Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9338–9342, December 1986
Biochemistry

Assembly of viral particles in *Xenopus* oocytes: Pre-surface-antigens regulate secretion of the hepatitis B viral surface envelope particle

(synthetic mRNAs/SP6/microinjection/22 nm particle)

DAVID N. STANDRING*†, JING-HSIUNG OU*, AND WILLIAM J. RUTTER*†

*Hormone Research Institute and †Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143

Contributed by William J. Rutter, August 19, 1986

ABSTRACT **Infection with hepatitis B virus (HBV) is associated with the production of a viral envelope particle that contains membrane lipids, surface antigen (S), and two pre-surface-antigens (pre-S) comprised of the entire S moiety with approximately 55 (pre-S2) and 174 (pre-S1) additional NH_2-terminal amino acids. We show here that *Xenopus* oocytes injected with synthetic S mRNA assemble and secrete characteristic 22-nm viral envelope particles. In contrast, pre-S1 and pre-S2 antigens are synthesized but not secreted. By coinjecting mRNAs, we found that synthesis of high levels of pre-S proteins specifically inhibits S antigen secretion. On the other hand, high levels of S synthesis can drive the secretion of small amounts of either pre-S antigen. These observations are consistent with a model for viral envelope assembly in which both S and pre-S proteins are incorporated into a multimeric particle, presumably via interactions between the S protein domains, while the pre-S amino-terminal moieties regulate the secretion of this structure. Our results indicate that *Xenopus* oocytes will provide a powerful system for studying the morphogenesis of simple structures of viral or cellular origin.**

Hepatitis B virus (HBV), on infection, produces two types of particles (1): the 42-nm virus, with a core containing HBV DNA and viral proteins surrounded by an envelope containing three related surface antigen (S) molecules, and 22-nm particles and filaments comprised of the elements of the viral envelope. The latter particles, either isolated from the sera of HBV carriers or produced in heterologous cells by recombinant DNA methods, form the basis of an effective HBV vaccine (2, 3). Whereas knowledge of the structure, transcription, and replication cycle of HBV has increased dramatically in recent years (4, 5), the details of the assembly and secretion of these viral particles are less clear. Recent studies (6–8) suggest that S particles bud into the lumen of the endoplasmic reticulum prior to secretion. Although the different classes of viral particles contain different ratios of pre-surface-antigens (pre-S) to S (9), the mechanism by which this is achieved and the role of pre-S in the assembly and secretion of viral particles is obscure.

To dissect the complex problem of assembly, we have developed an approach involving the microinjection of individual and combinations of synthetic viral mRNAs into *Xenopus* oocytes, which allows the analysis of the contribution of specific viral proteins to viral structures. These studies have revealed that the pre-S antigens can regulate the secretion of the S particle.

MATERIALS AND METHODS

Plasmid Construction and *in Vitro* Transcription. For construction of the SP6-HBV transcription plasmids, the following HBV DNA fragments were ligated to *Sma* I/*Bgl* II-double-digested pSP65 (Promega Biotec, Madison, WI): for S mRNA, *Eco*RI/*Bgl* II 2-kilobase (kb) fragment; for pre-S2 mRNA, *Mst* II/*Bgl* II 2-kb fragment; for pre-S1 mRNA, *Bst* EII/*Bgl* II 2.5-kb fragment (10). The *Eco*RI and *Bst*EII sites of S and pre-S1 DNA fragments were blunt-ended with DNA polymerase I Klenow fragment. For RNA transcription, *Sal* I linearized plasmids (50 μg/ml) were incubated at 40°C for 1 hr with 400 units of SP6 RNA polymerase (Boehringer Mannheim) per ml in 40 mM Tris·HCl (pH 7.5) containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 800 units of RNasin (Promega Biotec) per ml, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.2 mM GTP, and 1 mM 7-methylguanosine(5′)triphosphoguanosine (m^7GpppG). DNA was removed from the reaction mixture by treatment with 25 μg of DNase I (Worthington) per ml at 37°C for 10 min, followed by extraction with phenol/chloroform. RNA was precipitated with ethanol in the presence of RNasin, then resuspended in water at 0.4 mg/ml and stored at −70°C. Synthetic prolactin mRNA was a gift from P. Garcia and P. Walter (University of California, San Francisco).

