

Purification of a Soluble UmuD'C Complex from *Escherichia coli*

COOPERATIVE BINDING OF UmuD'C TO SINGLE-STRANDED DNA*

(Received for publication, January 19, 1996, and in revised form, February 25, 1996)

Irina Bruck[‡], Roger Woodgate[§], Kevin McEntee[¶], and Myron F. Goodman[‡]

From the [‡]Department of Biological Sciences, Hedco Molecular Biology Laboratories, University of Southern California, Los Angeles, California 90089-1340, the [§]Section on DNA Replication, Repair and Mutagenesis, National Institutes of Health, Bethesda, Maryland 20892-2725, and the [¶]Department of Biological Chemistry and Molecular Biology Institute, UCLA School of Medicine, Los Angeles, California 90024

The *Escherichia coli* UmuD' and UmuC proteins play essential roles in SOS-induced mutagenesis. Previous studies investigating the molecular mechanisms of mutagenesis have been hindered by the lack of availability of a soluble UmuC protein. We report the extensive purification of a soluble UmuD'C complex and its interactions with DNA. The molecular mass of the complex is estimated to be 70 kDa, suggesting that the complex consists of one UmuC (46 kDa) and two UmuD' (12 kDa) molecules. In contrast to its inability to bind to double-stranded DNA, UmuD'C binds cooperatively to single-stranded DNA as measured by agarose gel electrophoresis and confirmed by steady-state fluorescence depolarization. A Hill coefficient, $n = 3$, characterizes the binding of UmuD'C to M13 DNA and to a 600 nucleotide DNA oligomer, suggesting that at least three protein complexes may interact cooperatively when binding to DNA. The apparent equilibrium binding constant of UmuD'C to single-stranded DNA is approximately 300 nM. Binding of the complex to a short, 80 nucleotide, DNA oligonucleotide was detectable by fluorescence depolarization, but it did not appear to be cooperative. Binding of UmuD'C to single-stranded M13 DNA causes an acceleration of the protein-DNA complex, suggesting that the longer DNA may undergo compaction. The UmuD'C complex associates with RecA-coated DNA, and the UmuD'C complex remains bound to DNA in the presence of RecA.

The SOS regulon present in *Escherichia coli* is triggered as part of the cell's response to exogenous DNA damage (1, 2). As a consequence of attempting to replicate past DNA lesions, an inducing signal is generated that results in the activation of RecA protein (RecA*)¹ (3). In its activated state, RecA* enhances the self-cleavage of the LexA transcriptional repressor (4), thus leading to the expression of more than 20 proteins that help the cell to avoid the lethal effects of DNA damage. Recent reviews of the SOS response are contained in Refs. 5 and 6.

An important, yet poorly understood, feature of the SOS response is error-prone repair or "SOS-induced" mutagenesis.

The UmuD and UmuC proteins are essential participants in this process (7, 8), and the absence of either protein reduces mutagenesis by more than 100-fold to spontaneous background levels (2, 9). The UmuDC operon is induced following LexA cleavage, and the process is further regulated by the need for the UmuD protein to undergo a RecA*-mediated cleavage. This event is mechanistically similar to LexA autodigestion, but in the case of UmuD (and its homologs), cleavage leads to activation of its mutagenesis function(s) (10–12). Activated UmuD' forms homodimers and associates with UmuC to form a UmuD'C complex (13). Both UmuD' and UmuC are believed to interact with RecA* (14, 15) in such a way as to target the relatively small number of mutagenically active UmuD'C molecules to lesions within DNA (15–17).

Replicative bypass of the RecA*-UmuD'C-coated lesion is most likely performed by pol III holoenzyme (18, 19). Although pol II is induced in response to DNA damage as part of the SOS regulon (20, 21), its role in either lesion bypass or DNA repair has yet to be firmly established. Recent data suggest that pol II may be required for the bypass of abasic lesions provided that heat shock proteins are not induced (22). A two-step model describing error-prone synthesis was proposed by Bridges and Woodgate (23, 24) in which pol III incorporates a nucleotide opposite a template lesion but cannot continue synthesis. Generation of a UmuD'C-RecA* "mutasome" would enable the stalled pol III molecule to continue synthesis past the lesion (5, 13).

The ability of *E. coli* to facilitate error-prone translesion synthesis depends upon the cellular levels of the Umu proteins. Under normal conditions the proteins are expressed at low levels (25). Genetic experiments in which the *in vivo* levels of the UmuD'C proteins have been artificially manipulated leads to a variety of phenotypes. Overproduction of the Umu proteins in fully SOS-induced cells can cause a cold-sensitive phenotype that is associated with the rapid cessation of DNA replication (26). Expression of excess UmuC results in a nonmutable phenotype similar to what is seen when there is a deficiency of UmuC (27). Modest overproduction of UmuD'C appears to inhibit recombinational functions of RecA and promotes a switch from error-free recombinational repair pathways to those that are error prone (5, 28).

Testing of these models has been severely hampered by the lack of reconstituted "error-prone" and "error-free" lesion bypass assays. While pol III holoenzyme, RecA, and UmuD' have been extensively purified and characterized, UmuC has been more difficult to study because of its apparent insolubility and its lack of defined enzymatic activity. Previous studies of UmuC and UmuD'C complexes were performed with UmuC that had been purified from a denatured form and renatured in the presence of chaperone proteins (13, 29). In this paper we

* This research was supported by National Institutes of Health Grants GM21422, GM42554, AG11398, and GM29558. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The abbreviations used are: RecA*, RecA protein activated by the presence of either ATP or ATP_γS; ATP_γS, adenosine 5'-O-(3-thiotriphosphate); UmuD'C, a complex consisting of 2 UmuD' and 1 UmuC molecules believed to be the complex required to observe SOS-induced mutagenesis; pol III, DNA polymerase III from *E. coli*; pol II, *E. coli* DNA polymerase II; ssDNA and dsDNA, single- and double-stranded DNA and DNA, respectively.

describe the first purification of a soluble, intact UmuD' C complex and characterize its interactions with DNA.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Plasmids—The UmuD' C complex and UmuC proteins were purified from *E. coli* K-12 strain RW82F'IQ (30) containing plasmids expressing either UmuD' C, or UmuC alone, from the p_{lac} promoter. The UmuC overproducing plasmid, pRW282, was constructed by cloning a 1594-base pair *Eco*RI fragment from pRW124 (27) into p_{lac} expression vector pKK223-3 (Pharmacia Biotech Inc.). The UmuD' C overproducing plasmid, pOS1, was constructed in two steps. First, *umuD'* was placed under p_{lac} control by ligating an 801-base pair *Eco*RI-*Hind*III fragment from pEC42 (15) into pKK223-3-generating plasmid pEC44. The second step, in which the *umuC* gene was reconstructed, was achieved by cloning a ~2-kilobase pair *Mlu*I-*Sca*I fragment from pRW124 (27) into the *Mlu*I-*Hind*III (blunt-ended) vector pEC44.

