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Construction of Nondefective Adenovirus Type 5 Bearing a 2.8-Kilobase Hepatitis B Virus DNA Near the Right End of Its Genome

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A novel helper-free adenovirus type 5 (Ad5) vector system, which utilizes a cloning site 0.2 kilobase (kb) from the right end of the genome, has been developed. To construct a nondefective Ad5 bearing the 2.8-kb DNA fragment of hepatitis B virus (HBV) at this site, we deleted the 2.1-kb nonessential E3 fragment from cloned DNA covering the right one-fourth of the Ad5 genome (76 to 100 map units), inserted the HBV DNA into this site, ligated the recombinant DNA to the rest of the Ad5 genome, and transfected the ligated DNA into human embryo kidney cells. Most of the recovered virus clones had only the E3 deletion and no HBV insertion, suggesting that a homologous recombination occurs between transfected DNAs in these cells. The isolated Ad5 virus bearing the HBV DNA (Ad5-HBL) grew without helper virus in HeLa cells as efficiently as wild-type Ad5, although the 1.9-kb major E4 transcript was detected only poorly in the early phase in the Ad5-HBL-infected cells, suggesting that the HBV DNA inserted upstream of the E4 promoter reduces the E4 transcript. HBV mRNAs transcribed from the inserted DNA were at least as abundant as Ad5 early mRNAs in the late phase of Ad5-HBL infection, but the HBV surface antigen was barely detectable in the infected-cell lysate and culture medium. This result suggests that HBV mRNAs can be transcribed from the inserted genes but no protein can be translated from the HBV mRNAs, presumably because of the translational suppression of cellular mRNAs caused by adenovirus in its late phase.

Adenovirus may be a basis for valuable expression vector systems because its large genome size (35 kilobases [kb]) allows the substitution of large DNA fragments. Adenovirus vectors are usually either defective, requiring a helper such as wild-type (WT) virus, or can accept only selectable genes, such as simian virus 40 T antigen (19, 22–24). Helper-free adenovirus vectors have been developed recently (2, 9) and have several advantages, such as their high copy number in infected cells and good stability. In one of the vector systems, the foreign gene is substituted for the E1 region. Consequently, the recombinant virus can grow only in cell line 293 and not in HeLa cells, which are easy to culture on a large scale. In the other helper free vector system, the foreign gene is substituted for the nonessential E3 region. However, this system is not useful for studying the structure of RNA transcripts from genes such as the hepatitis B virus (HBV) genome (4, 25) because transcription from an inserted gene must be influenced by adjacent transcription from both the E3 and major late promoters (18). Therefore, it is desirable to find another site where an inserted gene can be expressed without suffering from adjacent transcription units and without reducing the viability of the vector in HeLa cells.

We describe here a new helper-free adenovirus type 5 (Ad5) vector system in which foreign DNA can be inserted near the right end of the genome, where no adenoviral transcription has been reported. We constructed a recombinant virus bearing 2.8 kb of HBV DNA that grows in HeLa cells as efficiently as WT Ad5. In the late phase the recombinant virus abundantly expresses a 2.0-kb RNA identical to the mRNA that codes for HBV surface antigen (HBsAg) but fails to produce HBsAg from the HBV mRNA. We think that this is the first clear analysis of the expression of a foreign gene inserted in the adenovirus genome together with its own promoter.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in suspension in Eagle minimal essential medium supplemented with 10% calf serum. Human embryo kidney (HEK) cells were cultured in monolayers in Eagle minimal essential medium supplemented with 10% fetal calf serum. WT Ad5 was originally obtained from T. Shenk.

Reagents and DNA preparation. All restriction enzymes were products of Takara Shuzo, Kyoto, Japan. Nuclease S1 and the Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim Biochemica, Mannheim, West Germany. Restriction fragments were electroeluted, purified by phenol-chloroform extraction and by spin columns (15) of hydroxyapatite and Sephadex G-25, and then used for the next enzymatic reaction. The other procedures used in plasmid construction have been described previously (7). The Ad5 DNA terminal protein complex (DNA-PC) was purified as described previously (6, 12), concentrated by CsCl equilibrium centrifugation in a Hitachi vertical rotor (RPV65Ti), dialyzed against buffer containing 2 mM Tris-hydrochloride (pH 8.0)–0.4 mM EDTA, and then stored at −80°C.

