 Preferential expression of the large hepatitis B virus surface antigen gene by an adenovirus–hepatitis B virus recombinant

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Using an adenovirus–hepatitis B virus (HBV) recombinant, expression of the HBV surface antigen (HBsAg) genes was examined in various cell lines using S1 nuclease mapping and radioimmunoassay. The steady-state level of the 2.4 kb RNA encoding the large HBsAg was much greater than, or the same as, that of the 2.0 kb RNA, encoding the middle and major HBsAgs, in primate cells, but was negligible in non-primate cells, as is the case in most expression systems. According to the amount of 2.4 kb RNA expressed, cells were classified into three groups: those in which (1) the amount of 2.4 kb RNA was much greater than that of 2.0 kb RNA (HepG2 and JHH-4), (2) the amount of 2.4 kb RNA was the same as that of 2.0 kb (Hul-1, HeLa and other non-hepatic primate cells), and (3) the amount of 2.4 kb RNA was less than one-tenth of that of 2.0 kb RNA (rodent cells). Radioimmunoassay revealed that most HBsAg is located intracellularly in primate cells, but is secreted into the culture medium of rodent cells. The expression of 2.4 kb RNA was unaffected by an inhibitor of DNA synthesis in HepG2 cells, which are of human liver origin, whereas it was strongly inhibited in human non-hepatic HeLa cells.

Introduction

The hepatitis B virus (HBV) particle consists of a nucleocapsid core, containing partially single-stranded, circular DNA, coated with an envelope made up of three surface antigens (HBsAg), designated large (p39/gp42), middle (gp33/gp36) and major (p24/gp27) (Heerman et al., 1984). Studies on the transcription of HBV DNA during the complete replication cycle have demonstrated that three species of cytoplasmic HBV RNA are present in HBV-infected chimpanzee liver tissue and hepatoma cell lines (Cattaneo et al., 1983, 1984; Will et al., 1987). One species, the 3-3 kb RNA [length without the poly(A) tail], is known to encode the HBV core antigen and to serve simultaneously as pregenomic RNA. The other two species of cytoplasmic HBV RNA, which are 2-4 and 2-0 kb in length without the poly(A) tail, are known to encode HBsAgs; the 2-4 kb RNA encodes the large HBsAg, which is translated from the pre-S1 initiation codon, and the 2-0 kb RNA encodes the middle HBsAg, which is translated from the pre-S2 initiation codon, and the major HBsAg, which is translated from the S region initiation codon (Tiollais et al., 1985). Although HBV has a strict host range, replicating only in human and chimpanzee liver cells, HBsAg can be expressed by transfecting cloned HBV DNA into cultured rodent cell lines (Dubois et al., 1980; Christman et al., 1982; Asselsberg et al., 1986; Yoneyama et al., 1988), in which the 2-0 kb RNA is expressed abundantly (Zelent et al., 1987). In the past few years several systems for propagating HBV in vitro in human cells have been reported (Sureau et al., 1986; Chang et al., 1987; Tsurimoto et al., 1987; Yaginuma et al., 1987; Sells et al., 1988; Ochiya et al., 1989); in these systems pre-S2 and S RNAs (2-0 kb) are expressed abundantly, and pre-S1 (2-4 kb) RNA is expressed poorly. Suzuki et al. (1989) reported abundant pre-S2 and S RNA (48-8% of the total HBV RNA), and low pre-S1 RNA (7-1% of the total HBV RNA) expression in human hepatoma cells (HepG2) transiently transfected with cloned HBV DNA.

In this report, in contrast to previous reports, we have shown abundant expression of the 2-4 kb large HBsAg RNA using an adenovirus type 5 (Ad5)–HBV recombinant (Ad5–HBL) bearing 87% of the HBV genome (Saito et al., 1985), not only in HepG2 cells, but also in all primate cells tested. In rodent cells, however, expression of the 2-4 kb large HBsAg RNA was poor, as reported previously. Since Ad5–HBL can efficiently infect a broad range of cells, we were able to compare the expression of HBV RNAs and proteins in various cell lines.

