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Expression of Hepatitis B Antigens with a Simian Virus 40 Vector

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Recombinant DNA molecules consisting of the simian virus 40 (SV40) early region and different subgenomic hepatitis B virus DNA fragments were constructed in vitro and packaged in vivo into SV40 capsids by using a complementing SV40 helper virus. Upon infection with these virus stocks the three known hepatitis B-specific antigens were expressed under SV40 control. The surface antigen was released into the medium, and the core antigen and its derivative hepatitis B e antigen were only detected intracellularly. Size analysis of the core gene product(s) by immunoblotting revealed the presence of a single protein species identical with the 21-kilodalton core antigen isolated from human liver. The hepatitis B core antigen expressing construct did not contain a putative precore sequence, indicating that such a sequence is not needed for hepatitis B core antigen synthesis in animal cells. S1 analysis demonstrated the use of SV40 signals for initiation and polyadenylation of the core gene transcripts. In addition, a processing-polyadenylation signal was identified within the core gene.

Due to the lack of an in vitro propagation system for hepatitis B virus (HBV), most of our knowledge of the structure and function of the HBV genome comes from the analysis of viral DNA cloned in bacteria (48, 53). DNA sequencing and expression studies in procaryotes localized the genes encoding the two major viral proteins, the surface antigen (HBsAg) and core antigen (HBcAg) on the HBV genome (12, 13, 32). Moreover, HBcAg has been converted by limited proteolysis or by treatment with denaturing agents (or both) into hepatitis B e antigen (HBeAg), a third serological marker correlated with HBV infection (27, 45, 46).

For further analysis of the functional organization of the HBV genome, a variety of eucaryotic expression systems has been employed. In all these systems and also in cell lines derived from human hepatocarcinomas the S-gene is efficiently expressed (2, 10, 16, 28, 31, 39, 43, 44), whereas C-gene expression has proven to be more difficult to achieve. In animal cells transformed with cloned HBV DNA, some investigators found no expression of the C-gene (2, 3, 10, 51), or only at low levels (6, 16, 20, 21), despite the fact that very similar techniques or even identical plasmids were used in some of these studies. Moreover, only a minor fraction of individual clones transformed with the same plasmid produced HBcAg-HBeAg (15, 16, 20). Furthermore, human hepatocarcinoma-derived cell lines do not produce HBcAg-HBeAg, although complete core gene sequences are present in some of the chromosomally integrated HBV DNA sequences (9, 22), possibly because of methylation of the C-gene (30). To overcome some of these difficulties and to study C-gene expression on episomally and actively replicating molecules that might mimic more closely the in vivo situation, we have employed a simian virus 40 (SV40) vector system. Here the SV40 late genes are replaced by the DNA segment to be analyzed (18). Such a lytic system offers distinct advantages for the functional analysis of eucaryotic genes. The encapsidated SV40 pseudovirus infects reproducibly and rapidly the great majority of the host cell population and, due to the efficient amplification of SV40 replicons, very high numbers of recombinant DNA molecules accumulate in the infected cells. In addition, the well-known elements that control SV40 transcription can be employed to express an inserted gene in a defined way. As a consequence, the search for insert-specific gene products and transcriptional signals is greatly facilitated.

MATERIALS AND METHODS

Construction of SV40-HBV hybrid plasmids. Vectors pSV010/1 and pA11SVL2 (18) were used to construct SV40 shuttle vector pA13 (Fig. 1). Vector pSV010/1 was derived from pSV010 (25) by deletion of a 54-base-pair (bp) *Bam*HI-*Bgl*II fragment from the polylinker region (H. Will, unpublished data). pSV010/1 was linearized with *Xba*I and ligated with the SV40 *Xba*I fragment obtained from pA11SVL2.

The 1.0-kilobase (kb) *Bam*HI DNA fragment used for construction of pHBc10 and pHBc10/0 was isolated from plasmid pH98 (S. Stahl, unpublished data) in which a *Bam*HI site was created 19 bp in front of the C-gene by BAL 31 treatment and *Bam*HI linker addition as described previously (42). The 1.5-kb *Bam*HI HBV DNA fragment used for construction of pHBc15 and pHBc15/0 was isolated from plasmid pSH14-3, which contains a sequenced full-length HBV genome of subtype ay (53) cloned into the *Pst*I site of the linker region of pSV010. The 1.4-kb *Bam*HI HBV DNA fragment used for construction of pHBs14 and pHBs14/0 was isolated from plasmid pA01 (8) containing a full-length HBV genome of subtype ad. The restriction map of this HBV genome (51) is very similar to the clone sequenced by Valenzuela et al. (49). Therefore, the exact position of the S-gene and of the *Bam*HI sites at the ends of the 1.4-kb fragment are given according to the sequenced HBV clone.