Purification of UmuD' C Complex—A 30-liter culture of RW82(pOS1) was grown in Tryptone-phosphate-rich medium (2% tryptone, 0.8% NaCl, 1.5% yeast extract, 0.2% Na₂HPO₄, 0.1% KH₂PO₄) at 37 °C supplemented with 100 µg/ml ampicillin, 70 µg/ml chloramphenicol, and 50 µg/ml kanamycin until it reached $A_{600} = 0.8$. The overproduction of UmuD' C operon was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside. The culture was allowed to overproduce overnight (about 16 h).

Cells were harvested and resuspended in equal weight/volume (gm/ml) amount of storage buffer (50 mM Tris-HCl (pH 7.5), 10% sucrose, 10 mM EDTA). The cells were then quickly frozen by dropwise addition of cell suspension into liquid nitrogen and stored at -70 °C. Cells were thawed at 4 °C in 2.5 volumes of lysis buffer (50 mM Tris-HCl (pH 7.5), 10% sucrose, 0.1 M NaCl). Once thawed, lysozyme was added to a final concentration of 0.4 mg/ml. The cell slurry was incubated for 1 h at 4 °C followed by a 4-min incubation at 37 °C and centrifuged at 11,800 rpm for 1 h in a Sorvall GSA rotor.

A solution of 10% polyethyleneimine HCl (pH 7.6) was added to lysate supernatant in 10-ml portions to a final concentration of 1.1%. The solution was stirred for 15 min after each addition. At the end of the last addition, the suspension was kept on ice for 20 min, and the precipitate was collected by centrifugation for 10 min at 9,000 rpm in a Sorvall SS34 rotor. Proteins were extracted by stirring the pellet for 15 min in 50 ml of R buffer (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) containing 1 M NaCl. Following centrifugation (10 min at 6,000 rpm) the supernatant was collected and the extraction repeated until all proteins were eluted from the pellet.

Supernatants were combined and dialyzed overnight against R buffer containing 50 mM NaCl (from this point on the concentration of glycerol was increased to 20% glycerol (v/v)). The dialyzed sample was loaded onto a 200-ml DEAE-Sephacel (Pharmacia) column (3 × 15 cm) at a flow rate of 1 ml/min. The column was washed with 2 column volumes of R buffer containing 50 mM NaCl, and protein was eluted from the column with three column volumes of R buffer containing 500 mM NaCl.

Fractions containing high concentrations of UmuD' C were combined, dialyzed overnight against R buffer containing 250 mM NaCl, loaded onto a 150–170-ml phosphocellulose (Whatman) column (3 × 24 cm), and extensively washed. UmuC was eluted from the column with 2 volumes of R buffer containing 1 M NaCl. Fractions containing protein were combined and concentrated to 6 ml using Centrplus-30 concentrators (Amicon), and the total protein was loaded onto a 600-ml Superdex-75 (Pharmacia) column (2 × 160 cm) equilibrated with R buffer containing 1 M NaCl. The column was run at a flow rate of 0.25 ml/min, and 2.0-ml fractions were collected. Fractions highly enriched for UmuD' C were combined and concentrated using Centrplus-30 concentrators to final concentration of at least 1 mg/ml. These fractions were divided into aliquots (100 µl) and quickly frozen at -70 °C. A final yield of 2–4 mg of UmuD' C complex was obtained from 30 liters of cells.

Microsequencing—The purified UmuD' C was loaded in duplicate on a 10% SDS-polyacrylamide gel and transferred onto a ProBlot membrane (Applied Biosystems).

Microsequencing was performed as suggested (31) on one sample, while the other was processed for Western blot analysis with UmuC antiserum. Amino-terminal analysis for putative UmuC was performed by Lynn Williams (USC Microchemical Facilities) and found to be: NH₂-MFALXDVFYASXE (X represents an unidentified residue), in agreement with the reported DNA sequence for UmuC (32, 33).

DNA Gel Mobility Shift Assay—The UmuD' C complex was incubated with either ssM13mp18 (NEB) or ss600-mer (*Hinf*I restriction digest

product of multiprimed ssM13mp18) in binding buffer (30 µl of 20 mM Tris-HCl (pH 7.5), 8 mM magnesium acetate, 4% glycerol) for 20 min at 30 °C. In all of the titration experiments, the salt concentration of the reaction was held at around 50 mM NaCl. All protein dilutions were done in 20 mM Tris-HCl (pH 7.5), 40 µg/ml bovine serum albumin, 5 mM dithiothreitol, 4% glycerol. An aliquot of each binding reaction (20 µl) was loaded onto 0.9% agarose gel and separated in LTAE buffer (6.7 mM Tris-HCl, 3.3 mM sodium acetate, 1 mM EDTA, (pH 7.5)) with buffer recirculation (29). Upon completion of electrophoresis the gel was soaked in transfer buffer (25 mM Tris base, 190 mM glycine, 0.04% SDS, (pH 8.5)) and electrotransferred to nitrocellulose membrane. Protein blots were incubated with rabbit polyclonal UmuC or UmuD' C antiserum followed by incubation with goat anti-rabbit IgG peroxidase conjugate (Pierce) and visualized by the ECL detection system (Amersham Corp.) according to manufacturer's directions. Properly exposed film (Hyperfilm ECL, Amersham) was subjected to densitometric analysis using a Hoefer scanning densitometer GS300. Different exposures were used to ensure that band intensities were within the linear range of the film.

Molecular Weight Determination by Gel Filtration—Gel filtration analysis was performed using an analytical Sephadex G-100 column equilibrated in R buffer + 1.0 M NaCl. Pharmacia LMW (low molecular weight) calibration kit was used for protein standards. K_{av} was calculated using $K_{av} = (V_e - V_0)/(V_t - V_0)$; where V_e is elution volume for the protein, V_t is total bed volume, and V_0 is column void volume or elution volume for blue dextran 2000. V_0 for the 86-ml column was 35 ml. ECL immunodetection was used to locate UmuC in the UmuC-only column fractions.

Etheno-M13 DNA/Rhodamine-X 80-Mer—Single-stranded M13mp18 DNA was purified by the hexadecyltrimethylammonium bromide method (34), extracted with phenol/chloroform twice, and ethanol-precipitated. The protocol used to make etheno-M13 DNA was based on methods described previously (35, 36). The reaction was as follows; 400 µg of ssM13mp18 DNA was reacted in 2 ml of 20 mM potassium phosphate (pH 7.0) and 0.2 M chloroacetaldehyde (Aldrich) at 37 °C for 48 h. The slightly yellow product was dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA overnight at 4 °C followed by dialysis at 50 °C for 7 h to bring derivatized bases to maturation. The final product had an absorption maximum at 264 nm (broad maximum 250–267 nm) and an absorption minimum at 244 nm with $A_{270}/A_{260} = 1.03$ (starting material absorption minimum was 230 nm and $A_{270}/A_{260} = 0.84$ (37)). The concentration of etheno-M13 DNA was determined using an extinction coefficient $A_{260} = 7000 \text{ M}^{-1} \text{ cm}^{-1}$, as described previously (37, 38). Rhodamine-X (Molecular Probes) labeling of the 80-mer oligonucleotide was carried out as described (39).

