Calf thymus Hsc70 and Hsc40 can substitute for DnaK and DnaJ function in protein renaturation but not in bacteriophage DNA replication

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Abstract Calf thymus (ct) Hsc70 has been shown previously to reactivate heat-inactivated prokaryotic and eukaryotic enzymes, while DnaK was able to reactivate solely prokaryotic enzymes. Here, we report on isolation from calf thymus of a DnaJ homolog, ctHsc40, and on testing of its cooperative function in three different assays: (i) reactivation of heat-inactivated DNA polymerases, (ii) stimulation of the ATPase activity of ctHsc70 chaperone, and (iii) replication of bacteriophage λ DNA. Surprisingly, ctHsc70/ctHsc40 chaperones were found to reactivate the denatured prokaryotic and eukaryotic enzymes but not to promote bacteriophage λ DNA replication, suggesting species specificity in DNA replication. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Hsc70; Heat shock protein 40; DnaK; DnaJ; Protein renaturation; DNA replication

1. Introduction

Molecular chaperones mediate correct folding, assembly and disassembly of other polypeptides ensuring their proper function in the cell. Many chaperones are constitutively expressed (Hsc proteins) while the rate of synthesis of other chaperones (Hsp proteins) transiently accelerates after a shift in temperature [1]. Under stress conditions, chaperones protect cellular proteins from heat denaturation, or once damage has occurred, disaggregate them and allow them to refold back to an active form [2]. Among the best-characterized heat shock proteins are those belonging to the Hsp70 family with their co-chaperones Hsp40 and Hsp20, as exemplified by the *Escherichia coli* DnaK/DnaJ/GrpE system.

The DnaK heat shock protein possesses a weak ATPase activity [3,4], which could be efficiently stimulated in the presence of both DnaJ and GrpE co-chaperones [5–8] and/or by the presence of protein substrates. Depending on whether DnaK is in an ATP or ADP bound form, it possesses a different conformation and protein substrate specificity [9–12]. The protein substrate forms a complex with DnaK(ADP) and DnaJ either by direct interaction with DnaK in the DnaJ–DnaK(ADP) complex that is facilitated by DnaJ, or is loaded onto DnaK(ADP) by the interaction of the protein substrates with DnaJ. Then, in the presence of ATP, GrpE accelerates the exchange of ADP for ATP in DnaK and after ATP hydrolysis the complex dissociates. A similar DnaK/DnaJ/GrpE cycle is likely to be employed in the release of properly folded proteins from a multiprotein complex, as it takes place in the initiation of DNA replication.

Direct involvement of molecular chaperones in the replication of many prokaryotic replicons was shown both by genetic and biochemical studies [13]. Bacteriophage λ DNA replication was the first system in which the synergistic and cooperative action of the DnaK/DnaJ/GrpE molecular chaperone machinery was demonstrated [14,15]. DnaK/DnaJ/GrpE chaperones function in release of λP protein form the preprimosomal complex. It is postulated that binding of DnaJ to the preprimosomal nucleoprotein structure sets that stage for ATP dependent partial complex disassembly [16–19]. DnaJ possesses moderate affinity to λP, λO and DnaB proteins [18–20]. In the presence of ATP, DnaJ facilitates binding of the DnaK chaperone to the λP protein [10]. The third member of the chaperone system, GrpE, triggers the release of λP from the DnaK complex [5–7,12,21–23], with concomitant hydrolysis of ATP. Thus, in this reaction chaperones DnaK/DnaJ/GrpE perform disassembly of a multiprotein complex consisting of native and properly folded proteins as in contrary to the disassembly of aggregates composed of misfolded or unfolded polypeptides.

A similar chaperone system has been found in eukaryotic cells. SV40 large T antigen (TAg), containing a J-domain typical for all DnaJ-like proteins, has been shown to interact with mammalian Hsc70 and to promote viral DNA replication [24]. Moreover, the J-domains of two human DnaJ homologs, HSJ1 and DNAJ2, could substitute functionally for the J-domain of TAg in promoting viral DNA replication.

