[1] Overproduction and Purification of RFC-Related Clamp Loaders and PCNA-Related Clamps from *Saccharomyces cerevisiae*

By GÖRAN O. BYLUND, JERZY MAJKA, and PETER M. J. BURGERS

Abstract

The replication clamp PCNA and its loader RFC (Replication Factor C) are central factors required for processive replication and coordinated DNA repair. Recently, several additional related clamp loaders have been identified. These alternative clamp loaders contain the small Rfc2–5 subunits of RFC, but replace the large Rfc1 subunit by a pathway-specific alternative large subunit, Rad24 for the DNA damage checkpoint, Ctf18 for the establishment of sister chromatid cohesion, and Elg1 for a general function in chromosome stability. In order to define biochemical functions for these loaders, the loaders were overproduced in yeast and purified at a milligram scale. To aid in purification, the large subunit of each clamp loader was fused to a GST-tag that, after purification could be easily removed by a rhinoviral protease. This methodology yielded all clamp loaders in high yield and with high enzymatic activity. The yeast 9-1-1 checkpoint clamp, consisting of Rad17, Mec3, and Ddc1, was overproduced and purified in a similar manner.

Introduction

The proliferating cell nuclear antigen PCNA is the processivity clamp that organizes and stabilizes DNA replication complexes and most DNA repair complexes on the DNA (reviewed in Majka and Burgers, 2004). Its function as a sliding clamp that encircles the DNA requires that PCNA is loaded around DNA by a clamp loader, RFC. The heteropentameric RFC uses the energy of ATP to open PCNA, bind the effector DNA, and reclose PCNA around this DNA. RFC consists of four small subunits of 36–40 kDa in all eukaryotes, Rfc2, Rfc3, Rfc4, and Rfc5, and one large subunit ~95–145 kDa in size, Rfc1. The 4-subunit Rfc2–5 complex is designated as the core complex because it functions in at least three other clamp loader complexes.

These alternative eukaryotic clamp loaders are designated Rad24-RFC, Ctf18-RFC and Elg1-RFC. Rad24-RFC, a complex of Rad24 and the Rfc2–5 core, functions in the DNA damage checkpoint, and this complex

METHODS IN ENZYMOLOGY, VOL. 409
0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(05)09001-4

loads an alternative clamp, the 9-1-1 complex (Rad17/3/1 in *S. cerevisiae*), at sites of DNA damage in order to mediate checkpoint activation (Bermudez *et al.*, 2003a; Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou *et al.*, 2003). Ctf18-RFC, a 7-subunit complex of Rfc2–5, Ctf18, Dcc1, and Ctf8, is required for the establishment of sister chromatid cohesion (Hanna *et al.*, 2001; Mayer *et al.*, 2001). Ctf18 functions as the Rfc1 homolog in this complex. Biochemically, Ctf18-RFC can both load and unload PCNA (Bermudez *et al.*, 2003b; Bylund and Burgers, 2005; Shiomi *et al.*, 2004). A distinct biochemical function for the 5-subunit Elg1-RFC complex, with Elg1 replacing Rfc1, has not yet been defined (Bylund and Burgers, 2005).

The discovery of these clamp loaders has created a demand for large quantities of highly pure and active complexes in order to facilitate biochemical studies. An important consideration during the development of our strategy was that overproduction of clamp loaders in *E. coli* met with varying success. While the canonical RFC could be overexpressed and purified from *E. coli* in high yield and with high activity, a similar strategy failed for the Rad24- and Ctf18-related loaders (Gomes *et al.*, 2000). Whereas it was relatively easy to overproduce these alternative clamp loaders in *E. coli*, and obtain them in pure form, the purified complexes lacked any detectable clamp loading activity (unpublished results from our laboratory). Therefore, all alternative clamp loader complexes were purified from yeast overproduction systems.

