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RNA- and DNA-Binding Activities in Hepatitis B Virus Capsid Protein: a Model for Their Roles in Viral Replication

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The hepatitis B virus capsid or core protein (p21.5) binds nucleic acid through a carboxy-terminal protamine region that contains nucleic acid-binding motifs organized into four repeats (I to IV). Using carboxy-terminally truncated proteins expressed in *Escherichia coli*, we detected both RNA- and DNA-binding activities within the repeats. RNA-binding and packaging activity, assessed by resolving purified *E. coli* capsids on agarose gels and disclosing their RNA content with ethidium bromide, required only the proximal repeat I (RRRDRGRS). Strikingly, a mutant in which four Arg residues replaced repeat I was competent to package RNA, demonstrating that Arg residues drive RNA binding. In contrast, probing immobilized core proteins with ^{32}P-nucleic acid revealed an activity which (i) required more of the protamine region (repeats I and II), (ii) appeared to bind DNA better than RNA, and (iii) was apparently modulated by phosphorylation in p21.5 derived from *Xenopus* oocytes. Deletion analysis suggested that this activity may depend on an SPXX-type DNA-binding motif in repeat II. Similar motifs found in repeats III and IV may also function to bind DNA. On the basis of these observations, together with a reinterpretation of recent studies showing that capsid protein mutants cause defects in viral genome replication, we propose a model suggesting that hepadnavirus capsid proteins participate directly in the intracapsid reverse transcription of RNA into DNA. In this model, repeat I binds RNA whereas the distal repeats are progressively recruited to bind elongating DNA strands. The latter motifs may be required for replication to be energetically feasible.

Packaging the viral pregenomic RNA into the hepatitis B virus (HBV) nucleocapsid (core particle) is a crucial function of the 21.5-kDa capsid or core protein (p21.5). The highly selective packaging of the RNA pregenome seen in replication-competent viral nucleocapsids (10) requires cooperation between the capsid protein, the viral *pol* gene product (4, 15), and a packaging signal in the pregenomic RNA (16, 17). However, RNA packaging is an intrinsic property of p21.5 (for a review of p21.5, see reference 28) since recombinant bacterial p21.5 assembles into capsids that contain nonpregenomic RNA (5). Recent genetic studies (27, 38) indicate that carboxy-terminal truncation of the duck hepatitis B virus (DHBV) capsid protein adversely affects reverse transcription of the viral pregenome RNA into DNA, suggesting that the role of the capsid protein may go beyond RNA packaging.

The first 149 residues of the 185-residue p21.5 capsid protein form a domain which is very resistant to proteolytic attack (36) and directs assembly of the 28-nm capsid shell (5, 13). The final 36 residues form an Arg-rich region that bears a striking similarity to protamines and mediates the interaction with nucleic acid detected by either an RNA encapsidation assay (5) or a Southwestern (DNA-protein) blot assay (13, 24). This region contains multiple motifs, particularly blocks of Arg residues and SPXX motifs (33), that are candidates for binding nucleic acid and neutralizing its charge, but the exact contribution of each motif to binding has not been evaluated. In this study we have addressed this question by using a series of bacterially expressed core protein mutants truncated to endpoints within the protamine region. The nucleic acid-binding motifs that we identify in p21.5 appear to be specifically configured for a role in HBV replication.

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MATERIALS AND METHODS

Reagents. Homogeneous preparations of recombinant capsids from *Escherichia coli* and from *Saccharomyces cerevisiae* were kindly provided by Chiron, Emeryville, Calif., and Merck, West Point, Pa., respectively. All chemical and biochemical reagents were of fine reagent grade. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim; radiochemicals were obtained from NEN. Oligonucleotides were synthesized on a Milligen Biosearch 8700 synthesizer. DNA sequencing was performed with an Applied Biosystems 373A DNA sequencer. Standard protocols were used for molecular biology procedures.

Construction of mutants and expression plasmids. HBV DNA of subtype *adw* (34) was used for all constructs. For expression in *E. coli*, the 185-amino-acid p21.5 coding region was mobilized by the polymerase chain reaction from plasmid 64-C (29) to the expression vector pAR3040 (31). The 5′ primer AAGCTTCATATGGACATTGACCCTTAT introduced an *Nde*I site next to the p21.5 AUG; the 3′ primer GGCCCGGGCTAACATTGAGATTCCCG introduced a *Sma*I site beyond the termination codon. The amplified fragment was recovered by elution from agarose, cut with *Nde*I and *Sma*I, and ligated into *Nde*I-*Sma*I-cleaved pAR3040, creating pARC, in which p21.5 expression is driven by the inducible T7 polymerase in *E. coli* BL21 (DE3)(pLysS) (31). To create the simplified vector pSPARC, which facilitates the transfer of mutants between the vectors used for the oocyte and *E. coli* systems, a 642-bp *Sph*I-*Ssp*I fragment carrying the T7 promoter and the 5′ half of the p21.5 gene was cleaved from pARC and inserted into

pSP64T-C (39) in place of an analogous *Sph*I-*Ssp*I fragment bearing the SP6 promoter and same region of p21.5.

Mutant Δ-149, truncated to residue Val-149, was created by polymerase chain reaction treatment of pSP64C by using the 3′ oligonucleotide CCCGGGAGCTCTAGACAACAG TAGTTTCCGG and the same 5′ oligonucleotide as for pARC (see above). Mutant Δ-157 (39) encodes a protein contiguous with p21.5 as far as residue Ser-157, which also incorporates the last two amino acids (QC) of wild-type p21.5. To create mutant Δ149-R4 an oligonucleotide adaptor was assembled from CCGGAAACTACTGTTGTTCGAA GAAGAAGATAG and GTAACCTATCTTCTTCTTCGAA CAACAGTAGTTT and inserted into *Bsp*EI-*Bst*EII-cleaved pSPARC. Δ-162 and Δ-172 were created by polymerase chain reaction treatment of plasmid pSP64T-C (39) by using, respectively, the 3′ synthetic oligonucleotides CCCCCGG TAACCCTAAGTTCTTCTTCTAGGGGACC and CCCC GGTAACCCTACGATTGAGATCTGCGTCTGC. A small *Bsp*EI-*Bst*EII restriction fragment carrying each mutation was then mobilized into pSPARC. All mutations, and the core protein-coding sequences of the original vectors, were verified by direct DNA sequencing.