Previous reports using plasmid vectors have shown that the secretion of HBsAgs is strongly inhibited when
major, middle and large HBsAgs are synthesized together. Using transgenic mice, Chisari et al. (1986) reported that the concentration of HBsAgs in serum decreases as the level of HBsAg expressed in the liver increases; Persing et al. (1986) also reported this using COS7 cells. A subsequent report showed that the pre-S1 region of HBsAg facilitates the retention of HBsAg in the cytoplasm and inhibits the secretion of HBsAg (Jing-Hsiung & Rutter, 1987). Our results show that the HBsAgs are secreted poorly by primate cells in which expression of the 2.4 kb large HBsAg RNA is abundant, but efficiently by rodent cells in which expression of the 2.4 kb large HBsAg RNA is poor, in agreement with the data obtained using plasmid vectors.

Methods

Cells and viruses. HeLa (human cervical carcinoma), HuL-1 [human foetal liver (Katsuta et al., 1980)], JHH-4 [HBV genome-negative, human hepatocellular carcinoma (Sujino et al., 1986)], HepG2 (HBV genome-negative, human hepatocellular carcinoma), KB (human pharyngeal carcinoma), CV-I (monkey kidney), Vero (monkey kidney), NIH 3T3 (3T3; mouse embryo), L (mouse connective tissue), C 127 (mouse mammary tumour), 3Y-I (rat embryo) and RLC-10 (rat liver) cell lines were used. Cellsm were grown as monolayers in Eagle's MEM or Dulbecco's modification of Eagle's medium supplemented with 10% foetal calf serum. The structure of the genome of recombinant Ad5-HBL is shown in Fig. 1; it contains the large BglII fragment [2.8 kb, nucleotides (nts) 2425 to 1986 of HBV (subtype adr) DNA at a site 0.2 kb downstream from the right terminus of the Ad5 genome (Saito et al., 1986). Ad5-dIX virus, which does not contain HBV DNA, was used as a control (Saito et al., 1985). Virus stocks were prepared from the infected KB cell monolayers. Plaque assays were performed on A549 cells (human lung carcinoma).

Reagents. All restriction enzymes were products of Takara Shuzo; S1 nuclease was purchased from Boehringer Mannheim; cytosine β-D-arabinofuranoside (ara-C) was purchased from Sigma.

Analysis of RNA and DNA. Unless otherwise stated, monolayers were infected with Ad5-HBL at a multiplicity of 20 p.f.u./cell and incubated for 2 h at 37 °C in culture medium containing ara-C (75 μg/ml). Cytoplasmic RNA was extracted from infected cells by the procedure of Berk et al. (1979) and between 50 and 100 μg was hybridized at 51 °C with 25 to 40 ng of specific DNA fragments (Fig. 1a). After S1 nuclease treatment (500 units/ml), DNA–RNA hybrids were fractionated on a gel (Saito et al., 1986) and the number of copies of Ad5-HBL DNA per infected cell was determined by dot blot hybridization (Gross-Bellard et al., 1973) using nick-translated HBV DNA as a probe.

Densitometric analysis of RNA. The quantity of RNA was determined by densitometry using the film scanner of a spectrophotometer (Chromoscan; Joyce Loebel). The linear range of the film density, which is dependent on the concentration of 32P-labelled DNA, was determined using a preflashed film and varying the autoradiographic exposure time.

Radioimmunoassay (RIA) for HBsAg. To detect intracellular HBsAg, cells were collected by centrifugation, suspended in fresh culture medium, and processed as described above.
medium, and lysed by three cycles of freezing and thawing and sonication. HBsAg in the cell lysate (intracellular HBsAg) and in the culture medium (extracellular HBsAg) was determined using an AUSRIA II RIA diagnostic kit (Abbott Laboratories). Absolute amounts of HBsAg were determined from the standard curve obtained using known amounts of HBsAg purified from human HBV carrier sera.

