

Inhibition of HBV Replication by Antisense Oligodeoxyribonucleotides in HepG2 Cells Transfected with a Cloned HBV DNA*

Sang-Hwan Oh, Byung-Il Yeh, and Seok-Hyun Kim

The effect of antisense oligodeoxyribonucleotides(oligo[dN]s) on hepatitis B virus(HBV) replication in HepG2 cells harboring a cloned HBV genome was examined. Antisense oligo[dN]s directed at translational initiation sites of S, pre C and P genes of HBV were treated to the cells and the amount of HBsAg and HBV DNA content were measured 72 hours after the treatment. HBsAg expressions in HepG2 cells harboring the HBV genome were inhibited 68%, 53%, and 46% by the treatment with antisense oligo[dN] directed at S, pre C, and P gene loci, respectively, and HBV DNA content in the cells was also reduced by the treatment of each antisense oligo[dN]. The doubling times of the cultured cells treated with 25 µg, 50 µg, and 100 µg of antisense oligo[dN]/ml medium were 43.3, 62.1, and 93.0 hours, respectively, compared with 37.5 hours of the untreated control cells. Cellular DNA synthesis was inhibited by the treatment with 100 µg/ml of antisense oligo[dN], however, no significant effect was observed by the treatment with 50 µg or less of antisense oligo[dN]/ml. These results suggested that antisense oligo[dN]s specific to the translational initiation sites of S, pre C, and P genes of HBV may have therapeutic potential for the suppression of HBV propagation in chronic HBV infected patients.

Key Words: Antisense oligodeoxyribonucleotides, HBsAg, HBV replication

Hepatitis B virus (HBV) is a small DNA virus belonging to the hepadnavirus family and the infection of this virus may cause acute or chronic hepatitis and liver cirrhosis (Ganem, 1982; Tiollais *et al.* 1985). Moreover, epidemiological studies have indicated that chronic HBV infection is closely associated with human hepatocellular carcinoma (Beasley *et al.* 1981; Szmuness, 1988).

Due to the restricted host range and human

pathogenicity of HBV, the investigation on the expression and replication of the HBV genome has been hampered. Several mammalian cell lines have been transfected with cloned HBV DNA in order to establish an in vitro tissue culture system in which HBV is propagated (Dubois *et al.* 1980; Wang *et al.* 1983; Will *et al.* 1984). However, these experiments have failed to yield cells which are able to produce the replicative DNA intermediates or Dane-like viral particles. Fortunately, an establishment of HepG2 cell line which can support the assembly and secretion of replicative intermediate of HBV has been successfully attained by transfection of a plasmid carrying the gene which compasses four 5'-3' tandem copies of HBV genome positioned such that two dimers of the genomic DNA are 3'-3' with respect to one another (Sells *et al.* 1987). Therefore, this cell line has been used in the study for HBV replication in vitro system.

Received November 6, 1995

Accepted December 14, 1995

Department of Biochemistry & Molecular Biology and the Institute of Genetic Science, Yonsei University College of Medicine, Seoul, Korea

*This study was supported by CMB-Yuhan Research Grant (1994) Yonsei University College of Medicine, Seoul 120-752, Korea

Address reprint requests to Dr. S.H. Oh, Department of Biochemistry & Molecular Biology, College of Medicine, C. P.O. Box 8044, Seoul 120-752, Korea

Now, the search for a therapeutic agent that can effectively inhibit HBV propagation is extensively encouraged. Antibiotics such as vidaravine, interferons, and phosphono compounds have been tested for the treatment of chronic hepatitis B patients but their therapeutic effects were temporal and unsatisfactory due primarily to their cytotoxicity (Chadwick *et al.* 1978; Bassendine *et al.* 1981; Gutterman *et al.* 1982; Weller *et al.* 1982; Scotto *et al.* 1983). Application of an immunosuppressant, prednisolone, has been tested for the control of HBV propagation, but this agent had also exerted a transient inhibitory effect on HBV replication (Scullard *et al.* 1981). Recently, antisense oligodeoxyribonucleotide (oligo[dN]) has been known to be a potential candidate agent for the selective inhibition of viral replication (Zamecnik *et al.* 1986; Goodchild *et al.* 1988; Sarin, 1988; Stein and Cohen, 1988). Inhibitory effect of antisense oligodeoxyribonucleotides on the expression of HBsAg in PLC/PRF/5 human hepatoma cell line has been demonstrated (Goodarzi *et al.* 1990; Blum *et al.* 1991).

