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12. C3H10T1/2 cells, in 20% serum, can suppress focus formation by some chemically induced C3H10T1/2 transformants. When grown in 5% serum, focus formation occurred in these mixed cultures of selected transformed C3H10T1/2 cells and C3H10T1/2 cells [J. S. Bertram, *Cancer Res.* **37**, 514 (1977)]. Cyclic adenosine 3′,5′ phosphodiesterase inhibitors greatly enhance suppression of focus formation in such mixed cultures [J. S. Bertram and M. B. Faletto, *Cancer Res.* **45**, 1946 (1985)].
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Inhibition of Secretion of Hepatitis B Surface Antigen by a Related Presurface Polypeptide

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The presurface (preS) proteins of hepatitis B virus are structural components of the viral envelope that may play important roles in virion assembly and infectivity. They are specified by a large open reading frame that includes the coding region for the major surface (S) protein in its 3′ half. Translation of the preS proteins initiates upstream from the S region, giving rise to proteins that are composed of the S domain and an additional 163 (preS1) or 55 (preS2) amino acids. Little is known about the biosynthesis and assembly of these proteins. The expression of the S and preS1 proteins was examined by transfecting cultured mammalian cells with viral DNA and injecting synthetic messenger RNA's into *Xenopus* oocytes. In contrast to the proteins encoded by the S region, the preS1 proteins are not detectably secreted into the culture medium. Furthermore, when the S and preS1 proteins are synthesized together, secretion of the S proteins is specifically and strongly inhibited. The results suggest a unique molecular interaction during secretion of the S and preS proteins that may be important for virus assembly.

A DISTINCTIVE FEATURE OF ACUTE or chronic hepatitis B virus (HBV) infection is the accumulation in the serum of large amounts of particulate hepatitis B surface antigen (HBsAg) (*1*). The major component of HBsAg particles is the product of the viral S gene, a protein of 226 amino acids that is present in nonglycosylated ($p24^S$) and glycosylated ($gp27^S$) forms (*2*). Two sets of less abundant HBsAg-related polypeptides were recently identified; these polypeptides are specified, in part, by the presurface (preS) region, a 522–base pair (bp) open reading frame (*3*) that is upstream and in phase with the S coding region. These larger proteins, termed the preS1 and preS2 proteins, are composed of the 226 residues encoded by the S region plus an additional 163 (preS1) or 55 (preS2) NH_2-terminal amino acids derived from the preS region (Fig. 1A) (*4, 5*). The function of the preS proteins is unknown, but they are present in greater abundance in virions than in the more numerous subviral HBsAg particles (*5*), suggesting a role for these protein domains in infectivity or virus assembly or both.

In order to examine HBV preS gene expression in mammalian cells, we first constructed two plasmids containing the HBV S and contiguous preS regions positioned downstream from the strong promoter in the long terminal repeat (LTR) of Rous sarcoma virus (RSV). These constructs, termed pSA10 and pSA41, differ only in the orientation of the HBV-specific insert relative to the LTR (Fig. 1A). Both plasmids are capable of directing the synthesis of the S and preS2 proteins by virtue of the HBV S gene promoter that lies within the preS1 open reading frame (*6, 7*); however, pSA41, unlike pSA10, can also promote the expression of the full preS1 reading frame from the RSV LTR. Mouse LTK^- cells, which are deficient in thymidine kinase, were transfected with these plasmids together with the thymidine kinase (TK) gene of herpes simplex virus, and TK^+ foci from each transfection were isolated. To confirm the presence of the predicted transcripts, we performed Northern blotting analysis of polyadenylated [poly(A)$^+$] RNA prepared from the two cell lines (Fig. 1B). Both cell lines exhibited the 2.2-kb message previously demonstrated to originate from the HBV S promoter (*6, 7*). In addition, pSA41 transformants had an abundant additional transcript of approximately 2.6 kb; this species originates from the RSV LTR, as shown by its annealing to probes from U5 but not U3 sequences of RSV, as well as to probes specific for the HBV preS1 region. We then tested the two cell lines for HBsAg expression by radioimmunoassay. As expected, pSA10 transfectants expressed HBsAg in the cytoplasm and in the culture medium (Fig. 1C). Surprisingly, however, cells transfected with pSA41, while expressing HBsAg in the cytoplasm, showed no detectable secretion of immunoreactive material into the medium.

