Efficient Hsp90-independent in Vitro Activation by Hsc70 and Hsp40 of Duck Hepatitis B Virus Reverse Transcriptase, an Assumed Hsp90 Client Protein*

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Hsp90 is a specialized chaperone that controls the activity of many key regulator proteins such as steroid hormone receptors (SHRs). Binding hormone, and therefore SHR activation, requires Hsp90, which is loaded onto the receptors by a series of events involving Hsp70, Hsp40, Hop, and p23. The reverse transcriptase (RT) of hepatitis B viruses, small DNA-containing viruses that replicate via an RNA intermediate, has been reported to depend similarly on Hsp90 for enzymatic activity. Using an in vitro reconstitution system consisting of recombinant duck hepatitis B virus RT, purified chaperones, and the authentic RNA template DΔe, we demonstrate here that this RT can be activated efficiently by just Hsp40 and Hsc70 plus energy, without the need for Hsp90 or other cofactors. The reaction appears to proceed selectively with the Hdj1 variant of Hsp40 but not Hdj2 or its yeast homolog Ydj1. The primary reaction product is a metastable, RNA binding-competent intermediate that decays quickly in the absence of its cognate RNA but, in its presence, accumulates in an initiation-competent form over several hours. Because deletion of the RNase H domain rendered the protein partly chaperone-independent, the chaperones may be needed indirectly to relieve occlusion of the RNA binding site by this domain. Our results do not exclude that other factors contribute to RT activation in vivo, but they challenge a fundamental SHR-like dependence on Hsp90. Thus Hsc70, mostly known for its role in general protein folding, is able to effect activation of a highly specialized target protein.

Molecular chaperones are essential for protein folding in vivo; in the mammalian cytosol, probably some 20% of the newly translated proteins gain their final three-dimensional structure through interactions with members of the heat shock protein (Hsp)170 family (the constitutively expressed Hsc70, and the stress-inducible Hsp70 proteins; DnaK in bacteria), assisted by chaperones of the Hsp40 family (DnaJ in bacteria) (1). A fraction of these Hsc70/Hsp40 substrates requires further interactions with the specialized chaperone Hsp90, which are mediated, and modulated, by chaperones such as Hop (Hsp-organizing protein), Hip (Hsp70-interacting protein), p23, and others (2). The best known Hsp90 client proteins are signaling kinases and, in particular, steroid hormone receptors (SHRs). Induction of their transcriptional trans-activation function depends on binding of the cognate hormone ligand. Binding competence is generated through interactions of the unstable ligand binding domain with Hsp90 (3–6). Once properly folded the ligand binding domain may bind hormone, or return to a binding-incompetent conformation for new cycles of activation. Hsp90 binding does not occur directly but via an energy-dependent multistep process that most likely involves transient interactions with Hsp40 and Hsp70 followed by formation of an intermediate complex containing Hsp70, Hop, and Hsp90, and finally conversion into a mature, ligand binding-competent receptor complex containing Hsp90, p23, and usually a member of the tetratricopeptide repeat immunophilin family. Ligand binding activity has been reconstituted from purified receptors, deprived of their cellular partners, by incubation in rabbit reticulocyte lysate lysate (RL) which is an abundant source for the aforementioned chaperones and co-chaperones and, more recently, entirely from purified components. Optimal activation required a minimal set of five factors, namely Hsp70, Hsp40, Hop, Hsp90, and p23 + ATP (7, 8); Hsp70 and Hsp90 are essential, and Hsp40, Hop, and p23 increase the rate or extent of receptor-Hsp90 complex formation (9, 10). A potential role of the newly identified Aha-1 Hsp90 cochaperones (11) has not yet been investigated.

Among a few other non-kinase and non-transcription factor proteins, two reverse transcriptases (RTs), telomerase (12), and the P protein of hepatitis B virus (HBV) are also thought to require Hsp90 for activation (13). HBV, the causative agent of acute and chronic hepatitis B in humans (14), is the type member of the hepadnaviruses, small DNA-containing viruses that replicate through an RNA intermediate (15). Related animal viruses, e.g. woodchuck hepatitis B virus and duck hepatitis B virus (DHBV), are important model systems. In all hepadnaviruses, a terminally redundant transcript, the pre-genomic RNA, acts as mRNA for the capsid protein and P protein, and the same RNA is packaged into viral capsids and reverse transcribed into partially double-stranded circular DNA (16, 17).

Both nucleocapsid assembly and replication initiation (Fig. 1) are triggered by the binding of P protein to an RNA stem-loop structure, close to the 5′-end of the pregenomic RNA (18, 19). The interaction induces conformational changes in the
RNA (20) and in the protein (21), leading to an initiation-competent ribonucleoprotein (RNP) complex. A specific RNA segment, forming a bulge in the free RNA, becomes a template for reverse transcription into a short (3 or 4 nucleotides) DNA segment, forming a bulge in the free RNA, becomes a template competent ribonucleoprotein (RNP) complex. A specific RNA RNA (20) and in the protein (21), leading to an initiation-

replication initiation by hepadnaviral P proteins. A. current model of SHR-like Hsp90-dependent P protein activation. DHBV P protein, with its TP and RT/RH domains linked through a dispensable spacer, is unable to bind its cognate De RNA unless it is activated by chaperones. Activation is thought to proceed via the Hsp70/Hsp40/Hop-mediated formation of a Hsp90/p23-containing complex. RNA binding is accompanied by structural changes in the RNA and P protein, enabling the synthesis of a short DNA oligonucleotide templated by a bulge region within De; its 5'-terminal nucleotide is covalently linked to a Tyr residue in the TP domain. This priming reaction can be reconstituted in vitro, B, detailed view of D. The RNA template region is highlighted by bold, larger lettering; the copied DNA primer is boxed. In the context of a whole virus genome, De is located close to the capped 5'-end of the pregenomic RNA (pgRNA); for synthesis of a complete (-)-strand DNA, the primer-P protein complex is translocated to a matching 3'-proximal acceptor site and extended from there to the RNA 5'-end; (+)-strand DNA synthesis is not shown.

DNA. The end product is a partially double-stranded, relaxed circular DNA in which the (-)-strand DNA is still covalently fixed to P protein.

