DNA polymerase of a basidiomycete fungus, *Coprinus cinereus*

Dorrit LOWE* and Dan LEWIS†

Department of Botany and Microbiology, University College London, Gower Street, London WC1E 6BT, U.K.

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DNA polymerase activity was studied in *Coprinus cinereus*, a basidiomycete fungus. Only one form of the enzyme could be demonstrated, whether by affinity or ion-exchange chromatography; this enzyme had a molecular weight of 185 000 on Sephadex G-200, and was inhibited by mercaptoethanol. *Coprinus*, a representative of the most advanced type of the filamentous fungi, resembles other eukaryotic micro-organisms in its lack of a mammalian β -type DNA polymerase. The properties of the polymerase are compared with those of two other fungi, and found to resemble most closely the yeast polymerase A in Mg^{2+} requirements and template preference.

Most work on the characterization of DNA-directed DNA polymerases has been carried out on either bacteria or mammals.

Escherichia coli has three enzymes; DNA polymerase I, although comprising 90% of the total activity, is believed to be responsible for DNA repair processes, and the minor DNA polymerase III is probably the replication enzyme; all three are of high molecular weight, 100 000 or greater, but polymerase I differs from forms II and III in that it is not inhibited by thiol-blocking reagents (Richardson *et al.*, 1964; Englund *et al.*, 1968; De Lucia & Cairns, 1969; Goulian, 1972; Holmes & Johnston, 1975). Other bacteria have DNA polymerases more or less analogous to those of *E. coli* (*Bacillus subtilis*: Okazaki & Kornberg, 1964; Gass & Cozzarelli, 1973; *Acinetobacter calcoaceticus*: Nes & Kleppe, 1974; Nes *et al.*, 1974; *Micrococcus luteus*: Hamilton *et al.*, 1974).

Mammals, on the other hand, have characteristically two main classes of DNA polymerase. Polymerase- α is of high molecular weight and is inhibited by thiol-blocking reagents, whereas polymerase- β has a molecular weight of 50 000 or less and is not affected by thiol-blocking reagents (nomenclature according to Weissbach *et al.*, 1975). This has been found for numerous tissues, for example rat liver (Haines *et al.*, 1971), rat ascites hepatoma (Tsuruo & Ukita, 1974), calf thymus

(Chang & Bollum, 1973), mouse tissues (Hecht, 1975) and various cell cultures (Sedwick *et al.*, 1972; Adams *et al.*, 1973; Matsukage *et al.*, 1974; Craig & Keir, 1975; Roufa *et al.*, 1975). Polymerase- β is tightly bound to the nucleus, whereas polymerase- α is usually found in the cytoplasmic fraction; however, there is evidence (Herrick *et al.*, 1976) that the latter is also associated with the nucleus *in vivo*. The two enzymes are now accepted as distinct forms (Spadari *et al.*, 1974; Holmes *et al.*, 1974; Hecht, 1975), although there have also been reports of their interconvertibility (Chang & Bollum, 1972; Hecht, 1973; Morioka & Terayama, 1974). There is also a third, cytoplasmic, enzyme, DNA polymerase- γ , comprising about 1% of the total, as well as a mitochondrial polymerase-mt (Holmes & Johnston, 1975).

The situation in other groups of organisms has been less well studied, but surveys (Holmes & Johnston, 1975; Chang, 1976) suggest that most multicellular eukaryotes, with the possible exception of insects, have DNA polymerase analogous to the two mammalian forms α and β . According to Chang (1976), plants have only polymerase- α , but it has since been shown that, for example, sugar beet (Tymonko & Dunham, 1977), pea seedlings (Stevens & Bryant, 1978; Stevens *et al.*, 1978) and soy-bean seedlings (D'Alessandro *et al.*, 1980) have both α - and β -type activities. Single-celled eukaryotes, on the other hand, all seem to lack the β -class of enzyme, for example Protozoa (*Tetrahymena*: Crerar & Pearlman, 1971; *Euglena*: McLennan & Keir, 1975a,b; *Paramecium*: Tait & Cummings, 1975; *Chlamydomonas*: Ross & Harris, 1976) and yeast (E. Wintersberger, 1974; Chang,

Abbreviation used: DNAase, deoxyribonuclease.

* Present address: Department of Biochemistry, King's College London, Strand, London WC2R 2LS, U.K.

