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Expression of Hepatitis B Virus Surface and Core Antigens: Influences of Pre-S and Precore Sequences†

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Amphotropic retroviral expression systems were used to synthesize hepatitis B virus surface antigen (HBsAg) and core antigen. The vectors permitted establishment of cell lines which expressed antigen from either the retroviral long terminal repeat or the mouse metallothionein-I promoter. HBsAgs were synthesized containing no pre-S sequences, pre-S(2) sequences alone, or pre-S(1) plus pre-S(2) sequences. Inclusion of pre-S(2) sequences did not affect the secretion or density of HBsAg particles but did reduce their mass by approximately 30%. Addition of pre-S(1) sequences almost completely abolished secretion of HBsAg and resulted in its localization in an aqueous-nonextractable pre- or early-Golgi cellular compartment. HBsAg was localized to the cytoplasm of the cell. This localization was unaffected by the presence of pre-S sequences in the antigen. Cell lines synthesizing hepatitis B antigens from core DNA fragments, containing or not containing precore sequences, secreted hepatitis B e antigen. However, the absence of precore DNA sequences resulted in additional synthesis of hepatitis core antigen, which was predominantly nuclear in localization.

Hepatitis B virus (HBV) is a 42-nm particle (the Dane particle) which comprises a 3.2-kilobase partially double-stranded DNA genome enclosed in a 27-nm nucleocapsid of hepatitis B core antigen (HBcAg) (39). The nucleocapsid is enveloped by a lipoprotein coat comprising cellular lipid and hepatitis B surface antigen (HBsAg). In addition, the Dane particle possesses an endogenous DNA polymerase activity and a polypeptide covalently attached to the 5′ end of the minus DNA strand (39). Because of the lack of a cell culture system to propagate HBV, much of the information regarding viral products has been derived from molecular analysis. HBV DNA has been cloned and sequenced, revealing four large open reading frames (ORFs) (10, 11, 27, 29, 40).

An ORF has been identified which codes for HBsAg (11, 29). The production of the major HBsAg, the 25-kilodalton (kDa) nonglycosylated and 28-kDa glycosylated polypeptides (P25/GP28), has been analyzed in a variety of expression systems (6, 7, 17, 25). Synthesis of these polypeptides in mammalian cells resulted in secretion of 22-nm particles with biophysical properties similar to those of subviral particles isolated from serum during HBV infection. In addition, particles can be synthesized and secreted which contain a relatively high proportion of the middle HBsAg polypeptide (GP33/GP36) (22). This polypeptide contains 55 additional amino acids [pre-S(2)] at the amino terminus of the P25/GP28 HBsAg which are encoded by a second in-frame translation initiation codon present in the HBsAg ORF. A third in-frame translation initiation codon exists in the HBsAg ORF. Translation from this initiation codon results in synthesis of the large HBsAg polypeptide (P43/GP46) containing 108 amino acids [pre-S(1)] at the amino terminus of the GP33/GP36 HBsAg (16).

Analysis of the core ORF revealed two in-frame translation initiation codons in several, but not all, HBV genomes (10, 11, 27, 29, 40). Expression systems using the second initiation codon have been analyzed and indicate that HBcAg is synthesized when this initiation codon is used (28, 32, 41). However, expression systems which include the first initiation codon, and therefore can code for the precore and core sequences, predominantly synthesize hepatitis B e antigen (HBeAg) (28, 32). This recombinant HBeAg may represent polypeptides similar to the HBeAg found in serum during HBV infection (38) of the HBeAg present cryptically on HBcAg particles (20).

In the current study, expression of HBsAg was extended to include each of the three in-frame initiation codons present in the HBsAg ORF. Syntheses of HBsAg, HBsAg containing pre-S(2), and HBsAg containing both pre-S(1) and pre-S(2) were compared in mouse fibroblasts. Synthesis of core antigens and consequences of inclusion of the precore sequence were also examined in the same system. In addition, this system, derived from an amphotropic retrovirus, was developed to examine the possibility of transmitting HBV antigen expression into a variety of different cell types by viral infection. This report describes the initial characterization of HBV antigens produced in mouse NIH 3T3 fibroblasts transfected with the amphotropic retroviral expression system.

MATERIALS AND METHODS

Plasmid constructions. The construction of amphotropic retrovirus vectors pARV1, pARV2, and pARV1MT is outlined in Fig. 1. The various steps in the production of these vectors were performed by standard techniques (21). The vector CistorNeo has been described previously (36). pARV1 was derived from CistorNeo by cloning the Moloney murine leukemia virus (Mo-MuLV) *Xba*I-*Sma*I fragment (coordinates 5325 to 5750) (34) from clone no. 48 (1) into the *Cla*I site 5′ to the neomycin phosphotransferase (*neo*) gene. This was achieved by converting the *Cla*I site 5′ to the *neo* gene in CistorNeo to an *Xba*I site by linker insertion. The *Sma*I site of the Mo-MuLV fragment was converted to a *Cla*I site by linker insertion. This generated an Mo-MuLV *Xba*I-*Cla*I DNA fragment which was used to replace the *Xba*I-*Cla*I *neo* fragment of the modified CistorNeo. From

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TABLE 1. HBV DNA fragments (subtype *ayw*) expressed in amphotropic retroviruses

HBV ORF cloned into retroviral vector	HBV DNA fragment coordinates[a]	Restriction enzyme sites[b]	Amphotropic retroviral vector	Expression vector[d]	G418-resistant cell line established after transfection with expression vector
			pARV2		3T3 pARV2
Surface (large)	2839–1990	*Bgl*II–*Bgl*II	pARV2	pARV2P1/2S	3T3 pARV2P1/2S
Surface (middle)	3166–1411	*Sau*I–*Fnu*DII	pARV2	pARV2P2S	3T3 pARV2P2S
Surface (major)	47–971	*Stu*I–*Stu*I	pARV1MT	pARV1MTS	3T3 pARV1MTS
Precore	1803–2804	*Hin*pI–*Hin*pI	pARV2	pARV2PC	3T3 pARV2PC
Core	≈1890–2804[c]	*Hin*pI (BAL31)–*Hin*pI[c]	pARV2	pARV2C	3T3 pARV2C
Core	≈1890–2804[c]	*Hin*pI (BAL31)–*Hin*pI[c]	pARV1MT	pARV1MTC	3T3 pARV1MTC

[a] The coordinates of the HBV fragments are derived from the DNA sequence (11) as reported in the GenBank genetic sequence data bank.
[b] Restriction enzyme sites defining the HBV fragments. These sites were modified to *Sal*I sites before cloning into the *Sal*I sites of the retoviral vectors.
[c] This 5′ coordinate is an estimate from detailed restriction enzyme analysis of this fragment, which represent a BAL31 5′ deletion of the *Hin*pI fragment with coordinates 1803 to 2804.
[d] The HBV DNA fragments were cloned into the amphotropic retroviral vectors such that the antigen ORF was oriented in the same direction as transcription from the LTR and the MT promoter.

