Large Complexes of β-Poly(L-malate) with DNA Polymerase α, Histones, and Other Proteins in Nuclei of Growing Plasmodia of *Physarum polycephalum*†

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Received May 3, 1995; Revised Manuscript Received August 3, 1995⊗

ABSTRACT: Of the various cell types in the life cycle of *Physarum polycephalum,* only the growing plasmodium contains the unusual polyester β-poly(L-malate). The nuclei exhibit large complexes of this polymer with nuclear proteins, among them DNA polymerase α, histones, and HMG-like proteins. The complexes are indicated by the results of size exclusion chromatography and chemical cross-linking with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC). After hydroxylaminolysis of the cross-linked polyester, the proteins are liberated and visualized on Western blots. The complexes of 1200−1400 kDa molecular mass exceed by far the size of free β-poly(L-malate) and proteins. The observed variation in mass appears to be mainly a function of the kind and stoichiometry of the protein constituents and may explain the relatively high molecular mass in S phase and the low molecular mass during G2 phase of the mitotic cycle. The complexes are considerably stable at moderate ionic strength (100 mM KCl). Also, endogenous β-poly(L-malate) does not exchange with added β-[^{14}C]poly(L-malate) during the lysis of the nuclei and the sample preparation. The complexes are dissociated at elevated concentrations of KCl, in the presence of spermine hydrochloride, or by treatment with DEAE/cellulose. Available evidence indicates that β-poly(L-malate) may be involved in the maintenance of the plasmodial state of *P. polycephalum.*

The plasmodium is a fascinating type of cell that manages to carry out a synchronous mitotic division of a large number of nuclei not separated by cellular membranes. Obviously, plasmodia must contain a physiological device allowing them to carry out everywhere in the large cell the same type of reactions at exactly the same time. *Physarum polycephalum* contains an unusual polyester, β-poly(L-malate),[1] which is specifically synthesized only in the plasmodium but not in any of the other (mononucleate) stages of its life cycle (Fischer et al., 1989). It may be possible that PMA is one such device.

The true slime mold *P. polycephalum* has been regarded as an interesting model organism due to its clear-cut life cycle consisting of mating-competent haploid mononuclear amoebae, vegetative diploid polynucleate plasmodia, spores, and specialized cellular forms that allow for survival under unfavorable environmental conditions (Rusch, 1980). The plasmodial cell can be very large, containing billions of nuclei. The nuclei divide with a high degree of synchrony, and the S phase directly follows mitosis in the absence of a G1 phase.

PMA is highly soluble in water and is concentrated in the nuclei at amounts comparable to that of DNA (Holler et al., 1992a). It also occurs at much lower concentrations in the cytoplasm and is secreted into the culture medium. The physicochemical properties of the polymer, such as the high negative charge and a certain degree of isosterism with the DNA deoxyribose phosphate backbone, strongly suggest that the biological function of PMA is to interact with nuclear proteins by mimicking DNA and that this is of physiological significance for the plasmodium (Holler et al., 1992a). Such interactions have been demonstrated by *in vitro* studies showing that PMA forms reversible complexes with DNA polymerase α/primase, histones, and also biogenic amines (Holler et al., 1992b). The DNA polymerase is inhibited by the complex formation. The inhibition is reversed by histones and biogenic amines, suggesting that this may serve as a mechanism to couple the generation of DNA polymerase activity to the synthesis of histones/biogenic amines under *in vivo* conditions (Fischer et al., 1989). PMA may function also as a mobile matrix, storage device, molecular buffer, and/or chaperone by binding these and other basic nuclear proteins (Holler et al., 1992a).

The slime mold as one of the earliest eukaryotes (Johansen et al., 1988; Cavalier-Smith, 1993) represents an interesting model organism for studying the evolution of the DNA replication apparatus. In this regard, it is possible that PMA is a primitive nonproteinaceous device for maintaining a particular organization in the plasmodium, that lateron in evolution is carried out by acidic nuclear proteins.

The present investigation provides evidence for hitherto merely assumed nuclear complexes between PMA and proteins. We have succeeded in showing by size exclusion chromatography and by chemical cross-linking that the polymer forms large complexes with DNA polymerase α, histones, and other proteins in the nuclei of the plasmodia.

MATERIALS AND METHODS

Materials

Microplasmodia of *P. polycephalum* strains M_3CVII and M_3CVIII were cultivated according to Daniel and Baldwin

† Supported by the Deutsche Forschungsgemeinschaft Grant Ho 416/21-1.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMA, β-poly(L-malate); Pol α, DNA polymerase α.

0006-2960/95/0434-14741$09.00/0

(1964) and amoebae, strain LU352, according to Dee et al. (1989). Macroplasmodia were cultured and the cell cycle was followed as described by Nygaard et al. (1969). For the isolation of nuclei, an amount of cells corresponding to 10 mL (2 mL) of settled plasmodia was seeded into 100 mL of culture medium in 500 mL dentated Erlenmeyer flasks and grown under shaking for 1 day (2 days). Plasmodia were collected on a cotton sieve, washed with 2−3 volumes of distilled water, and dried briefly by removal of excess water with blotting paper. DNA polymerase α was obtained according to a modified purification method of Achhammer et al. (1992), involving batch chromatography on DEAE/cellulose and gradient chromatography on phosphocellulose and Mono Q. PMA (peak average molecular mass of 30 kDa) was purified as described (Fischer et al., 1989). Metabolically labeled [^{14}C]PMA was purified from culture medium of 3-day-old microplasmodia grown in [^{14}C]D-glucose (12 μCi/mmol) and had a specific activity of 8.6 μCi/mmol and a peak average molecular mass of 30 kDa. Synthetic [^{14}C]PMA was a gift of Dr. M. Vert, Montpellier (France), and had a specific activity of 0.94 μCi/mmol (peak average molecular mass of 62 kDa).

Polyclonal antibodies against highly purified DNA polymerase α/primase (Achhammer et al., 1992) and purified mixtures of *P. polycephalum* histones (Holler et al., 1992b) were raised in rabbits following the method of Vaitukaitis (1981). Antisera R 279 and H 284 against histones and AS1 and AS2 of *P. polycephalum* were gifts of Dr. Loidl (Innsbruck, Austria).

Spermine hydrochloride and molecular mass markers were purchased from Sigma, USA. Dextran (4000−6000 kDa) was from Serva (Germany). Phosphocellulose and DEAE/cellulose were obtained from Whatman (England). Superose 6 16/50, Superose 6 HR 10/30, and Superose 12 HR 10/30 were from Pharmacia (Sweden). All other reagents have been obtained as described previously (Holler et al., 1987, 1992a; Weber et al., 1988). All chromatographies were carried out at 4 °C with a Pharmacia FPLC system equipped with a 2210 two-channel UV recorder.

Buffers

Homogenization buffer consisted of 15 mM Tris/HCl (pH 7.5), 15 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM EGTA, 500 mM hexylene glycol, 10% dextran, and 14 mM 2-mercaptoethanol. Nuclei extraction buffer consisted of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM KCl, 0.5% Triton X100, 50 mM EGTA, 20% glycerol, 14 mM 2-mercaptoethanol, and a protease inhibitor cocktail [5 mM sodium bisulfite, 2 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine (Sigma), 1 μM pepstatin A (Merck, Germany), 10 μM leupeptin (Sigma), 1 mg/mL aprotinin (Merck), 10 μM tosyl-L-lysine chloromethyl ketone (Calbiochem, USA), 100 μM Pefabloc SC (Merck), and 2 μg/mL E 64 (Boehringer-Mannheim, Germany)]. Combined cell lysis and extraction buffer was the same as the nuclei extraction buffer except 300 mM NaCl instead of 200 mM KCl. Standard buffer consisted of 50 mM Tris/HCl (pH 7.5), 0.3 mM dithioerythritol, 20% glycerol, 5 mM sodium bisulfite, 1 mM EDTA, and 0.2 mM phenylmethanesulfonyl fluoride.

Methods

Preparation of Leakproof Nuclei. Plasmodia suspended in homogenization buffer (2 mL/g of cells) were lysed by 8−10 strokes in a Dounce homogenizer at 4 °C and pelleted for 5 min at 500*g*. The supernatant was cleared by centrifugation at 2500*g* (cytosolic extract). The first pellet was resuspended in homogenization buffer (4 mL/g of cells) containing 25% Percoll (v/v). Microscopically clean nuclei were obtained after centrifugation for 10 min at 2500*g*. They were washed four times each with 4 mL of homogenization buffer and pelleted at 700*g*. Occasionally observed mitochondria were lysed by inclusion of 0.02% Triton X100 in the wash buffer. For extraction, the nuclear pellets (3×10^8 nuclei) were resuspended in 1 mL of extraction buffer and incubated for 15 min on ice with occasional agitation. After centrifugation, the extraction was repeated and the supernatants were combined (nuclear extract).

