Nucleic Acids Research, Vol. 20, No. 10 2525–2532

Purification and characterization of TnsC, a Tn7 transposition protein that binds ATP and DNA

Pascal Gamas[+] and Nancy L.Craig*
Department of Microbiology and Immunology and Department of Biochemistry and Biophysics, George W.Hooper Foundation, University of California, San Francisco, CA 94143, USA

Received January 21, 1992; Revised and Accepted April 9, 1992

ABSTRACT

The bacterial transposon Tn7 encodes five transposition genes *tnsABCDE*. We report a simple and rapid procedure for the purification of TnsC protein. We show that purified TnsC is active in and required for Tn7 transposition in a cell-free recombination system. This finding demonstrates that TnsC participates directly in Tn7 transposition and explains the requirement for *tnsC* function in Tn7 transposition. We have found that TnsC binds adenine nucleotides and is thus a likely site of action of the essential ATP cofactor in Tn7 transposition. We also report that TnsC binds non-specifically to DNA in the presence of ATP or the generally non-hydrolyzable analogues AMP-PNP and ATP-γ-S, and that TnsC displays little affinity for DNA in the presence of ADP. We speculate that TnsC plays a central role in the selection of target DNA during Tn7 transposition.

INTRODUCTION

Transposons are discrete DNA segments that can move from one genetic location to another. Most elements transpose at low frequency and insert at many different sites (see 1 for review). The bacterial transposon Tn7 (2; see 3,4 for review) is remarkable in its ability to transpose at high frequency to a specific site. This specific target site is present in the chromosomes of many bacteria and in *Escherichia coli* is called *attTn7* (5 ,6). When *attTn7* is unavailable, Tn7 transposes at low frequency to other target sites. Another interesting feature of Tn7 is that it encodes an elaborate array of transposition genes, *tnsABCDE*, that mediate two distinct but overlapping transposition pathways (7,8,9). *tnsABC* + *tnsD* promote high-frequency insertion into *attTn7* and low-frequency insertion into pseudo-*attTn7* sites whereas *tnsABC* + *tnsE* promote low-frequency insertion into many different sites that are unrelated to *attTn7* and to each other.

What are the roles of the Tns proteins? Identification of the proteins that participate directly in recombination and elucidation of the steps underlying the transfer of DNA strands from one site to another demands a biochemical approach. Biochemical dissection of many transposition reactions has been hampered by the fact that recombination occurs at low frequency. By contrast, the ability of Tn7 to transpose at high frequency to a specific site has facilitated the development of an efficient cell-free system for Tn7 transposition (10). In this system, Tn7 translocates from a donor DNA molecule to a target DNA molecule containing *attTn7* upon incubation of exogenous DNA substrates with ATP and four protein fractions; each fraction is derived from a strain containing one of the *tns* genes required for *in vivo* transposition to *attTn7*. We show here that purified TnsC protein can replace the fraction from cells containing *tnsC* extract in the cell-free Tn7 transposition system. This finding demonstrates that TnsC protein participates directly in Tn7 recombination. We also show that TnsC is an ATP-dependent DNA binding protein, suggesting that it interacts directly with a DNA substrate during recombination and likely mediates the role of ATP which is an essential cofactor for transposition.

MATERIALS AND METHODS

Plasmids

The TnsC expression plasmid pPA101 was constructed by inserting a *NcoI-HindIII tnsC* fragment, obtained from a plasmid similar to pKAO53 (11) after partial digestion with *HindIII*, between the *NcoI* and *HindII* sites of pGD108 (12). In this construction, the proposed *tnsC* initation ATG (11,13,14) has been modified to include an *NcoI* site. DNAs containing Tn7 transposition sequences (pEM, which is a donor plasmid containing a miniTn7 element, and pKAO4-3, which is an *attTn7* target plasmid) are described in detail in Bainton *et al* (10). Bluescript pKS[+] (Stratagene) was used as DNA lacking Tn7 transposition sequences.

* To whom correspondence should be addressed at Howard Hughes Medical Institute and Department of Molecular Biology & Genetics, 615 PCTB, Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore MD 21205-2185, USA

[+] Present address: Laboratoire de Biologie Moleculaire des Relations Plantes-Microorganismes, CNRS-INRA, BP27-31326, Castanet Tolosan Cedex, France

Bacteria

TnsC was purified from an *E. coli* strain defective in proteolysis, CAG456 ($lacZ_{am}$ trp_{am} pho_{am} $supC_{ts}$ *rpsL htpR*165; 15), carrying the pPA101 *tnsC* plasmid. Strains containing the other *tns* genes from which the *tns* fractions used for Tn7 transposition *in vitro* are prepared are described in Bainton *et al* (10).

Purification of TnsC

Unless indicated, all steps were performed at 4°C. Fractions were frozen in liquid nitrogen and stored at −80°C.

Cell Growth. Cells were grown at 30°C in a 200 liter fermentor in LB broth (16) supplemented with 100 μg/ml carbenicillin. At OD 600 = 0.4, IPTG was added to 100 μM. Growth was continued for an additional 2 hours, the cells harvested by centrifugation, the cell paste was frozen in liquid nitrogen and stored at −80°C.

Cell Lysis and Preparation of Fraction I. 2 ml/gm cells of buffer A (50 mM Hepes pH 7.5, 1 mM EDTA, 100 mM NaCl) was added to 5 gm of cell paste and the mixture thawed at room temperature. Lysozyme was added to 300 μg/ml, the suspension incubated for 20 minutes on ice, frozen with liquid nitrogen, thawed for about 4 min at 30°C and then subjected to sonication. The resulting lysate was centrifuged for 30 min at 4°C in an SW60 rotor at 40,000 rpm. The clear portion of the resulting supernatant was collected and supplemented with ATP to 1 mM to form Fraction I. This procedure yields about 50−60 mg soluble protein per gm cells.