the resulting plasmid, pARV1 was produced by cloning the *Cla*I *neo* DNA fragment of CistorNeo into the unique *Cla*I site. These cloning steps resulted in introduction of the Mo-MuLV 3′ splice acceptor site into CistorNeo such that splicing between this site and the 5′ splice donor site should produce a transcript coding for the *neo* gene product. In addition, a unique *Xba*I cloning site was generated for expression of ORFs from the long terminal repeat (LTR) promoter in the absence of transcript splicing. pARV2 was derived by insertion of a *Sal*I linker into the *Xba*I site of pARV1. pARV1MT was derived by cloning the *Stu*I-*Hin*dIII mouse metallothionein-I (MT) gene fragment, deleted for the internal *Bgl*II-*Sst*II fragment (coordinates −700 to +64 and +930 to +1241) (13, 33), into the *Xba*I site of pARV1. Before cloning the MT DNA fragment into pARV1, we modified the *Stu*I and *Hin*dIII sites to *Xba*I sites by linker insertion and cloned a *Sal*I linker into the site where the *Bgl*II-*Sst*II fragment was deleted. This generated a *Sal*I cloning site for expression of the ORFs from the MT promoter, which directs transcription in the same orientation as that of the retroviral LTR. The HBV DNA fragments described in Table 1, which were cloned into the *Sal*I site of either pARV2 or pARV1MT, were derived from the plasmid pCP10 (8) by modification of appropriate restriction enzyme sites to *Sal*I sites by linker insertion.

Cells and transfections. Mouse NIH 3T3 fibroblasts were grown, transfected, and selected as previously described (36), except that 15 μg of plasmid DNA without carrier DNA was used for each transfection. A cell line derived from multiple aminoglycoside antibiotic G418-resistant colonies was established from each transfection.

Antigen assays. HBsAg was measured by solid-phase radioimmunoassay (AUSRIA II; Abbott Laboratories, North Chicago, Ill.) with the HBsAg positive control (20 ng/ml) as a standard. HBeAg was measured by enzyme-linked immunosorbent assay (ELISA) (HBe EIA Diagnostic Kit; Abbott) with the HBeAg positive control (arbitrarily defined as 1 U/ml undiluted) as a standard. For all experiments, a single batch of control HBeAg was used as the standard. Pre-S(1) and pre-S(2) antigens were measured by ELISA as previously described (23). Briefly, pre-S(1)- and pre-S(2)-specific monoclonal antibodies (MA18/7 [16] and 4408 [19], respectively) were used on the solid phase, and horseradish peroxidase-labeled monoclonal antibody to HBsAg, provided by P. Kaplan (Ortho Laboratories, Raritan, N.J.), was used as the probe. HBcAg was measured by ELISA with a monoclonal antibody to HBcAg (3120) on the solid phase, and a horseradish peroxidase-labeled monoclonal antibody to HBcAg (3105) as the probe. This assay is HBcAg specific and does not detect HBeAg (38).

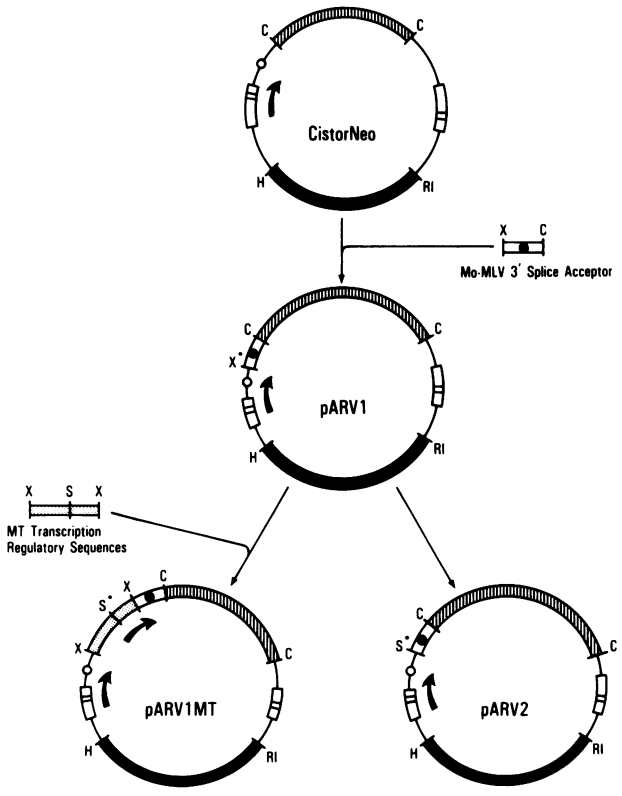

FIG. 1. Construction of amphotropic retrovirus vectors pARV1, pARV2, and pARV1MT. CistorNeo has been described previously (36). Sequence elements in the expression vectors are the Ampho8 LTR and the 5′ splice donor site ( ), the Ampho8 LTR ( ), the Mo-MuLV 3′ splice acceptor site ( ), the 5′ and 3′ splice sites (○ and ●, respectively), the neomycin resistance gene ( ), the MT gene transcription regulatory sequences ( ), and pUC9 ( ). The symbol ( ) indicates the direction of transcription from the LTR and the MT promoter. Restriction enzyme sites: C, *Cla*I, H, *Hin*dIII, RI, *Eco*RI, S, *Sal*I, and X, *Xba*I. The unique restriction sites used to clone ORFs are indicated (*).

Antigen assays were performed on cell culture media or cell lysates after suitable dilutions in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.4], 145 mM NaCl) containing 1% (wt/vol) bovine serum albumin, 5% (vol/vol) fetal calf serum, and 0.005% (vol/vol) Tween 20. Cell lysates were prepared by washing 5×10^6 cells in PBS and suspending the cells in 1 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride. The cell suspensions were subjected to three cycles of freezing and thawing followed by centrifugation for 30 min at 12,800 × *g*. The supernatant represented the cell lysate.

Analysis of HBsAg. Secreted HBsAg was partially purified by ammonium sulfate precipitation and CsCl density gradient centrifugation as previously described (22). The partially purified HBsAg was characterized further by immunogold electron microscopy (4) and sucrose gradient analysis. HBsAg (0.2 ml) was sedimented through 4.2 ml of 5 to 20% (wt/vol) sucrose gradients prepared in PBS containing 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged for 45 min at 50,000 rpm in an SW60 rotor at 20°C.

Sucrose gradient analysis of HBeAg and HBcAg. Cell culture media and cell lysates (0.4 ml), derived as described in the antigen assay section, were sedimented through 4 ml of 5 to 20% (wt/vol) sucrose gradients prepared in 10 mM Tris hydrochloride (pH 7.6)–1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged for various times at 50,000 rpm in an SW60 rotor at 20°C.