We have shown previously that the eukaryotic chaperone ctHsc70 (from calf thymus) possesses a broader substrate range than the prokaryotic DnaK protein, since it was able to protect and to reactivate heat-inactivated prokaryotic and eukaryotic enzymes [25]. In this paper we address two questions: (i) how does the ctHsc40 co-chaperone modulate ctHsc70 function in reactivation of heat-denatured enzymes, and (ii) can the eukaryotic chaperones ctHsc70 and ctHsc40 substitute for function of their prokaryotic counterparts, DnaK and DnaJ, in replication of bacteriophage λ DNA?

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2. Materials and methods

2.1. Proteins and DNA

ctHsc70 and calf thymus DNA polymerase α were purified as described previously [25,30]. Bacterial proteins DnaK, DnaJ, GrpE, SSB, gyrA, gyrB, DnaB, DnaG, and DNA polymerase III holoenzyme, as well as bacteriophage λ proteins λO and λP were purified as described earlier [16]. λdv plasmid DNA was isolated as described previously [26].

2.2. Western blot assay

An immunoblot assay [27] with antibodies raised against *E. coli* DnaJ protein, generated as described previously for anti-DnaK antibodies [25], was employed for detection of Hsc40 protein during its purification from calf thymus tissue. Specifically, we used an Immobilon P membrane that was blocked with 3% (w/v) BSA and incubated with the primary antibodies diluted 1:1000 in RIPA buffer. The secondary antibodies were directed against rat IgG and conjugated to alkaline phosphatase. Western blot analyses with anti-K–Y–H–P–D–K peptide, anti-Hsp40 and anti-HSJ1 antibodies were performed as described above with the difference that secondary antibodies were directed against rabbit IgG.

2.3. Purification of DnaJ homolog from ctHsc40

For the isolation of ctHsp40, previously published protocols devised for bovine brain tissue and HeLa cells were adapted [28,29]. All purification steps were performed at 4°C and all buffers contained the two protease inhibitors: pepstatin (final concentration: 1 μg/ml) and leupeptin (final concentration: 1 μg/ml). 120 ml of cytosolic crude extract from calf thymus were dialyzed overnight against buffer A (50 mM Tris–HCl, pH 7.3; 10% (v/v) glycerol; 0.5 mM EDTA; 1 mM DTT; 0.2 mM PMSF) and loaded onto a 30 ml DEAE–Sephacel column pre-equilibrated with the same buffer. The column was washed with 300 ml of buffer A and proteins were eluted with 300 ml of a 0–300 mM KCl gradient in buffer A. Fractions containing the DnaJ homolog, ctHsc40 (detected with anti-DnaJ antibodies) were pooled, dialyzed overnight against buffer B (20 mM KPO_4, pH 7.0; 10% (v/v) glycerol; 0.5 mM EDTA; 1 mM DTT; 0.2 mM PMSF) and loaded onto 10 mM hydroxyapatite column pre-equilibrated with the same buffer. The column was washed with 30 ml of buffer B. The flow through and the wash from the column were pooled, dialyzed overnight against buffer B containing 1 M ammonium sulfate and loaded onto 10 ml phenyl–Sepharose 4B Cl column pre-equilibrated with the same buffer. The column was washed with 100 ml of buffer B containing 0.3 M ammonium sulfate and the proteins were eluted with 100 ml of a 0.3–0 M ammonium sulfate gradient in buffer B. Fractions containing ctHsc40 were pooled, dialyzed overnight against buffer C (50 mM Tris–HCl, pH 7.3, 10% (v/v) glycerol; 1 mM EDTA; 1 mM DTT; 50 mM KCl) and loaded onto 1 ml MonoQ FPLC column pre-equilibrated with the same buffer. The column was washed with 10 ml of buffer C and the proteins were eluted with 14 ml of 100–800 mM KCl in buffer C. Fractions containing ctHsc40 were pooled, dialyzed overnight against storage buffer (25 mM HEPES–KOH, pH 7.5; 10% (v/v) sucrose; 1 mM DTT; 0.2 mM PMSF) and stored in small portions in liquid nitrogen until use. From 40 g of calf thymus tissue approximately 0.8 mg of ctHsc40 was obtained.

