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Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids

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Assembly of hepadnaviruses depends on the formation of a ribonucleoprotein (RNP) complex comprising the viral polymerase polypeptide and an RNA segment, ε, present on pregenomic RNA. This interaction, in turn, activates the reverse transcription reaction, which is primed by a tyrosine residue on the polymerase. We have shown recently that the formation of this RNP complex in an avian hepadnavirus, the duck hepatitis B virus, depends on cellular factors that include the heat shock protein 90 (Hsp90). We now report that RNP formation also requires ATP hydrolysis and the function of p23, a recently identified chaperone partner for Hsp90. Furthermore, we also provide evidence that the chaperone complex is incorporated into the viral nucleocapsids in a polymerase-dependent reaction. Based on these findings, we propose a model for hepadnavirus assembly and priming of viral DNA synthesis where a dynamic, energy-driven process, mediated by a multi-component chaperone complex consisting of Hsp90, p23 and, potentially, additional factors, maintains the reverse transcriptase in a specific conformation that is competent for RNA packaging and protein priming of viral DNA synthesis.

Keywords: hepadnavirus/Hsp90/molecular chaperone/reverse transcription/ribonucleoprotein complex

Introduction

Assembly and initiation of reverse transcription in hepadnaviruses are coupled reactions that depend on the formation of a ribonucleoprotein (RNP) complex. It is known that the viral polymerase, a reverse transcriptase, and a sequence, ε, on the viral pregenomic RNA are both required for the formation of this RNP (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990, 1991; Junker-Niepmann *et al.*, 1990; Bartenschlager and Schaller, 1992; Pollack and Ganem, 1993, 1994; Wang and Seeger, 1993; Wang *et al.*, 1994). Furthermore, recent results obtained with the duck hepatitis B virus (DHBV) indicated that this reaction also depends on host factors that include the heat shock protein Hsp90 (Hu and Seeger, 1996). These observations suggested a model for the role of Hsp90 in assembly and initiation of reverse transcription in hepadnaviruses in which Hsp90 stabilizes a transient conformation of the viral polymerase that permits the formation of an RNP complex between the polymerase and ε RNA. This reaction triggers the *de novo* initiation of reverse transcription, which is primed by a tyrosine residue located within the polymerase polypeptide (Wang and Seeger, 1992; Zoulim and Seeger, 1994). During this so-called protein priming reaction, the polymerase synthesizes a three or four nucleotide long DNA strand using sequences of the ε RNA located near the 5′ end of the pregenome as a template. To continue minus strand DNA synthesis, the polymerase must switch template from the 5′ to the 3′ end of the pregenomic RNA (Wang and Seeger, 1993; Tavis *et al.*, 1994). In addition to its role in protein priming, RNP formation also initiates packaging of pregenomic RNA and the polymerase into viral nucleocapsids, the site of viral DNA replication (Bartenschlager and Schaller, 1992; Pollack and Ganem, 1994).

At least three distinct activities have been attributed to Hsp90. They include the stabilization of functional conformers representing a 'transient native' structure, such as the hormone binding conformation of steroid receptors (Picard *et al.*, 1990; Pratt, 1993; Smith and Toft, 1993; Bohen and Yamamoto, 1994; Bohen *et al.*, 1995), the intracellular trafficking of certain target polypeptides, such as the v-src tyrosine kinase (Brugge *et al.*, 1981; Courtneidge and Bishop, 1982; Hutchison *et al.*, 1992; Xu and Lindquist, 1993) and, *in vitro*, the refolding of denatured proteins (Wiech *et al.*, 1992; Jakob *et al.*, 1995; Freeman and Morimoto, 1996). The mechanism by which Hsp90 interacts with such a diverse group of target proteins and exerts its specific functions remains unknown. It is clear that Hsp90 forms high molecular weight complexes with various other proteins, and recent studies indicate that there are multiple, distinct Hsp90 complexes each with different constituents, which pre-exist in the cell independently of their association with target proteins (Perdew and Whitelaw, 1991; Smith, 1993; Chang and Lindquist, 1994; Jakob and Buchner, 1994; Johnson *et al.*, 1996). It is possible that these Hsp90-associated factors may target the various Hsp90 complexes to different substrate proteins and, furthermore, that different complexes may perform distinct functions. For example, the Hsp90 complex involved in maintaining the steroid receptors in a hormone binding state contains an acidic phosphoprotein p23, which is an essential component of the aporeceptor complex (Johnson and Toft, 1994, 1995; Johnson *et al.*, 1994, 1996; Hutchison *et al.*, 1995; Smith *et al.*, 1995). On the other hand, the Hsp90 complex associated with the v-src and raf kinases apparently lacks p23 but contains a protein called p50 (Brugge *et al.*, 1981; Courtneidge and Bishop, 1982; Hutchison *et al.*, 1992). p50 has been identified recently as the mammalian homolog of CDC37 in yeast, which interacts with several protein kinases and appears to function as the kinase-targeting subunit of an Hsp90–p50 complex (Stepanova *et al.*, 1996). The function of this Hsp90–p50 complex is to

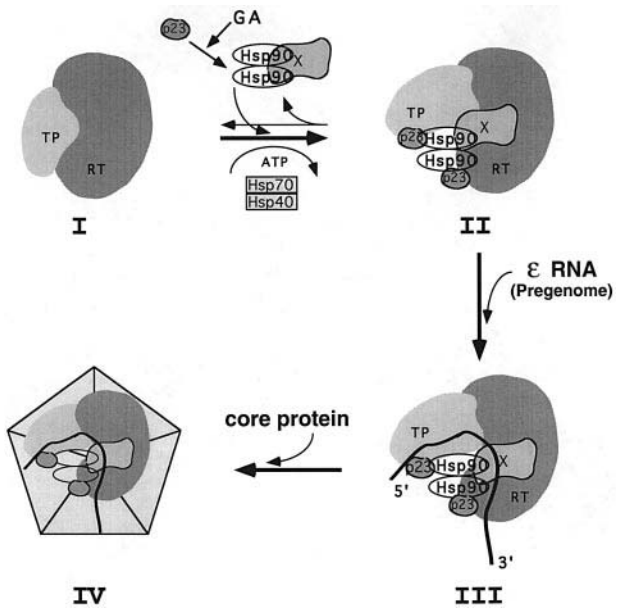

Fig. 1. Model for the assembly of nucleocapsids in hepadnaviruses. The polymerase is translated in an apo-structure (I) that provides a substrate for the Hsp90 pathway. TP denotes the N-terminal domain of the polymerase (the so-called terminal protein), which is unique to the hepadnavirus reverse transcriptases and harbors the primer tyrosine residue for initiation of reverse transcription. RT indicates the reverse transcriptase domain which is conserved among all known reverse transcriptases. The Hsp90 complex is depicted together with the chaperone partner p23 and possible additional factors (X) as described in the Discussion. The function of Hsp90 also depends on the ATPase Hsp70 and its partner Hsp40. Interaction of the polymerase with the chaperone complex induces a conformational change (II) that triggers the binding of the polypeptide to ε RNA (III), which in turn provides the signal for nucleocapsid assembly and initiation of viral DNA synthesis (IV). A detailed description of this pathway is provided in the text.

facilitate the intracellular trafficking of the associated kinases and to maintain their stability. Furthermore, recent results have demonstrated that the association between the Hsp90 complexes and their targets is not static but rather is dynamic; thus the hormone-free steroid receptors undergo a dynamic, energy-driven process through ordered association with distinct Hsp90 complexes until ligand binding dissociates the chaperone complex (Smith, 1993; Smith *et al.*, 1995; Johnson *et al.*, 1996). In order to maintain the aporeceptors in a hormone binding state, Hsp90 also depends on the function of another chaperone and a known ATPase, Hsp70 and its partner, the DnaJ homolog Hsp40 (Smith *et al.*, 1992; Hutchison *et al.*, 1994; Kimura *et al.*, 1995), which undergo cycles of substrate binding and release that are coupled with ATP hydrolysis (Hendrick and Hartl, 1993; Hartl, 1996).