† Present address: Department of Plant Biology and Microbiology, Queen Mary College, Mile End Road, London E1 4NS, U.K.

1977). Where there is a distinct nuclear enzyme, it is also of the α -type (U. Wintersberger, 1974; McLennan & Keir, 1975c).

Two groups of organisms which fall between the unicellular/multicellular classification are the slime moulds and the filamentous fungi. Both the true slime mould (*Physarum*: Schiebel & Bamberg, 1973) and the cellular slime mould (*Dictyostelium*: Loomis *et al.*, 1976) have only one DNA polymerase, of the α -type. The most advanced class of filamentous fungi is the Basidiomycetes, and very little seems to be known of their DNA polymerases, with the exception of the parasitic smut fungus *Ustilago maydis*. This organism again has only α -type polymerase (Banks *et al.*, 1976); a similar minor polymerase was also found by this group (G. R. Banks, unpublished work, cited by McLennan & Keir, 1977).

The Basidiomycete *Coprinus cinereus*, one of the ink-caps, is well known genetically, and from this species two well-characterized strains were selected for the study of DNA polymerase activity and properties: a wild type, in the hope that the findings would be typical for the species and possibly the family, and a methionine-requiring mutant which had been used previously in our laboratory to demonstrate mutagenicity of the amino acid analogue *p*-fluorophenylalanine (Talmud & Lewis, 1974a,b; Talmud, 1977). In the latter case it was hoped that enough DNA polymerase activity would be obtained to demonstrate misincorporation of bases by the polymerase from mutagen-treated cultures.

Experimental

Materials

Deoxynucleotides were obtained from Boehringer Corp. (London) Ltd., Lewes, E. Sussex, U.K., and [^3H]dTTP ([methyl- ^3H]thymidine 5'-triphosphate) from The Radiochemical Centre, Amersham, Bucks., U.K. Poly(dA)·d(pT)₁₂ was from PL Biochemicals (through International Enzymes Ltd., Windsor, Berks., U.K.) and DNA (calf thymus, type I), bovine pancreatic DNAase (deoxyribonuclease) (Type I), DNA polymerase (*Micrococcus lysodeikticus*) and the molecular-weight markers for Sephadex chromatography were all from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Strains

Two strains of *C. cinereus* were used: H5, a wild-type collected from Hertfordshire, U.K., and PR2301, a methionine-requiring mutant derived by u.v. treatment of strain H5 (Lewis, 1961); both had been maintained in culture for several years.

Growth media

The liquid medium (based on that of Fries, 1953) contained (per litre) asparagine (2g), ammonium tartrate (0.5g), KH_2PO_4 (1.0g), Na_2HPO_4 (2.25g), Na_2SO_4 (anhydrous) (145mg), thiamin (0.04mg), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5g) and glucose (10g). The solid medium also contained 15g of agar/litre. For strain PR2301, media were supplemented with methionine.

Growth of liquid cultures

The fungus was grown on agar plates for 4–5 days at 37°C. A dense mycelial mat formed on the surface; this was scraped into a small volume of liquid medium and macerated in a Waring blender at top speed for 30s. The homogenate was inoculated into conical flasks of liquid medium, at four or five plates per flask, and incubated at 37°C with shaking, for 23–28h. This procedure gave a yield of 0.13–0.23g dry wt. per flask for strain H5, and 0.03–0.10g dry wt. per flask for strain PR2301.

Preparation of postmicrosomal supernatant

Liquid cultures were harvested, blotted and homogenized in ice-cold homogenizing medium (0.05M-Tris/HCl, pH 7.5, 1mM-EDTA and 0.05M-NaCl; sometimes 0.5mM-dithioerythritol was also present) at 9ml/g wet wt., in a VirTis blender for 2min at top speed. The homogenate was centrifuged for 1h at 40000 rev./min. (190000g_{max.}) in the 8 × 50μl rotor of the MSE 65 centrifuge, to give a clear supernatant, referred to here as 'post-microsomal', by analogy with standard cell-fractionation techniques. This supernatant could be stored frozen for months with little or no loss of enzyme activity.

All operations from homogenization onwards were carried out at 0–5°C except where specified.