In this report, we describe a robust procedure to overproduce and purify the alternative clamp loaders, Rad24-RFC, Ctf18-RFC, and Elg1-RFC and the damage response clamp, Rad17/3/1, yielding milligram amounts of pure complexes. One protein in each of the complexes is expressed as an N-terminal fusion to a protease cleavable form of glutathione S-transferase (GST) and the complexes are affinity purified by the use of glutathione sepharose chromatography (Smith and Johnson, 1988). The advantage of using GST-tagged fusion proteins and glutathione sepharose chromatography is that protein with a purity of $>80\%$ can readily be obtained in this single purification step that does not require harsh elution conditions. The GST-tag is cleaved off by treatment with a human rhinovirus protease, which has a highly unique substrate recognition site, and the complexes are further purified by FPLC either on a heparin, monoS or monoQ column (Cordingley *et al.*, 1990). Neither the protease nor the cleaved GST moiety bind to these columns and are therefore readily washed away. The Ssa1 chaperone protein appears as a consistent contaminant during these purification procedures; however, it can be eluted from the columns by washing with a Mg-ATP buffer prior to elution of the complex.

Overproduction of Complexes in Yeast

Expression Plasmids and Strains

In our laboratory, we have successfully used the pRS424-GAL (*TRP1*), pRS425-GAL (*LEU2*), and pRS426-GAL (*URA3*) plasmids for the overproduction of a variety of replication proteins in yeast, including clamps and clamp loaders (Fig. 1A) (Burgers, 1999). These plasmids contain a 2μ origin for high copy maintenance in yeast and a Bluescript SKII$^+$ backbone for amplification in *E. coli*. Expression of genes cloned into these plasmid vectors is driven from the bidirectional *GAL1–10* promoter. The expression of genes under *GAL1–10* control is high when galactose is present in the growth medium and low in its absence. Glucose represses expression from the *GAL1–10* promoter and must therefore be excluded from the growth medium, or kept at a low concentration ($\leq$0.1%) in order to achieve rapid and efficient induction of expression upon addition of galactose (Johnston *et al.*, 1994).

We have extended the utility of these vectors by making GST-fusion derivatives. Vectors pRS424-GALGST, pRS425-GALGST, and pRS426-GALGST all contain a GST-tag (encoding the 26.6 kDa **g**lutathione **S**-**t**ransferase from *Schistosoma japanicum*) cloned into the respective GAL vector. The GST tag is separated from the target gene by a recognition sequence for the human rhinovirus protease (Fig. 1B). Vectors and

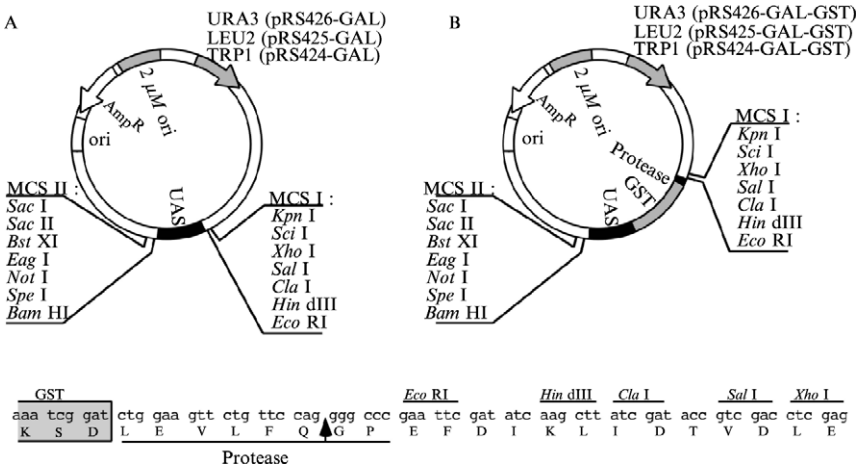

FIG. 1. Vectors used to overexpress alternative clamp loaders and clamps in *S. cerevisieae*. A. GAL vectors. B. GAL-GST vectors. The sequence around the protease cleavage site is shown. The vectors are available upon request from the author (burgers@biochem.wustl.edu).

sequences are available from the corresponding author upon request. In order to overproduce a complex in yeast, we routinely clone one of the genes of the particular complex into the GAL–GST vector via an N-terminal fusion to GST, while the other genes are cloned without modification into the regular GAL vectors. If desired, multiple genes can be cloned into a single GAL vector by cloning multiple *GAL1–10* sites into the plasmid (e.g., see Gerik *et al.*, 1997). Alternatively, or in addition, genes can be cloned into multiple 2μ plasmids, each with a different selectable marker, as these are readily maintained in yeast, (e.g., see Bylund and Burgers, 2005).