Until recently, attempts to generate hepadnaviral P proteins in simple heterologous systems such as Escherichia coli were unsuccessful. Most of the replication details described above were therefore derived genetically. The first cell-free system allowing us to address more mechanistic questions was the in vitro translation of P protein in RL (28); there, the RT of DHBV (but not that of HBV) is capable of performing the authentic initiation reaction, i.e. synthesis of the short DNA primer when provided with DHBV e (De) RNA and dNTPs. Initially based on antibody depletion of several of the chaperones present in RL it was concluded that P protein requires Hsp70, Hsp90, and p23 for activity (13). A second line of evidence was the proposed packaging into nucleocapsids of a complex of P protein with Hsp90 and p23, although only p23 was directly detected, possibly for a lack of high affinity anti-Hsp90 antibodies (29). The frequent association of p23 with Hsp90 suggested, nonetheless, the simultaneous presence of Hsp90, although a Hsp90-independent, geldanamycin-resistant association of p23 with P protein was described in the same study. Geldanamycin, a drug that interferes with Hsp90-p23 interaction, also reduced DHBV replication in virus-transfected cells. However, hepadnavirus replication involves several phosphorylation steps, and kinases are a major Hsp90 target family; hence, various reasons for replication inhibition beyond a specific action of Hsp90 on the RT can be imagined. Several of these data sets may thus be open to alternative interpretations, but altogether they strongly suggested P protein as an Hsp90 client protein that might be activated in a manner similar to that of SHRs (Fig. 1A).

More thorough testing this model was hampered by the low yields of full-length P protein obtainable by in vitro translation (in the range of 1 ng/μl) and the high abundance (in the range of 100 ng/μl) of most of the chaperones in question. Reconstituting a priming-competent P protein complex from purified components offers a potentially more feasible approach, given that a reliable source for recombinant P protein, preferentially free of mammalian chaperones, can be established. Previously we had shown that the TP domain and, after removing the C-terminal 25 amino acids, also the RT/RH domain of P protein could be expressed in E. coli as separate polypeptides in significant amounts. Although only a fraction of RT/RH was soluble, this was sufficient to reconstitute a priming-active complex from purified TP, RT/RH, De, and RL (30), confirming the ability of the domains to complement each other in trans (31). However, initial experiments to activate these separate domains with purified chaperones failed, prompting us to reinvestigate recombinant expression of a single chain P protein.

Using two different E. coli protein, NusA and GrpE, as fusion partners we were indeed able to produce and purify soluble, nearly full-length DHBV P proteins in substantial amounts. During this study, the successful reconstitution with purified chaperones of a more severely truncated P protein fused to glutathione S-transferase and expressed in E. coli, though in low amounts, was reported (32). In that study Hsp70, the yeast homolog of Hsp40 Ydj1, Hop and Hsp90 were found to be the minimal set of factors necessary and sufficient to activate the fusion protein; p23, previously considered to be crucial (29), increased the kinetics of, but was not essential for, complex formation. These results further supported the model of an SHR-like activation mechanism for the hepadnaviral RTs.

Below we show that our new fusion proteins can be reconstituted into a priming-active complex with purified chaperones. Unexpectedly, Hsc70 and the human Hsp40 Hdj1 were necessary and sufficient to activate efficiently both P protein derivatives for De-dependent priming; only under special conditions could a very modest activity increase by the additional presence of Hop, Hsp90, and p23 be detected. Hence there is no fundamental requirement for Hsp90 in hepadnaviral P protein activation.

**EXPERIMENTAL PROCEDURES**

**Cloning of DHBV P Fusion Proteins**

The NusDP expression construct was obtained by cloning a modified P protein open reading frame (ORF) from DHBV strain 16 into the ScaI
and XhoI sites of pET34a (Novagen). The P protein part of the encoded NusDP fusion protein (see Fig. 2), preceded by the bacterial NusA ORF, consists of amino acids 1–220 of the TP domain, a Ser-Pro-Gly linker linking the spacer amino acids 221–348, and the complete RT/RH domain from amino acid 349, except that the nonessential 25 amino acids 762–786 from the C terminus were replaced by the peptide Val-Glu-His6. The NusDP/RH construct, encoding a shortened P protein, was sequenced downstream of amino acid 575, was obtained by PCR-mediated mutagenesis. The GrpE expression vector was constructed by replacing the NusA ORF between the NdeI and SpeI sites in the NusDP construct with a PCR product containing the 197-amino-acid ORF of GrpE from E. coli.

Expression and Purification of DHBV P Fusion Proteins

The fusion proteins were expressed in E. coli BL21 Codon Plus RIL cells (Stratagene). Cultures of 200-ml volume were grown to an OD600 of 0.6, and expression was induced by adding isopropyl-1-thio-galactopyranoside to a final concentration of 1 mM. After 4 h at 23 °C, the cells were harvested by centrifugation. For cell lysis the pellet was resuspended in 5 ml of cold lysis buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 10 mM imidazole, 10 mM β-mercaptoethanol, with 1 mg/ml lysozyme, 1 mg/ml DNase I) supplemented with 100 μl of complete EDTA-protease inhibitor mixture (Roche Applied Science). After incubation on ice for 1 h the lysate was sonicated and cleared by centrifugation and filtration through a 0.45-μm filter.

The His-tagged fusion proteins were purified by nickel affinity chromatography using HiTrap columns and FPLC equipment (Amersham Biosciences). After equilibrating the column with 5 volumes of column buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 10 mM imidazole, 10 mM β-mercaptoethanol) the lysate was applied, and the column was washed with 10 volumes of washing buffer (column buffer containing 20 mM imidazole). The fusion proteins were eluted with a linear imidazole gradient. Peak fractions were pooled, dialyzed against storage buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol), and stored in small aliquots at −80 °C. Protein concentrations as estimated by Coomassie Blue staining were in the range of 50–200 μg/ml.

Chaperones

Hsp40—The complete human Hsp40 (Hdj1) ORF (33) from plasmid pET-Hsp40 (kindly provided by J. Hohfeld) was amplified by PCR using a primer that replaced the stop codon by an HpaI site. The PCR product was digested with NdeI and XhoI and cloned into the corresponding restriction sites of the vector pET30a+ (Novagen). The resulting plasmid pET30-Hsp40His encodes full-length Hsp40 with a C-terminal His6 tag. Protein expression and purification by nickel affinity chromatography were carried out essentially as described for NusDP, except that MgCl2 was omitted from all buffers. Peak fractions were pooled, dialyzed against storage buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol), and stored in small aliquots at −80 °C. The yield of Hsp40 was in the range of 40 mg/liter E. coli culture. This preparation of Hsp40 was routinely used. We also tested commercially available purified recombinant human Hsp40 expressed in E. coli (Stressgen, Victoria, BC, Canada). No significant differences in activity were observed.

Hsc70/Hsp70—Purified recombinant bovine Hsc70 and human and rat Hsp70 were purchased from Stressgen. A plasmid construct encoding d壳cuck Hsp70 was kindly provided by M. Hild. The complete ORF was cloned into the pET30a+ vector. Expression and purification of the C-terminally His-tagged d壳cuck Hsp70 protein by nickel affinity chromatography was performed as described above for Hsp40.