*Immunoprecipitation of HBsAg.* Monolayers were infected with Ad5-HBL or Ad5-dlX at a multiplicity of 5 p.f.u./cell. Cell cultures were maintained for 40 min in cysteine-depleted medium and then labelled with [35S]cysteine (ICN, > 500 Ci/mmol) between 48 and 72 h post-infection (p.i.). Cell lysates were prepared by the procedure of Goh *et al.* (1985) and incubated with normal serum and Protein A (*Staphylococcus aureus* Cowan I) for 30 min at 0 °C. After centrifugation, the supernatants were incubated with anti-HBsAg serum for 1 h at 0 °C and Protein A-bound Igs were eluted in 2.3 M SDS, 5% 2-mercaptoethanol. The samples were analysed by 12.5% SDS-PAGE (Heerman *et al.*, 1984). For immunoprecipitation, either an anti-major HBsAg guinea-pig serum or a mouse monoclonal antibody produced against a synthetic peptide consisting of 13 amino acids from positions 95 to 107 in the pre-S1 region was used (Okamoto *et al.*, 1985).

**Results**

*Detection of HBsAg RNAs in a human hepatoma cell line infected with Ad5–HBL.*

HepG2 cells, differentiated human hepatoma cells, are known to support replication of and virion formation by HBV (Sells *et al.*, 1988). To examine the expression of HBsAg RNAs in HepG2 cells infected with Ad5–HBL, cytoplasmic RNA was hybridized with the 5' end-labelled SalI–XbaI (nts 2426 to 252) probe (Fig. 1a) and hybrids protected against S1 nuclease digestion were analysed on a urea–polyacrylamide gel (Fig. 2). We detected abundant 2-4 kb HBV RNA and less 2-0 kb HBV RNA at both 20 h and 48 h p.i.; the 2-4 kb RNA was detected as a single 660 nt band, whereas the 2-0 kb RNA was separated into three discrete bands of 280, 250 and 245 nts (Fig. 2) because of the heterogeneity of its 5' end, as described previously (Saito *et al.*, 1986; Suzuki *et al.*, 1989) and shown in Fig. 1 (b). Densitometric analysis showed that the steady-state level of the 2-4 kb RNA was almost 12-fold and eightfold greater than that of the 2-0 kb RNA at 20 h and 48 h p.i., respectively.

Relative abundance of HBsAg RNA

To determine whether the abundance of the 2-4 kb RNA relative to the 2-0 kb RNA was dependent on the origin of the cells (tissue or species), cytoplasmic RNA was prepared 48 h p.i. from various cell lines infected with Ad5–HBL. This RNA was hybridized with an unlabelled 2-8 kb HBV DNA fragment containing the entire HBV DNA insert in Ad5–HBL. After digestion with S1 nuclease, the DNA–RNA hybrids were electrophoresed on a neutral agarose gel, blotted onto a nitrocellulose filter and hybridized with the 32P-labelled S gene probe. Nuclear DNAs were prepared from the same cells for the dot blot test (c). Each column contains 10-fold serial dilutions of 200 ng of DNA (columns 1 to 12) or 20 ng of DNA (column R). The blot was probed with 32P-labelled HBV DNA. Cell lines used were: columns 1, HeLa; 2, HuI-1; 3, JHH-4; 4, KB; 5, WI-38; 6, CV-1; 7, HeLa; 8, 3T3; 9, L; 10, C127; 11, 3Y-1; 12, RLC-10; R, reference HBV DNA.

Fig. 3. S1 nuclease analysis of HBsAg RNA and number of copies of Ad5–HBL. Cytoplasmic RNA was extracted from Ad5–HBL-infected primate cells (a) or rodent cells (b) 48 h p.i. The RNA was hybridized with an unlabelled 2-8 kb HBV DNA containing the entire HBV DNA insert in Ad5–HBL. After treatment with S1 nuclease, the DNA–RNA hybrids were electrophoresed on a neutral agarose gel, blotted onto a nitrocellulose filter and hybridized with the 32P-labelled S gene probe. Nuclear DNAs were prepared from the same cells for the dot blot test (c). Each column contains 10-fold serial dilutions of 200 ng of DNA (columns 1 to 12) or 20 ng of DNA (column R). The blot was probed with 32P-labelled HBV DNA. Cell lines used were: columns 1, HeLa; 2, HuI-1; 3, JHH-4; 4, KB; 5, WI-38; 6, CV-1; 7, HeLa; 8, 3T3; 9, L; 10, C127; 11, 3Y-1; 12, RLC-10; R, reference HBV DNA.