Expression and purification of recombinant core particles. Proteins were expressed in *E. coli* as described previously (5). Briefly, 2-ml overnight cultures were used to inoculate 500 ml of LB–glucose (4 g/liter). When the cultures reached an A_{600} of 0.7 to 1.0, expression was induced by addition of 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation (Sorvall GS3 rotor; 3,500 rpm for 30 min) after 4 h (Δ-149) or 12 to 16 h (the remaining mutants) and frozen. The thawed cell pellets were resuspended in 50 mM Tris (pH 7.5)–10 mM EDTA and disrupted on ice by multiple 30-s bursts of sonication. Debris were removed by centrifugation (Sorvall HS4 rotor; 3,500 rpm for 30 min), and ammonium sulfate was added slowly to 40% saturation. After 30 min on ice, the precipitated proteins were pelleted (Sorvall HS4 rotor; 3,500 rpm for 30 min), resuspended in 50 mM Tris–250 mM NaCl, and clarified (Sorvall SS34 rotor; 15,000 rpm for 60 min). Core particles were pelleted in the Beckman TL100 centrifuge (TLA100.3 rotor; 100,000 rpm for 60 min) and then resuspended and centrifuged for 18 to 20 h at 25,000 rpm through 10 to 60% sucrose gradients (Beckman LM8 centrifuge, SW28 rotor). Fractions containing capsids were identified by Western immunoblotting, and the particles were collected by centrifugation (TLA 100.3 rotor; 100,000 rpm for 60 min), resuspended in 50 mM Tris (pH 7.5)–250 mM NaCl and stored at 4°C. The purity of each preparation was assessed by Coomassie blue staining after polyacrylamide gel electrophoresis (PAGE) on discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (19). The protein concentration was estimated by the method of Bradford (6).

Determination of RNA packaging. Samples of purified native capsids (3 to 5 μg of protein) were electrophoresed at 70 V through 1.2% agarose gels equilibrated in 45 mM Tris-borate–1 mM EDTA and containing 10 μg of ethidium bromide per ml. Gels were destained, photographed with Polaroid 667 film under UV transillumination, and then restained with Coomassie blue to reveal the core proteins. The destained gels were photographed after being dried onto a transparent membrane.

Detection of DNA and RNA binding to immobilized core proteins. Capsid proteins were resolved by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon; Millipore Corp). For DNA-binding studies, the membranes were blocked by incubation for 60 min at room temperature with 5% nonfat milk–yeast tRNA (10 μg/ml) in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–2.5 mM $MgCl_2$–75 mM KCl (HMK). The blocked membranes were incubated for 60 min at 22°C with 500,000 cpm of a high-specific-activity (>10^8 cpm/μg) ^{32}P-labeled DNA probe per ml, derived from plasmid 64-C (29), which contains the whole HBV genome. The probe was derived by random priming and was not denatured before binding. The blots were washed three times (10 min per wash) in HMK and then exposed to X-ray film. For RNA binding, the blots were blocked in 50 mM Tris chloride (pH 7.5)–100 mM NaCl–5× Denhardt's solution–10 μg of yeast tRNA per ml (100 μg of *E. coli* DNA per ml was used in some experiments not reported here), and incubated with a high-specific-activity ^{32}P-labeled p21.5 RNA probe synthesized by transcribing plasmid pSP64T-C (see below) in vitro with SP6 RNA polymerase. The blot was then processed as described above for the DNA probe.

Following autoradiography, the membranes were reprobed with anti-core antibody to detect core proteins. After blocking for 1 h at 22°C with 5% nonfat milk in 20 mM Tris hydrochloride (pH 7.5)–500 mM NaCl–0.05% Tween 20 (TNT), the blots were incubated for 30 min at 22°C with a 1:5,000 dilution of anti-p21.5 antibody (Dako) in TNT, washed three times for 10 min each in TNT, incubated as above with a 1:5,000 goat anti-rabbit alkaline phosphatase- or goat anti-rabbit horseradish peroxidase-coupled antibody (Bio-Rad), and rewashed as above. Phosphatase and peroxidase were detected with a Nitro Blue Tetrazolium–bromochloro-indolyl phosphate stain (Bio-Rad) or by chemiluminescence with an ECL disclosure kit (Amersham), respectively. The kits were used as specified by the manufacturers.

Synthesis of p21.5 in *Xenopus* oocytes. Standard methods (8, 9) were used to handle, microinject, and culture oocytes. Further details of these methods (29, 30) and of the synthetic p21.5 mRNA (39) used for p21.5 expression have been reported elsewhere. Injected oocytes were incubated for ~48 h and then homogenized in 50 mM Tris hydrochloride (pH 7.5)–1% Nonidet P-40–10 mM EDTA. For Southwestern analysis (see above), unlabeled p21.5 was immunoprecipitated from ~72 oocytes' worth of lysate with rabbit anti-core antiserum (Dako).

To prepare metabolically labeled core proteins, batches of 10 to 20 healthy injected oocytes were transferred, within 3 h of injection, to modified Barth's medium supplemented with 1 to 2 mCi of either $^{32}P_i$ (NEN) or [^{35}S]Met and [^{35}S]Cys (Expresslabel; NEN) per ml and incubated for a further 44 h at 17°C. The [^{32}P]p21.5 or [^{35}S]p21.5 was immunoprecipitated from five or one oocyte's worth of homogenate, respectively, and analyzed by SDS-PAGE.

Two-dimensional electrophoretic analysis of p21.5. Purified bacterial core particles from the *E. coli* expression system, or [^{35}S]p21.5 immunoprecipitated from about two oocytes, was analyzed by two-dimensional PAGE, essentially as described previously (22), by using a mini two-dimensional electrophoresis system (Bio-Rad). Samples were dissolved in 9.5 M urea–2.0% Triton X-100–5% 2-mercaptoethanol–1.6% ampholytes (pH 5 to 7)–4% ampholytes (pH 3 to 10) and electrophoresed at 750 V for 3.5 to 5 h through 4% polyacrylamide tube gels containing the same buffer. The gels were extruded into Laemmli sample buffer, placed onto 15% polyacrylamide slab gels, and subjected to SDS-PAGE for the second dimension as described above.