Chemical Cross-Linking and Hydroxylamination. Aliquots of $3-4 \times 10^8$ nuclei were incubated for 90 min at room temperature with 10 mg/mL 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride in homogenization buffer where Tris has been replaced by 50 mM Mops buffer (pH 7.4). The reaction was terminated by the addition of Tris/HCl buffer at a final concentration of 50 mM. The resulting nuclei were extracted eight times with nuclei extraction buffer, containing 1.5 M KCl in the last two washes. The washed nuclei were ruptured by sonication and repeatedly washed again. The residual solid material was resuspended in 1 mL of freshly prepared 10% (mass by volume) aqueous hydroxylamine (pH 9.2) and incubated for 2 h at room temperature. Longer incubation times were unfavorable because of the degradation of proteins. The samples were pelleted and the supernatants 4-fold diluted in Tris/HCl buffer (pH 7.5), desalted over Centricon 100, and 5-fold concentrated. After additional concentration on 20 μL of StrataClean (Stratagene, Germany) (Nielson et al., 1993), the samples were analyzed by Western blots. The yield in DNA polymerase α polypeptides was 5−10% of the amount obtained directly from untreated nuclei, not corrected for degradation and hydroxylamine-insensitive cross-linking with other proteins.

Size Exclusion Chromatography. The size of samples was 300 μL for chromatography on Superose 12 HR and 6 HR columns (both 24 mL bed volume), corresponding to the amount of extract from 1.5×10^8 nuclei (15−30 units of DNA polymerase activity; 30−60 μg of PMA), and 0.5−1.0 mL for chromatography on Superose 6 (100 mL bed volume), corresponding to the amount of extract from 5×10^8 nuclei. The chromatography was carried out in standard buffer containing 100 mM KCl at a rate of 0.3 mL/min (the HR columns) or 0.5 mL/min (Superose 6). Eluates were monitored for absorbance at 280 nm. Columns were standardized with regard to blue dextran (exclusion volume), GroEl (14-mer) (798 kDa), thyroglobuline (669 kDa), ferritin (450 kDa), catalase (245 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carboanhydrase (29 kDa). Standards had positions that were similar for 50−500 mM KCl in the elution buffer.

Protein Gels and Western Blots. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using a 3 or 5% polyacrylamide stacking gel and a separation gel of varying pore size. Dilute samples (quantities of 200−500 μL) from chromatographic eluates were frequently concentrated by adsorption to 5−10 μL of StrataClean before the SDS-containing sample buffer was added. After electrophoresis, the protein bands were either silver stained using the mehod of Heukeshoven and

Dernick (1988) or electrophoretically transferred to Immobilon (Millipore) membranes and immunostained according to the method of Towbin et al. (1979). After blotting, the molecular mass markers were stained with Ponceau S in 3% trichloroacetic acid.

Assay of DNA Polymerase α and of Primase Activities. The standard DNA polymerase α assay with activated salmon testis DNA as template/primer (Holler et al., 1987) was used if not mentioned otherwise. For reverting the inhibition by β-poly(L-malate), 0.4 mM spermine hydrochloride was added to the polymerization mixture. In a modified assay, 3.3 μg/mL poly(dA)/dT_{10} (10:1) was used as template/primer, and the four nucleoside 5′-triphosphates were replaced by 3.3 μM [α-^{32}P]dTTP (0.33 mCi/mmol). One unit of DNA polymerase activity measured by the standard assay corresponded to the incorporation of 1 nmol of bases into acid precipitable DNA during 1 h. Protein was measured by the method of Bradford (1976). DNA primase was assayed in the presence of 0.5 unit of Klenow fragment using poly(dT) as template (Holler et al., 1992). One unit of primase activity corresponded to the incorporation of 1 nmol of dAMP into acid precipitable DNA during 1 h.

RNA polymerase was assayed according to Hildebrandt and Sauer (1973), with one unit referring to an incorporation of 1 nmol of [^{32}P]UMP into acid-insoluble material in 1 h at 37 °C. Lactate dehydrogenase was assayed at 37 °C in 0.1 M phosphate buffer (pH 7.6) containing 0.75 mM pyruvate and 0.25 mM nicotinamide adenine dinucleotide (NAD). One unit of activity refers to the turnover of 1 μmol of NADH in 1 min.

Assay for β-Poly(L-malate). Samples were hydrolyzed in the presence of 0.5 M NaOH as described (Fischer et al., 1989). Samples with a high protein content were deproteinized by acidification (pH 2 with 7 M $HClO_4$) and removal of the precipitate by centrifugation before hydrolysis. β-Poly(L-malate) in higly diluted samples was precipitated in the presence of 2.5 volume of ethanol (−20 °C for 12 h), pelleted at 19000*g*, and hydrolyzed in 100 μL of 0.5 M NaOH. After hydrolysis, L-malate was quantified according to Gutmann and Wahlefeld (1974). The mass calculated for PMA refers to its potassium salt (156 Da/L-malate residue).

RESULTS

Preparation of Leakproof Nuclei from Plasmodia. The preparation of leakproof nuclei has been optimized with regard to 5 mM EGTA contained in the plasmodium homogenization buffer. That the nuclei were leakproof was indicated by the enzymatic activities of DNA polymerase and marker enzymes. The activities were in the cytosol: 3.9 ± 0.4 units/g of plasmodia DNA polymerase when spermine was included in the standard assay, 1.8 ± 0.2 units/g of plasmodia when spermine was absent, 6.0 ± 0.5 units/g of plasmodia lactate dehydrogenase, and 0.04 ± 0.02 unit/g of plasmodia RNA polymerase. The activities were in the nuclei: 110 ± 8 units/g of plasmodia DNA polymerase when spermine was included in the standard assay, 12 ± 2 units/g of plasmodia when spermine was absent, 0.7 ± 0.1 unit/g of plasmodia lactate dehydrogenase, and 3.5 ± 0.5 units/g of plasmodia RNA polymerase. Nuclei from both microplasmodia and macroplasmodia were indistinguishable. This should be kept in mind since samples of microplasmodia were a mixture of plasmodia, randomly distributed over the cell cyle (8 h cell cycle, of which 3 h is S phase).

Nuclei were extracted under mild conditions, i.e. in the presence of moderate ionic strength (less than 200 mM KCl, see Buffers) and with little physical agitation. Because of this protocol, the extract was devoid of detectable amounts of DNA [detection limit: 1 μg/mL, according to the assay of Thomas and Farquhar (1978)]. The extracts contained a variety of different proteins as indicated after silver staining (not shown). Western blots revealed the 140 kDa active DNA polymerase α (Figure 1; Figure 7, lanes 1, 9, 16, and 24) besides smaller fragments (especially the 110 kDa species), which most probably had been derived from the 140 kDa polypeptide by proteolytic nicking (Weber et al., 1988). The higher molecular mass species, especially the 220 kDa polypeptide, do not have polymerase activity and are candidates for ubiquinated forms of the polymerase (work in progress). The following experiment revealed that endogenous DNA polymerase α activity was completely suppressed by an excess of inhibitor (PMA). After addition of increasing amounts of purified DNA polymerase α to a fixed amount of nuclear extract, the polymerase activity was not detectable until a reproducible, minimal amount of the polymerase had been added. From there on, the activity increased in parallel with the added amount of polymerase. This amount required for the "neutralization" of the inhibitor referred to (30 ± 10)% of the endogenous polymerase activity (100%) in the extract (measured in the presence of spermine). Extracts of amoebae which were devoid of PMA did not show the inhibition. We ascribe the inhibition to the activity of free (or loosely bound) PMA.

β-Poly(L-malate)/Protein Complexes Detected by Size Exclusion Chromatography. When nuclear extract of plasmodia was passed over a size exclusion column, such as Superose 12 HR in Figure 1A, the DNA polymerase activity together with PMA appeared near the exclusion volume. The pronounced activation, seen when spermine was included in the assay mixture, was typical for the inhibitory effect of PMA on the activity of DNA polymerase α (Fischer et al., 1989). A very similar activation and an identical position were observed, when PMA in each eluate fraction was removed by adsorption on DEAE/cellulose (300 mM KCl) before the polymerase assay. The high-molecular mass position was independent of whether acitvated salmon testis DNA or poly(dA)/(dT) was used as template/primer (Figure 1C). Since the activity of purified DNA polymerase α with the synthetic template primer was almost negligible, the activity most likely referred to other types of DNA polymerases known to efficiently accept this template primer. For activated salmon testis DNA, the polymerase activity was inhibited by 0.5 mg/ml aphidicolin, an inhibitor of DNA polymerase α. On Superose 6 HR, with a higher resolution than for Superose 12 HR, the polymerase activity and PMA coeluted in the position of 1400 kDa (Figure 1B). With this eluate, the reversibility of the spermine effect was demonstrated by the addition of superstoichiometric amounts of purified PMA to the spermine-containing assay mixture. The resulting activity profile and the original profile before the inclusion of spermine in the assay mixture were similar. The possibility was tested whether the observed elution profiles originated from the binding of DNA polymerases to nucleic acids. But the nucleic acid content of the eluates was below the detection limit [below 1 μg/mL according to Thomas and Farquhar (1978)]. Benzonase (an unspecific endonuclease; 25 units, 1 h incubation at 37 °C), which had been added to the nuclear extract, had no effect on the elution