Immunoblotting. Cell lysates were prepared by washing 5×10^6 cells in PBS and homogenizing them in 0.3 ml of sample buffer (18). Samples were boiled for 10 min and then centrifuged for 15 min at 12,800 × *g*. The supernatants were separated on 12.5% polyacrylamide gels as previously described (18). Medium samples were boiled with an equal volume of 2× sample buffer before electrophoresis. Transfer of the separated proteins to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.; BA-85; 0.45-μm pore size) was as previously described (16). Membranes were rinsed in 25 mM Tris hydrochloride (pH 7.5)–500 mM NaCl (TN) for 10 min at 22°C and blocked in TN containing 3% (wt/vol) gelatin and 0.05% (vol/vol) Tween 20 for 8 h at 22°C. The membranes were probed with rabbit anti-peptide antiserum specific for HBsAg (anti-P49a) (12) and HBcAg (unpublished data) in 20 ml of TN containing 1% (wt/vol) gelatin and 0.05% (vol/vol) Tween 20 for 16 h at 22°C. The membranes were washed four times in 100 ml TN containing 0.05% (vol/vol) Tween 20 for 15 min at 22°C and developed with 5 μCi of [^{125}I]protein A (Amersham Corp., Arlington Heights, Ill.) in 50 ml of TN containing 1% (wt/vol) gelatin and 0.05% (vol/vol) Tween 20 for 1 h at 22°C. After washing the membranes four times in TN containing 0.05% (vol/vol) Tween 20 for 15 min at 22°C, we dried the immunoblot and subjected it to autoradiography.

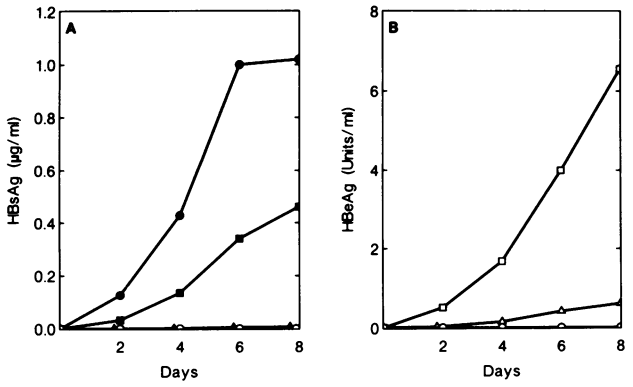

FIG. 2. Time course of the secretion of (A) HBsAg and (B) HBeAg into cell culture medium. Cells (10^6) were plated on a 10-cm (diameter) tissue culture plate, and the medium was changed on the following day. Samples (0.5 ml) were collected 2, 4, 6, and 8 days later and assayed for HBV antigens. Cells were confluent by day 2. Cell lines: 3T3 pARV2P1/2S (▲), 3T3 pARV2P2S (■), 3T3 pARV1MTS (●), 3T3 pARV2PC (□), 3T3 pARV2C (△), and 3T3 pARV2 (○).

Immunofluorescence. The localization of HBV antigens in cell lines was determined by immunofluorescence microscopy (4). Cells were grown on microscope slides, fixed for 1 min in ice-cold acetone, and stained with appropriate antibodies. Pre-S(1) polypeptides, pre-S(2) polypeptides, HBsAg, HBcAg, and HBeAg were assayed indirectly by probing with specific mouse monoclonal antibodies and staining with a fluorescein-conjugated, affinity-purified goat antibody to mouse immunoglobulin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The specificity of the various antibodies was assessed by examining the staining of the 3T3 pARV2 cell line.

RESULTS

Construction of retroviral vectors. The construction of the amphotropic retroviral vectors pARV1, pARV2, and pARV1MT is described in Fig. 1. Insertion of the Mo-MuLV 3′ splice acceptor site into CistorNeo resulted in the production of pARV1. pARV2 is identical to pARV1 except the unique *Xba*I site was modified by linker insertion so that a unique *Sal*I site was present at the same location. This *Sal*I site can be used for cloning and expressing exogenous ORFs. After transfection of this vector into mammalian cells, expression from the retroviral LTR produces both the product of the *neo* gene from a spliced transcript and the product of the ORF from an unspliced transcript. The *neo* gene product confers G418 resistance to the cells and permits their selection (37). Antigen synthesis occurs from the first translation initiation codon present in the ORF. The vector, pARV1MT, was derived by insertion of the MT gene regulatory sequences into the *Xba*I site of pARV1 (Fig. 1). The direction of transcription from the MT promoter is the same as that from the retroviral LTR. pARV1MT codes for the *neo* gene product from a spliced transcript in a manner similar to those of pARV1 and pARV2, but antigen synthesis from an ORF is directed by the MT promoter rather than the retroviral LTR. ORFs expressed from the MT promoter are cloned into the unique *Sal*I site of pARV1MT. The DNA fragments containing the various HBV ORFs were cloned into the retroviral vectors as indicated in Table 1.

Secretion of HBV antigens. After transfection of the expression vectors into NIH 3T3 cells, G418-resistant cell lines were established and examined for expression of HBV antigens. Secretion of HBsAg and HBeAg into the culture medium was examined (Fig. 2). Cell lines 3T3 pARV1MTS and 3T3 pARV2P2S secreted relatively large amounts of HBsAg, whereas cell line 3T3 pARV2P1/2S secreted HBsAg at approximately 1% of the level of these two cells lines (Fig. 2a; Table 2). Cell lines 3T3 pARV2PC and 3T3 pARV2C both secreted HBeAg (Fig. 2b). However, inclusion of the precore sequence in the expression vector resulted in an approximately 10-fold higher level of HBeAg secretion (Table 2).

Analysis of soluble intracellular HBV antigens. The levels of soluble intracellular HBsAg in the cell lines were approx-

TABLE 2. Estimate of intracellular and extracellular HBV antigens

Cell line	Avg rate of secretion of[a]:		Amt of soluble intracellular[b]:		Rate of secretion/intracellular HBsAg ratio	Rate of secretion/intracellular HBeAg ratio
	HBsAg (ng/10^6 cells per 24 h)	HBeAg (U/10^6 cells per 24 h)	HBsAg (ng/mg of protein)	HBeAg (U/mg of protein)		
3T3 pARV2P1/2S	2		5		0.4	
3T3 pARV2P2S	115		99		1.2	
3T3 pARV1MTS	255		174		1.5	
3T3 pARV2PC		1.64		0.19		8.6
3T3 pARV2C		0.15		3.16		0.05

[a] Rate of secretion was calculated from the following formula: (HBV antigen concentration × 10)/(5 × 8), based on the HBV antigen concentration on day 8, a tissue culture medium volume of 10 ml, and 5 × 10^6 cells per confluent plate of cells.