Fluorescence Intensity and Anisotropy Measurements—All fluorescence intensity and anisotropy experiments were performed with a PTI Quantamaster model QM-1 fluorimeter. Reactions were performed in an 180-µl ultra-microcuvette (Hellma) at 21 °C. Reaction buffer used in all titrations was 20 mM Tris-HCl (pH 7.9), 8 mM magnesium acetate, 4% glycerol. Proteins were allowed to bind for 1 min before measurements were taken. For both measurements, the excitation wavelength was selected to be 315 nm and emission wavelength was monitored at 410 nm with a 6 nm bandpass. The excitation wavelength was selected for lowest interference from intrinsic protein fluorescence and highest signal emission by etheno-M13 DNA. Intensity correction calculations were made by subtracting UmuD' C complex titration on unmodified ssM13 DNA from intensity measurements of UmuD' C complex titration on etheno-M13 DNA. Anisotropy was determined by using a vertically oriented polarizer for the incident beam and a vertical and 90° rotation of the emission polarizer (dichroic sheet polarizers, Oriol). Three anisotropy measurements of 10-s duration were averaged for each titration point. Contaminating fluorescence signal from buffer was subtracted from all measurements. The fluorescence anisotropy is $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$; r_f and r_b refer to anisotropies of free and bound fluorophores, and f_f and f_b are the fraction of total fluorescence due to each form of the fluorophore. The fraction of etheno-DNA bound by UmuD' C complex (f_b) was calculated using $f_b = (r - r_f) / (r_b - r_f + r - r_f)$ (where $R = q_b/q_f$, the ratio of the quantum yield of the bound and free fluorophore). This equation corrects for the decrease in quantum yield of etheno-DNA when UmuD' C binds DNA (40).

SDS-Polyacrylamide Gel Electrophoresis—Proteins were separated in a SDS-15% polyacrylamide gel according to Laemmli (41). Proteins were visualized following a 20-min staining with a 10% solution of Coomassie Brilliant Blue R-250 in 20% methanol, 10% acetic acid and destaining in a solution of 10% methanol, 10% acetic acid. Fractionated proteins were transferred to nitrocellulose, probed with antiserum against UmuD' C or UmuC and visualized using an ECL detection

system as described above. Polyclonal antibodies were raised in rabbits to affinity purified UmuC protein (27) and UmuD/D' proteins (13) by Hazelton Laboratories (Washington, D. C.). In all immunodetection experiments, antiserum was used at dilution of 1:20,000.

Protein Determination—Protein concentrations were measured according to the Bradford assay (Bio-Rad protein assay kit) using bovine plasma γ -globulin (protein assay standard I, Bio-Rad) as a standard.

RESULTS

Previous attempts to purify UmuC involved extracting insoluble UmuC protein from inclusion bodies, solubilizing in the presence of 8 M urea and subsequently refolding of the purified polypeptide using either S9 ribosomal protein (13) or by sequential incubation with Hsp70 and Hsp60 heat shock chaperones (29). The effects of denaturation and renaturation on enzymatic and biochemical properties of UmuC are not known. Therefore, a major goal of this study was to purify and characterize a complex containing UmuC and UmuD' (UmuD'C complex) while maintaining the complex in a soluble form throughout purification.

Purification of a UmuD' C Complex—The UmuD'C proteins were overexpressed and purified from an *E. coli* K-12 strain (RW82F'IQ) (30) carrying a chromosomal deletion of the *umuDC* operon (42). Overproduction of UmuD'C in its native form was accomplished by placing the operon on a plasmid under *ptac* control. Typical yields of UmuD'C from a 30-liter culture (150 g wet cell weight) were 2–4 mg. We estimate that the recovery of soluble UmuC is 5–10-fold greater in cell extracts made from strain RW82F'IQ compared with the strain (MC1000 containing pRK248(λ Clt₈) and pSB13) originally described for UmuC overproduction (11). Most of the soluble material obtained from crude cell lysates that cross-reacts with anti-UmuC antiserum was comprised of a tightly bound protein complex containing UmuC and UmuD' (*i.e.* UmuD'C complex, see Fig. 1). RecA was not present in the complex based on the absence of detectable cross-reactivity to anti-RecA antibody.

In the initial purification step, nucleic acids were precipitated by addition of polyethyleneimine. UmuC and UmuD' coprecipitated with the nucleic acid and were extracted in the presence of 1 M salt and dialyzed into 50 mM NaCl (see "Experimental Procedures"). The remaining purification steps involved separation on DEAE-Sephacel, phosphocellulose, and Superdex-75 columns (see "Experimental Procedures"). UmuD'C was present in 1 M NaCl following fractionation on Superdex-75 and attempts to reduce the salt concentration subsequent to gel filtration, *e.g.* by dialysis, were hampered by the formation of an insoluble protein precipitate containing both UmuC and UmuD'.

The UmuD'C fractions from each stage of purification were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by Western immunodetection (Fig. 1). The UmuD'C complex appeared to be greater than 80% pure (based on integration of densitometric band intensities in the linear range) following fractionation with Superdex 75 (Fig. 1A, lane 5). Microsequencing was used to confirm the true identity of the soluble UmuC protein (see "Experimental Procedures"): 15 residues identified from the NH₂ terminus coincided with the published UmuC amino acid sequence (32, 33).

Antibodies to both UmuD' and UmuC showed that at least a fraction of the overproduced UmuD' protein copurified with UmuC (Fig. 1B). Gel filtration of the phosphocellulose pool on a Superdex-75 column in 1 M NaCl failed to separate UmuD' from UmuC and analytical gel filtration using a Sephadex G100 column in the presence of 1 M NaCl, suggested that the proteins associated in a complex with a molecular weight of approximately 70 kDa (Fig. 2). This molecular mass is consistent with a composition of one UmuC (46 kDa) and two UmuD' (12 kDa) molecules (13). The elution volumes of the protein