Construction of pX2S-HBV recombinant plasmids. Figure

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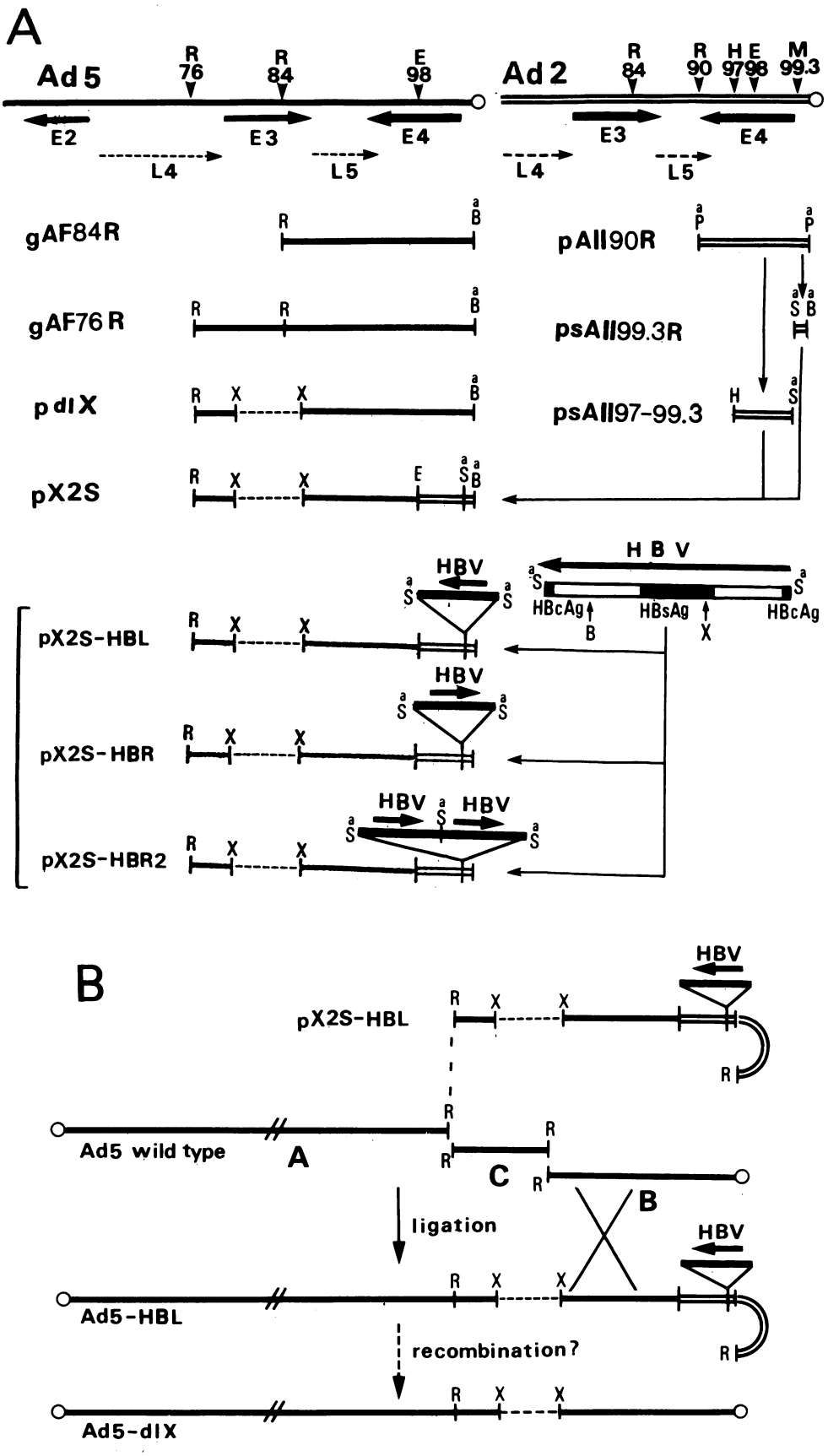
A
Ad5
R 76
R 84
E 98
E2
E3
E4
L4
L5
Ad2
R 84
R 90
H E 97 98
M 99.3
E3
E4
L4
L5
gAF84R
gAF76R
pdlX
pX2S
pAll90R
psAll99.3R
psAll97-99.3
HBV
H B V
HBcAg
HBsAg
HBcAg
B
X
pX2S-HBL
pX2S-HBR
pX2S-HBR2
B
pX2S-HBL
Ad5 wild type
A
C
B
ligation
Ad5-HBL
recombination?
Ad5-dlX

1A summarizes the strategy used for construction of the Ad5-HBV recombinant plasmids. gAF84R, a generous gift from H. Kato, is a pSV2gpt (16) derivative containing the Ad5 *Eco*RI B fragment (84 to 100 map units [m.u.]). To clone this fragment, the right end of Ad5 DNA was treated with nuclease S1 to remove the terminal protein together with several nucleotides and was ligated with *Bam*HI linker. We constructed gAF76R, covering 76 to 100 m.u., by inserting the Ad5 *Eco*RI C fragment (76 to 84 m.u.) into the unique *Eco*RI site in gAF84R. We then prepared pdlX by removing both the *Escherichia coli gpt* gene and the 2.1-kb *Xba*I fragment in the E3 region from a gAF76R plasmid that had been amplified in a *dam* mutant *E. coli* strain, GM33. pAII90R, a generous gift from H. Ariga, bears the Ad2 right-end *Eco*RI fragment (90 to 100 m.u.), inserted at the pBR322 *Pst*I site by using dG · dC homopolymer tailing. pX2S was constructed from pdlX and pAII90R: we converted the pAII90R *Mbo*II site at 99.3 m.u., 0.2 kb downstream of the Ad2 right end, to a *Sal*I site by linker insertion, subcloned the resulting fragments (psAII99.3R and psAII97-99.3), and then substituted them for the corresponding Ad5 regions
of pdlX by using the common *Bst*EII sites (98 m.u.). The HBV *Bgl*II fragment of 2.8 kb, originally derived from pBRHBadr125 (5), was ligated with *Sal*I linkers and then inserted into the *Sal*I site of pX2S. We obtained three plasmids (pX2S-HBL, pX2S-HBR, and pX2S-HBR2) which differed in the orientation and copy number of the HBV insert.

Construction of Ad5-HBV recombinant virus. Figure 1B shows our strategy in constructing Ad5-HBV recombinants through a modification of the method of Stow (20, 21) and our earlier work (7). We mixed each recombinant plasmid (0.6 μg) with Ad5 DNA-PC (0.2 μg), digested them with *Eco*RI (8 U) at 37°C for 3 h in a volume of 10 μg as specified by the supplier, and ligated them at 4°C overnight after simply adding T4 DNA ligase (final concentration, 4 U/ml) and ATP (final concentration, 1 mg/ml) to the mixture without inactivating the *Eco*RI. The ligated mixture was carried on ice, mixed quickly with 1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid)-buffered saline and 50 μl of 2 M calcium chloride (10) at room temperature, and transfected into monolayer cultures of HEK cells in two 6-cm plastic dishes. Virus plaques appeared in the dishes overlaid with agar in 5 to 7 days. The agar over the plaques was picked, suspended in 1 ml of culture medium, frozen and thawed five times, and used to infect HeLa cells in a 24-well dish. After 3 to 5 days, the infected cells were pelleted, suspended in 50 μl of buffer containing 20 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per ml and incubated at 55°C for 1 h. The mixture was extracted once with phenol-chloroform, gel-filtered with a spin column, and subjected directly to cleavage with restriction enzymes without removing the cellular DNA. Usually, staining the gel with ethidium bromide was sufficient to identify the desired clone.