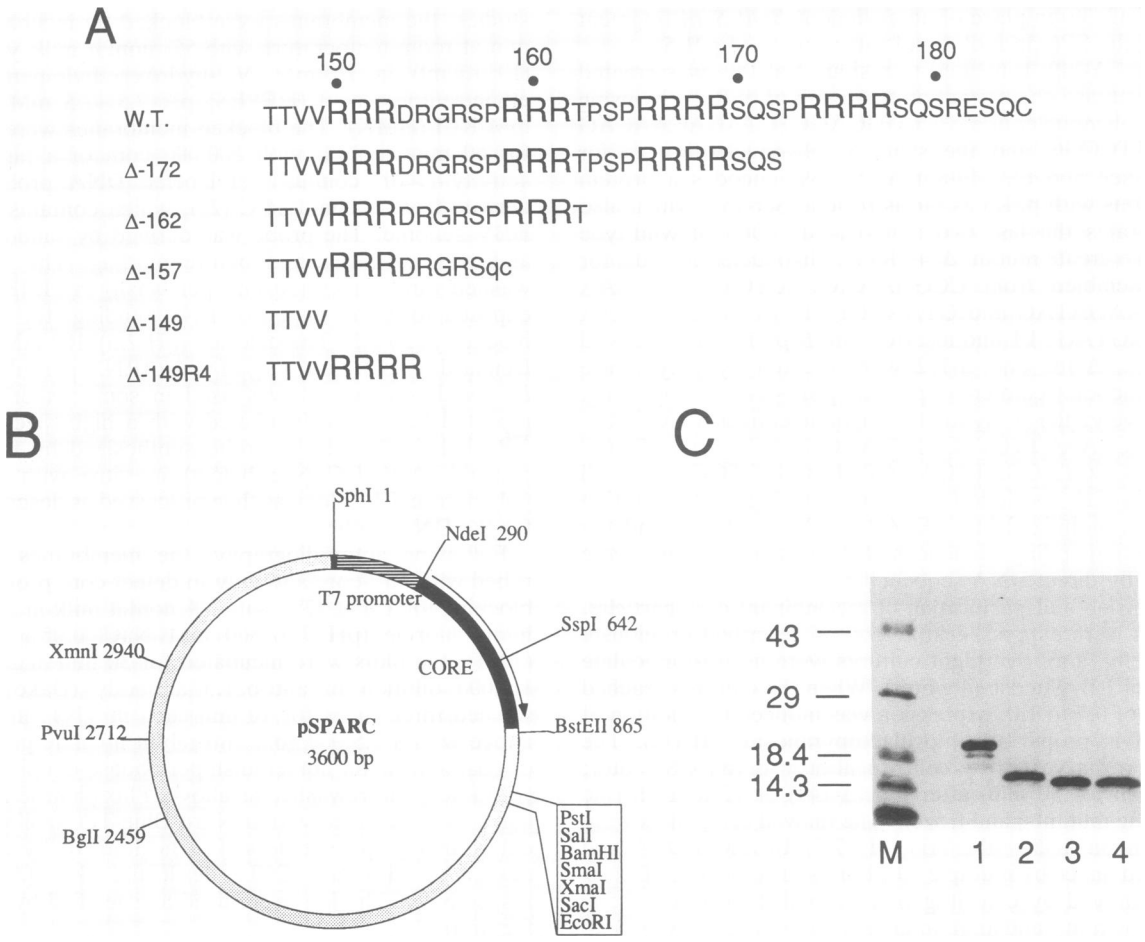

FIG. 1. Structure and expression of core protein mutants. Core particles were expressed in *E. coli* and purified as described in Materials and Methods. (A) Comparison between the carboxy-terminal sequences of the mutants and wild-type (w.t.) p21.5 (top line). (B) Structure of the *E. coli* p21.5 expression vector pSPARC showing important restriction sites, the T7 promoter (hatched), and the p21.5 coding sequence (black), with an arrow indicating the 5′-to-3′ direction of the gene. (C) Coomassie blue-stained SDS-polyacrylamide gel showing purified capsid preparations from wild-type p21.5 (lane 1), Δ-157 (lane 2), Δ-149R4 (lane 3), and Δ-149 (lane 4). The sizes (in kilodaltons) of protein molecular mass markers (lane M) are indicated at the left.

RESULTS

Structure, expression, and purification of capsids. The carboxy-terminal amino acid sequences of p21.5 and the various mutant core proteins are shown in Fig. 1A. The last 36 amino acids (residues 150 to 185) of p21.5 resemble protamine molecules (34) and include four evenly spaced blocks of three or four Arg residues which effectively divide this region into four repeats (see Discussion for more details). The mutants specify core proteins progressively truncated to endpoints between the Arg blocks. Mutant Δ-172 encodes a 20-kDa core protein (p20) that retains three of the four Arg blocks; Δ-162 encodes a 19-kDa protein (p19) that includes two Arg blocks; Δ-157 encodes an 18-kDa protein (p18) with only the first Arg block; and Δ-149 encodes a 17-kDa protein (p17) from which the protamine region has been precisely deleted. Mutant Δ-149R4, constructed to test the nucleic acid binding properties of a single Arg block, encodes a core protein with just four Arg residues appended onto the carboxyl terminus of the Δ-149 core protein.

The inducible T7 polymerase-driven system (31) was used to express these proteins in *E. coli*. The parental vector pSPARC (Fig. 1B) contains the T7 promoter (hatched) and the p21.5 coding sequence (black) in a pSP64 (18) vector background. The mutants were all expressed with similar efficiency, and, as expected since the protamine region is dispensable for particle assembly (5, 13), all assembled into capsids (data not shown). Capsids were purified by an ammonium sulfate precipitation step, a high-speed pelleting step, and sedimentation through 10 to 60% (wt/vol) sucrose. Yields typically ranged from 1 to 7 mg of capsids per liter of culture. A representative analysis (Fig. 1C) of the purity of the wild-type p21.5 (lane 1), Δ-157 (lane 2), Δ-149R4 (lane 3), and Δ-149 (lane 4) capsid preparations by SDS-PAGE and Coomassie blue staining shows that the core proteins migrated mainly as single bands with the expected molecular masses. Silver staining revealed several minor but no major impurities in these preparations (data not shown).

A single Arg block mediates RNA binding and encapsidation. Birnbaum and Nassal (5), in a study aimed at investigating the p21.5 polypeptide requirements for core particle assembly, described a convenient method for examining the RNA content of HBV capsids in which native capsids are stained with ethidium bromide after electrophoresis through agarose. We used this simple assay, which measures the

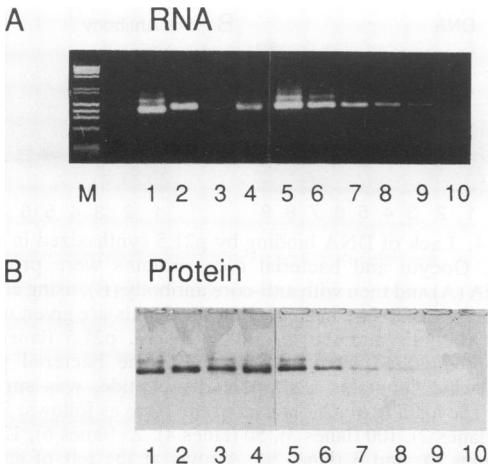

FIG. 2. Minimal protamine sequences required for encapsidation of nucleic acid into core particles. Purified preparations of core particles from *E. coli* were electrophoresed through agarose gels which were stained first with ethidium bromide to reveal RNA (A) and then with Coomassie blue to reveal protein (B) as described in Materials and Methods. Lanes 1 to 4 in both panels depict ~5 μg of particles corresponding to wild-type p21.5 (lanes 1), Δ-157 (lanes 2), Δ-149 (lanes 3), and Δ-149R4 (lanes 4). Lanes 5 to 10 show a twofold serially diluted set of p21.5 capsid standards with dilutions as follows: undiluted (lanes 5, ~5 μg of p21.5), 2-fold (lanes 6), 4-fold (lanes 7), 8-fold (lanes 8), 16-fold (lanes 9), and 32-fold (lanes 10). Lane M (panel A) shows a mixture of *Hin*dIII-cut phage lambda and *Hae*III φX DNA size standards.