Activated DNA

DNAase (0.17ml; 400ng/ml) was added to 1.5ml of DNA (2mg/ml), both solutions containing 10mM-Tris, 5mM-MgSO₄, 10mM-KCl and 1mg of bovine serum albumin/ml. The mixture was incubated at 37°C for 8min, then at 77°C for 10min.

Denatured DNA

DNA in water was heated for 15min at 95°C.

DNA polymerase assay

The method was based on those of Johnston's group (Haines *et al.*, 1970; Holmes & Johnston, 1973) and Shepherd & Keir (1966). The enzyme (0.1ml) was added to 0.15ml of an incubation mixture to give final concentrations as follows: Tris/HCl (pH 7.5), 50mM; MgSO₄, 5mM; KCl, 1mM; EDTA, 0.4mM; albumin, 1mg/ml; dATP, dGTP, dCTP, dTTP, 0.1mM each; activated DNA, 200μg/ml; [^3H]dTTP, 4μCi/ml. Incubation was for

30 min at 37°C. The reaction was stopped by chilling the tubes in ice and adding 0.5 ml of cold 0.1 M-Na₄P₂O₇, then 1 ml of 17% (w/v) trichloroacetic acid. After 10 min, the precipitate was spun down in a bench centrifuge, washed twice with 5% trichloroacetic acid, and dissolved in 0.15 ml of 98% (v/v) formic acid.

In some experiments, poly(dA)·d(pT)₁₂ was used instead of DNA, at a final concentration of 18 μM. It was dissolved initially in 10 mM-Tris/HCl/0.1 M-NaCl, and the concentration checked by measuring the A₂₆₀.

One unit of enzyme activity was defined as 1 nmol of [³H]dTTP incorporated in 30 min.

Radioactivity measurement

The solution in formic acid was plated in 0.05 ml portions on to Whatman GF/C glass-fibre discs, dried, and counted for radioactivity in 5 ml of toluene scintillant containing 6 g of butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,5-diazole]/litre. The efficiency of counting was 20%, estimated by internal standard.

Protein assay

Protein was assayed by the method of Lowry *et al.* (1951). Appropriate standards were run where interfering substances such as Tris, NaCl or dithioerythritol were present.

DNAase assay

The method used was that of Polman *et al.* (1974). Assay mixtures (15 ml) contained 10 mg of DNA, 0.2 mM-Tris/HCl, pH 7.6, 50 mM-MgCl₂ and enzyme; after incubation at 30°C, 1.5 ml samples were withdrawn at various times and added to 0.1 ml of ice-cold albumin (5 mg), followed by 1 ml of 2.5 M-HClO₄. The precipitate was centrifuged down and the A₂₆₀ of the supernatant measured. Bovine pancreatic DNAase I (22.4 Kunitz units) was used as a standard.

DNA assay

DNA was assayed by the diphenylamine method of Burton (1956).

DNA-cellulose affinity chromatography

DNA-cellulose was made by the method of Alberts & Herrick (1971) and was a generous gift from Dr. R. Holliday's laboratory (NIMR, Mill Hill).

The process of removing DNA from the enzyme sample by DNAase treatment followed by dialysis to remove Mg²⁺ (Alberts & Herrick, 1971) was found to inactivate the DNA polymerase completely (see the Results section) and was therefore omitted.

After removal of fines, the DNA-cellulose was poured to give a column of bed dimensions 1.4 cm × 11 cm, and washed with homogenizing medium. The

sample (15–20 ml) was loaded, non-binding proteins were washed off with 25 ml of the same medium, then DNA polymerase was eluted with a 200 ml linear gradient of 0.05–0.5 M-NaCl in the same buffer. On one occasion a gradient of 0.05–2.0 M-NaCl was used, but no enzyme was eluted by the higher NaCl concentrations. The A₂₈₀ and DNA polymerase activity of the 5 ml fractions were measured. Dithioerythritol at 0.5 mM was present in some experiments.

DEAE-cellulose ion-exchange chromatography

DEAE-cellulose (DE-52; Whatman) was equilibrated with homogenization medium, and, after removal of fines, poured to give a column of 1.4 cm × 10 cm bed dimensions. After washing with the same medium, the sample (15–20 ml) was loaded, followed by 25 ml of medium, and then a 200 ml linear gradient of 0.05–0.5 M-NaCl in the same medium. All solutions contained 0.5 mM-dithioerythritol.