Polyethylenimine and Ammonium Sulfate Precipitations. The protein concentration of Fraction I was adjusted to 20 mg/ml with buffer A + 1 mM ATP. Polyethylenimine (Sigma; adjusted to pH 7.5 with HCl) was added to 0.8%, the mixture incubated for 10 minutes, centrifuged for 10 minutes at 10,000 rpm in a JS-13 rotor, and the supernatant collected. Ammonium sulfate was added (176 mg/ml) with stirring over 20 minutes, the mixture incubated an additional 30 min and the resulting pellet collected by centrifugation (20 min at 8000 rpm in a JS-13 rotor). The pellet was washed with 3 ml buffer A + 1 mM ATP to which 176 mg/ml ammonium sulfate was added and collected after centrifugation for 3 min in the same rotor. The pellet was resuspended with 3 ml buffer B (25 mM Hepes pH 7.5, 2.5 mM DTT, 0.1 mM EDTA, 1 mM ATP, 10 mM CHAPS <Sigma>, 10% (v/v) glycerol) + 1.0 M NaCl, incubated for 20 min on ice and aggregates removed by centrifugation (10 min at 10,000 rpm in a JS-13 rotor). The resulting supernatant is Fraction II.

Low-salt Protein Precipitation. The concentration of Fraction II was adjusted to 150−180 μg/ml with buffer B, then dialyzed for 90 min against (at least) 100 vol buffer C (25 mM Hepes pH 7.5, 2.5 mM DTT, 0.1 mM EDTA, 100 μM ATP, 1 mM CHAPS, 10% (v/v) glycerol) + 0.3 M NaCl and then for 3 hr against 100 vol buffer C + 0.1 M NaCl. Precipitated protein was collected by centrifugation (10 min at 10,000 rpm in a JS-13 rotor), rinsed with buffer C + 0.1 M NaCl, recentrifuged and dissolved in buffer C + 1.0 M NaCl + 10 mM $MgCl_2$. After incubation for 20 min, aggregates were removed by centrifugation (10 min at 10,000 rpm in a JS-13 rotor) and the resulting supernatant saved as Fraction III.

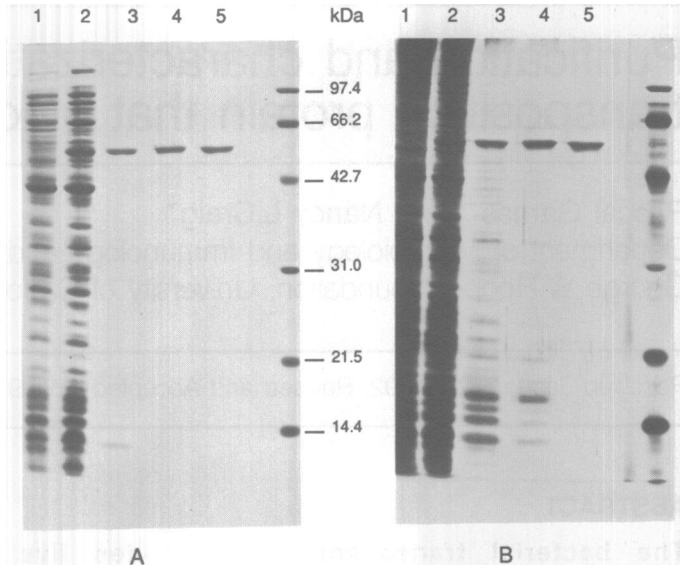

Figure 1. SDS-PAGE Analysis of TnsC Fractions. The indicated amounts of protein from various TnsC fractions were separated by SDS-PAGE and stained with Coomassie Blue R-250 (Panel A) or silver (Panel B). The flanking lanes contains marker proteins of the indicated molecular weights. lane 1: 40 μg Fraction I from a strain lacking TnsC. lane 2: 40 μg Fraction I. lane 3: 3 μg Fraction II. lane 4: 3 μg Fraction III. lane 5: 3 μg Fraction IV.

Biorex-70 Chromatography. Fraction III was dialyzed for 90 min against 500 vol buffer C + 0.3 M NaCl + 5 mM $MgCl_2$, loaded onto a Biorex-70 column equilibrated in the same buffer and the column washed with 7 column vol of the same buffer. TnsC was present in the flow-through and wash fractions. TnsC-containing fractions were pooled and concentrated by centrifugation with a Centricon-30 filter (Amicon). This concentration step also likely reduces contaminating low molecular weight polypeptides. The resulting material is Fraction IV.

Purity of TnsC: Lack of Nuclease or Topoisomerase Activity

We looked for endonuclease or topoisomerase activities by incubation of TnsC fractions with supercoiled plasmids containing Tn7 transposition sequences (pEM and pKAO4−2A) or with circular single-stranded M13mp 18 (+) DNA. Reactions (25 μl) contained 25 mM Hepes pH 7.5, 2 mM DTT, 100 mM KCl, 15 mM MgAc, 100 μg/ml BSA, 1 μg DNA and 1 μl TnsC fraction (about 200 ng TnsC). After incubation for 60 min at 30°C or 37°C, the DNAs were examined by agarose gel electrophoresis; no nuclease or topoisomerase activity was detectable in either Fraction III or IV.

Amino-terminal Sequence Analysis of TnsC

10 μg Fraction III TnsC was subjected to SDS-PAGE and then electro-transfered (buffer: 25 mM Tris-192 mM glycine pH 8.3, 15% methanol) onto an Immobilon-P membrane (Millipore). The membrane was stained for 10 min in 0.2% Coomassie Blue R-250, 10% acetic acid and 45% methanol, and then destained in 7% acetic acid and 90% methanol. The portion of the membrane containing TnsC was excised and kept in water. Amino-terminal sequencing of the membrane bound-protein was performed by the Biomolecular Resource Center, UCSF.

Table 1. TnsC Purification.