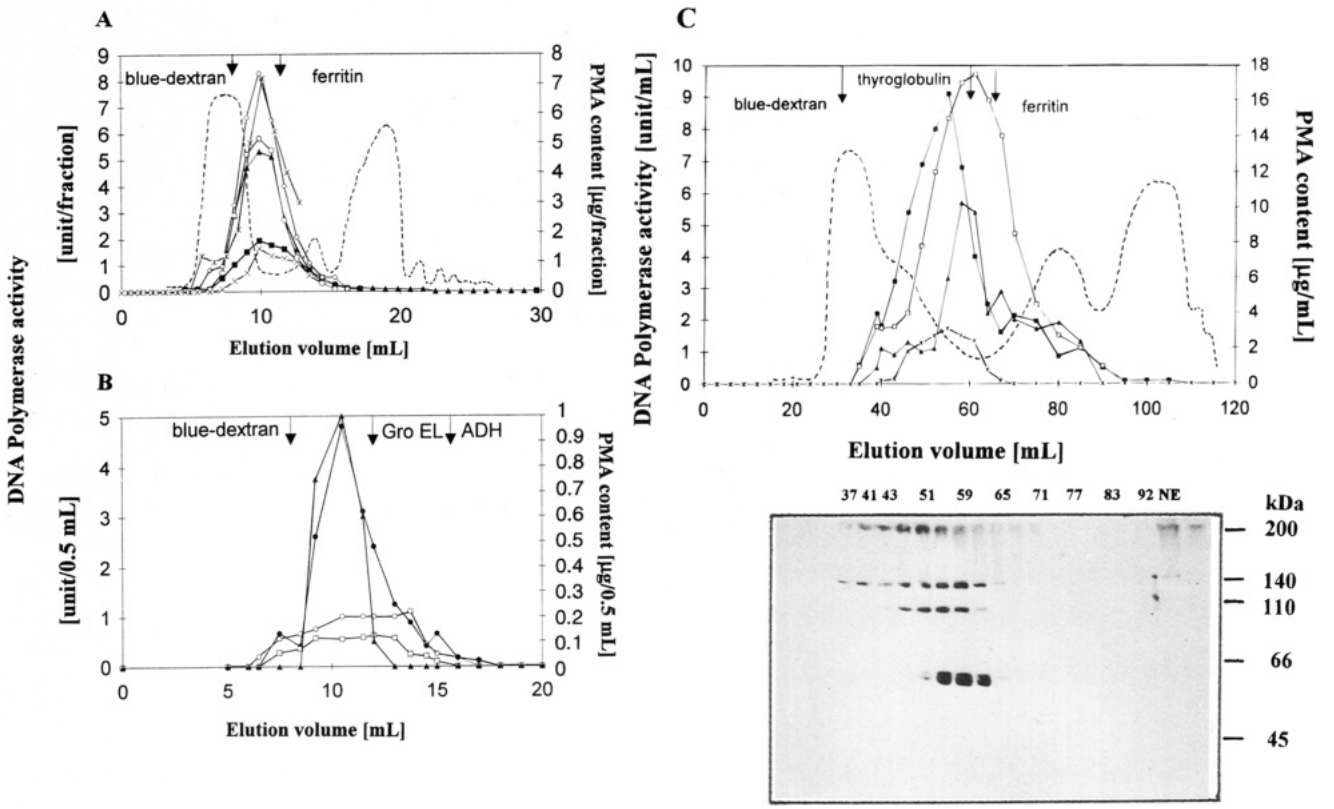

FIGURE 1: β-Poly(L-malate)/DNA polymerase complexes detected by size exclusion chromatography of nuclear extracts. (A) Chromatography on Superose 12 HR (300 kDa size exclusion): (- - -) relative absorbance at 280 nm wavelegnth, (■) DNA polymerase activity, (▲) DNA polymerase activity in the presence of 0.2 mM sperminium chloride, (○) DNA polymerase activity after passage over DEAE/cellulose, (◇) DNA polymerase activity in the presence of 0.2 mM sperminium chloride after passage over DEAE/cellulose, (×) DNA polymerase activity in the presence of 0.2 mM sperminium chloride and 0.5 mg/mL aphidicolin, and (*) PMA content. (B) Chromatography on Superose 6 HR (5000 kDa size exclusion): (○) DNA polymerase activity, (●) DNA polymerase activity in the presence of 0.2 mM sperminium chloride, (□) DNA polymerase actvity in the presence of 0.2 mM sperminium chloride and 1 μg/mL PMA, and (▲) PMA content. ADH refers to alcohol dehydrogenase. (C) Chromatography on Superose 6 (5000 kDa size exclusion): (- - -) relative absorbance at 280 nm wavelength, (■) DNA polymerase activity, (▲) DNA primase activity, (□) PMA content, and (*) DNA polymerase activity with poly(dA)/dT_{10} as template/primer. Fractions (100 μL) were concentrated with StrataClean and analyzed by SDS−7.5% polyacrylamide gel electrophoresis/Western blot (panel below) with antiserum against DNA polymerase α/primase (Achhammer et al., 1992). The numbers on top of the gel refer to the chromatographic elution volume above. NE refers to the sample of the unfractionated nuclear extract.

profile, indicating that neither DNA nor RNA were involved in the formation of the high-molecular mass complexes. The stimulation of the polymerase activity was referred to the dissociation of the complexes of PMA with DNA polymerase α and/or other types of DNA polymerases.

Using the preparative Superose 6 column, the resolution and capacity were improved (Figure 1C). PMA and polymerase activity were now eluting in different but overlapping regions, suggesting that a large portion of PMA complexes contained proteins other than the polymerase. Results of a Western blot using anti-DNA polymerase α/primase antiserum confirmed the presence of the DNA polymerase α 140 kDa protein in the position of maximum enzyme activity (the 110 kDa polypeptide refers to a coexisting nicked form of the DNA polymerase). Since the antiserum had been raised against DNA polymerase α/primase, it also detected primase subunits corresponding to polypeptides of 53 and 58 kDa in the position of primase activity. The staining densities indicated that primase polypeptides were contained in these fractions in superstoichiometric amounts over the DNA-synthesizing polymerase units of 140 and 110 kDa and eluted in positions shifted toward lower molecular masses as compared with the DNA-polymerizing subunit. A portion of DNA primase was apparently not firmly complexed with the DNA polymerase α-synthesizing subunit. The elution position of 600−700 kDa, in comparison to the calculated intrinsic molecular mass of 109 kDa for the heterodimer (Achhammer et al., 1992), indicated that primase was probably also complexed with PMA.

Identification of Histones Comigrating with PMA. Samples of the nuclear extract after passage over Superose 6 (Figure 2A) were analyzed by Western blots with a specific antiserum against histones of *P. polycephalum* (Figure 2B). Bands were identified as histones H1 (Smolarz et al., 1988; Mende et al., 1983) and CP1, a species identified as a cleavage product of histone H1 (Côté & Pallotta, 1985). It should be mentioned that *Physarum* histone H1 migrates in an unusual position when compared with H1 from other organisms (Côté & Pallotta, 1985; Loidl & Gröbner, 1987a). In Figure 2A, a few other unidentified bands also comigrate with the complexes such as band X or the band(s) located between H1 and CP1. The antiserum R279 (Figure 2B) indicated bands attributed to histones H1, H2B/H3, and probably AS1 and AS2, corresponding to HMG poteins of higher eukaryotes (Smolarz et al., 1988). Antiserum R284 indicated protein bands of histones H1, H2B/H3, and the HMG-like proteins AS1 and AS2 (Figure 2C). The identified proteins coeluted with PMA in the range of 200−1.000 kDa. These molecular masses were much higher than those indicated in the gels after SDS gel electrophoresis (Figure 2). The elution at high-molecular mass positions/comigration with PMA was indicative of PMA/histone and PMA/HMG-like complexes.