In the present study, the inhibitory effect of antisense oligo[dN] directed at translational initiation sites of HBsAg, HBcAg, and HBV DNA polymerase genes on the replication of HBV in HepG2 cells harboring HBV genome (HepG2-HBV) was tested in order to evaluate their possible application for the repression of HBV propagation in the chronic HBV infected patients.

MATERIALS AND METHODS

Cells and Transfection

A human hepatoblastoma cell line, HepG2,

transfected with a plasmid containing two head to tail dimers of HBV gene in a tail-to-tail orientation (HepG2-HBV) was obtained from Dr. Han K (Yonsei University College of Medicine, Seoul, Korea). Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum at 37°C in a moist atmosphere containing 5% CO₂. The cultured cells were detached from culture dishes (100×20 mm) and divided into wells of cell culture cluster dish (25 mm, 12 wells, 5×10⁵ cells/well). They were grown for 48 hours and 25 μg, 50 μg, or 100 μg of antisense oligo[dN] per ml of the freshly changed culture media were added to each well. The cells were cultured for another 72 hours. Cultured media were removed from each well of the cluster dish and saved for analysis. Cells were harvested and separately saved for analysis.

Preparation of Antisense Oligo (dN)

Antisense oligo[dN]s directed at the translational initiation sites of S, pre C, and P genes of HBV (subtype Adr; Kim *et al.* 1988) were synthesized by DNA synthesizer (ABI 381A). The sequences of these three oligomers are as follows: ① antisense S gene locus, 5'-CTC CAT GTT CGG TGC-3' (nucleotide No. 160-146); ② antisense pre C gene locus, 5'-TAC AAA GAT CAT TAA-3' (nucleotide No. 1768-1754); ③ antisense P gene locus, 5'-GGG CAT TTG GTG GTC-3' (nucleotide No. 2310-2296). The positions of the antisense oligo[dN]s in HBV genome are shown in Fig. 1. The synthesized antisense oligo[dN]s were purified by SEP-PAK and a portion of them were end labeled with ³²P using γ-³²[P]ATP by the method of Davis *et al.* (1994).

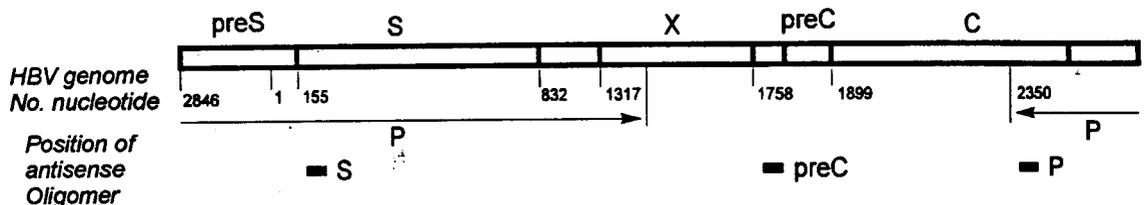


Fig. 1. Schematic diagram of HBV genes and position of antisense oligodeoxyribonucleotides.

Measurement of HBsAg

HBsAg in the culture media of HepG2-HBV cells was measured by AUSRIA II-125 kit (Abbott Laboratories, North Chicago, IL, U.S. A.). The amount of HBsAg was represented as the amount of radioactivity in ^{125}I -labeled antibody bound to HBsAg in the medium.