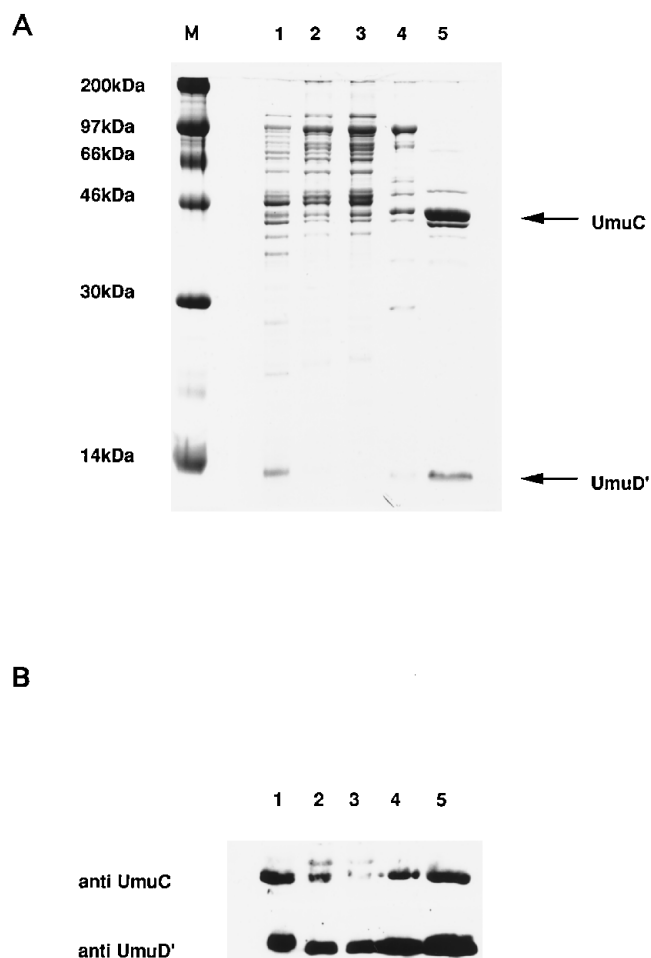


FIG. 1. Purification and detection of UmuD'C complex. Fractions from each purification step were separated on a 15% SDS-polyacrylamide gel. **A**, Coomassie Brilliant Blue G-250-stained purification gel. **Lane M**, prestained molecular weight markers; **lane 1**, crude lysate (20 μ g of total protein); **lane 2**, polyethyleneimine extract (20 μ g of total protein); **lane 3**, DEAE pool (20 μ g of total protein); **lane 4**, phosphocellulose pool (10 μ g of total protein); **lane 5**, Superdex 75 concentrated pool (10 μ g of total protein). **B**, Western immunodetection of the proteins in the purification steps. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with either UmuC antiserum or UmuD'.D antiserum, and visualized with enhanced chemiluminescence. **Lane 1**, crude cell lysate; **lane 2**, polyethyleneimine extract; **lane 3**, DEAE pool; **lane 4**, phosphocellulose pool; **lane 5**, Superdex 75 concentrated pool. Five micrograms of each protein fraction were loaded.

standards (Fig. 2) were determined by running a mixture of the standards alone and in the presence of the Superdex 75 fraction. It was possible to purify a small amount of soluble UmuC from RW82F'IQ harboring a plasmid that overproduced UmuC in the absence of UmuD'. Elution of UmuC on an analytical Sephadex G100 column gave a value of K_{av} slightly larger than that of ovalbumin (43 kDa). The apparent molecular weight for UmuC in the range of 45–47 kDa is in agreement with a previous determination (13).

The properties of UmuC and the UmuD'C complex were investigated using a low ionic strength native agarose gel. Proteins were resolved by gel electrophoresis, transferred to nitrocellulose, and incubated with UmuC antiserum. UmuC is a basic protein having a calculated isoelectric point of about 9.6 based on amino acid composition. Therefore, UmuC should migrate toward the cathode in an agarose gel. When UmuC and two highly acidic UmuD' molecules ($pI \sim 4.5$) combine to form a UmuD'C complex, migration should be toward the anode. A fraction of soluble UmuC, concentrated after purification

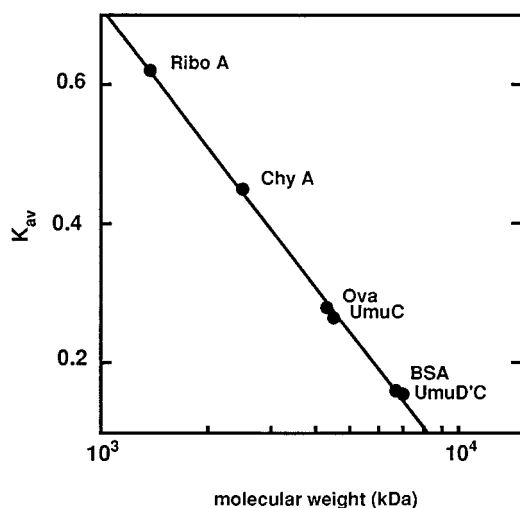


FIG. 2. **Size analysis of UmuD' C complex.** The purified UmuD' C complex was gel-filtered on a Sephadex G-100 column, as described under "Experimental Procedures." The apparent molecular weights of the UmuD' C protein complex and UmuC protein were estimated based on the migration of protein standards of known molecular weight. The position of gel filtration standards was determined separately from purified protein runs. Ribonuclease A (*Ribo A*) (13.7 kDa), chymotrypsinogen A (*Chy A*) (25 kDa), ovalbumin (*Ova*) (43 kDa), bovine serum albumin (*BSA*) (67 kDa).

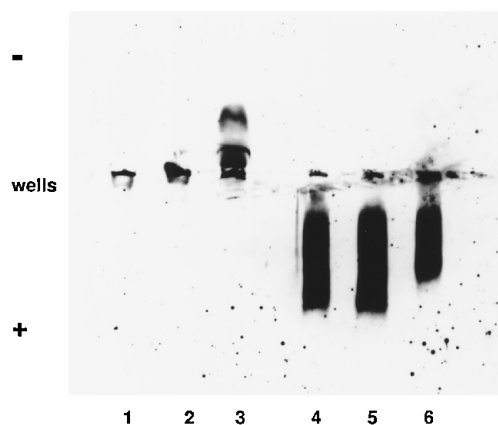


FIG. 3. **Charge-dependent gel mobility of the UmuD' C complex.** UmuC protein and UmuD' C protein complexes were electrophoresed on a 0.9% agarose gel and immunodetected with UmuC antiserum. *Lane 1*, UmuC protein untreated; *lane 2*, UmuC protein incubated with DNase I (RNase-free); *lane 3*, UmuC protein incubated with RNase A (DNase-free); *lane 4*, UmuD' C protein complex untreated; *lane 5*, UmuD' C protein complex incubated with DNase I (RNase-free); *lane 6*, UmuD' C protein complex incubated with RNase A (DNase-free).

through a phosphocellulose column (see "Experimental Procedures"), migrated toward the cathode, but only after treatment with RNase (DNase-free) (Fig. 3, *lane 3*). An untreated sample of UmuC or one treated with DNase (RNase-free) remained in the wells (*lanes 1* and *2*, respectively). It appears that in the absence of UmuD', and especially when UmuC was extracted from a crude cell lysate at low salt concentration (~50 mM NaCl), it was tightly bound to RNA. The UmuD' C complex migrated toward the anode as expected (Fig. 3, *lane 4*), and this pattern was essentially unaffected by treatment with DNase or RNase (Fig. 3, *lanes 5* and *6*, respectively).

Cooperative Binding of UmuD' C to ssDNA—Single-stranded M13 DNA and a 600-nucleotide ssDNA product, prepared from a restriction digest of partially double-stranded M13 DNA (see "Experimental Procedures"), were used to measure the binding of UmuD' C. UmuD' C was incubated at varying ratios with

ssDNA, and reactions were analyzed by agarose gel electrophoresis followed by Western blotting with anti-UmuC and anti-UmuD' antiserum. In separate experiments staining of the gels with ethidium bromide prior to electroblotting was used to show that the position of the bound UmuD' C, *i.e.* UmuD' C incubated in the presence of ssDNA migrated as a protein-DNA complex.

