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Plasmid-Directed Synthesis of Hepatitis B Surface Antigen in Monkey Cells

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We introduced the gene encoding the hepatitis B virus surface antigen (HBsAg) into simian virus 40 (SV40)-based plasmids capable of autonomously replicating in both *Escherichia coli* and permissive monkey cells. After introduction into monkey cells by transfection, these plasmids directed the synthesis of high levels of HBsAg, as determined by immunofluorescence, radioimmunoassays, and identification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the polypeptides comprising the antigen. Expression was dependent upon the presence of an SV40 promoter, with both the early and late promoters able to effectively initiate transcription. Using expression of HBsAg to assay promoter function, we demonstrated that an intact copy of the SV40 72-base pair repeat, which constitutes an essential element of the SV40 early promoter during the lytic SV40 cycle and which can enhance the transcriptional activity of heterologous promoters, was not required for HBsAg expression, suggesting that the hepatitis genome contains an enhancer element capable of complementing that provided by the 72-base pair repeat element of SV40. The antigen appears to be glycosylated after synthesis in transfected cells and is apparently secreted, as evidenced by the localization of [^{35}S]cysteine-labeled antigen to the medium of transfected cultures. Using constructions in which the first ATG sequence appearing in HBsAg mRNA was that corresponding to the gene encoding the mature form of the antigen, we demonstrated that these post-translational events could occur without the involvement of a putative precursor peptide suggested by the DNA sequence of the viral genome. In view of the inability of hepatitis B virus to propagate in vitro, this strategy offers a convenient approach for further characterizing the biosynthesis of this antigen and may provide a means to identify additional polypeptides encoded by this virus.

Compared with the life cycles and genetics of other human pathogenic viruses, those of hepatitis B virus (HBV) are poorly understood. This ignorance is largely a consequence of the inability of HBV to propagate in any tissue culture system or to infect convenient experimental animals (for reviews, see references 47 and 55). Recently, the 3,200-base pair (bp) viral genome has been molecularly cloned from infectious virus isolated from serum (8, 50), and the determination of the complete nucleotide sequence of the cloned DNA has provided definitive evidence that at least two viral antigens are encoded by the genome of the virus (7, 15, 42, 57). These include the core antigen, a basic polypeptide of 19,000 daltons which represents the major viral nucleocapsid protein, and the surface antigen (HBsAg). HBsAg normally circulates as particles of different sizes and forms in the sera of infected individuals. The predominant spherical and filamentous particles (mean diameter, 22 nm) represent free envelopes of the virus. The less common 42-nm Dane particles constitute the infectious virion. HBsAg represents both the major envelope protein and the neutralizing antigen of the infectious virion and as such has proven effective as a vaccine against the disease, which is estimated to chronically afflict 200 million persons worldwide, or 5% of the world population (53). Owing to the limited host range of the virus, however, the source of this antigen has been confined to the sera of infected individuals (53).

Recently, several groups have reported the introduction and expression in mammalian cells of the gene encoding HBsAg. These efforts have utilized either dominant selectable markers (9, 12) or lytic vectors based on simian virus 40 (SV40) (28, 35). One advantage of virus-based vectors such as SV40 is that, by exploiting the viral replication machinery, a high vector copy number is assured and a high level of heterologous gene expression can be observed (16, 23, 28, 35, 36). The general strategy involves the

replacement of an essential viral gene (in either the early or the late region) with the foreign gene and propagation of the resulting replication-defective virus by complementation with helper virus. There are several drawbacks to this approach, however. First, because SV40 has strict packaging constraints, inserts of foreign DNA are confined to sizes not significantly larger than the sequences removed, precluding the use of this approach for the expression of many genes. Second, because the process involves a fully lytic infection, strict limits on the duration of the experiment are imposed. Third, the lytic nature of the infection seriously hampers the interpretation of many molecular events (see below). Recently, nonlytic vectors capable of replicating in both bacteria and mammalian cells have been described (29, 32, 38). These vectors are comprised of sequences from the bacterial plasmid pBR322 and SV40 sequences spanning the viral origin of DNA replication. Such vectors are capable of replicating in monkey cells, provided large T antigen is present to initiate successive rounds of DNA synthesis. Expression of heterologous genes has been observed in such vectors (21, 32, 34), an expression which can be augmented when sequences encompassing the 72-bp repeated elements of SV40 are present to activate the promoter of the foreign gene (4, 34). Such sequences, known as enhancers because of their ability to activate the promoter of a remote gene (4), constitute an essential element of the SV40 early promoter, without which SV40 is non-conditionally defective (5, 22). We have positioned SV40 restriction fragments which encompass the region comprising the origin of DNA replication, the enhancer sequences, and the promoter for either the early or late region proximal to the gene encoding HBsAg. After transfection of permissive monkey cells, such plasmids replicate to high copy number while concomitantly directing the synthesis of HBsAg under SV40 promoter control. This strategy affords several advantages over previous approaches. Since expression does not involve virus propagation, synthesis of HBsAg is observed within 24 h after transfection in a high percentage of cells, in contrast to the significant lag time characteristic of lytic vectors upon primary transfection (28, 33, 35, 36). Expression continues for several weeks and reaches cumulative levels 10 to 100 times those observed in lytic systems (28, 35).

Although the amino terminus of mature HBsAg has been determined (44), an open reading frame sufficient to encode an additional 163 amino acids precedes the structural gene (15, 57, 58). It has been postulated that these sequences may encode a cleavable leader peptide (45, 55, 58) which could ensure the proper assembly of antigen or its secretion across the plasma membrane (or both). We investigated this possibility by constructing expression vectors in which the first ATG sequence in HBsAg mRNA was that corresponding to the first amino acid in the mature form of antigen. HBsAg so produced had the antigenic properties and molecular weight characteristic of authentic HBsAg, could be localized intracellularly, and, after a 24-h period, could be found extracellularly, indicating that its secretion can proceed without involvement of a leader peptide.