Detection of HBV DNA and HBV DNA polymerase activity

HBV DNA in the media of the cultured cells transfected with antisense oligo[dN]s was detected by the Southern blot hybridization method (Southern, 1975). HBV particles in the media(10 ml) of the cultured HepG2-HBV cells were concentrated by ultracentrifugation($40,000 \times g$, 1 hour). The concentrated HBV particles were digested with Xho I restriction enzyme and subjected to agarose gel (1.0%) electrophoresis. The DNA fragments were transferred onto the nylon membrane and blotted. HBV DNA was hybridized according to a method described by Sambrook *et al.*(1989) using a synthetic oligodeoxyribonucleotide probe corresponding to S gene locus of HBV(5'-ATTACCAATTTT-CTTTTGTCT-3';nucleotide No. 800-820). The probe was 5'-end-labeled with ^{32}P using [γ - ^{32}P]ATP according to the method of Davis *et al.*(1994). HBV DNA polymerase activity was determined by the method of Kaplan *et al.*(1973).

Measurement of the cytotoxicity

The cytotoxic effect of antisense oligo[dN] was determined by the measurement of the growth rates and DNA synthesis of cells transfected with antisense oligo(dN). The number of viable cells was counted by trypan blue staining after the treatment of antisense oligo(dN). For the measurement of the rate of DNA synthesis, ^3H -labeled thymidine ($2 \mu\text{Ci}/10^5$ cells) was added to the cell culture medium at the time of the treatment of antisense oligo [dN] and the amount of radioactivity incorporated into the chromosomal DNA of HepG2-HBV cells was counted by a liquid scintillation counter(Packard TriCarb 300).

RESULTS

Effect of antisense oligo[dN]s on the expression of HBsAg

The amount of HBsAg in the media of HepG2-HBV cells decreased by the treatment of antisense oligo(dN)s directed at the translational initiation sites of S, pre C, and P genes of HBV (Fig. 2). HBV DNA polymerase activity was not detected in the cultured media of HepG2-HBV cells and the enzyme activity could not be used as a criterion of HBV replication in the present study. Antisense oligo(dN) directed at the translational initiation site of S gene in HBV was the most effective one among the antisense oligo[dN]s tested for the inhibition of HBsAg expression.

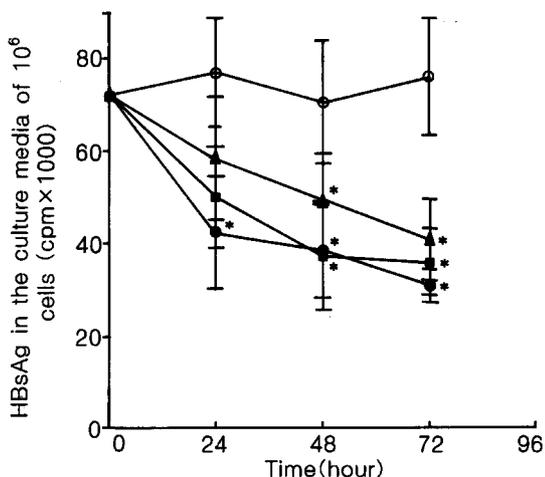


Fig. 2. Effect of antisense oligodeoxyribonucleotides on the expression of HBsAg in HepG2 cells harboring a cloned HBV genome.

HBsAg in the culture media of the control cells(○) treated with each antisense oligodeoxyribonucleotide($25 \mu\text{g}$) directed at S(●), preC(■) and P(▲) were measured by AUSRIA II-125 kit, and ^{125}I -labeled anti-HBs bound to the antigen in the culture media was counted.

*: Values are significantly($p < 0.05$) different from that of the control group

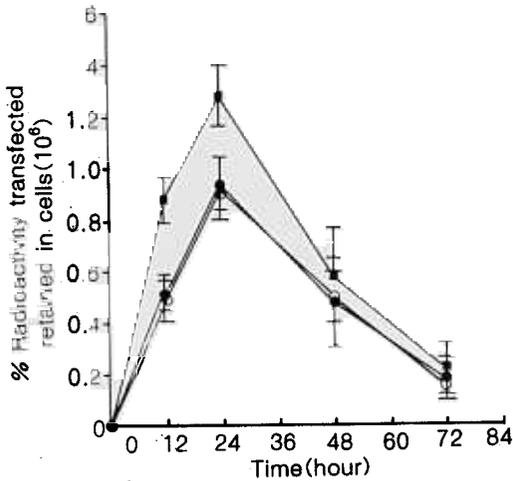


Fig. 3. Uptake and retention of antisense oligodeoxyribonucleotides treated to HepG2 cells harboring a cloned HBV genome.