[b] Soluble intracellular HBV antigen was determined in cell lysates prepared by freezing and thawing as described in Materials and Methods. Protein concentrations were determined by the Bradford assay (3).

imately proportional to the rates of secretion of HBsAg from these cells (Table 2). This suggested that the rate of secretion may be related to the amount of soluble HBsAg inside the cell. Cell line 3T3 pARV2C had greater than 10 times more soluble intracellular HBeAg than did cell line 3T3 pARV2PC (Table 2). This contrasts with the rate of secretion of HBeAg from these two cell lines. These observations suggest that inclusion of the precore sequence in the expression vector results in synthesis of polypeptides which are preferentially secreted compared with polypeptides lacking this sequence.

Antigenicities of secreted and soluble intracellular HBV antigens. The products of the three HBsAg-producing cell lines were analyzed by ELISA for synthesis of soluble pre-S polypeptides (Table 3). As expected, cell line 3T3 pARV1MTS produced HBsAg containing no pre-S sequences. The pre-S(2) content of the HBsAg produced by cell line 3T3 pARV2P2S was estimated at approximately 20% for soluble intracellular antigen and 50% for secreted antigen. This is consistent with inclusion of the pre-S(2) DNA sequence in the expression vector used to generate this cell line. Because of the low level of soluble intracellular antigen produced by the 3T3 pARV2P1/2S cell line, the presence of pre-S sequences was not detectable on this antigen. However, a low level (5%) of pre-S(2)-containing polypeptides was detected on the HBsAg secreted from this cell line. In addition, pre-S(1) sequences were detected on the secreted HBsAg. As each large HBsAg polypeptide contains the pre-S(2) sequence in addition to the pre-S(1) sequence, it is likely that this secreted HBsAg contains no more than 5% pre-S(1)-containing molecules.

The antigens produced by the 3T3 pARV2PC and 3T3 pARV2C cell lines were analyzed for HBeAg and HBcAg activities (Table 4). Since the Abbott HBe EIA detects both HBeAg and HBcAg, the HBcAg-specific assay was required to differentiate between these two antigens. HBcAg was detected only in the soluble intracellular compartment of the 3T3 pARV2C cell line. In combination with the data in Table 2, this indicates that the presence of the precore sequence results in the synthesis of HBeAg, which is readily secreted. In the absence of the precore sequence, HBcAg is synthesized and accumulates inside the cell. Furthermore, since HBeAg was detected in the cell culture medium of the 3T3 pARV2C cell line, production of HBeAg does not necessarily require the precore sequence.

CsCl and sucrose density gradient analysis of HBsAg. Secreted HBsAg from cell lines 3T3 pARV1MTS, 3T3 pARV2P2S, and 3T3 pARV2P1/2S was precipitated from cell culture medium with 45% $(NH_4)_2SO_4$, pH7.5, and analyzed by CsCl isopycnic density gradient centrifugation (Fig. 3A to C). The densities of the HBsAg particles from these cell lines were estimated to be 1.21, 1.22, and 1.26 g/ml, respectively. This indicated that the presence of approximately 50% pre-S(2)-containing polypeptide did not significantly alter the density of the HBsAg. However, it appeared that a relatively small amount of pre-S(1)-containing polypeptide increased the density of the HBsAg. This HBsAg also showed a broad range of densities, suggesting a spectrum of HBsAg particles containing larger proportions of pre-S(1) sequences as the density of the HBsAg increased (Fig. 3C). Electron microscopy of the CsCl gradient fractions containing HBsAg revealed particles having estimated diameters of 23 and 21 nm for HBsAg lacking pre-S sequences and HBsAg containing pre-S(2) sequences, respectively (Fig. 3A and B). The low yield of secreted HBsAg from the 3T3 pARV2P1/2S cell line did not permit detection of pre-S(1)-containing particles by electron microscopy.

HBsAg particles from the CsCl gradients were further characterized by sucrose gradient sedimentation analysis (Fig. 4A to C). This demonstrated that the pre-S(2)-containing particles (Fig. 4B) sedimented at approximately 70% of the rate of HBsAg particles lacking pre-S sequences (Fig. 4A). This has been confirmed in similar experiments in which the HBsAgs were sedimented for longer times (unpublished data). The difference in the rates of sedimentation of these particles presumably reflects a difference in their

TABLE 3. Pre-S polypeptide compositions of HBsAgs

Cell line	Secreted HBsAg		Soluble intracellular HBsAg	
	Pre-S(1)[a]	% Pre-S(2)[b]	Pre-S(1)[a]	% Pre-S(2)[b]
3T3 pARV2P1/2S	+	5	ND[c]	ND
3T3 pARV2P2S	0	50	0	20
3T3 pARV1MTS	0	0	0	0

[a] The symbol + means that the HBsAg contained detectable levels of pre-S(1) sequences. Estimation of the percentage of polypeptides containing pre-S(1) sequences was not possible because of lack of a suitable standard.

[b] The amount of pre-S(2)-containing polypeptides was estimated by comparison with recombinant HBsAg with ≈35% pre-S(2)-containing polypeptides (22).

[c] ND, Not detectable.

TABLE 4. Antigens produced by 3T3 pARV2PC and 3T3 pARV2C cell lines

Cell line	Secreted antigen		Soluble intracellular antigen	
	HBeAg[a]	HBcAg[b]	HBeAg[a]	HBcAg[b]
3T3 pARV2PC	+	−	+	−
3T3 pARV2C	+	−	+	+

[a] Determined by the Abbott HBe EIA Diagnostic Kit.

[b] Determined by the HBcAg-specific ELISA described in Materials and Methods.

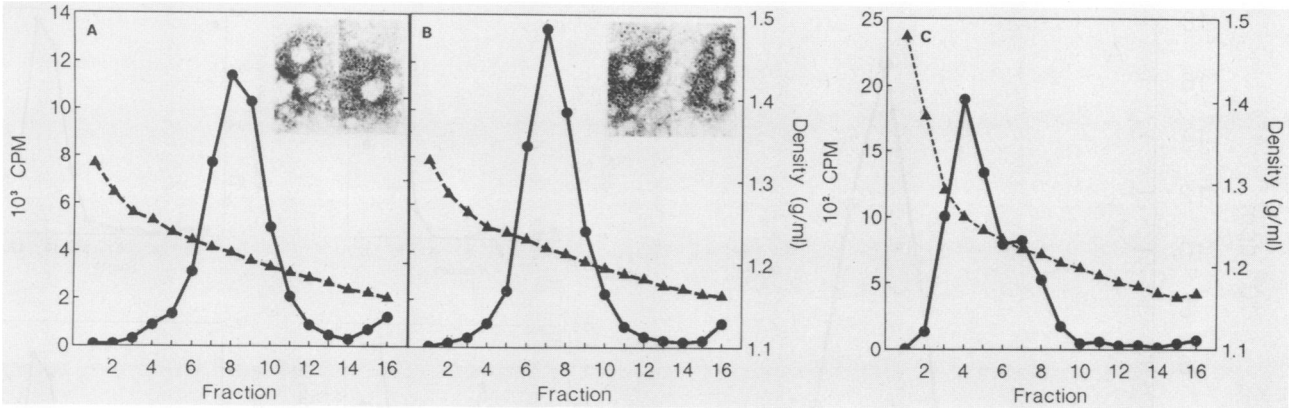

FIG. 3. CsCl density gradient analysis of secreted HBsAg from cell lines (A) 3T3 pARV1MTS, (B) 3T3 pARV2P2S, and (C) 3T3 pARV2P1/2S. Gradients were fractionated from the bottom (fraction 1), and HBsAg was determined by the AUSRIA II assay. Symbols: ●, counts per minute; ▲, density. The inserts show electron microscopic analyses of the negatively stained HBsAg particles isolated from the CsCl gradients.