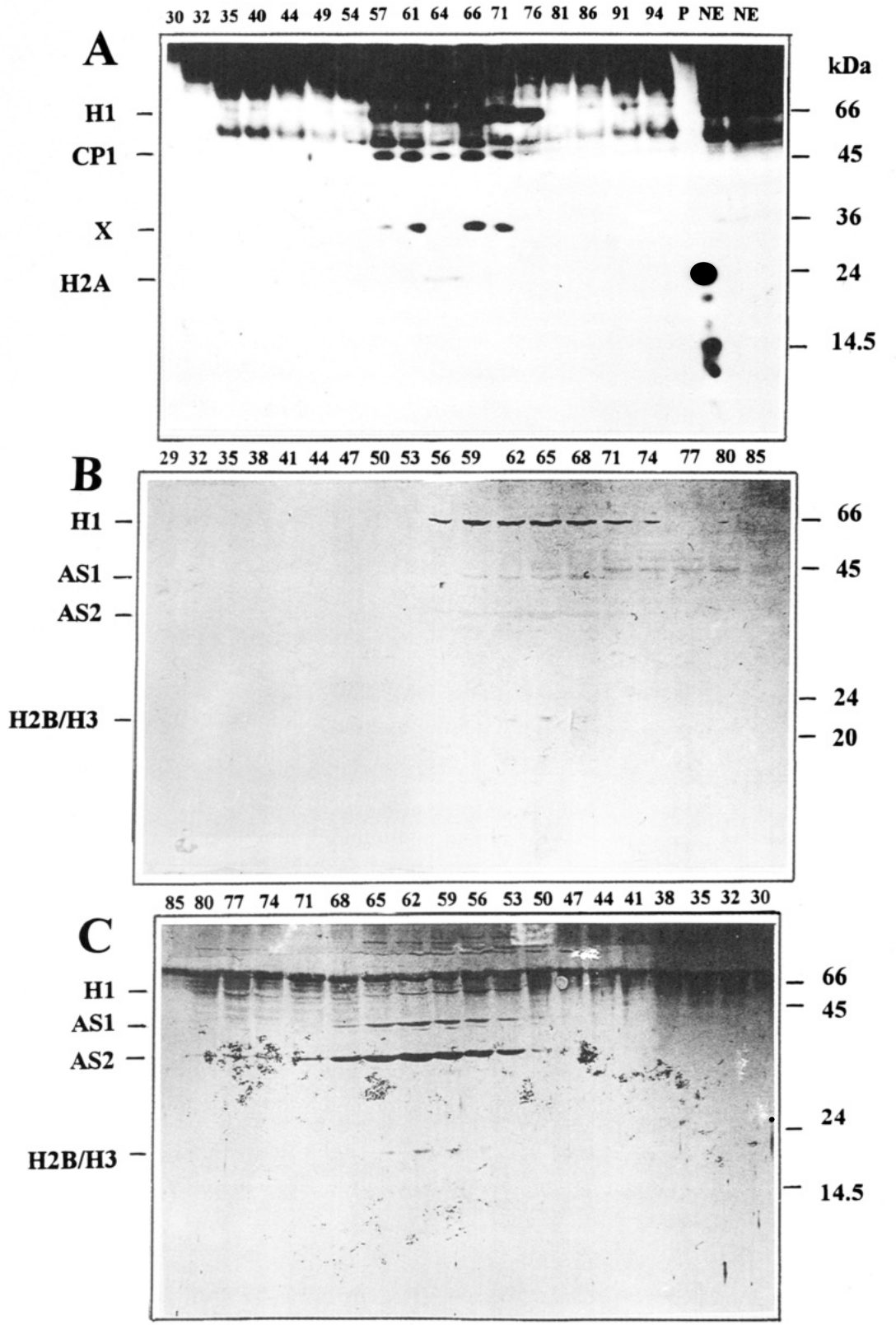

FIGURE 2: Detection of histones and HMG-like proteins complexed with PMA in the eluate after Superose 6 size exclusion chromatography of plasmodial nuclei. Western blots after SDS−15% polyacrylamide gel electrophoresis. Numbers on the top of each panel refer to the chromatographic elution volume as in Figure 1C. NE refers to the unfractionated nuclear extract. P is a positive control containing histones H2A and H4. (A) Antiserum against purified histones H1, H2A, and H2B from *P. polycephalum*. Note that histone H1 behaves unusually with regard to electrophoresis (see text). CP1 refers to a cleavage product of H1. The identity of protein X is not known. (B) Antiserum R279. (C) Antiserum R 284. AS1 and AS2 refer to HMG-related proteins.

That not all nuclear proteins were complexed to PMA is shown by the different locations of the PMA peak and the A_{280} peaks as shown in Figure 1A,C.

Spermine- and Salt-Induced Dissociation of DNA Polymerase/PMA Complexes. The routinely applied stimulation of DNA polymerase α by spermine has been referred to as the dissociation of the polymerase/PMA complex by the binding of spermine to the polymer (Fischer et al., 1989). This dissociation was now demonstrated by a shift of the polymerase activity from the position of 1200 ± 200 kDa

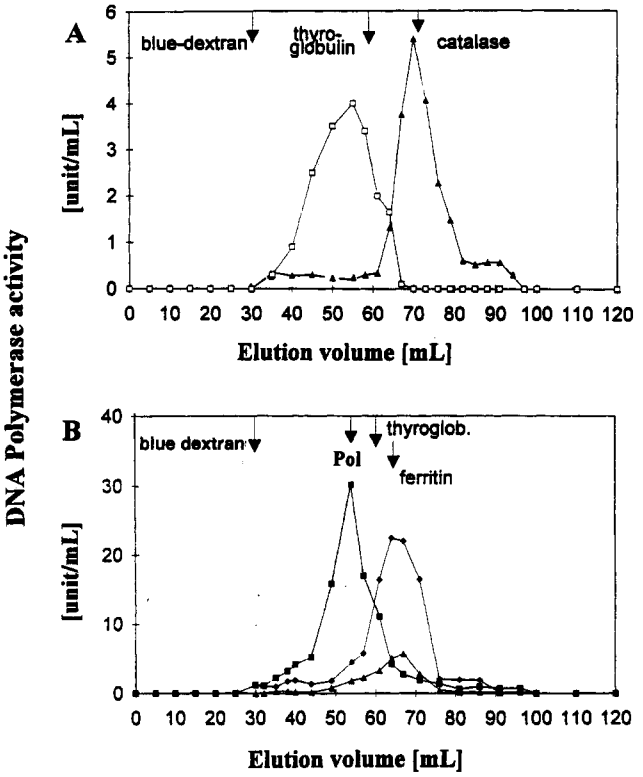

FIGURE 3: Dissociation of DNA polymerase/PMA complexes, detected by size exclusion chromatography and DNA polymerase activity. (A) (▲) Dissociation by pretreatment of the nuclear extract with DEAE/cellulose before chromatography on preparative Superose 6 and (□) untreated nuclear extract. (B) Dissociation during chromatography on preparative Superose 6 in the presence of 150 mM KCl (▲) or 500 mM KCl (◆). Dissociation was not observed in the presence of 50 mM KCl (■) or 100 mM KCl (standard chromatography condition as in Figure 1C; the peak position of the activity is indicated by Pol). The results shown refer to an amount of extract from 10−12 g of plasmodia, except for experiment (▲) where the extract was from 3 g of plasmodia.

to the position of 250 ± 20 kDa when the extract was mixed with spermine before size exclusion chromatography (not shown). A dissociation was also observed when PMA was removed on DEAE/cellulose in the presence of 200 mM KCl before size exclusion chromatography, leading to a very similar shift in the elution position (Figure 3A).

Binding of the polymerase to PMA was likely to be electrostatic in nature, and high ionic strengths were supposed to dissociate the complex. Figure 3B shows the effect of 50, 150 mM, and 500 mM KCl in the elution buffer during size exclusion chromatography. The increase in KCl concentration resulted in a shift toward low molecular masses, indicating the dissociation of the PMA/DNA polymerase complex. In parallel, the PMA maximum shifted from position 650 kDa (50 mM KCl) to 350 kDa (150 mM KCl) and 250 kDa (500 mM KCl) (proteins as calibration standards), with a concomitant broadening of the elution peaks. The shifts were consistent with an electrostatic attraction as the major driving force for the complex formation. The broadening for PMA was interpreted as indicating that proteins are bound to the polyanion with widely differing affinities and thus varying susceptibilities for a salt-induced dissociation of the complexes.

Amoebae of *P. polycephalum* have been reported to be devoid of PMA, and extracts of amoebae were considered as negative controls when demonstrating PMA protein complexes. The results in Figure 4 show the polymerase

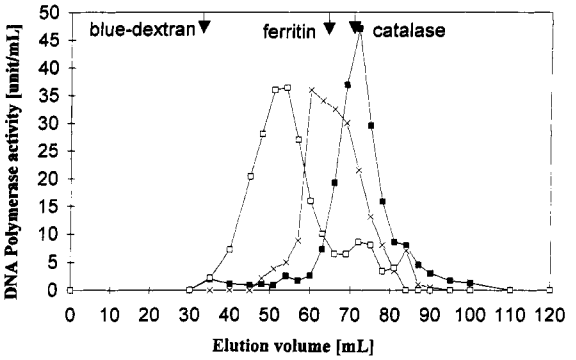

FIGURE 4: Size exclusion chromatography on preparative Superose 6 of an amoebal extract of *P. polycephalum:* (■) DNA polymerase activity with activated salmon testis DNA as template/primer, (□) DNA polymerase activity (activated salmon testis DNA) in the nuclear extract from plasmodia, and (×) DNA polymerase activity in the amoebal extract with poly(dA)/dT_{10} as template/primer.

activity in a position corresponding to 230 kDa, which is close to 255 ± 15 kDa reported for the size exclusion chromatography of purified DNA polymerase α (Weber et al., 1988). The correspondence in molecular mass indicates that the high-molecular mass complexes for plasmodia are not due to the binding of the proteins to DNA (for instance, replicationally active complexes) since these are very likely to occur also in amoebae. The positive control in Figure 4 was from the nuclear extract of plasmodia and eluted in the position of 1200−1400 kDa as shown before. In Figure 1C, an activity was contained in the plasmodial nuclei that was distinct from DNA polymerase α because it accepted poly(dA)/(dT) as template/primer. This activity from the plasmodial extract eluted in the position of high-molecular mass complexes. In contrast, the synthetic template/primer-accepting activity of the amoebal extract eluted in the range of 250−700 kDa (Figure 4), indicating that such DNA polymerase(s) of plasmodia in a high-molecular mass position was also complexed to PMA.