Sephadex gel filtration

Sephadex G-100 and G-200 were swollen and run in homogenizing medium containing 0.5 M-dithioerythritol. Bed volumes were 1 cm × 45 cm and 1.3 cm × 65 cm respectively. Sample volumes were 1.2–1.7 ml. The Sephadex G-200 column was calibrated with: cytochrome c (horse heart, type III), mol.wt. 12400; bovine serum albumin (Cohn fraction V), 67000; glucose oxidase (*Aspergillus niger*, type V), 153000; catalase (bovine liver), 247500; Blue Dextran, 2000000.

Results

DNA polymerase activity

For both *C. cinereus* strains, enzyme yield (units/g dry wt. of mycelium) and specific activity (units/mg of protein) were greatest when the liquid culture had been grown for the least time. Presumably this is because the enzyme is only active where nuclear division is taking place, at the hyphal tips.

DNA measurements on the homogenate and the 'postmicrosomal supernatant' showed that nuclear breakage must have occurred during homogenization, because 26–39% of the total DNA was found in the supernatant (values from three experiments with the H5 strain). The enzyme activity in the supernatant was 60–94% of the total; however, this extra activity may still have had a nuclear origin, because leakage can occur from intact nuclei (Herrick *et al.*, 1976).

In common with that of most organisms, *C. cinereus* DNA polymerase prefers (as a template) activated DNA to both native and denatured DNA (2–4 times and 6–8 times respectively). Increasing

the DNA concentration did not result in increased activity. The minimum deoxyribonucleotide concentration for full activity was 0.1 mM; a similar result was found by Haines *et al.* (1972) for rat liver. The Mg^{2+} and K^+ concentrations used (5 mM and 1 mM respectively) were based on published mammalian methods; for *U. maydis*, however, the optima were found to be 12 mM and 120 mM respectively (Banks *et al.*, 1976). When *C. cinereus* polymerase was assayed at either of these concentrations (keeping that of the other salt as before), activity was decreased by 36% and 80% for Mg^{2+} and K^+ respectively; when both concentrations were changed, a synergistic inhibition of 96% was achieved. The original concentrations were therefore retained, although later experiments showed the Mg^{2+} optimum of more purified fractions to be 3 mM.

Thiol-protecting reagents (mercaptoethanol and dithioerythritol) were found to have no protective or stimulating effect. However, a thiol-blocking agent, *p*-hydroxymercuribenzoate, inhibited polymerase activity by 50% at 0.03 mM, with 98% inhibition at 0.10 mM. The latter inhibition was partly (20%) reversed by 0.5–5.0 mM-dithioerythritol, but this

Fig. 1. Effect of enzyme concentration on enzyme activity of various DNA polymerase preparations

The enzyme was assayed as described in the Experimental section, with DNA as the template. All points are the average of two or three determinations and the curves are typical of experiments repeated up to five times. Crude postmicrosomal supernatants: ○, strain H5; ●, strain PR2301. DNA-cellulose peak fraction: □, strain PR2301. Pure bacterial polymerase: ■, *M. lysodeikticus* enzyme.

reversal appeared not to depend on concentration of the latter.

Glycerol, which is widely used to stabilize polymerases, showed no protective action here, either during storage or in assays. In the latter case, the bovine serum albumin present in the incubation mixture probably gives enough protection.

The crude postmicrosomal supernatants of both strains showed a plateau or even sometimes a decline in activity with time, when incubated beyond the standard time of 30 min. Their activities were also not linear with respect to sample concentration, especially at high concentrations (Fig. 1). This non-linearity was not due to a rate-limiting reagent being used up, because bacterial DNA polymerase showed linearity over a wide range of concentration in the same assay system, achieving an incorporation of 1.40 nmol of dTTP per assay at the highest concentration used, compared with a maximum of 0.14–0.25 nmol of dTTP per assay for the fungal polymerase. Non-linearity with time of incubation was not due to gradual inactivation of the polymerase at 37°C, because preincubation of the enzyme at this temperature for 30 min and 1 h resulted in a decrease in activity of only 7% and 20–30% respectively; this is not enough to account for the plateaux observed. When the postmicrosomal supernatant was assayed for DNAase, some activity was observed. Indirect evidence is presented below that endogenous DNAase activity may be the cause of the plateaux.