The methods for separation, isolation, and ligation of DNA fragments are described elsewhere (19). Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Laboratories or purified as described by Roberts et al. (37). Plasmid DNA was amplified and isolated from *Escherichia coli* C600 by standard procedures (4).

Growth, transfection, and infection of AGMK cells. AGMK cells were grown at 37°C in basal Eagle medium with Eagle salt (Flow Laboratories) supplemented with 10% newborn

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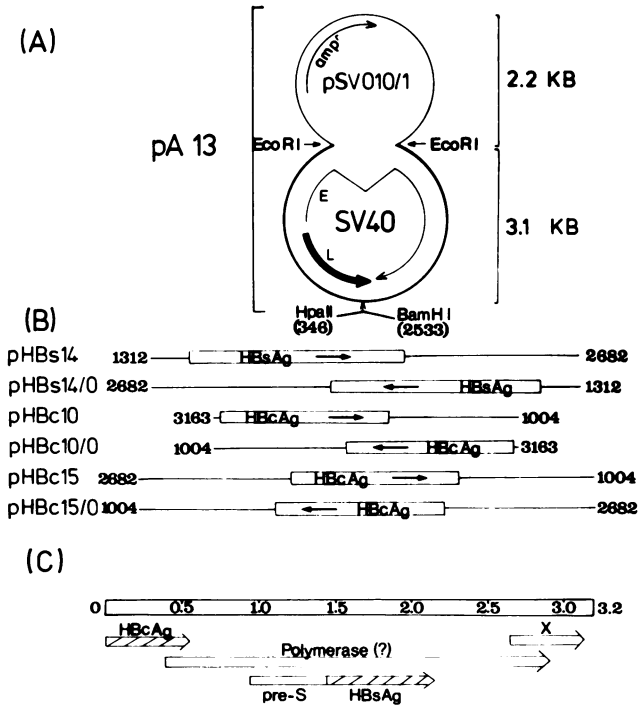

FIG. 1. Structure of SV40-HBV recombinants used. (A) Vector pA13 consists of two parts, a bacterial plasmid, pSV010, and an SV40 late gene deletion mutant (*Hpa*II to *Bam*HI), which can be separated by *Eco*RI or *Xba*I cleavage. Plasmid pSV010/1 is inserted in the intron of SV40 larger T gene. The unique *Bam*HI cleavage site of the vector located at the SV40 early (E) and late (L) junction region which was used for HBV DNA fragment insertion is indicated. Nucleotide positions of SV40 cleavage sites are given as described by Buchman et al. (1). (B) Three different HBV DNA fragments with *Bam*HI cohesive ends inserted into vector pA13 in both orientations resulted in six plasmids as indicated. The localization and orientation of the HBsAg and HBcAg coding sequences are indicated by open boxes and arrows. Nucleotide positions on the HBV genome are according to the nomenclature of Pasek et al. (32). (C) To facilitate the localization of the HBV fragments on the HBV genome a schematic drawing of the preliminary map of the HBV genome organization is presented.

calf serum and passaged at 1 to 5 dilutions. Before transfection the pSV010/1 part of the SV40-HBV plasmids was released by *Eco*RI cleavage. Transfections were performed by a modified DEAE-dextran procedure (40): subconfluent AGMK cells in an 80-cm^2 Falcon flask were washed with basal Eagle medium with Eagle salt and incubated for 8 to 12 h in 2.5 ml of transfection cocktail (1× basal Eagle medium with Eagle salt, 40 mM Tris [pH 7.3], 200 μg of DEAE-dextran [molecular weight, 2,000,000] per ml) in which 1 pmol of DNA was dissolved at 37°C in 1× basal Eagle medium with Eagle salt. After 24 h cells were infected with SV40 tsA58 helper virus to provide SV40 late proteins for encapsidation and incubated at 40.5°C until cell lysis occurred. These lysates were used as virus stocks (designated vpHBs14, vpHBc10, etc.) for infection of AGMK cells.

Detection of HBsAg, HBcAg, and HBeAg in culture medium and cell extracts of AGMK cells. Samples of culture medium of infected cells (3×10^6 cells were incubated in 10 ml of medium) were taken at different times after infection. Cell extracts from 3×10^6 cells were prepared in 2.0 ml of phosphate-buffered saline (10 mM sodium phosphate [pH 7.4], 150 mM NaCl) by freeze-thawing of the sonic extract. HBsAg and HBcAg were determined by commercially available radioimmunoassay (Ausria II and HBeAg test kit). In the Ausria II, 200 μl of extract was incubated with antibody-coated glass beads (guinea pig anti-HBsAg) for 3 or 20 h, and the antigen bound was detected by ^{125}I-labeled human anti-HBsAg. The HBeAg test kit uses essentially the same procedure, but in both steps a mixture of human antibodies directed against HBeAg and against HBcAg is used. The results of these assays are given as the ratio of the radioactivity of the positive sample versus that of the negative control (*P/N*). HBsAg was quantitated with a standard human HBsAg preparation.