Using the expression of HBsAg as an assay, we examined the role of the SV40 enhancer sequences on a promoter function. In contrast to the involvement of the enhancer sequences both in SV40 promoter function during the lytic cycle and in enhancing the activity of heterologous promoters, we found that an intact copy of the SV40 72-bp repeat element was not required for either early or late promoter activity when the role of SV40 enhancer sequences was assayed in this fashion. Possible explanations of this observation are discussed.

MATERIALS AND METHODS

DNA constructions. All restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from Bethesda Research Laboratories or New England Biolabs and were used according to the prescribed reaction conditions. Oligonucleotides were synthesized by previously described methods (10). The procedures for plasmid DNA isolation (11), electrophoresis (11), and electroelution (49) were as described previously. DNA used in the constructions included that of SV40 (strain 776 [14, 59]) and plasmids pML (29) and pHBV (28). *Escherichia coli* 294 (3) was used as the recipient in all bacterial transformations.

DNA transfections and replication assays. To validate replication of plasmid DNA in monkey cells, we grew monolayers of COS1 cells (monkey CV-1 cells transformed with an origin-defective mutant of SV40) to 50 to 60% confluency in 6-cm plastic dishes and transfected the cells with DNA as described previously (51). Briefly, the cells were washed with Dulbecco modified medium, and 2 ml of medium containing 50 ng of plasmid DNA and DEAE-dextran at 200 μg/ml was applied for 12 h at 37°C. The DNA solution was removed, the cells were washed twice with medium, 5 ml of medium containing 10% fetal calf serum was added, and the cells were incubated at 37°C for various times before DNA extraction. At these times, small supercoiled plasmid DNA was isolated by the method of Hirt (24). The DNA was subjected to agarose gel electrophoresis and transferred to nitrocellulose filters (52). To visualize replicating plasmids, we probed the nitrocellulose filters with HBV [^{32}P]DNA (52).

Immunofluorescence. For visualization of HBsAg by immunofluorescence, COS cells were transfected with appropriate plasmid DNA as described above. The cells were trypsinized 3 days after transfection and replated on glass cover slips in medium containing 2%

fetal calf serum. The cover slips were washed with phosphate-buffered saline (PBS) 3 h later, fixed in 100% methanol at −20°C, and incubated for 30 min at 37°C with a 1:50 dilution of rabbit antibody prepared against purified HBsAg (subtype Ad) derived from human serum (North American Biologicals, Inc.). After a PBS wash, the cover slips were incubated for 30 min with a 1:30 dilution of rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories). After additional washes with PBS, the cover slips were mounted and observed immediately with a Zeiss photomicroscope (equipped with epifluorescence optics), using a ×20 lens and filter modules as appropriate. Fluorescence results were recorded on Kodak Tri-X panchromatic film (ASA 400).

Radioimmunoassays. HBsAg was assayed with the Ausria II radioimmunoassay kit (Abbott Laboratories) and quantitated by serial dilution of the unknown sample and comparison with the positive control supplied.

S1 analysis. For determination of the 5′ ends of RNA, five 150-cm plates of COS cells were transfected with appropriate plasmid DNA. The cells were washed with PBS 48 h after transfection and lysed with buffer (3 ml per plate) containing 10 mM Tris-hydrochloride (pH 8.6), 0.5% Nonidet P-40, 1.5 mM $MgCl_2$, and 140 mM NaCl. The lysate was clarified by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 20 min, and the supernatant was mixed with an equal volume of 2% sodium dodecyl sulfate (SDS)–200 mM Tris-hydrochloride (pH 7.5)–25 mM EDTA–300 mM NaCl–400 μg of proteinase K per ml. After incubation at 37°C for 30 min, the solution was extracted once with phenol and once with chloroform and precipitated by the addition of 2 volumes of ethanol. The precipitated nucleic acid was pelleted by centrifugation, washed once with 70% ethanol, and suspended in 2 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. The polyadenylate-containing mRNA was purified by oligodeoxythymidylic acid-cellulose as described previously (13). To prepare the ^{32}P-labeled probe, we digested plasmid DNA with an appropriate restriction nuclease, dephosphorylated the terminus with calf intestine alkaline phosphatase (Boerhinger Mannheim Corp.), and treated it with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The labeled DNA was subsequently cleaved with a second nuclease and purified by electroelution after polyacrylamide gel electrophoresis. DNA-RNA heteroduplex formation and S1 nuclease digestion were carried out by the procedures described by Berk and Sharp (6).

Isotopic labeling of HBsAg. To monitor HBsAg synthesis in COS cells by autoradiography, we transfected COS cells in 6-cm dishes with appropriate plasmid DNA as described above. The medium was replaced 72 h after transfection with medium lacking serum and cysteine. [^{35}S]cystine (523 Ci/mmol; New England Nuclear Corp.) was then added to a final concentration of 100 μCi/ml for 60 min, and proteins present in the cell and medium were analyzed separately by electrophoresis on 12% SDS-polyacrylamide gels (26) either immediately after the labeling period or after a 24-h chase period during which the labeled medium was replaced with regular medium. For analysis of extracellular proteins, medium was collected and mixed with SDS-sample buffer. For analysis of cellular proteins, the dishes were washed twice with PBS, and the cells were directly lysed with SDS-sample buffer. Serum-derived HBsAg run as a standard (North American Biologicals Inc.) and molecular weight markers (BioRad Laboratories) were visualized by silver staining (41).

RESULTS

Construction of vectors. The efficient replication of recombinant SV40-pBR322 plasmids in monkey cells depends upon several factors. First, the SV40 origin of replication is required (29, 38). Second, pBR322 sequences inhibitory to replication must be absent (29). Finally, SV40 T antigen, which is involved in the initiation of viral DNA synthesis during the course of normal lytic infection (40, 54), must be present (29, 38). For the replication of recombinant SV40-pBR322 plasmids, T antigen may be provided either by expression from the vector itself or by cells endogenously expressing the protein (29, 38). To obviate the requirement for vectors which encode and express SV40 T antigen, we used the COS cell line as the recipient. These cells synthesize SV40 T antigen capable of supporting the replication of SV40 *A* gene mutants (18).