In an effort to elucidate the mechanism by which Hsp90 controls DNA synthesis in hepadnaviruses, we have begun to examine the nature of the Hsp90 complex associated with the viral polymerase and to investigate the dynamics of this complex during various stages of viral DNA replication. Here, we report that p23 and ATP hydrolysis are essential for the protein priming reaction, thus revealing similarities between this system and steroid receptors (Figure 1). Our results further indicate that the chaperone complex required by hepadnaviruses remains associated with the polymerase during a complete cycle of viral DNA replication.

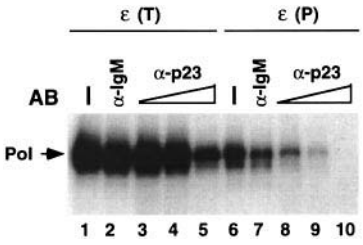

Fig. 2. Blocking of the protein priming reaction by anti-p23 antibody. An autoradiogram of ^{32}P-labeled polymerase from a protein priming reaction is shown. The polymerase was translated in RL in the presence [ε (T)] or absence of the ε RNA. After the translation was completed, 4 µl of the translation mixtures were either used directly in the priming reaction (lanes 1 and 6) or were incubated with 4 (lanes 3 and 8), 2 (lanes 4 and 9) or 1 µl (lanes 5 and 10) of the anti-p23 mAb, or the control anti-IgM antibody (4 µl, lanes 2 and 7) for 1 h on ice. The priming reaction was then initiated by addition of the reaction buffer and nucleotides. ε (P) denotes that ε was only added to the polymerase translation mixture during the priming reaction, i.e. after the incubation with antibodies. The arrow (Pol) indicates the ^{32}P-labeled polymerase.

Results

To investigate the nature of the Hsp90 complex associated with the DHBV polymerase, we asked whether p23 and Hsp70, two components of the Hsp90 complex known to be required for the chaperone function in the steroid receptor system, are also part of the polymerase complex and what role, if any, they may play in the polymerase function.

p23 is required for RNP formation

Initial evidence of a role for p23 in RNP formation was obtained when the p23-specific monoclonal antibody (mAb) JJ3 was shown to inhibit the protein priming reaction (Figure 2). The DHBV polymerase was translated in the rabbit reticulocyte lysate (RL) and used in the *in vitro* protein priming reaction, with or without the addition of mAb JJ3. As shown previously with Hsp90-specific antibodies (Hu and Seeger, 1996), the anti-p23 mAb was effective in inhibiting the protein priming reaction provided that it was added to the translated polymerase prior to RNP formation with ε RNA [Figure 2, ε (P)]. In contrast, incubation of pre-formed RNP with the anti-p23 antibody had only a slight effect on DNA synthesis at the highest antibody concentration used [Figure 2, ε (T)]. This result demonstrated that p23, like Hsp90, was required specifically for the interaction between the polymerase and ε RNA. The association of p23 with the polymerase was confirmed by co-immunoprecipitation of the polymerase expressed in the RL with the anti-p23 antibody (Figure 3A). Hsp90 was also co-immunoprecipitated by the anti-p23 antibody (Figure 3B), consistent with the previous observation that almost all the p23 in the cell is complexed with Hsp90, independent of the target proteins (Johnson *et al.*, 1994). To determine if the association of p23 with the polymerase was dependent on Hsp90, as is the case with the steroid receptors (Johnson and Toft, 1995; Smith *et al.*, 1995), immunoprecipitation was performed in the presence of the drug geldanamycin (GA). GA is known to bind Hsp90 and to disrupt Hsp90–p23 complexes (Whitesell *et al.*, 1994; Johnson and Toft, 1995; Smith *et al.*, 1995). Because p23 binds indirectly, via interaction with Hsp90, to the steroid

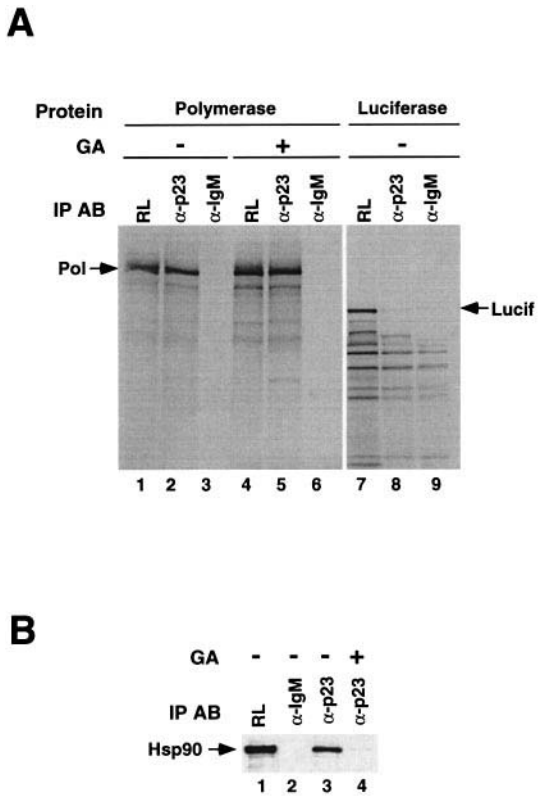

Fig. 3. Co-immunoprecipitation of DHBV polymerase with anti-p23 antibody. The RL *in vitro* translation system was programed with the RNA template for the DHBV polymerase or with the luciferase RNA (Promega) in the presence of [^{35}S]methionine. The translation reactions were then immunoprecipitated with an anti-p23 mAb (JJ3) or with a control antibody (anti-IgM) and the immunoprecipitates were then resolved on SDS–PAGE. (**A**) An autoradiogram of the immunoprecipitates with the anti-p23 mAb JJ3 (lanes 2, 5 and 8) or with the control antibody (lanes 3, 6 and 9) from the polymerase (lanes 2, 3, 5 and 6) or luciferase (lanes 8 and 9) translation mixture. Lanes 1, 4 and 7 (labeled RL) show the ^{35}S-labeled polymerase (lanes 1 and 4) and luciferase (lane 7) before immunoprecipitation. GA (100 μg/ml) was added to the translation mixture shown in lanes 4–6 during the translation reaction. In (**B**), Hsp90 from the RL (lane 1) or from the immunoprecipitates with the anti-p23 mAb (lanes 3 and 4) or anti-IgM (lane 2) was detected by Western blot analysis. GA (100 μg/ml) was added to the translation reaction shown in lane 4. The positions of the polymerase (Pol), luciferase (Lucif) and Hsp90 are indicated.