HBcAg was analyzed by an enzyme-linked immunoassay (38) with 50 μl of extract that was coated onto microtiter plates and detected by peroxidase-labeled human anti-HBcAg immunoglobulins that contained less than 0.1% anti-HBeAg antibodies. All extracts were analyzed twice; values varied up to 20%. HBsAg was quantitated with a standard human HBsAg preparation. Since there is no HBcAg reference sample of defined specific antigenicity available, highly purified hepatitis B core particles synthesized in *E. coli* from clone pR1-11 (5, 42) were used as a standard (a gift from P. Wingfield, BIOGEN S.A.). Partially purified core particles from human liver were used as reference source of HBcAg for immunoblotting.

Characterization of HBcAg-HBeAg by immunoblotting. Total cellular proteins of virus-infected AGMK cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24) and electrophoretically transferred onto nitrocellulose filters by the blotting system of Bio-Rad Laboratories. After transfer the nitrocellulose filter was incubated with highly purified bovine serum albumin (Behringwerke AG, Marburg) (3% in phosphate-buffered saline) for 2 h and then incubated with 6 ml of ^{125}I-labeled anti-HBcAg–anti-HBeAg immunoglobulins (Abbott HBeAg test kit) for 12 h at room temperature. After 2 h of intensive washing with phosphate-buffered saline the nitrocellulose filter was dried and exposed with an X-ray film.

RNA extraction and S1 mapping. Cytoplasmic RNA was extracted (11) from CV1 cells 48 to 60 h after infection. Probes A and B were double-stranded DNA restriction fragments purified from pHBc10, and probe C was purified from pSH2.6 (2). Probe A was a 0.99-kb *Xba*I fragment, probe B was a 2.97-kb *Xba*I fragment, and probe C was a 0.76-kb *Hpa*II fragment. Probe A was labeled (29) at both 5′ ends with a specific activity of $\sim 5 \times 10^5$ cpm/pmol. Probes B and C were labeled at both 3′ ends with a specific activity of $\sim 2 \times 10^6$ cpm/pmol. For hybridization ~0.02 pmol of probes was dried down in an Eppendorf tube with RNA from $\sim 5 \times 10^5$ cells. The residue was then suspended in 10 μl of 80% formamide–0.4 M NaCl–0.04 M piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (pH 6.4)–1 mM EDTA. The RNA-DNA mixture was denatured by heating for 10 min at 80°C, and hybridization was performed at 48°C (probe A) or 52°C (probes B and C) overnight. Samples were diluted by the addition of 100 μl of chilled S1 buffer containing 0.3 M NaCl, 0.03 M sodium acetate (pH 5.4), 0.003 M $ZnSO_4$, 10 μg of denatured calf thymus DNA per ml, and 100 U of nuclease S1 (Sigma Chemical Co.) and incubated for 30 to 60 min at 24°C. Samples were then ethanol precipitated and analyzed on 6% acrylamide sequencing gels as described previously (52).

RESULTS

Construction of SV40-HBV recombinants and their replication in AGMK cells. Plasmid pA13, a modification of the