The specific activity obtained from *Coprinus* postmicrosomal supernatants was comparable with that from other organisms cited in the literature, in the range 0.8–1.3 units/mg of protein for strain H5 and 0.25–0.47 unit/mg of protein for strain PR2301. However, the yield, expressed as units/mg dry wt. of mycelium, was relatively low, probably because fungi are slow growing with a low rate of cell division.

Before running the postmicrosomal supernatants on DNA-cellulose columns, attempts were made to remove endogenous DNA (see the Experimental section). Two methods were used. The first involved DNAase treatment followed by dialysis to remove Mg^{2+} , and resulted in complete inactivation of the polymerase. Analysis of the steps involved showed that inactivation was due to the DNAase treatment; probably the DNAase was re-activated by the Mg^{2+} used in the polymerase assay. The second method was to treat with poly(ethylene glycol), then dialyse against homogenizing medium. Half the activity was lost, and the poly(ethylene glycol) could not easily be removed. Therefore DNA-cellulose columns were used without pre-treatment of the sample.

DNA polymerase purification

On DNA-cellulose, both strains gave one peak, at

0.12–0.20 M-NaCl, as well as some breakthrough material (Fig. 2a). The presence of the latter may be due to endogenous DNA. The two strains differed from each other in two ways. Some 85–95% of the enzyme activity of the mutant PR2301 was in the main peak, whereas about one-third of the activity of strain H5 was in the breakthrough fraction. The enzyme from strain PR2301 consistently showed activation, in a range of 2.8–4.7-fold compared with the starting material (three experiments), but this was never observed for strain H5 (three experiments). DNAase assay showed that strain PR2301 had five times as much DNAase as strain H5. The

effects of enzyme concentration and time of incubation of the crude extract (mentioned above) were also more pronounced for strain PR2301 than for strain H5. After column chromatography, the enzyme activity was linear with respect to enzyme concentration (Fig. 1) and time, showing that the inhibition had been removed. These differential effects are probably related to the difference in DNAase content of the two strains, removal of DNAase during purification giving rise both to activation and to linearity with time and concentration.

Since high salt concentration can inhibit some

Fig. 2. Chromatography of DNA polymerase from strain PR2301

Postmicrosomal supernatant was loaded on to a DNA-cellulose (a) or a DEAE-cellulose (b) column, and eluted with a 0.05–0.5 M-NaCl gradient as described in the Experimental section. The arrows show the start and finish of the gradient. O, A_{280} ; ●, enzyme activity.

DNA polymerases (Holmes *et al.*, 1974), the column fractions were dialysed against homogenizing medium to remove the elution salt, in the hope that removal of inhibition might reveal a further peak of activity. However, all the activity was lost on dialysis. Postmicrosomal supernatants could be dialysed with no loss of activity, so the proteins removed on chromatography must have had a protective effect.

Chromatography of the post-microsomal supernatant of strain PR2301 on DEAE-Sephadex (Fig. 2*b*) gave the same enzyme pattern as on DNA-cellulose, again with activation (2.0–2.6-fold in three experiments). Further purification of this peak on DNA-cellulose necessitated removal of excess NaCl. Since conventional overnight dialysis had been shown to inhibit the enzyme, a different method was used this time. The sample was placed in a Bio-fiber 50 Minibeaker (Bio-Rad, Watford, Herts., U.K.) and homogenizing medium was passed through. Equilibration, as checked by conductivity measurements, was complete in 40 min and the enzyme retained all its activity. However, active DEAE-cellulose fractions treated in this way lost their activity after passage through DNA-cellulose; this second purification must have rendered the enzyme even more susceptible to damage.