S1 mapping. We infected HeLa cells in suspension culture with Ad5-HBL at 20 PFU per cell and extracted cytoplasmic RNA from the infected cells at various times. The preparation of viral DNA probes labeled in vivo with ^{32}P and the procedure for S1 mapping have been described previously (1, 17). Hybridization was carried out at 58°C for detecting Ad5 mRNAs and at 52°C for detecting HBV mRNAs.

Radioimmunoassay for HBsAg. HeLa cells were infected for the periods indicated. The cells were separated from the culture medium by centrifugation. Each cell pellet was suspended in 1 ml of culture medium, freeze-thawed three times, and sonicated. Cell extracts and culture media were tested for HBsAg with an AUSRIA II radioimmunoassay kit (Abbott Laboratories, North Chicago, Ill.) (14). Known amounts of HBsAg purified from human carrier sera were included as controls.

Computer analysis of DNA sequence. We screened the restriction enzyme sites in Ad2 and Ad5 DNAs with a computer program, FLEXP (13), and the possible secondary structure in the Ad2 E4 promoter region with a program described previously (27).

RESULTS

Construction of Ad5-HBV recombinant virus. The deletion of the 2.1-kb *Xba*I fragment from the nonessential E3 region of the cloned Ad5 genome (Fig. 1A) served two purposes. It allowed the insertion of a large foreign DNA fragment into the Ad5 vector without hampering its packaging and also removed the *Eco*RI site at 84 m.u. which was an obstacle to restoring the plasmid to the Ad5 genome. To insert the *Sal*I linker into the region between the right inverted terminal repeat and the E4 promoter, we adopted the *Mbo*II site at 99.3 m.u. in Ad2 DNA because Ad5 DNA has no restriction site in this region. (The Ad2 and Ad5 genomes are 98% homologous in their nucleotide sequences.) We then inserted the HBV DNA fragment of 2.8 kb into the *Sal*I site of pX2S and obtained three recombinant plasmids with different orientations and copy numbers of the insert. To restore each recombinant plasmid to the Ad5 genome, we ligated an excess amount of the *Eco*RI-digested plasmid with the total *Eco*RI digest of Ad5 DNA-PC without isolating the restriction fragment (about 15 times more plasmid than Ad5 DNA-PC by molar ratio) (Fig. 1B). The ligated mixture was transfected into HEK cells, and virus clones were isolated from the plaques.

When the plasmids containing Ad5 or Ad5-HBV DNA were used to restore the Ad5 genome, the plaque-forming efficiency was at least 10 times higher than when pSV2gpt DNA was used as a control (Table 1). The result suggests that the Ad5 sequence of each plasmid may be efficiently

FIG. 1. (A) Strategy used for the construction of pX2S-HBV recombinant plasmids. Top lines show the Ad5 (heavy black line) and Ad2 (open line) genomes. Restriction sites and map units are indicated above the lines, and the directions of transcription are shown by arrows below the lines. The constructed plasmid DNAs are shown under the genomes; pBR322 and pSV2gpt sequences are not shown. The HBV DNA insert is shown under the Ad2 genome, indicating the coding regions for HBsAg and HBV core antigen (HBcAg) as solid boxes; the direction of transcription is shown by an arrow. The insert lacks most of the HBV core antigen coding region. The deleted sequences in the Ad5 E3 region are shown as dotted lines. Restriction site abbreviations: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; M, *Mbo*I; X, *Xba*I; S, *Sal*I; E, *Bst*EII. An a above the restriction site shows that the site is not present in the original sequences but has been introduced artificially by DNA linker or dG · homopolymer tailing. (B) Strategy used for restoring the Ad5-HBV recombinant plasmids to the Ad5 genome. The inserted HBV DNA is indicated. The Ad5 terminal protein is shown as an open circle, and the pBR322 sequence is represented by the open-line semicircle at the right end of pX2S-HBL and Ad5-HBL.

TABLE 1. Structures of the isolated recombinant viruses[a]

Plasmid	No. of plaques/μg[b]	No. of isolated recombinant viruses:				
		Examined	Desired[c]	WT Ad5	Ad5-dlX	Other
pdlX	80	10	9	0	9	1[d]
pX2S	55	6	1	1	4	0
pX2S-HBL	80	7	1	0	6	0
pX2S-HBR	65	7	0	0	7	0
pX2S-HBR2	60	15	0	0	15	0
pSV2gpt[e]	<5					
Ad5 DNA-PC[f]	3,400					

[a] The recombinant plasmids were restored to Ad5 by in vitro ligation with the *Eco*RI digest of Ad5 DNA-PC and transfection into HEK cells.
[b] Number of plaques observed per 1 μg of the *Eco*RI digest of Ad5 DNA-PC.
[c] Desired recombinant virus, such as Ad5-dlX when pdlX was used.
[d] A defective virus plus Ad5-dlX (refer to the text).
[e] pSV2gpt DNA was used instead of the Ad5-HBV recombinant.
[f] Ad5 DNA-PC was transfected without *Eco*RI digestion.

restored to the Ad5 genome. However, it was surprising that pX2S-HBR2 gave about the same efficiency of plaque formation as did pdlX and pX2S, because the HBV insert of this plasmid (5.6 kb) seems to be too large for the recombinant virus to be packaged properly.