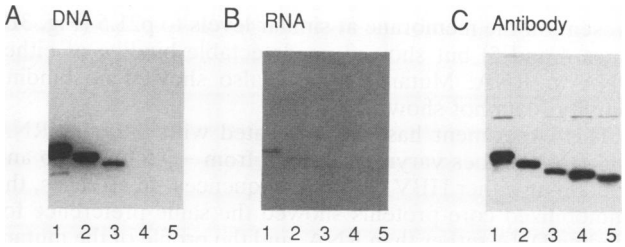

FIG. 3. Binding of nucleic acid by immobilized core proteins. Purified core proteins (~500 ng) derived from wild-type p21.5 (lanes 1), Δ-172 (lanes 2), Δ-162 (lanes 3), Δ-157 (lanes 4), or Δ-149 (lanes 5) were resolved by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and probed in parallel with either [^{32}P]DNA (A) or [^{32}P]RNA (B) as described in Materials and Methods. The membranes were exposed to X-ray film with an intensifying screen at −70°C for 3 h (panel A) or 18 h (panel B). The core proteins on the membrane in panel A were reprobed with rabbit anti-core antibody, followed by a horseradish peroxidase secondary, and visualized by a chemiluminescent detection system (C). The membrane was exposed to X-ray film for ~1 min.

ability of the core proteins to package (encapsidate) RNA in solution, to delineate the minimal protamine segment required for efficient RNA binding and encapsidation.

Purified capsids were electrophoresed through agarose and stained with ethidium bromide to reveal RNA (Fig. 2A) and then with Coomassie blue to visualize protein (Fig. 2B). Both profiles showed the p21.5 (lane 1), Δ-157 (lane 2), Δ-149 (lane 3), and Δ-149R4 (lane 4) capsids migrating mainly as single species with similar mobilities; traces of slower-migrating species may represent aggregated capsids. Nucleic acid and protein comigrated in all cases, indicating that all capsids contained nucleic acid; however, although similar amounts of protein (~5 μg) were seen in lanes 1 to 4, the RNA content of the capsids clearly differed. To confirm that the encapsidated nucleic acid was indeed RNA, as reported by Birnbaum and Nassal (5), we heated the capsids to 65°C for 15 min and then 22°C for 30 min in the absence or presence of RNase A prior to electrophoresis. Heating in the absence of RNase A resulted in the release of nucleic acid that ran on the agarose gel as a low-molecular-mass smear of ethidium bromide-stained material; this product was eliminated when the heat treatment was conducted in the presence of RNase A (data not shown).

RNA-packaging levels were estimated by comparing the RNA staining intensities against those of a panel of 2-fold serially diluted p21.5 capsid standards (lanes 5 to 10), ranging from undiluted (5 μg of p21.5; lane 5) to 32-fold diluted (lane 10). Δ-149 capsids (Fig. 2A, lane 3) contained slightly less RNA than the 16-fold-diluted p21.5 standard (lane 9) but more than the 32-fold-diluted standard (lane 10), indicating that removal of the protamine region diminished RNA packaging roughly 20-fold. This confirms the role of the protamine region in packaging and is in excellent agreement with the results of Birnbaum and Nassal (5).

Δ-157 capsids (Fig. 2A, lane 2) contained RNA levels close to those of p21.5 capsids (compare lanes 1 and 5), with packaging being reduced by roughly twofold (compare lanes 2 and 6). A similar reduction in RNA packaging (~50%) was seen for both the Δ-162 and Δ-172 capsids (data not shown). Whether this slight decrease in RNA packaging reflects deletion of a binding activity from the carboxyl terminus of the protamine segment or whether it occurs for indirect reasons (e.g., perturbation of the capsid structure) remains to be determined.

Our deletion analysis suggests that the first 8 amino acids, RRRDRGRS (residues 150 to 157), of the protamine region possess most of the RNA-binding and encapsidation activity of the entire region. This minimal sequence is Arg rich and includes the proximal Arg block.

The crucial role of Arg residues in encapsidating RNA into core particles was demonstrated by using the Δ-149R4 capsid, in which just four Arg residues follow the 149-amino-acid hydrophobic domain of p21.5. This mutant yielded capsids containing roughly 25% as much RNA as wild-type p21.5 capsids did (Fig. 2A, compare lanes 4, 6, and 7). This is close to the RNA content of the Δ-172, Δ-162, and Δ-157 capsids, suggesting that Arg residues are largely responsible for driving the binding and hence the encapsidation of RNA.

Nucleic acid binding by immobilized core proteins. For an independent evaluation of the nucleic acid-binding activity of the protamine region, we examined the nucleic acid-binding properties of immobilized core proteins (Fig. 3). Identical samples of core proteins were resolved in parallel by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with a ^{32}P-labeled HBV genome probe (>10^8 cpm/μg) containing mainly double-stranded DNA (Southwestern; Fig. 3A), or with a ^{32}P-labeled p21.5 RNA probe of comparable specific activity (Northwestern [RNA-protein]; Fig. 3B). The Southwestern membrane was subsequently reprobed with anti-core antiserum (Western immunoblot; Fig. 3C). Lanes 1 to 5 in each panel show wild-type p21.5, Δ-172, Δ-162, Δ-157, and Δ-149, respectively.

In this assay, p21.5, Δ-172, and Δ-162 displayed a strong affinity for [^{32}P]DNA (Fig. 3A, lanes 1 to 3) but bound RNA only weakly (Fig. 3B, lanes 1 to 3). Mutants Δ-157 (Fig. 3A and B, lanes 4) and Δ-149 (Fig. 3A and B, lanes 5) were

present on the membrane at similar levels to p21.5 (Fig. 3B, lanes 4 and 5) but showed no detectable binding of either DNA or RNA. Mutant Δ-149R4 also showed no binding activity (data not shown).

This experiment has been repeated with different RNA and DNA probes varying in length from ~0.5 to 3.4 kb and containing either HBV or vector sequences. In all cases, the immobilized core proteins showed the same preference for binding DNA rather than RNA, and the profile of the mutant core proteins with the different probes was analogous to that seen in Fig. 3 (data not shown). RNA probes incorporating the pregenome RNA encapsidation signal (17) were also tested but showed no sign of an increased affinity for immobilized core proteins (data not shown).