Molecular-weight determination

Postmicrosomal supernatant was concentrated 25-fold by high-pressure ultrafiltration, by using an Amicon ultrafiltration cell with a PM-10 membrane (molecular-weight cut-off 10000). When analysed on a Sephadex G-100 gel-filtration column, the activity all came out with the void volume, indicating a molecular weight of 150 000 or more. On Sephadex G-200, the molecular weight appeared to be 185 000. No activity was seen in the 45 000-mol.wt. region, indicating a lack of mammalian DNA polymerase- β -type activity. To check against the possibility of aggregation caused by the ultrafiltration process, a sample that had not been concentrated was chromatographed on a Sephadex G-200 column, but the result was the same. Aggregates of the mammalian low-molecular-weight enzyme occur at ionic strengths below 0.07 mol/l (Holmes & Johnston, 1975). The ionic strength used here was 0.1 mol/l, which is only just above this critical value. Assaying in the presence of 0.5 M NaCl or 0.5 M KCl resulted in inhibition by 90% and 84% respectively, and made the enzyme much less stable on storage. Subsequent addition of $MgCl_2$ to 0.25 M did not reverse the inhibition, suggesting that the enzyme was irreversibly inactivated. Thus the enzyme could not be analysed on Sephadex G-200 at high ionic strength.

Activity with synthetic template

Enzyme from the mutant PR2301 was tested with poly(dA)·d(pT)₁₂ instead of DNA, in the hope of setting up an assay for miscoding. The peak fraction from DNA-cellulose purification was used as the enzyme source. The activity was variable, being at best the same as that with DNA as template, compared on the basis of nmol of [³H]dTTP incorporated/ μ mol of template nucleotide. Alteration of the Mg^{2+} concentration suggested an optimum of 2 mM rather than the 5 mM used with DNA. The activity was linear with time, implying the absence of nuclease activity. Bacterial polymerase in the same system was $2\frac{1}{2}$ – $5\frac{1}{2}$ times as active as the fungal enzyme, although the two polymerases performed comparably with each other when DNA was used as a template in the same experiment.

The activity obtained would not have been sufficient to detect misincorporation at the low extents expected.

Discussion

C. cinereus is shown here to possess only one DNA polymerase, of high molecular weight, and sensitive to thiol-blocking reagents, like the α -polymerase of mammals. This organism thus resembles other fungi in its lack of a β -type enzyme. *Ustilago* (Banks *et al.*, 1976) also has only one major polymerase, but yeast has two (E. Wintersberger, 1974; Chang, 1977).

The *Coprinus* enzyme has a Mg^{2+} optimum of 3–5 mM, and is inhibited by high KCl concentration. In this respect it differs from the *Ustilago* enzyme, but the Mg^{2+} optimum resembles that of the yeast enzymes. The activity with poly(dA)·d(pT)₁₂ as a template is no greater than that with activated DNA; in this respect the *Coprinus* enzyme resembles the polymerase A or I of yeast, whereas the other yeast enzyme, and the *Ustilago* enzyme, have a pronounced preference for the synthetic template (E. Wintersberger, 1974; Chang, 1977; Banks *et al.*, 1976).

The activity of the crude postmicrosomal supernatant showed non-linearity with enzyme concentration and with time, sometimes resulting in a decrease in incorporation in the latter case. This was not due to exhaustion of substrate, nor to heat-inactivation of the enzyme. After purification, the enzyme activity was linear with respect to both concentration and time. These results have also been observed by Robinson & Bryant (1975) in peas, and by Sherman & Kang (1973) in mouse embryo; the latter authors ascribed this effect to nucleases. Strain PR2301, which shows the non-linearity effect most markedly, also has five times as much DNAase activity as strain H5. A conclusion consistent with

the data is that a contaminating DNAase is removed by DNA-cellulose chromatography.

Most prokaryotic DNA polymerases have associated DNAase activity, whereas most eukaryotic polymerases do not. Among eukaryotic micro-organisms, however, *Ustilago maydis* has such activity, and yeast and *Tetrahymena* may do (for references and *Ustilago* work, see Banks & Yarranton, 1976). It would be tempting to conclude from the linearity of activity of the purified *Coprinus* DNA polymerase that this enzyme has no associated DNAase activity, but Banks & Yarranton (1976) showed that the nuclease activity of *Ustilago* polymerase could not take place in the presence of DNA synthesis, because it is inhibited by deoxynucleotides, and so would not be detectable during DNA polymerase assays.

Thus the fungi so far studied all resemble each other in their lack of a mammalian β -type DNA polymerase. Within the fungi, *Coprinus cinereus* resembles *Ustilago* in having only one DNA polymerase, but the properties of this enzyme resembles those of the yeast A enzyme more than the *Ustilago* enzyme. This is unexpected, because *Ustilago* and *Coprinus* both belong to the same fungal group, the Basidiomycetes, whereas yeast is an Ascomycete.

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