Stability of the Nuclear PMA/Protein Complexes. The stability of the nuclear PMA/protein complexes was of crucial importance for the credibility of the observed complexes; these could have been artificially formed after lysis of the isolated nuclei. In order to test the authenticity of the complexes, purified radioactively labeled PMA was mixed with the nuclei before lysis. After size exclusion chromatography, the elution position of the radioactivity in the extract was compared with the position of the purified radioactive PMA alone. Added synthetic [^{14}C]PMA in amounts of 0.3 or 1.5 mg did not appear in the peak corresponding to the protein/PMA complexes. In Figure 5, [^{14}C]-PMA purified from the culture medium of growing plasmodia had been added to the nuclei in a 6-fold excess over the endogenous (unlabeled) PMA. Again, the radioactivity did not integrate into the DNA polymerase α/PMA complex, which corresponded to the shoulder in the range of 40−60 mL elution volume. The results proved that the protein/PMA complexes were stable for at least 150 min, the period required for extract preparation and size exclusion chromatography.

PMA/Protein Complexes Detected by Chemical Cross-Linking with 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide Hydrochloride (EDC). In order to obtain additional support for the existence of PMA/protein complexes in nuclei, PMA and proteins were chemically cross-linked with EDC in isolated nuclei. This reagent preferentially cross-

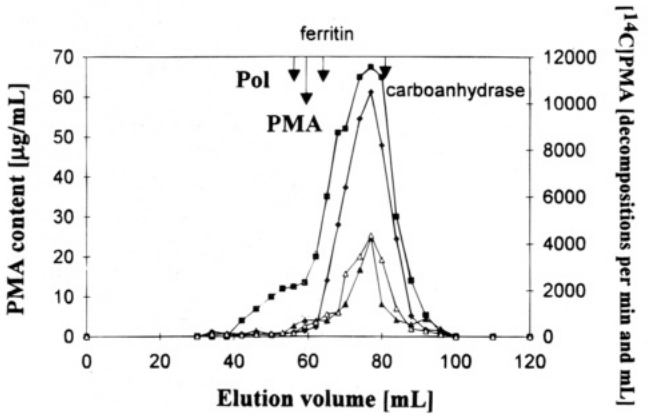

FIGURE 5: Stability of plasmodial PMA/protein complexes detected by the absence of [^{14}C]PMA exchange. The chromatography was performed on preparative Superose 6. The nuclei were mixed with 1.5 mg of [^{14}C]PMA (from *Physarum* culture medium) before they were lysed in the usual fashion: (◆) radioactivity, and (■) total PMA content. Pol denotes the peak position of DNA polymerase activity in the eluate and PMA the peak position for endogenous PMA, both measured in the absence of added [^{14}C]PMA. In a control, a sample of purified [^{14}C]PMA (0.5 mg) was chromatographed under exactly the same conditions: (△) radioactivity and (▲) PMA content.

links biomolecules interacting by salt bridges between carboxylate and ammonium groups (Mauk & Mauk, 1989). After the removal of unreacted proteins and PMA, the cross-linked constituents were investigated.

At first, the cross-linking reaction was studied using purified DNA polymerase α and PMA. It is seen in Figure 6A (lanes 1−6) that the presence of PMA resulted even in the absence of cross-linking in a broad smear due to the reversible binding of PMA to the polypeptides. The smear reflected the chain length inhomogeneity of the polymer (Fischer et al., 1989). The intensity of the smear decreased to some degree during cross-linking, as was expected if some of the products had become too large to enter the electrophoretic gel. The protein in the absence of the polymer did not show this tendency (Figure 6, lanes 8−13). After cross-linking, the product was separated from unreacted material by binding to Q-Sepharose and gradient elution. The cross-linked material emerged as a single peak in the presence of 1 M KCl that ran again as a smear after electrophoresis (results not shown). Upon treatment with hydroxylamine, the product was converted to L-malylhydroxamate and to malylated, enzymatically inactive polypeptides derived from 140 kDa DNA polymerase α and its 110 kDa nicked form (Figure 6B). The recovery after the treatment with hydroxylamine was estimated to be in the percent range, as could be noted by a comparison of the protein intensities in Figures 1 and 7B. This loss of protein was due to intermolecular cross-links, which rendered aggregates to be irreversibly insoluble, and also to fragmentation after prolonged incubation as is seen by comparison of lanes 3 and 5 in Figure 7B. The solubilized DNA polymerase α such as in Figure 7B shows an increased electrophoretic mobility. We ascribe this to an increased overall negative charge of the protein after acylation of amino groups and to an increase in the protein compactness due to intramolecular cross-linking.

For the cross-linking of nuclear PMA and proteins, isolated leakproof nuclei were treated with EDC. Samples taken during the cross-linking reaction showed a progressive disappearance of the DNA polymerase activity and of the protein bands in the nuclear extracts and in the residual

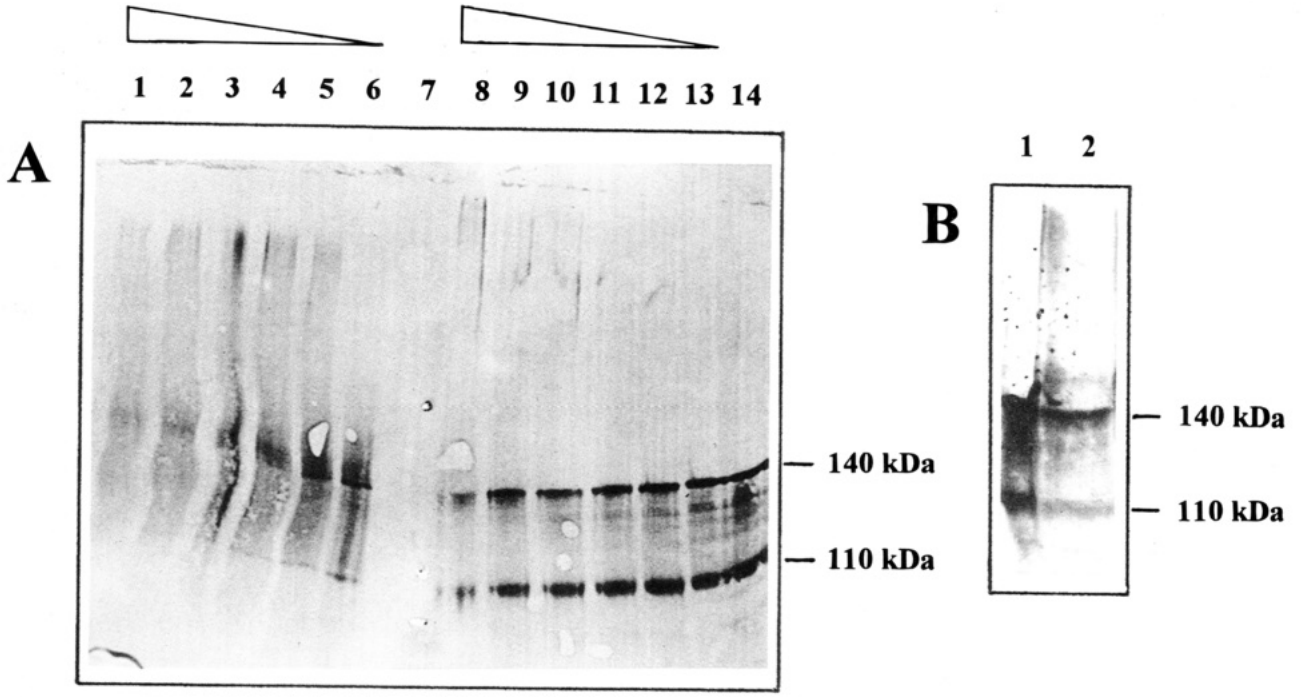

FIGURE 6: Cross-linking of purified DNA polymerase α and PMA in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and regeneration of the protein by treatment of the cross-linked product with hydroxylamine. (A) Western blot after SDS−5% polyacrylamide gel electrophoresis showing the 140 kDa DNA polymerase α core protein (30 units/mL) and its 110 kDa proteolytic fragment during the reaction with EDC. Lanes 1−6, in the presence of PMA (3 mg/mL) and EDC (5 mg/mL). Reaction times were 120 min (lane 1), 60 min (lane 2), 45 min (lane 3), 30 min (lane 4), 15 min (lane 5), and 0 min (lane 6). Lanes 8−13, the same time points in the absence of PMA (starting with 120 min in lane 8). Lane 14, DNA polymerase α in the absence of PMA and EDC. Lane 7, without sample. (B) Cross-linked PMA/DNA polymerase α complex purified by chromatography on Q Sepharose and treated with 10% (w/v) hydroxylamine (pH 9.5) for 2 h at room temperature. Lane 1, purified DNA polymerase α/PMA complex before cross-linking. Lane 2, the same but after cross-linking. The 140 kDa band refers to the intact core polymerase and the 110 kDa band to a proteolytic fragment.