The general approach for the construction of vectors involved the incorporation of SV40 sequences spanning the viral origin of replication into plasmid pML (a pBR322 derivative lacking sequences which inhibit replication in monkey cells [29]) (Fig. 1). These sequences not only provide an origin of replication but, because the SV40 early and late promoters overlap with the origin region (56, 60), should also permit an evaluation of promoter function if positioned appropriately before a suitable gene. In the experiments to be described, we introduced the gene encoding HBsAg distal to the SV40 origin-promoter sequences. Two different but overlapping SV40 DNA fragments which contain all sequences necessary to direct the replication of recombinant plasmids in COS cells (38) were used in our constructions. A 342-bp fragment resulting from *Pvu*II and *Hin*dIII digestion of SV40 72-bp repeat (enhancing sequences [4, replication (38) and two complete copies of the SV40 72-bp repeats (enhancing sequences [4, 34]) believed to be responsible for activating expression of both SV40 T antigen and heterologous genes (4, 5, 22, 34). The 311-bp fragment resulting from *Eco*RII digestion of SV40 DNA also contains a functional origin (38) but lacks sequences within the 72-bp repeat required for enhancer activity (4, 34). After the addition of *Eco*RI linkers to the ends of both fragments, the fragments were inserted in both possible orientations into plasmid pML-HBs preceding the gene encoding HBsAg, permitting the separate evaluation of early and late SV40 promoter functions (Fig. 1).