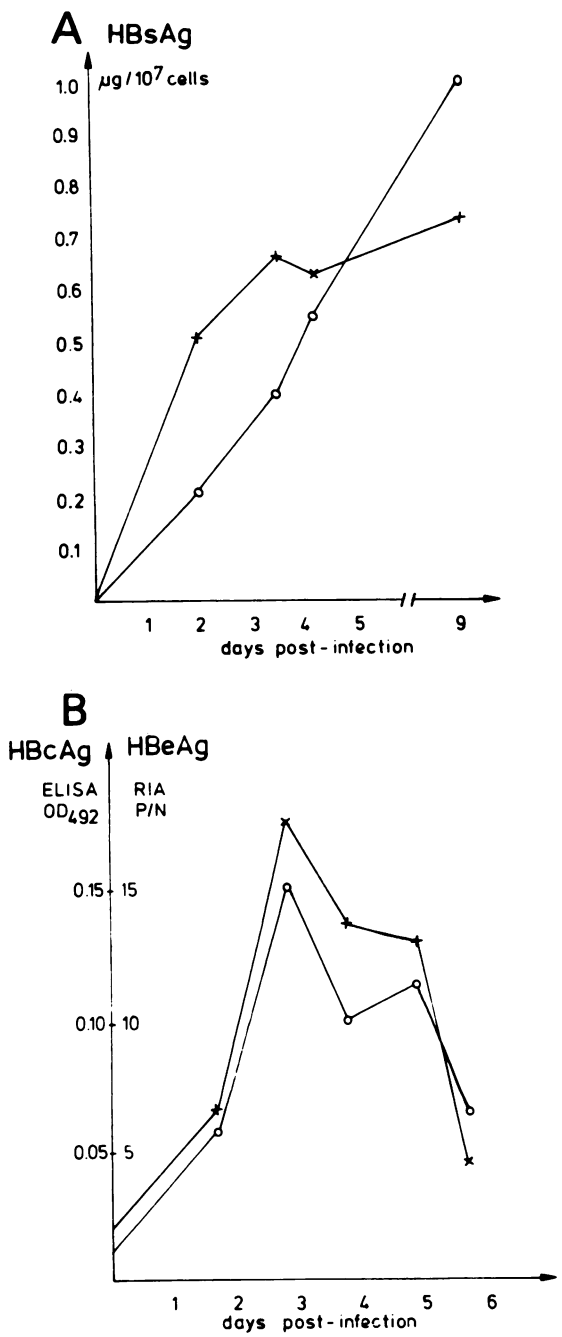

FIG. 2. (A) Kinetics of HBsAG expression by AGMK cells infected with vpHBs14. At different times after infection, HBsAg was measured in the culture medium (○) and in cell extracts (×) of AGMK cells. (B) Kinetics of HBcAG-HBeAg expression by AGMK cells infected with vpHBc10. HBcAg was measured by enzyme-linked immunoassay (×), and HBeAg was determined by radioimmunoassay (○).

SV40 late genes replacement vector pA11SVL2 (18), was employed to construct SV40-HBV recombinants, which, in the presence of a helper virus providing the SV40 late gene products, can replicate and form progeny carrying the recombinant genome. As shown in Fig. 1A, pA13 is a shuttle vector consisting of fused SV40 and pBR322 replicons, which can be amplified in *E. coli* and subsequently specifically released by cleavage with restriction endonuclease *Eco*RI. For our purpose the *Eco*RI release in pA13 is preferable to the *Xba*I release used with pA11SVL2 (18), since *Eco*RI does not cleave our HBV DNA inserts.

As outlined in the legend to Fig. 1B, three different HBV DNA fragments were inserted in either orientation into pA13 at the unique *Bam*HI site replacing the SV40 late genes. Plasmids pHBc10 and pHBc15 both contain the C-gene and 455 bp of 3′-flanking DNA sequence but differ in the length of their 5′-flanking HBV DNA sequences (506 and 19 bp, respectively). Plasmid pHBs14 contains the S-gene, 125 bp of the pre-S region, and 568 bp of 3′-flanking HBV DNA.

The HBV-SV40 DNA fusions contained in these plasmids were introduced into AGMK cells, amplified, and encapsidated by using SV40 tsA58 as a helper virus (see above). The resulting cell lysates (designated as virus pools vpHBs and vpHBc) provided a mixed virus stock of recombinant and helper virus used for infection of AGMK cells.

To determine the amount of recombinant DNA present in AGMK cells infected with these mixed virus stocks, total cellular DNA was extracted 6 days after infection and analyzed by agarose gel electrophoresis for the size distribution of viral DNA before or after cleavage with *Bam*HI. With all virus pools viral DNA was detected as strong bands of the predicted size after staining with ethidium bromide or by Southern blotting (41) with an appropriate DNA probe (data not shown), indicating that the viral DNA was the dominant DNA species in the infected AGMK cells. From the intensity of these bands relative to that of the 5.2-kb band representing the SV40 helper virus, the fraction of recombinants present in the replicating virus stocks could be estimated. These estimates varied from 10% (vpHBc15) up to 30 to 50% (vpHBc10 and vpHBs14). They indicate that the SV40-HBV recombinants replicate in AGMK cells with rates comparable to that of the SV40 helper virus.

Expression of immunoreactive HBcAg, HBeAg, and HBsAg in AGMK cells. Initially, we tested our vector system using an HBV DNA segment carrying the S-gene, since essentially similarly designed, although less well defined, SV40-HBV recombinants had been reported to efficiently express and secrete HBsAg from AGMK cells (31). Synthesis of HBsAg was induced by infection of AGMK cells with vpHBs14, and increasing levels of the antigen were detected until 9 days after infection (Fig. 2A). Final yields reached approximately 500 ng per plate (3×10^6 cells). Immunoreactive HBsAg was detected in both cell extracts and the culture medium (Fig. 2A). Since only 125 bp precede the S-gene in our recombinant, neither synthesis nor secretion of HBsAg can depend on the presence of the extended pre-S sequence (48) preceding the C-gene (Fig. 1B). The same conclusion has also been obtained by another group (7, 26). No HBsAg was synthesized with virus pool HBs14/0 in which the HBV DNA insert is inverted (data not shown). This indicates that recombinant vpHBs14 retaining 125 bp of HBV DNA upstream of the HBsAg gene is most likely transcribed under SV40 late promoter control.