Oocyte Microinjection and Labeling. Fragments of ovary were surgically removed from an anesthetized *Xenopus laevis* frog and rinsed extensively with modified Barths medium containing 100 μg of penicillin, 100 μg of streptomycin, and 50 μg of gentamicin per ml (MBSH medium) (11). Individual stage 5 or 6 oocytes were isolated manually, rinsed well, and stored overnight at 17–19°C in MBSH medium. Healthy oocytes were injected in the vegetal pole with 20–30 nl of the appropriate synthetic mRNA (0.4 mg/ml in distilled H_2O). For studies on the translation of synthetic mRNAs in oocytes, see refs. 12 and 13. Batches of 20 healthy oocytes were transferred next day to 0.5 ml of MBSH medium containing 10% fetal calf serum and 0.7–1.4 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (Amersham, ≈1360 Ci/mmol) per ml. After 48 hr at 17–19°C, the medium was carefully removed and frozen: the oocytes were rinsed thoroughly with MBSH medium and then homogenized in aliquots (500–800 μl) of homogenization buffer (50 mM Tris·HCl, pH 7.6/1% Triton X-100/10 units of aprotonin per ml/1 mM phenylmethylsulfonyl fluoride). Medium and homogenate were kept at −70°C until required.

Immunoprecipitation and Gel Analysis. Prior to immunoprecipitation, the samples were thawed and centrifuged (2 min in an Eppendorf centrifuge) to remove yolk and debris. Aliquots (250 μl) were boiled with $NaDodSO_4$, diluted with 4 vol of dilution buffer as described (14), and preabsorbed with washed Pansorbin (Calbiochem) prior to overnight incubation at 4°C with monoclonal mouse anti-S supernatant (a gift of George Kuo, Chiron Corporation, Emeryville, CA).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; S, surface antigen; pre-S, pre-surface-antigens; kb, kilobase(s); P/N ratio, positive/negative ratio; GP, glycosylated protein; P, nonglycosylated protein.

The antibody was absorbed onto Pansorbin by using rabbit anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA) as a bridge. The centrifuged Pansorbin was washed extensively, and the ^{35}S-labeled antigens were released by boiling with Laemmli sample buffer and analyzed on a denaturing 12.5–15% polyacrylamide gel (15). After staining and enhancement with sodium salicylate, the gel was dried and autoradiographed at −70°C. To quantitate these data, appropriate exposures of the autoradiograms were scanned with a laser densitometer (LKB). The relative peak intensities were determined by digital integration or by cutting and weighing the peaks on the chart paper. These values were then corrected for the number of methionines in each species to yield molar data.

Experiments with synthetic prolactin mRNA were performed essentially as described above by using rabbit anti-prolactin antiserum (a gift of P. Garcia and P. Walter, University of California, San Francisco) in place of the mouse anti-S antibody.

Kinetics of Secretion. Batches of 20 oocytes injected with the appropriate RNAs were incubated at 17–19°C in 500-μl aliquots of MBSH medium supplemented with 5% fetal calf serum. After 24 ± 1.5 hr, this medium was removed and stored at 4°C with 0.1% sodium azide. Fresh medium was added to the oocytes. This process was repeated each day for up to 6 days. Aliquots (25–200 μl) of the medium were assayed for S in duplicate by using a commercial RIA kit (Ausria II; Abbott) according to the manufacturer's instructions. Results are presented as a positive/negative (P/N) ratio in which the activity of the sample is compared to that of a negative control supplied by the manufacturer. P/N ratios of <2.1 are considered negative. Controls and pre-S1-injected or 1:1 pre-S1/S-injected oocytes gave P/N ratios of ≈1.5; pre-S2 RNA gave P/N ratios of 2.1–2.4. P/N ratios >10 were determined from serially diluted samples to ensure that the assay was in the linear range.

Electron Microscopy of 22-nm Particles. Twenty oocytes were injected with S mRNA, and medium containing secreted proteins was generated over a 6-day period as described for the kinetic experiments. The medium (≈3 ml) was pooled and passed through a 30-μl anti-HBV surface antigen-conjugated Sepharose CL-4B (Pharmacia) column. Bound HBV surface antigen particles were eluted with 3 M potassium thiocyanate. One-drop fractions were collected. For electron microscopy, the particles were negatively stained with 2% uranyl acetate.