receptors, GA is able to block the association of p23 with the receptors (Johnson and Toft, 1995; Smith *et al.*, 1995). As shown in Figure 3, while addition of GA disrupted the interaction between Hsp90 and p23, it apparently did not dissociate p23 from the polymerase (compare Figure 3B, lane 4 with A, lane 5). Thus, this result indicated that p23 can bind to the polymerase independently of Hsp90 and may, in fact, bind directly to the polymerase. The residual amount of Hsp90 that was co-immunoprecipitated by the anti-p23 mAb in the presence of GA (Figure 3B, lane 4) could be attributed to the Hsp90 bound to the polymerase, consistent with the observation that GA does not inhibit binding of Hsp90 to the polymerase (Hu and Seeger, 1996) or the steroid receptors (Johnson and Toft, 1995; Smith *et al.*, 1995). On the other hand, as we have reported previously (Hu and Seeger, 1996), GA is able to inhibit the binding of the polymerase to ε RNA. Thus, binding *per se* of p23 and Hsp90 to the polymerase, which is not affected by GA, is not sufficient to enable the polymerase to bind ε. Rather, these results suggest that the Hsp90–p23 interaction, which is disrupted by GA, is critical for the formation of the viral RNP complex.

Reconstitution of a functional Hsp90–polymerase complex with exogenous p23

So far, a precise analysis of the Hsp90 pathway has been hampered by failure to reconstitute a complete functional Hsp90 complex *in vitro*. However, recent results have demonstrated that partial reconstitution of the complex with purified p23, expressed in bacteria, is possible (Johnson and Toft, 1994; Hutchison *et al.*, 1995). These results revealed that the deficiency of the Hsp90 pathway in the wheat germ (WG) extracts is due, in part, to the lack of p23, which can be complemented by bacterially expressed p23. In addition, it has been demonstrated that the Hsp90 function in reconstituting a steroid binding-competent aporeceptor complex can be restored in p23-depleted RL through addition of exogenous p23 (Johnson and Toft, 1994). Thus, the availability of a functional, purified component of the Hsp90 system provides an important means to investigate directly the significance of this complex for the assembly and DNA synthesis in hepadnaviruses.

For this purpose, the polymerase was expressed in WG extracts followed by incubation of the WG-expressed polymerase with RL. Previously, we have shown that the polymerase expressed in a WG extract is not competent for RNP formation with ε due to a lack of functional Hsp90 complexes in WG extracts (Hu and Seeger, 1996). Furthermore, addition of RL to the WG-expressed polymerase is able to activate the polymerase for ε binding and thus for protein priming. In agreement with the proposed role of p23, p23-depleted RL, in contrast to complete RL, was deficient in activating the WG-expressed polymerase as measured by the protein priming reaction (Figure 4A, lanes 2–4). Addition of p23, purified from bacteria, to the depleted RL restored ~80% its activity as compared with complete RL (Figure 4A, lanes 4–7). Furthermore, addition of purified p23 alone to the WG translation mixture could activate the polymerase partially (Figure 4B). As in the case with RL (see below), activation of the protein priming activity by purified p23 was also dependent on the presence of an ATP-regenerating system (data not shown). The optimal concentration of p23 for activation of protein priming activity was between 5 and 10 ng of p23/μl, which is in good agreement with results obtained from a similar experiment with the steroid receptor (Johnson and Toft, 1994; Hutchison *et al.*, 1995).

Together with the data described in the previous section, these results provided strong evidence that p23 is essential for the protein priming reaction and constitutes a critical component of the functional Hsp90 complex in the hepadnavirus system.

Hsp70 and ATP are required for RNP formation

In contrast to the results shown with p23, we were not able to detect a stable association between Hsp70 and the polymerase by co-immunoprecipitation using an Hsp70-specific antibody (data not shown). This was not unexpected, as Hsp70 is also not a stable component of the glucocorticoid aporeceptor complex, although it is essen-

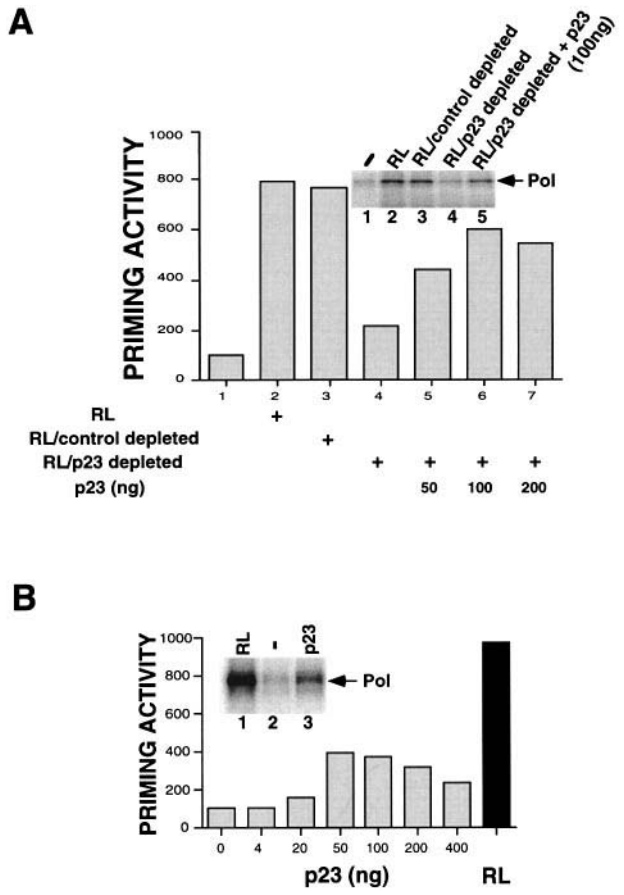

Fig. 4. Effect of p23 depletion and reconstitution on protein priming activity. In (**A**), 4 µl of the polymerase expressed in WG was either used directly in the priming reaction (lane 1) or was activated by addition of either 4 µl of RL (lane 2), the control PR22-depleted RL (lane 3) or p23-depleted RL (lanes 4–7). In the reactions shown in lanes 5, 6 and 7, the priming reaction (10 µl final volume) was supplemented further with 50, 100 and 200 ng of purified recombinant p23. The priming activity was quantified as described in Figure 2; the control, non-supplemented priming activity is set as 100 (lane 1). The inset shows an autoradiogram of ^{32}P-labeled polymerase from a priming reaction, with additions to the priming reaction shown above each lane. Note that the lane numbers in the inset correspond to those shown in the bar graph except that lane 5 corresponds to lane 6 in the bar graph. In (**B**), the WG-synthesized polymerase was activated by addition of purified recombinant p23. The indicated amounts of p23 were added to the WG translation mixture together with the ATP-regenerating system. The last lane shows activation of WG-translated polymerase by addition of RL, which served as a positive control for polymerase activation. The inset shows an autoradiogram of ^{32}P-labeled polymerase. WG-translated polymerase (4 µl) was used either directly in the priming reaction (lane 2) or was supplemented with 4 µl of RL (lane 1) or 50 ng of p23 (lane 3). The arrow (Pol) indicates the ^{32}P-labeled polymerase.