Overexpression is routinely carried out in the protease-deficient strain BJ2168 (*MAT*a, *ura3–52*, *trp1–289*, *leu2–3*, *112*, *prb1–1122*, *prc1–407*, *pep4-3*). In this strain, the *GAL1–10* promoter is strongly induced by the addition of galactose to the growth medium. However, using galactose as the only carbon source gives very poor growth. Instead, a combination of glycerol and lactate, allowing slow but satisfactory cell growth, is used as the carbon source prior to induction.

Media

Indicated quantities are per liter of media. Solid media contain in addition 20 g of agar. SCD: 1.7 g of yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g ammonium sulfate, 1 g amino acid drop-out mix (from a blended mixture of equal quantities of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine), 20 g glucose. When needed, 20 mg each of uracil and tryptophan, 50 mg of adenine, and 100 mg of leucine are added. SCGL: 1.7 g of yeast nitrogen base without amino acids and ammonium sulfate, 5 g ammonium sulfate, 1 g glucose, 30 ml glycerol, 20 ml lactic acid, and amino acids as for SCD. Prior to autoclaving the pH of the media is adjusted to 5–6 with concentrated sodium hydroxide. YPGLA: 10 g yeast extract, 20 g peptone, 2 g glucose, 30 ml glycerol, 20 ml lactic acid, 20 mg adenine. Prior to autoclaving, the pH of the media is adjusted to 5–6 with concentrated sodium hydroxide. If bacterial contamination of the media is a concern, kanamycin can be added to a final concentration of 50 μg/ml. Kanamycin will not affect the growth of yeast.

Procedure

Strain BJ2168 is transformed with the appropriate plasmids using the LiAc procedure. Transformants are selected on selective SCD plates and purified by restreaking on the same medium.

A single colony is streaked out onto an entire selective SCD plate and grown for 1–2 days at 30°.

A 150 ml starter culture of selective SCGL media is inoculated with a large amount of cells from the SCD plate and grown overnight with shaking at ~200 rpm.

The next morning, the starter culture generally has reached an OD_{660} of about 5. The starter culture is split over 6 × 1 liter selective SCGL, in 3 liter flasks, and growth is continued overnight with shaking.

Next morning the cultures generally have reached an $OD_{660} = 2–3$. To each flask, one liter of YPGLA is added, and growth is continued for another 2–3 h.

Expression is induced by adding 40 g of solid galactose to each 2-liter culture, and shaking is continued for 3–5 h.

The cells are harvested by centrifugation at 4000*g* for 10 min and washed with 500 ml of ice-cold water.

The cell paste is resuspended in 1–2 ml of ice-cold water per 5 g of wet cells, and frozen by slowly pouring with stirring into liquid nitrogen. This popcorn yeast is stored at −70°. Yields are typically 50–100 g of cells, wet weight, per 12 liters of culture.

Extract Preparation and Purification

Buffers and Inhibitors

Hepes-based buffers containing sodium or potassium chloride are used. For the purification of the checkpoint clamp/clamp loader complexes (Rad17/3/1 and Rad24-RFC, respectively) usage of the KCl buffer results in a better yield, whereas for the Ctf18-RFC and Elg1-RFC complexes, the NaCl buffer proved to be more suitable.

Composition of buffer 1 × HEP_{100}: 50 m*M* Hepes-NaOH/KOH (pH 7.5), 100 m*M* NaCl/KCl, 10% glycerol, 0.1% Tween-20, 3 m*M* DTT. The suffix refers to the m*M* NaCl/KCl concentration. For the preparation of the extract the buffer is made as 2× HEP_{100} (contains all components at twice the concentration of buffer 1× HEP_{100}) supplemented with a mixture of protease and phosphatase inhibitors (final concentration in 1× HEP_{100}: 1 m*M* EDTA, 1 m*M* EGTA, 2.5 m*M* Na-pyrophosphate, 1 m*M* β-glycerophosphate, 10 m*M* Na-bisulfite, 2.5 m*M* benzamidine, 5 μg/ml chymostatin, 5 μM pepstatin A, 10 μM leupeptin, and 1 m*M* PMSF).