![Fig. 2: S1 nuclease analysis of the HBsAg RNA from Ad5 HBL-infected HepG2 cells. Cytoplasmic RNA was extracted from cells 20 h (lane 1) or 48 h p.i. (lane 2). The probe used was the 5' end-labelled SalI–XbaI fragment. The S1 nuclease-protected hybrids were electrophoresed on an 8 M-urea-polyacrylamide gel. Lane M, 1 kb marker ladder (Bethesda Research Laboratories).](image-url)

![Fig. 3: S1 nuclease analysis of HBsAg RNA and number of copies of Ad5–HBL. Cytoplasmic RNA was extracted from Ad5–HBL-infected primate cells (a) or rodent cells (b) 48 h p.i. The RNA was hybridized with an unlabelled 2-8 kb HBV DNA containing the entire HBV DNA insert in Ad5–HBL. After treatment with S1 nuclease, the DNA–RNA hybrids were electrophoresed on a neutral agarose gel, blotted onto a nitrocellulose filter and hybridized with the 32P-labelled S gene probe. Nuclear DNAs were prepared from the same cells for the dot blot test (c). Each column contains 10-fold serial dilutions of 200 ng of DNA (columns 1 to 12) or 20 ng of DNA (column R). The blot was probed with 32P-labelled HBV DNA. Cell lines used were: columns 1, HeLa; 2, HuI-1; 3, JHH-4; 4, KB; 5, WI-38; 6, CV-1; 7, HeLa; 8, 3T3; 9, L; 10, C127; 11, 3Y-1; 12, RLC-10; R, reference HBV DNA.](image-url)
expressed a strikingly large amount of the 2.4 kb RNA (Fig. 3a, lane 3), whereas another liver cell line, Hul-1 (from human foetal liver), produced a little less 2.4 kb RNA than 2.0 kb RNA (Fig. 3a, lane 2), similarly to other non-hepatic human cells. In contrast to the different levels of the 2.4 kb RNA in the primate and rodent cells, the steady-state level of the 2.0 kb RNA in the three rodent cell lines, 3T3, L and 3Y1, was comparable with that in human cells [except C127 (lane 10) and RCL-10 (lane 12) in Fig. 3b, where very low-level expression was observed]. The five human cell lines (permissive for Ad5-HBL) and the monkey CV-1 cell line (semi-permissive) contained about 10^4 DNA copies/cell (Fig. 3c, lanes 1 to 6), whereas 3T3, L and 3Y1 rodent cells contained 10^3, 10^2 and 10^1 copies of Ad5-HBL DNA/cell, respectively (Fig. 3c, lanes 8, 9 and 11). The number of copies of Ad5-HBL DNA was the same in 3T3 mouse cells and CV-1 primate cells, and yet the level of expression of the 2.4 kb RNA differed, so the poor expression of 2.4 kb RNA in rodent cells was probably not due to poor replication of the Ad5-HBL vector, but was species-specific.

To compare the ratio of 2.4 kb RNA to 2.0 kb RNA more quantitatively, another S1 nuclease protection experiment was carried out. Cytoplasmic RNA was hybridized with the 5′ end-labelled XbaI probe (Fig. 1a) and S1-protected hybrids were analysed on a urea-polyacrylamide gel. The 2.4 kb RNA was detected weakly but clearly in rodent cells as a band of 660 nt (Fig. 4, lanes 5 to 7). Densitometric analysis showed that the steady-state level of 2.4 kb RNA was 50 to 150% of that of 2.0 kb RNA in all primate cells except JHH-4, in which the level of 2.4 kb RNA was fivefold greater than that of 2.0 kb RNA (Fig. 4, lane 3). In contrast, in rodent cells the steady-state level of 2.4 kb RNA was only 6% (3T3) or 12% (L) of that of the 2.0 kb RNA (Fig. 4, lanes 5 to 7). Mouse C127 cells expressed detectable amounts...
Preferential expression of large HBsAg gene