To examine further the generality of this finding and to develop a system in which constructions could be rapidly screened without the necessity for selecting stable transformants, we turned to a transient expression system based upon SV40 transcription and replication signals (*8*). Briefly, plasmids containing the S (pSV24H) or the entire preS and S (pSV45H) coding regions cloned downstream from the SV40 early promoter (and *ori*) sequences (Fig. 2A) were constructed and used to transfect COS7 cells. These cells contain integrated SV40 genomes that supply T antigen in trans, resulting in the amplification of transfected DNA bearing the viral replication origin. Forty-eight hours after transfection of cultures with each construct, cell extracts and media were assayed for HBsAg by quantitative radioimmunoassay. As expected, cells transfected with pSV24H produced abundant quantities of HBsAg, which was readily demonstrable in both medium and cytoplasm (Fig. 2B). Parallel cultures similarly transfected with pSV45H synthesized comparable quantities of cytoplasmic antigen, but the levels of extracellular antigen were 1/12 to 1/10 those of the intracellular fraction.

These studies suggest that the preS1 anti-

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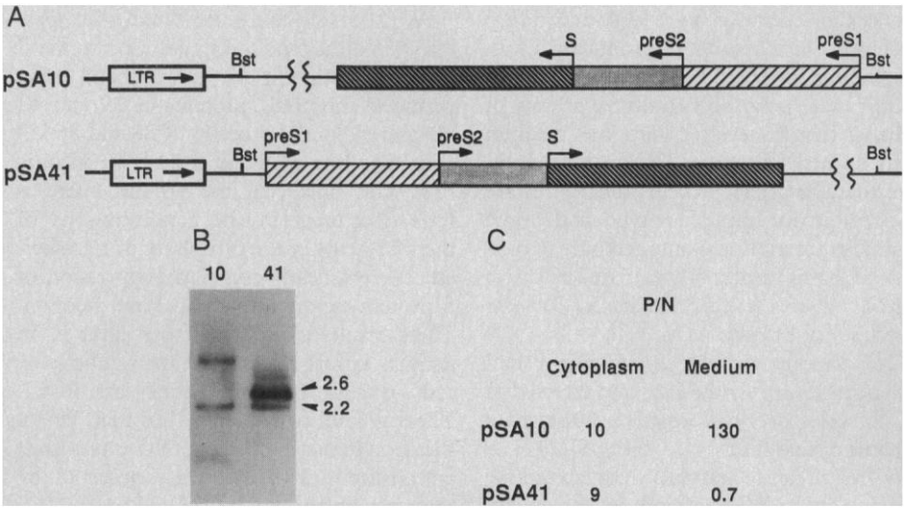
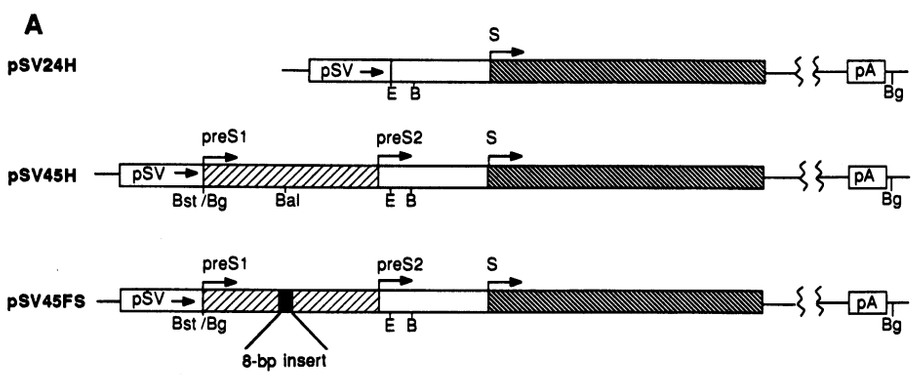