Breakage of Cells with Dry Ice

In order to reduce the activity of proteases the frozen yeast cells are cracked open by blending the popcorn yeast with dry ice in a commercial blender (Waring Commercial Laboratory blender, Torrington, CT).

The stainless steel chamber is filled to 1/4-1/3 volume with crushed dry ice. Blending is started and continued until the dry ice is a fine powder.

Thereafter 2× HEP_{100} buffer, containing inhibitors, is poured on top of the dry ice in 20–30 ml portions and blending is continued between additions of buffer. The total volume of 2× buffer added should be equal to the amount of popcorn yeast in grams that will be cracked open. 70–100 grams of popcorn yeast plus buffer and dry ice is the maximum load for the model 51BL30 blender.

After addition of the popcorn yeast, blending is continued for 10 min. When the blender motor gives off a high whining noise, this is an indication that the blade is no longer properly blending the mixture, and manual mixing of the chamber contents becomes necessary. In general, every 20–30 sec, the blender is turned off and the sides scraped with a steel spatula. It also helps to knock the walls of the chamber with the spatula while blending. More dry ice is added if the chamber appears to warm up (melting on the sides).

Ammonium Sulfate Fractionation

The dry ice mixture is thawed at room temperature in a glass beaker, which is submerged in water. From now on all steps are performed at +4° or on ice.

Ammonium sulfate (4 *M*) is added to a final concentration of 200–300 m*M* to the extract. This step prevents the coprecipitation of DNA binding proteins with the nucleic acids during the next step.

Nucleic acids are precipitated from the cell extract by the addition of 45 μl of 10% Polymin P per ml cell extract, and the mixture gently stirred on ice for ~10 min.

Precipitated nucleic acids are pelleted at 40,000*g* for 45 min, in several tubes in a Sorvall SS34 rotor, and the pellets discarded.

The volume of the supernatant is measured and proteins are precipitated by slowly adding 0.35 g fine powdered ammonium sulfate per ml of extract while stirring on ice. When all of the ammonium sulfate has gone into solution, stirring on ice is continued for another 10 min.

The precipitated protein is pelleted at 40,000*g* for 45 min. The supernatants are discarded and the tubes are spun for an extra 5 min for residual supernatant removal. At this point the protein pellets can be frozen at −70°.

Glutathione Sepharose Chromatography

Glutathione Sepharose 4B (Amersham Biosciences), ~1 ml per 50 g of cells, is transferred to a column that should be able to hold at least 10 ml, and washed with 10 vol of MQ water.

The glutathione sepharose is equilibrated with at least 10 vol of HEP_{150}.

The protein pellets are carefully dissolved in a total of 25–50 ml of HEP_0 using a wide-bore pipette to stir and suck the suspension up and down. Avoid foaming of the protein solution.

Thereafter, the protein solution is diluted with HEP_0 until it reaches a conductivity of HEP_{150}. The final volume is usually 150–250 ml.

Equilibrated glutathione sepharose is transferred to the protein solution and GST-fusion proteins are batch bound to the glutathione-sepharose for 1–3 h by gentle rotation at 4°.

The sepharose is pelleted briefly at 300*g* for 1 min in a clinical centrifuge (equipped with a swing-out rotor) and the supernatant discarded (Fig. 2, lane 1).

The sepharose is transferred back into the column and washed with at least 10 vol of HEP_{150} (lane 2).