relative masses. A 30% difference in mass would predict a spherical particle diameter of 19.7 nm for pre-S(2)-containing particles, assuming a diameter of 22.0 nm for HBsAg particles. This is consistent with the observed electron microscopy data (Fig. 3A and B). In addition, it was apparent that approximately half of the pre-S(1)-containing HBsAg sedimented at the same rate as HBsAg lacking pre-S sequences, and the remainder represented larger particles (Fig. 4C). These larger particles may represent filaments which preferentially contain pre-S(1) polypeptides (16).

Sucrose density gradient sedimentation analysis of HBeAg and HBcAg. The relative sizes of HBeAg and HBcAg were investigated by sucrose gradient analysis. HBeAg synthesized in each of the various cell lines was slow sedimenting and therefore nonparticulate in nature (Fig. 5A to D). However, a significant proportion of the HBcAg activity measured in 3T3 pARV2C cell lysate was fast sedimenting, suggesting that this HBcAg has a particulate nature (Fig. 5C and E). The rate of sedimentation of the fast-sedimenting HBcAg in this cell lysate was similar to that observed for purified recombinant HBcAg (Biogen S.A., Geneva, Switzerland), indicating that it may be similar in nature to the 27-nm HBcAg particles synthesized in *Escherichia coli* or isolated from HBV-infected human liver (5). In addition, some of the HBcAg activity was slower sedimenting, suggesting the presence of subparticulate HBcAg in this cell line. The relative amounts of HBcAg- and HBeAg-reactive material in the various gradient fractions also suggested that the lysate of 3T3 pARV2C cells contained significant amounts of slow-sedimenting HBeAg.

Immunoblotting analysis of HBV antigens. Characterization of HBV antigens by immunoblotting was performed to determine the total intracellular antigen content and to estimate the relative sizes of the various polypeptides. Analysis of intracellular HBsAg indicated that cell line 3T3 pARV1MTS contained polypeptides of 25 and 27 kDa, cell line 3T3 pARV2P2S contained polypeptides of 25, 27, and 32 kDa, and cell line 3T3 pARV2P1/2S contained polypeptides of 25, 27, and 43 kDa, whereas the secreted CHO HBsAg particles contained polypeptides of 25, 28, and 33 kDa (Fig. 6A). The differences in polypeptide sizes were confirmed by immunoblotting analysis of mixtures of intracellular and secreted HBsAgs synthesized by these cell lines (data not shown). The presence of polypeptides of 27 rather than 28 kDa and 32 rather than 33 kDa suggested that the glycosylated intracellular HBsAgs contain only high-mannose, rather than complex, oligosaccharide chains, as has been previously demonstrated for this antigen (30, 31). This indicated that the intracellular HBsAg resides in a pre- or early-Golgi cellular compartment. Cell line 3T3 pARV2P2S produced the 25-kDa HBsAg in addition to the 32-kDa HBsAg, possibly because of inefficient utilization of the pre-S(2) translation initiation codon. Cell line 3T3 pARV2P1/2S also produced the 25- and 27-kDa HBsAgs in addition to the expected 43-kDa HBsAg. However, as no 32-kDa HBsAg was apparent, it seems unlikely that inefficient translation initiation can explain this observation. The 25- and 27-kDa HBsAgs were probably produced from transcripts initiating at the endogenous HBV promoter located in the pre-S(1) region of the viral genome (35). The levels of intracellular HBsAg in cell lines 3T3 pARV1MTS and 3T3 pARV2P2S correlated with the amounts of soluble antigen (Table 2). However, it was apparent that the total antigen present in cell line 3T3 pARV2P1/2S was approximately equivalent to that in the other two cell lines. This observation, together with the relatively low level of soluble HBsAg present in the 3T3 pARV2P1/2S cell line (Table 2), indicated that approximately 95% of the total intracellular HBsAg was present in an aqueous-nonextractable cellular compartment of this cell line. This change in compartmentation is attributable to a property located, at least in part, in the pre-S(1) region of the 43-kDa polypeptide since the 32-kDa HBsAg was in the soluble intracellular compartment. The coexistence of the 25- and 27-kDa and 43-kDa HBsAgs resulted in the major polypeptides residing in the aqueous-nonextractable compartment, whereas when the major polypeptides were synthesized alone they were present in the soluble cellular compartment. This indicated that the 25- and 27-kDa polypeptides interact with the 43-kDa HBsAg and are thereby trapped in the aqueous-nonextractable cellular compartment, preventing secretion of the major polypeptides.

Analysis of the product of the 3T3 pARV2C cell line indicated that, in the absence of the precore sequence, the predicted 21-kDa HBcAg polypeptide was synthesized and accumulated within the cell (Fig. 6B, lanes 1 and 4). The presence of this polypeptide and the fast-sedimenting HBcAg in lysates of these cells (Fig. 5C) is consistent with this polypeptide forming a particulate structure similar to that observed in other systems (5). In addition, it suggests that this polypeptide can also form the slow-sedimenting