	Volume (ml)	Total Protein (mg)	TnsC[a] (mg)	TnsC Yield (%)	TnsC Purity (% Total Protein)	Purification (fold)	Specific[b] Transposition Activity of TnsC (units/mg TnsC)
FI Cleared Lysate	12.5	250	6.25	(100)	(2.5)	–	1.0×10^6
FII PEI/AS Precipitations	3.0	2.0	1.5	24	75	30	nd
FIII Low-salt Precipitation	1.0	0.52	0.50	8.0	95	38	1.0×10^6
FIV Biorex-70	0.25	0.25	0.245	3.9	98	39	0.5×10^6

[a] The amount of TnsC protein in each fraction was determined by immunoblot analysis.
[b] One unit of TnsC activity is arbitrarily designated as the amount of TnsC fraction required to generate 1 ng of simple insertion product in the cell-free Tn7 transposition system. The specific activity of the TnsC protein in each fraction was determined by evaluating the recombination activity of dilutions of each fraction and by determining the amount of TnsC protein in each fraction by immunoblot analysis.

Sucrose Gradient Sedimentation

A 5 ml 5–20% sucrose gradient in 25 mM Hepes pH 7.5, 2 mM DTT, 1 mM EDTA, 0.4 M NaCl and 2 mM ATP was prepared in a polyallomer tube prerinsed with 1% sterile gelatin. 100 µl of TnsC fraction was layered onto the gradient. The gradient was centrifuged for 16 hours at 2°C at 40,000 rpm in a SW50.1 rotor. About 25 fractions were collected from the bottom of the gradient into tubes containing 10 µl 25 mM Hepes pH 7.5, 2 mM DTT, 0.4 M NaCl, 2 mg/ml BSA and 5% sucrose.

Tn7 Transposition *in vitro*

Reactions were performed and protein fractions other than TnsC were produced as described in Bainton *et al* (10). Reaction mixtures (100 µl) contained 1.3 mM Tris pH 7.5, 26.5 mM Hepes pH 8.5, 2.5 mM KPO_4, 0.07 mM EDTA, 2.1 mM DTT, 20 µg BSA, 0.11% (v/v) glycerol, 5% PVA, 2 mM ATP, 15 mM MgAc, 100 ng miniTn7 donor plasmid (pEM), 2.5 µg *attTn7* target plasmid (pKAO4-3), 40–50 µg *tnsA* crude lysate, 45 ng Fraction IV TnsB protein, 6 µg TnsD fraction and various amounts of TnsC fraction. For the experiments shown in Figure 2 and Table 1, reactions also contained 3.5 mM NaCl, 56 mM KCl and 0.02 mM CHAPS. For the experiments shown in Figure 3, reactions also contained 24.5 mM NaCl, 36 mM KCl and 0.3% (w/v) sucrose. 4 µl of 375 mM MgAc was added after the other components were mixed and incubated at 30°C for 7 minutes; after MgAc addition, the incubation was continued at 30°C for an additional 30 minutes. Reactions were stopped by the addition of 400 µl of 10 mM Tris.HCl pH 7.4, 5 mM EDTA, 100 mM KCl and 6 M urea. As described in Bainton *et al* (10), DNA was recovered by spermine precipitation, an aliquot of the recovered DNA digested with *Eco*RI, electrophoresed through an agarose gel, electro-transfered to Nytran and detected by Southern hybridization using as a probe a fragment specific to the miniTn7 element. Reaction products were quantified by tracing of autoradiograms with a LKB Ultroscan XL laser densitometer.

ATP Crosslinking

To reduce the amount of endogenous ATP in the TnsC fractions to be analyzed, Fraction III was prepared in a slightly different way: the protein pellet resulting from the low-salt dialysis of Fraction II was resuspended in Buffer B–ATP + 1.0 M NaCl + 10 mM $MgCl_2$. 1.0 µl of this fraction (about 0.45 µg TnsC) was added to 9 µl of reaction buffer (25 mM Hepes pH 7.5, 2 mM DTT, 200 mM NaCl, 1 mM $MgCl_2$, 8% sucrose and 0.25 µM α-^{32}P ATP <400 Ci/mmol; 10 µCi> in a microtiter plate well and then incubated for 10 min at room temperature. The mixture was then UV irradiated in a Stratalinker (Stratagene); the optimal dose for crosslinking ATP to TnsC was observed to be about the same as that recommended by the manufacturer for DNA crosslinking to a nylon membrane (0.12 J). 2.5 µl of SDS-PAGE sample buffer was added, then the mixture boiled for 5 minutes and separated by SDS-PAGE. Following staining, the gel was dried and autoradiographed.

TnsC–DNA Binding Assays

Non-specific substrate DNA was Bluescript pKS$^+$, linearized by digestion with *Eco*RI, and end-labeled by incubation with the Klenow fragment of DNA Polymerase I and α-^{32}P dATP. All binding reactions were 50 µl and contained 5 ng DNA (about 10,000 cpm). The reactions shown in Figure 3 contained 26.5 mM Hepes pH 7.5, 2.1 mM DTT, 106 µg/ml BSA, 8.2% (w/v) sucrose, 24 mM NaCl, 80 mM KCl, 0.12 mM ATP, 0.1 mM ATP-γ-S and various amounts of TnsC protein from the sucrose gradient sedimentation. The reactions shown Figure 5 contained 26 mM Hepes pH 7.5, 2.1 mM DTT, 136 µg/ml BSA, 7.5% (w/v) sucrose, 0.04% (v/v) glycerol, 0.04 mM CHAPS, 4 mM NaCl, 116 mM KCl, 4 µM ATP, 0.04 mM $MgCl_2$, and Fraction III TnsC protein (60 ng in panels A and C and various amounts as indicated in panel B); where indicated, reactions were also supplemented with an additional 100 µM of various adenine nucleotides and various amounts of $MgCl_2$. The reactions were incubated for 20 min at 30°C and then filtered at about 1 ml/min through 0.45 µm 24 mm nitrocellulose filters (Hoefer) which were

presoaked in binding buffer (25 mM Hepes pH 7.5, 80 mM KCl, 100 μg/ml BSA and 7.5% sucrose); the filters were washed 3 times with 0.5 ml binding buffer. Radioactivity was measured by Cerenkov counting.

Protein Analysis

Protein concentration was determined using the Bio-Rad Protein Assay with BSA as a standard. SDS-PAGE using 12.5% gels was performed by the method of Laemmli (17). Proteins were detected either by staining with Coomassie Blue R-250, which was sometimes followed by staining with silver, or by Western analysis using affinity-purified anti-TnsC antibodies (11).