Virus pools from the C-gene containing construct pHBc10 induced synthesis of both HBcAg and HBeAg in infected AGMK cells. In contrast to HBsAg synthesis, both antigens were detected only in cell extracts, not in the culture medium. The level of HBcAg and HBeAg increased in parallel during the first 3 days after infection and then decreased (Fig. 2B). This decrease is probably due to proteolysis of the antigens in the lysate. The level of immunoreactive HBcAg-HBeAg varied considerably between different experiments probably related to variations in

TABLE 1. HBeAg and HBcAg expression in infected AGMK cells[a]

Expt	vpHBc10		vpHBc10/0	
	HBeAg *P/N*	HBcAg A_{492}	HBeAg *P/N*	HBcAg A_{492}
1	20.1	0.16	1.5	0.04
2	67.5	0.24	1.1	0.04
3	18.1	0.27	1.0	0.01
4	48.6	0.23	1.2	0.02
5	43.1	0.16	1.3	0.02
6	28.2	0.12	1.1	0.04
7	29.3	0.15	1.1	0.01
8	6.4	0.06	1.4	0.02

[a] HBeAg was measured by radioimmunoassay, and HBcAg was measured by enzyme-linked immunoassay (see the text). *P/N* ratios of <2 in the HBeAg assay and an absorbance at 492 nm (A_{492}) of ≤0.04 in the HBcAg assay as determined in cell extracts from uninfected AGMK cells are considered negative. For the bacterial HBcAg reference (42; Materials and Methods), HBeAg *P/N* ratios were 5.9 and 32.7 in two experiments, and for HBcAg the absorbancy values at 492 nm were 0.06 and 0.29 (2.5 and 25 ng, respectively).

confluency of cells and multiplicity of infection. However, consistently positive *P/N* ratios with values up to 67.5 were obtained (Table 1). When HBcAg synthesized in *E. coli* was used for quantitation these optimal levels corresponded to approximately 50 ng per plate (3×10^6 cells) of HBcAg, which is about 1/10 of the HBsAg synthesized in vpHBs14-infected cells.

No expression of HBcAg or HBeAg was detected with virus pools from the control construct pHBc10/0 containing the HBV DNA insert in the opposite orientation of pHBc10 (Table 1). Surprisingly, C-gene expression was also not detectable with vpHBc15 (HBeAg by radioimmunoassay, $P/N \leq 2$; HBcAg by enzyme-linked immunosorbent assay, absorbancy at 492 nm of ≤0.04), which contained an additional 481 bp of HBV DNA upstream of the C-gene. The apparent lack of HBcAg synthesis observed cannot be attributed to the three- to fivefold-lower level of intracellular vpHBc15 DNA compared with vpHBc10 since a corresponding lower level of the antigens would have still been detected in our assays.

To analyze the nature of the antigens detected, we compared extracts from cells infected with virus pool vpHBc10 with partially purified HBcAg from liver cells of HBV-infected human patients. The proteins were separated according to size on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose filters, and probed with ^{125}I-labeled anti-HBcAg and anti-HBeAg immunoglobulins. In such an experiment (Fig. 3) HBcAg derived from human liver cells and the immunoreactive protein produced in AGMK cells were found to have identical electrophoretic mobilities, corresponding to a molecular mass of 21 kilodaltons (kd). No signal was observed with extracts from vpHBc15-, vpHBc10/0-, vpHBs14-, or mock-infected cells (data not shown).

It should be noted that the procedure used can detect HBcAg-HBeAg only by the anti-HBeAg-specific antibodies since HBcAg is converted completely into HBeAg by sodium dodecyl sulfate-mercaptoethanol treatment (27, 45, 47). This was confirmed in control immunoblots with anti-HBcAg immunoglobulins, which did not give a positive signal with authentic HBcAg after the blotting procedure (data not shown).