Fig. 1. HBsAg production by L-cell transformants bearing preS sequences. (A) Maps of plasmids pSA10 and pSA41. Construction of plasmid pSA10 has been described (*6*). Plasmid pSA41 was prepared by repairing the termini of a Bst EII–permuted unit-length HBV genome with AMV polymerase followed by blunt-end ligation to a similarly treated Bst EII (Bst)–cleaved plasmid (pAV2) bearing the RSV LTR. (B) HBsAg transcripts in transfected cells. Poly(A)$^+$ RNA (4 μg) from each of the clones presented was subjected to electrophoresis in parallel through 1% agarose–2.2*M* formaldehyde, transferred to nitrocellulose, and probed with ^{32}P-labeled HBV DNA. Size estimates (in kilobases) are shown at the right, derived from reference to 18*S* and 28*S* ribosomal RNA bands detected by ethidium bromide staining of the same lanes. The top band in the pSA10 lane 10 also anneals to pBR322 and arises by promotion from or readthrough into DNA flanking the viral insert. Lane 41 is for pSA41. (C) HBsAg production by L-cell transformants. LTK$^-$ cells were cotransfected with HSV *tk* DNA and pSA10 or pSA41, and TK$^+$ foci were selected in hypoxanthine-aminopterin-thymidine medium (*6*); DNA from selected foci was screened by Southern blotting with HBV probes to identify cotransfected colonies. Colonies were grown to saturation in 60-mm plates in 0.6 ml of medium. After 3 days at confluence, medium and cells were harvested. Cells were lysed in 0.6 ml of 0.4% NP40 and then 0.2 ml each of medium and the NP40 lysates were assayed by solid-phase radioimmunoassay (Ausria-II, Abbott Laboratories). Results are expressed as *P/N* [the ratio of bound ^{125}I-labeled sample (counts per minute) to bound negative control]; values greater than 2.1 are considered positive. One set of data from a typical experiment is shown.

gen is not efficiently exported from cells; furthermore, since both pSA41 and pSV45H have the potential to produce secretory S proteins from the HBV S promoter, they suggest that the production of preS1 polypeptides might inhibit the export of S polypeptides. Alternatively, the abundant transcription of preS1 sequences in pSA41 and pSV45H could interfere in cis with S gene transcription from the internal S promoter. Several lines of evidence indicate that the observed effect is not the result of transcriptional interference. First, direct analysis of messenger RNA (mRNA) species in stable L-cell transformants bearing pSA10 or pSA41 reveals comparable levels of the 2.2-kb S mRNA (Fig. 1B) (*6*). Second, the effect is relieved by mutations that specifically affect the translation of preS1 sequences. To demonstrate this, we introduced a frameshift mutation into the preS1-specific portion of the preS reading frame in plasmid pSV45H (Fig. 2A); this plasmid (pSV45FS), although identical to pSV45 in



B

HBsAg expression

	Cytoplasm (ng/ml)	Medium (ng/ml)	C/M
pSV24H	48	37	1.3
pSV45H	46	3	15
pSV45FS	19	40	0.5