The results indicate that UmuD' C bound cooperatively to a single-stranded 600 mer (Fig. 4). No detectable protein was bound at low UmuD' C/DNA ratios (Fig. 4A, *lanes 1–3*) while in the "steep binding" range, binding increased from about 1 UmuD' C bound per 300 nucleotides to about 1 per 50 nucleotides. Saturation of the 600-mer occurred when approximately one UmuD' C was bound per 20 nucleotides. Binding of UmuD' C to the ss600-mer resulted in measurable retardation as a function of increasing UmuD' C/DNA ratios. To quantitate the binding of UmuD' C to the ss600-mer, gel band intensities corresponding to free UmuD' C and UmuD' C complexed to DNA were integrated in the linear region of the film (see "Experimental Procedures"). A logarithmic Hill plot was fit by a straight line with a slope corresponding to a Hill coefficient $n = 3$ (Fig. 4B). We estimated a value of $K_D = 260$ nM for the apparent binding constant of the UmuD' C complex to the 600-mer.

The qualitative binding profile of UmuD' C to M13 DNA was similar to that for the 600 mer. There was no detectable binding of UmuD' C to the DNA at low UmuD' C/DNA ratios (Fig. 5, *lanes 1–5*). Saturation of the M13 DNA (7250 nt) was difficult to quantitate in the gel binding study, caused, in part, by an accelerated migration of the UmuD' C-M13 DNA complex; the maximum clear attainable binding was roughly 1 UmuD' C per 100 nucleotides occurring at an input level of about 1 UmuD' C per 40 nucleotides. Since the binding measurements were carried out using salt concentrations near 50 mM NaCl, the high salt concentration of the UmuD' C sample coupled with the requirement to maintain a low reaction volume limited the amount of UmuD' C that could be used in attempting to saturate the M13 DNA.

A more sensitive measurement for the binding of UmuD' C to M13 DNA was performed using fluorescence depolarization, see *e.g.* Refs. 40 and 43. In this assay, the protein was incubated in the presence of chemically modified M13 DNA containing a random distribution of the fluorescent base etheno-adenine and to a lesser extent ethenocytosine (see "Experimental Procedures"). Protein-DNA binding was observed as an increase in the steady-state anisotropy of the protein-DNA complex with increasing amounts of UmuD' C. Much more protein can be used in the fluorescence assay because of the increased reaction volume (180 μ l) compared with the gel assay (30 μ l). We were able to observe a saturation in the fraction of UmuD' C bound to M13 DNA (Fig. 6). The data fit by a Hill plot gives an apparent binding constant in the range of 300–350 nM and a slope of 3, similar to the Hill coefficient determined for the 600-mer. A plot for the increase in fluorescence intensity with increasing UmuD' C overlapped with the anisotropy plot offering independent evidence for the cooperative nature of the binding of UmuD' C to DNA (data not shown). In contrast to the retardation observed when UmuD' C was bound to a 600-mer, binding of UmuD' C to M13 DNA appeared to accelerate migration of the protein-DNA complex on an agarose gel at high UmuD' C/DNA nucleotide ratios (Fig. 7) suggesting that the longer DNA may undergo compaction.

We observed no detectable binding of UmuD' C to ssDNA less than 600 nucleotides long in the agarose gel assay. However, using fluorescence depolarization we observed binding of UmuD' C to an 80-mer, and in contrast to the binding of

A

FIG. 4. Cooperative binding of the UmuD'C protein complex to an ss600-mer. A, the UmuD'C-600-mer complex was separated on a 0.9% agarose gel and immunovisualized with UmuC antiserum. Each lane represents addition of UmuD'C in increasing increments from 25 to 450 nM added protein complex with a constant amount of 600-mer (5.4 nM). The last lane on the right-hand side is free UmuD'C complex. B, Hill plot for the data in A. Y is the amount of bound UmuD'C for a given input concentration (UmuD'C), Y_{\max} is the maximum amount of UmuD'C complex bound to the DNA. The slope of the line fit by linear least squares is the Hill coefficient, $n = 3$.

FREE UmuD'C
600 MER/ BOUND UmuD'C

UmuD'C

B

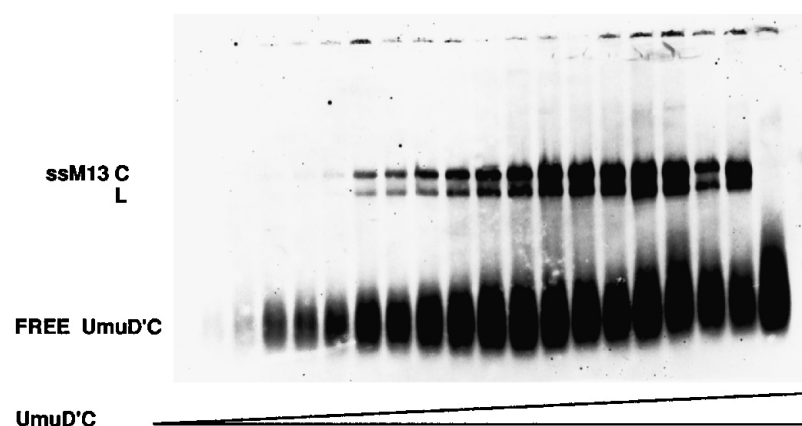
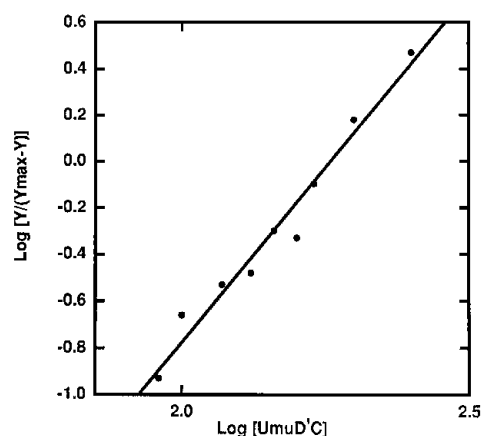


FIG. 5. Cooperative binding of the UmuD'C protein complex to ssM13 DNA. The UmuD'C-ssM13 complex was separated on a 0.9% agarose gel and immunovisualized with UmuC antiserum. Each lane represents addition of UmuD'C in increasing increments from 25 to 200 nM added protein complex with constant amount of ssM13 DNA (0.8 nM). The last lane on the right-hand side is free UmuD'C complex. M13 DNA C and L forms on the left-hand side refer to circular and linear forms of ssM13 DNA, respectively.

UmuD'C to either the 600-mer or to M13 DNA, binding to the 80-mer did not appear to be cooperative (data not shown). The K_D for binding the 80-mer was near 150 nM, and ATP was not required for protein-DNA binding. We were unable to detect any significant difference in the binding of UmuD'C to either UV-irradiated or unirradiated M13 single-stranded DNA using the agarose gel assay, and binding of UmuD'C to irradiated DNA did not protect against degradation by T4 endonuclease V (data not shown). No detectable binding of UmuD'C was observed either to UV-irradiated or unirradiated double-stranded DNA (data not shown).