2.4. Reactivation reaction

Reactivation reaction was performed essentially as described previously [25]. Calf thymus DNA polymerase α (0.2 units, 10.7 nM, 25 ng of protein per assay) and *E. coli* DNA polymerase III (pol III) (0.12 units, 10.7 nM, 30 ng of protein per assay) were diluted 15-fold and inactivated by incubation for 15 min at 45°C. Then, ctHsc70 or DnaK protein (at a molar ratio to DNA polymerase of 150:1 or 15:1, e.g. 1.6 mM or 0.16 mM), ctHsc40 or DnaJ protein (at a molar ratio to DNA polymerase of 1:1, e.g. 11 nM), ATP and an energy regenerating system (final concentration of 40 mM creatine phosphate and 0.1 mg/ml creatine kinase) were added. The reaction mixtures were incubated for various times at 37°C, that was followed by measuring of DNA polymerase activity as outlined previously [30]. Relative DNA polymerase activity was calculated in comparison to the untreated control (defined as 100%) in the presence of the same amounts of chaperone proteins.

2.5. ATPase assay

ATPase activity of the DnaK and ctHsc70 proteins was determined as described previously [5] using DnaK or ctHsc70 alone, or in the presence of DnaJ or ctHsc40 and GrpE, in different combinations (as indicated in the figure legends).

2.6. Replication assay

The λdv replication assays were done as described previously [16] with few modifications. A final volume of 25 μl reaction mixture contained: 40 mM HEPES–KOH, pH 8.0; 25 mM Tris–HCl, pH 7.4; 80 μg/ml BSA; 4% (w/v) sucrose; 4 mM DTT, 11 mM Mg-acetate, 2 mM ATP, 400 ng λdv plasmid DNA, 0.5 mM each of GTP, CTP and UTP; 10 mM phosphocreatine; 20 μg/ml creatine kinase; 0.1 mM each of dATP, dGTP and dCTP; [^{3}H]dTTP (50 cpm/pmol); 670 ng SSB, 470 ng DNA gyrase subunit A (GyrA); 600 ng DNA gyrase subunit B (GyrB); 125 ng DnaB helicase; 10 ng DnaG primase; 270 ng DNA polymerase III holoenzyme; 65 ng λP protein; 140 ng λO protein and chaperones in the amounts indicated in the figures. The reaction mixtures were incubated for 30 min at 30°C, precipitated with trichloroacetic acid and the radioactivity of insoluble material was determined in a scintillation counter.

3. Results and discussion

Previously, we had purified a DnaK chaperone homolog from calf thymus tissue, the ctHsc70 protein, and showed that it was able to protect and to reactivate heat-denatured prokaryotic and eukaryotic enzymes [25]. In order to widen our investigation of the function of this protein as chaperone, we decided also to test one of its co-chaperones, a homolog of the DnaJ protein.

3.1. Isolation of a DnaJ homolog from calf thymus tissue

First, we isolated a DnaJ homolog from calf thymus tissue using antibodies raised against DnaJ to detect its homolog during purification. The purification procedure included four columns being DEAE–Sephacel, hydroxyapatite, phenyl–Sepharose 4B Cl and FPLC MonoQ (Fig. 1). From the DEAE–Sephacel column the DnaJ homologous protein eluted

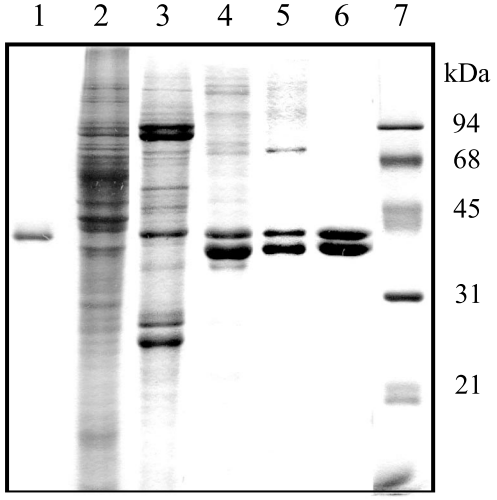

Fig. 1. Purification of ctHsc40 co-chaperone. 12% SDS–polyacrylamide gel, stained with Coomassie blue, shows the subsequent purification steps (For details see Section 2). Lane 1: DnaJ (1 μg); lane 2: crude extract from calf thymus tissue (20 μg); lane 3: elution from DEAE–Sephacel column (20 μg); lane 4: flow through from hydroxyapatite column (10 μg); lane 5: elution from phenyl–Sepharose 4B Cl column (5 μg); lane 6: elution from MonoQ FPLC column (5 μg); lane 7: molecular weight markers.