RESULTS

Expression and Secretion of S, pre-S1, and pre-S2. A single contiguous HBV open reading frame encodes all three antigens; translation of each antigen initiates from a distinct methionine residue. The antigen-encoding sequences were independently cloned into vector SP65 so as to juxtapose the appropriate methionine and the SP6 promoter. Capped S, pre-S1, and pre-S2 transcripts were generated from these vectors *in vitro* (12) and injected into oocytes. [^{35}S]Methionine-labeled antigens were immunoprecipitated from the oocyte (Fig. 1*A*) or medium (Fig. 1*B*) and resolved electrophoretically. The S, pre-S2, and pre-S1 antigens synthesized inside the oocyte (Fig. 1*A*, lanes 1, 2, and 3, respectively) correspond to the following authentic glycosylated (GP) and nonglycosylated (P) viral protein components described by Heerman *et al.* (9): P24/GP27 for S antigen (lane 1); traces of P30 and GP33/GP36 for pre-S2 (lane 2); and P39/GP42 for pre-S1 (lane 3). This confirms the ability of *Xenopus* oocytes to appropriately glycosylate proteins (17). *In vitro* translation of these RNAs yields mainly P24, P30, and P39 (indicated by open arrowheads in Fig. 1*A*, lane 8); only these three species were immunoprecipitated by anti-S antibody (data not shown). Pre-S2 RNA also generated S (perhaps by internal translational initiation) at various levels from 34% to 63% of those for pre-S2.

The secreted proteins are displayed in Fig. 1*B*, lanes 1–3. S antigen secretion occurred (Fig. 1*B*, lane 1), but inefficiently: only 2.5% of the total S was exported in 48 hr versus 90% for prolactin (D.N.S., unpublished data). In contrast to S, very little pre-S2 and no pre-S1 reached the medium.

Pre-S Antigens Can Inhibit the Secretion of S. Unexpectedly, pre-S2 synthesis inhibited S export. Whereas pre-S2 mRNA generated 38% as much S within the oocyte as did S mRNA (compare lanes 1 and 2 in Fig. 1*A*), the secreted S from pre-S2 mRNA was only 1% of that from S mRNA (Fig. 1*B*, lanes 1 and 2). We next tested if pre-S1 affects S secretion

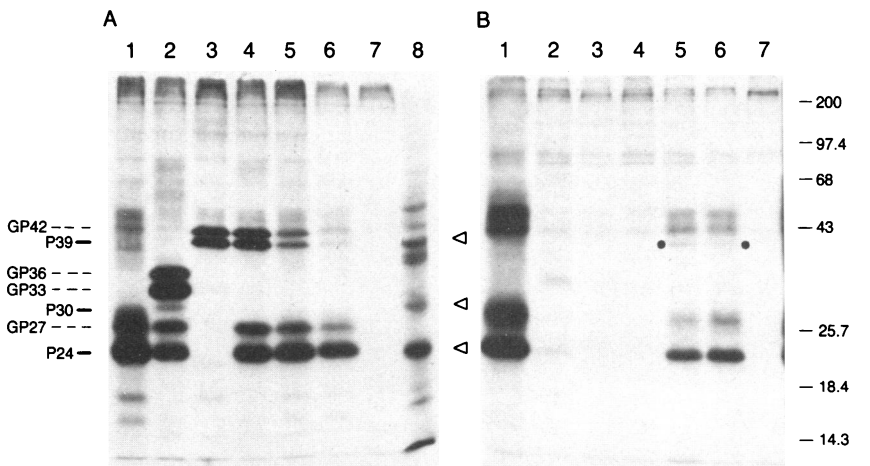

FIG. 1. Intracellular (*A*) and secreted (*B*) forms of pre-S and S synthesized by microinjected *Xenopus* oocytes. The synthetic mRNAs used were: S (lanes 1), pre-S2 (lanes 2), pre-S1 (lanes 3), 1:1 pre-S1/S (lanes 4), 0.2:1 pre-S/S (lanes 5), 0.04:1 pre-S1/S (lanes 6), and no-RNA controls (lanes 7). *In vitro* translation products from the three mRNAs are displayed in lane 8: P24, P30, and P39 are indicated by arrows. The identities of the antigens are indicated to the left of *A*, and molecular weight standards (shown in kDa), are to the right of *B*. In addition, diffuse high molecular weight S species, which are less prominent in some experiments (see Fig. 2), are visible (lanes 1 and 2). These presumably represent hypermodified or dimeric (16) species. Each lane in *A* represents immunoprecipitated products from 6 oocytes versus 10 oocytes per lane in *B*. *A* and *B* represent 10-hr and 8-day autoradiographic exposures of the gel, respectively. For mixing experiments (lanes 4–6), 1 μl of S mRNA was mixed immediately prior to injection with 1 μl of pre-S1 mRNA, which was undiluted (lane 4) or diluted 1:5 (lane 5) or 1:25 (lane 6) with distilled water.