32 P-labeled antisense oligodeoxyribonucleotide (15mer) directed at p gene with (■) or without (●) polybrene and 32 P-labeled antisense oligodeoxyribonucleotide (21mer) without polybrene (○) were treated to the cells. The cells were cultured for 72 hours, harvested and washed 3 times with 10 volumes of saline. The radioactivities retained in the cells were counted.

Retention of antisense oligo(dN) transfected into cells

The change in the radioactivity in HepG2-HBV cells treated with 32 P labeled antisense oligo[dN] is shown in Fig. 3. The amount of radioactivity uptaken by the cells increased gradually for the first 24 hours and then decreased to a low level (0.15% of the amount treated) at 72 hours after the treatment. The addition of an equal amount (W/W) of polybrene to the antisense oligo[dN] increased the cellular uptake of antisense oligo[dN] with similar pattern of retention.

Effect of antisense oligo(dN) on the HBV DNA content in HepG2-HBV cells

HBV DNA content in HepG2-HBV cells treated with each antisense oligo[dN] was

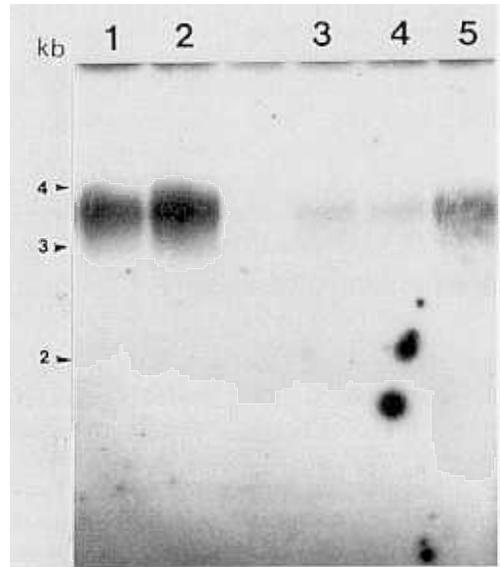


Fig. 4. Southern blot hybridization of HBV DNA in the culture media of HepG2 cells harboring a cloned HBV genome after the treatment with antisense oligodeoxyribonucleotides.

1 & 2: Control group (untreated)

3: Antisense oligomer treated group (directed at S gene locus)

4: " " " " " (" preC " ")

5: " " " " " (" P " ")

much less than that in the untreated control cells (Fig. 4). The inhibitory effect of antisense oligo[dN] directed at S and preC genes on the replication of HBV in HepG2-HBV was prominent compared to that directed at the p gene of HBV.

Cytotoxic effect of antisense oligo (dN)

The inhibition of growth and DNA synthesis in HepG2-HBV cells by the treatment of antisense oligo[dN] was evidenced by the decreased incorporation of 3 H-labeled thymidine into chromosomal DNA (Table 1). The inhibition of 3 H-thymidine incorporation into chromosomal DNA was significant ($p < 0.05$) at the antisense oligo[dN] concentration of 25 μ g/ml in both the antisense S locus and preC locus groups. A significant reduction in the

Table 1. Effect of antisense oligodeoxyribonucleotides on the DNA synthesis in HepG2 cells harboring a cloned HBV genome

Group	³ H-thymidine incorporation (CPM/mg DNA)	Chromosomal DNA content (μg/flask)
Control (untreated)	86,400 ± 9,600	66.2 ± 12.7
Antisense (S locus)	81,400 ± 10,200	45.1 ± 7.4*
" (preC ")	82,850 ± 4,150	38.8 ± 8.0*
" (P ")	82,300 ± 7,800	47.3 ± 15.1