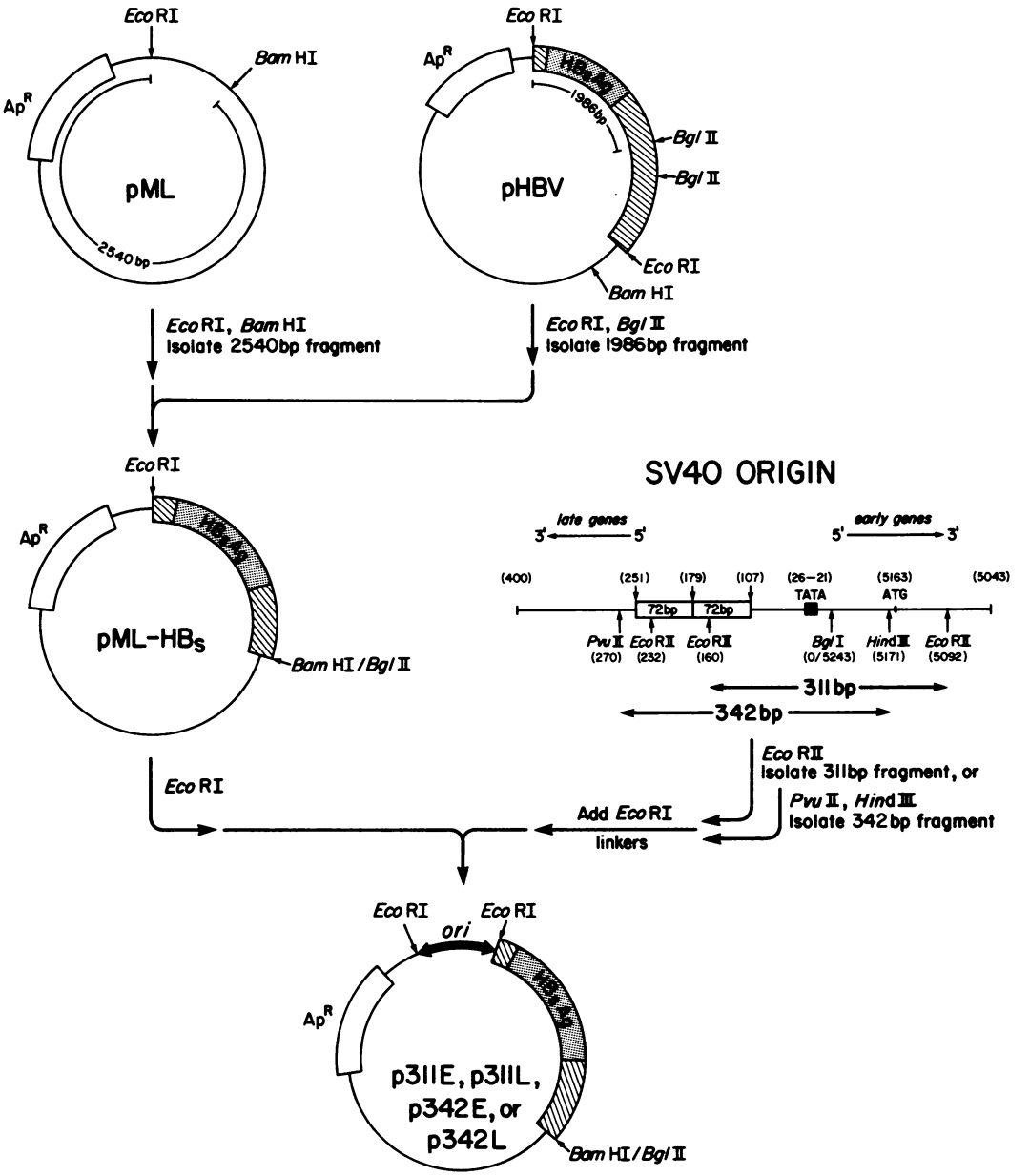

FIG. 1. Construction of HBsAg expression plasmids. Cloned pML DNA was digested with *Eco*RI and *Bam*HI, and the 2,540-bp fragment was isolated from polyacrylamide gels by electroelution. pHBV (a plasmid in which HBV DNA was cloned into the *Eco*RI site of pBR322) was digested with *Eco*RI and *Bgl*II, and the 1,986-bp fragment (which spans the sequences encoding HBsAg) was isolated. After ligation and transformation into *E. coli*, plasmid pML-HBs was isolated. For the isolation of fragments which contained the origin and promoter regions of SV40, SV40 DNA was digested with *Pvu*II and *Hin*dIII, and the 342-bp fragment spanning the origin was isolated. After addition of *Eco*RI linkers, the fragment was inserted into the *Eco*RI site of pML-HBs. The 311-bp fragment was isolated after digestion of SV40 DNA with *Eco*RII; *Eco*RI linkers were then added and the fragment was similarly introduced into the *Eco*RI site of pML-HBs. The early and late SV40 promoters overlap one another in opposite orientations; we therefore isolated plasmids which contained the 311-bp fragment oriented such that either the early promoter (p311E) or the late promoter (p311L) was positioned before the HBsAg coding sequences. Similarly, plasmids were isolated which contained the 342-bp fragment oriented with either the early promoter (p342E) or the late promoter (p342L) preceding the HBsAg gene. The origin-promoter region of SV40 is depicted and illustrates the positions of the 72-bp repeats, the Goldberg-Hogness (TATA) box involved in promoting the specific initiation of early transcription (5, 19), and the translational initiation codon (ATG) for T antigens. The SV40 sequence (14, 59) is numbered according to the BBB system (56).

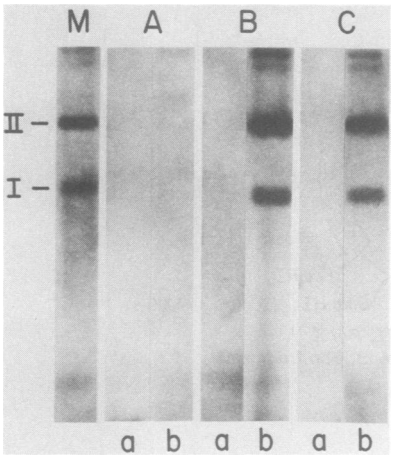

FIG. 2. Replication of plasmid DNA in monkey cells. Monolayers of COS cells were grown to 50 to 60% confluency in 6-cm plastic dishes. The cells were washed with Dulbecco modified medium and transfected with DNA as described in the text. After removal of DNA, the cells were incubated at 37°C for 12 (lanes a) or 72 (lanes b) h before DNA extraction. At these times, small supercoiled plasmid DNA was isolated, and the DNA was subjected to agarose gel electrophoresis. After transfer to nitrocellulose filters, the replicating DNA was visualized by probing the filters with HBV [P^{32}]DNA (33). (A) DNA from Hirt lysates of cells transfected with pML-HBs DNA; (B) DNA from Hirt lysates of cells transfected with p342L DNA; (C) DNA from Hirt lysates of cells transfected with p311L DNA; (M) 10 ng of p342L DNA; bars indicate the positions of form I and form II DNA.

Replication of vectors in COS cells. To confirm that replication of the recombinant plasmids occurred in permissive cells, we transfected monolayers of COS cells with p311L, p342L, or pML-HBs DNA, using DEAE-dextran. Using the increase in supercoiled DNA with time as a measure of DNA replication in COS cells, we isolated low-molecular-weight DNA at various times after transfection and analyzed the DNA by electrophoresis in agarose gels and Southern blot hybridization (52). Replication of the recombinant pML-SV40-HBV plasmids occurred, as reflected by the amount of supercoiled p342L DNA (Fig. 2B) and p311L DNA (Fig. 2C) which accumulated during the 72 h after transfection. The control plasmid pML-HBs, which lacked the SV40 origin fragment (but was otherwise identical to p311L and p342L), did not replicate in COS cells (Fig. 2A). Replication of the early promoter recombinant plasmids proceeded, as expected (29, 38), as efficiently as late promoter construct replication (data not shown).

Expression of HBsAg in CV-1 and COS cells. To establish that synthesis of HBsAg was occurring and to determine the efficiency with which cells incorporated DNA and expressed antigen, we transfected COS cells with DNA and monitored HBsAg expression by indirect immunofluorescence with antisera raised against purified HBsAg. Approximately 40 to 50% of COS cells transfected with plasmid p311L displayed a bright cytoplasmic fluorescence 72 h after transfection, after fixation and staining with antibody specific for HBsAg (Fig. 3). No bright fluorescence was observed in cells which received pML-HBs DNA as a control (Fig. 3A), nor was fluorescence observed in either case when preimmune serum was used (data not shown).

To quantitatively measure HBsAg synthesis directed by the recombinant plasmids in the presence and absence of SV40 T antigen, we transfected COS cells and CV-1 cells separately with plasmid DNA and at various times assayed by radioimmunoassay (RIA) the amount of HBsAg present in the medium. Within 48 h of transfection, significant levels of HBsAg expression were observed in COS cells receiving p342E, p342L, and p311L DNAs (Fig. 4A). It is evident that expression of HBsAg was dependent upon the presence of SV40 sequences, since cells transfected with pML-HBs DNA (which lacked the SV40 sequences that provide both a replication origin and a promoter) failed to synthesize detectable amounts of HBsAg. p311E DNA, which lacked an intact copy of the SV40 72-bp repeat, did not direct the expression of detectable levels of HBsAg. Although this result is consistent with the conclusion that the sequences comprising the 72-bp repeats (enhancer sequences) constitute an essential element of the SV40 early promoter (5, 22, 34), our results indicate that the failure to observe secretion of mature HBsAg in cells transfected with p311E DNA was not a consequence of transcriptional considerations (see below). Interestingly, comparable levels of synthesis were observed in COS cells with the p342L and p311L constructions. Since the SV40 sequences present in p311L lacked an intact 72-bp repeat, the results indicate that these enhancer sequences, which serve to activate SV40 early gene expression as well as transcription of heterologous genes, are not, in contrast, a prerequisite for SV40 late promoter function when assayed in this manner.