Fig. 2. HBsAg production by COS7 cells after short-term transfection with SV40-based vectors containing S and preS sequences. (A) The target plasmid used in all of the above constructs (pSV65) was constructed by inserting the origin-containing 342-bp Pvu II–Hind III fragment of SV40 into pSP65 (Promega Biotech). Plasmid pSV24H was constructed by inserting the 2.3-kb Pst I–Bgl II fragment of HBV DNA into the Pst I and Bam HI sites of pSV65. Plasmid pSV45H (Fig. 1) was constructed by first converting the unique Bst EII site of the HBV genome into a Bgl II (Bg) site by Bgl II linker addition. The resulting genome was then cleaved with Bgl II to liberate a 2.3-kb Bgl II fragment containing the entire preS-S region and the HBV polyadenylation signal embedded in the core gene. This fragment was then inserted into the Bam HI site in the polylinker region of pSV65, and clones of the proper orientation were identified by restriction analysis. Plasmid pSV45FS was constructed by inserting an 8-bp Bgl II linker into the Bal I site of the preS1 region. (B) COS7 cells were grown to 80% confluence in 60-mm culture dishes and transfected with the indicated plasmids in the presence of DEAE-dextran (*8*). After 6 to 8 hours of transfection, the DNA-containing medium was removed, the cells were washed once in isotonic tris buffer, *p*H 7.4 (TBS), and 1.5 ml of fresh medium was added. At 48 hours after transfection, the medium was collected and the cell monolayer was washed three times in chilled TBS and harvested. Harvested cells were then subjected to sonication for 15 seconds at maximum power in a sonicator (Contes Micro-ultrasonic); serial 1:5 dilutions of portions of the medium and cytoplasm from each set of transfections were assayed for HBsAg by radioimmunoassay (Ausria-II). Values for HBsAg concentration within the linear range of the assay were quantitated with reference to a standard of serum-derived HBsAg of known concentration. HBsAg concentrations for the cytoplasmic sonicates (left column) and medium (middle column) are expressed in nanograms per milliliter; the right column expresses the concentrations of HBsAg in the medium (*M*) and cytoplasm (*C*) as a simple ratio, *C*/*M*. One set of data from a typical experiment is shown.

the organization of its SV40 and HBV promoters, is incapable of producing a full-length preS1 protein. When introduced into COS7 cells, pSV45FS directs synthesis of HBsAg that is secreted into the medium with normal efficiency. These results indicate that the preS1 proteins themselves are responsible for the phenotype and argue against transcriptional interference, a conclusion that is further substantiated by direct mRNA injection experiments in *Xenopus* oocytes (see below).

To demonstrate which HBsAg-related polypeptides are synthesized and secreted in COS7 cells, we performed a pulse-chase analysis of cells transfected with pSV24H or pSV45H. Transfected cells were labeled for 1 hour with [^{35}S]methionine and either harvested directly or incubated with excess unlabeled methionine for an additional 24 hours; medium and cells from each time point were examined by precipitation with antibody to HBsAg (Fig. 3). After 1 hour of labeling with [^{35}S]methionine, the cytoplasm of cells transfected with pSV24H showed the expected products of the HBV S gene, p24^{S} and gp27^{S} (Fig. 3A). After 24 hours of "chase" in unlabeled methionine, there was a slight decrement in the intracellular level of these proteins and corresponding amounts of both products appeared in the medium (Fig. 3, B and C).

As expected, cells transfected with plasmid pSV45H produce, in addition to the S proteins, both sets of preS-encoded polypeptides: the preS1 proteins of 39 and 42 kD and the preS2 proteins of 33 and 36 kD (Fig. 3A, lane 3). After a 24-hour incubation with unlabeled methionine, there is little decrement in the concentrations of these proteins in the cytoplasm of transfected cells and nearly complete suppression of S protein export into the culture medium. These results indicate that the effect is on antigen export rather than on synthesis or stability (Fig. 3, B and fourth lane in C). The low level of secretion (less than 7% of that seen in the absence of preS1 expression) is consistent with the levels observed by radioimmunoassay (Fig. 2B).