Mapping of transcripts from the C-gene region by S1 protection. To identify the signals responsible for initiation, termination, or processing of mRNA from the C-gene expressing recombinant pHBc10, transcripts produced after infection with this virus stock were analyzed by S1 mapping. With a 5′-labeled DNA probe covering the SV40-HBV junction (probe A, Fig. 4a) several protected DNA bands, 335, 305, and 205 to 215 bp in length, were detected as shown in Fig. 4b (lane 1). The 5′ ends of the S1-resistant fragments map on SV40 sequences around positions 150, 180, and 270 to 280. Both the positions and the heterogeneity of these potential transcription initiation sites add further support to the notion that the C-gene is expressed in vpHBc10 under the control of the SV40 late promoter. However, compared with transcription in SV40 virus (14) the positions of the 5′ ends of the SV40-HBV transcripts had shifted 50 to 100 nucleotides toward the SV40 origin of replication. A similar shift has been observed in SV40 mutants (33) and in SV40-preproinsulin (17) and SV40-HBsAg (2) hybrid constructions.

To identify the 3′ ends of transcripts downstream of the C-gene we used probe B, which covers the 3′-terminal part of the gene, the following 455 bp of HBV DNA, and the flanking SV40 DNA segment (Fig. 4a). RNA from vpHBc10-infected AGMK cells protected a DNA species of about 780 nucleotides from S1 digestion (Fig. 4b, lane 10). This size correlated with an HBcAg-specific mRNA covering all HBV sequences downstream of the C-gene and extending into SV40 up to the AAUAAA polyadenylation signal for SV40 late mRNA (35) at position 2657. No discrete bands shorter than 660 nucleotides that would correspond to a 3′ end mapping on HBV DNA could be detected indicating the absence of HBV-encoded termination or splice donor sites in the sequence analyzed.

To test whether the absence of C-gene expression in vpHBc15 could be explained at the level of transcription, RNA from vpHBc15-infected cells was analyzed with probe C covering the appropriate HBV DNA segment (Fig. 4a).

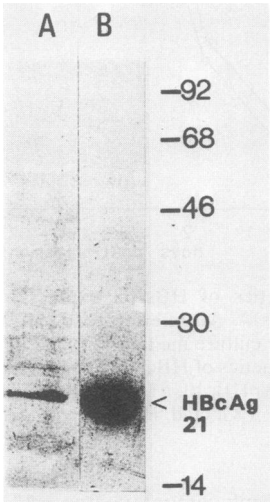

FIG. 3. Identification of HBcAg-HBeAg produced in AGMK cells by immunoblotting with ^{125}I-labeled anti-HBcAg and anti-HBeAg antibodies. (A) Cell extract from vpHBc10-infected AGMK cells. (B) HBcAg partially purified from an HBV-infected human liver. ^{14}C-labeled protein standards (phosphorylase *b* [92 kd], bovine serum albumin [68 kd], ovalbumin [46 kd], carbonic anhydrase [30 kd], and α-lactalbumin [14.3 kd]) are indicated.