Hsp90—Recombinant human Hsp90α expressed in E. coli and human Hsp90 purified from HEla cells were purchased from Stressgen. Alternatively, human and yeast Hsp90 were provided by J. Buchner (University of Munich, Germany). Yeast Hsp90 was shown, in-house, to have ATPase activity comparable with that reported in the literature (34); the human Hsp90, expressed in insect cells (35), had been characterized by Dr. Buchner’s laboratory to be dimeric in gel filtration and to have ATPase activity with a kcat of 0.04 ± 0.02 min−1, similar to the kcat of 0.09 min−1 reported in the literature (36).

Other Chaperones—His-tagged human p23 was expressed in E. coli and purified by nickel affinity chromatography followed by gel filtration on a Superdex 75 HR column (37). A cDNA for human Hsc70 was obtained from the Resource Center/Primary Data Base (RZPD, Berlin, Germany). The Hdj2 ORF was cloned into pET30a+, and the full-length, C-terminally His-tagged Hdj2 protein was expressed and purified essentially as described above for Hsp40. Human Hsp and yeast Ydj1 were also provided by J. Buchner.

De RNA Synthesis

De RNA was obtained by in vitro transcription (T7 MEGASHortscript kit, Ambion, Austin, TX) from plasmid pDe1 linearized with Clal as described previously (38). The transcript is nominally 76 nucleotides in length and contains the DHBV sequence from position 2557 to 2624. The DNA template was digested with DNase I. The RNA was phenol extracted, precipitated, and the RNA pellet was resuspended in H2O to a final concentration of 40 μM.

Reconstitution of Priming-competent De-P Protein Complexes with RL

Reconstitution reactions were assembled from 10 to 50 ng of purified recombinant P protein, 4 μl of RL (microcolloidal nucleate-treated rabbit RL, Promega), in vitro transcribed De RNA at a final concentration of 1 μM, 10 units of RNasin (Promega), and H2O to a final volume of 10 μl. All components were mixed and incubated at 30 °C for 2 h. Subsequently initiation-competent complexes were detected using priming assays (see below).

Reconstitution of Priming-competent De-P Protein Complexes with Purified Chaperones

Standard reconstitution reactions were assembled, in a final volume of 10 μl, by mixing the following components: 50 ng of purified recombinant P protein, 1.8 μg of recombinant bovine Hsc70, 2 μg of recombinant human Hsp90, 40 μl of DHBV DNA, 80 μl of TKD buffer (100 mM Tris, pH 7.5, 150 mM KCl, 25 mM dithiothreitol), 1 μl of an ATP-regenerating system (50 mM ATP, 25 mM MgCl2, 250 mM creatine phosphate, 100 units/ml phosphocreatine kinase), and 10 units of RNasin. The samples were incubated at 30 °C for 2 h and subsequently subjected to priming assays. The separately expressed TP plus RT/RH domains (30) and the truncated NusDP2/H domain protein were analyzed accordingly.

Kinetic Analysis of the Generation and Decay of Activated NusDP (NusDP*)

For the analysis of NusDP* synthesis a 10-μl standard reconstitution reaction lacking De RNA was assembled and incubated at 30 °C. At the indicated time points 1-μl aliquots were taken and mixed immediately with 4 μl of dilution buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 30 mM KCl, 2.5 mM MgCl2, 5 mM dithiothreitol) containing De RNA to give a final concentration of 1 μM. After 1 h at 30 °C, the samples were analyzed by priming assays. To investigate the stability of NusDP* a standard reconstitution reaction of 30-μl volume lacking De RNA was analyzed in the presence of 0.6 μl of an ATP-regenerating system (50 mM ATP, 25 mM MgCl2, 250 mM creatine phosphate, 100 units/ml phosphocreatine kinase), and 10 units of RNasin. The samples were incubated at 30 °C for 2 h and subsequently subjected to priming assays. The separately expressed TP plus RT/RH domains (30) and the truncated NusDP2/H domain protein were analyzed accordingly.

Efficient Expression in E. coli of Near Full-length DHBV P Protein—Our previous study had shown that the TP domain and a RT/RH domain lacking the last 25 amino acids (but not a protein containing the complete C terminus) could be expressed at high levels in E. coli (30), and confirmed earlier data that the spacer region connecting the domains in the authentic protein is dispensable for function (39). In a first attempt toward a recombinant single-chain P protein we covalently connected

RESULTS

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tion products were separated by SDS-PAGE, and 32P-labeled NusDP at a concentration of 50 μg/ml from 1 liter of E. coli cells transformed with the Nu

...tein was expressed efficiently in mostly soluble form in E. coli (40). The resulting fusion protein, termed NusDP (Fig. 2A), was monitored subsequently by priming assay in the presence of [α-32P]dATP as described under “Experimental Procedures.”

The domains by replacing the central 128 amino acids of the 180-amino acid spacer with a 3-amino acid linker; the 25 C-terminal residues of RT/RH were replaced by a His6 tag. This protein was expressed efficiently in E. coli but was totally insoluble. To overcome this problem, the same construct was then fused, at its N terminus, to the 491-amino acid domains by replacing the central 128 amino acids of the 180-amino acid spacer with a 3-amino acid linker; the 25 C-terminal residues of RT/RH were replaced by a His6 tag. This protein was expressed efficiently in E. coli but was totally insoluble. To overcome this problem, the same construct was then fused, at its N terminus, to the 491-amino acid

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that NusDP can be specifically activated by just Hsc70 and by whole RL.

The above described experiments established that NusDP was visualized by autoradiography. Numbers below the gel indicate fold-activation relative to the sample without chaperones (left lane). B, specificity for the D NusDP template. Aliquots of 50 ng of NusDP protein were mixed with 90 μg/ml Hsc70, 200 μg/ml Hsp40, an ATP-regenerating system, and the indicated D RNAs. After 2 h at 30 °C the samples were subjected to priming assays in the presence of ω-32PdTATP with or without a mixture of dGTP and dTTP (50 μM each) as indicated. 32P-Labeled NusDP was visualized by autoradiography. wt, wild type. C, ATP dependence. Aliquots of 50 ng of NusDP protein were mixed with 45 μg/ml Hsc70, 200 μg/ml Hsp40, D NusDP, and the indicated nucleotides. After 2 h at 30 °C priming-competent NusDP complexes were monitored by priming assays.

ATPγS. As shown in Fig. 3C significant priming activity required ATP, with the strongest signal at high ATP concentrations. Neither ADP nor ATPγS could replace ATP, demonstrating that ATP hydrolysis is necessary for NusDP activation and suggesting that the chaperone activity of Hsc70 is involved.