Binding of UmuD'C to ssDNA in the Presence of RecA*—Two experiments were performed to visualize the binding of

UmuD'C to M13 ss DNA in the presence of activated RecA protein: preincubation of RecA with DNA followed by the addition of UmuD'C (Fig. 8A) and preincubation of UmuD'C with DNA followed by addition of varying levels of RecA* (Fig. 8B). An agarose gel was used to separate the UmuD'C-ssDNA (Complex I), UmuD'C-RecA*-ssDNA (Complex II) and free UmuD'C. Complexes containing UmuD'C were visualized using UmuC antiserum, and bound and free ssDNA were located by staining with ethidium bromide.

The UmuD'C-M13 DNA complex formed in the absence of RecA* caused an acceleration of the DNA (Fig. 8A, lane 1, Complex I). Under these conditions, the amount of UmuD'C complex in the reaction was limiting as all of it appeared to be

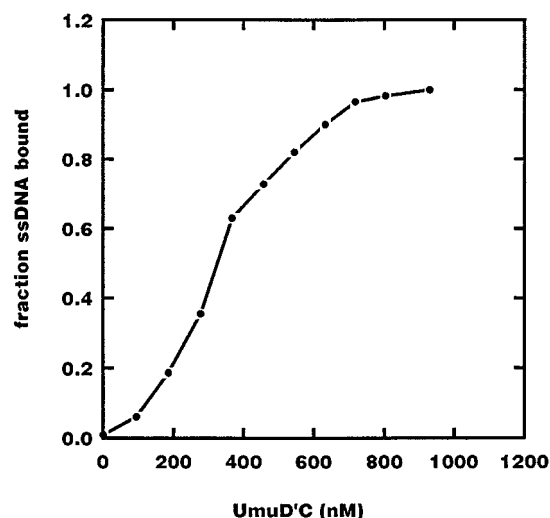


FIG. 6. **Cooperative binding of the UmuD'C complex to etheno-M13 DNA.** Titration of the UmuD'C complex (0.1–1.0 μ M) on etheno-M13 DNA (1.0 nM) was performed as described under "Experimental Procedures." The calculation of the fraction of UmuD'C bound to DNA obtained from the raw anisotropy data is described under "Experimental Procedures."

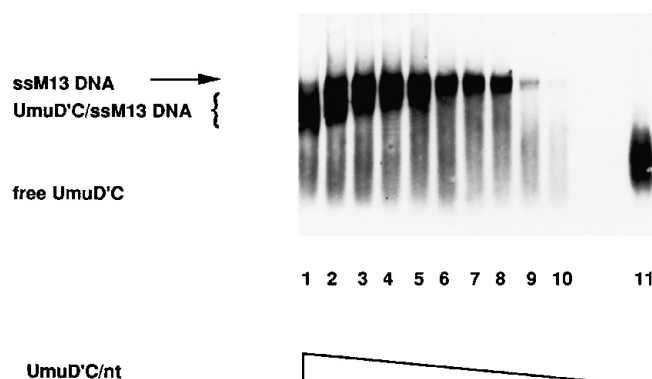


FIG. 7. **Accelerated mobility of the UmuD'C-ssM13 complex in agarose gels.** Binding of UmuD'C to ssM13 DNA is shown as a function of DNA concentration. UmuD'C-ss M13 DNA complexes were separated on a 0.9% agarose gel and immunodetected with UmuC antiserum. A constant concentration of UmuD'C (300 nM) complex was used in each lane in which ssM13 DNA concentration (nucleotide ssM13 DNA) was increased as follows: lane 1, 560 nM ssM13 DNA; lane 2, 1.1 μ M ssM13 DNA; lane 3, 1.6 μ M ssM13 DNA; lane 4, 2.2 μ M ssM13 DNA; lane 5, 2.8 μ M ssM13 DNA; lane 6, 3.2 μ M ssM13 DNA; lane 7, 3.9 μ M ssM13 DNA; lane 8, 4.5 μ M ssM13 DNA; lane 9, 5 μ M ssM13 DNA; lane 10, 5.6 μ M ssM13 DNA; lane 11, represents migration of the free UmuD'C complex.

bound to the ssDNA. Addition of excess UmuD' to the reaction gave an essentially similar result, although there was a slight increase in the amount of detectable free UmuD'C complex (Fig. 8A, lane 2). In contrast, however, when RecA was preincubated with DNA (in the presence of ATP γ S) to form a RecA*-nucleoprotein filament and UmuD'C subsequently added to the reaction, a retarded complex was observed (Complex II) that is consistent with it being a UmuD'C-RecA*-DNA complex (Fig. 8A, lane 3). Further addition of excess UmuD' to the reaction had no measurable effect on the formation of complex II (Fig. 8A, lane 4). It is interesting to note that under these conditions, where a significant fraction of the ssDNA would be expected to be bound by RecA*, there was a significant increase in the amount of free UmuD'C complex (Fig. 8A, lanes 3 and 4). This result suggests that UmuD'C is unable to compete RecA from ssDNA once a RecA*-nucleoprotein filament has formed.

Preincubation of UmuD'C with DNA followed by the addi-

tion of three different levels of RecA produced a series of complexes that were consistent with UmuD'C-DNA complexes associated with variable amounts of RecA protein (Fig. 8B, lanes 2–4). Since UmuD'C remained bound to the DNA following the addition of RecA, and UmuD'C was limiting in the reactions, it is likely that RecA does not compete off the preassembled UmuD'C-DNA complex but simply binds regions free from UmuD'C. The complex formed by preincubating DNA with RecA (Fig. 8A, lanes 3 and 4) migrated more slowly than the complex II formed by preincubation with UmuD'C (Fig. 8B, lanes 2–4), suggesting that prior binding of UmuD'C to DNA limited the ability of RecA to form a nucleoprotein filament.

DISCUSSION

Previously, Echols and co-workers succeeded in purifying denatured UmuC (13); upon renaturation it was shown that UmuC bound ssDNA (29). Although these results represented an important initial step for reconstituting lesion bypass *in vitro* (44), it is necessary to isolate and purify UmuC alone or complexed with UmuD' in soluble form to investigate the full range of biological interactions of these proteins.

We coexpressed the UmuD'C proteins from a ptac promoter and purified the UmuC protein from a strain carrying a deletion of the entire chromosomal *umuDC* operon. Under these conditions a sizable fraction of a 46-kDa protein was soluble, and this protein was shown to be UmuC by antibody binding and microsequencing.

UmuC remained soluble throughout each purification step and copurified as a tightly bound complex with UmuD' (Fig. 1). The UmuD'C complex with an estimated molecular mass of 70 kDa remained intact during chromatography on Superdex 75 in the presence of 1 M NaCl. These results suggest that this complex is composed of a UmuC monomer (46 kDa) and UmuD' dimer (24 kDa), which are tightly associated.

It was also possible to purify a small amount of soluble UmuC by overproducing UmuC alone in the absence of UmuD'. Free UmuC has an apparent molecular mass of 46 kDa based on gel filtration (Fig. 2), a finding suggesting that it exists as a monomer when in solution (13). UmuC is a basic protein and should therefore migrate in an electric field toward the cathode in a neutral agarose gel. However, the UmuC fraction obtained from the phosphocellulose column remained in the wells during electrophoresis unless it was first treated with RNaseA (Fig. 3). Following a pretreatment of the phosphocellulose fraction with RNaseA, UmuC migrated toward the cathode (Fig. 3, lane 3), suggesting that free UmuC is bound to RNA in crude cell lysates. UmuC can be released from RNA in the presence of 1 M NaCl (Fig. 2), and this property can be exploited in the future to purify substantially larger quantities of soluble UmuC. Our data suggest that UmuC binds preferentially to UmuD' precluding formation of significant amounts of the UmuC-RNA complex. Pretreatment of the UmuD'C complex with RNase had negligible effect on its migration toward the anode in a neutral agarose gel (Fig. 3, compare lanes 5 and 6).