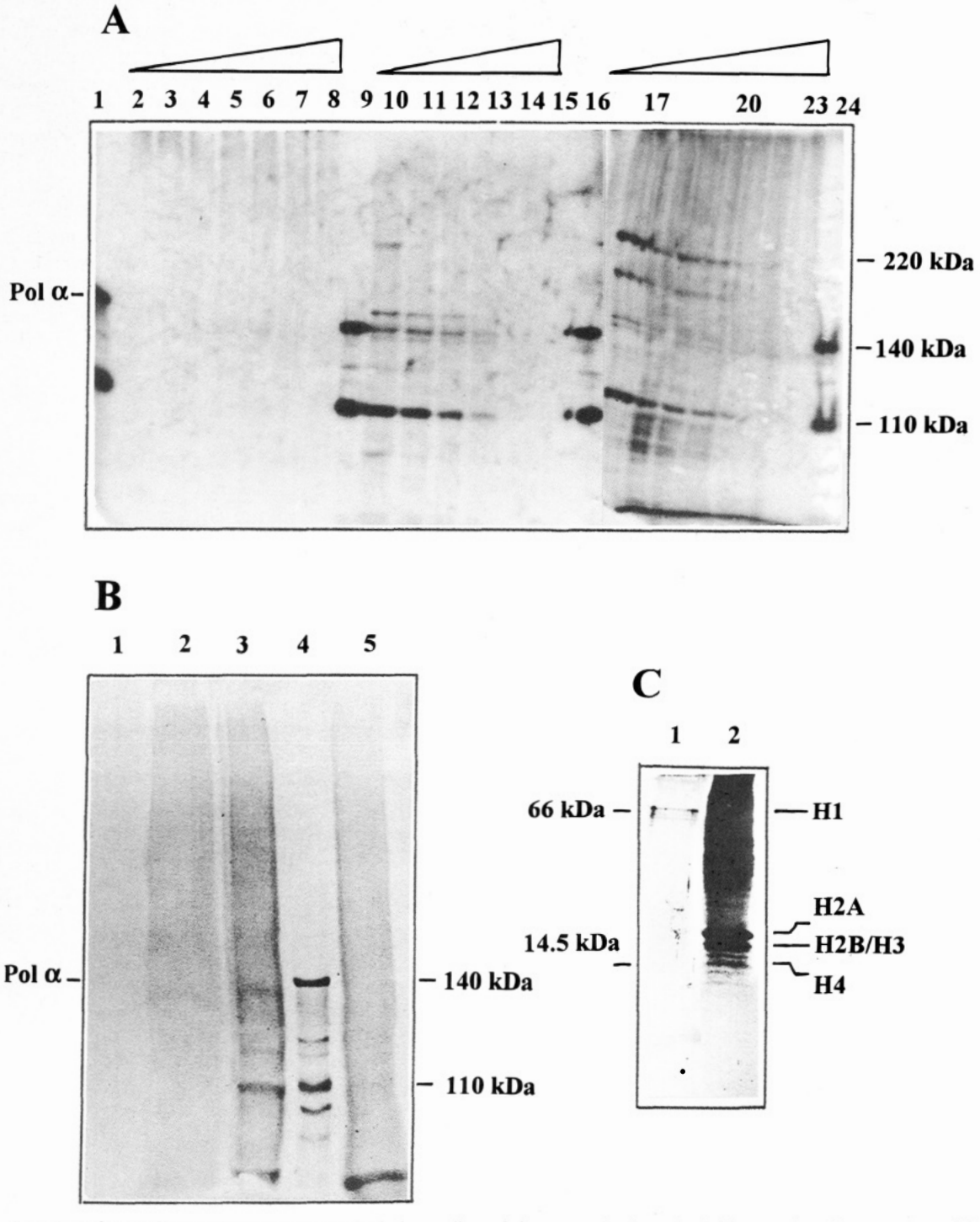

FIGURE 7: Progress of cross-linking in isolated nuclei of plasmodia and the reconstitution of soluble proteins after treatment of the cross-linked nuclei with hydroxylamine. Pol a refers to the 140 kDa DNA polymerase α polypeptide in the positive control prepared according to Achhammer et al. (1992). The immuno positive bands of higher molecular mass refer to polymerase inactive forms with not yet defined functions (see text), and the 110 kDa protein refers to a proteolytically nicked form of the DNA polymerase. (A) Cross-linking of 2.5×10^7 purified nuclei with 5 mg/mL EDC was carried out for 0, 15, 30, 45, and 90 min. Samples were taken from the supernatant of the reaction mixture after pelleting the nuclei (lanes 2−8), from nuclear extracts (lanes 11−15), and from the residual pellets after nuclear extraction (lanes 17−23). They are presented as Western blots for DNA polymerase α after SDS−5% polyacrylamide gel electrophoresis. Controls of purified DNA polymerase α according to Achhammer et al. (1992) in the absence of EDC are shown in lanes 1, 9, 16, and 24. (B) Cross-linking of 3.8×10^8 purified nuclei with 10 mg/mL EDC for 90 min. All samples were analyzed by Western blot for DNA polymerase α (SDS−5% polyacrylamide gel electrophoresis). Lane 1, rupture of cross-linked nuclei by sonication and analysis of the nuclear extract. Lane 2, same as in lane 1, but after a 2 h treatment of the ruptured nuclei with hydroxylamine. Lane 3, same as in lane 2 after desalting and concentrating. Lane 5, same as in lane 3, however after 18 h treatment with hydroxylamine. Lane 4, control of purified DNA polymerase α without cross-linking and hydroxylamine treatment. (C) Nuclei (10^7) after cross-linking and treatment with hydroxylamine for 2 h as above for the sample in lane 3. Western blots are shown for the *Physarum* histone antiserum R284 in lane 1 and the antiserum against a mixture of *Physarum* histones (Holler et al., 1992b) in lane 2.

pellets. Lanes 2−8 in Figure 7A demonstrate that the nuclei were leakproof under the conditions of the cross-linking reaction. The disappearance of the polymerase bands seen in lanes 10−15 of Figure 7A for the nuclear extract and in lanes 17−23 of Figure 7A for the residual nuclear pellet was due to immobilization within the nuclei due to cross-linking.

PMA also became insoluble such that 85−90% of the total nuclear amount was immobilized after 90 min. The missing 10−15% is referred to L-malate residues bearing cross-links, which were not recognized as substrates by L-malate dehydrogenase in the PMA assay. The degree of retention in the nuclei was a function of the EDC concentration and was complete for 10 mg/mL EDC after 90 min. These nuclei consisted of completely insoluble material. They were exhaustively washed with extraction buffer, to ensure removal of traces of protein and PMA. After ultrasonic rupture, the fractured nuclei were washed again. Lane 1 of Figure 7B shows that the wash was devoid of staining material. After treatment of the insoluble material with hydroxylamine, bands appeared in the Western blot that migrated in the positions attributed to the 140 kDa DNA polymerase α, the 110 kDa polypeptide, and another, smaller peptide, which was attributed to a proteolytic fragment. This band remained after prolonged hydroxylaminylation (Figure 7B, lanes 3 and 5) (note that DNA primase cannot be detected because it has left the gel). By the same technique, the cross-links of PMA with proteins H1, and H2B/H3 could be demonstrated in Figure 7C. Here, the Western blot with anti-histone antiserum R284 in lane 1 reveals a set of faint bands attributed to histone H1, and in lane 2 with antiserum against a histone mixture (Holler et al., 1992b) shows a smear of probably cross-linked proteins together with bands attributed to histones H2A, H2B/H3, and H4. The solubilization of DNA polymerase and histones by the hydroxylamine treatment after cross-linking is valued as an independent indication for the existence of protein/PMA complexes in the nuclei.