The pattern of HBsAg expression directed by the various plasmids in CV-1 cells was markedly different from that in COS cells (Fig. 4). Apart from differences in overall expression levels (which are most likely the result of differences in plasmid copy number due to replication of the plasmids in COS cells), it was clear that the early promoter construction (p342E) directed the synthesis of larger amounts of HBsAg in CV-1 cells than did either type of late promoter construction (Fig. 4B), in contrast to the results observed

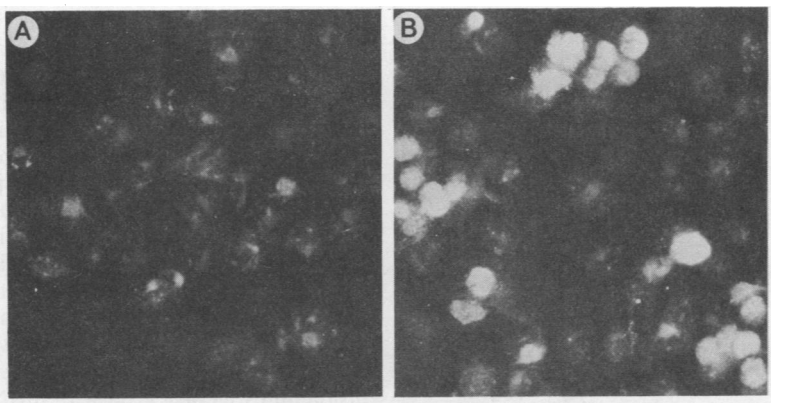

FIG. 3. Visualization by immunofluorescence of HBsAg produced in COS cells. COS cells were transfected with pML-HBs (A) or p311L (B) as described in the legend to Fig. 2. The cells were trypsinized 3 days after transfection and replated on glass cover slips. The cover slips were then washed with PBS and fixed, and HBsAg was visualized by indirect immunofluorescence with rabbit antibody prepared against purified HBsAg derived from human serum as described in the text.

for COS cells. The simplest interpretation of these results is that the early promoter, not being subject to T-antigen repression (25, 46) in CV-1 cells, functioned more efficiently in CV-1 cells than in COS cells. The fact that COS cells transfected with pML-HBs failed to synthesize detectable quantities of HBsAg indicates that expression was dependent on either the replication origin or promoter function of SV40. Since expression remained dependent on SV40 sequences in CV-1 cells (Fig. 4), which are not permissive for the replication of these plasmids (29), it appeared that the failure to express is a consequence of a transcriptional block resulting from the absence of a functional promoter, rather than from a failure to replicate.

Localization of the 5′ ends of HBsAg mRNA. To confirm that transcription of the HBsAg gene was indeed initiating from within the SV40 promoter, we mapped the 5′ terminus of HBsAg mRNA synthesized in COS cells transfected with various plasmid DNAs by hybridizing RNA to the coding strand of a radiolabeled *Pvu*I-*Xba*I restriction fragment that was expected to overlap the 5′ terminus of the HBsAg gene. RNA-DNA hybrid molecules were digested with S1

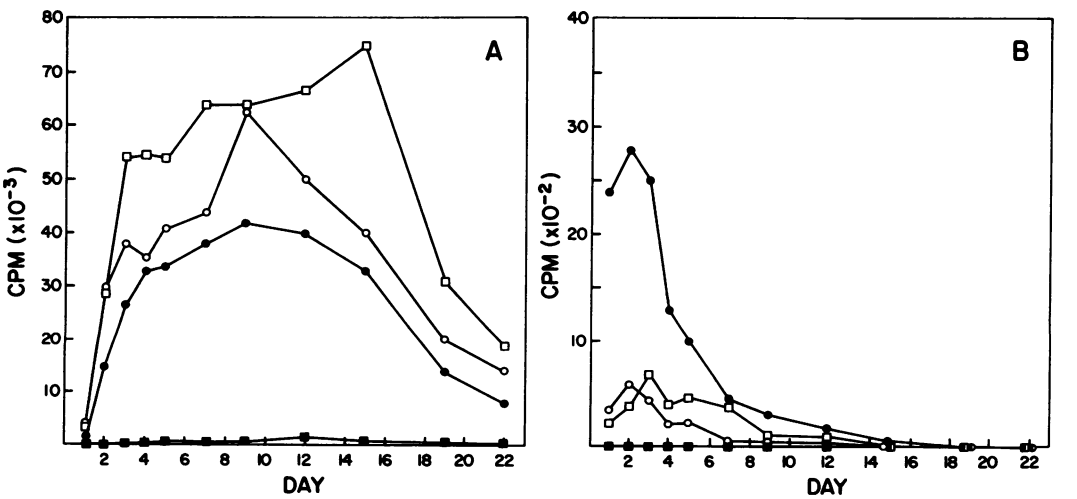

FIG. 4. Time course of HBsAg expression in COS and CV-1 cells. COS and CV-1 cells were transfected with p311E (■), p311L (□), p342E (●), or p342L (○) DNA as described in the legend to Fig. 2. Every 24 h the medium was changed, and HBsAg which accumulated in the medium was assayed by RIA after dilution as appropriate to ensure linearity of assay results. No expression of HBsAg was observed with pML-HBs DNA at any time point, paralleling the results for p311E. (A) HBsAg expression in COS cells; (B) HBsAg expression in CV-1 cells.