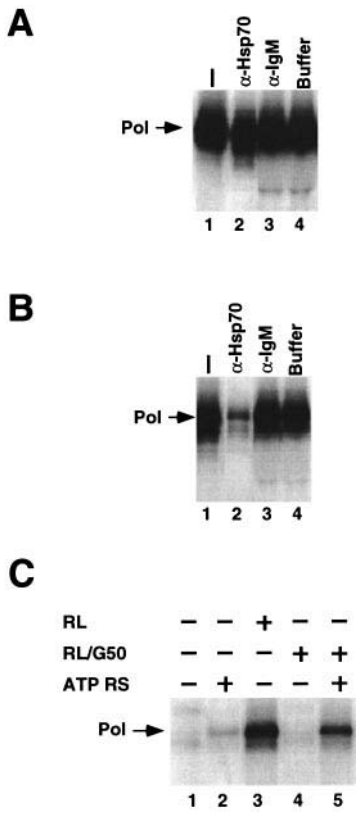

Fig. 5. Hsp70 and ATP are required for the protein priming reaction. (**A**) and (**B**) The effect of the anti-Hsp70 mAb on protein priming. The polymerase was translated in RL in the presence (A) or absence (B) of the ε RNA. After the translation was completed, 4 µl of the translation mixtures were either used directly in the priming reaction (lanes 1) or were incubated with 4 µl of the anti-Hsp70 mAb SPA-815 (lanes 2), the control IgG antibody (anti-IgM, lanes 3) or buffer (0.2% bovine serum albumin in phosphate-buffered saline, lanes 4) for 1 h on ice. The priming reaction was then initiated by addition of the reaction buffer and nucleotides. In the reactions shown in (B), ε was added to the polymerase translation mixture during the priming reaction, i.e. after the incubation with antibodies. (**C**) The effect of ATP on the protein priming reaction. The polymerase was translated in the WG extract and 4 µl of translation mixture was then used for the *in vitro* protein priming reaction by addition of ε RNA and the reaction buffer as described in Materials and methods. The priming reaction was supplemented further with an ATP-regenerating system as described in Materials and methods (ATP RS, lane 2), 4 µl of complete RL (lane 3), 4 µl of desalted RL (RL/G50, lane 4) and the desalted RL plus the ATP-regenerating system. Lane 1 shows a non-supplemented control priming reaction. The arrow (Pol) indicates the ^{32}P-labeled polymerase.

tial for assembly of the receptor complex with Hsp90 (Pratt, 1993; Smith and Toft, 1993; Bohen and Yamamoto, 1994). However, we were able to demonstrate a functional role for Hsp70 in the protein priming reaction. For example, incubation of the polymerase expressed in the RL with an Hsp70-specific mAb inhibited the protein priming reaction ~5-fold (Figure 5B, lane 2). Furthermore, as in the case with the Hsp90 or p23 mAbs, this inhibitory effect was only observed when the Hsp70 mAb was added to the priming reaction before, but not after, the formation of an RNP complex between the polymerase and ε RNA (compare Figure 5A and B). These results indicated that Hsp70 and, by inference, its partner Hsp40 were also required for the interaction between the polymerase and ε, even though Hsp70 did not appear to be a stable component of the polymerase complex.

In the course of identifying the cellular factors required for protein priming, we also discovered a requirement for ATP hydrolysis for this reaction. For example, the ability of RL to activate the polymerase expressed in the WG extract was abolished by depletion of small molecules by passing over a desalting Sephadex G50 column (Figure 5C, lanes 3 and 4), which could be restored fully by adding back an ATP-regenerating system (Figure 5C, lane 5). Even addition of the ATP-regenerating system to the WG-expressed polymerase without RL (Figure 5C, lane 2), and, to a lesser degree, ATP alone, stimulated the

protein priming activity somewhat (results not shown). In contrast, addition of a non-hydrolyzable ATP analog, ATP-γ-S, could not activate the polymerase. Furthermore, the essential role of ATP in the protein priming reaction correlated well with a requirement for ATP for the binding of the polymerase to ε (data not shown), as determined with an RNA binding assay (Wang *et al.*, 1994; Hu and Seeger, 1996). These results indicated that the need for ATP hydrolysis in the protein priming reaction reflected its requirement in RNP formation. Since Hsp70 is the only known component of the Hsp90 complex that displays an ATPase activity, these results provide further support for a role for Hsp70 in viral RNP formation.

Association of chaperones with viral nucleocapsids

A characteristic feature of the Hsp90 complex is that it interacts transiently with a given substrate. For example, binding to steroid receptors ceases upon ligand binding (Pratt, 1993; Smith and Toft, 1993; Bohen and Yamamoto, 1994; Bohen *et al.*, 1995). Contrary to this general observation, we found that binding of ε to the polymerase *in vitro* did not induce the release of Hsp90 or p23 from the polymerase and that both polypeptides remained associated with the polymerase following completion of the protein priming reaction (results not shown). These results raised the question of whether the chaperone complex is incorporated into virus particles or whether it is displaced, perhaps by the viral core proteins, during nucleocapsid assembly.

To address this issue, we examined cytoplasmic nucleocapsids for the presence of Hsp90 and p23. For this purpose, we purified nucleocapsids from cells that were transfected with a DHBV clone (pCMVDHBV-1s) that is defective for the expression of the viral envelope proteins. This construct was chosen to avoid potential contamination of the nucleocapsid preparations with surface antigen aggregates, with which several chaperone proteins appear to associate (see below). To differentiate between possible fortuitous association of chaperones with nucleocapsids and their specific incorporation as a consequence of binding to the viral polymerase, we also purified capsids from cells that were transfected with a second construct (pCMVDHBV-1s/dB) that is defective for expression of both the polymerase and envelope genes. Nucleocapsids prepared from either pCMVDHBV-1s- or pCMVDHBV-1s/dB-transfected cells behaved similarly in the sucrose and CsCl density gradient centrifugation steps employed. When purified nucleocapsids were examined for the presence of Hsp90 and p23 by Western blot analyses, we found that p23 could only be detected in preparations of nucleocapsids purified from cells that expressed the polymerase, but not from cells transfected with the polymerase-minus construct (Figure 6). Quantitation of the signal corresponding to p23 on Western blots with known amounts of purified recombinant p23 as standards showed that ~2–4 copies of p23 were present per nucleocapsid (Figure 6C). The concentration of nucleocapsids was estimated based on the amount of viral DNA present in the core particles, as determined by DNA dot-blot analysis. This stoichiometry is consistent with a model which predicts that p23 is incorporated into the nucleocapsids through an association with the polymerase at a ratio of one or two molecules of p23 per polymerase polypeptide