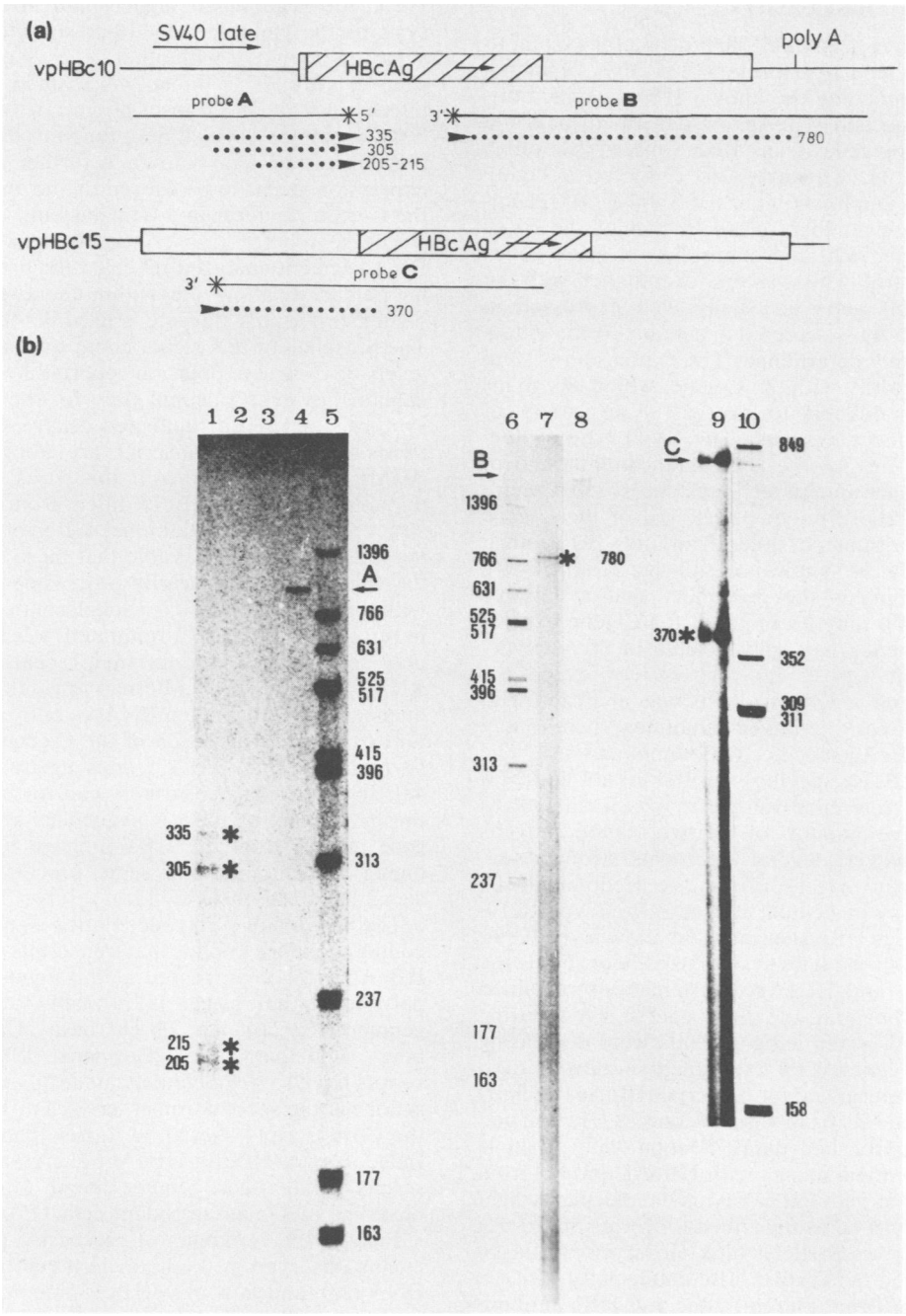

FIG. 4. S1 analysis of the transcripts produced by virus pool vpHBc10 and vpHBc15 in CV1 cells. (a) Map of pHBc10 and pHBc15, of the probes used for S1 analysis, and of the transcripts produced in CV1 cells with vpHBc10 and vpHBc15. HBV DNA is represented by boxes, and SV40 DNA is represented by thin lines. The position and orientation of the C-gene are indicated. Probes are indicated as lines; nucleotides at HBV positions 94, 432, and 2852 were labeled in probes A, B, and C, respectively. RNA-protected DNA from probes A, B, and C are depicted as dotted lines. (b) S1 analysis of the SV40-HBV hybrid RNAs produced in CV1 cells by vpHBc10 and vpHBc15. Lanes: 1, 2 and 3, RNA from vpHBc10-infected CV1 cells, SV40-infected cells, and mock-infected CV1 cells, respectively, hybridized with probe A and subjected to S1 digestion; 7, RNA from vpHBc10-infected CV1 cells hybridized with probe B and S1 digested; 9, RNA from vpHBc15-infected cells hybridized with probe C (arrow) and S1 digested; 4 and 8, probes A and B, respectively; 5, 6, and 10, molecular weight markers. Protected fragments are indicated with an asterisk.

With this probe a specific RNA 3′ terminus was detected as a strong S1-resistant DNA band of 370 ± 10 nucleotides (Fig. 4b, lane 9). This corresponds to RNA species with 3′ termini at HBV position 40 ± 10, at the appropriate distance of 20 nucleotides from the variant polyadenylation signal (35) TATAAA in position 16 to 22. This position is situated within the HBcAg gene and not in the segment deleted in vpHBc10.

DISCUSSION

In this study we have used an SV40 lytic vector system to transfer, to amplify, and to express HBV DNA segments carrying the genes encoding the known HBV-specific antigens HBsAg, HBcAg, and HBeAg. As described for SV40 recombinants carrying genes from other sources (see reference 36 for a review), we have also observed a highly efficient transfer and amplification of the SV40-HBV recombinants to yield copy numbers close to that of the SV40 helper virus ($\sim 10^5$ per cell) and expression of HBV genes under late SV40 control. This was first exemplified with the HBs gene, since this gene is usually well expressed in eucaryotic cells; HBsAg is secreted in a stable form, and its amounts can be readily determined. The expression system worked also satisfactorily with the C-gene, which has so far proven to be very difficult to express in a variety of eucaryotic gene transfer systems. The SV40 lytic system allowed for the first time synthesis of significant amounts of HBcAg-HBeAg in an animal cell system so that more detailed analysis of these polypeptides and of the C-gene specific transcripts became possible. In addition to this main objective of our study the synthesis of full-size 21-kd HBcAg from vpHBc10 eliminates the possibility that a putative precore sequence (49) may be required for C-gene expression since this sequence is largely deleted in this actively expressing construct.