GST-fusion protein-containing complex is eluted with HEP_{150} supplemented with 0.05% ampholytes pH 3–10, 20 m*M* glutathione (reduced form), and protease inhibitors, omitting benzamidine and PMSF. Since glutathione is acidic, 5 *M* NaOH is added to adjust the pH to 8.0. The

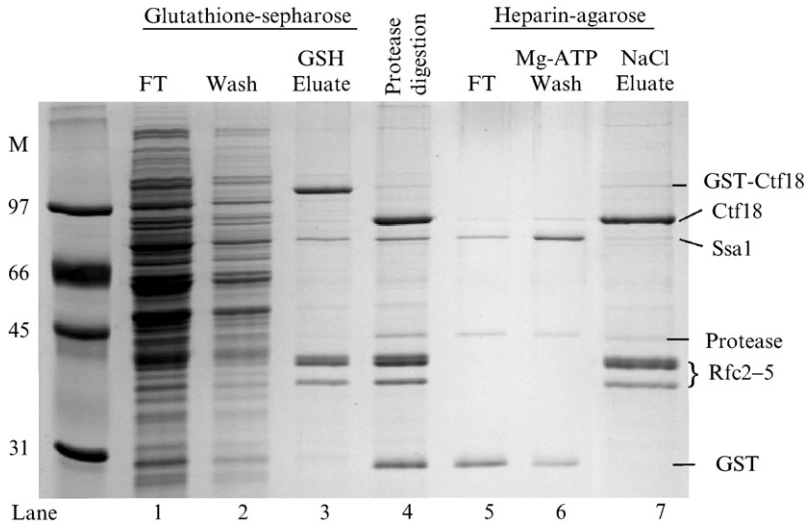

FIG. 2. Purification of a RFC-like clamp loader. During the course of purifying the 5-subunit Ctf18-RFC, samples were collected from the different purification steps and separated on a 13% polyacrylamide-SDS gel. FT, Wash, and GSH Eluate denote the flow through, wash, and elution, respectively, from the glutathione sepharose column (lanes 1–3). Lane 4, after PreScission protease treatment. Lanes 5–7 are the flow through, Mg-ATP wash, and peak eluate fraction, respectively, of the heparin column. Molecular weight marker proteins are indicated in the left lane of the gel.

HEP buffers are supplemented with 0.05% ampholytes to stabilize the clamp loaders (Gomes *et al.*, 2000). Benzamidine is omitted to allow detection of protein peaks at A_{280} during the subsequent FPLC purification step. PMSF is omitted since it has a tendency to precipitate on FPLC columns resulting in clogging of the columns.

To improve the efficiency of elution, 1 ml of elution buffer is mixed by pipetting with 1 ml of resin. After sitting for 15 min the column is drained, and the next ml of elution buffer mixed in. This procedure is repeated 4 times. Alternatively, the column can be hooked up to a peristaltic pump and ~5 ml of elution buffer very slowly pumped through at 100 μl/min (lane 3).

Proteolytic Removal of the GST-Tag

In order to cleave off the GST-tag from the GST-fusion proteins the glutathione column eluates are treated with human rhinovirus protease (commercially available as PreScission™ protease, Amersham Biosciences). The cleavage efficiency differs between different GST-fusion proteins and the optimal amount must be determined in a small-scale experiment. Meanwhile, the bulk of the preparation can be frozen on dry ice and stored at -70°.

Typically, a 5 ml fraction is treated with 5–25 units of PreScission protease overnight on ice.

Purification of Ctf18-RFC and Elg1-RFC

After overnight proteolytic cleavage, the treated fractions are pooled and diluted with HEP_0 to HEP_{100} (lane 4).

The protein is loaded on a 1 ml monoS column (Amersham Biosciences), equilibrated in HEP_{100}, for purification of the 7-subunit Ctf18-RFC complex or Elg1-RFC. The 5-subunit Ctf18-RFC is loaded on a 5 ml HiTrap Heparin column (Amersham Biosciences), equilibrated in HEP_{100}. Most of the GST and PreScission protease flow through (lane 5). The column is washed with 5–10 column vol of HEP_{100}.

The yeast chaperone Ssa1 purifies together with the overexpressed complexes both during glutathione-sepharose chromatography and MonoS or heparin chromatography. It is removed during the latter step by washing the column with 5–10 column vol of HEP_{100} containing 5 m*M* Mg-acetate and 100 μM ATP (lane 6). Subsequently the column is washed with 2 vol of HEP_{100}.