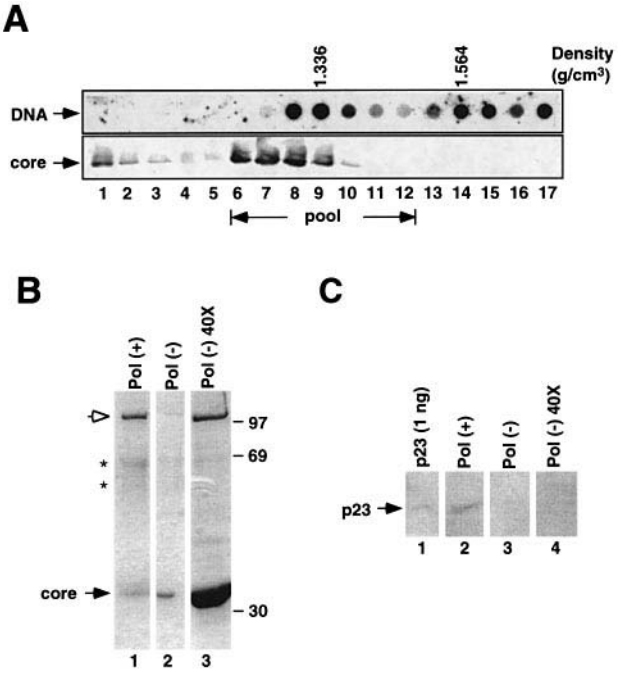

Fig. 6. Detection of p23 associated with purified viral nucleocapsids. LMH cells were transfected with constructs expressing either the core protein plus the polymerase [Pol(+)] or the core protein alone [Pol(–)]. Cytoplasmic nucleocapsids were extracted from transfected cells and purified by sequential sucrose and CsCl density gradient centrifugation. (**A**) Viral DNA and core proteins present in the CsCl fractions prepared from the Pol(+) transfectants, as detected by Southern blot and Western blot analysis respectively. (**B**) and (**C**) The indicated CsCl gradient fractions shown in (A) and the corresponding fractions obtained from the Pol(–) transfectants were pooled and analyzed by SDS–PAGE; total proteins were detected by Coomassie blue staining (B) and p23 was detected by Western blot analysis using mAb JJ3 (C). Capsids prepared from the Pol(+) transfectants were shown in lane 1 of (B) and lane 2 of (C) and equivalent amounts of capsids from the Pol(–) transfectants in lane 2 of (B) and lane 3 of (C). Forty-fold more core proteins, as compared with the amount from the Pol(+) pool, were loaded in the lanes labeled Pol(–) 40X (B, lane 3 and C, lane 4). One nanogram of purified recombinant p23 was loaded in lane 1 of (C). Open arrow, an ~110 kDa protein that co-sedimented with the capsids during the sucrose and CsCl gradient centrifugation; stars, staining artifacts that were apparent even in lanes where no protein sample was loaded. The buoyant density of the appropriate fractions from the CsCl gradient are indicated in (A). The positions for viral DNA, core protein and p23 are indicated. The identity of the core protein was confirmed by Western blot analysis.

and assumes that one or two molecules of polymerase are present per nucleocapsid, as has been estimated previously (Bartenschlager and Schaller, 1992). In agreement with these observations, we could also detect p23 in preparations of partially purified nucleocapsids from DHBV-infected duck liver (results not shown).

Hsp90 could not be detected definitively in the same nucleocapsid preparations. However, our Western blot assay to detect Hsp90 using the available Hsp90 antibodies was at least 10-fold less sensitive as compared with detection of p23 using the p23-specific JJ3 antibody, and it produced higher background signals. Assuming that Hsp90 was present in the nucleocapsids at the same level as p23 (as would be predicted if it was incorporated as a complex with p23 and the polymerase), the amount of Hsp90 associated with the nucleocapsids would have been below the level of detection under these conditions. Although it remains formally possible that only p23, but

not Hsp90, is associated with the nucleocapsids, we consider it to be unlikely since virtually all p23 in the cell is associated with Hsp90 (Johnson *et al.*, 1994) and there is no evidence to indicate that Hsp90 is dissociated from p23 or the polymerase during protein priming *in vitro*.

In addition, we also attempted to determine whether the chaperone complex is present in the extracellular virions, as would be predicted from its association with the viral nucleocapsids. For this purpose, we purified serum-derived DHBV particles by CsCl gradient centrifugation. The partially purified particles were then examined by Western blot analyses using mAbs against Hsp90 and p23. These experiments showed that both Hsp90 and p23 could be detected in the DNA-containing virion fractions. Unexpectedly, however, we could also detect these proteins in fractions that were essentially devoid of viral DNA and were enriched for the so-called surface antigen particles, aggregates of the envelope polypeptides which are present in serum at 10^3- to 10^6-fold molar excess over virions (Ganem and Varmus, 1987). The distribution of Hsp90 and p23 in the gradient overlapped but did not coincide with the viral surface proteins, whereas the heat shock cognate protein 70 (Hsc70), another molecular chaperone protein recently shown to associate with the viral large surface protein (Swameye *et al*., 1994), did co-migrate with the surface proteins. As compared with Hsc70, Hsp90 and p23 appeared to be enriched in the DNA-containing virion fractions, suggesting that Hsp90 and p23 were indeed associated with extracellular virions as predicted.

In summary, these results indicated that, in contrast to many of the known Hsp90 target proteins, the polymerase does not dissociate from the chaperone complex upon ε RNA binding; instead, the complex is incorporated into viral particles presumably via its association with the polymerase.

Is there a role for Hsp90 in viral DNA synthesis following RNA packaging and protein priming?

Having established that p23 and, by inference, the entire Hsp90 complex remain associated with the viral nucleocapsids, we asked whether the complex plays any detectable role in viral DNA synthesis that occurs subsequent to the protein priming/RNA packaging reaction. Previously, we demonstrated that the Hsp90 inhibitor GA can prevent RNA packaging by inhibiting RNP formation between the polymerase and ε RNA (Hu and Seeger, 1996). Since GA is cytotoxic following prolonged incubation of tissue culture cells with the drug, its specific effects on viral DNA synthesis have to be determined within a relatively short time frame of 24–48 h. To optimize our assay conditions, we employed a temperature-sensitive variant of DHBV, CA51, and a pyrophosphate analog phosphonoformate (PFA). CA51 bears two point mutations in the polymerase gene and is competent for DNA synthesis only at the permissive temperature (33°C) (Seeger *et al.*, 1996). A stable cell line derived from LMH hepatoma cells by transfection with the CA51 variant can thus be maintained at the non-permissive temperature (39°C) and viral DNA synthesis induced rapidly (within 24 h) upon shifting to 33°C. PFA is known to inhibit elongation of the viral DNA strands but does not block the protein priming reaction *in vitro* (Wang and Seeger, 1992) and thus should not affect RNP formation between the polymerase and ε or RNA packaging. Therefore, when the CA51 cells are shifted from 39 to 33°C in the presence of PFA, viral RNA packaging and protein priming occur normally but subsequent viral DNA synthesis is blocked. Removal of PFA then leads to the rapid induction of viral DNA synthesis (Seeger *et al.*, 1996). With the help of this system, we determined the effects of GA on viral replication in a 24 h time frame. As expected from our previous observation (Hu and Seeger, 1996), the drug inhibited viral DNA replication when it was present prior to packaging of the pregenomic RNA (Figure 7, lane 2). In contrast, viral DNA synthesis was not affected when GA was added to cells that contained already packaged pregenomes (Figure 7, lane 4). Apparently, only the first steps in viral DNA synthesis, namely the assembly of viral nucleocapsids and priming of minus DNA synthesis, were sensitive to treatment with GA. It thus appears that Hsp90 function may only be required for the interaction of the polymerase with ε RNA, as depicted in our model (Figure 1).