RESULTS

Purification of TnsC

Our source of TnsC protein for purification was an *E. coli* strain containing an expression plasmid (pPA101) in which the proposed *tnsC* initiation codon (11,13,14) is fused to a heterologous ribosome binding site located a few nucleotides downstream of the stop codon of a small, highly-expressed, IPTG-inducible polypeptide. This construction was designed to promote high-level TnsC expression through translational coupling to the upstream open reading frame (12). When this plasmid is expressed in an *htpR*$^-$ strain, which is defective in proteolysis (15), TnsC, a 58 kd polypeptide (11), is evident and is present as about 15% of total cell protein (data not shown). Most of the TnsC protein in the *htpR*$^-$ strain is found in aggregates ('inclusion bodies') that can be pelleted from cell lysates by low-speed centrifugation. In such a cleared cell lysate, soluble TnsC is about 2.5% of total protein (Figure 1, lane 2) and can easily be detected by Coomassie Blue staining after SDS-PAGE. The cleared cell lysate was highly active in Tn7 transposition *in vitro* (see below). We were unable to recover active TnsC from the inclusion bodies using a variety of procedures.

Purification of TnsC from the cleared cell lysate was monitored by following the TnsC polypeptide as visualized by Coomassie Blue staining or Western analysis after SDS-PAGE (Figure 1), and by evaluating *tns*C-dependent activity in the cell-free Tn7 transposition system (see below). We have exploited the fact that TnsC readily precipitates in low-salt solutions to design a rapid and simple purification procedure. A typical purification is described in Table 1 and Figure 1. Preparation of at least 0.25 mg of highly (greater than 98%) purified TnsC from 5 g of cells can be readily accomplished in less than 15 hrs.

Following thawing of a washed cell paste, cells are lysed by incubation with lysozyme, followed by another cycle of freezing and thawing and then by sonication. The resulting lysate is clarified by a high-speed centrifugation. ATP is added to this material and is present in all other TnsC fractions (see below). The cleared cell lysate is Fraction I. Nucleic acids and some proteins are removed from the supernatant by polyethylenimine (PEI) precipitation and centrifugation. (Although a substantial fraction, approximately 75%, of the TnsC also precipitates with the PEI and is thus lost, this step is useful as it removes contaminants that are otherwise difficult to separate from the remainder of the TnsC). TnsC is then precipitated from the resulting supernatant by the addition of ammonium sulfate (AS) to 30% saturation, collected by centrifugation and the resulting pellet solubilized with high-salt (1.0 M NaCl) buffer which also contains the non-denaturing detergent CHAPS to give Fraction II. The principal purification of TnsC occurs at this step. Fraction II is then dialyzed against low-salt (0.1 M NaCl) buffer which results in the selective precipitation of TnsC, which is then resolubilized in high salt (> 0.5 M NaCl) buffer to yield Fraction III. In Fraction III, TnsC is approximately 95% pure; no nuclease or topoisomerase activity was evident with this material using a variety of DNA substrates (see Materiaals and Methods). The majority of the remaining contaminants in Figure III can be removed by passage over an ion exchange column (Biorex-70); TnsC (Fraction IV) is found in the flow-through.

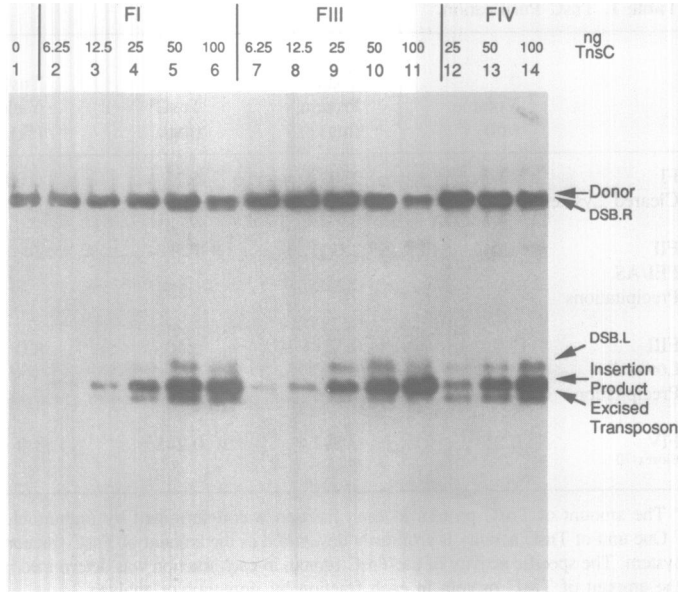

Figure 2. Purified TnsC is Active In and Required For Tn7 Transposition *in vitro*. An autoradiogram of a gel is shown which displays DNAs from Tn7 transposition reactions carried out *in vitro* in which various TnsC fractions were used. DNAs were detected by Southern hybridization with a transposon-specific probe which recognizes the substrate donor DNA containing the miniTn7 transposon, several transposition intermediates (the DSB.R and DSB.L species are donor molecules cut by a double-strand break at either transposon end), an excised transposon and the product of simple insertion into *attTn7*. Reactions contained the indicated amount of TnsC (as determined by Western analysis) from various fractions; note that TnsC is only a portion of the total protein added with each TnsC fraction.

Both ATP and $MgCl_2$ have considerable effects on the solubility of TnsC. In the presence of ATP, the resolubilization of TnsC to produce Fractions II and III is much improved. Also, the presence of ATP appears to stabilize the activity of purified TnsC (data not shown). When 10 mM $MgCl_2$ is present in the low-salt dialysis buffer for the preparation of Fraction III, no precipitation of TnsC is observed. We imagine that TnsC adopts different conformations resulting in different properties in the presence of these various cofactors (see also below).

The sequence of the amino terminus of purified TnsC was determined to be: X-Gly-Ala-Thr-Arg-Ile-Gln-Ala. This sequence is as expected for inserting the proposed *tnsC* ATG (11,13,14) into the expression vector. It should be noted that, because of the expression vector used, the second amino acid of the TnsC protein we have purified is different than that encoded by wild-type *tnsC* (Ser to Gly). This change does not detectably affect the activity of TnsC in Tn7 transposition *in vitro* (data not shown).