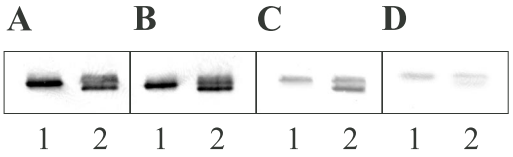

Fig. 2. Identification of ctHsc40 by using immuno-assay. Western blot analysis was performed as described in Section 2, by using antibodies raised against DnaJ from *E. coli* (A), against conserved K–Y–H–P–D–K peptide (B), against heat-inducible Hsp40 from HeLa cells (C) or against HSJ1 from human brain (D). Lanes 1: DnaJ (0.8 μg); lanes 2: ctHsc40 (1.3 μg).

as a broad peak at a KCl concentration between 150–300 mM (Fig. 1, lane 3). The eluted proteins were then passed through hydroxyapatite column that resulted in enrichment in proteins of molecular weight of approximately 42 and 40 kDa (Fig. 1, lane 4). The phenyl–Sepharose purification step was the most powerful one, since the majority of other proteins did not bind to this column (Fig. 1, lane 5). The final purification step on FPLC MonoQ removed most of the remaining contaminants resulting in a 42/40 kDa protein of more than 95% homogeneity (Fig. 1, lane 6). The smaller protein was likely a result of partial degradation of the 42 kDa protein that occurred in spite of the use of protease inhibitors (Fig. 1, compare lanes 3 and 4). Identification of these two proteins by microsequencing analysis failed due to a block at the N-termini of the proteins.

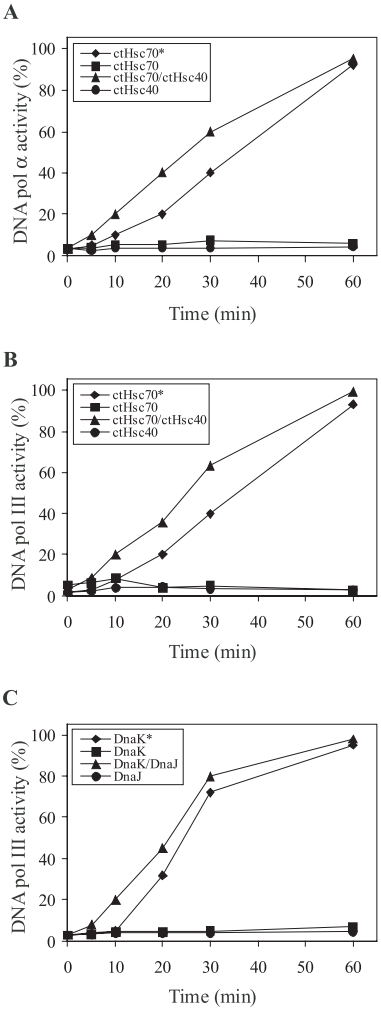

Fig. 3. Reactivation of calf thymus DNA polymerase α and *E. coli* DNA polymerase III by ctHsc70, DnaK and their co-chaperones. A: Reactivation of pol α by eukaryotic chaperones. B: Reactivation of pol III by eukaryotic chaperones. C: Reactivation of pol III by prokaryotic chaperones. ctHsc70 and DnaK were used either at 150:1 (marked with*) or 15:1 molar ratio to DNA polymerase, ctHsc40 and DnaJ were used at 1:1 molar ratio to DNA polymerase. For details see Section 2.

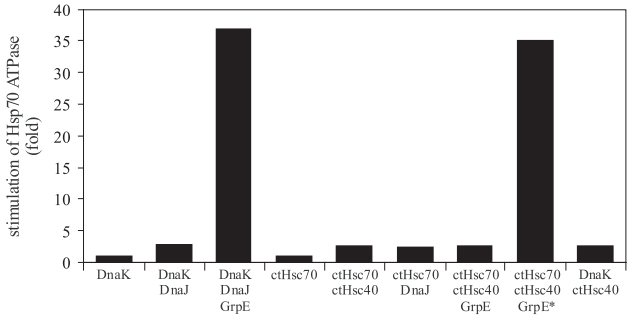

Fig. 4. Stimulation of ATPase activity of Hsp70s by co-chaperones. ATPase activity of DnaK (0.4 μg) or ctHsc70 (0.65 μg) chaperones was measured in the absence or in the presence of co-chaperones: DnaJ (0.78 μg), ctHsc40 (0.84 μg) and GrpE (0.2 μg) or GrpE* (5 μg) alone, or in combinations, as indicated on the figure.