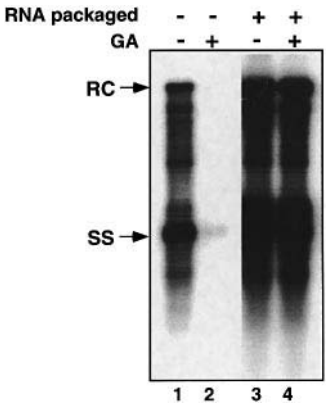

Fig. 7. Geldanamycin does not inhibit viral DNA synthesis following RNA packaging. The CA51 cell line, derived from stable transfection of the LMH hepatoma cells with a DHBV replication construct expressing a temperature-sensitive polymerase gene (mutant CA51) (Seeger *et al.*, 1996), was shifted from the non-permissive temperature (39°C) to the permissive temperature (33°C) to induce viral DNA synthesis. At the same time, GA (1 μg/ml, lane 2) or PFA (1 mM, lanes 3 and 4) was added to the culture medium. At 24 h after the temperature shift, a control, untreated culture (lane 1) and the GA-treated culture were harvested for viral DNA extraction. The PFA-treated cells were maintained at 33°C for 2 days to allow for packaging of the pregenomic RNA. PFA was then removed and, at the same time, GA (1 μg/ml) was added to one of the PFA-treated cultures (lane 4). The other PFA-treated culture was left untreated after removal of the drug (lane 3) and 24 h later the cells were harvested. Viral DNA intermediates were extracted and analyzed by Southern blot analysis. RC, relaxed circular DNA; SS, single-stranded DNA; both of which are characteristic replicative intermediates in hepadnavirus DNA replication.

Discussion

The purpose of this study was to investigate the nature of the Hsp90 complex, which was identified previously as an essential component of a hepadnavirus reverse transcriptase, and to determine the role of this complex during a complete cycle of viral DNA replication. Our results showed that in hepadnaviruses, as in other systems that depend on Hsp90 for function, Hsp90 acts together with certain partner proteins that include p23 and Hsp70 and, most likely, additional components that await identi-

fication. What is the role of these proteins for the function of Hsp90 and, most importantly, for the activity of hepadnavirus reverse transcriptases? The proposed model for viral DNA synthesis in hepadnaviruses predicts multiple functions for the viral polymerase during DNA synthesis that include the formation of an RNP, priming of DNA synthesis with the help of a hydroxyl group of a tyrosine residue, polymerization of a 3–4 nucleotides long nascent DNA strand, a template switch and, finally, elongation of the nascent DNA strand (Seeger and Mason, 1996). In addition, translocation of an RNA primer and a second template switch are required for second strand DNA synthesis. Based on previous results (Hu and Seeger, 1996) and observations described in this report, we propose a role for Hsp90 and its partner p23 in stabilizing transient conformations of the polymerase required during distinct steps in viral DNA synthesis. In analogy with a model proposed for steroid aporeceptors (Pratt, 1993; Smith and Toft, 1993; Bohen and Yamamoto, 1994; Bohen *et al.*, 1995), we propose a role for the chaperones Hsp70 and Hsp40 in facilitating Hsp90 activities through a dynamic energy-driven process (Figure 1).

The availability of purified p23 derived from bacteria permitted a direct demonstration of the requirement for a known cellular protein in RNP formation (Figure 4). Identification of p23 as an essential factor for the binding of the polymerase with ε RNA was important for several reasons. The requirement for p23 explains why GA, which inhibits binding of Hsp90 to p23, can act as an inhibitor of RNP formation *in vitro* and DNA replication *in vivo* (Hu and Seeger, 1996). The antiviral activity of GA could also be detected with the human hepatitis B virus (HBV) (J.Hu and C.Seeger, unpublished results), which supports our view that results obtained with avian hepadnaviruses can, as expected, be extended to their mammalian counterparts. The demonstration of p23 as an essential factor for viral RNP formation also suggests that the Hsp90 complexes involved in hepadnavirus replication and in steroid receptor function share similar components and activities. Nevertheless, there appear to be fundamental differences in the way that the Hsp90–p23 complex associates with different protein targets. For example, p23 is thought to associate indirectly with steroid receptors through its association with Hsp90 (Johnson *et al.*, 1994; Johnson and Toft, 1995). However, as GA did not disrupt p23 binding to the polymerase (Figure 3), we propose that p23 can bind to the polymerase independently of Hsp90. Because GA is able to strongly inhibit viral RNP formation without affecting the association of either Hsp90 or p23 with the polymerase, binding *per se* of either chaperone to the polymerase apparently is not sufficient for RNP formation. Rather, the block in RNP formation caused by GA is correlated with its ability to disrupt Hsp90–p23 association. Together, these results suggest that Hsp90 and p23 have to communicate directly with each other in order to facilitate RNP formation. Interestingly, Hsp90 mutants have been identified that are competent in binding to steroid receptors but are nevertheless deficient in facilitating hormone binding (Bohen and Yamamoto, 1993; Bohen, 1995). In light of our findings, this phenotype could be explained by the failure of these Hsp90 mutants to communicate with p23.

The current model for hepadnavirus replication implies that the polymerase is maintained in several alternate conformations and raises questions about the mechanism by which the necessary transitions may occur. We have shown here that, in addition to Hsp90 and p23, another chaperone, Hsp70, and ATP hydrolysis are required for the interaction between the polymerase and ε RNA (Figure 5). So far, we have not tested directly whether Hsp40, the Hsp70 partner, plays a role in RNP formation. However, biochemical and, recently, genetic evidence have implicated Hsp40, which is known to regulate the ATPase activity of Hsp70, as an essential constituent of the Hsp90 pathway in the formation of signaling-competent steroid receptors (Bohen and Yamamoto, 1994; Bohen *et al.*, 1995; Kimura *et al.*, 1995; Hartl, 1996). Thus it seems likely that Hsp70, together with Hsp40, facilitates the conformational transitions of the polymerase through ATP-dependent cycles of polypeptide binding and release and thus feeds an appropriate substrate into the Hsp90 pathway to stabilize a given conformation as required for ε binding and, possibly, other functions during the viral replication cycle (see below).