Table 1. Secretion of HBsAg from Ad5–HBL-infected cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Species†</th>
<th>Tissue</th>
<th>HBsAg (ng/10^7 cells)*</th>
<th>Intra-cellular</th>
<th>Extracellular</th>
<th>Amount secreted (%)‡</th>
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<td>70</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>KB</td>
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<td>Pharyngeal carcinoma</td>
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<td>15</td>
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<tr>
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<td>4</td>
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<td>Kidney</td>
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<td>303</td>
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<tr>
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<td>M</td>
<td>Connective tissue</td>
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<td>203</td>
<td>65</td>
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<td>79</td>
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<tr>
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<td>Embryo</td>
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<td>89</td>
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<td>R</td>
<td>Liver</td>
<td>0.5</td>
<td>2.3</td>
<td>-</td>
<td></td>
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</table>

* Cells were infected with Ad5–HBL at a multiplicity of 20 p.f.u./cell. At 72 h p.i., extracellular HBsAg in the culture medium and intracellular HBsAg were titrated separately using an RIA diagnostic kit.
† H, Human; S, simian; M, mouse; R, rat.
‡ Calculated using the formula [(extracellular HBsAg)/(intracellular HBsAg + extracellular HBsAg)].

of 2·0 kb RNA in this sensitive assay, but 2·4 kb RNA was still undetectable (data not shown). These results showed that in rodent cells the level of 2·4 kb RNA is as low as one-tenth of that of 2·0 kb RNA.

Expression of HBsAg

To determine whether the observed species-specific difference in RNA expression is reflected in its translation into HBsAgs, the time course of HBsAg production and secretion was studied. Eight cell lines were infected with Ad5–HBL and the amount of intracellular HBsAg and extracellular HBsAg was measured at intervals of 24 h (Fig. 5); the amounts of HBsAg detected at 72 h p.i. are shown in Table 1. In all primate cell lines, HBsAg was mainly intracellular (60 to 85%), whereas in rodent cell lines the antigen was found mainly in the culture medium (65 to 79%). The total amount of HBsAg detected differed among the cell lines, being relatively high in the human hepatoma JHH-4 cells, and the mouse 3T3 and L cells (about 500 ng/10^7 cells at 72 h p.i.). Some rodent cells, RLC-10 (rat liver cell) for example, produced very little HBsAg (Table 1), probably due to the low number of copies of Ad5–HBL DNA/cell. All human cell lines except JHH-4 produced only a small amount of HBsAg, although 10^4 copies of Ad5–HBL DNA/cell were present 48 h p.i.

The fact that the distribution of HBsAg was different in primate and rodent cell lines suggested that the HBsAgs expressed in these cells were qualitatively different. To test this possibility, lysates of JHH-4 and 3T3 cells infected with Ad5–HBL were immunoprecipitated with two different anti-HBsAg antibodies (Fig. 6). We detected six polypeptides of 24K, 27K, 33K, 36K, 39K and 42K in JHH-4 cells using anti-major HBsAg guinea-pig serum (a and c) or anti-pre-S1 mouse monoclonal antibody (b), and electrophoresed on a polyacrylamide gel. Control preparations infected with Ad5–dlX (lanes 1); preparations infected with Ad5–HBL (lanes 2); the positions of 14C-labelled methylated protein markers (Amersham) are shown at the left.
HBV RNA expression with limited Ad5–HBL DNA replication

The abundant expression of the 2.4 kb RNA in primate cells was shown to be regulated in a species-specific manner. To study the effect of replication of the Ad vector on HBsAg RNA expression in primate cells, expression of the 2.4 kb RNA was examined in the presence or absence of a DNA synthesis inhibitor. HepG2 or HeLa cells were infected with Ad5–HBL in the absence or presence of ara-C (75 µg/ml), which blocks Ad DNA replication, late gene expression and the virus-induced shut-off of cellular gene expression (Feldman & Rapp, 1966).