Because the HBV S promoter is embedded within the preS1 open reading frame, preS1 expression exclusive of S expression is not possible in vectors of the type we have described above. In order to examine the secretory phenotype of preS1 proteins alone, we obtained the separate expression of preS and S proteins by injection of synthetic mRNA's for each polypeptide into *Xenopus* oocytes (*9*). Plasmids capable of directing synthesis of either S or preS1 mRNA's in vitro were constructed by inserting the HBV S coding region (pSP24H) or preS and S open reading frames (pSP45H) downstream from the *Salmonella* phage SP-6 promoter (legend to Fig. 4). Capped mRNA's produced by transcription in vitro were injected into *Xenopus* oocytes, resulting in cytoplasmic synthesis of S proteins from pSP24H and preS1 proteins from pSP45H (Fig. 4). Inspection of the medium of oocytes 3 days after injection shows that while oocytes injected with S mRNA are capable of secreting immunoprecipitable S proteins, the oocytes injected with preS1 RNA do not export the preS1 polypeptide. Injection of equimolar quantities of each mRNA resulted in the synthesis of both sets of viral proteins; again, as in mammalian cells, the secretion of all antigen forms in coinjected oocytes was inhibited by the presence of the preS1 polypeptides. The inhibitory effect of preS1 expression on export was not the result of a global inhibition of protein secretion in injected eggs; analysis of unprecipitated medium from oocytes injected with S or preS1 RNA's revealed identical quantities and species of secreted cellular proteins.

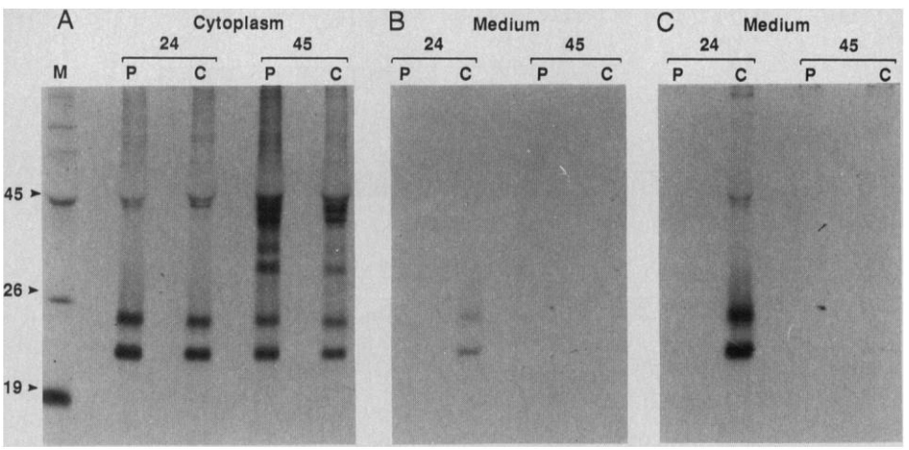


Fig. 3. Pulse-chase analysis of S and preS1 polypeptides produced in transfected COS7 cells. (A) Immunoprecipitation from cytoplasmic extracts; (B) immunoprecipitation from the medium. P designates the 1-hour pulse with the labeled sample, C indicates the 24-hour chase sample. Transfected plasmids are indicated above each lane. (C) 72-hour exposure of data in (B). Lane M contains the molecular weight standards. Sets of two plates of COS7 cells were transfected with either pSV24H (lane 24) or pSV45H (lane 45) as described legend to Fig. 2. At 48 hours after transfection, the cells were washed three times in TBS and incubated in 1.0 ml of methionine-free medium for 1.5 hours. [^{35}S]methionine (150 μCi) was then added to the contents of each dish, and the plated cells were allowed to incubate for 1 hour. At the end of the hour, the medium and cytoplasm from a plate of cells transfected with each plasmid were harvested; cell lysates were obtained by incubation of cell monolayers at 4°C with 1.0 ml of radioimmunoprecipitation assay buffer (50 m*M* tris, *p*H 8.15, 0.1% SDS, 0.5% NP40, and 0.5% sodium deoxycholate) followed by centrifugation of the supernatants in a centrifuge (Eppendorf). The two remaining plates were incubated with medium containing unlabeled methionine (30 μg/liter) for 24 hours, then harvested as above. Portions (400 μl) of both medium and cytoplasmic extracts from each plate were taken for immunoprecipitation with antiserum to HBsAg (Calbiochem) as previously described (*17*). Immunoprecipitated samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on a 14% polyacrylamide gel. The gel was then fixed, fluorographed, dried, and exposed to film for 18 hours (A) and (B), or 72 hours (C).