To examine the genome structures of the viral clones derived from each recombinant plasmid, the viral DNAs were digested with *Xba*I and *Sal*I and analyzed on agarose gels (Fig. 2; Table 1). All viral DNAs examined derived from pdlX showed the expected deletion of the *Xba*I D fragment (Fig. 2A), indicating that at least a part of the viral sequence was definitely of pdlX origin. We designated the deletion mutant virus Ad5-dlX; it must be identical to a virus previously constructed and designated Ad5Δ(78.9-84.3) by Berkner and Sharp (2, 3). An unexpected band was seen in the gel (Fig. 2A, lane 6), which was probably due to a defective virus which coexists with Ad5-dlX. One of the *Sal*I-digested viral DNAs from pX2S-HBL showed the expected bands of 2.8 and 0.2 kb (Fig. 2B, lane 5), showing that the pX2S-HBL sequence was accurately restored to the Ad5 genome (designated Ad5-HBL). We also obtained the recombinant virus whose right-hand one-fourth was of pX2S origin (Ad5-X2S), because the 0.2-kb band was detected in the *Sal*I digest (Fig. 2B, lane 1). However, most of the virus DNAs lacked both the HBV DNA insert and the *Sal*I linker sequence that we inserted into the recombinant plasmids. Interestingly, almost all of these viruses were not WT Ad5 but Ad5-dlX-like, because the *Xba*I-digested DNAs lacked *Xba*I-D (Fig. 2C). This result was confirmed by an experiment with *Xho*I digests (data not shown), suggesting that the right one-fourth of these viruses is derived only partly from the recombinant plasmid used and partly from the Ad5 DNA-PC fragment (discussed below) (Fig. 1B). All the recombinant viruses isolated, Ad5-dlX, Ad5-X2S, and Ad5-HBL, grew as efficiently as WT Ad5 in HeLa cells without helper virus in terms of titer (about 10^9 PFU/ml), plaque size, time course of cytopathic effect, and yield of viral DNA and were stable for at least four passages throughout our experiments.

Ad5-HBL mRNAs transcribed from adenoviral genes. To examine whether the 2.8-kb HBV insert changed the expression of adenoviral mRNA species, we carried out nuclease S1 protection experiments between the cytoplasmic RNA extracted from infected HeLa cells at various periods and a vast excess of uniformly ^{32}P-labeled whole viral DNAs. Figure 3 shows the effect on the RNA in the early phase (8 h postinfection [p.i.]) tested with an Ad5-dlX probe, and that in the late phase (20 h p.i.) tested with a WT Ad5 probe. No significant change was observed in most of the Ad5 early and late cytoplasmic mRNAs in the cells infected with WT Ad5, Ad5-dlX, and Ad5-HBL. Ad5 major early transcripts were previously identified and mapped on the WT Ad5 genome by Berk et al. (1), and we have confirmed the results by using each *Xho*I fragment as probes (18; Saito, unpublished data). The early transcripts from Ad5-dlX and Ad5-HBL were detected in sizes apparently identical to those of WT Ad5 transcripts except for a novel early transcript of 1.45 kb (Fig. 3A). This transcript may have been derived from the *Xba*I-deleted E3 region of Ad5-dlX and Ad5-HBL, because a transcript of 1,410 nucleotides has been mapped in the *Xba*I-deleted E3 region of Ad5Δ(78.9-84.3), a virus identical to Ad5-dlX (3). The presence of this transcript in the late phase is uncertain because of the presence of a late transcript of similar size. The major E3 mRNA of 3.1 kb (1) was detected only in the cells infected with WT Ad5 (Fig. 3B, lane W), again because of the E3 deletion in Ad5-dlX and Ad5-HBL.

The Ad5 major E4 transcript is 1.9 kb long (1, 18); this was confirmed by using the uniformly labeled *Xho*I-B probe (83 to 100 m.u.) (data not shown). This transcript was detected only poorly in the Ad5-HBL-infected cells in the early phase (Fig. 3A, lane H). The band of the E4 transcript in lane H is at most one-fifth as intense as that in lane X. This difference was also observed in the analyses of RNAs prepared at 12 h and 16 h p.i. (data not shown), although it was not clear in the late phase (20 h p.i., Fig. 3B) owing to the high background of the gel. It should be noted that the 1.9-kb E4 transcript covers m.u. 91 to 97 of the Ad5 genome (1, 11) and consequently does not overlap the Ad2 region of Ad5-HBL (98 to 100 m.u.). Thus, the results suggest that the 2.8-kb HBV DNA insert reduces the amount of the E4 transcript.

Ad5-HBL mRNAs transcribed from the inserted HBV gene. We detected and mapped three major (2.4, 2.0, and 0.7 kb; Fig. 4C) and several minor HBV mRNAs from Ad5-HBL-infected HeLa cells. The major 2.0-kb mRNA was identical in its 5′ end, coding region, and 3′ end to that reported to encode HBsAg (Saito et al., submitted). Here, we report the quantitative aspects of the major HBV mRNAs expressed in this Ad5 vector. To determine the time course of the cytoplasmic HBV mRNAs, we carried out an S1 protection experiment between the HeLa RNAs extracted at various periods after Ad5-HBL infection and used a DNA probe labeled at its 3′ *Bam*HI end in the HBV gene (Fig. 4A). This probe should detect the 3′ sequences common to the three major HBV mRNAs as a doublet of bands, owing to their 3′ heterogeneity (Fig. 4C). The doublet of bands was detected faintly and abundantly in RNAs at 16 and 20 h p.i., respectively (Fig. 4A, lanes 16 and 20), showing that the HBV mRNAs are efficiently accumulated in the cytoplasm only in the late phase, in parallel with adenoviral late mRNAs (data not shown).