Antisense oligodeoxyribonucleotides (25 μg/ml) were treated to 5×10^5 cells in each well of the cluster dishes and incubated at 37°C for 72 hours. ³H-thymidine (0.5 μCi/well) was added to the media at the same time of the treatment of antisense oligodeoxyribonucleotides. All values are means ± S.D. *: Significant difference from the control value (P < 0.05)

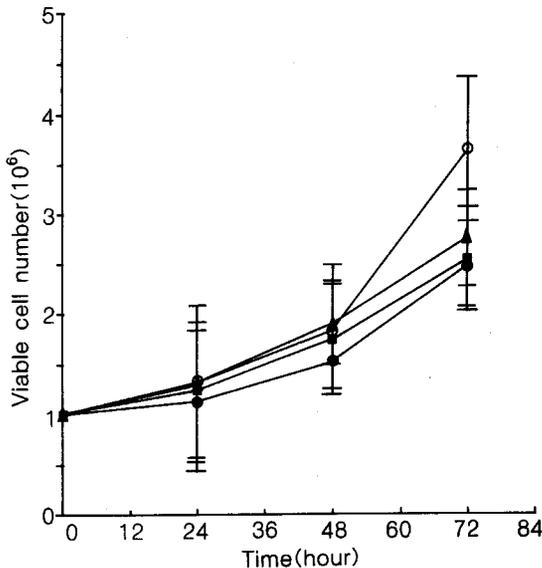


Fig. 5. Changes in viable cell number after the treatment of anti-S gene antisense oligodeoxyribonucleotides (15mer) to HepG2 cells harboring a cloned HBV genome. Control cells (○) were treated with 25 μg (△), 50 μg (■) and 100 μg (●)/ml of antisense oligodeoxyribonucleotides (15 mer) directed at S gene of HBV genome.

cell number was observed 72 hours after the treatment of 50 μg/ml and 100 μg/ml of antisense oligo[dN] (Fig. 5).

DISCUSSION

In the present study, the treatment of antisense oligo[dN] directed at the translational initiation sites of S, pre C, and P genes of HBV decreased HBsAg expression and HBV DNA content in the cultured media of HepG2-HBV cells indicating that translation of HBsAg, HBV DNA polymerase, and HBcAg were effectively prevented by these corresponding antisense oligo [dN].

Since the inhibitory effect of an antisense oligo[dN] toward its mRNA target depends on a number of factors such as secondary structure, tertiary structure, and the length of nucleotides, we have chosen 5'-terminal regions of these target genes where circularization of intermediate transcripts may occur. Therefore, the target regions of HBV genome for antisense oligo[dN]s used in this study were thought to be the favorable sites for the antisense hybridization with less chance of secondary structure formation. The inhibitory effect of antisense oligo[dN] on the translation of the target gene has been known to be sequence specific and both initiation and termination sites, either upstream or downstream were thought to be more effective (Goodchild *et al.* 1988). Antisense oligo[dN] has been shown to be most effective when directed at the initiation site of the S gene of HBV whereas it was least effective when it was directed at the inside of the gene (Goodarzi *et al.* 1990). Antisense oligo[dN] directed against the translation initiation site of S gene inhibited HBsAg expression more than that of the pre C or P gene indicating that the inhibition of a gene expression by its corresponding antisense oligo (dN) is sequence specific. The inhibitory effect of antisense oligo[dN] against pre C and P on HBsAg expression might be resulted from the allosteric hindrance of HBsAg translation or from the inhibition of viral replication by the insufficient supply of HBV core protein and HBV DNA polymerase.

The presence of HBV DNA as well as

HBsAg in the culture media of HepG2-HBV cells in the present study confirmed that these cells can serve as an in vitro model system to test the effect of the drugs which could interfere with viral replication. This result confirmed the fact that HepG2-HBV cells are capable of supporting some phases of HBV replication and are amenable to the study of the replication of HBV (Sells *et al.* 1987). The present result that HepG2 cell transfected with a cloned HBV DNA produced much less HBsAg and HBV DNA in the culture media by an antisense oligo[dN] treatment is consistent with the earlier observation by Sells *et al.*(1987).