by coinjecting various proportions of pre-S1 and S mRNAs. Pre-S1 mRNA was added to a constant amount of S mRNA to bring the ratio to 1:1 (Fig. 1*A*, lane 4), 0.2:1 (Fig. 1*A*, lane 5), and 0.04:1 by weight (Fig. 1*A*, lane 6). The respective pre-S1/S molar ratios determined for the intracellular antigens (Fig. 1*A*) are 0.5:1; 0.13:1; and 0.03:1. Thus, allowing for the lower translational efficiency of pre-S1 mRNA, the antigen ratio parallels the input mRNA ratio. At a 0.5:1 ratio, pre-S1 virtually eliminated S secretion (Fig. 1*B*, lane 4); at a ratio of 0.13:1, secretion of S was again observed (Fig. 1*B*, lane 5); at a ratio of 0.03:1, secretion increased a further 25% (compare lanes 5 and 6 in Fig. 1*B*). Under these conditions the secreted particles (see below) included P39 (marked by dots in lanes 5 and 6 in Fig. 1*B*) and presumably GP42, which was obscured by high molecular weight derivatives of S (compare lane 1). The internal molar P39/P24 ratio is 3-fold higher in lane 5 than in lane 6 (0.068 vs. 0.021): the latter P39 content approximates that of 22-nm spherical particles *in vivo* (9). Secreted GP27 was larger and more heterogeneous than the intracellular GP27 (compare lanes 5 and 6 in Fig. 1*B* vs. Fig. 1*A*), indicating more extensive glycosylation of the secreted GP27 (18). This suggests that intracellular GP27 resides largely in the endoplasmic reticulum or early Golgi. Patzer *et al.* (18) in studies on S secretion in mammalian cells have noted that S secretion is very slow ($t_{1/2}$ = 5 hr) and that intracellular S is retained in the endoplasmic reticulum or early Golgi prior to secretion.

Pre-S2 also inhibited S secretion, although less than did pre-S1. Pre-S2 mRNA directed synthesis of pre-S2 (GP33/GP36) and S (P24/GP27) in a molar ratio of 3:1 (Fig. 2*A*, lane 1), but little S was secreted (Fig. 2*B*, lane 1). A 1:1 mixture of pre-S2 and S mRNAs decreased the intracellular pre-S2/S ratio to 0.59:1 and increased intracellular S by 130% (Fig. 2*A*, lane 2) and secreted S by 14-fold (Fig. 2*B*, lane 2; note that S secretion was totally inhibited by 0.5:1 pre-S1/S). At a 0.2:1 pre-S2/S mRNA ratio, S secretion increased an additional 3.5-fold (Fig. 2*B*, lane 3) to the level found for S mRNA alone (Fig. 2*B*, lane 4). The amount of pre-S2 in the secreted particles clearly correlates with the level of secreted S (Fig. 2*B*, lanes 1–3) and not with the intracellular level of pre-S2 (Fig. 2*A*, lanes 1–3).

Pre-S1 Does Not Influence the Secretion of Prolactin. Pre-S1 proteins did not indiscriminately block protein secretion: electrophoretic profiles of secreted, ^{35}S-labeled, endogenous