Similarly Efficient Activation of NusDP by Hsc70/Hsp40 as by Whole RL—The above described experiments established that NusDP can be specifically activated by just Hsc70 and Hsp40 + ATP; the reaction efficiency, however, was rather moderate. To optimize the reaction conditions, the concentration of Hsc70 was varied, and, in addition, the time course of the reaction was investigated. Reconstitution reactions containing NusDP, D NusDP, RNA, an ATP-regenerating system, a constant concentration of Hsp40 (200 μg/ml) plus different concentrations of Hsc70 were assembled and incubated at 30 °C. At various time points aliquots were taken, and the amount of priming-competent complexes was monitored by subsequent priming assays. As shown in the inset of Fig. 4, only marginal signals were obtained in the absence of Hsc70, which did not increase even after very long incubation times (20 h, data not shown). However, increasing concentrations of Hsc70 and increased incubation times led to a corresponding increase in 32P-labeled NusDP protein. For a quantitative analysis, the signal intensities were determined using a PhosphorImager and plotted against the incubation times (Fig. 4). After an initial lag phase (see below) of about 30 min, the reaction rates remained constant for each specific setting over at least 4 h. The priming signals continued to increase after 24 h, and in some experiments even 48 h, incubation, although at these very late time points the rate of product formation gradually decreased. The signal intensities eventually reached in such long term reactions were up to 1,000-fold higher than in the chaperone minus controls. Thus priming-competent complexes accumulated with a constant rate over several hours, and this rate was strongly dependent on the concentration of Hsc70.

To compare the efficiencies of the Hsc70/Hsp40 versus whole RL-mediated reactions the same amount of NusDP as above was incubated in RL in the presence of D NusDP, and aliquots were taken and analyzed as described above (see inset, panel marked RL). Because RL contains an endogenous dNTP pool that reduces the specific radioactivity of the added ω-32PdTATP we first determined the extent of this effect in the specific batch of RL used. To this end, the priming signals obtained with whole RL, and with RL depleted of small molecules by rapid gel filtration, were compared side by side. Depletion increased the 32P priming signal by about 2-fold (data not shown). Therefore the measured signal intensities from RL-containing samples (inset in Fig. 4) were multiplied by a factor of 2 before plotting them in the diagram. Three points of interest became evident from a quantitative comparison with the Hsc70/Hsp40 reactions. (i) During the 1st h of the reaction in RL the amount of priming-competent complexes increased linearly, and the rate of complex formation (slope of the curve) was very similar to that observed in the presence of 360 μM Hsc70 in the steady-state phase. (ii) The lag phase of about 30 min was absent (Fig. 4, right panel). (iii) Priming-competent complexes did not continue to accumulate over several hours but rather reached a maximum after 2–3 h and then declined. Thus, at sufficiently high Hsc70 concentrations, the overall efficiency of NusDP complex formation in the two-chaperone system was even higher than in RL.

Two Molecules of Hsc70 Cooperate in NusDP Activation—The usual function of Hsp40 is to stimulate the ATPase activity of Hsc70, thus stabilizing the Hsc70-substrate interaction (43). We therefore investigated the NusDP-D NusDP complex reconstitution rate at different Hsc70/Hsp40 ratios. Plotting the priming signal intensities obtained with increasing Hsc70 concentrations and 200 μg/ml Hsp40 led to a curve whose slope
Hsc70 was held constant at 180 μg/ml Hsp40. Reconstitution reactions were performed as in and the amounts of 32P-labeled NusDP were plotted against the Hsp40 concentration in the presence of 180 μg/ml Hsc70 on NusDP where both must be occupied to allow for binding of De. By contrast, no evidence for cooperativity was seen by increasing the Hsp40 concentration in the presence of 180 μg/ml Hsc70. Here the plot (Fig. 5B) showed a hyperbolic curve with a steep initial slope that was directly proportional to the Hsp40 concentration but then leveled off. Half-maximal activity was observed at 20–25 μg/ml Hsp40 and near saturation above 50 μg/ml. Further doubling the Hsp40 concentration to 100 and 200 μg/ml yielded only minor increases in signal intensity.

**Hsc70/Hsp40 Activity Is Required to Establish and Maintain an Equilibrium between De RNA Binding-competent and -incompetent NusDP Conformations**—A likely function for Hsc70/Hsp40 in NusDP activation is the induction of structural changes that lead to a conformation that is able to bind De RNA (termed NusDP°). In the experiments described above De RNA was present throughout the incubation period, and priming-competent NusDP°-De complexes accumulated with steady-state kinetics over several hours. A possible explanation is that the NusDP° generated by the chaperone activity is, on its own, metastable but converted into a more stable RNP complex by De RNA binding; alternatively, NusDP° may accumulate without De RNA. To distinguish between these possibilities, a three-step assay was employed (schematically outlined in Fig. 6A). First, NusDP was preincubated with Hsc70/Hsp40 plus ATP as described above but without De RNA. At different time points aliquots from this first protein-only preincubation (activation phase) were taken, and a saturating concentration of De RNA was added for a second incubation period of 1 h (De binding phase); this is necessary because RNA binding does not occur in priming buffer. To prevent further generation of NusDP° during this RNA binding step we took advantage of the second order Hsc70 kinetics determined above and diluted the first incubation mix 5-fold before adding the De RNA; this should result in a 25-fold reduction of chaperone activity. Finally, the amounts of priming-competent complexes were determined by quantitative priming assays, and the signal intensities were plotted against the time of the protein-only incubation (Fig. 6C). The data fit to a hyperbolic curve with a steep initial slope that levels off after about 30 min, reaching near saturation around 60 min. Hence, in the absence of De RNA, the concentration of NusDP° increased only during the 1st h of incubation and then approached a maximum; this is in sharp contrast to incubation in the presence of De RNA where priming-competent complexes continued to be produced at a constant rate (Fig. 4).

This result suggested the existence of a dynamic equilibrium between inactive NusDP and RNA binding-competent NusDP°, generated by the continued chaperoning activity of Hsc70/Hsp40. Because ATP hydrolysis is essential for Hsc70 activity ATP depletion should inhibit this activity and allow measurement of the half-life of NusDP°. As outlined schematically in Fig. 6B, NusDP was therefore incubated with the two chaperones for 90 min to reach saturating concentrations of NusDP° (activation), then ATP was removed by rapid gel filtration. The reaction mix was incubated further at 30 °C (decay phase), and aliquots were taken at several time points and incubated with De RNA for 1 h. Thereafter, the amounts of priming-competent complexes were monitored by quantitative priming assays as above. A plot of the priming signals against the time after ATP depletion (Fig. 6D) showed a rapid decline that followed first-order kinetics. Hence in the absence of De RNA and of continued chaperone activity, NusDP° decayed with a half-life of about 12 min.