Reduced HBV DNA content in the cultured media of HepG2-HBV cells by the treatment with each antisense oligo[dN] in the present study indicates that they inhibited the replication of HBV and the release of HBV-like particles into the media (Fig. 4). Although the mechanisms by which HBV gene infected into HepG2-cells enter the replicative cycle are unknown, it is probable that HepG2 cells in culture are supporting HBV replication.

HBV DNA polymerase, the P gene product of HBV has been known to be responsible for the filling of single-stranded gap present at (+) strand of HBV DNA (Summers *et al.* 1975) and to be involved in the multiplication cycle of HBV since it possesses a reverse transcriptase activity (Tiollais *et al.* 1985; Seeger *et al.* 1986).

The absence of a detectable amount of HBV DNA polymerase activity in the culture media of HepG2-HBV implicates that most of HBV DNA in Dane-like particles in the media are in double stranded circular forms without the single stranded gap and that they may not be used as the template for HBV DNA polymerase. The radioactivity incorporated into HepG2-HBV cells treated with ³²P-labeled antisense oligo[dN] increased for the first 24 hours after the treatment followed by a gradual decrease thereafter indicating that a single treatment of antisense oligo[dN] to the cells was not sufficient to bring about the persistent inhibitory effect beyond 24 hours.

Goodarzi *et al.*(1990) have observed that the inhibitory effect of antisense oligo(dN) persist-

ed as long as 120 hours after the second administration of 24 hours interval. Therefore, it is assumed that multiple administration of antisense oligo[dN] in 24 hours interval will be beneficial to get a persistent inhibitory effect on the viral replication. In the present study, cytotoxic effect of antisense oligo[dN] was not severe in the concentration of 25 µg/ml media, however, its effect was dramatic at 50 µg or more per ml of media indicating that a limitation for the dose of administration of antisense oligo (dN) should be critically considered.

In considering the cytotoxicity of antisense oligo[dN], the efficient internalization into the cells with a limited amount of antisense oligodeoxyribonucleotides is an important factor to get a maximum inhibitory effect on the viral replication with the least toxicity. A soluble DNA carrier system that is targetable to hepatocytes via asialoglycoprotein receptors has been developed by Wu and Wu(1992). They have demonstrated an efficient cellular uptake of oligonucleotide complementay to the polyadenylation signal of HBV complexed with asialoglycoprotein DNA carrier system. The administration of small amount of antisense oligo[dN] mixed with an efficient transfection mediator such as polybrene or lipofectin may be considered as an another alternate method to alleviate the cytotoxicity of antisense oligo[dN].

REFERENCES

- Bassendine MF, Chadwick RG, Salmeron J, Shipton U, Thomas HC, Sherlock S: Adenine arabinoside therapy in HBsAg-positive chronic liver disease:A controlled study. *Gastroenterology* 80: 1016-1021, 1981
- Beasley RP, Linn CC, Hwang JY, Chen CS: Hepatocellular carcinoma and hepatitis B virus, a progressive study of 22,707 men in Taiwan. *Lancet* 2: 1129-1133, 1981
- Blum HE, Galun E, Weizsacker F, Wands JR: Inhibition of hepatitis B virus by antisense oligodeoxyribonucleotides. *Lancet* 337: 1230, 1991
- Chadwick RG, Bassendine MF, Crawford E, Thomas HC, Sherlock S: HBs antigen positive chron-