Cytoplasmic RNA and nuclear DNA were prepared from cells at 20 h p.i. Ara-C treatment reduced the number of copies of Ad5–HBL by 100-fold in both HepG2 and HeLa cells (Fig. 7b, lanes 1 and 2; Fig. 7d, lanes 1 to 4). In HepG2 cells, the ratio of 2.4 kb RNA to 2.0 kb RNA, which was 13 in untreated cells, decreased to 7 in ara-C-treated cells (Fig. 7a, lanes 2 and 1), whereas in HeLa cells the ratio (about 3 in untreated cells) decreased to as low as 0.6 in ara-C-treated cells (Fig. 7c, lanes 1 to 4), i.e. the expression of 2.4 kb RNA was strongly inhibited by the DNA inhibitor in HeLa cells whereas the effect was limited in HepG2 cells. Thus, the 2.4 kb RNA was efficiently expressed in differentiated human hepatoma HepG2 cells without extensive virus replication, whereas in non-hepatic HeLa cells the efficient expression of the 2.4 kb RNA required extensive Ad DNA replication.

Discussion

We have compared the amounts of HBsAg RNA expressed by an Ad–HBV recombinant in human, monkey and rodent cells. In rodent cells, the major transcripts are the 2.0 kb species, the promoter of which is located within the pre-S gene, in agreement with previous reports (Dubois et al., 1980; Christman et al., 1982; Asselsberg et al., 1986). In primate cells, in addition to the 2.0 kb RNA, there is abundant expression of the 2.4 kb RNA, the promoter of which precedes the pre-S region. According to the amount of 2.4 kb RNA expressed, cells have been classified into three groups: (1) HepG2 and JHH-4 cells, which express the 2.4 kb RNA more efficiently than the 2.0 kb RNA, (2) Hul-1 and non-hepatic primate cells, which express the same amount of 2.4 kb RNA relative to 2.0 kb RNA and (3) rodent cells, which express the 2.4 kb RNA poorly. A similar description of cell specificity was made by Raney et al. (1990) using a chloramphenicol acetyltransferase assay, but the amount of 2.4 kb RNA relative to 2.0 kb RNA appeared to be higher in our system. The cells of groups (1) and (2) secrete the expressed HBsAg poorly, whereas those of group (3) secrete it efficiently (Table 1). This is consistent with the results of RNA expression experiments, because the large HBsAg, encoded by the 2.4 kb RNA, is known to inhibit the secretion of HBsAg.
RNA was abundant in groups (1) and (2).

Chang et al. (1989) have reported that the promoter for the large hepatitis B Ag is preferentially active in differentiated hepatoma cell lines which express hepatocyte nuclear factor 1 (HNF-1), which regulates the preferential use of the pre-S1 promoter. Our results showing that the HepG2 and JHH-4 cells express abundant 2-4 kb RNA are consistent with the idea that differentiated HepG2 and JHH-4 cells must express HNF-1 abundantly. Hul-1 cells, of human foetal liver origin, may well be less differentiated, thus expressing less HNF-1 and consequently less 2-4 kb RNA.

The experiments with ara-C show that the expression of the 2-4 kb RNA is hardly affected by the DNA inhibitor in differentiated human hepatoma HepG2 cells but is significantly reduced by ara-C in non-hepatic HeLa cells; high level expression of the 2-4 kb RNA in HeLa cells requires Ad DNA replication. This result is consistent with our observation that the expression of HBsAg RNA in HepG2 cells is abundant at an early stage of Ad5-HBL infection, whereas in HeLa cells it is abundant at a later stage (data not shown). It is possible that in HeLa cells expression of the 2-4 kb RNA is regulated in the same way as that of Ad late genes, which begin to be transcribed efficiently after viral DNA replication. The HBsAg RNAs are not transcribed from the Ad late promoter in Ad5-HBL, because the direction of HBV transcription in Ad5-HBL is opposite to that of the Ad late promoter (Saito et al., 1986). Ad early proteins, such as E1A, trans-activate cellular genes in the late stage of infection (Hearing & Shenk, 1983); perhaps a factor similar in function to HNF-1 is trans-activated by an Ad protein and regulates the use of the pre-S promoter late in infection. This observation will be useful in the analysis of factors which enhance the expression of the 2-4 kb RNA.

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