Size Distribution of PMA/Protein Complexes. The width of the PMA elution peaks suggested that the high-molecular mass complexes were highly heterogeneous with regard to their protein content and/or the size of PMA. Isolated PMA had been reported to be heterogeneous in size (Figure 5C; Fischer et al., 1989; Holler et al., 1992), and whether the elution profiles reflected this molecular mass heterogeneity was tested. PMA in various positions of the eluate was precipitated with ethanol and rechromatographed after removal of the denatured protein. The positions referred to molecular masses above 1200 kDa and to ranges of 700−1200 kDa, 300−700 kDa, and 150−300 kDa. After rechromatography on the same column (Superose 6), all the samples eluted in positions of 150−200 kDa with proteins as standards or 40−60 kDa with poly(styrene sulfonate) as standards. According to the standard activity assay, these fractions inhibited DNA polymerase α at identical concentrations (50% inhibition at 0.2 μg/mL PMA), also arguing for similar polymer sizes, as inhibition was dependent on the polymer length (Fischer et al., 1989). It is concluded that the molecular mass reflects mainly the protein composition of the complexes.

Changes in the Molecular Mass of PMA/Protein Complexes during the Mitotic Cycle. Both the catalytic function of DNA polymerase α/primase in S phase within a high-molecular mass replication complex such as the "replication factories" (Coverly & Laskey, 1994) and the differential synthesis pattern of *Physarum* histones in late G2 and early S phase (Loidl & Gröbner, 1987a) suggested a cell cycle dependent composition of the PMA complexes. The results for different periods of the mitotic cycle obtained by size exclusion chromatography are shown in Figure 8. As judged by both the DNA polymerase activity and the PMA content,

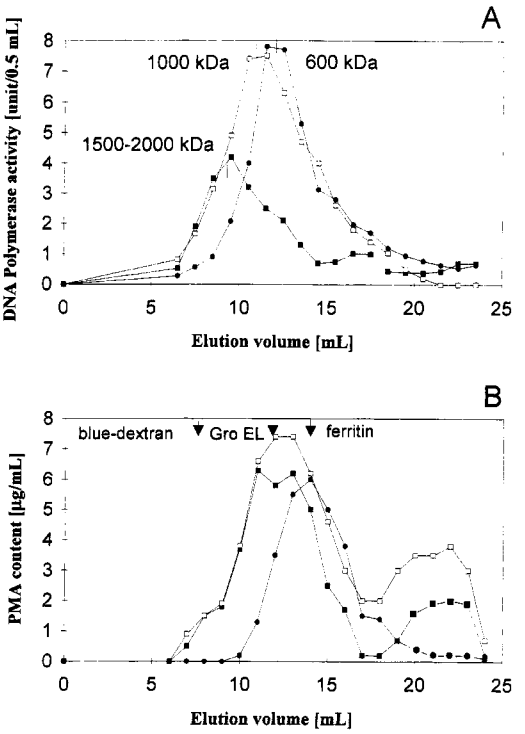

FIGURE 8: Effects of the mitotic cycle on the elution positions of DNA polymerase activity and PMA during size exclusion chromatography of nuclear extracts. (A) DNA polymerase activity in the presence of 0.2 mM sperminium chloride: (■) S Phase, (□) early G2 phase, and (●) late G2 phase. (B) PMA content. Symbols are as in panel A.

the complexes were largest during S phase. Moreover, during S phase and early G2 phase a peak of PMA was seen in positions of 10 kDa and below [corresponding to β-oligo-(L-malate) of 2 kDa or less when poly(styrene sulfate) was used as standard] (Figure 8B). These fractions did not contain detectable amounts of protein.

DISCUSSION

PMA was discovered in plasmodia of *P. polycephalum* by virtue of its inhibitory activity on DNA polymerase α which could be eliminated by passing the cellular extract over a DEAE/cellulose column or by supplementing the assay mixture with biogenic amines such as spermine (Fischer et al., 1989). The effect was specific for DNA polymerase α of *P. polycephalum* since polymerases from other organisms were not inhibited. This phenomenon and, later on, the demonstration of PMA/histone complexes (Holler et al., 1992b) led to the assumption that PMA bound to the DNA polymerase and to histones in plasmodia of *P. polycephalum.* Support for this assumption came from the discovery that PMA had some degree of isosterism with the DNA deoxyribose phosphate backbone and the finding that the inhibition of the polymerase was competitive in nature. It is believed that PMA in *P. polycephalum* has a number of physiological functions that copied those of the DNA backbone and had evolved for the plasmodium.

The investigation here is aimed at a verification of PMA complexes with DNA polymerase α and histones in isolated leakproof nuclei of plasmodia. Nuclei are used in order to focus on the compartment where the concentration of PMA is highest, allowing the preparation of concentrated extracts to be used in the preparative chromatography of complexes, and also for cross-linking. The prepared nuclei were

leakproof by the criteria of marker enzymes and contained more than 90% of the DNA polymerase activity found in total cell extracts (0.2 mM sperminium chloride). The conditions of cross-linking were thus well-defined for nuclei but would have been circumstantial for whole cells.

The endogenous polymerase activity was virtually suppressed in the nuclear extract and was contained in PMA/protein complexes of high molecular mass in the 10^6 Da range. The complexes were revealed by size exclusion chromatography. They could not be detected in the extracts of amoebae of *P. polycephalum*, which did not contain PMA and displayed full DNA polymerase activity. The polymerase activity (activated salmon testis DNA) of amoebal extracts eluted in positions comparable to the one seen for PMA-stripped DNA polymerase α such as after chromatography on DEAE/cellulose (Figures 4 and 3B) or after protein purification. Assuming 10^5 molecules of DNA polymerase α per nucleus (Weber et al., 1988), 3×10^8 nuclei per sample of nuclear extract, and an average chain of β-poly(L-malate) of 5 kDa [an approximate minumum size of the polymer allowing its firm binding to the polymerase (Fischer et al., 1989)], we calculated an amount of 0.15 μg of PMA in the complex with the polymerase. This amount was much less than the 200−400 μg of PMA actually measured in the fractions after size exclusion chromatography, indicating that the majority of PMA was bound to proteins other than DNA polymerase α. The conclusion from this estimate is supported by the observation that the peak of the DNA polymerase activity and β-poly(L-malate) did not elute in the same position (Figure 1C).

The size of the PMA/protein complexes depended on the time of harvest in the mitotic cycle. Peak molecular masses of the PMA/DNA polymerase complexes were 1500−2000 kDa in the S phase and 400−600 kDa in the late G2 phase (Figure 8). We found that the observed size variation of the PMA/protein complexes was likely to reflect in the first place a varying composition of the protein constituents. PMA may function as a "glue" without contributing much to the overall molecular mass of the complex. Regarding its small diameter and the high flexibility of the polymer chain, PMA may easily wind around proteins (unpublished results from molecular modeling). As the efficiency to function as a glue depends on the polymer length, short β-oligo(L-malate) may occasionally not be bound at all. We think that this happens during late S phase and early G2 phase of the mitotic cycle when free histones become rare, leading to the appearance of the protein-free fractions of PMA in Figure 8B. In late G2 phase, 40% of the total histone is synthesized (Loidl & Gröbner, 1987a), which seems to be enough to bind the low-molecular mass PMA.

The PMA/protein complexes appeared to be long-lived and stable under the conditions of sample preparation and size exclusion chromatography. This could be demonstrated by showing that added radioactively labeled PMA did not exchange with unlabeled PMA in the complexes during size exclusion chromatography. The result strongly favors the assumption that the complexes were not artificially formed during the extraction of the nuclei. It could be argued that the complexes had been formed during the preparation of the nuclei, but we consider this possibility unlikely due to the high nuclear concentration of PMA, its solubility, its electric charge, and its known tendency for complex formation under *in vitro* conditions (Fischer et al., 1989; Holler et al., 1992b). As a polyanion, PMA can bind proteins by electrostatic forces between pending PMA carboxylate groups and protein ammonium groups. The strong destabilization of the PMA/DNA polymerase α complex (Figure 3B) effected by the narrow increase in salt from 50 to 150 mM KCl suggested that the contribution by the electrostatic linkages was cooperative. The disruption of the complex by spermine hydrochloride or DEAE/cellulose supports this assumption of electrostatic binding.

The results obtained with the nuclear extract were hampered by the fact that the experimental condition was far from nuclei located within intact plasmodia. One step further toward the *in vivo* situation was the demonstration of complexes in leakproof nuclei. It was assumed that after EDC-mediated cross-linking, PMA and proteins were interconnected by amide bonds between pending carboxylates and protein amino groups. The cross-linking reaction was exemplified with purified DNA polymerase α and PMA (Figure 6). The immediate polymerase/PMA complex as well as the cross-linked product was indicated by a smear after SDS gel electrophoresis. The cross-linking of whole nuclei resulted in physically compact, rubber ball-like material that released only traces of material under a variety of extreme elution conditions (high salt, sonication). The cross-linked nuclei were thus hardly accessible for an analysis. Digestion with proteinase K, in contrast to the treatment with Benzonase, allowed for the solubilization of PMA (data not shown), at least indicating its immobilization by means of cross-links with proteins. The solution of the analytic problem was that the immobile cross-linked PMA/protein complexes could be disrupted by the splitting of the ester bonds with hydroxylamine. This allowed for the resolubilization of the proteins, now cross-linked to malic acid hydroxamate, and, thus, the demonstration of their previous complexes with PMA. Silver-stained electrophoretic gels revealed several faint bands of resolubilized proteins (results not shown). DNA polymerase α and histones H1, H2A, and H2B/H3 could be identified by Western blots (Figure 7C). These proteins were also contained in the high-molecular mass fractions after size exclusion chromatography (Figure 2). The disadvantage of the cross-linking method is, however, its experimental difficulty and the low yield of hydroxylamine-solubilized single proteins.