- ic liver disease: Inhibition of DNA polymerase activity by vidarabine. *Br Med J* 2: 531-537, 1978
- Davis LG, Dibner MD, Battey JF: *End-labeling of synthetic probes*. In Davis LG, Dibner MD, Battey JF, eds. *Basic Method in Molecular Biology*. New York, Elsevier, 1994, 72-76
- Dubois MF, Porcel C, Rausset S, Chang C, Tiollais P: Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proc Natl Acad Sci(USA)* 77: 4549-4553, 1980
- Ganem D: Persistent infection of human with hepatitis B virus: Mechanisms and consequences. *Rev Infect Dis* 4: 1026-1047, 1982
- Goodchild J, Agrawal S, Civeria MP, Sarin PS, Sun D, Zamecnik PC: Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. *Proc Natl Acad Sci* 85: 5507-5511, 1988
- Goodarzi G, Gross SC, Tewari A, Watabe K: Antisense oligodeoxyribonucleotides inhibit the expression of the gene for hepatitis B virus surface antigen. *J Gen Virol* 71: 3021-3025, 1990
- Gutterman JU, Fine S, Quesadar J, Horning SJ, Levine JF, Alexanian R, Bernhardt L, Kramer M, Spiegel H, Colburn W, Trown T, Merigan T, Dziewanowski Z: Recombinant leukocyte A interferon: Pharmacokinetics, single dose tolerance and biologic effects in cancer patients. *Ann Intern Med* 96: 549-556, 1982
- Kaplan PM, Greenman RL, Gerin JL, Purcell RH, Robinson WS: DNA polymerase associated with human hepatitis B antigen. *J Virol* 12: 995-1005, 1973
- Kim KT, Hyun SW, Kim YS, Rho HM: Complete nucleotide sequence of hepatitis B virus (sub-type Adr). *Korean Biochem J* 21:319-331, 1988
- Sambrook J, Fritsch EF, Maniatis T: *Hybridization of radiolabeled probes to nucleic acids immobilized on nitrocellulose filters or nylon membrane*. In Sambrook J, Fritsch EF, Maniatis T, eds. *Molecular cloning, a laboratory manual, second edition*. New York, Cold Spring Harbor Laboratory Press, 1989, 9.52-9.55
- Sarin PS: Molecular pharmacologic approaches to the treatment of AIDS. *Ann Rev Pharmacol* 28: 411-428, 1988
- Scott G, Secher D, Flowers D, Bute J, Cantell K, Tyrell D: Toxicity of interferon. *Br Med J* 282: 1345-1348, 1983
- Scullard G, Andres L, Greemgerg H: Antiviral treatment in chronic hepatitis B virus infection: Improvement in liver disease with interferon and adenine arabinoside. *Hepatology* 1: 228-232, 1981
- Seeger C, Ganem D, Varmus HE: Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science* 232: 477-484, 1986
- Sells MA, Chen ML, Acs G: Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci (USA)* 84: 1005-1009, 1987
- Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98: 503-517, 1975
- Stein A, Cohen JS: Oligodeoxynucleotides as inhibitors of gene expression. *Cancer Res* 48: 2659-2668, 1988
- Summers J, O'Connell A, Millman I: Genome of hepatitis B virus: Restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc Natl Acad Sci (USA)* 72: 4597-4601, 1975
- Szmunes W: Recent advances in the study of the epidemiology of hepatitis B. *Am J Pathol* 81: 629-649, 1988
- Tiollais P, Pourcel C, Dejean A: The hepatitis B virus. *Nature* 317: 489-495, 1985
- Wang Y, Stratowa C, Schaefer-Ridder M, Doehmer J, Hofschneider PH: Enhanced production of hepatitis B surface antigen in NIH 3T3 mouse fibroblasts by using extrachromosomally replicating bovine papilloma virus vector. *Mol Cell Biol* 3: 1032-1039, 1983
- Weller IVD, Bassendine MF, Craxi A, Fowler MJF, Monjardino J, Thomas HC, Sherlock S: Successful treatment of HBs and HBeAg positive chronic liver disease: Prolonged inhibition of viral replication by highly soluble adenine arabinoside 5'-monophosphate(ARA-AMP). *Gut* 23: 717-723, 1982
- Will H, Cattaneo R, Pfaff E, Kuhn C, Roggendorf M, Schaller H: Expression of hepatitis B antigen with a Simian virus 40 vector. *J Virol* 50: 335-342, 1984
- Wu GY, Wu CH: Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides. *J Biol Chem* 267: 12436-12439, 1992
- Zamecnik PC, Goodchild J, Tayuchi Y, Sarin P: Inhibitor of replication and expression of human T-cell lymphotropic oligonucleotides complementary to viral RNA. *Proc Natl Acad Sci (USA)* 83: 4143-4146, 1986