Protein is eluted from the MonoS column with a 15–20 ml linear gradient of 100–500 m*M* NaCl, Ctf18-RFC eluting at ~0.35 *M* NaCl and Elg1-RFC at ~0.3 *M* NaCl. The 5-subunit Ctf18-RFC complex is eluted

from the heparin column with a 20–30 ml linear gradient from 100–1000 m*M* NaCl, the complex eluting at ~0.5 *M* NaCl. 0.3–0.5 ml fractions are collected, analyzed by SDS-PAGE (lane 7), and stored frozen at −70°.

Complex-containing fractions are generally divided in smaller aliquots since activity is gradually lost upon repeated freezing and thawing. A tube is usually discarded after five uses.

Purification of Rad24-RFC and Rad17/Mec3/Ddc1

After overnight proteolytic cleavage, the treated fractions are pooled and diluted with HEP_0 to HEP_{125} (KCl).

The protein is loaded on a 1 ml monoQ column (Amersham Biosciences), equilibrated in HEP_{125}. Most of the GST and PreScission protease flow through. The column is washed with 10 column vol of HEP_{125}, followed by 5–10 column vol of HEP_{125} containing 5 m*M* Mg-acetate and 100 μM ATP, and 2 column vol of HEP_{125}.

Protein is eluted with a 15 ml linear gradient of 125–500 m*M* KCl. Rad24-RFC elutes at ~0.3 *M* KCl and Rad17/3/1 at ~0.25 *M* KCl.

Discussion

Saccharomyces cerevisiae has proved to be a very useful organism for the overproduction of not only yeast proteins but also other eukaryotic proteins, which in other organisms are hard or impossible to overexpress or purify. Available protease deficient yeast strains and the wide variety of inducible promoters, together with the development of several affinity chromatography techniques in recent years, has simplified the purification of eukaryotic multi-protein complexes. Of the several methodologies available to prepare extracts, we prefer the dry ice method because of the ease with which this procedure can be carried out on a relatively large scale while inhibiting proteolysis. The only caveat of this method is that it is associated with a drop in pH, generally to 6.5–7, however, buffers can be used to limit this pH drop (Burgers, 1999). Using this methodology together with a GST-affinity purification step, all known yeast clamp loader complexes were purified to close to homogeneity

The use of N-terminal GST-protein fusions for the overproduction and purification of various proteins/multi-protein complexes is a very valuable tool in the biochemical research field. The conditions used for column chromatography and protein elution are very mild, and are compatible with reducing agents such as DTT. This is an important consideration because the RFC-like complexes are rapidly inactivated in the absence of reducing agent. Generally, we use 3 m*M* DTT in our buffers. However,

there are some possible limitations to this approach that have to be taken into consideration.

First, the presence of the GST tag can alter the properties of the protein of interest that it is fused to. These include the size of GST itself (26.6 kDa) and the ability of GST to form dimers (Kaplan *et al.*, 1997). To overcome these potential problems GST can be proteolytically removed by the human rhinovirus protease, whose recognition site is often embedded in a linker in the cloning vectors, including the pRS420-GALGST series (Fig. 1B).

Second, the human rhinovirus protease has a unique recognition sequence. It cleaves between the glutamine and glycine in the amino acid sequence: Leucine-glutamic acid-X-leucine-phenyl alanine-glutamine-glycine-proline, where X is valine, alanine, or threonine. None of the many complexes we have purified thus far contained an internal cleavage site. Upon digestion of the GST-fusion protein, the released protein moiety of interest will, at the minimum, contain an N-terminal extension of GPEF, if the EcoRI site is used for cloning (Fig. 1B).

Third, when GST-fusion proteins are overexpressed in yeast, the Ssa1 chaperone often copurifies with these proteins. In order to remove this contamination, an ATP-Mg wash step can be included in the purification procedure, as indicated.

Acknowledgments

This work was supported in part by Grant GM32431 from the National Institutes of Health. G.O.B. was supported in part by a stipend from the Washington University-Umeå University Exchange program.

References

Bermudez, V. P., Lindsey-Boltz, L. A., Cesare, A. J., Maniwa, Y., Griffith, J. D., Hurwitz, J., and Sancar, A. (2003a). Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex *in vitro*. *Proc. Natl. Acad. Sci. USA* **100,** 1633–1638.