We were interested in whether DNA polymerases other than of the type α were also part of the complexes. This question was investigated with the PMA/protein complexes from size exclusion chromatography. Using the poly(dA)/dT_{10} template/primer, which is only efficiently used by DNA polymerases δ and ε, the results in Figures 1C and 4 revealed that such types of polymerases also appeared to be part of the complexes. Partially purified DNA polymerases of these types from *P. polycephalum* were inhibited by PMA following inhibition constants of 1 μg/mL (type δ) and of 0.4 μg/mL (type ε) (concentrations of PMA at 50% inhibition, unpublished results).

We have proposed that one of the physiological roles of PMA was to function as an anchorage for the transient storage of histones (Holler et al., 1992b). Histones H1, H2A, and H2B, which we have identified here as constituents of the PMA/protein complexes, are synthesized during G2 phase (Loidl & Gröbner, 1987a). Similarly, nucleoplasmin and the protein N1 have been reported to function in the storage of histones in oocytes of *Xenopus laevis* (Adamson & Woodland, 1974). The stockpiled histones are utilized during

replication after fertilization of the oocyte (Laskey et al., 1993). Nucleoplasmin and protein N1 have also been shown to function as molecular chaperones, facilitating the formation of nucleosomes. Their sequences show repeated stretches of glutamic acid (Dingwall et al., 1987). Under *in vitro* conditions, the assembly of nucleosomes is also chaperoned by poly(L-aspartate) [for a review, see McGee and Felsenfeld (1980)], which appears to be structurally and functionally similar to PMA (Holler et al., 1992b). Similarly, as nucleoplasmin and factor N1 in the early embryonic stages of *X. laevis*, PMA may function in *Physarum* plasmodia, when, imbedded in a common cytoplasm, all the nuclei progress synchronously through the mitotic cycle. PMA may facilitate not only the assembly of nucleosomes but also reversely their dissociation. For nucleoplasmin, this has been reported by Chen et al. (1994), showing that it stimulated transcriptional activation by facilitating the dissociation of histones H2A and H2B from nucleosomes that otherwise blocked the binding of transcription factors. Any of such functions has yet to be proven for PMA.

Because of the mild extraction conditions used in our preparations (i.e. 0.5% Triton X100 and 200 mM KCl), the protein/PMA complexes are considered as soluble in the nuclei and not bound to chromatin or nuclear scaffold. Much more rigorous conditions (for instance, 1 M $CaCl_2$ and 80 °C) have been reported for the extraction of histones from chromatin (Loidl & Gröbner, 1987b). DNA polymerase α of higher eukaryotes has been referred to as a soluble form and a nuclear matrix-bound form, which is the one active in replication (Cook, 1991). We believe that the PMA/DNA polymerase complexes contain part of the replication factory in an inactive soluble form. After a scheduled dissociation of PMA in response to an abundance of newly synthesized free histones (and possibly biogenic amines), these protein complexes can react with DNA and/or nuclear matrix to form the replication factories. The occurence of PMA may be a physiological peculiarity of *Physarum* plasmodia, which lack G1 phase, have a closed nuclear division, and are devoid of cytokinesis. We consider PMA to be a primitive ancestor of the more sophisticated proteins such as nucleoplasmin, which are active in nuclear transport, histone storage, and chromosome assembly/disassembly.

PMA is not the only unusual polymer found in nuclei of plasmodia. An organic polyphosphate has been reported, which, in contrast to PMA, is enriched in nucleoli, is isolated together with chromosomal DNA during the first steps of its purification, has no effect on DNA polymerases, and, under conditions of starvation, inhibits the initiation of transcriptional activity of RNA polymerase I [for a review, see Braun and Seebeck (1982)]. Also, a sulfated and phosphorylated β-D-galactan has been purified from chromatin under similar conditions; however, only little is known about its physiological properties (Farr & Horisberger, 1978; Horisberger et al., 1978). The future will have to show how these different polymers are organized and how they eventually cooperate in the plasmodium.

REFERENCES

Achhammer, G., Angerer, B., Windisch, C., Uhl, A., & Holler, E. (1992) *Cell Biol. Int. Rep. 16*, 1047−1053.

Adamson, E. D., & Woodland, H. R. (1974) *J. Mol. Biol. 88*, 263−285.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Braun, R., & Seebeck, T. (1982) in *Cell Biology of Physarum and Didymium* (Aldrich, H. C., & Daniel, J. W., Eds.) pp 393−435, Academic Press, New York.

Cavalier-Smith, T. (1993) *Microbiol. Rev. 57*, 953−994.

Chen, H., Li, B., & Workman, J. L. (1994) *EMBO J. 13*, 380−390.

Cook, P. (1991) *Cell 66*, 627−635.

Côté, S., & Pallotta, D. (1985) *Biochim. Biophys. Acta 828*, 22−28.

Coverley, D., & Laskey, R. A. (1994) *Annu. Rev. Biochem. 63*, 745−776.

Daniel, J. W., & Baldwin, H. H. (1964) *Methods Cell Physiol. 1*, 19−41.

Dee, J., Foxon, J. L., & Anderson, R. W. (1989) *J. Gen. Microbiol. 125*, 1567−1588.

Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., & Laskey, R. A. (1987) *EMBO J. 6*, 69−74.

Farr, D. R., & Horisberger, M. (1978) *Biochim. Biophys. Acta 539*, 37−40.

Fischer, H., Erdmann, S., & Holler, E. (1989) *Biochemistry 28*, 5219−5226.

Gutmann, I., & Wahlefeld, A. W. (1974) *Methoden Enzym. Anal. (3. Aufl.) 2*, 1632−1639.

Heukeshoven, J., & Dernick, R. (1988) *Electrophoresis 9*, 28−32.

Hildebrandt, A., & Sauer, H. W. (1973) *FEBS Lett. 35*, 41−44.

Holler, E., Fischer, H., Weber, C., Stopper, H., Steger, H., & Simek, H. (1987) *Eur. J. Biochem. 163*, 397−405.

Holler, E., Angerer, B., Achhammer, G., Miller, S., & Windisch, C. (1992a) *FEMS Microbiol. Rev. 103*, 109−118.

Holler, E., Achhammer, G., Angerer, B., Gantz, B., Hambach, C., Reisner, H., Seidel, B., Weber, C., Windisch, C., Braud, C., Guerin, P., & Vert, M. (1992b) *Eur. J. Biochem. 206*, 1−6.

Horisberger, M., Farr, D. R., & Vonlanthen, M. (1978) *Biochim. Biophys. Acta 542*, 308−314.

Johansen, T., Johansen, S., & Haugli, F. B. (1988) *Curr. Genet. 14*, 265−273.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Laskey, R. A., Mills, A. D., Philpott, A., Leno, G. H., Dilworth, S. M., & Dingwall, C. (1993) *Philos. Trans. R. Soc. London, Ser. B 339*, 263−269.

Loidl, P., & Gröbner, P. (1987a) *J. Biol. Chem. 262*, 10195−10199.

Loidl, P., & Gröbner, P. (1987b) *Nucleic Acids Res. 15*, 8351−8366.

Mauk, M. R., & Mauk, A. G. (1989) *J. Biochem. 186*, 473−486.

McGee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115−1156.

Mende, L. M., Waterborg, J. H., Mueller, R. D., & Matthews (1983) *Biochemistry 22*, 38−51.

Nielson, K., Ehret, M., & Mathur, E. (1993) *Strategies 6*, 29.

Nygaard, O. P., Guttes, S., & Rush, H. P. (1960) *Biochim. Biophys. Acta 38*, 298−306.

Rusch, H. P. (1980) in *Growth and differentiation in Physarum polycephalum* (Dove, W. F., & Rusch, H. P., Eds.) pp 1−8, Princeton University Press, Princeton, NJ.

Smolarz, E., Gröbner, P., & Loidl, P. (1988) *Biochemistry 27*, 4142−4147.

Thomas, T. S., & Farquhar, M. N. (1978) *Anal. Biochem. 89*, 35−44.

Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.

Vaitukaitis, J. L. (1981) *Methods Enzymol. 73*, 46−52.

Weber, C., Fischer, H., & Holler E. (1988) *Eur. J. Biochem. 176*, 199−206.

BI950994J