The binding studies with ssDNA offer potentially important insights into the properties of the UmuD'C complex. Binding of the protein complex, either to a 600-mer fragment of M13 DNA (Fig. 4), or to full-length M13 DNA (Fig. 5), occurred cooperatively with a Hill coefficient of 3. Such cooperativity was observed using two independent measurements: (i) agarose gel electrophoresis and immunodetection of free UmuD'C or UmuD'C complexed with DNA (Figs. 4 and 5) and (ii) steady-state fluorescence depolarization of ethenoadenine-labeled M13 DNA at increasing concentration of UmuD'C (Fig. 6). The depolarization data were also confirmed by an independent measurement of an increase in the fluorescence of ethenoadenine as a function of increasing UmuD'C concentration (data

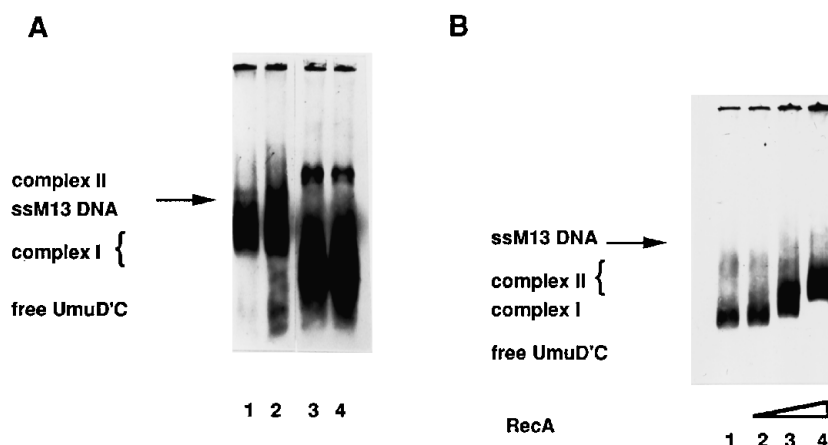


FIG. 8. **Binding of the UmuD'C complex and RecA* protein to ssM13 DNA.** Binding of UmuD'C to ssM13 DNA in presence of RecA* is examined using a mobility gel shift assay (0.9% agarose gel) probed with UmuC antiserum. *A*, lane 1, UmuD'C (300 nM) complex incubated with DNA in presence of 1 mM ATP γ S; lane 2, same as lane 1 except that 30 nM UmuD' protein was added to the reaction; lane 3, RecA* (560 nM) was preincubated with ssM13 DNA (560 nM in the DNA nucleotide) for 10 min at 37 °C before the UmuD'C complex was added to the reaction; lane 4, same as lane 3 except that 30 nM UmuD' protein was added to the reaction. *B*, UmuD'C (300 nM) was preincubated with ssM13 DNA (560 nM in the DNA nucleotide) for 10 min at 37 °C prior to the addition of RecA protein. Lane 1, UmuD'C complex bound to ssM13 DNA; lane 2, UmuD'C-ssM13 DNA with 46 nM RecA protein; lane 3, UmuD'C complex with 140 nM RecA protein; lane 4, UmuD'C-ssM13 DNA complex with 560 nM RecA protein. The three levels of RecA protein used correspond to 1 RecA per 12, 4, and 1 nucleotide(s), respectively. The position of free ssM13 DNA on the gel was determined by staining with ethidium bromide (indicated by the arrow).

not shown). As reported previously (29), in the gel assay UmuD'C did not bind irradiated or unirradiated double-stranded DNA.

Fluorescence properties of etheno-modified DNA have been used successfully to calculate DNA binding parameters of RecA protein (37, 38, 45) and bacteriophage T4-coded Gene 32 protein (46). Since parameters for most activities attributed to these proteins have been confirmed by other methods, it is believed that higher affinity of binding to etheno-modified DNA over natural ssDNA for RecA and gp32 is caused by a disruption of secondary structure of ssDNA. In our experiments, using two independent techniques, we found that UmuD'C bound cooperatively to both ethenoadenine-modified ssDNA (Fig. 6) and to unmodified ssDNA (Fig. 4) with similar values for the Hill coefficient.

In addition to its ability to interact with free ss DNA, UmuD'C interacts with ssDNA that had been pre-coated with RecA (Fig. 8A). These two properties, direct cooperative binding of UmuD'C to DNA, and binding to a RecA nucleoprotein filament, may help to explain some of the intriguing phenotypes exhibited when the Umu proteins are overexpressed *in vivo* (26–28). Under physiological conditions, the UmuD'C proteins are maintained at low basal levels (25). Since UmuD' is normally expressed at about 12-fold higher levels than UmuC (25), most, if not all, of the UmuC is likely to exist as a UmuD'C complex. Thus, even in fully SOS-induced cells there may be only about 200 UmuD'C molecules per cell (25).

In contrast, under similar conditions, there are likely to be 30,000–50,000 molecules of RecA protein/cell (3, 25). Thus, under normal cellular conditions, UmuD'C may associate with a RecA nucleoprotein filament, perhaps through an interaction between UmuD'-RecA* (15) or UmuC-RecA* (14). When a RecA* nucleoprotein filament has formed at a lesion in double-stranded DNA (47), a UmuD'C-RecA* interaction would provide an efficient way to target Umu proteins to locations within the cell where they are required to facilitate error-prone translesion DNA synthesis (15).

Under conditions where UmuD'C is moderately overproduced, *e.g.* when expressed from an operator constitutive mutant on a low copy number plasmid, some UmuD'C might begin to bind directly to regions of ssDNA and inhibit the extent of RecA*-nucleoprotein filament formation. This interaction

might be sufficient to inhibit RecA's recombinatorial activities directly (28) while allowing for the formation of a lesion-localized nucleoprotein structure (a putative mutasome) containing RecA, UmuD'C and pol III (5, 13) or perhaps pol II (20, 22).

The nonmutable phenotype of cells that slightly overexpress UmuC alone (27) may result from increased formation of UmuD'C-DNA complexes at regions of ssDNA generated when a cell attempts to replicate damaged DNA, which may hinder continued replication rather than enhance it and ultimately lead to killing of cells with premutagenic lesions, thereby reducing the number of mutants. Significant overproduction of UmuD'C, *e.g.* from a multicopy plasmid in fully SOS-induced cells, may result in levels of UmuD'C that can bind directly and cooperatively to regions of ssDNA, even in undamaged cells. Encountering UmuD'C coated DNA rather than *E. coli* single-stranded binding protein-coated DNA may impede replication and thus result in the rapid cessation of DNA synthesis that is associated with cold sensitivity (26).