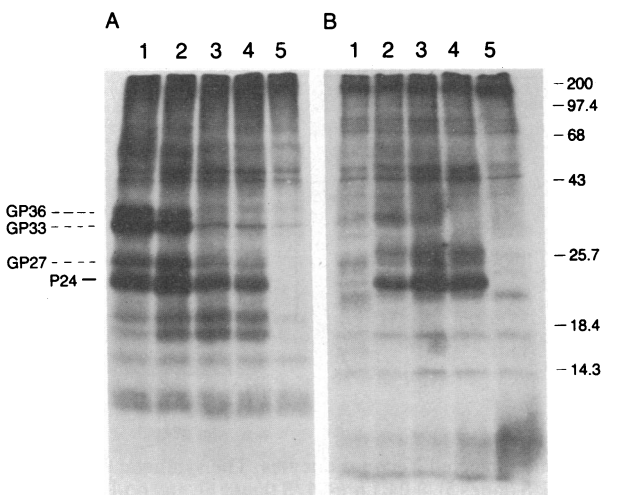

FIG. 2. The effect of pre-S2 on S secretion. The mRNAs injected were as follows: pre-S2 (lanes 1), 1:1 pre-S2/S (lanes 2), 0.2:1 pre-S2/S (lanes 3), 1:1 S/distilled H_2O (lanes 4), and no RNA (lanes 5). The intracellular (*A*) and secreted (*B*) antigens derived from 10 oocytes are shown. The autoradiograms were exposed for 18 hr (*A*) and 20 days (*B*).

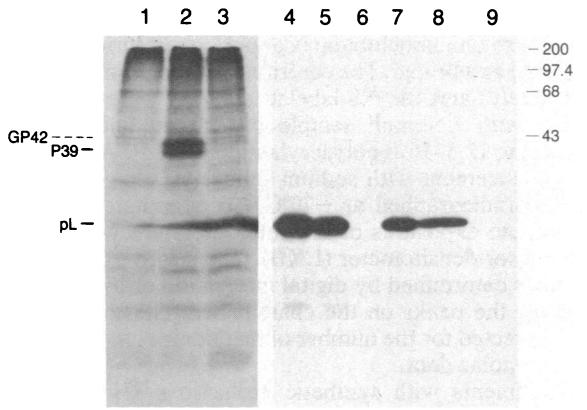

FIG. 3. Pre-S1 does not influence prolactin (pL) secretion. Pre-S1 was immunoprecipitated from extracts of oocytes injected with the following mRNAs: pL (lane 1), 1:1 pL/pre-S1 (lane 2), and no RNA (lane 3). pL was immunoprecipitated from the intracellular (lanes 4–6) or secreted (lanes 7–9) fraction of oocytes injected with the following mRNAs: pL (lanes 4 and 7), 1:1 pL/pre-S1 (lanes 5 and 8), and no RNA (lanes 6 and 9). The positions of pL and pre-S1 proteins are indicated to the left, and of molecular weight standards (shown in kDa), to the right. Lanes: 1–3, 16-hr autoradiogram of protein derived from four oocytes; 4–9, 5-hr autoradiogram of protein equivalent to 0.1 oocyte per lane.

oocyte proteins did not change on injection with S, pre-S1, or pre-S2 mRNAs (data not shown). Furthermore, a 1:1 mixture of pre-S1 and prolactin mRNAs directed the synthesis of less of both intracellular (Fig. 3, lane 5) and secreted (Fig. 3, lane 8) prolactin by a factor of 1.7–1.8 compared to prolactin mRNA alone (Fig. 3, lanes 4 and 7); thus, pre-S1 does not inhibit prolactin secretion. Immunoprecipitation with anti-S antibody (Fig. 3, lanes 1–3) confirmed that oocytes injected with pre-S1 plus prolactin mRNAs contain intracellular levels of pre-S1 (Fig. 3, lane 2) comparable to those seen in Fig. 1, lane 3. We also showed that pre-S1 does not influence the secretion of HBV precore antigen (data not shown).

Pre-S1 and Pre-S2 Do Not Change the Kinetics of Secretion of S. The observed attenuation of secretion by pre-S is a true inhibition rather than a change in the kinetics of secretion. The secretion kinetics for pre-S2/S and pre-S1/S are presented in Fig. 4. These and similar experiments showed that S secretion increased modestly for 2–3 days then slowly decreased. Coinjection with pre-S1 or pre-S2 mRNAs reduced the level of secreted antigen, but the profiles remained similar. Within experimental error, these data are consistent