Synthesis of C-gene specific proteins was analyzed with an HBcAg-specific enzyme-linked immunosorbent assay and an HBcAg-HBeAg-specific radioimmunoassay. The lack of a strictly HBeAg-specific sensitive assay and of a quantitated HBeAg reference sample makes it difficult to determine the relative amounts of the two related antigens with accuracy. However, several arguments strongly suggest that the 21-kd full-size HBcAg is the predominant C-gene product present in cellular extracts from vpHBc10-infected cells, whereas HBeAg remains at a low level due to its rapid breakdown in the lytic system used here. First, the ratio of the HBcAg and HBeAg determinations remained rather constant throughout the time course even during progressing cells lysis when antigen levels were decreasing (Fig. 2B), and between several experiments, and this ratio was similar to that determined for bacterial HBcAg (Table 1) and for HBcAg standards from various sources (H. Will and M. Roggendorf, unpublished data). Second, only a single protein of 21 kd, identical in size with HBcAg derived from human liver, was observed, but none in the molecular weight range between 19 to 14 kd as described for forms of HBeAg not associated with core particles (46). However, the 21-kd protein still contained the cryptic HBe antigenicity, since it was recognized after denaturation by the anti-HBe antibodies in the immunoblot experiment. Third, neither antigen was detectable in the culture medium, whereas HBeAg is normally predominantly found in the serum of HBV-infected patients. Finally, it should be kept in mind that HBcAg can be rapidly converted into HBeAg by proteolysis or denaturation or both (27, 45, 46). Thus, the most likely interpretation of our results is that, although constantly being formed from HBcAg, HBeAg is rapidly degraded further so that it is present only as a minor fraction. In contrast to the lytic system analyzed here, HBeAg was found to be the predominant C-gene product synthesized in HBV DNA containing stable cell lines, and in this case the antigen was detected in the culture medium only (15, 16).

To summarize this part of our study, we have shown that the SV40 lytic system used makes it possible to synthesize reasonable amounts of full-size HBcAg in animal cells in vitro. Although much higher than in other gene transfer systems the yields obtained are still well below the levels achieved in vivo or with other genes, e.g., the hepatitis B S-gene in vitro. This difficulty can in part be explained by interference on the transcriptional or translational level of HBV sequences, which may function differently in a heterologous system (see below). A further reduction of C-gene expression seems to be caused by the apparent instability of the C-gene products in a lytic system.

The search for transcriptional signals within the HBV DNA segment analyzed revealed the presence of a processing-polyadenylation site within the C-gene, and we have demonstrated that this site is used in the infected liver (2a). The presence of this signal could explain the relatively low levels of C-gene expression reported here and by others. It cannot, however, account fully for the absence of HBcAg synthesis in vpHBc15-infected cells, since only about two-thirds of the C-gene transcripts are processed at this signal in AGMK cells infected with this virus pool (R. Cattaneo, unpublished data). pHBc15 differs from the expressing construct pHBc10 by an additional 481 bp of HBV DNA preceding the C-gene. It is possible that these sequences influence the efficiency of the nearby processing-polyadenylation site within the C-gene. This is reminiscent of a similar situation in retroviruses, where a reiterated polyadenylation signal is only active in the long terminal repeat at the 3′ end of the RNA genome (50). Alternatively, these interfering sequences may influence mRNA stability or secondary structure or reduce translation of the C-gene by the presence of several additional AUG codons upstream of the gene (23, 48). In any case, our results lead to the question whether these "interfering" DNA sequences are also absent in C-gene mRNA from the HBV-infected liver, where a much higher efficiency of the same processing-polyadenylation signal has been observed (2a).

The position of a polyadenylation signal within the C-gene coding sequence implies that transcripts expressing HBcAG-HBeAg are not processed at this signal. Since the C-gene polyadenylation signal is probably unique in the HBV genome (R. Cattaneo, Ph.D. thesis, University of Heidelberg, Heidelberg, West Germany, 1983) and the C-transcripts have to be polyadenylated, it is conceivable that this is done at the second transit through it. If this were the case, the C-transcripts should be longer than the HBV genome. Indeed, two HBcAg-HBeAg expression-associated transcripts greater than genome length (4.4 and 4.0 kb) were observed by Gough in rodent cells (15).

Finally, the variability of processing-polyadenylation efficiency observed in the liver, in a cell line containing HBV DNA (2a), and now in vpHBc15-infected cells supports the speculation that modulation of processing-polyadenylation efficiency could be used to modulate gene expression in the HBV life cycle.

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