The apparent molecular weight of TnsC as determined by SDS-PAGE is 58 kDa (Figure 1; 11): this value is not

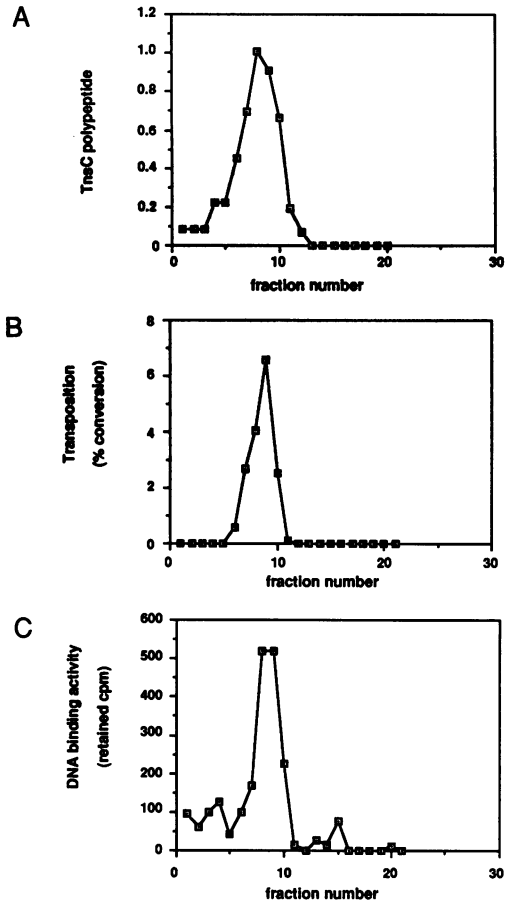

Figure 3. Copurification of TnsC Polypeptide with *tnsC*-Dependent Tn7 Transposition. and TnsC-dependent DNA Binding. TnsC was fractionated on a sucrose gradient and each fraction evaluated for *tnsC*-dependent transposition activity and ATP-γ-S-dependent DNA binding. The amount of TnsC in each fraction (Panel A) was determined by Western analysis, followed by densitometric scanning of a photograph of the resulting blot. The amount of protein in Fraction 8 was designated arbitrarily as 1.0; the actual concentration of this fraction was about 10 μg/ml. The transposition activity (Panel B) of 3 μl of each fraction was evaluated using the *in vitro* transposition assay, followed by densitometric scanning of an autoradiogram after Southern hybridization. Transposition activity is shown as the ratio of the simple insertion product to remaining donor plasmid. The DNA binding activity (Panel C) of 3 μl of each gradient fraction was evaluated by filter binding assay in the presence of ATP-γ-S using DNA lacking Tn7 transposition sequences as a substrate. DNA binding activity (Panel C) is shown as the amount of radioactivity retained on the filter.

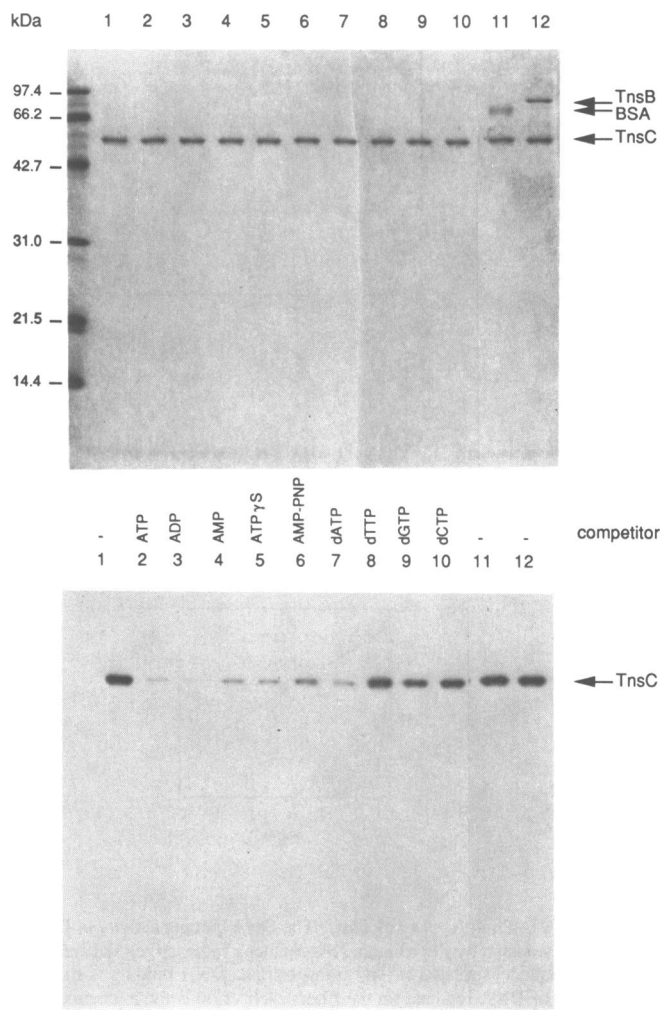

Figure 4. TnsC Interacts Specifically with Adenine Nucleotides. ATP was UV-crosslinked to Fraction III TnsC in reactions containing 2.5 μM α-^{32}P ATP and, as indicated, other non-radioactive nucleotides at 200 μM. The mixture was separated by SDS-PAGE and the gel analyzed by staining with Coomassie Blue R-250 (top panel) and by autoradiography (bottom panel). To evaluate the specificity of ATP labeling, the reaction in lane 11 also contained 0.5 μg BSA and that in lane 12 also contained 0.5 μg TnsB.

significantly different from the value of 63 kDa predicted from the proposed amino acid sequence of TnsC (14). TnsC behaves as a monomer when sedimented in a sucrose gradient in 0.3 M NaCl (data not shown); these ionic conditions are somewhat different from those for *in vitro* transposition and other assays.