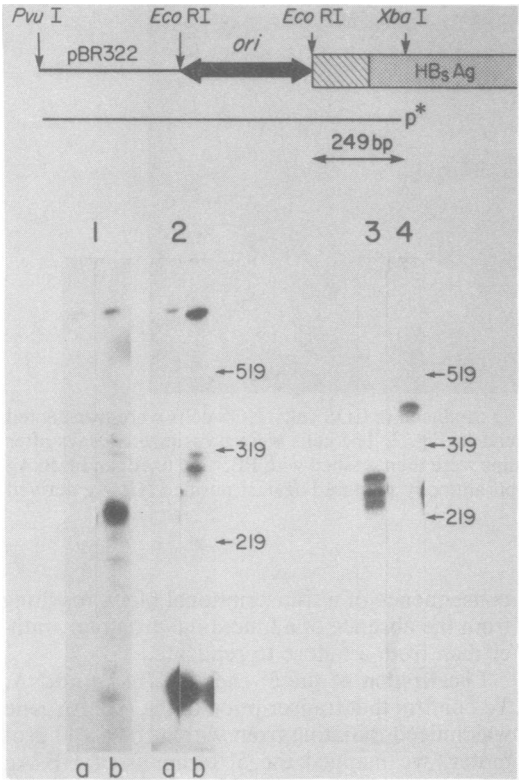

FIG. 5. Determination of 5′ ends of mRNA by S1 nuclease mapping. The diagram at the top depicts the origin-promoter region of SV40 plus adjoining HBV and pBR322 sequences as detailed in the legend to Fig. 1. A DNA probe was obtained by digesting p342L (lane 1), p342E (lane 2), p311L (lane 3), and p311E (lane 4) with *Xba*I, labeling with [γ-^{32}P]ATP, and isolating the labeled fragment from a polyacrylamide gel after digestion with *Pvu*I. A total of 25 μg of RNA from COS cells transfected with p342L DNA (lane 1b), p342E DNA (lane 2b), p311L DNA (lane 3), and p311E DNA (lane 4) was hybridized with labeled probe as described previously (6). Control hybridizations were performed with RNA extracted from COS cells transfected with DNA lacking HBV sequences but otherwise identical to p342L (lane 1a) and p342E (lane 2a). Samples were heat denatured and electrophoresed on an 8% polyacrylamide gel containing 7 M urea (30) and then autoradiographed. Molecular weight markers were generated by treating fragments of p342L DNA with kinase after digestion with various restriction nucleases.

nuclease, and S1-resistant DNA fragments were sized by gel electrophoresis. Figure 5 shows an autoradiographic exposure of a gel that was used to map DNA fragments protected from S1 digestion by various RNA samples. It is evident that mRNA prepared from COS cells transfected with p342L protected approximately 240 nucleotides of the majority of probe (Fig. 5, lane 1b), which indicates that the major 5′ terminus of HBsAg mRNA was at the position expected if initiation occurred from within the SV40 late promoter (56, 60). As in the case with late mRNA synthesized during the course of a lytic infection (60), additional start sites were observed, although they occurred at a considerably reduced frequency. mRNA derived from cells transfected with plasmid p342E protected approximately 300 nucleotides of probe (Fig. 5, lane 2b), consistent with a bona fide start from the SV40 early promoter (60). Similarly, start sites indicative of transcription initiating from the SV40 promoter were seen in mRNA prepared from cells transfected with plasmids p311L (Fig. 5, lane 3) and p311E (lane 4). No protection was observed with mRNA from cells which were transfected with DNA lacking HBV sequences but otherwise identical to p342L (Fig. 5, lane 1a) or p342E (lane 2a).

Identification of HBsAg polypeptides in COS cells. The two major polypeptides found in the HBV envelope (termed PI and PII) represent two forms of HBsAg protein which differ in the extent of glycosylation (47, 55). The molecular weights of PI and PII are 25,000 and 29,000, respectively (55), and the polypeptides are believed to represent the major neutralizing antigen of the virus. The antigen is present not only in infectious viral envelopes but is often found associated with noninfectious particles (free viral envelopes) in the plasma, where it accumulates after secretion from infected hepatocytes (31, 47). To further characterize HBsAg expression directed by the recombinant plasmids, we analyzed proteins synthesized in transfected COS cells by SDS-polyacrylamide gel electrophoresis. COS cells were transfected with either pML-HBs or p311L DNA; 3 days later, [^{35}S]cysteine was added for 1 h (pulse), and the cells and medium were then harvested directly, or incubation was continued for an additional 24 h in the presence of excess unlabeled cysteine (pulse-chase). Total proteins were then visualized by autoradiography after SDS-polyacrylamide gel electrophoresis. After the 1-h labeling period, a protein with an apparent molecular weight of 25,000 was observed in cellular extracts derived from cultures transfected with p311L DNA (Fig. 6A, lane c). This protein was not apparent in control cultures receiving pML-HBs DNA (which lacks SV40 sequences but is otherwise identical to p311L) (Fig. 6A, lane a). After the 24-h chase period, the presence of this protein within the cells was diminished (Fig. 6A, lane d), indicating a short intracellular half-life relative to the half-lives of the host proteins. Conversely, when proteins secreted into the medium were analyzed, two major polypeptides (molecular weights, 25,000 and 29,000) were observed specifically in cultures transfected with p311L DNA, but only after a 24-h chase period (Fig.

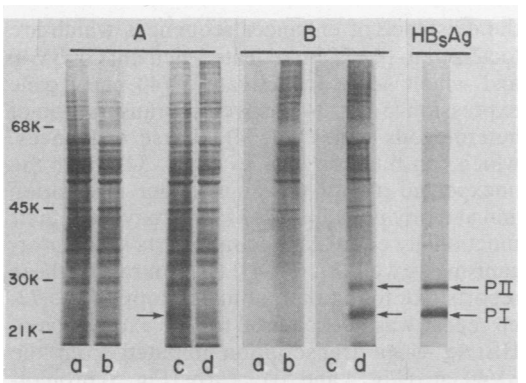

FIG. 6. Synthesis and secretion of HBsAg in COS cells. COS cells were transfected with pML-HBs DNA (lanes a and b) or with p311L DNA (lanes c and d) in duplicate as described in the legend to Fig. 2. The cells were labeled 72 h later with [^{35}S]cystine for 60 min, and proteins present in the cells (A) and medium (B) were analyzed separately by electrophoresis on 12% SDS-polyacrylamide gels (26) as described in the text. Samples were collected immediately after the labeling period (lanes a and c), or after a 24-h chase period (lanes b and d) during which the labeled medium was removed and replaced with regular medium. For the analysis of extracellular proteins, medium was collected and mixed with SDS-sample buffer. For the analysis of cellular proteins, the dishes were washed twice with PBS, and the cells were directly lysed with SDS-sample buffer. After electrophoresis, the gel was silver stained to visualize purified HBsAg (subtype Ad) (derived from human serum), which was run on a parallel lane. The gel was photographed, dried, and exposed to film for 24 h for visualization of [^{35}S]cysteine-labeled proteins. PI and PII. Positions of nonglycosylated and glycosylated HBsAg determined from silver stain (see text). Proteins serving as molecular weight markers were electrophoresed in parallel.