It is likely that the polymerase–Hsp90 complex contains additional components that may include one or more proteins belonging to the family of immunophilins. Several immunophilins have been found associated with Hsp90 complexes in steroid receptors (Pratt, 1993; Smith and Toft, 1993; Bohen and Yamamoto, 1994; Jakob and Buchner, 1994; Sanchez and Ning, 1996). Whether a peptidyl-prolyl isomerase activity generally associated with immunophilins is important in the polymerase system remains to be elucidated. Other candidates include p60, which appears to mediate the interaction between Hsp90 and Hsp70 (Chen *et al.*, 1996), and p48 (Hip), a component of the aporeceptor complex which binds to Hsp70 and appears to stimulate the chaperoning activity of Hsp70/Hsp40 (Hohfeld *et al.*, 1995; Prapapanich *et al.*, 1996).

The incorporation of the Hsp90 chaperone complex into the viral nucleocapsids and extracellular virions raises the possibility that these factors may play additional roles in the viral life cycle, in addition to their role in protein priming and nucleocapsid assembly. Critical subsequent steps in viral DNA synthesis such as its arrest prior to the translocation of the polymerase from the 5′ to 3′ end of pregenomic RNA may, indeed, depend on the ability of the polymerase to undergo specific structural changes. Evidence for such a conformational change during the transition from a ‘protein priming’ to a ‘DNA elongation mode’ has been obtained recently with certain reverse transcriptase inhibitors. For example, PFA, dideoxynucleotide triphosphates or 3′ azidodeoxythymidine triphosphate act as efficient inhibitors of DNA elongation but apparently do not affect the protein priming reaction (Wang and Seeger, 1992; Staschke and Colacino, 1994). However, results obtained with the CA51 variant and PFA treatment (Figure 7) suggested that a p23-containing chaperone complex is unlikely to be involved in steps of viral DNA synthesis subsequent to RNA packaging and protein priming, because GA showed no measurable effect on elongation of the viral minus and plus strand DNAs. A possible caveat to this interpretation is that PFA may allow the translocation of the polymerase during minus-strand DNA synthesis—a transition which may require chaperone assistance—but simply arrest the elongation of

nascent minus strands at the 3′ end of pregenomic RNA. Thus, it remains a possibility that the packaged chaperone complex may play a role in facilitating conformational changes of the polymerase that are critical for viral DNA synthesis following the packaging reaction. An alternative possibility is that the chaperones may be dissociated from the elongating polymerase during viral DNA synthesis but remain trapped within the nucleocapsids.

The characteristics of the substrate proteins that are recognized by Hsp90 or p23 remain undetermined at the present time. There is no apparent consensus sequence motif that is common to the known Hsp90 target proteins, which are diverse in structure and function (Bohen and Yamamoto, 1994; Jakob and Buchner, 1994; Bohen *et al.*, 1995). We have attempted to identify the determinants on the polymerase that are responsible for Hsp90 and p23 binding by mutagenesis studies. Our results indicated that two regions of the polymerase, one in the N-terminal terminal protein (TP) domain and the other in the reverse transcriptase (RT) domain, can bind independently to Hsp90 and p23 (data not shown). The TP and RT domains do not bear any apparent sequence similarities to each other or to the other known Hsp90–p23 target proteins, which is consistent with the notion that the Hsp90 complexes mostly likely recognize some tertiary structural features of the substrate proteins that cannot be recognized yet based on the amino acid sequences. Recently, it has been reported that Hsp90 recognizes a 'mutant conformation' of the tumor suppresser protein p53, which can be exhibited by either mutant or wild-type p53 proteins (Blagosklonny *et al.*, 1996; Sepehrnia *et al.*, 1996), lending support to the proposal that Hsp90 recognition is mainly at the global conformational level. Alternatively, we cannot rule out the possibility that Hsp90 is recruited to the polymerase with the help of an as yet unidentified component analogous to p50 (CDC37), which appears to target Hsp90 to the cyclin-dependent kinase Cdk4 (Stepanova *et al.*, 1996).

A critical role for chaperones in viral replication was reported more than two decades ago. These studies identified the bacterial chaperonin GroEL and the Hsp70 homolog DnaK/DnaJ as essential host factors in bacteriophage assembly and DNA replication, respectively (Georgopoulos *et al.*, 1972, 1973; Georgopoulos, 1977; Friedman *et al.*, 1984). While subsequent studies with animal viruses have documented the association of various chaperones with different viruses, evidence for a functional correlation was lacking (Santoro, 1994). However, recent studies with human immunodeficiency virus 1 have identified cyclophilin A, an immunophilin, as an essential factor in the production of infectious virus (Franke *et al.*, 1994; Thali *et al.*, 1994). These observations highlight the role of the cellular protein folding machinery in viral replication and may reflect the requirement for certain viral proteins to undergo obligatory conformational changes in order to carry out multiple functions at different stages of the viral replication cycle. As shown in this report, the hepadnavirus reverse transcriptase represents an example of such a class of proteins which depend on specific cellular protein folding pathways in order to play multiple roles during viral replication.

Materials and methods

Plasmids

pHP was used for expression of the DHBV polymerase *in vitro* and has been described previously (Wang *et al.*, 1994; Zoulim and Seeger, 1994). pCMVDHBV directs expression of the DHBV pregenome from the cytomegalovirus immediately-early promoter (Seeger and Maragos, 1989; Wu *et al.*, 1991). pCMVDHBV-1s was derived from pCMVDHBV and harbors two engineered stop codons in the S gene which eliminate expression of both the large and small surface proteins (Horwich *et al.*, 1990; Summers *et al.*, 1990). pCMVDHBV-1s/dB was derived from pCMVDHBV-1s by filling-in the *Bgl*II site at nucleotide 391, creating a 4 nt insertion and a frameshift mutation in the polymerase gene after codon 74.

Antibodies and other reagents

The JJ3 monoclonal antibody against p23 has been described before (Johnson *et al.*, 1994). The anti-Hsp90 mAb (clone AC16) and goat anti-mouse IgM (IgG fraction) were purchased from Sigma, St Louis. The mAb (SPA815) against Hsc70 was purchased from Stressgen (Victoria, BC, Canada). The polyclonal rabbit antibody against the DHBV core antigen was kindly provided by William Mason (Fox Chase Cancer Center, PA) (Jilbert *et al.*, 1992) and the mAb against DHBV surface antigen was a generous gift from John Pugh (Fox Chase Cancer Center, PA). GA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. PFA was from Sigma. The RL and WG extract were purchased from Promega.

Recombinant p23 and p23-depleted RL

Purified recombinant human p23 from bacteria was obtained as described before (Johnson and Toft, 1994; Johnson *et al.*, 1994) with the following modification. Following DEAE-cellulose chromatography, p23 was purified further by chromatography on phenyl–Sepharose using a 1.5–0 M ammonium sulfate gradient. At this stage, SDS gel electrophoresis of 10 μg of p23 showed no detectable contaminants. Depletion of p23 from the RL was accomplished by adsorbing the RL twice with the JJ3-coated resin; ~90–95% of the p23 was removed from the RL by the immunodepletion procedure (Johnson and Toft, 1994). An antibody against the progesterone receptor, PR22, was used as the negative control for the immunodepletion procedure.