For identification of the purified protein as a member of the Hsp40 chaperone family an immuno-assay was employed, since the heat shock proteins are highly conserved within each family. Immunoblot experiments with polyclonal antibodies raised against (i) *E. coli* DnaJ, (ii) a peptide containing K–Y–H–P–D–K motif from J-domain conserved among all known Hsp40 members, (iii) heat-inducible cytoplasmic Hsp40 from HeLa cells [31], and (iv) non-conserved region of HSJ1, a homolog of DnaJ protein isolated from human brain [32] were performed. The purified 42/40 kDa protein strongly cross-reacted with anti-DnaJ and anti-peptide antibodies (Fig. 2A,B) and only weakly cross-reacted with anti-human Hsp40 antibodies (Fig. 2C), while its cross-reaction with anti-HSJ1 antibodies was extremely weak (Fig. 2D). Based on these data and on the fact that this protein was isolated from the cytoplasm of the untreated calf thymus tissue, we named it ctHsc40.

3.2. *ctHsc40 protein modulates function of ctHsc70 protein in reactivation of heat-denatured enzymes*

We showed previously that ctHsc70 was able to reactivate heat-inactivated eukaryotic (calf thymus DNA polymerases α and ε) and prokaryotic (pol III and RNA polymerase) enzymes, while the *E. coli* DnaK chaperone was exclusively able to reactivate enzymes from *E. coli* but not from eukaryotic cells [25]. We employed heat-denatured calf thymus DNA polymerase α (pol α) and *E. coli* DNA polymerase III (pol III) to study the concert action of the Hsp70 chaperones and their respective Hsp40 co-chaperones. For efficient reactivation of DNA polymerases (pol α and pol III) a large amount of ctHsc70 was required (molar ration of 150:1) when used alone (Fig. 3A,B). A 10-fold reduction in the amount of ctHsc70 resulted in only marginal reactivation of pol α and pol III (Fig. 3A,B). Addition of ctHsc40 to this limiting amount of ctHsc70 not only allowed reactivation by the chaperone but also improved the reaction kinetics. The same effect of the *E. coli* DnaJ protein on reactivation of pol III (Fig. 3C)

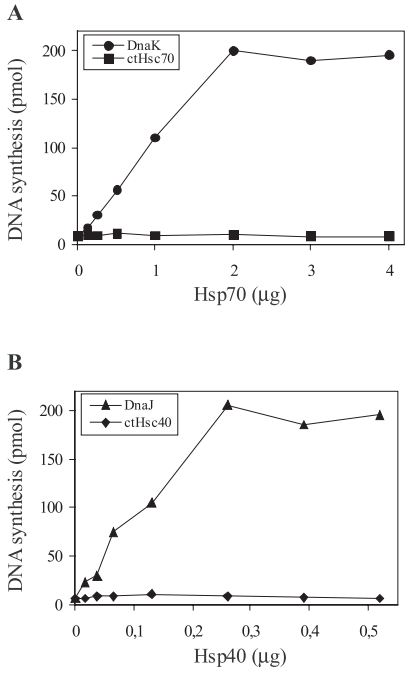

Fig. 5. Replication of bacteriophage λ DNA mediated by ctHsc70, DnaK and their co-chaperones. A: Titration of ctHsc70 or DnaK chaperone in the presence of DnaJ (0.25 μg) and GrpE (0.2 μg). B: Titration of ctHsc40 or DnaJ co-chaperone in the presence of DnaK (2.2 μg) and GrpE (0.2 μg). For details see Section 2.

and RNA polymerase [33] by DnaK was observed. These results prove that the purified ctHsc40 protein is functionally active in one of the major aspects of the stress-induced response, namely in reactivation of heat denatured enzymes crucial for the cell function.