A conceivable difference between the Hsc70/Hsp40 only and
Hsp90-independent Hepadnavirus P Protein Activation

Assessing a potential role of Hsp90, Hop, and p23 in P protein activation. A, effect of additional chaperones on Hsc70/Hsp40 reconstitution. Reconstitution reactions consisting of 50 ng of NusDP, De RNA, an ATP-regenerating system plus the indicated chaperones at the indicated concentrations were incubated for 2 h at 30 °C and subjected to standard priming assays. Priming signals were quantified, and for lanes 1–3 and lanes 4–6 were normalized separately to the control reactions in lanes 1 and 4, respectively (shown as -fold activation). B, absence of detectable amounts of Hsp90 in the Hsc70/Hsp40 reconstitution system. 10 μg of Hsp40, 10 μg of Hsc70, 5 μl of an ATP-regenerating system (ARS), and 0.5 μg of NusDP were screened for contaminating Hsp90 by Western blotting with the anti-Hsp90 monoclonal antibody AC88. A serial dilution of human Hsp90 (lanes 1–5) indicated a detection limit of below 12.5 ng.

The whole RL reconstitution system could be that other factors, present only in RL, stabilize the NusDP* complex. Therefore, the stability in whole RL of NusDP* was investigated by a similar experiment performed in parallel (Fig. 6D). However, NusDP* was again unstable and decayed even faster with a half-life of about 4 min.

These data indicate that NusDP* corresponds to a metastable intermediate that builds up in the early phase of the reaction until an equilibrium between NusDP and NusDP* is established where the decay of NusDP* is compensated for by its regeneration via continuous ATP-consuming chaperone activity; the presence of De RNA apparently stabilizes these activated molecules such that they accumulate, over several hours, at a nearly constant rate.

Effects of Hsp90, Hop, and p23 on the Efficacy of Hsc70/Hsp40-mediated Reconstitution.—The efficient reconstitution of a priming-active P protein complex by just Hsc70 and Hsp40 appears discordant with a recent report that in vitro activation of a truncated DHBV P protein fused to glutathione S-transferase did not only require Hsp70 and Ydj1 (a Hsp40 homolog from yeast used instead of mammalian Hsp40 in those experiments) but, with occasional exceptions, was also strictly dependent on Hsp90 and Hop and was boosted by p23 (32). We therefore investigated whether inclusion of these chaperones would enhance the activity beyond that observed with only Hsp40 and Hsc70. Recombinant human Hsp90, functionally active according to several criteria including ATPase activity (36), together with Hop and p23 at concentrations reported to be optimal for P activation (32), did not significantly increase the signal strength compared with Hsp40 and Hsc70 only (Fig. 7A, lanes 1–3). At higher concentrations of Hsc70, sufficient to generate strong priming signals with just Hsp40, a moderate 2–2.5-fold enhancement by the Hsp90 machinery was reproducibly detected (Fig. 7A, lanes 4–6). Hence under certain conditions Hsp90 and its cochaperones exerted a beneficial effect on P activation. However, compared with the up to several hundredfold activation by Hsc70 and Hsp40, the contribution of the Hsp90 system appeared to be marginal. Similar results were obtained with four different batches of Hsp90 from different sources; occasionally, a reduction rather than an enhancement of the Hsc70/Hsp40 mediated priming signal was observed with some batches of human but not yeast Hsp90.

Because Ydj1 is a yeast protein and, in addition, is more closely related to human Hdj2 than to the Hdj1 used in our study (Ydj1 and Hdj2, but not Hdj1, contain an additional zinc binding domain), we repeated the two-chaperone reconstitution experiments with Hsc70 plus either Ydj1 or human Hdj2. However, neither of these two Hsp40s could replace Hdj1 (data not shown). We then wondered whether Hsp90 and Hop could boost activation by Hsc70/Ydj1. However, neither yeast nor human Hsp90 led to an enhancement of priming signals (data not shown).

A potential concern was the unintentional presence of some Hsp90 in one of the components of our two-chaperone reconstitution system, although most of the Hsc70 and Hsp40 preparations were obtained by recombinant expression in E. coli. We therefore tested all components of our reconstitution system for contamination with Hsp90. By Western blotting we were not able to detect any Hsp90 protein in aliquots of Hsp40, Hsc70, an ATP-regenerating system (which contains creatine kinase from mammalian tissue), or purified NusDP (Fig. 7B). Given a detection limit of Hsp90 below 12.5 ng and accounting for the relative amounts of the individual components in the reconstitution reactions, less than 2.5 ng of contaminating Hsp90 could have been present in our 10-μl assays. This represents only 0.2% of the amount (120 ng/10-μl reaction) required to be present to generate a significant priming signal (32) and therefore cannot account for the strong activity observed in our experiments.

Hsc70/Hsp40 Activation of Recombinant DHBV P Protein Is Not Restricted to NusDP—To exclude that Hsp90-independent activation is a unique feature of the NusDP fusion protein caused by some property of NusA rather than the P protein part we replaced the NusA domain by a different fusion partner, the GrpE protein from E. coli (Fig. 8A). GrpE displays no similarities to NusA in terms of size, sequence, structure, or function, except that it has been reported also to enhance solubility of heterologous proteins expressed in E. coli (40). GrpE acts as a nucleotide exchange factor for the bacterial DnaK but is known not to interact with mammalian Hsp70s (44). Like NusDP, the GrpE fusion protein (termed GrpDP) could be produced in substantial amounts in E. coli and be purified in soluble form. Reconstitution of priming-competent complexes from GrpDP in our two-chaperone system revealed the same features as described above for NusDP (Fig. 8B).

In the presence of Hsc70, Hsp40, De RNA, ATP-regenerating system, and unlabeled dGTP/dTTP, [α-32P]dATP was incorporated with similar efficiency as into NusDP (lane 3). In the absence of chaperones, or De RNA, or dGTP/dTTP, or only very weak priming signals were observed. As with GrpDP, chaperones of the Hsp90 machinery stimulated the Hsc70/Hsp40 activity only 2–3-fold, and mammalian Hsp40 could not be replaced by yeast Ydj1 (data not shown). Hence GrpDP displayed essentially the same characteristics as NusDP, strongly arguing that the P protein parts per se rather than the heterologous domains are responsible for these properties. In addition, deletion of the spacer region in NusDP and GrpDP was not responsible for Hsp90 independence because a GrpE-P protein fusion containing the contiguous P sequence from amino acids 1 to 761, including the authentic spacer region, was also activated efficiently without Hsp90 (data not shown).