To compare the amount of HBV mRNAs with that of Ad5 mRNAs in the late phase of Ad5-HBV infection, we performed another S1 protection experiment between the infected HeLa cell cytoplasmic RNA at 20 h p.i. and uniformly labeled Ad5-HBL whole DNA. However, we found it difficult to detect both kinds of mRNAs simultaneously, because the melting temperatures of Ad5 (58% G+C) and HBV (49% G+C) DNAs are significantly different. The best result was obtained when hybridization was performed at 56°C (Fig. 4B), when the 2.0-kb band, corresponding to the major HBV mRNA, was detected in the Ad5-HBL-infected cells at

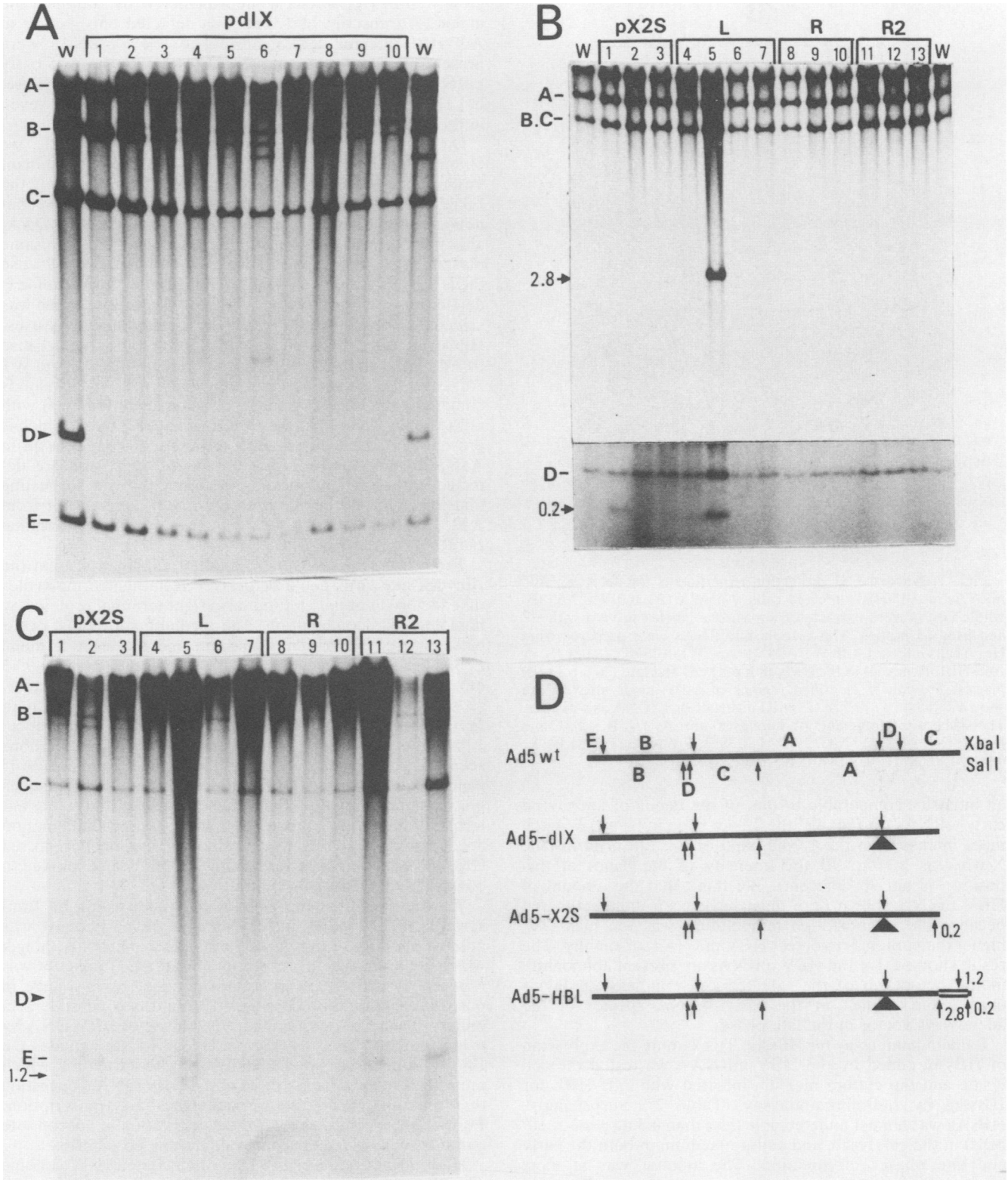

FIG. 2. Restriction enzyme assay of DNAs prepared from the isolated viral clones. The plasmid restored to the Ad5 genome is shown at the top for each panel. (A) pdlX. (B and C) pX2S and its derivatives bearing HBV DNA: L, pX2S-HBL; R, pX2S-HBR; R2, pX2S-HBR2. Viral DNAs were labeled in vivo with $^{32}P_i$, digested with *Xba*I (A and C) or *Sal*I (B), and subjected to electrophoresis on a 1.2% agarose gel (A and C) or a discontinuous gel of 1.2% agarose (upper) and 8% polyacrylamide (lower) (B). Lane W, WT Ad5 DNA; WT Ad5 DNA restriction fragments are indicated at the left. (D) Restriction maps of the constructed Ad5 genomes. Solid line, Adenovirus (Ad5 and Ad2) DNA; open line, HBV DNA; solid triangle, E3 deletion. The *Xba*I and *Sal*I restriction fragments are shown above and below the WT Ad5 genome, respectively. The sizes of extra bands are also shown (in kilobases).