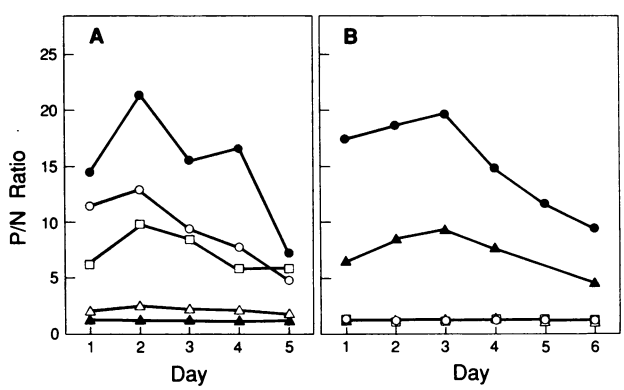

FIG. 4. Kinetics of secretion of S and pre-S antigens from oocytes. (*A*) Effect of pre-S2 on S secretion. The mRNAs injected were: S diluted 1:1 with distilled H_2O (●), 0.2:1 pre-S2/S (○), 1:1 pre-S2/S (□), pre-S2 (△), and no RNA (▲). (*B*) Effect of pre-S1 on S secretion. The mRNAs were: 0.04:1 pre-S1/S (●), 0.2:1 pre-S1/S (▲), 1:1 pre-S1/S (△), pre-S1 (□), and no RNA (○).

with the immunoprecipitation data of Figs. 1 and 2. From the data in Fig. 4, we estimate that each oocyte can secrete up to 0.25 ng of S per day, and a 1:1 mixture of pre-S1/S RNAs results in a reduction by at least a factor of 14 in the amount of secreted S levels relative to a 0.04:1 mixture.

DISCUSSION

By injecting synthetic mRNAs into *Xenopus* oocytes, we have elucidated a complex interaction between HBV pre-S and S antigens that can lead to either the cosecretion of these antigens or the inhibition of S secretion, depending on the relative levels of pre-S and S. Thus, pre-S and S regulate each other's secretion.

These results can be rationalized in terms of the formation of the various HBV viral particles. As anticipated, S secretion in oocytes yields a 22-nm spherical particle (Fig. 5) similar to that found in mammalian systems and in the serum of infected patients. These particles apparently originate via a budding process within the membrane of the endoplasmic reticulum (6–8). They contain ≈100 S molecules (9) presumably held together by intermolecular interactions (e.g., hydrophobic, electrostatic, and disulfide bridge interactions), which probably help to direct the formation of the viral envelope structure and may also serve to exclude cellular membrane proteins. Unlike S, pre-S proteins are poorly, if at all, secreted. S can drive the secretion of pre-S proteins presumably because low levels of these molecules can be accommodated within the S matrix of 22-nm particles. Secretion then proceeds, albeit at a reduced rate, yielding particles containing pre-S. However, as the pre-S/S ratio increases to high levels (0.5:1 for pre-S1/S and 3:1 for pre-S2/S), particle secretion is attenuated and pre-S and S molecules remain within the oocyte. At these high levels, we presume that the NH_2-terminal domains of pre-S1 or pre-S2 interact with each other and destabilize the S matrix that forms the 22-nm particle. The finding that higher levels of pre-S1 are present in filamentous particles or 42-nm virions versus 22-nm particles (9) supports the hypothesis that pre-S1 influences particle morphology. *In vivo* a single promoter drives the expression of pre-S2 and S mRNAs (19–22); a second promoter must be used for pre-S1 mRNA formation (4, 23–25). Thus, differential expression of pre-S1 and pre-S2/S could regulate the production and perhaps the morphology of the viral particles.

It has been reported that mammalian expression vectors containing both pre-S and S sequences synthesize high levels of HBV mRNA but produce few particles (24, 26). We surmise that this reflects failure to secrete the hybrid particles. Our results suggest that there are intrinsic limitations in the ability to incorporate the highly immunogenic (27) pre-S2 and pre-S1 proteins into S particles used as HBV vaccines (28, 29). Multivalent vaccines comprised of hybrid particles containing S antigen fused to other viral antigens also may be subject to similar limitations.