Hsp90 Is Not Required to Orient the TP and RT/RH Domains Specifically—The function of Hsp90 in SHR activation is to make the ligand binding cleft accessible to the hormone. A
Hsp40, an ATP-regenerating system, and D/H9280ing 20 GrpDP upon Hsc70/Hsp40 activation. Reconstitution reactions contain- 
and ologous domains.

protein fusions are caused by the P protein part, not the heter-
priming (21, 46) and thus should allow addressing the role of 
2 . 5ha t3 0 the presence of 
° , were incubated for 2ha t3 0 /H9262 RT/RH, D/H9280 dependent on TP, RT/RH, and D/H9280 consequently subjected to priming assays. Priming was absolutely 
RNA were incubated with Hsp40, Hsc70, and ATP, and subse-
posed in that Hsp90 might appropriately orient the TP and 
similar role for Hsp90 in P protein activation has been pro-
chaperones by priming assays. The truncated protein, termed 
verifying TP, RT/RH, and D/H9280 expressed in and purified from 
C. Recombinant P protein was subjected to dGTP + dTTP (only lanes 1–3) as described under “Experimental Procedures.” C. Hsc70/Hsp40-mediated 
activation of separately expressed TP and RT/RH domains. Reconsti-
tution reactions containing, as indicated, 25 µg/ml TP, 10 µg/ml 
RT/RH, De RNA, and an ATP-regenerating system plus either 200 
µg/ml Hsp40 and Hsc70 (concentrations in µg/ml as indicated), or RL (lane 7), were incubated for 2 h at 30 °C, and active complexes were detected by priming assays.

similar role for Hsp90 in P protein activation has been pro-
proteins to become independent of Hsp90. To address this point we resorted to the separate expression of TP and RT/RH; there, 
domains could obviously not be forced into any specific 
orientation by the linker. Because the separate domains previ-
ously appeared not to be activatable with isolated chaperones (30) we now repeated these experiments using the improved 
conditions established above; reconstitution with whole RL 
served as control. As shown in Fig. 8C, the 30-kDa TP domain 
was indeed 32P-labeled when recombinant TP, RT/RH, plus De 
RNA were incubated with Hsp40, Hsc70, and ATP, and subse-
cutively subjected to priming assays. Priming was absolutely 
dependent on TP, RT/RH, and De RNA, and the signal strength increased with increased Hsc70 concentration. Thus, Hsc70 
and Hsp40 were sufficient to mediate the formation of productive 
complexes among TP, RT/RH, and De RNA, without need for a specific orienting function of Hsp90. The absence in the 
trans-complementation system of any foreign domain further 
corrobates that Hsc70/Hsp40 activation is caused by the P 
protein part.

Chaperone-independent Priming by a NusDP Protein Lack-
ing the RNase H Domain—To start mapping of the potential 
Hsc70/Hsp40 target sites within the P protein sequence we 
extended a large segment of the C-terminal P protein part, 
including the entire RH domain. The remaining sequence still 
contains all of the sequence motifs universally present in RTs 
although it lacks part of the predicted thumb region. Similar 
deletions have previously been shown to support De-dependent 
priming (21, 46) and thus should allow addressing the role of

FIG. 8. Chaperone-dependent priming characteristics of the P 
protein fusions are caused by the P protein part, not the heter-
ologous domains. A, domain organization of GrpDP protein. Numbers 
and abbreviations are as in the legend to Fig. 2A, B, priming activity of 
GrpDP upon Hsc70/Hsp40 activation. Reconstitution reactions containing 
20 µg/ml GrpDP and, as indicated, 45 µg/ml Hsc70, 200 µg/ml 
Hsp40, an ATP-regenerating system, and De RNA were incubated for 2.5 h at 30 °C, and active complexes were detected by priming assays in the presence of [α-32P]dATP and unlabeled dGTP/dTTP (only lanes 1–3) as described under “Experimental Procedures.” C. Recombinant P protein was subjected to dGTP + dTTP (only lanes 1–3) as described under “Experimental Procedures.” C. Hsc70/Hsp40-mediated 
activation of separately expressed TP and RT/RH domains. Reconsti-
tution reactions containing, as indicated, 25 µg/ml TP, 10 µg/ml 
RT/RH, De RNA, and an ATP-regenerating system plus either 200 
µg/ml Hsp40 and Hsc70 (concentrations in µg/ml as indicated), or RL (lane 7), were incubated for 2 h at 30 °C, and active complexes were detected by priming assays.

Reverse transcription of hepadnaviruses is regulated strictly 
on the level of initiation of DNA synthesis through highly 
specific RNA template-RT interactions. Although the natural 
tRNA primed initiation process of retroviral RTs is probably 
similarly complex (47), two unique features in hepadnaviral 
reverse transcription are the protein priming mechanism and 
its requirement for cellular factors. The prevailing model is 
that P protein activation proceeds, analogously to SHRs, 
through sequential interactions with the Hsp70 and Hsp90 
machineries; in particular, a strict dependence on Hsp90, a 
hallmark of SHR activation, has been postulated. The results of 
this study show that specific, and efficient, initiation of DNA 
synthesis from the authentic RNA template by a recombinant P 
protein can be reconstituted in vitro, without any Hsp90, by a 
simple two-chaperone system consisting of only Hsc70 and 
Hsp40 plus energy.

Using this system we obtained direct evidence that (i) a 
metastable RNA binding-competent form of P protein (P*) 
is generated through the ATP- and Hsp40-dependent chaperon-
ing activity of Hsc70 which is stabilized by binding of e RNA; 
(ii) by using appropriate conditions, a similar rate of formation

FIG. 9. Partially chaperone-independent priming activity of a 
C-terminally truncated NusDP protein, NusDPΔRH. Reconstitu-
tion reactions were set up with NusDPΔRH without chaperones, 
or with Hsc70/Hsp40, or with whole RL, and priming activities with 
[α-32P]dATP were determined in the presence and absence of De RNA 
and dGTP + dTTP as indicated. The relative signal intensities, normal-
ized to the signal obtained in the nonassisted reaction with De RNA but 
without dGTP + dTTP, are shown below each lane.
of P* by Hsc70/Hsp40 is achievable as by reconstitution with whole RL; (iii) activation most likely involves two molecules of Hsp70; (iv) in this system, Hsp90 and its cofactors are neither required to generate P* from a single-chain derivative nor from separately expressed TP and RT/RH domains; (v) the C-terminal RH domain is involved in mediating the Hsc70/Hsp40 dependence of P* formation. Below we discuss this two-chaperone reconstitution system with respect to its specificity, efficiency, and mechanistic implications compared with reconstitution in whole RL.