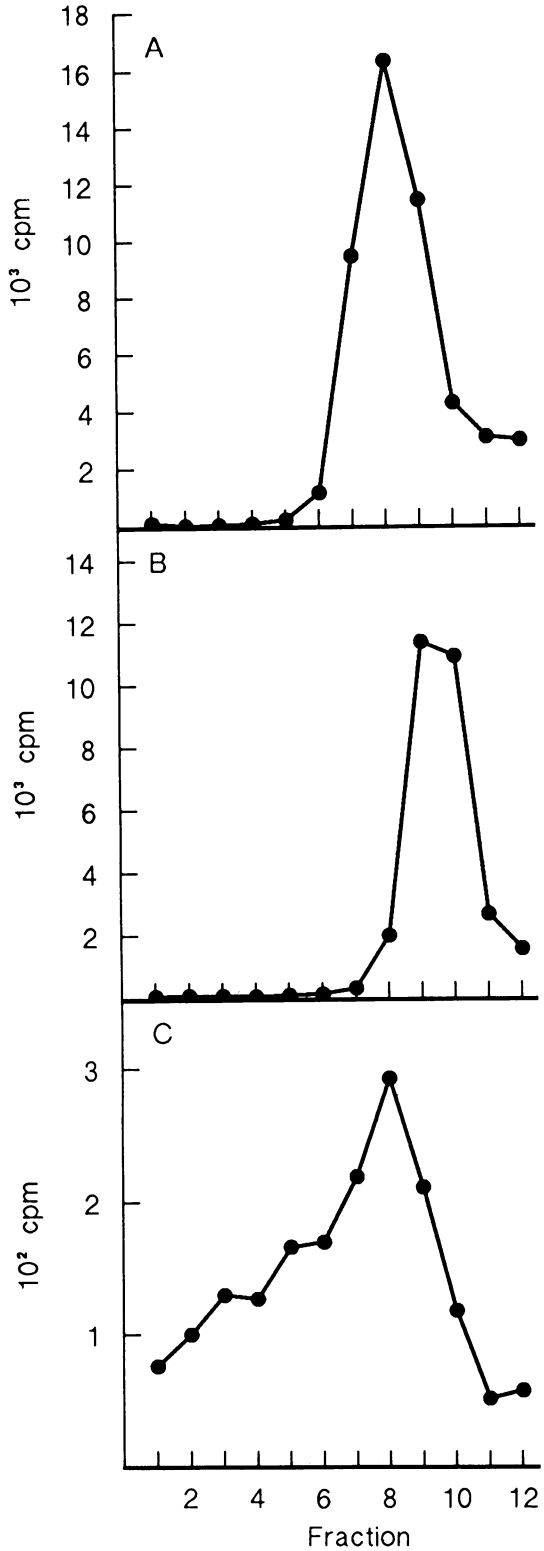

FIG. 4. Sucrose gradient analysis of secreted HBsAg from (A) 3T3 pARV1MTS cell line CsCl gradient fractions 7 to 9 (Fig. 3A), (B) 3T3 pARV2P2S cell line CsCl gradient fractions 6 to 8 (Fig. 3B), and (C) 3T3 pARV2P1/2S cell line CsCl gradient fractions 4 and 5 (Fig. 3C). The pooled CsCl gradient fractions were dialyzed against PBS containing 1 mM phenylmethylsulfonyl fluoride before sucrose gradient analysis. Gradients were fractionated from the bottom (fraction 1), and HBsAg was determined by the AUSRIA II assay. cpm, Counts per minute.

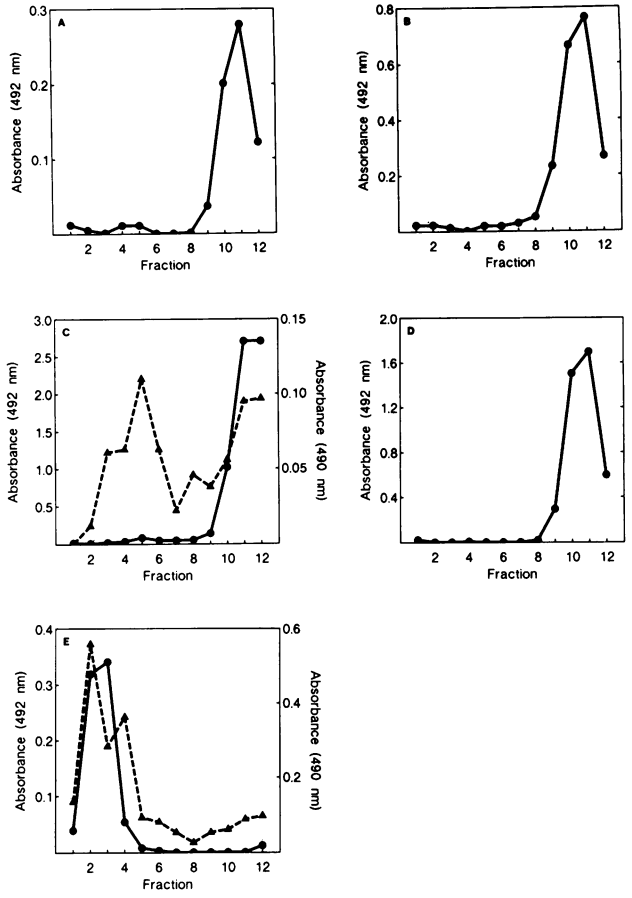

FIG. 5. Sucrose gradient analysis of HBeAg and HBcAg from (A) medium of 3T3 pARV2C cells, (B) medium of 3T3 pARV2PC cells, (C) cell lysate of 3T3 pARV2C cells, (D) cell lysate of 3T3 pARV2PC cells, and (E) recombinant HBcAg (Biogen). HBeAg plus HBcAg was determined by HBe EIA (A_{492}, ●), and HBcAg was measured with the HBcAg-specific ELISA (A_{490}, ▲). Samples A, B, and D were centrifuged for 2 h, and samples C and E were centrifuged for 45 min as described in Material and Methods. Gradients were fractionated from the bottom (fraction 1).

HBeAg and subparticulate HBcAg (Fig. 5C), although it is possible that smaller polypeptides of limited abundance account for a proportion of the observed antigens. The 3T3 pARV2C cell line secreted HBeAg (Table 4) and, from longer exposures (data not shown) of the autoradiogram shown in Fig. 6B, lane 7, this correlates with the presence of equal amounts of core polypeptides of 15 and 21 kDa in the cell culture medium.

The inclusion of the precore sequence in the 3T3 pARV2PC cell line was predicted to result in synthesis of a 24-kDa polypeptide (24). However, the observed intracellular antigen was a polypeptide of approximately 21 kDa (Fig. 6B, lane 3). This antigen had no HBcAg activity (Table 4) and therefore is probably different from the 21-kDa polypeptide synthesized by the pARV2C cell line. This suggests that the 21-kDa polypeptide synthesized in the 3T3 pARV2PC cell line is a processed product of the predicted 24-kDa polypeptide coded for by the precore ORF. The secreted products of this cell line were a major polypeptide of approximately 15 kDa and two minor polypeptides of approximately 18 and 21 kDa (Fig. 6B, lane 6). The size of the major secreted polypeptide is similar to that found in HBeAg from serum (38) and further suggests that a larger precore

polypeptide was processed in 3T3 cells to yield secreted HBeAg. In addition, it should be noted that the relative abundance of the total intracellular core-specific polypeptides (Fig. 6B, lanes 3 and 4) correlates with the levels of soluble intracellular antigen (Table 2) in these cell lines. This suggests that, unlike the case of pre-S(1)-containing polypeptides, the precore sequence does not partition antigen into an aqueous-nonextractable cellular compartment but rather promotes secretion of HBeAg.

Cellular localization of HBV antigens. The distribution of the HBV antigens in the various cell lines was analyzed by immunofluorescence (Fig. 7A to G). HBsAg was observed in the cytoplasm of the three cell lines producing this antigen (Fig. 7A to C). In addition, pre-S(2) sequences were detected in the 3T3 pARV2P2S and 3T3 pARV2P1/2S cell lines (Fig. 7D and E), whereas pre-S(1) sequences were detected only in the 3T3 pARV2P1/2S cell line (Fig. 7F), in agreement with the immunoblotting analysis (Fig. 6A). In addition, it appeared that the antigen containing pre-S(1) sequences had a coarser granular appearance than HBsAg lacking this sequence. As expected, the pre-S sequences were localized to the cytoplasm in a manner similar to that of HBsAg.