Although these studies have been carried out in nonhepatic cells, it is likely that similar interactions occur in infected hepatocytes, since the effect is observed in cells of widely differing tissues and species (Figs. 1, 2, and 4). Standring *et al.* (*10*) have independently observed the effect of preS1 expression on S export in *Xenopus* oocytes. Likewise, using vaccinia virus vectors engineered for the separate expression of preS1 and S proteins, Cheng *et al.* (*11*) showed that cells infected with both vectors display reduced S secretion compared to cells infected with the S recombinant alone. Similar interactions have been observed in intact animals; in transgenic mice whose cells contain a preS-S coding region in which preS1 expression is controlled by a metallothionein promoter, induction of preS1 expression results in a marked decrease in serum levels of HBsAg (*12*).

How might the ability of preS1 proteins to inhibit the secretion of the related S antigen be explained? In other systems, trans-dominant phenotypes caused by mutations in otherwise identical polypeptides typically result from subunit mixing in multimeric assemblies (*13*). The secretory form of HBsAg is such an assembly, composed primarily of multiple S promoters organized into a lipoprotein particle. The initial product of S biosynthesis is an integral transmembrane protein (*14*); HBsAg particles are believed to be generated by aggregation of these monomers in the endoplasmic reticulum (ER) membrane, followed by budding into the ER lumen. This model is consistent with electron micrographs of antigen-producing cells (*15*) and is supported by recent

pulse-chase experiments demonstrating that transmembrane S antigen is the precursor to the luminal particulate form (*16*). We also showed earlier that cells producing both preS2 and S polypeptides can synthesize and secrete particles of mixed subunit composition (*17*). We propose that the inhibition of S secretion in cells expressing both preS1 and S polypeptides is due to similar formation of mixed aggregates in which the block to export that is specific to preS1 (Fig. 4) is conferred upon the mixed assembly. The degree of this inhibition should be proportional to the relative quantities of preS1 and S subunits in the assembly. Consistent with this is the fact that in HBV infection in vivo, in which large quantities of S antigen particles are secreted, preS polypeptides generally constitute only 1% to 10% of the total antigen pool (*5*).

The precise nature and site of action of the inhibitory influence of preS1 proteins are not known. The proteins clearly enter the secretory pathway, since they are glycosylated as efficiently as the S proteins (Figs. 3 and 4). Recent findings indicate that the preS1 proteins, like their S protein counterparts, are initially synthesized as transmembrane proteins. However, protease protection and endoglycosidase H sensitivity experiments indicate that the preS1 proteins remain in a transmembrane configuration in the ER or cis-Golgi; unlike the S proteins, they do not undergo conversion to a particulate form that is fully within the lumen (*16*). The structural features of the preS1 domain that are responsible for this behavior are currently being investigated. Recently we discovered that the NH_2-terminus of the preS1 proteins is post-translationally modified by the addition of myristic acid (*18*); this modification could help to anchor the protein in the ER and might contribute to the phenotype described here. Additional experiments, however, will be necessary to determine whether this or some other feature of the protein accounts for its secretory behavior.

Does the secretory behavior of preS1 proteins reported here play a role in authentic viral replication? At present, no simple answer can be given to this question since the function of preS1 proteins in vivo is unknown. However, it is clear that, unlike S proteins, these polypeptides are preferentially localized in virions rather than in the more numerous subviral particles (*5*). In this respect they resemble the traditional surface proteins of other enveloped animal viruses (for example, the G protein of vesicular stomatitis virus or influenza virus hemagglutinin). Such proteins do not undergo the spontaneous budding process proposed for the S antigen of HBV; rather, the initiation of budding is triggered by interaction with nucleocapsid or matrix components of the virus (*19, 20*). The intracellular retention of preS1 polypeptides (and coaggregated S proteins) may be a reflection of a similar requirement for such interactions with other HBV proteins; if so, this property of the preS1 region may be important for virion assembly.