The nature of the interactions between pol III or pol II and the SOS-induced proteins described above, that lead to either error-free or error-prone lesion bypass, remain entirely speculative. Our aim is to elucidate the precise interactions between polymerase holoenzymes, RecA*, UmuD'C, and any additional proteins that facilitate bypass of DNA template lesions by reconstituting an SOS lesion bypass system *in vitro*. The availability of an extensively purified source of soluble UmuD'C complex is therefore a critical step toward achieving this goal.

Acknowledgments—We thank Ekaterina Frank and Olga Kulaeva for help in constructing the UmuD'C-overproducing plasmid pOS1. Linda Bloom and John Petruska provided incisive comments concerning the protein-DNA binding studies and analysis. We are grateful to Steven Lloyd for his generous gift of T4 endonuclease V. The Echols' laboratory including Marie-Agnes Petit, Wendy Bedale, Chi Lu, Peter McInerney, Malini Rajagopalan, and Harrison Echols played a central role in the development of this project. Most of the key concepts concerning lesion bypass involving a mutasome complex are based on models proposed by Hatch Echols and Bryn Bridges.

REFERENCES

1. Radman, M. (1975) in *Molecular Mechanisms for the Repair of DNA, Part A* (Hanawalt, P., and Setlow, R. B., ed) pp. 355–367, Plenum Press, New York
2. Witkin, E. M. (1976) *Bacteriol. Rev.* **40**, 869–907
3. Sassanfar, M., and Roberts, J. W. (1990) *J. Mol. Biol.* **212**, 79–96
4. Little, J. W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1375–1379
5. Echols, H., and Goodman, M. F. (1990) *Mutat. Res.* **236**, 301–311

6. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, American Society of Microbiology, Washington, D. C.
7. Woodgate, R., and Sedgwick, S. G. (1992) *Mol. Microbiol.* **6**, 2213–2218
8. Murli, S., and Walker, G. C. (1993) *Curr. Opin. Genet. Dev.* **3**, 719–725
9. Walker, G. C. (1984) *Microbiol. Rev.* **48**, 60–93
10. Shinagawa, H., Iwasaki, H., Kato, T., and Nakata, A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1806–1810
11. Burckhardt, S. E., Woodgate, R., Scheuremann, R. H., and Echols, H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1811–1815
12. Nohmi, T., Battista, J. R., Dodson, L. A., and Walker, G. C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1816–1820
13. Woodgate, R., Rajagopalan, M., Lu, C., and Echols, H. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 7301–7305
14. Freitag, N., and McEntee, K. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8363–8367
15. Frank, E. G., Hauser, J., Levine, A. S., and Woodgate, R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8169–8173
16. Sweasy, J. B., Witkin, E. M., Sinha, N., and Roegner-Maniscalco, V. (1990) *J. Bacteriol.* **172**, 3030–3036
17. Bailone, A., Sommer, S., Knezevic, J., Dutreix, M., and Devoret, R. (1991) *Biochimie (Paris)* **73**, 479–484
18. Bridges, B. A., Mottershead, R. P., and Sedgwick, S. G. (1976) *Mol. & Gen. Genet.* **144**, 53–58
19. Hagensee, M. E., Timme, T. L., Bryan, S. K., and Moses, R. E. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 4195–4199
20. Bonner, C. A., Randall, S. K., Rayssiguier, C., Radman, M., Eritja, R., Kaplan, B. E., McEntee, K., and Goodman, M. F. (1988) *J. Biol. Chem.* **263**, 18946–18952
21. Iwasaki, H., Nakata, A., Walker, G., and Shinagawa, H. (1990) *J. Bacteriol.* **172**, 6268–6273
22. Tessman, I., and Kennedy, M. A. (1993) *Genetics* **136**, 439–448
23. Bridges, B. A., and Woodgate, R. (1984) *Mol. & Gen. Genet.* **196**, 364–366
24. Bridges, B. A., and Woodgate, R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4193–4197
25. Woodgate, R., and Ennis, D. G. (1991) *Mol. & Gen. Genet.* **229**, 10–16
26. Marsh, L., and Walker, G. C. (1985) *J. Bacteriol.* **162**, 155–161
27. Woodgate, R., Singh, M., Kulaeva, O. I., Frank, E. G., Levine, A. S., and Koch, W. H. (1994) *J. Bacteriol.* **176**, 5011–5021
28. Sommer, S., Bailone, A., and Devoret, R. (1993) *Mol. Microbiol.* **10**, 963–971
29. Petit, M.-A., Bedale, W., Osipiuk, J., Lu, C., Rajagopalan, M., McInerney, P., Goodman, M. F., and Echols, H. (1994) *J. Biol. Chem.* **269**, 23824–23829
30. Lawrence, C. W., Borden, A., and Woodgate, R. (1996) *Mol. & Gen. Genet.* in press
31. Yuen, S. W., Chui, A. H., Wilson, K. J., and Yuan, P. M. (1989) *BioTechniques* **7**, 74–82
32. Kitagawa, Y., Akaboshi, E., Shinagawa, H., Horii, T., Ogawa, H., and Kato, T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4336–4340
33. Perry, K. L., Elledge, S. J., Mitchell, B. B., Marsh, L., and Walker, G. C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4331–4335
34. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) *Methods Enzymol.* **204**, 125–139
35. Kohwi-Shigematsu, T., Enomoto, T., Yamada, M.-A., Nakanishi, M., and Tsuboi, M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4689–4693
36. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181**, 281–295
37. Silver, M. S., and Fersht, A. R. (1982) *Biochemistry* **21**, 6066–6072
38. Kowalczykowski, S. C., Clow, J., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193**, 81–95
39. Perez-Howard, G. M., Weil, P. A., and Beechem, J. M. (1995) *Biochemistry* **34**, 8005–8017
40. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp. 145–147, Plenum Press, New York
41. Laemmli, U. K. (1970) *Nature* **227**, 680–685
42. Woodgate, R. (1992) *Mutat. Res.* **281**, 221–225
43. Chabbert, M., Lami, H., and Takahashi, M. (1991) *J. Biol. Chem.* **266**, 5395–5400
44. Rajagopalan, M., Lu, C., Woodgate, R., O'Donnell, M., Goodman, M. F., and Echols, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10777–10781
45. Chabbert, M., Cazenave, C., and Helene, C. (1987) *Biochemistry* **26**, 2218–2225
46. Newport, J. W., Lonberg, N., Kowalczykowski, S. C., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145**, 105–121
47. Rosenberg, M., and Echols, H. (1990) *J. Biol. Chem.* **265**, 20641–20645

**Purification of a Soluble UmuD'C Complex from *Escherichia coli*: COOPERATIVE
BINDING OF UmuD 'C TO SINGLE-STRANDED DNA**

Irina Bruck, Roger Woodgate, Kevin McEntee and Myron F. Goodman

J. Biol. Chem. 1996, 271:10767-10774.

doi: 10.1074/jbc.271.18.10767

Access the most updated version of this article at <http://www.jbc.org/content/271/18/10767>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 43 references, 24 of which can be accessed free at
<http://www.jbc.org/content/271/18/10767.full.html#ref-list-1>