Specificity and Efficiency of P Protein Activation by Hsc70 Plus Hsp40—A critical prerequisite for this study was the establishment of a reliable source for soluble recombinant P protein. Covalently linking the derivatives of TP and RT/RH which had previously been optimized for high expression in E. coli (30), and N-terminal fusion to either the NusA or GrpE protein yielded single-chain P proteins that were expressed at high levels, were mostly soluble, and could substantially be enriched by a single metal ion affinity chromatography step. Both fusion proteins displayed essentially the same characteristics in our in vitro reconstitution system, indicating that the salient features observed reflected genuine properties of the P protein part.

The restriction to e RNA as the specific template was fully reproduced by the recombinant P proteins, both by reconstitution in whole RL and in the two-chaperone system. No significant priming signals were observed without De RNA or with a mutant De RNA known to be priming-defective with in vitro translated authentic P protein in RL. Furthermore, efficient incorporation of [α-32P]dATP required the simultaneous presence of dGTP and dTTP, as expected for a DNA primer copied from the authentic template region within De.

Regarding chaperone specificity all our data are congruent with a fundamental requirement for Hsc70 and Hsp40 plus energy but no further factors; we have also excluded that any of the reconstitution components fortuitously contains significant amounts of Hsp90. Another concern was that the P protein preparations, although substantially enriched for full-length NusDP and GrpDP, still contained minor amounts of additional proteins (Fig. 2B). It is unlikely, however, that these contaminating proteins contributed to Hsp90 independence. Given that this phenotype was common to both fusion proteins, the potentially responsible factor should be present in both preparations; however, upon direct comparison by SDS-PAGE and Coomassie Blue staining only three of the several minor bands visible had an identical mobility (data not shown). Immunoblots suggest that two of them represent E. coli Hsp70 (DnaK) and Hsp60 (GroEL), but the very same bacterial chaperones have been found in apparently Hsp90-dependent RT preparations (45). Regarding HtpG, the only known, though nonessential, Hsp90-like E. coli protein, we cannot rule out that some of it is present in our preparations. However, HtpG has been reported to be unable to substitute for mammalian Hsp90 in P protein activation (32). Furthermore, HtpG contains a functionally essential ATPase activity that is sensitive to geldanamycin (34), yet we could not observe any inhibitory effect of the drug on Hsp40/Hsc70-mediated P protein activation (data not shown). This result strongly argues against a contribution of HtpG to RT activation in our system.

In most assays we used a commercial preparation of recombinant bovine Hsc70 produced in E. coli. Although an occasional batch of this protein was inactive, several other Hsc70s and Hsp70s tested were also active; these included home-made recombinant duck Hsc70 and commercial preparations of rat and human Hsp70. Hence both the constitutively and the inducibly expressed Hsp70 members from a variety of organisms can support P protein activation. This suggests that the notorious inactivity of P protein from the human HBV is not caused by an incompatibility with the rabbit Hsp70 present in RL.

In contrast, the specific nature of the Hsp40 used did matter; in our hands, only human Hdj1, both a commercial and a home-made preparation, exerted activity. Hsp40s form a large family of related but distinct proteins, characterized by a common J domain (named after the bacterial prototype DnaJ), which interacts with Hsp70, and differing by the presence or absence of additional domains (48). Hdj1 is a major human type II Hsp40 that lacks a zinc binding domain present in type I Hsp40s such as Hdj2 and yeast Ydj1. Interestingly, neither human Hdj2 nor Ydj1 could replace Hdj1 in our assays; in fact, in control reactions containing Hsc70 and an equimolar mixture of Hdj1 and Ydj1 no P protein activation was observed, i.e. Ydj1 behaved as a dominant negative inhibitor (data not shown). One possibility is that the inactivity of the type I Hsp40s was related to the specific batches of protein preparations, as mentioned above for commercial Hsc70. We note, however, that the Hdj2 was expressed and purified using the same vector and protocol as Hdj1, which routinely yielded active protein. Alternatively, the zinc-binding Hsp40s may be unable to cooperate productively with Hsc70 in P protein activation. The different Hsp40s are functionally redundant in some aspects but not in others; for instance, no difference in the ability of Ydj1, Hdj1, and Hdj2 to promote in vitro binding of Hsp70 to Hop has been detected (49). However, only Hdj2 but not Hdj1 could suppress the ligand binding defect of human androgen receptor expressed in a yeast strain deleted for the Ydj1 gene (50). Hence the presence or absence of the zinc binding domain may well influence the specificity of Hsp40/Hsc70 toward different target proteins. Also, differences in the interactions between individual combinations of J domain proteins and Hsp70s have been reported. For instance, several Hsp40s bind only weakly to Hsp70, whereas auxilin, a J domain protein that catalytically promotes Hsc70 binding to, and depolymerization of, clathrin baskets, binds strongly (51). Hsc70 and Hsp70 differ in Hsp40 induced homopolymerization (52), and because polymerization prevents substrate binding (53), the specific combination of Hsc70/Hdj1 in our study versus Hsp70/Ydj1 used by others (32) may account, in part, for the different results. It is also conceivable that, in vivo, a specialized J domain protein rather than Hsp40 is involved in P protein activation; an example is hTid-1 which appears to be important for herpes simplex virus (54) and human T cell leukemia virus (55).

Our initial experiments revealed a specific yet modest (64-fold) activation of P protein by Hsc70/Hsp40 and ATP which was lower than that observed in whole RL or that described for in vitro activation in the additional presence of Hop, Hsp90, and p23 (32); there, about 60–70% of the activation obtained with whole RL was achieved with Hsp70/Ydj1 concentrations of 35 and 100 μg/ml, or, alternatively, 100 μg/ml Hsp70 and 20 μg/ml Ydj1. However, increased Hsc70 concentrations and prolonged incubation periods in our assays led to priming signals whose strengths eventually exceeded those obtained with whole RL; moreover, the rate of product formation at the highest Hsc70 concentrations tested (360 μg/ml; 5 μM) combined with ≥50 μg/ml Hsp40 was comparable with that in RL. Hence Hsc70/Hsp40 activation represents a major and efficient pathway, at least in vitro. The concentrations of Hsc70 and of Hsp40 required may appear very high; it should, therefore, be emphasized that the chaperone concentration in RL is also very high; Hsc70 has been estimated to be present at about 7 μM concentration (i.e. about 500 μg/ml) in neat RL (56), corresponding to about 200 μg/ml in our RL reconstituted reactions. Further-
more, the Hsp70 plus Ydj1 concentrations reported for optimal in vitro reconstitution of the SHRs in the five-chaperone system were 100 μg/ml + 8 μg/ml for the progesterone receptor (7) and 300 μg/ml + 8 μg/ml for the glucocorticoid receptor (57). Thus the Hsc70/Hsp40 concentrations used in our assays are in a comparable range.