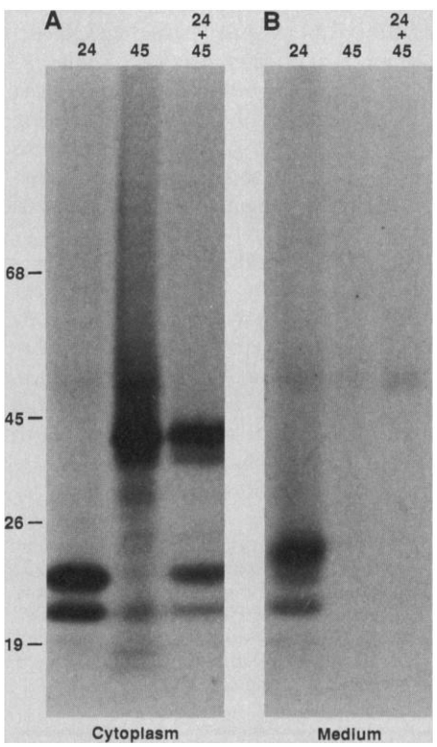


Fig. 4. Synthesis of S and preS1 proteins by injection of synthetic mRNA's into *Xenopus* oocytes. (A) Immunoprecipitates of oocytes injected with S-specific mRNA (lane 24), oocytes injected with preS1-specific mRNA (lane 45), and oocytes injected with both S- and preS1-specific mRNA's (lane 24+45). (B) Immunoprecipitates of medium of oocytes injected with S-specific mRNA (lane 24), medium after injection with preS1-specific mRNA (lane 45), and medium after injection with both S- and preS1-specific mRNA's (lane 24+45). The numbers to the left indicate the positions of the molecular weight standards (in kilodaltons). Plasmid pSP24H was constructed by ligating the 2.3-kb Pst I–Bgl II HBV-specific fragment into the Pst I and Bam HI sites of pSP65 (Promega Biotech). Plasmid pSP45H was constructed by inserting the 2.8-kb Bgl II fragment of HBV into the Bam HI site of pSP65 and screening the resultant clones for orientation by restriction analysis. Next, a clone bearing an insert in the correct transcriptional orientation was digested with Sma I and Bst EII to liberate most of the HBV sequences upstream from the preS1 AUG. The single-stranded termini were repaired with the Klenow fragment of *Escherichia coli* Pol I followed by blunt-end ligation. For transcription in vitro, 1 μg of SP6 promoter-containing plasmid that had been previously linearized with Pvu II was incubated with 20 units of SP6 polymerase in a 50-μl reaction containing 10 m*M* tris (*p*H 7.5), 10 m*M* $MgCl_2$, 10 m*M* spermidine, 50 m*M* NaCl, 1.0 m*M* each of the ribonucleotides ATP, GTP, CTP, UTP, and the 5′me7GpppG3′ cap analog (Pharmacia Biochemicals), 2 units of RNASIN placental ribonuclease inhibitor (Promega Biotech), and 10 m*M* dithiothreitol. The reaction was allowed to proceed for 1 to 1.5 hours at 40°C. Injection of *Xenopus* oocytes was carried out by the procedure of Gurdon *et al.* (*9*). A 40-nl volume containing 60 to 120 ng of transcript in transcription buffer was injected into each of 15 freshly dissected oocytes, after which the transcript was allowed to equilibrate for 12 hours. The medium was then removed and replaced with medium containing [^{35}S]methionine and [^{35}S]cysteine (each at 5 mCi/ml), followed by incubation at 18°C for 72 hours. The labeled medium was removed, and the oocytes were washed in chilled TBS, resuspended in chilled RIPA buffer, and then homogenized with a glass pestle. Both medium and cell homogenates were centrifuged for 10 minutes, and the resulting supernatants were taken for immunoprecipitation with antibody to HBsAg. Samples were subjected to SDS-PAGE on a 10% to 14% continuous gradient gel, which was then fixed, fluorographed, dried, and exposed to film for 8 hours (A) or 36 hours (B).

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