TnsC participates directly in Tn7 transposition

We monitored the activity of TnsC fractions during purification by examining their ability to stimulate Tn7 transposition *in vitro*. We have developed a cell-free Tn7 transposition system (10). Recombination occurs when exogenous substrate plasmids, a donor molecule containing a miniTn7 element and a target plasmid containing *attTn7*, are incubated with ATP and extracts from cells carrying *tnsA*, *tnsB*, *tnsC* and *tnsD* which are required for *in vivo* Tn7 insertion into *attTn7*. Several new DNA species are generated during Tn7 transposition *in vitro* (Figure 2). The most prominent new species is the transposition product, a simple insertion of the transposon into *attTn7*. Also evident are recombination intermediates which include donor molecules cut by a double strand break at either end of the transposon (DSB.L and DSB.R) and an excised transposon. Figure 2 shows transposition in the presence of different amounts of various TnsC fractions. No transposition intermediates or products are detected in the absence of TnsC (lane 1). By contrast, efficient recombination is observed with various TnsC fractions, including highly purified TnsC (Fraction IV) (lanes 6,11,14). It is notable that the specific activity of TnsC itself, i.e. the amount of transposition promoted by a defined amount of TnsC polypeptide, was identical with either TnsC crude lysate (Fraction I—lanes 2−6) or highly purified TnsC (Fraction IV—lanes 12−14); thus the ability to provide *tnsC*-dependent transposition activity copurfied with TnsC (Table 1). Moreover, transposition activity also cosedimented with TnsC on a sucrose gradient (Figure 3,

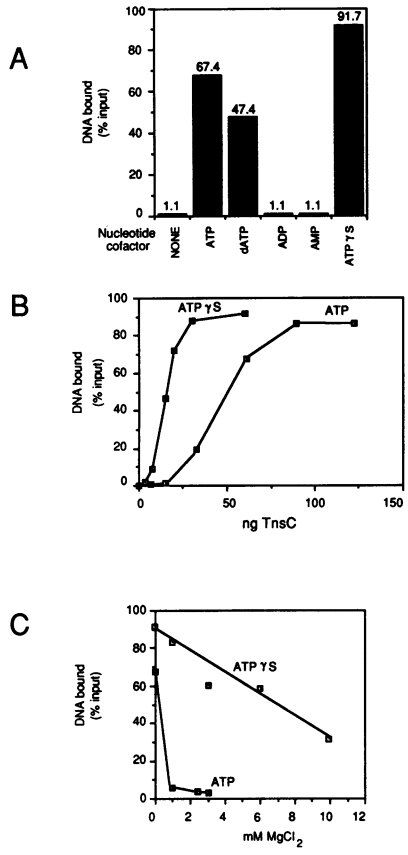

Figure 5. DNA Binding Activity of TnsC. The DNA binding activity of Fraction III TnsC was evaluated by filter binding assays using radioactively-labeled DNA which lacks sequences related to Tn7 transposition. DNA binding is measured as the fraction of DNA retained on the filter; each value is the average of three to five independent assays. Panel A: DNA binding activity of 60 ng TnsC in the presence of various nucleotides and the absence of added $MgCl_2$. Panel B: DNA binding of various amounts of TnsC in the presence of either ATP or ATP-γ-S without $MgCl_2$ addition. Panel C: DNA binding activity of 60 ng TnsC in the presence of either ATP or ATP-γ-S in the presence of various concentrations of $MgCl_2$.

panel A and B). These experiments provide strong evidence that TnsC participates directly in Tn7 transposition.

The amount of Tn7 transposition observed *in vitro* was sensitive to the amount of TnsC in the reaction mixture; maximum recombination is observed with approximately 100 ng TnsC in a 100 μl reaction (about 16 nM TnsC). By contrast, the concentration of the miniTn7 substrate DNA is 0.25 nM and the concentration of the *attTn7* target substrate is 10 nM.

TnsC is an ATP binding protein

Tn7 transposition *in vitro* requires ATP (10). We suspected that TnsC might interact with ATP because a Type A nucleotide binding domain is present in the predicted amino acid sequence of TnsC (13,14). Also, as noted above, we observe differences in the behavior of TnsC in the presence and absence of ATP. We demonstrated directly that TnsC does bind ATP by UV crosslinking experiments (18,19).

We incubated purified TnsC (Fraction III) with α-^{32}P-ATP, irradiated this mixture with UV, separated the mixture by SDS-PAGE and examined the distribution of protein by Coomassie Blue staining and nucleotide by autoradiography (Figure 4). TnsC was efficiently labeled with ATP (lane 1). No labeling was observed in the absence of irradiation (data not shown), no other ATP binding proteins were detected in the TnsC fraction nor was labeling of control proteins (BSA and TnsB—lanes 11 and 12) observed even upon longer exposure (data not shown). Thus TnsC is specifically recognized by ATP. Incubation mixtures included 1 mM $MgCl_2$ but ATP binding could also occur in the presence of 10 mM $MgCl_2$; ATP binding was reduced but not abolished by the presence of 10 mM EDTA (data not shown).

We also examined ATP binding to TnsC as detected by crosslinking of radioactively-labeled ATP in the presence of an 80-fold molar excess of other unlabeled nucleotides (Figure 4). ATP binding to TnsC was markedly decreased in the presence of adenine nucleotides including ATP, ADP, AMP, AMP-PNP, ATP-γ-S and dATP (lanes 2–7); a modest diminution of ATP binding was observed in the presence of dGTP (lane 9) but ATP binding to TnsC was insensitive to the presence of dTTP (lane 8) and dCTP (lane 10). Thus TnsC interacts specifically with adenine nucleotides.

