV gene expression. The results obtained support the potential for an antisense RNA strategy against HBV infection. The advantage of the antisense RNA approach is its potential for specificity and lack of host toxicity. In the traditional avenues of anti-viral drug research, protein functions are targeted; targeting is largely empirical and effective drugs are usually discovered after screening of thousands of agents. In contrast, in the antisense RNA approach, specific target sites on the virus can be selected and targeted with ease. All viral proteins can be targeted, even if the functions are obscure. In addition, antisense therapy can be directed at different levels of viral function, e.g. the nucleic acid sequences that control replication, transcription or translation. Therefore, based on the present data and the advantages of antisense RNA technology, we believe that antisense RNAs should be pursued as therapeutic agents for chronic HBV infection.

We thank Ms Deborah Sullivan and Dr Srikanta Dash for helpful discussions and technical advice throughout our research, and Mr Ellis Diaz for photographic assistance. This work was supported by NIH Grant CA54576.

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Received 27 August 1996; Accepted 4 November 1996