The nucleic acid-binding activity displayed by immobilized core proteins differs from the previously characterized RNA encapsidation activity in two important respects. First, immobilized core proteins seemed to interact poorly with RNA but exhibited a higher affinity for DNA probes. Second, more of the protamine region was required for the manifestation of Southwestern binding activity. Although it is possible that these differences arise for purely experimental reasons, two lines of argument persuade us that these assays both measure intrinsic p21.5 binding activities that are different. First, the RNA- and DNA-binding activities of immobilized core proteins were tested in parallel by using comparable conditions and a variety of probes (Fig. 3 and data not shown). Although it could be argued that our choice of experimental conditions somehow suppressed RNA binding, analogous conditions failed to eliminate DNA binding, suggesting that the latter is a distinct activity. Second, Δ-162 bound DNA whereas Δ-157 did not, placing an element that confers Southwestern binding activity between amino acids 157 and 162 (157-SPRRRT-162). This segment contains a known DNA-binding motif, SPRR, a member of the SPXX family of motifs (33) that bind to the minor groove of DNA and are found in DNA-binding proteins such as histones or RNA polymerase II (7, 33). Thus, it seems likely that the Southwestern assay is detecting a true DNA-binding activity in p21.5 which depends on SPRR and presumably recognizes double-stranded DNA (although we have not excluded the possibility that immobilized core proteins actually bind to single-stranded DNA).

Residues 162 to 185 of the protamine region contain two further SPRR copies at positions 164 to 167 and positions 172 to 175. Comparing the binding activity of wild-type p21.5 (Fig. 3A, lane 1), Δ-172 (lane 2), and Δ-162 (lane 3), we note that the DNA-binding signal plunged sharply with each truncation. Immunoblotting revealed that this decrease was partly due to decreased loadings of the mutant core proteins. (Fig. 3C, lanes 2 and 3) compared with p21.5 (lane 1), but the Southwestern signal still appeared to fall off more rapidly than the Western signal. These data suggest the possibility that the distal repeats also contribute to DNA binding, presumably via the additional SPRR motifs.

DNA binding is extinguished in p21.5 from *Xenopus* oocytes. Since we have been routinely expressing p21.5 in *Xenopus* oocytes under the direction of a synthetic mRNA for studies of capsid assembly (39–41), we compared the Southwestern binding activity of oocyte p21.5 with that of the *E. coli* core protein (Fig. 4). The bacterial core protein used in this experiment (provided by Chiron) bears a short amino-terminal leader peptide, a remnant of the expression system, and migrates as a ~23-kDa protein (p23) in SDS-PAGE. This leader peptide does not appear to influence the DNA-binding properties of the capsid protein; authentic p21.5 from *S. cerevisiae* (provided by Merck) or from our *E. coli* expression system (see above) gave the same results (Fig. 3A; data not shown).

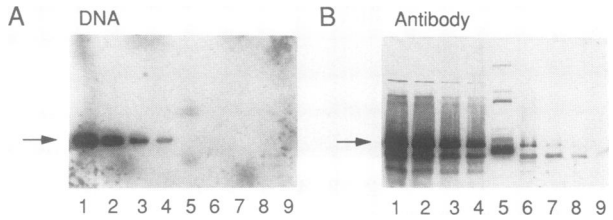

FIG. 4. Lack of DNA binding by p21.5 synthesized in *Xenopus* oocytes. Oocyte and bacterial core proteins were probed with [^{32}P]DNA (A) and then with anti-core antibody (B), using an alkaline phosphatase disclosure system. Further details are given in Materials and Methods and the legend to Fig. 3. p21.5 (lanes 5) was immunoprecipitated from ~72 oocytes. The bacterial p23 core protein, which contains a short leader peptide, was supplied by Chiron. The loading of p23 (in nanograms) was as follows: 500 (lanes 1), 250 (lanes 2), 100 (lanes 3), 50 (lanes 4), 25 (lanes 6), 12.5 (lanes 7), 6 (lanes 8), and 3 (lanes 9). Arrows at the left of each panel indicate the position of p23.

The immobilized proteins were probed with [^{32}P]DNA (Fig. 4A) and then reprobed with anti-core antiserum (Fig. 4B). DNA binding by bacterial p23 (arrow) was clearly visible for loadings of 500 (lane 1), 250 (lane 2), 100 (lane 3), and 50 (lane 4) ng of protein, although not for 25, (lane 6), 12.5 (lane 7), 6 (lane 8), or 3 (lane 9) ng. However, immunoprecipitated oocyte p21.5 (lane 5) showed no detectable DNA-binding activity, even though the immunoblot data suggest that the membrane contained ~250 ng of p21.5.

Various control experiments have ruled out trivial explanations for the reduction in binding activity seen for oocyte p21.5. The result has been verified (data not shown) by using bacterial and oocyte p21.5 preparations originating from vectors with identical p21.5 coding sequences (verified by DNA sequencing); thus, there is no genetic explanation. Moreover, bacterial capsids retained DNA-binding activity following injection into oocytes and recovery by immunoprecipitation (data not shown), so that oocyte handling and immunoprecipitation procedures were not responsible for the activity loss. This leaves posttranslational modification of oocyte p21.5 as the most plausible explanation for the loss of DNA-binding activity.

Phosphorylation of core proteins in *Xenopus* oocytes. Phosphorylation is the only modification documented for hepadnaviral capsid proteins (1, 11, 14, 26, 27). We have recently demonstrated that p21.5 is phosphorylated in oocytes (36). Here we examined whether phosphorylation could account for the extinction of the DNA-binding activity in p21.5.

We first investigated where phosphorylation occurs within the p21.5 polypeptide. Oocytes expressing wild-type or truncated core proteins were labeled in parallel with either [^{35}S]Met and [^{35}S]Cys (Fig. 5A) or $^{32}P_i$ (Fig. 5B), and the core proteins were analyzed by immunoprecipitation and SDS-PAGE. The lanes in each autoradiogram show p21.5 (lanes 1), Δ-172 (lanes 2), Δ-162 (lanes 3), Δ-157 (lanes 4), Δ-149R4 (lanes 5), and Δ-149 (lanes 6). The inclusion of more protamine sequence resulted in a gradual decrease in the ^{35}S signal (two- to threefold overall), an effect we have routinely observed (40a). This is not caused by any obvious decrease in RNA levels or capsid protein stability (data not shown) but, rather, may reflect a negative influence of the Arg-rich protamine region on translation.

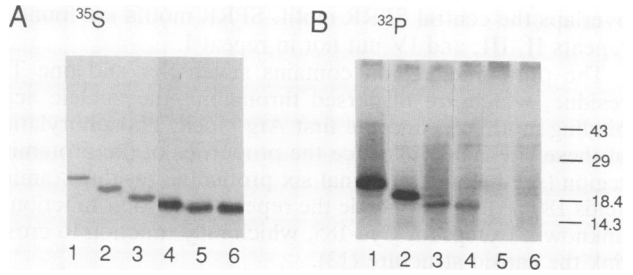

FIG. 5. Profile of (A) ^{35}S- or (B) ^{32}P-labeled core proteins immunoprecipitated from oocytes. Oocytes were injected with the following RNAs: wild-type p21.5 (lanes 1), Δ-172 (lanes 2), Δ-162 (lanes 3), Δ-157 (lanes 4), Δ-149R4 (lanes 5), and Δ-149 (lanes 6); they were then labeled metabolically with either [^{35}S]Met and [^{35}S]Cys or $^{32}P_i$. Core proteins were immunoprecipitated from either one (A) or five (B) oocytes and analyzed by SDS-PAGE and autoradiography. The dried gels were exposed to X-ray film at −70°C with a screen for 12 h (panel A) or 48 h (panel B). The positions of the molecular mass markers (in kilodaltons) are shown at the right of panel B.