In vitro transcription, translation and protein priming reaction

The *in vitro* expression construct pHP was first linearized with *Afl*II (position 2527 on the DHBV genome) and then transcribed with an *in vitro* transcription kit (MEGAscript, Ambion). The RNA template for translation of the firefly luciferase was from Promega. Purified RNAs were then translated, in the presence or absence of [^{35}S]methionine, for 60 min at 30°C in the RL or 120 min at 25°C in the WG as previously described (Hu and Seeger, 1996). Translation was stopped by addition of cycloheximide (final concentration of 20 μg/ml). For synthesis of the DHBV ε RNA, a synthetic DNA template containing the ε coding sequences (Wang *et al.*, 1994) was used for *in vitro* transcription. Various antibodies and the antibiotic GA were added to the translation mixture either before or after the ε RNA, as indicated. To activate the polymerase expressed in the WG, various supplements including complete RL (Promega), desalted RL obtained by passing the RL through a Sephadex G50 spin column, p23-immunodepleted RL, purified recombinant p23 or an ATP-regenerating system consisting of 5 mM ATP, 10 mM creatine phosphate and 50 μg/ml creatine phosphokinase were added to the protein priming reaction, essentially as described before (Hu and Seeger, 1996). The *in vitro* protein priming reaction was performed as described (Wang and Seeger, 1992; Hu and Seeger, 1996).

Immunoprecipitation

The DHBV polymerase was translated in the RL in the presence of [^{35}S]methionine and the translation mixture was then immunoprecipitated with the p23-specific mAb JJ3 or with the control antibody essentially as described (Johnson *et al.*, 1994). The immunoprecipitates were resolved on SDS–PAGE gels. Immunoprecipitated p23 and co-precipitated Hsp90 were detected by Western blot analyses using the JJ3 and AC16 antibodies, respectively. Western blots were developed using the chemiluminescence reagents (Amersham). Co-precipitated, ^{35}S-labeled translation products were detected by autoradiography.

Purification of viral nucleocapsids from transfected cells and extracellular virions from infected duck serum

To prepare cytoplasmic DHBV nucleocapsids, the chicken hepatoma cell line LMH (Kawaguchi *et al.*, 1987) was transfected with pCMVDHBV-1s or pCMVDHBV-1s/dB by calcium phosphate precipitation using a kit from 5 Prime — 3 Prime, Boulder. Cytoplasmic capsid particles were purified by a procedure adapted from published methods used to purify capsid particles from infected liver (Molnar-Kimber *et al.*, 1984; Oberhaus and Newbold, 1993). Briefly, cells were lysed with NP-40-containing buffer 5 days after transfection, and plasmid DNA was removed by nuclease digestion as described before (Pugh *et al.*, 1988; Seeger and Maragos, 1991; Hu and Seeger, 1996). The cytoplasmic extract was then treated with 20 mM EDTA and 160 μg/ml of RNase A for 1 h at 37°C and precipitates were removed by centrifugation at 10 000 r.p.m. for 20 min at 4°C in an HB4 rotor. The supernatant (22 ml per gradient) was loaded onto an 8 ml 10%–8 ml 20% sucrose cushion made in HCB2 buffer [20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 0.01% (v/v) Triton X-100 (Molnar-Kimber *et al.*, 1984)] and the capsids were pelleted by spinning at 20 000 r.p.m. for 16 h at 4°C in an SW28 rotor. The pellet was resuspended in HCB2 buffer and treated again with 20 mM EDTA and 100 μg/ml RNase for 30 min at 37°C. Precipitates were removed by spinning at 3000 r.p.m. for 10 min in a table top centrifuge. The cleared supernatant (4 ml per gradient) was loaded onto a 30 ml continuous 15–30% sucrose gradient made in HCB2 buffer and spun at 27 000 r.p.m. for 4 h at 4°C in an SW28 rotor. One ml fractions were then collected from the top using an automated gradient fraction collector and assayed for the presence of core protein by Western blot analysis using the rabbit anti-DHBV core antiserum. Core protein-positive fractions were pooled, diluted in HCB2 buffer and pelleted through a 20% sucrose cushion. The capsid pellet was resuspended in 4 ml of CsCl solution (0.4725 g/ml in HCB2 buffer plus an additional 2 μl of Triton X-100 per 4 ml) and centrifuged at 45 000 r.p.m. for 62 h at 20°C in an SW60 rotor. Fractions of 250 μl each were collected from the top and assayed for the presence of core protein by Western blot analysis using the core antiserum and for DHBV DNA by dot-blot analysis. Appropriate fractions positive for viral DNA and/or core protein (as indicated) were pooled, diluted in HCB2 buffer and pelleted through a 20% sucrose cushion. The pellet was resuspended in SDS sample buffer and resolved on an SDS–12% polyacrylamide gel. Total proteins were detected by Coomassie blue staining, and the core protein, p23 and Hsp90 were detected by Western blot analyses using the core antiserum or the monoclonal antibodies against p23 or Hsp90.

DHBV-positive serum from congenitally infected ducks was kindly provided by William Mason (Fox Chase Cancer Center). DHBV virions were purified from the serum as described (Molnar-Kimber *et al.*, 1984) with minor modifications. Briefly, virions were first pelleted twice through a 10–20% sucrose step gradient and the resuspended pellets were then fractionated on an isopycnic CsCl gradient (initial density of 1.18 g/cm^3) in an SW60 rotor at 50 000 r.p.m. for 27 h. Fractions were collected from the top and assayed for the presence of viral DNA by dot-blot analysis using ^{32}P-labeled cloned DHBV DNA as probe; for detection of the viral core protein and surface protein or Hsc70, Hsp90 and p23 in the fractions, Western blot analyses were performed using the appropriate polyclonal or monoclonal antibodies.

Treatment of the CA51 cell line with PFA and GA

The CA51 cell line was derived by stable transfection of the LMH chicken hepatoma cells with a DHBV replication construct expressing a temperature-sensitive polymerase gene (Seeger *et al.*, 1996). The CA51 cells were maintained at the non-permissive temperature (39°C) to block viral DNA replication. Upon shifting to the permissive temperature (33°C), viral DNA synthesis is induced rapidly. PFA (1 mM) was added to the culture medium at the time of temperature shift-down to block viral DNA replication (Seeger *et al.*, 1996) while allowing protein priming (Wang and Seeger, 1992) and RNA packaging. After 2 days of PFA treatment to allow RNA packaging, PFA treatment was stopped. To examine the effect of GA on viral DNA replication, GA (1 μg/ml) was added to the culture medium at the end of the 2 day PFA treatment. As a control, GA was added directly at the time of temperature shift-down (before viral RNA packaging) without the intervening PFA treatment. At 24 h after GA treatment, the cells were harvested and cytoplasmic viral DNA replicative intermediates were isolated and analyzed by Southern blot analysis as previously described (Hu and Seeger, 1996).

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