With an HBcAg-specific monclonal antibody, the 3T3 pARV2C and 3T3 pARV1MTC cell lines exhibited positive immunofluorescence localized predominantly to the nucleus (Fig. 7G). Qualitatively, these two cell lines had the same distribution of immunofluorescence, but quantitatively the 3T3 pARV1MTC cell line showed a greater signal. These results are consistent with the presence of intracellular HBcAg in these cell lines (Table 4; Fig. 5C). The distribution of HBcAg in these cell lines indicates that, at least in this heterologous expression system where HBV replication is absent, HBcAg can be transported to the nucleus, presumably by a mechanism analogous to that occurring during

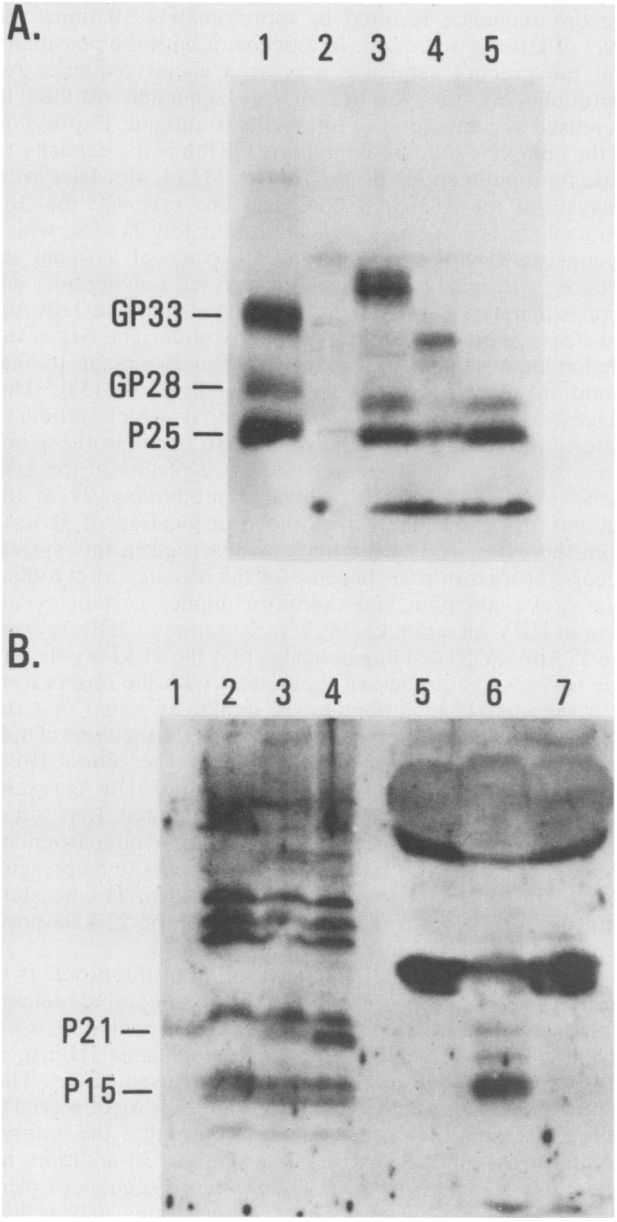

FIG. 6. Immunoblotting analysis of HBV antigen. (A) Immunoblot probed with HBsAg-specific anti-peptide antiserum. Lanes: 1, 60 ng of CHO cell-derived, secreted HBsAg (22); 2 to 5, 200 μg of protein from a cell line lysate. Cell line (lanes: 2, 3T3 pARV2; 3, 3T3 pARV2P1/2S; 4, 3T3 pARV2P2S, and 5, 3T3 pARV1MTS. (B) Immunoblot probed with HBcAg specific anti-peptide antiserum. Lanes 1, 60 ng of recombinant HBcAg (Biogen); 2 to 4, 200 μg of protein from a cell line lysate; 5 to 7, 50 μl of cell culture medium. Cell line (lanes): 2 and 5, 3T3 pARV2; 3 and 6, 3T3 pARV2PC, and 4 and 7, 3T3 pARV2C.

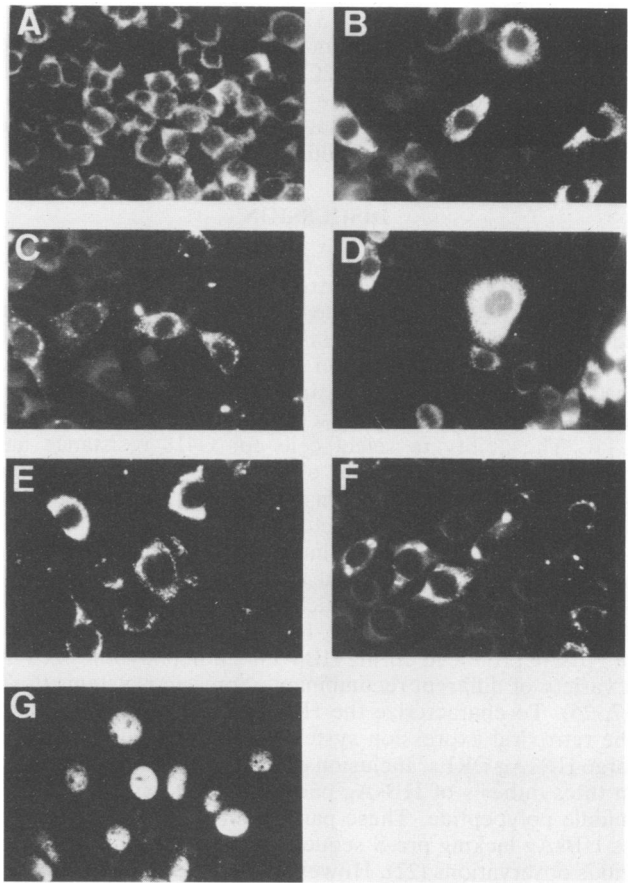

FIG. 7. Immunofluorescence analysis of HBV antigen-expressing cell lines. Panels: A, B, and C, 3T3 pARV1MTS, 3T3 pARV2P2S, and 3T3 pARV2P1/2S cells, respectively, stained with monoclonal antibody to HBsAg (provided by P. Kaplan [Ortho Laboratories]); D and E, 3T3 pARV2P2S and 3T3 pARV2P1/2S cells, respectively, stained with pre-S(2)-specific monoclonal antibody 5520 (26); F, 3T3 pARV2P1/2S cells stained with pre-S(1)-specific monoclonal antibody MA18/7 (16); G, 3T3 pARV1MTC cells stained with monoclonal antibody to HBcAg, 3120 (38).