TnsC is a non-specific DNA binding protein

We also asked if TnsC can bind to DNA. We incubated TnsC (Fraction III) with radioactively-labeled linear DNA, passed the mixture through a nitrocellulose filter and measured the retained radioactivity to evaluate protein-DNA binding. Although little DNA retention was observed in the absence of adenine nucleotide, substantial DNA binding by TnsC was observed in the presence of ATP, dATP and ATP-γ-S (Figure 5, panel A). We infer that the retention of DNA by the filter reflects DNA binding to TnsC because the DNA binding activity cofractionates with TnsC polypeptide when TnsC is sedimented through a sucrose gradient (Figure 3, panels A & C). We note that in this experiment, the substrate DNA was non-specific, i.e. lacked either *attTn7* or Tn7 end sequences. We have been unable to detect any preferential interaction of Tn7 with DNAs containing Tn7 transposition sequences; we observed no significant differences in the amount of TnsC required for maximal binding of DNAs containing or lacking Tn7 sequences (data not shown).

These experiments show that TnsC can form a ternary complex with DNA and the adenine nucleotides ATP, dATP and ATP-γ-S (and also AMP-PNP, data not shown) However, not all nucleotides that can bind to TnsC support the formation of such a ternary complex with DNA. No binding of DNA to TnsC was observed in the presence of ADP or AMP (Figure 5, panel A) although these nucleotides can interact with TnsC (Figure 3). We also found that ATP and ATP-γ-S differ considerably in their ability to form a ternary complex with TnsC and DNA as evaluated by the concentration of TnsC required for maximal DNA binding in the presence of each of these nucleotides: ATP-γ-S appears to be a better effector of TnsC binding to DNA than does ATP (Figure 5, panel B). AMP-PNP is also a better cofactor than ATP for TnsC binding to DNA (data not shown). That the usually non-hydrolyzable analogues AMP-PNP and ATP-γ-S are better effectors than ATP and that ADP was unable to support DNA binding suggests that TnsC may have an ATPase activity. We have been unable to detect such an activity although it should be noted that our assays would not have detected a single round (i.e. stoichiometric) hydrolysis of ATP by TnsC (data not shown).

We also examined the effect of $MgCl_2$ on the binding of DNA to TnsC. The DNA binding assays described above contained

low $MgCl_2$ (<0.04 mM). The presence of high $MgCl_2$ has a dramatic effect on DNA binding to TnsC in the presence of ATP; addition of 1 mM $MgCl_2$ decreased DNA binding more than 15-fold (Figure 5, panel C). The effect of high $MgCl_2$ in the presence of ATP-γ-S is less dramatic; DNA binding was decreased only about 2-fold in the presence of 8 mM $MgCl_2$. One attractive explanation for these results is that the presence of $MgCl_2$ provokes ATP hydrolysis which results in the dissociation of TnsC from DNA; a reasonable hypothesis is that no hydrolysis of ATP-γ-S occurs and that TnsC remains bound to DNA in the presence of this analogue even in the presence of $MgCl_2$.

DISCUSSION

We present here a simple procedure for purification of the Tn7-encoded transposition protein TnsC. This procedure allows the rapid preparation of nearly homogenous protein in amounts sufficient for most biochemical studies. A major result from our characterization of purified TnsC is the demonstration that this protein participates directly in and is required for Tn7 transposition. We have determined that purified TnsC protein can be substituted for the extract from *tnsC*-containing cells which is an essential component in a cell-free Tn7 transposition system. The direct participation of TnsC protein in transposition explains the requirement for the *tnsC* gene in Tn7 transposition *in vivo* (7−9). Other Tns proteins also participate directly in Tn7 transposition: TnsB is a sequence-specific DNA binding protein that interacts with the ends of Tn7 (20,21,22), TnsD is a sequence-specific DNA binding protein that recognizes *attTn7* (23, K.Kubo and N.L.Craig, in preparation) and TnsA has a direct but as yet undefined role in Tn7 transposition (R.Bainton, K.Kubo and N.L.Craig, in preparation).

We have identified two activities of TnsC that are likely intimately related to its role(s) in transposition. We have found that TnsC interacts specifically with ATP and other adenine nucleotides including the generally non-hydrolyzable ATP analogues AMP-PNP and ATP-γ-S, dATP, ADP and AMP. The specific interaction of TnsC with ATP was not unexpected as the amino acid sequence of TnsC as predicted from the *tnsC* sequence contains a consensus nucleotide binding site sequence (13,14).

We have also determined that TnsC is a DNA binding protein. The interaction of TnsC with DNA appears to be sequence-independent; in particular, no special interaction of TnsC with DNA sequences we know to be involved in Tn7 transposition was discernible. The ability of TnsC to bind DNA was highly influenced by the presence of particular nucleotide cofactors. Little binding of TnsC to DNA was detected in the presence of ADP and AMP, adenine nucleotides we have established do bind to TnsC, or in the absence of nucleotide. By contrast, TnsC binding to DNA was strongly and specifically stimulated by the presence of ATP and, to a lesser extent, dATP. Notably, even more effective binding of TnsC to DNA was observed in the the presence of generally non-hydrolyzable ATP analogues AMP-PNP and ATP-γ-S.

The overall picture of TnsC that has emerged from this work is of an essential transposition protein whose interaction with DNA is modulated by adenine nucleotide cofactors; TnsC binds to DNA in the presence of ATP but is unable to bind to DNA in the presence of ADP. It is attractive to speculate that TnsC may hydrolyze ATP but we have been unable to directly detect such an activity. Whether such hydrolysis may occur at a stoichiometric level or may by modulated by other components of the transposition machinery are interesting questions for future studies.

The proposed amino acid sequence of TnsC has been compared to protein data bases (14). Other than the region proposed to be directly involved in ATP binding, no other extensive structural similarities between TnsC and other proteins have been identified except a modest similarity to MalT, an *E. coli* ATP-dependent transcriptional activator (24,25). In the presence of ATP, MalT forms a higher-order nucleoprotein complex with specific DNA regions and it is likely that this complex mediates transcriptional activation by an as yet undefined mechanism (25).

Another site-specific recombination reaction, transposition of bacteriophage Mu also involves a protein, MuB, that can interact with both ATP and DNA (26,27). Non-specific binding of MuB to DNA is stimulated by ATP; upon ATP hydrolysis, the protein dissociates from DNA. One role of MuB is to mediate the interaction of the transposon ends, and their cognate binding protein MuA, with the target DNA. The modulation of MuB interaction with target DNA by ATP hydrolysis, which can be influenced by MuA protein, plays a critical role in target DNA selection (28).