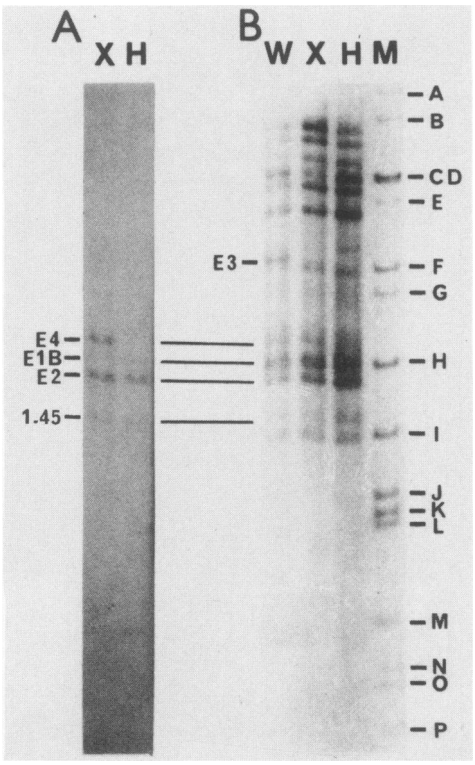

FIG. 3. Nuclease S1 protection experiments for detecting Ad5 mRNAs in Ad5-HBL-infected cells. Ad5-dlX (A) and WT Ad5 (B) whole DNAs were extracted from virions labeled in vivo with ^{32}P and used as probes. The cytoplasmic RNAs were prepared from HeLa cells infected with WT Ad5 wt (lane W), Ad5-dlX (lanes X), or Ad5-HBL (lanes H) in the early (8 h p.i.) (A) and late (20 h p.i.) (B) phases. Previously identified regions of early major mRNAs are shown at the left. Lane M, *Hin*dIII digest of Ad12 DNA as a marker. The sizes of the fragments (in kilobases) are: A, 5.4; B, 4.8; C and D, 3.7; E, 3.34; F, 2.52; G, 2.32; H, 1.79; I, 1.39; J, 1.11; K, 1.04; L, 0.99; M, 0.68; N, 0.53; O, 0.48; and P, 0.35.

an intensity comparable to that of the bands of adenoviral early mRNAs (in Fig. 4B, the 2.0-kb band in lane H is much more intense than the 1.9-kb band of E4 transcript in lane X,whereas in Fig. 3B the intensity of the bands at this position is not so different). We think that the amount of HBV mRNA shown here must have been underestimated because the hybridization temperature was too high (4°C higher than optimal) to detect HBV mRNAs sufficiently. The result showed that the HBV mRNAs are present abundantly in the cytoplasm of the infected cells, at least at levels comparable to that of the early mRNA species of the adenovirus vector in the late phase.

Radioimmunoassay for HBsAg. To examine the expression of HBsAg coded by the HBV mRNAs, we tested the cell lysate and the culture medium infected with Ad5-HBL for HBsAg by radioimmunoassay (Table 2). Surprisingly, HBsAg was almost undetectable (less than 0.3 ng per 3×10^6 cells) in the cell lysate and culture medium in both the early and late phases of infection. The amount was at most one-thousandth of that expressed in a human hepatoma cell line, PLC/PRF/5 (14), suggesting that the HBV mRNAs are barely translated in the late phase of Ad5-HBL infection.

DISCUSSION

We constructed Ad5-HBL, a nondefective Ad5 bearing 2.8 kb of HBV DNA (87% of the HBV genome). Ad5-HBL grew well in HeLa cells without helper virus, although the major E4 transcript of 1.9 kb was detected only poorly in Ad5-HBL-infected HeLa cells. The HBV mRNAs were present in an amount comparable to that of the Ad5 early mRNAs in Ad5-HBL-infected HeLa cells at the late phase, but HBsAg was barely detectable in the infected cells even in the late phase.

During the construction of Ad5-X2S and Ad5-HBL, we obtained an unexpected result: most of the recombinant virus clones were Ad5-dlX-like virus which retained only the E3 deletion derived from pX2S and pX2S-HBV plasmids but not the insertions of the *Sal*I linker or the HBV DNA, although both the deletion and the insertion lay on the same plasmid molecule. One plausible explanation is that, because the HBV DNA insertion near the right end of Ad5 genome is deleterious to virus growth, a virus lacking this insertion was selected during the isolation of these recombinant viruses. However, this explanation does not seem to be true because (i) Ad5-HBL and Ad5-X2S grew as well as Ad5-dlX and WT Ad5, (ii) the plaque-forming efficiency with the plasmids containing the HBV DNA insert was no lower than that with pdlX (Table 1), and (iii) the *Hin*cII site at 98.3 m.u., which is present in Ad2 but not in Ad5 and consequently present in Ad5-X2S and Ad5-HBL but not in Ad5-dlX, was not detected in the virus in question (data not shown), suggesting that the right end of the virus genome is derived not from Ad2 DNA in the plasmids but from the right terminal *Eco*RI-B of Ad5 DNA-PC.

The last reason leads us to another explanation, that the Ad5-dlX-like virus could be derived from ligated molecules that acquired not only left but also right terminal proteins by homologous recombination. The recombination must occur between Ad5 DNA in the plasmid and the right terminal *Eco*RI-B of Ad5 DNA-PC within the region from 84 to 98.3 m.u. (Fig. 1B). This recombination might be intermolecular, as shown in Fig. 1B, or intramolecular after ligation of *Eco*RI-B with the *Eco*RI end of the pBR322 sequence. Although both inter- and intramolecular recombinations seem to be infrequent, it is likely that a small number of Ad5 genomes carrying both terminal proteins could form plaques much more efficiently than those carrying only the left terminal protein. A model of homologous recombination accompanying Ad5 DNA transfection has been reported (26), and intramolecular recombination has been exploited to construct an adenovirus vector system (23, 24).

We observed that the amount of 1.9-kb major E4 transcript in the Ad5-HBL-infected cells in the early phase was at most one-fifth of that in Ad5-dlX-infected cells. Although we do not know whether the amount of the E4 transcript was reduced by a decrease in transcription or by a change in posttranscriptional processing, we found two possible secondary structures around the insertion site of HBV DNA by using computer analysis (Fig. 5). One structure includes the E4 TATA box and is preceded by the other structure, which must be destroyed by insertion of the HBV DNA, suggesting that the upstream structure influences E4 transcription. Further approaches, such as the construction of appropriate mutants, will be required to evaluate this possibility.