6B). These proteins comigrated with the two forms (PI and PII) of authentic HBsAg (derived from HBV carriers), which were run in parallel and visualized by silver staining, strongly indicating that the 25,000- and 29,000-molecular-weight proteins synthesized under plasmid control represent the translational products of the HBsAg gene.

DISCUSSION

Owing to the inability of HBV to propagate in tissue culture, comparatively little is known about its life cycle. The only viral gene products to have been rigorously identified are the core antigen (7, 42) and HBsAg (15, 57), although the coding capacity of the genome is clearly sufficient to specify additional polypeptides (15, 57, 58). In addition, the mechanisms of synthesis, glycosylation, assembly, and secretion of HBsAg are poorly understood. To overcome this host range restriction for the study of HBV-encoded products, the HBV genome has been placed under the control of SV40, exploiting the ability of SV40 both to replicate and to direct the transcription of HBsAg in permissive cells (28, 35). One significant drawback of this approach is that, by virtue of the requirement for helper virus, a lytic infection is involved. This both limits the duration of the experiment and complicates the analysis of certain molecular processes. We sought to expand upon a system which obviates the requirement for a fully lytic infection, yet preserves the ability of the vector to extensively replicate. It has been shown previously that a deletion mutant of plasmid pBR322 (pML) which carries the SV40 origin of DNA replication is capable of replicating in CV-1 monkey cells transformed with an origin-defective mutant of SV40 (COS cells) (18). These cells constitutively express the product of the SV40 *A* gene (T antigen), which presumably initiates the replication cycle of such plasmids in these cells (40, 54). Since both the early and the late promoters of SV40 overlap the region encompassing the origin of DNA synthesis, it was possible with suitable restriction enzymes to incorporate into pML SV40 DNA fragments which preserved both origin and either early or late promoter function (Fig. 1). When the gene encoding HBsAg was placed distal to these fragments, the resultant plasmids replicated after transfection into permissive cells (Fig. 2). On the basis of the intensity of hybridization relative to known quantities of plasmid DNA (data not shown) and assuming that 40% of transfected cells replicate plasmid, as judged by immunofluorescence (Fig. 2), we estimated the plasmid copy number to be approximately 5×10^5 per cell by day 3 after transfection, in good agreement with data obtained by others (29, 32). Accompanying the replication of the recombinant plasmids was an extensive synthesis of HBsAg, as documented by immunofluorescence, quantitative RIA, and isotopic labeling of proteins synthesized in transfected cells. Expression of this heterologous gene product could be achieved under the control of either the early or the late promoter of SV40 (Fig. 4 and 5), which functioned in COS cells with approximately equal efficiencies (Fig. 4). Expression depended on the presence of SV40 sequences; plasmids lacking the SV40 origin-promoter region but containing the HBsAg gene failed to direct the synthesis of detectable levels of HBsAg. Although the SV40 sequences provided a replication function for the plasmids (in COS cells), it appeared that their critical role in HBsAg expression resided at the transcriptional level. This conclusion results from the observation that in CV-1 cells, as well as in COS cells, expression of HBsAg remained dependent on the SV40 sequences, as evidenced

by the failure of plasmids lacking SV40 sequences (but otherwise identical to the parent plasmids) to direct the synthesis of detectable amounts of HBsAg after transfection (Fig. 4). Since CV-1 cells are nonpermissive for the replication of plasmids containing the SV40 origin of replication (since the cells do not express SV40 T antigen [29; unpublished data]), differences in expression levels of HBsAg exhibited by the various constructions in this cell line were not a reflection of differences in plasmid copy number resulting from de novo replication of the plasmids. The conclusion that the SV40 sequences were providing a promoter without which the expression of the HBsAg gene could not occur is in accord with the recent finding that the HBV promoter for HBsAg is located approximately 560 bp upstream from the structural gene (45); since only 160 nucleotides of HBV precede the structural gene in these constructions, the SV40 promoter represents the only known promoter present on the plasmids capable of functioning in mammalian cells.

To directly confirm the involvement of the SV40 promoter in the transcription of HBsAg mRNA, we mapped the 5′ ends of HBsAg mRNA present in COS cells transfected with various HBsAg-expressing plasmids. S1 nuclease analysis clearly indicated that, within the limits of the assay (≈10 nucleotides), the 5′ ends were located at the positions expected, assuming transcription is initiated from within the SV40 promoter. This conclusion applies to constructions utilizing both the SV40 early promoter and the late promoter (Fig. 5). It has been observed that during lytic infection, the SV40 late mRNAs display heterogeneous capped 5′ termini, indicating that late transcription can initiate at any of several locations spanning a 400-bp region adjacent to the replication origin. Early transcription, on the other hand, initiates with much greater fidelity, although a shift in the initiation site reportedly occurs during the course of lytic infection (17). We have noticed a similar phenomenon in the transcripts directed from the recombinant plasmids: mRNA initiated from the late promoter displayed heterogeneous 5′ ends, a phenomenon which was largely independent of the presence of enhancer sequences (Fig. 5). Transcripts initiating from the early promoter exhibited, on the other hand, a more uniform pattern of cap sites. Why the transcripts initiating from the SV40 late promoter have heterogeneous 5′ ends, both in the lytic cycle and when promoting the expression of heterologous genes, is unclear; perhaps this is a consequence of the absence of a TATAA sequence, which is thought to be involved in establishing transcriptional initiation sites (5, 19).