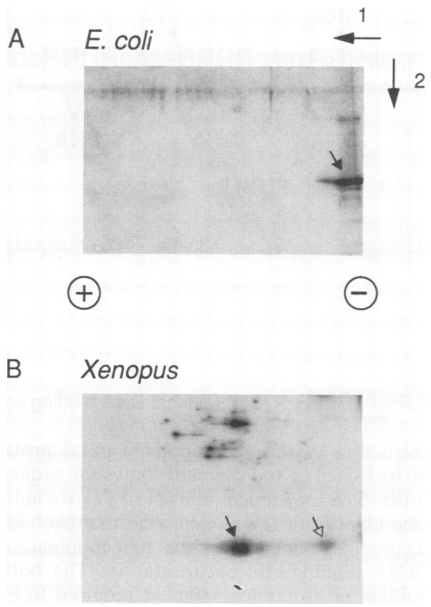

FIG. 6. Two-dimensional PAGE analysis of *E. coli* (A) and oocyte (B) p21.5. Analysis was performed on ~2 μg of pure *E. coli* p21.5, which was detected by Coomassie blue staining, or on [^{35}S]p21.5 immunoprecipitated from two oocytes, which was detected by autoradiography (3 days at −70°C with a screen). Experimental details are provided in Materials and Methods. The first (isoelectric focusing) and second (SDS-PAGE) electrophoretic dimensions are indicated by arrows at the top of the figure. The polarity of the electrodes used for isoelectric focusing is indicated by the + and − signs beneath panel A. Care was taken to use comparable electrophoresis conditions for the first dimension; the oocyte p21.5 was run slightly farther than the bacterial p21.5 in the second dimension. The positions of major (solid arrows) and minor (open arrows) p21.5 species are indicated. Prestained protein markers were used to provide molecular mass calibration for the SDS-PAGE second dimension, but these have been omitted for the sake of clarity.

In contrast, inclusion of more protamine sequence dramatically increased the ^{32}P signal (Fig. 5B). Labeling was nonexistent for the Δ-149R4 (lane 5) and Δ-149 (lane 6) proteins, weak for the Δ-157 (lane 4) and Δ-162 (lane 3) proteins, and very strong for the Δ-172 (lane 2) and p21.5 (lane 1) proteins. These data suggest that phosphorylation of p21.5 in oocytes is restricted to target residues in the protamine region and that increasing segments of protamine region bring in additional phosphorylation targets. Ser residues in the protamine region are the proposed (2) targets for the kinase activity found in core particles from virions, and the oocyte data are consistent with this notion. The first Ser residue (Ser-157) is evidently phosphorylated in the Δ-157 capsid protein. One or more of the Ser residues at positions 164 or 170 and at least one of four Ser residues between positions 172 and 183 are apparently also phosphorylated in oocytes.

We next used two-dimensional PAGE (Fig. 6) to examine to what extent p21.5 polypeptide chains were modified in *E. coli* compared with oocytes. p21.5 was expressed in *E. coli* by using the pSPARC vector and purified essentially to homogeneity as described above; ^{35}S-labeled oocyte p21.5 was translated from pSP64T-C mRNA (pSPARC and pSP64T-C carry genetically identical p21.5 sequences) and recovered by immunoprecipitation. These p21.5 preparations were subjected to isoelectric focusing to resolve the proteins on the basis of their net charge in the first dimension followed by SDS-PAGE to resolve the proteins by size in the second dimension.

The electrophoretic profile of pure *E. coli* p21.5 (Fig. 6A) showed that this protein behaved as expected in SDS-PAGE, migrating mainly as a Coomassie blue-stainable species of ~21.5 kDa (indicated by an arrow); a minor band of higher mass (~40 kDa) probably represents a p21.5 dimer. In the first dimension, the bacterial p21.5 remained close to the basic end of the isoelectric focusing gel, migrating as a slightly smeared band. These data suggest that bacterial p21.5 is a highly basic protein, as expected from the protein sequence. Immunoprecipitated oocyte [^{35}S]p21.5 (Fig. 6B) also migrated as a 21.5-kDa species in SDS-PAGE, but the autoradiogram showed that the bulk of the oocyte [^{35}S]p21.5 species (solid arrow) migrated in the first dimension as a protein far more acidic than bacterial p21.5. Only a minor peak of more basic [^{35}S]p21.5 (open arrow) was seen in the autoradiogram. These p21.5 spots were not seen in the two-dimensional profile of proteins brought down by anti-core antibody from uninjected control oocytes, but the higher-molecular-mass species seen in Fig. 6B were visible in the control and apparently represent endogenous oocyte proteins brought down under the nonstringent immunoprecipitation conditions of this experiment (data not shown).

Although we have not formally established that phosphorylation is the modification detected in this experiment, the acidic character of the oocyte p21.5 is certainly consistent with this possibility. The data from Fig. 5 and 6 suggest that the bulk of the p21.5 polypeptide chains synthesized in oocytes may carry multiple phosphate groups, presumably attached to protamine region Ser residues. In contrast, bacterial p21.5 is not phosphorylated, confirming the observations of others (21, 27). Thus, our data provide indirect evidence that phosphorylation down regulates the DNA-binding activity of p21.5 (at least in Southwestern assays). Since this work was completed, Machida et al. (21) have reported that authentic virion-derived p21.5, unlike bacterial p21.5, is unable to bind DNA in a Southwestern assay. These authors provided formal proof that phosphorylation is responsible for this phenomenon by enzymatically dephosphorylating virion p21.5, thus restoring its DNA-binding activity.

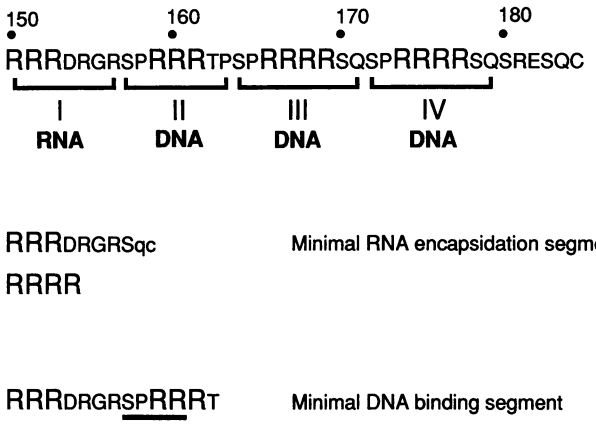

FIG. 7. Structure of the 36-amino-acid p21.5 protamine region (residues 150 to 185). The top segment shows the sequence of amino acids 150 to 185. The four repeat units (I to IV) are indicated below, along with the identity of the nucleic acid that each is proposed to bind. The center segment shows the two minimal sequences required for RNA binding and encapsidation. The bottom segment shows the minimum protamine segment required to bind DNA on Southwestern blots, with the SPRR motif underlined. More details are given in the Discussion.