HBV mRNAs transcribed from the inserted HBV genome were detected abundantly only in the late phase, that is, after DNA replication of Ad5-HBL. These HBV mRNAs are transcribed from their own promoter sites (Saito et al., submitted). This result suggests that the increase in cytoplasmic mRNA transcribed from the inserted foreign gene depends mainly on the copy number of the virus genome in the infected cells. Interestingly, the time course of HBV mRNA

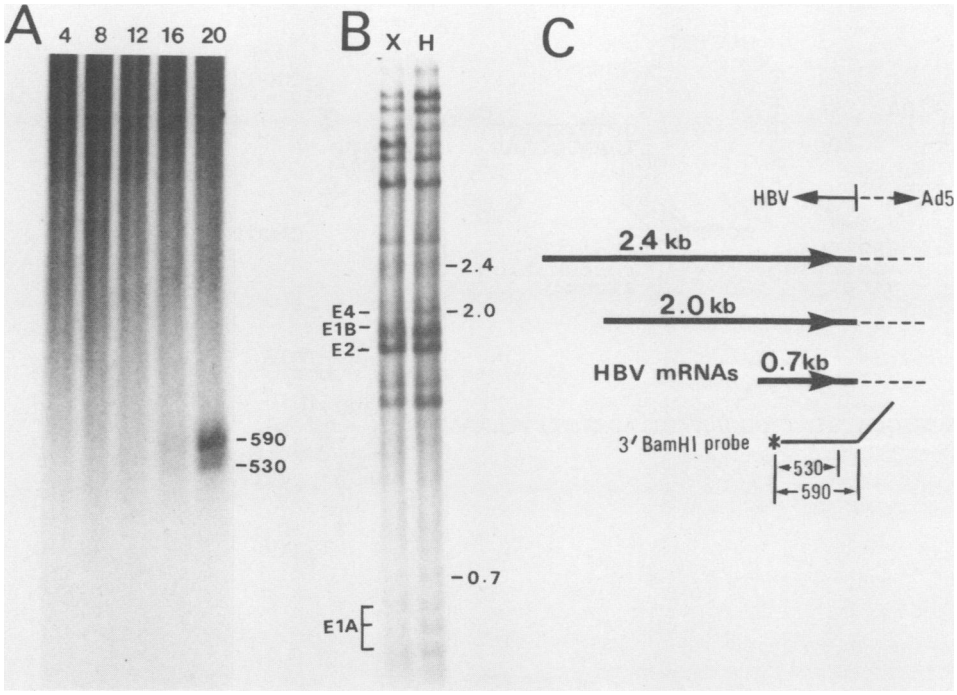

FIG. 4. Nuclease S1 protection experiments for detecting HBV mRNAs in Ad5-HBL-infected cells. (A) Cytoplasmic RNAs were extracted from HeLa cells infected with Ad5-HBL at various times (indicated above the lanes in hours p.i.). The probe was the 3'-end-labeled *Bam*HI fragment of pX2S-HBR which is colinear up to the end of the HBV insert. (B) Cytoplasmic RNAs were extracted from HeLa cells infected with Ad5-dlX (lane X) or Ad5-HBL (lane H) at 20 h p.i. The probe was Ad5-HBL whole DNA labeled uniformly in vivo. (C) The major HBV mRNAs transcribed in Ad5-HBL-infected cells detected with the probe used in (A). The length of the mRNA is shown above the arrow. The ^{32}P-labeled end is indicated by an asterisk. About two-thirds of the mRNAs were not terminated within the HBV DNA but traversed the Ad5-HBV junction.

appearance was essentially identical to that of Ad5 late mRNAs, suggesting that no Ad5-specific transcriptional regulation is necessary for an adenoviral late gene to be transcribed and transported efficiently during the late phase when it is located on the Ad5 genome.

Although the HBV mRNAs were present abundantly in the late phase, HBsAg was barely detectable in the infected cell lysate or in the culture medium. It should be noted that the 2.0-kb HBV mRNA, a major HBV species expressed in this vector system, was identical in its 5' end, coding region, and 3' end to that reported to encode HBsAg and that this mRNA did not have any base change detectable in S1 protection experiments with our original HBV DNA clone, the integrity of which was proved by nucleotide sequencing (5). Moreover, our HBV DNA clone did express HBsAg when it was introduced into hamster cells with the pSV2gpt vector (Masuda et al., unpublished data). These results suggest that proteins are barely translated from the non-Ad5 mRNAs owing to so-called translational suppression of "cellular" mRNAs, even though the HBV mRNAs are transcribed from the genes located on the Ad5 genome. The result seems to be consistent with the classic report that Ad2 does not impede transcription of coinfecting vaccinia virus but suppresses translation of vaccinia virus mRNA (8) and the recent report that the tripartite leader sequence of Ad5 late mRNAs is required for efficient translation of an inserted simian virus 40 T antigen gene in the late phase of Ad5 infection (24).

TABLE 2. Detection of HBsAg in Ad5-HBL-infected HeLa cells[a]

Infecting virus	Material tested	HBsAg detected (P/N)[b] at time p.i.:							
		4 h	8 h	12 h	16 h	20 h	24 h	28 h	32 h
Ad5-HBL	Cells	ND[c]	ND	ND	ND	2.0	2.4	1.9	2.5
	Medium	1.5	1.4	1.4	1.4	1.3	ND	ND	1.3
WT Ad5	Cells	ND	ND	ND	ND	1.8	1.7	1.5	1.6
	Medium	1.3	ND	1.4	ND	1.3	ND	ND	1.2

[a] 1×10^6 to 3×10^6 HeLa cells were infected with Ad5-HBL or WT Ad5, and the infected cells and the culture medium were examined by radioimmunoassay.