Our studies indicate the value of the oocyte system for studying protein–protein interactions between selected proteins such as S and pre-S. A key characteristic of this system is the quantitative relationship that exists between the input mRNA and the protein produced; thus, the ratios of the desired proteins can be controlled (see Fig. 1). This feature was important in discovering the role of S and pre-S moieties in particle secretion. By using this method, labeled viral envelopes with predetermined proportions of pre-S antigens can be generated for testing the proposed role (30) of pre-S2 (and pre-S1?) in mediating the uptake of viral particles by the liver. The extension of these studies to other HBV mRNAs may elucidate important features of the replicative pathway

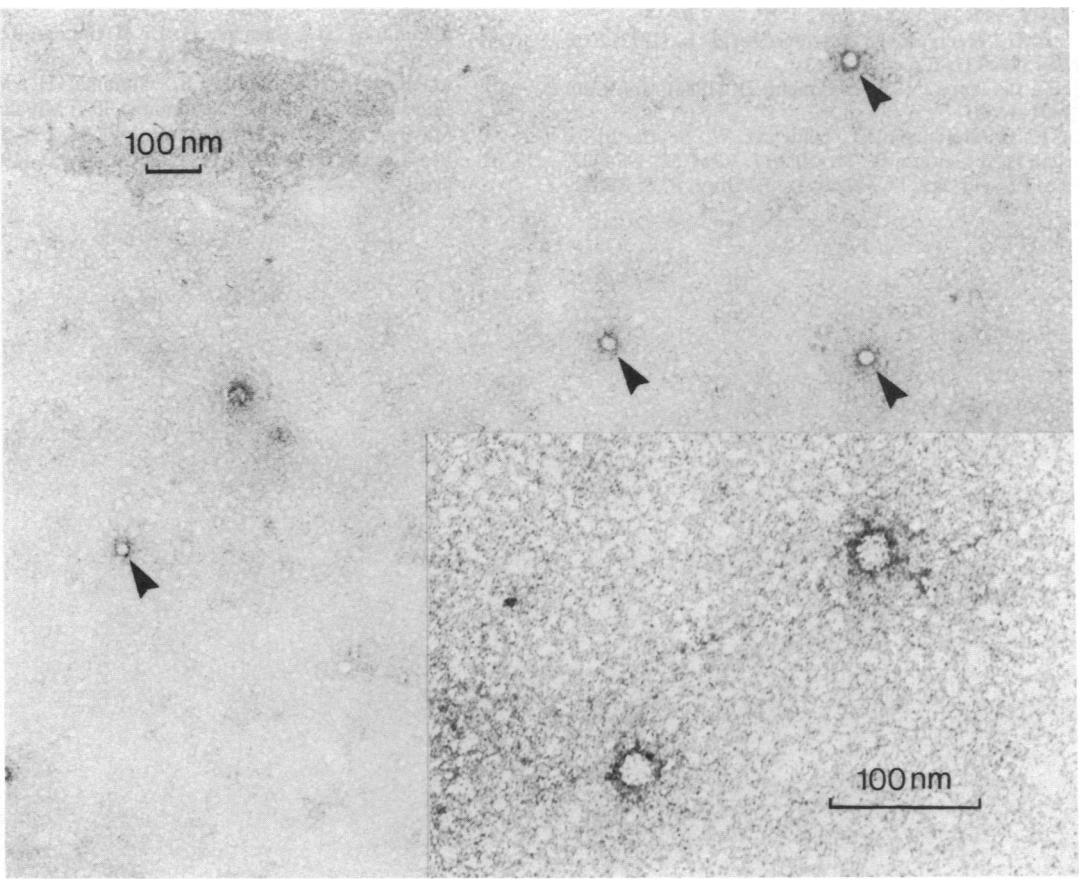

FIG. 5. Electron micrograph of 22-nm viral particles secreted from oocytes.

of HBV and the constitution, function, and assembly of the various components of the 42-nm viral particle.

We believe that the *Xenopus* system provides a powerful means to elucidate the morphogenesis of simple structures of viral or cellular origin. Selective addition of single or mixtures of appropriate mRNA species should provide information concerning the various intermediate structures and, therefore, reveal the essential features of morphogenetic pathways.

Note Added in Proof. Since this manuscript was completed, we have learned that the ability of pre-S1 to inhibit S secretion has been demonstrated in mammalian cells by D. H. Persing and colleagues (31) and in transgenic mice by F. Chisari (personal communication) and colleagues.

We thank Leslie Spector for impeccable manuscript preparation. We thank Pablo Garcia, Barry Gumbiner, Mark Selby, Peter Walter, and Judy White of University of California, San Francisco, for valuable discussions and critical reading of the manuscript and George Kuo of Chiron Corporation for a generous gift of monoclonal antibody. This research was supported by National Institutes of Health Grant AI 19744.