DISCUSSION

Little is known about the interactions between the HBV p21.5 capsid protein and nucleic acid. In the work described in this report we created HBV capsid proteins bearing truncations within the p21.5 protamine region and tested their ability to encapsidate RNA in solution, leading to a detailed characterization of the interaction between p21.5 and RNA. We also examined the ability of these proteins to bind ^{32}P-nucleic acid probes in a Southwestern assay, which revealed that p21.5 contains an activity apparently directed to binding DNA. These observations led us to consider the possibility that p21.5 interacts with both RNA and DNA to facilitate the intracapsid process of viral replication. This idea was reinforced by a reanalysis (see below) of recent genetic studies on the role of the capsid protein in DHBV. In this section we propose a model outlining how such facilitated replication may occur.

Organization of the protamine region. The 36-amino-acid sequence of the p21.5 protamine region is shown in Fig. 7. The first 30 residues are organized into four repeats (I to IV) of seven or eight residues. There is more than one way to view this organization, but we chose SPRRRRSQ (amino acids 172 to 179, repeat IV) as the prototype repeat. Repeat III contains a second SPRRRRSQ copy (amino acids 164 to 171). Closely related SPRRRTP (amino acids 157 to 163) forms repeat II. Proximal repeat I (RRRDRGR; amino acids 150 to 156) is the least related member of the family. The endpoints of the Δ-149, Δ-157, Δ-162, and Δ-172 mutants closely approximate the repeat boundaries.

The salient charge neutralization or nucleic acid-binding elements of the protamine region are found in the repeats. These include four Arg blocks (amino acids 150 to 152, 159 to 161, 166 to 169, and 174 to 177) and three overlapping SPRR motifs (amino acids 157 to 160, 164 to 167, and 172 to 175). The SPRR motifs are members of the SPXX family of motifs (33), which are found in DNA-binding proteins such as histones and RNA polymerase and are thought to bind in the minor groove of DNA (7, 33). A TPXX motif (TPSP; amino acids 162 to 165) which may possess similar activity (7, 33) overlaps the central SPRR motif. SPRR motifs are found in repeats II, III, and IV but not in repeat I.

The protamine region contains seven Ser and one Thr residue, which are dispersed throughout the nucleic acid-binding motifs beyond the first Arg block. Phosphorylation of these sites may influence the properties of the protamine region (see below). The final six protamine residues (amino acids 180 to 185) lie outside the repeats, and their function is unknown, except for Cys-185, which may function to cross-link the capsid structure (13).

Intriguingly, the Arg blocks and overlapping SPRR motifs occur at intervals of 7 to 9 residues, a periodicity that may have structural and biological significance. Since SPRR motifs adopt a β-turn structure, the protamine region may comprise reiterated β-turns. In glutenins, the proteins responsible for the elasticity of wheat flour dough, successive hexa- and nonapeptide β-turn configurations form a helix of stacked β-turns which has elastic properties (12). In p21.5, such a structure could also serve to align the binding motifs for optimal interaction with nucleic acid, much as a 7- to 9-residue spacing would do in an α-helical structure.

Arg residues in repeat I direct RNA encapsidation. The p21.5 sequences required to bind and package RNA into capsids were determined by using a recently described encapsidation assay (5). Birnbaum and Nassal (5) showed that the p21.5 protamine region is essential for efficient RNA encapsidation. They narrowed the segment required for RNA encapsidation down to Arg-164 for p21.5 from the *ayw* HBV subtype (Arg-166 in our subtype), leading to the suggestion that the SPXX motif 157-SPRR-160 mediates RNA packaging (5). The present work extends these results by showing that core proteins truncated as far as Ser-157 encapsidated RNA at roughly 50% of wild-type levels. This defines repeat I (150-RRRDRGRS-157 [Fig. 7]) as the minimal region required for RNA binding and packaging and shows that SPRR is not required for RNA binding.

Simple Arg-rich motifs have recently been shown to mediate the RNA-binding properties of bacteriophage transcriptional antiterminators (20) and small nuclear ribonucleoproteins (3). An 11-residue consensus RNA-binding motif, featuring clustered Arg or Lys residues flanking a negatively charged Asp or Glu, has been proposed (20). Except for its length, RRRDRGRS seems to fit this consensus well. However, the central Asp-Arg (DR) dipeptide is an insertion found uniquely in the *adw* HBV subtype and is thus unlikely to be a crucial part of the RNA-binding motif. This leaves little more than a few Arg residues to mediate RNA binding.

A striking demonstration of the RNA-binding power of Arg residues was provided by mutant Δ-149R4, which specifies a core protein containing just four Arg residues following the first 149 residues of p21.5. This protein encapsidated levels of RNA close to those seen for the core proteins truncated to residues 172, 162, or 157 at RNA, suggesting that four Arg residues provide an effective RNA-binding motif.

The distal repeats can bind DNA. We also examined the interaction between immobilized core proteins and nucleic acid probes. We initially presumed that this approach would verify the nucleic acid-binding activity identified by the RNA encapsidation assay, but the experimental data soon led us to believe that a fundamentally different binding activity was being recognized.

First, immobilized core proteins showed a strong preference for binding probes that consisted mainly of double-stranded DNA, rather than RNA. Second, repeats I and II were both required for activity in this assay, whereas only

repeat I was required for RNA encapsidation. These observations can be covered by a single unifying explanation if the 157-SPRR-160 motif (underlined in Fig. 7) is responsible (at least in part) for the activity seen in Southwestern blots. As mentioned above, this motif is found at the start of repeat II (but does not occur in repeat I) and SPXX motifs are known to interact with double-stranded DNA. The influence of phosphorylation on the DNA-binding activity (see below) is also consistent with the known properties of SPXX motifs, and additional support for the idea that hepadnavirus capsid proteins bind DNA through SPXX motifs is provided by our reanalysis of genetic studies in the DHBV system (see below).

In addition to repeat II, repeats III and IV each contain a single SPRR copy, suggesting that the protamine region may contain up to three elements that can bind DNA. Some support for this idea is provided by the observation that immobilized Δ-172 and p21.5 display increased DNA-binding activity compared with Δ-162 and by the DHBV genetic studies (see below). Presumably, the DNA-binding activity of the protamine region is directed toward double-stranded DNA, although we do not rule out the possibility of single-stranded binding activity.

It is not clear why the two assays used in this work should detect different nucleic acid-binding activities, but, given the different characters of the assays, it is perhaps not surprising. The RNA encapsidation assay measures an interaction that occurs in solution between native core proteins, probably in the form of dimers (40), and RNA, probably the most abundant nucleic acid available for packaging in *E. coli*. Since many p21.5 molecules may participate cooperatively in the encapsidation process (41), RNA binding need not involve a high-affinity interaction. In contrast, the binding of nucleic acid to immobilized core proteins may reflect the properties of isolated (perhaps denatured) core proteins. The preference for binding DNA could be an indication that immobilized core proteins have a higher intrinsic affinity for DNA (which may also explain why immobilized Δ-157 and Δ-149R4 proteins do not bind RNA).