Mechanistic Implications of Hsc70/Hsp40-mediated P Protein Activation—The dependence on Hsp40 and ATP hydrolysis are common features of Hsc70 chaperoning. The most likely role of Hsp40 in P protein activation is to stimulate the ATPase activity of Hsc70, thus converting it from the ATP-bound to the ADP-bound form, which has a much higher substrate affinity (40); that substoichiometric amounts of Hsp40 were sufficient indicates its only transient involvement, similar to Hsp40-mediated loading of Hsp70 onto Hsp (49). Whether Hsc70-ATP binds to P and is then stimulated by Hsp40, or whether Hsp40 binds first to P and then recruits Hsc70, as shown, e.g. for denatured luciferase (58) and recently also for progesterone receptor activation (59), remains to be determined. Notably, the latter mechanism could provide an explanation for the selective activation by Hsp70 over Hsp60, given that DnaJ proteins are now regarded as the major class of Hsp70 specificity factors (60).

The product of the Hsp40-mediated Hsc70 action on P protein is an activated state, P*, which is able to interact with De RNA, and to use the RNA as template subsequently for protein-primed DNA synthesis. Such specialized chaperoning functions are frequently associated with Hsp90 rather than Hsc70; it might then be argued that the activation seen in the two-chaperone system simply reflects that our recombinant fusion proteins are partially denatured and then subject to the general protein folding activity of the Hsc70 system. This would be consistent with the continuous accumulation of priming-competent P* molecules observed in the presence of De RNA but not with the data obtained in its absence: there, P* accumulated for only about 0.5–1 h, consistent with the establishment of an equilibrium between P* and inactive P molecules. The decay of P* implicated by this process was demonstrated directly by preventing further Hsc70 chaperoning activity by ATP depletion, which led to the disappearance of P* with first order kinetics. Thus, P* is a metastable intermediate rather than a finally folded stable structure. In the presence of De RNA, however, P* is converted into much more stable P*-De complexes that accumulate, in a priming-competent state, over several hours.

The second order dependence on Hsc70 concentration of P* formation indicates that two molecules of Hsc70/P protein are involved. One explanation would be that dimerization of Hsc70 is required; however, there is no precedence for an increased activity of dimeric or higher order Hsc70 complexes; rather the substrate binding sites may be occupied by other Hsc70 molecules and thus be unavailable for true substrates (52). Alternatively, dimerization of P could explain the observed Hsc70 kinetics. However, several lines of evidence suggest that only one molecule of P protein is packaged per nucleocapsid (61) where authentic reverse transcription occurs. Hence, P dimerization cannot be formally excluded but appears rather unlikely.

We therefore favor a model, summarized in Fig. 10, according to which two distinct Hsc70 binding sites exist on P protein, both of which must be occupied, at least transiently, for De RNA binding. The location of these two sites is not yet clear; however, the priming Tyr residue in the TP domain and the polymerase active site in RT/RH must both be close to the De RNA template, hence the RNA binds most likely in a cleft between the two domains. Opening this cleft could be achieved by Hsc70a interacting with both TP and RT/RH, similar to the role ascribed previously to Hsp90 (45); apart from more global structural alterations the recently discovered amide peptide bond isomerase activity of Hsc70 (62) might be involved. A probable mediator of chaperone action within RT/RH is the RH domain, or possibly the thumb region of the RT domain, because their deletion led to a protein, NusDP RH, with much reduced chaperone dependence. A substantial fraction of molecules interacted productively with De in the absence of any chaperone, although formation of priming-active complexes was enhanced further, with similar efficiency, by Hsc70/Hsp40 and whole RL. This is congruent with the truncation rendering the closed complex less stable such that a significant proportion of the molecules exists, per se, in the open form, whereas release of occlusion in the full-length protein requires chaperones. Activity of the truncated protein was strictly De-dependent, but its specificity for the authentic template region appeared to be relaxed because some priming occurred with dATP in the absence of dGTP and dTTP. The ratio of priming signals with and without dGTP/dTTP was not affected by chaperones, hence it probably reflects an intrinsic property of the truncated P protein. The increase in the presence of dGTP and dTTP suggests, nonetheless, that NusDP ARH, different from the reported activity of a similarly truncated P protein (46), is able to incorporate more than 1 nucleotide into the DNA primer. However, further experimentation will be required to establish this point thoroughly.

Comparison between DP Activation by Hsc70/Hsp40 and by Whole RL—The lack of a fundamental requirement for Hsp90 in P protein activation is in contrast to SHR activation where, despite some debate on the importance of various cochaperones (10, 56), Hsp90 is considered to be absolutely indispensable. Although this indicates a basic difference in the activation mechanisms in vitro, contributions from other factors, including Hsp90, to P protein activation in more complex environments such as RL or live cells, are by no means ruled out; in fact, they are likely to occur not only because Hsp90 is one of the most abundant proteins in the eukaryotic cytosol but because many of the chaperones exist in various heterocomplexes, e.g. the foldosome or Hsp90/Hsp70-based chaperone
machinery in RL which, besides some Hsp40, contains Hsp70, Hop, Hsp90 (49, 56).

Furthermore, additional chaperones and factors that regulate chaperone activity are absent from the in vitro reactions. Indeed, three differences between the Hsc70/Hsp40 versus whole RL reconstitution assays were reproducibly observed. (i) The two-chaperone priming reactions always displayed a lag phase of about 30 min which did not occur with RL. (ii) The velocity of priming product formation was faster in RL compared with the lower Hsc70 concentrations tested (although, after the lag phase, similar rates could be achieved at 360 μg/ml Hsc70). (iii) Priming product formation in RL ceased after 2–3 h but increased continuously in the two-chaperone system. In addition, the half-life of priming-competent complexes in RL upon ATP removal (and in the absence of De RNA) was even shorter (about 4 min versus 12 min). Given that P* formation involves cycling of Hsc70 through different states, the presence in RL of factors influencing the kinetics of Hsc70 cycling is likely to also affect the kinetics of P* generation and decay. Bag-1, for instance, can act like a nucleotide exchange factor and thus accelerate the ATP for ADP exchange on Hsc70 (44, 63, 64), an effect that may be counteracted by Hip (65). A factor and thus accelerate the ATP for ADP exchange on Hsc70.

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Does Hsc70 remain bound during RNA binding itself to screening for compounds that interfere with P protein

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Efficient Hsp90-independent in Vitro Activation by Hsc70 and Hsp40 of Duck Hepatitis B Virus Reverse Transcriptase, an Assumed Hsp90 Client Protein

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