There have been several reports documenting the existence of enhancer sequences which are localized to the 72-bp repeated elements of SV40 and which serve to activate SV40 early gene expression (5, 22, 34), as well as transcription of heterologous genes (4, 34). These sequences, which can function only in *cis* (4, 34), have the unexpected ability to act in either orientation and at many positions (4, 34) and may have their functionally equivalent counterparts in other organisms as well (37, 39, 43). In contrast to these reports, we found that an intact copy of the 72-bp repeat was not required for the expression of HBsAg when transcription initiated from the SV40 late (Fig. 4 and 5) or early (Fig. 5) promoter. Although no expression of HBsAg protein was seen with p311E, which lacks an intact copy of the 72-bp repeat, normal levels of HBsAg mRNA were observed (Fig. 5; unpublished data), suggesting that failure to express HBsAg is not a consequence of transcriptional impairments. Indeed, analysis of the DNA sequence spanning the SV40-HBV junction of p311E (38; data not shown) reveals that the HBsAg gene in this construction follows in frame 231 nucleotides from the initiator ATG sequence of T antigen, with no intervening termination codons. Thus, any protein synthesized with HBsAg determinants would appear as a fused polypeptide with an amino terminus consisting of the first 24 amino acids of T antigen followed by 52 amino acids specified by the sequence immediately preceding the HBsAg gene. Since our assay detected only immunologically active HBsAg secreted into the medium, such a fusion would have likely escaped our attention. Consistent with this interpretation is the observation that the introduction of an in-frame translational termination codon into this plasmid immediately after the sequences encoding the first 24 amino acids of T antigen restores expression of an actively secreted HBsAg molecule, presumably by permitting translation to reinitiate at the HBsAg gene (C.-C. Liu, unpublished data).

Although no data specifically address the role of the SV40 enhancer sequences in late transcription, there is suggestive evidence based on deletion mutants in the 72-bp repeat region which fail to complement late mutants of SV40 that this region may be essential for late gene expression as well as early gene expression (22). Thus, the observation that HBsAg gene expression under the control of the SV40 early or late promoter was independent of these sequences was somewhat unexpected. Considering, however, that functionally equivalent sequences from heterologous sources can complement the SV40 enhancers during productive infection (27), we find that our results are compatible with the hypothesis that the HBV genome may also contain enhancer sequences capable of comple-

menting those of SV40. In accord with this suggestion, we have recently found that removing all but 12 bp of the 72-bp repeat from p342E (which eliminates the SV40 enhancer function [4, 34]) results in at most only a slight reduction (<20%) of HBsAg expression, as determined by quantitative RIA measurements (Liu, unpublished data).

It is apparent that more HBsAg was produced in COS cells than in CV-1 cells (Fig. 4); this is presumably a reflection of the fact that the copy number of these vectors in COS cells increased because of amplification due to T-antigen-induced replication (29; Fig. 2). The degree of expression augmentation in COS cells, as compared with that in CV-1 cells, depended on the characteristics of the plasmid used. Whereas vectors with the SV40 late promoter were more effective in directing HBsAg synthesis in COS cells than were vectors with the SV40 early promoter, the opposite holds true in CV-1 cells. The simplest interpretation of these results is that in CV-1 cells, the early promoter, not being subject to T-antigen repression (25, 46), can function more efficiently than it can in COS cells, where T antigen is abundant. Although the early promoter appeared to be more efficient than the late promoter in CV-1 cells, the observation of even low levels of HBsAg synthesized under control of the late promoter is surprising, given the reports that *A* gene function is required (either directly or indirectly) for expression of late viral functions, at least under the usual conditions of infection (1, 2, 20, 48). We calculate, on the basis of quantitative RIA measurements (Fig. 4), that approximately 10^8 molecules of HBsAg were synthesized per cell per day by 72 h after transfection of p311L DNA into COS cells. This compares well with the 10^8 molecules of VP1 (the major capsid protein of SV40) which are synthesized per cell during the entire productive cycle of SV40 virus (56) and represents 2 to 10 times as much HBsAg as has been observed previously with more conventional SV40-based vectors (28, 35). Of course, this comparison becomes even more favorable in light of the fact that HBsAg synthesis continued for a period of several weeks with the vectors described (Fig. 4), in contrast to the 48- to 72-h synthesis period characteristic of lytic SV40 expression vectors.

HBsAg as it is found in naturally occurring 22-nm particles exists in two forms, termed PI and PII, which differ in molecular weight and extent of glycosylation (55). The molecular weights of PI and PII are 25,000 and 29,000, respectively (55). To establish that HBsAg synthesized in COS cells had the molecular weight characteristic of authentic HBsAg, COS cells were transfected with a vector which directed the expression of HBsAg from the SV40 late promoter (p311L); 3 days later proteins were isotopically labeled with [^{35}S]cysteine and analyzed by SDS-polyacrylamide gel electrophoresis. After pulse-labeling, a protein with an apparent molecular weight of 25,000 was observed in extracts of cells transfected with p311L DNA but not in control extracts. After a 24-h chase, the presence of this protein within the cells was considerably diminished, indicating a relatively short intracellular half-life. Conversely, when proteins secreted into the medium were analyzed, two major polypeptides with molecular weights of 25,000 and 29,000 were observed specifically from cultures transfected with p311L DNA, but only after a 24-h chase. These proteins comigrated with the two forms (PI and PII) of authentic HBsAg, strongly indicating that the 25,000- and 29,000-molecular-weight proteins synthesized under plasmid control represent the translational products of the HBsAg gene.

Because there is not an experimentally suitable host, little is known about the molecular events involved in the biosynthesis of this antigen. For example, an open reading frame precedes the structural gene for HBsAg, and it has been proposed that these sequences could encode a leader peptide which would ensure proper processing of the antigen (45, 55, 58). On the basis of the constructions described here, it is evident that antigenically active HBsAg could be produced without the contribution of these sequences. This conclusion follows from the fact that in the p348E, p348L, and p311L constructions, the first ATG codon in HBsAg mRNA was that corresponding to the first amino acid in the mature form of HBsAg. It thus appears that HBsAg is one of the few secreted proteins whose transport across the plasma membrane does not require the processing of a cleavable signal peptide.

In addition to establishing that synthesis of HBsAg proceeded with considerable efficiency, these results suggest that the post-translational modifications and transport typifying the synthetic process in vivo are reproduced in the in vitro system described here. Since synthesis of large quantities of a heterologous gene product can occur over a protracted period of time with vectors such as those described here, this approach offers, apart from more general applications, the prospect of identifying new HBV-encoded polypeptides and characterizing in a convenient fashion the processes associated with post-translational modification, assembly, and transport of HBsAg.

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