1. Dane, D. S., Cameron, C. H. & Briggs, M. (1970) *Lancet* **i**, 695–698.
2. Stevens, C. E., Taylor, P. E., Tong, M. J., Toy, P. T. & Vyas, G. N. (1984) in *Viral Hepatitis and Liver Disease*, eds. Vyas, G. N., Dienstag, J. L. & Hoofnagle, J. H. (Grune & Stratton, Orlando, FL), pp. 275–291.
3. Jilg, W., Schmidt, M., Zoulek, G., Lorbeer, B., Wilske, B. & Deinhardt, F. (1984) *Lancet* **ii**, 1174–1175.
4. Standring, D. N. & Rutter, W. J. (1986) in *Progress in Liver Disease*, eds. Popper, H. & Schaffner, F. (Grune & Stratton, Orlando, FL), Vol. 8, pp. 311–333.
5. Tiollais, P., Pourcel, C. & Dejean, A. (1985) *Nature (London)* **317**, 489–495.
6. Kamimura, T., Yoshikawa, A., Ichida, F. & Sasaki, H. (1981) *Hepatology* **1**, 392–397.
7. Dunn, A. E. G., Peters, R. L. & Schweitzer, I. L. (1973) *Exp. Mol. Pathol.* **19**, 113–126.
8. Eble, B. E., Lingappa, V. R. & Ganem, D. (1986) *Mol. Cell. Biol.* **6**, 1454–1463.
9. Heerman, K. H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52**, 396–402.
10. Valenzuela, P., Quiroga, M., Zaldivar, J., Gray, P. & Rutter, W. J. (1980) in *Animal Virus Genetics*, eds. Fields, B., Jaenisch, R. & Fox, C. F. (Academic, New York), 57–70.
11. Colman, A. (1984) in *Transcription and Translation: A Practical Approach*, eds. Hames, D. & Higgins, S. (IRL, Oxford), pp. 271–301.
12. Drummond, D. R., Armstrong, J. & Colman, A. (1985) *Nucleic Acids Res.* **13**, 7375–7393.
13. Krieg, P. A. & Melton, D. A. (1984) *Nucleic Acids Res.* **12**, 7057–7070.
14. Anderson, D. J. & Blobel, G. (1983) *Methods Enzymol.* **96**, 111–120.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Mishiro, S., Imai, M., Takahashi, K., Machida, A., Grotanda, T., Miyakawa, Y. & Mayumi, M. (1980) *J. Immunol.* **124**, 1589–1593.
17. Lane, C. D. (1983) *Curr. Top. Dev. Biol.* **18**, 89–116.
18. Patzer, E. J., Nakamura, G. R. & Yaffe, A. (1984) *J. Virol.* **51**, 346–353.
19. Cattaneo, R., Will, H., Hernandez, N. & Schaller, H. (1983) *Nature (London)* **305**, 336–338.
20. Standring, D. N., Rutter, W. J., Varmus, H. E. & Ganem, D. (1984) *J. Virol.* **50**, 563–571.
21. Cattaneo, R., Will, H. & Schaller, H. (1984) *EMBO J.* **3**, 2191–2196.
22. Enders, G., Ganem, D. & Varmus, H. (1985) *Cell* **42**, 297–308.
23. Rall, L. B., Standring, D. N., Laub, O. & Rutter, W. J. (1983) *Mol. Cell. Biol.* **3**, 1766–1773.
24. Laub, O., Rall, L. B., Truett, M., Shaul, Y., Standring, D. N., Valenzuela, P. & Rutter, W. J. (1983) *J. Virol.* **48**, 271–280.
25. Ou, J. H. & Rutter, W. J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 83–87.
26. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 193–197.
27. Milich, D. R., Thornton, G. B., Neurath, A. R., Kent, S. B., Michel, M. L., Tiollais, P. & Chisari, F. V. (1985) *Science* **228**, 1195–1199.
28. Michel, M. L., Pontisso, P., Sobczak, E., Malpiece, Y. & Streeck, R. E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7708–7712.
29. Persing, D. H., Varmus, H. E. & Ganem, D. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3440–3444.
30. Machida, A., Kishimoto, S., Ohnuma, H., Miyamoto, H., Baba, K., Oda, K., Nakamura, T., Miyakakawa, Y. & Mayumi, M. (1983) *Gastroenterology* **85**, 268–274.
31. Persing, D. H., Varmus, H. E. & Ganem, D. (1986) *Science*, in press.