HBV liver infection. The 3T3 pARV2PC cell line was negative for HBcAg. Immunofluorescence analysis of the 3T3 pARV2PC, 3T3 pARV2C, and 3T3 pARV1MTC cell lines for HBeAg was negative with three different monoclonal antibodies, suggesting that this assay was not sensitive enough to detect the intracellular antigen.

DISCUSSION

An amphotropic retroviral vector system was developed for the expression of ORFs introduced into mammalian cells. This system represents three retroviral vectors which, in addition to expressing antigens from ORFs cloned into the appropriate unique restriction site, can confer resistance to the antibiotic G418 by expressing the product of the bacterial *neo* gene from a spliced transcript initiating in the retroviral LTR. The ability to select cells for G418 resistance has permitted the establishment of stable cell lines expressing various HBV antigens. In this study, the properties of the various antigens were characterized.

The 3T3 pARV1MTS cell line synthesizes and secretes the major HBsAg. It is localized to the cytoplasm of the cells and is secreted as 22-nm particles with a density of 1.2 g/ml. The major HBsAg, therefore, has properties similar to those of HBsAg produced during HBV infection or synthesized by a variety of different recombinant expression systems (6, 7, 17, 25). To characterize the HBsAg ORF further, we used the retroviral expression system to express the middle and large HBsAg ORFs. Inclusion of the pre-S(2) region resulted in the synthesis of HBsAg particles comprising about 50% middle polypeptide. These particles have the same density as HBsAg lacking pre-S sequences, in agreement with previous observations (22). However, sucrose gradient analysis indicated that the HBsAg containing pre-S(2) sequences forms particles smaller than those of HBsAg lacking pre-S sequences. This suggests that the synthesis of a high percentage of pre-S(2)-containing polypeptides can affect subunit packaging and, therefore, the nature of the assembled particle. This might be important in the HBsAg interactions involved in virion assembly. In addition, the intracellular HBsAg containing pre-S(2) sequences is soluble and localized to the cytoplasm. Inclusion of the pre-S(1) sequence dramatically affects the properties, but not the cytoplasmic location, of the HBsAg. The limited amount of HBsAg secreted from the 3T3 pARV2P1/2S cell line contains a small amount of pre-S(1)- and pre-S(2)-containing polypeptides. Furthermore, secreted particles from the pARV2P1/2S cell line exhibited increased density, indicating 3T3 that the presence of pre-S(1) sequences alters the nature of the secreted HBsAg. This antigen is particulate, with approximately half of the HBsAg showing the same sedimentation profile as HBsAg lacking pre-S sequences. This may, therefore, represent particles similar to those observed for HBsAg lacking pre-S sequences except that they contain a small amount of pre-S(1)- and pre-S(2)-containing polypeptides. The larger particulate HBsAg containing pre-S(1) sequences may represent filaments of various sizes. The intracellular HBsAg containing pre-S(1) sequences does not partition into the soluble cellular compartment despite total intracellular antigen levels similar to those of the 3T3 pARV1MTS cell line. This indicates that inclusion of the pre-S(1) sequence results in HBsAg becoming intimately associated with an aqueous-nonextractable cellular fraction, possibly the membranes of the endoplasmic reticulum, the pre- or early-Golgi compartment. This localization is implicated since the glycosylated intracellular HBsAg contains only high-mannose, rather than complex, oligosaccharide chains (9, 30, 31). In addition, the major polypeptide, which is soluble and secretable when synthesized alone, is trapped when coexpressed with the large polypeptide. This may reflect interactions between the large and major polypeptides similar to those which presumably occur in the Dane particle (16). It is possible that these properties of the recombinant HBV large polypeptide reflect functions associated with the pre-S(1) region, which may be relevant in the life cycle of the virus.

Expression of the core ORF with or without the precore sequence results in secretion of HBeAg. Inclusion of the precore sequence resulted in approximately 10 times the level of HBeAg secretion. In agreement with the possibility that the precore sequence acts as a signal sequence for secretion (28), the absence of this sequence resulted in increased accumulation of intracellular antigen. Expression of the complete precore-containing ORF has the capacity to code for a polypeptide of 24 kDa (10, 11, 24, 40). However, analysis of the 3T3 pARV2PC cell line revealed that the intracellular HBeAg peptide is approximately 21 kDa, which is consistent with cotranslational cleavage of a signal sequence by a signal peptidase. The secreted polypeptides are approximately 15, 18, and 21 kDa, suggesting that HBeAgs are cleavage products of a larger polypeptide. The size of the predominant secreted HBeAg (15 kDa) is similar to that found in sera from HBV-infected individuals (38). This suggests that a process similar to that which produces HBeAg during HBV infection may also occur in these cell lines. Absence of the precore sequence results in the synthesis of HBcAg, which is located predominantly in the nucleus. It seems likely that the translocation of HBcAg from the cytoplasm to the nucleus observed in this system occurs, at least in part, because of the absence of cytoplasmic viral replication and therefore mimics certain events seen in HBV infection (2, 14, 15). Secretion of HBeAg from the 3T3 pARV2C cell line indicates that the 21-kDa polypeptide may also be processed, consistent with the observation of a secreted 15-kDa peptide. It should be noted that the origin of the HBeAg present in the culture medium of this cell line is presumed to result from antigen secretion. However, the possibility that this small amount of HBeAg results from spontaneous cell death cannot be excluded. Regardless of the origin of the HBeAg, it exists as a nonparticulate antigen, in contrast to HBcAg, which appears to be present, at least in part, as a particulate antigen. This is consistent with the formation of core particles from the 21-kDa polypeptide during HBV infection.

Recent reports on the characterization of the products of the HBV core and precore ORFs are in general agreement with the results reported here. However, Ou et al. (28), using a simian virus 40 vector, observed cytoplasmic HBcAg in cells expressing both the precore and core sequences. This contrasts with the nuclear localization of HBcAg observed in cells expressing the core sequence from either the amphotropic retroviral LTR or the MT promoter. In addition, no detectable HBcAg was seen when precore sequences were expressed in this system. These observations may reflect differences in the cell lines, expression systems, or assays used to analyze these HBV antigens. In particular, replication of the simian virus 40 vectors in COS cells and resultant cell death due to virus production may have affected HBcAg cellular localization. Roossinck et al. (32) have examined the synthesis of HBeAg and HBcAg from the endogenous HBV core promoter. They report HBeAg synthesis and secretion, in the absence of HBcAg synthesis, from cells containing

precore sequences and HBcAg synthesis only when the precore sequences have been deleted. This is in agreement with the results observed with this retroviral expression system.

It is apparent that transfection of mouse NIH 3T3 fibroblasts with recombinant amphotropic retroviral constructs permits generation of stable cell lines efficiently expressing HBV antigens. This has permitted characterization of several properties of these antigens. In addition, infection of these cells lines with helper virus or transfection of retrovirus-packaging cell lines with these recombinant retroviral constructs may permit recovery of recombinant retroviruses which could be used to infect, select, and express HBV antigens in a wide variety of cell types.

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