Does phosphorylation modulate p21.5 DNA binding? Most p21.5 polypeptide chains synthesized in oocytes appear to be phosphorylated within the protamine region, probably at multiple Ser residues. Phosphorylation occurs before capsid assembly (data not shown), either during or soon after p21.5 synthesis, and is presumably mediated by a cellular kinase. This picture is somewhat different from the conventional view of p21.5 phosphorylation, in which a kinase activity present in capsids phosphorylates only a small fraction of the p21.5 subunits (14). The two views can be reconciled if the capsid-associated kinase is a vestige of the earlier, more efficient, phosphorylation step.

Phosphorylation reportedly modulates the DNA-binding activity of SPXX motifs (7, 33). Our data and those of Machida et al. (21) suggest that phosphorylation diminishes the interaction between p21.5 and DNA detected by Southwestern assay, although the implications of this for the interaction between p21.5 and DNA (or RNA) in capsids remain uncertain. An intriguing possibility (particularly in light of the direct role for the capsid protein in HBV replication proposed below) is that modulation of binding activity by phosphorylation could be used to regulate viral replication. Intriguingly, phosphorylation within the carboxy-terminal domain of subunit IIa of RNA polymerase II is required for this enzyme to make the transition from the initiation to the elongation phase of transcription (23). Since this carboxy-terminal domain comprises 17 to 52 repeats (depending on the organism) of a simple 7-residue peptide that includes an SPXX motif (37), the p21.5 protamine region appears to resemble a greatly simplified carboxy-terminal domain. This analogy certainly highlights the potential for p21.5 phosphorylation to play a regulatory role. To date no direct evidence has been presented to support this idea, although a correlation exists between capsid protein dephosphorylation and DHBV genome maturation (24a).

Model for the role of capsid proteins in hepadnaviral replication. We have shown that an RNA-binding element located in repeat I of the p21.5 protamine region suffices to direct RNA encapsidation, while the remaining three-quarters of this region apparently exhibits DNA-binding activity, presumably dependent (at least in part) on SPRR motifs in repeats II to IV. Thus the protamine region potentially possesses a series of nucleic acid-binding elements arranged in the order RNA-DNA-DNA-DNA (Fig. 7).

Although it is no surprise that the p21.5 protamine region binds RNA, the possibility that p21.5 possesses specialized DNA-binding elements seems to have been largely overlooked since the discovery that hepadnaviruses replicate by reverse transcription of an RNA pregenome (32). Since cellular protamines, the molecules which most closely resemble the p21.5 carboxyl terminus, are used in sperm to condense DNA genomes into their most compact form, this oversight is perhaps surprising.

Do these DNA-binding elements play any biological role? Transcription regulation is one possibility, and the p21.5 protamine region reportedly inhibits beta interferon gene expression (35). However, we envisage a more central role for DNA-binding activity in hepadnaviral replication, which occurs within the nucleocapsid and proceeds by reverse transcription of a pregenome RNA template, generating first single-stranded (minus-strand) DNA and then partially double-stranded DNA. Here we propose a model in which hepadnaviral capsid proteins bind both the RNA substrate and DNA products of reverse transcription (using the motifs described above), thereby facilitating replication. This model implies that the hepadnaviral capsid is specifically designed to accommodate intracapsid replication, in effect becoming part of a replication machine.

Although the *pol* gene product may be primarily responsible for selectively recognizing the 3.4-kb pregenomic RNA (4, 15), most of the binding, condensation, and charge neutralization required for RNA encapsidation is presumably provided by p21.5. Our data suggest that these functions may be adequately supplied by Arg residues in repeat I; each repeat would handle 19 bases of RNA on average, assuming contributions from all 180 capsid p21.5 subunits. As replication converts viral RNA to DNA, it is less clear that repeat I can interact well with DNA (particularly double-stranded DNA); repeat I carries no known DNA-binding motif (7), and the Δ-157 mutant was unable to bind DNA in the Southwestern assay. We propose that the distal repeats with their SPRR motifs are recruited during replication specifically for the purpose of binding DNA.

The idea that DNA binding and SPXX motifs may be important for hepadnaviral replication derives strong support from a reanalysis of recent elegant genetic studies (27, 38). This work showed that stepwise loss of double-strand and then single-strand DHBV DNA synthesis accompanies progressive carboxy-terminal truncation of the DHBV capsid protein (p34). It was originally suggested (38) that this phenotype might arise because the mutant capsids are physically too small to accommodate replication. However, inspection of the carboxy-terminal sequence of p34 (38),

which displays little similarity to the p21.5 protamine region beyond richness in basic residues, reveals two SPXX motifs and a TPXX motif. The observed DNA synthesis defects (38) correlate well with deletion of the two proximal motifs. Thus, second-strand DNA synthesis was lost as the truncation boundary entered an SPLP motif (amino acids 244 to 247); first-strand DNA synthesis was maintained until a TPQR motif (amino acids 238 to 241) was eliminated; and p34 mutants truncated to amino acid 226 still packaged RNA (27). Presumably the genetic replication defects arise because elongation of DNA strands is energetically unfavorable unless SPXX motifs are present to supply additional binding energy.

Thus, the genetic analysis suggests that p34 contains a similar array of RNA- and DNA-binding motifs to p21.5. By analogy with p34, we speculate that the SPRR motif in repeat II of p21.5 is involved in the handling of first-strand DNA, while the SPRR motif in repeat III binds the second DNA strand, leaving the distal SPRR motif in repeat IV as an apparently redundant motif (the distal SPXX motif in p34 also appears to be redundant). Whether repeat I is involved in interactions with DNA remains to be determined, as does the precise way the different repeats interact with specific replicative structures, such as RNA-DNA hybrids, single-stranded DNA, and duplex DNA.

In the above model, the role of the capsid protein is limited to binding and charge neutralization. We can envision more dynamic roles. It is conceivable that replication of hepadnaviral genomes requires the polymerase (pol) protein and capsid protein to act in concert, with the capsid protein effectively becoming a subunit of pol. According to this view, the entire capsid serves as a replication machine and capsid assembly may be a prerequisite for hepadnaviral replication. In support of this, we note that specific interactions between the pol and capsid proteins may be built into the assembly of replication-competent hepadnaviral nucleocapsids (4, 15) and that it has so far proved impossible to release an active reverse transcriptase from the hepadnaviral core particles (25).

An advantage of our model is that it can be tested by site-directed mutagenesis of specific motifs, coupled with an evaluation of the mutant phenotypes by both biochemical and genetic tests. If correct, the model suggests that novel strategies designed to disrupt the interaction between the HBV capsid protein and DNA may provide effective ways to curtail HBV replication and thus limit the progress of infection.

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