3.3. ctHsc40 stimulates the ATPase activity of both ctHsc70 and DnaK chaperones

Prokaryotic co-chaperones, DnaJ and GrpE, are known to stimulate the ATPase activity of DnaK [5–8], see also Fig. 4. We tested the prokaryotic and eukaryotic co-chaperones for their ability to stimulate the ATPase activity of both prokaryotic and eukaryotic Hsp70 chaperones. The efficiency of stimulation of the ATPase activity of ctHsc70 by ctHsc40 was comparable to the effect exerted by DnaJ on DnaK ATPase (Fig. 4). Interestingly, ctHsc40 was able to stimulate also the prokaryotic Hsp70 ATPase, DnaK (Fig. 4). The second co-chaperone, GrpE, showed a stimulatory effect on DnaK ATPase (in the presence of DnaJ; Fig. 4), but not on eukaryotic Hsp70, the ctHsc70 ATPase in the presence of ctHsc40 (Fig. 4) or DnaJ (data not shown), when used in molar ratio of 1:1 to the main chaperone. 25-fold increase in the amount of GrpE in this reaction resulted in efficient stimulation of ctHsc70 ATPase activity (Fig. 4). It is not clear, however, whether this effect is due to GrpE function as a chaperone or as a substrate. In the cytosol of eukaryotic cells a typical GrpE homolog has not been identified up to date, suggesting that cytosolic Hsp70 chaperones may not require any GrpE homolog for their function. These results, however, prove that the Hsp40 co-chaperones in both prokaryotic and eukaryotic Hsp70 chaperone systems exert similar effects on their main chaperones.

3.4. ctHsc70 and ctHsc40 proteins cannot substitute for DnaK and DnaJ function in bacteriophage λ DNA replication

The ctHsc40 co-chaperone appeared to function not only in ctHsc70-mediated reactivation of heat-denatured enzymes but also in stimulation of the ctHsc70 ATPase activity (see above). Therefore, we next addressed the question whether the eukaryotic chaperones can replace their prokaryotic counterparts in a process that is specific for the prokaryotic cells. We employed the bacteriophage λ DNA replication assay, since in this reaction chaperones interact with a substrate, which structure differs from the structure of denatured enzymes. First, the ctHsc70 was tested in the λdv replication system depleted of DnaK. It is evident that ctHsc70 was unable to perform chaperone function in this system, even when used in amounts higher than 2 μg (the optimum for DnaK; Fig. 5A). Next, the function of ctHsc40 was investigated in the same assay. No DNA synthesis could be detected when ctHsc40 was used instead of DnaJ (Fig. 5B), even though higher amounts of ctHsc40 (in comparison to DnaJ required) were used. Finally, addition of both the ctHsc70 and ctHsc40 chaperones was not sufficient for bacteriophage DNA replication (data not shown). These results indicate that eukaryotic chaperones are not able to substitute the function of DnaK and DnaJ in bacteriophage λ DNA replication. One explanation of these results could be that the eukaryotic chaperones ctHsc70 and ctHsc40 do not interact with GrpE in this particular reaction. However, this cannot be the reason of ctHsc70/40 failure in promoting λ DNA replication. It is known that GrpE is not absolutely required for λdv replication in vitro, since DnaK and DnaJ can, to some extend, perform their chaperone function in this system even in the absence of GrpE [16].

In sum, our results indicate that the mechanisms by which Hsp70 proteins function in bacteriophage λ DNA replication and in reactivation of heat-inactivated enzymes are different. This could be due to usage of different types of substrates in these two assays, and would indicate different reaction specificities of prokaryotic and eukaryotic chaperones pronounced as difference in the substrate recognition observed in the two assay systems. In case of reactivation reaction, chaperones interact with heat-denatured enzymes exposing hydrophobic groups [34], whereas in the replication system chaperones dissociate one of the preprimosome complex proteins at the λ origin of replication. In this case chaperones interact with native folded proteins, which probably expose some structural elements typical for an unfolded polypeptide, or alternatively, the preprimosome complex is recognized by the chaperones as an aggregate that must be disassembled to allow activation of DNA helicase. In fact, the nature of substrate recognition by DnaK/Hsp70 chaperones in DNA replication still remains unknown [34]. These results can also indicate on higher complexity of chaperone function in DNA replication in comparison to reactivation of heat-denatured proteins and/or suggest species specificity in DNA replication.

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