What role does TnsC play in Tn7 transposition? We are attracted to the view that TnsC interacts with both ATP and substrate DNA during recombination. Other experiments suggest that the interaction of TnsC with target DNA plays a critical role in recombination. In particular, the efficiency of recombination is greatly enhanced by incubation of a mixture of TnsC, ATP, TnsD and the target *attTn7* DNA prior to the addition of the other reactions components (Bainton, R., Kubo, K. and N.L.Craig, in preparation). It is notable that little recombination is observed when this 'preincubation' mixture contains MgAc (10), a condition that our observations here suggest promotes the dissociation of TnsC from DNA. The finding that effficient transposition does occur when MdAc is added to recombination mixtures after the preincubation step suggests that a key event during this step is the stable attachment of TnsC to DNA . We have also found that the substitution of ATP-γ-S or AMP-PNP for ATP has a profound impact on target selection during transposition (Bainton, R., Kubo, K. and N.L. Craig, in preparation); thus we imagine that the modulation of the interaction of TnsC with DNA by adenine nucleotides plays a critical role in recombination.

A large number of proteins interact with both ATP (or another nucleotide) and nucleic acids and are involved in complex transactions such as DNA replication, recombination, transcription, RNA processing and translation. Although diverse in their mechanisms and biological roles, two features which unify many of these processes are that they generally involve multiple proteins which assemble in an ordered fashion and they also often involve changes in the structure of nucleic acids. An attractive idea is that ATP hydrolysis may play a critical role in directing the ordered and appropriate assembly of particular nucleoprotein complexes. Interaction of proteins with alternative nucleotide cofactors could mediate changes in protein conformation and thereby mediate changes in nucleoprotein structure. TnsC is likely a component of an elaborate recombination machine that contains multiple proteins and the DNA recombination substrates (10). We speculate that TnsC, through conformational changes and interactions with DNA as determined by adenine nucleotides, plays a central role in the functional assembly of such a complex.

ACKNOWLEDGEMENTS

We are particularly grateful to Roland Bainton for numerous helpful discussions. We thank Lidia Arciszewska and Ken Kubo for providing TnsB, TnsD and also helpful comments about protein purification, and Karina Orle for plasmids and antibodies. We also thank Ann Hagemann, Ken Kubo and Anne Stellwagen for comments on the manuscript. PG was supported the NIH-CNRS program and a Nato Fellowship. The work was supported by a grant from the National Institute of Allergy and Infectious Disease to NLC.

REFERENCES

1. Berg, D.E. and Howe, M.M. (1989) (eds) Mobile DNA American Society forMicrobiology, Washington, D.C.
2. Barth, P.T., Data, N., Hedges, R.W. and Grinter, N.J. (1976). *J.Bacteriol.* 125, 800–810.
3. Craig, N.L. (1989). In Berg, D.E. and Howe, M.M. (eds.), Mobile DNA, (American Society for Microbiology, Washington, D.C., pp. 211–226.
4. Craig, N.L. (1991) *Mol Micro* **5**, 2569–2573.
5. Lichtenstein, C. and Brenner , S. (1982) *Nature* **297**, 601–603.
6. McKown, R.L., Orle, K.A., Chen, T. and Craig, N.L. (1988). *J. Bacteriol.* **170**, 352–358.
7. Rogers, M., Ekaterinaki, N., Nimmo, E. and Sherratt, D.J. (1986). *Mol. Gen. Genet.* 205, 550–556.
8. Waddell, C.S. and Craig, N.L. (1988). *Genes & Develop.* **2**, 137–149.
9. Kubo, K. and Craig, N.L. (1990). *J. Bacteriol.* **5**, 2774–2778.
10. Bainton, R., Gamas, P. and N.L. Craig (1991) *Cell* 65, 805–816.
11. Orle, K.A and Craig, N.L. (1991) *Gene* **104**, 125–131.
12. Grayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M. (1984). *EMBO J.* **3**, 2437–2442.
13. Smith, G. and Jones, P. (1986) *Nucleic Acids Res.* **14**, 7915–7927.
14. Flores, C., Qadri, M.I. and Lichtenstein, C. (1990). *Nucl. Acids Res.* **18**, 901–911.
15. Baker, T.A., Grossman, A. and Gross, C.A. (1984). *Proc. Natl. Acad. Sci. USA* 81, 6779–6783.
16. Miller, J. (1972). Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
17. Laemmli, U.K. (1970). *Nature* **227**, 680–685.
18. Yue, V.T. and Schimmel, P.R. (1977). *Biochemistry* 3, 297–317.
19. Biswas, S.B. and Kornberg, A. (1984). *J. Biol. Chem.* **259**, 7990–7993.
20. McKown, R.L., Waddell, C.S., Arciszewska, L.K. and Craig, N.L. (1987). *Proc.Natl. Acad. Sci. USA* 84, 7807–7811.
21. Arciszewska, L., McKown, R. and Craig, N.L. (1991) *J. Biol. Chem.* **266**, 21736- 21744.
22. Tang, Y., Lichtenstein, C. and Cotterill, S. (1991) *Nucl. Acids Res.* **19**, 3395–3402.
23. Waddell, C.S. and Craig, N.L. (1989) *Proc. Natl. Acad. Sci. USA* 86, 3958–3962.
24. Richet, E. and Raibaud, O. (1987). *J. Biol. Chem.* **262**, 12647–12653.
25. Richet, E. and Raibaud, O. (1989). *EMBO J.* **8**, 981–987.
26. Richet, E., Vidal-Ingigliardi, D. and Raibaud, O. (1991). *Cell* **66**, 1185–1195.
27. Chaconas, G., Gloor, G. and Miller, J.L. (1985). J. Biol. Chem. **260**, 2662–2669.
28. Maxwell, A., Craigie, R. and Mizuuchi, K. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 699–703.
29. Adzuma, K. and Mizuuchi, K. (1988). *Cell* 53, 257–266.