Bermudez, V. P., Maniwa, Y., Tappin, I., Ozato, K., Yokomori, K., and Hurwitz, J. (2003b). The alternative Ctf18-Dcc1-Ctf8-replication factor C complex required for sister chromatid cohesion loads proliferating cell nuclear antigen onto DNA. *Proc. Natl. Acad. Sci. USA* **100,** 10237–10242.

Burgers, P. M. (1999). Overexpression of multisubunit replication factors in yeast. *Methods* **18,** 349–355.

Bylund, G. O., and Burgers, P. M. (2005). Replication protein A-directed unloading of PCNA by the Ctf18 cohesion establishment complex. *Mol. Cell. Biol.* **25,** 5445–5455.

Cordingley, M. G., Callahan, P. L., Sardana, V. V., Garsky, V. M., and Colonno, R. J. (1990). Substrate requirements of human rhinovirus 3C protease for peptide cleavage *in vitro*. *J. Biol. Chem.* **265,** 9062–9065.

Ellison, V., and Stillman, B. (2003). Biochemical characterization of DNA damage checkpoint complexes: Clamp loader and clamp complexes with specificity for 5′ recessed DNA. *PLoS. Biol.* **1,** 231–243.

Gerik, K. J., Gary, S. L., and Burgers, P. M. (1997). Overproduction and affinity purification of *Saccharomyces cerevisiae* replication factor C. *J. Biol. Chem.* **272,** 1256–1262.

Gomes, X. V., Gary, S. L., and Burgers, P. M. (2000). Overproduction in Escherichia coli and characterization of yeast replication factor C lacking the ligase homology domain. *J. Biol. Chem.* **275,** 14541–14549.

Hanna, J. S., Kroll, E. S., Lundblad, V., and Spencer, F. A. (2001). *Saccharomyces cerevisiae* CTF18 and CTF4 are required for sister chromatid cohesion. *Mol. Cell. Biol.* **21,** 3144–3158.

Johnston, M., Flick, J. S., and Pexton, T. (1994). Multiple mechanisms provide rapid and stringent glucose repression of GAL gene expression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14,** 3834–3841.

Kaplan, W., Husler, P., Klump, H., Erhardt, J., Sluis, C. N., and Dirr, H. (1997). Conformational stability of pGEX-expressed Schistosoma japonicum glutathione S-transferase: A detoxification enzyme and fusion-protein affinity tag. *Protein Sci.* **6,** 399–406.

Majka, J., and Burgers, P. M. (2003). Yeast Rad17/Mec3/Ddc1: A sliding clamp for the DNA damage checkpoint. *Proc. Natl. Acad. Sci. USA* **100,** 2249–2254.

Majka, J., and Burgers, P. M. (2004). The PCNA-RFC families of DNA clamps and clamp loaders. *Progr. Nucl. Acids Res. Mol. Biol.* **78,** 227–260.

Mayer, M. L., Gygi, S. P., Aebersold, R., and Hieter, P. (2001). Identification of RFC(Ctf18p, Ctf8p, Dcc1p): An alternative RFC complex required for sister chromatid cohesion in S. cerevisiae. *Mol. Cell.* **7,** 959–970.

Shiomi, Y., Shinozaki, A., Sugimoto, K., Usukura, J., Obuse, C., and Tsurimoto, T. (2004). The reconstituted human Chl12-RFC complex functions as a second PCNA loader. *Genes Cells* **9,** 279–290.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene* **67,** 31–40.

Zou, L., Liu, D., and Elledge, S. J. (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc. Natl. Acad. Sci. USA* **100,** 13827–13832.

[2] Functional Assays for Replication Protein A (RPA)

By SARA K. BINZ, ANNE M. DICKSON, STUART J. HARING, and MARC S. WOLD

Abstract

Replication protein A (RPA) is a heterotrimeric, single-stranded DNA-binding protein. RPA is conserved in all eukaryotes and is essential for DNA replication, DNA repair, and recombination. RPA also plays a role in coordinating DNA metabolism and the cellular response to DNA damage. Assays have been established for many of these reactions. This

METHODS IN ENZYMOLOGY, VOL. 409 0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(05)09002-6