[b] P/N value, counts per minute in material divided by counts per minute in the negative control. If the value was less than 2.1, we judged the material negative (14). The negative control value of the kit was 1. With medium from uninfected cells, the value obtained was 1.4. The HBsAg controls gave P/N values of 7.7, 4.5, and 3.0 at HBsAg concentrations of 1.5, 0.75, and 0.375 ng/ml, respectively.

[c] ND, Not determined.

We adopted a position near the right end of the Ad5 genome as the site where foreign DNA can be inserted in our helper-free Ad5 vector system, whereas the E3 and E1 regions were adopted in earlier experiments (2, 9). However, we have observed that only small amounts of mRNAs are transcribed from the Ad12 E1A gene inserted in the E3 region of Ad5 *dl*312 (18), and the Ad5 vector with the E1 deletion can grow in cell line 293 but not in HeLa cells. Therefore we expected that our vector system could have the potential to produce a large amount of mRNA and protein from the inserted gene in a suspension culture of HeLa cells (see below). Foreign DNA longer than 4 kb may be acceptable in our system because of the 2.1-kb E3 deletion and the flexibility of adenovirus packaging. Our system should be useful for studying transcription in vivo

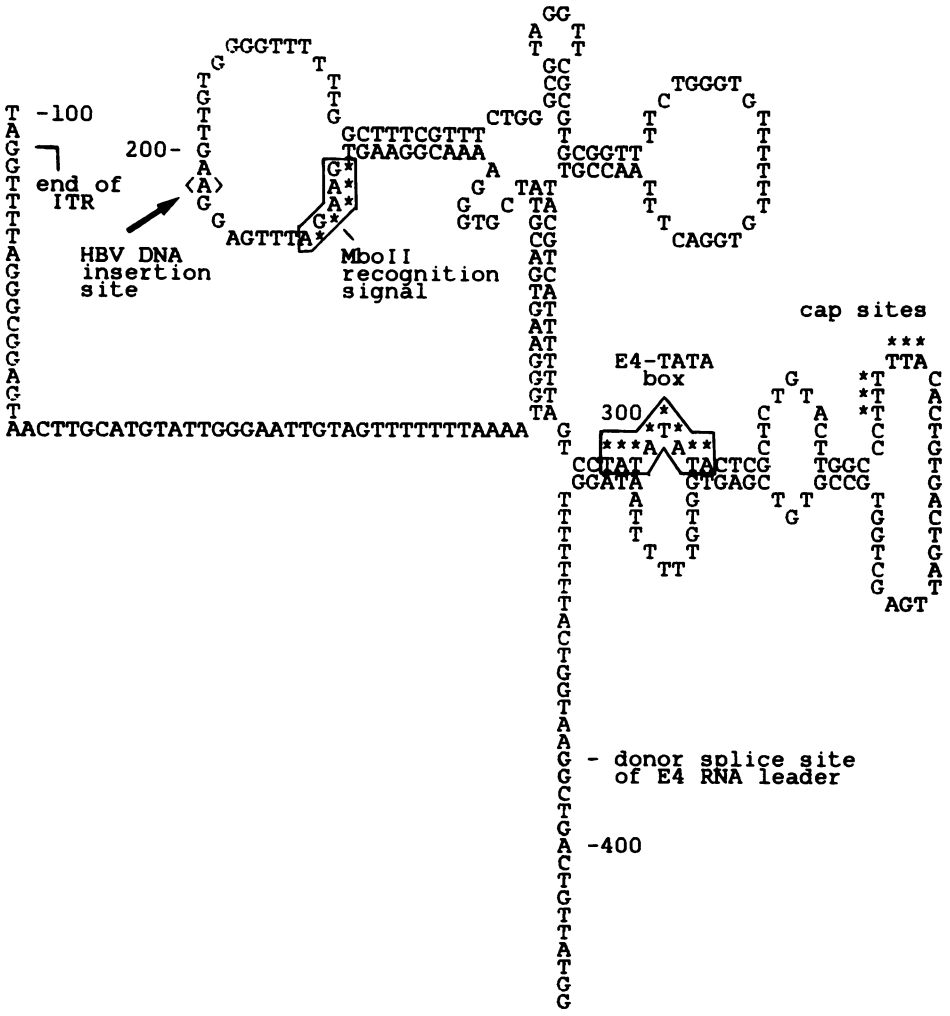

FIG. 5. Possible secondary structure of Ad2 DNA l-strand near the right end of the genome. An arrow indicates the *Mbo*II cleavage site where the HBV DNA was inserted. A nucleotide (<A>) at the arrow must have been removed by the exonuclease activity of the Klenow fragment before insertion of the *Sal*I linker and HBV DNA. The nucleotide sequence and the E4 transcriptional signals are from reference 11. Nucleotide numbering starts from the right end of the genome. ITR, Inverted terminal repeat.

and identifying mRNAs transcribed from foreign genes, because the inserted DNA is apparently isolated from any Ad5 transcription unit. It should be more useful if the target gene is of human or human virus origin, because this vector propagates in human cells. Accordingly, we have used this vector to identify and map HBV mRNAs. We have also tried to establish a rat cell line transformed with Ad5-HBL that expresses HBsAg persistently. Ad5-HBL transformed a rat cell line (3Y1) as efficiently as WT Ad5, but HBsAg was not expressed. Further investigation is necessary on this system.

At present, our vector system has two problems, (i) low frequency of the desired recombinant virus due to generation of Ad5-dlX-like virus and (ii) no protein synthesis from the inserted gene. We are now constructing Ad5-2R, an Ad5 bearing an additional *Eco*RI site at the 99.3 m.u. position described above. The first problem should be overcome by using Ad5-2R DNA-PC instead of WT Ad5 DNA-PC if our explanation for the low frequency is true. We are also constructing an Ad5-HBV recombinant virus in which the HBV promoter and 5′ flanking sequence are replaced by the Ad5 major late promoter and leader sequences in order to express HBV proteins efficiently.

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