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Use of gene fusions and protein–protein interaction in the isolation of a biologically active regulatory protein: The replication initiator protein of plasmid R6K

(protein tagging/subunit interaction/protein sequence analysis/affinity chromatography)

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ABSTRACT **The initiation of DNA replication of plasmid R6K is triggered by a 35-kilodalton initiator protein. The initiator protein had been elusive because of its lability and the lack of a convenient assay procedure to aid its purification. Using recombinant DNA techniques, we have fused the cistron of the initiator near its COOH-terminal end, in the correct reading frame, to the *lacZ* cistron of *Escherichia coli* at the ninth codon from the NH_2 terminus. The fused cistron yielded a protein that was not only stable *in vivo* but also had dual activities: initiation of DNA replication *in vivo* and *in vitro* and hydrolysis of β-galactoside. Using an affinity column that is specific for β-galactosidase, we have demonstrated the rapid purification of the hybrid protein to near homogeneity. Exploiting the polymeric structure of the initiator, we have also isolated the nonfused form of the initiator protein, associated through subunit interaction with the β-galactosidase-fused protein, which permits its purification by affinity chromatography. NH_2-terminal amino acid sequence analysis of the heteropolymer has not only shown that the fused and nonfused initiators have the same sequence but also confirmed the protein sequence of the initiator as predicted from its nucleotide sequence. The techniques described here should be generally useful for the isolation of other proteins that are difficult to purify by conventional procedures.**

Initiation of replication of chromosomes that replicate in the Cairns-type mode is triggered by the interaction of replication initiator proteins with specific sequences on the DNA (1). The biochemical elucidation of the initiation process still remains a major goal in molecular biology. Purification of replication initiator proteins, obviously, is a key step in the biochemical dissection of the initiation process.

We have used the drug-resistance plasmid R6K (2), which replicates in the Cairns-type configuration from multiple origins of replication (3–5), as a model system to study replication initiation. The replication of R6K is triggered by a 35-kilodalton (kDa) plasmid-encoded initiator protein (6–8). The apparent lability and lack of an adequate assay procedure posed serious problems during previous attempts to purify this protein. We have previously reported that the initiator can be tagged by fusion with the 89-amino-acid-long α-donor peptide of β-galactosidase to yield a fused protein that retains its ability to initiate DNA replication *in vivo* (9). However, this approach failed to yield a homogeneous preparation of stable hybrid-initiator protein.

In this communication we describe the fusion of the replication initiator gene of R6K with a larger β-galactosidase-coding sequence in such a way that the 3′ end of the initiator gene is covalently attached to the ninth amino acid codon from the NH_2 terminus of the *lacZ* cistron, in the correct translational frame. We further show that the fusion apparently stabilizes the initiator protein without destroying its biological activity, as measured by *in vivo* and *in vitro* DNA replication initiated from the region of replication origin γ. Using an affinity matrix that specifically retains β-galactosidase, we have demonstrated the rapid purification of the hybrid initiator to near homogeneity.

Exploiting the multimeric structure of the initiator, we have also shown that the nonfused initiator protomer can be rapidly isolated by affinity column chromatography because of subunit interaction between the fused and the nonfused initiator proteins produced from plasmids carrying a second nonfused copy of the initiator gene.

MATERIAL AND METHODS

Bacterial and Plasmid Strains. The *Escherichia coli* strain MC1000 (*ara* D139, Δ (*ara, leu*), 7697 Δ *lac* × 74, *galU galK*, *StrA*) was obtained from Malcom Casadaban (University of Chicago). The *lacZ* fusion vectors pORF5 and pMLB1031 were obtained from M. L. Berman through C. Turnbough (University of Alabama at Birmingham). The plasmid pJG100, that contains the initiator protein cistron of R6K in a *Bam*HI fragment, has been described (9).

Construction of Recombinant DNA Clones. These were carried out by using standard procedures as described (9).

Preparation of the Affinity Matrix. The diaminohexane linker was attached to cyanogen bromide-activated Sepharose 4B and then succinylated with succinic anhydride. The substrate *p*-aminophenyl-β-D-thio-galactoside was then attached to the succinyl diaminohexane arm with water-soluble carbodiimide. The entire procedure has been described (10). The chemicals used and ready-made affinity matrix are available from Sigma.

Purification of β-Galactosidase-Tagged Protein. Bacterial cells (strain MC1000) containing the plasmid pJG10 or pJG11 (see *Results*) were grown in standard Luria broth to a cell density of 2–5 × 10^8 cells per ml and then were harvested. All subsequent operations were carried out at 4°C. The cell pellet was then suspended in a suitable volume of buffer B (0.2 M Tris·HCl, pH 7.6/0.25 M NaCl/0.01 M magnesium acetate/10 mM 2-mercaptoethanol/5% glycerol; 10-ml volume per liter of culture). Lysozyme was added to 0.2 mg/ml and the suspension was kept on ice for 30 min. The cell suspension was frozen at −70°C and thawed quickly in a 30°C water bath, taking care not to allow the temperature of the thawing suspension to rise above 4°C. After brief sonication to reduce the viscosity, phenylmethylsulfonyl fluoride was added to 1 mM. The lysate was

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Abbreviations: NH_2PhSGal-Sepharose, *p*-aminophenyl-β-D-thio-galactosidyl succinyldiaminohexyl-Sepharose; kDa, kilodalton(s).

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clarified by centrifugation at 35,000 rpm in a type 60 Ti rotor (Beckman) for 30 min at 4°C. The β-galactosidase activity in the cleared lysate was determined as described (11). Nucleic acids were precipitated by adding slowly 0.1 vol of 30% (wt/vol) streptomycin sulfate. Precipitated material was removed by centrifugation. An equal volume of 80% (of saturation) ammonium sulfate solution was added slowly with stirring. The precipitate was collected by centrifugation and the supernatant was checked for β-galactosidase activity to make sure that it had <10% of the original activity. The supernatant was then discarded. The pellet was then suspended in buffer D (0.25 M NaCl/10 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/1 mM EDTA/10 mM 2-mercaptoethanol/0.1% Triton X-100) and dialyzed against 100 vol of the same buffer for 3 hr. The dialysate containing 90% of the original β-galactosidase activity was passed through a 10-ml bed volume of *p*-aminophenyl-β-D-thio-galactosidyl succinyldiaminohexyl-Sepharose (NH_2PhSGal-Sepharose) in a column at 4°C. Although ≈90% of the applied protein appeared in the flow-through, <10% of the input β-galactosidase activity was detectable in that fraction. The column was washed with 10 bed vol of buffer D, followed by 5 bed vol of buffer D without Triton X-100. The protein was eluted from the column with 0.1 M sodium borate (pH 10) and *promptly* precipitated with ammonium sulfate.

NH_2-Terminal Amino Acid Sequence. The NH_2-terminal sequence was analyzed in a Beckman sequencer by using a 0.55 M Quadrol program and the resulting phenylthiohydantoin amino acids were analyzed by high-pressure liquid chromatography as described by Vanaman and co-workers (12).

Two-Dimensional Gel Electrophoresis. This was carried out as described (13).

***In Vitro* DNA Replication.** Cell extracts of *E. coli* K12 (strain W3110) were prepared as described (14). The cell extract was supplemented with various amounts of purified β-galactosidase-tagged initiator protein, purified supercoiled DNA, and other components as described in the legend to Fig. 3.

RESULTS

Construction of Strains Overproducing the β-Galactosidase-Tagged Initiator. The plasmid pJG10, which contained the initiator protein of R6K fused to the *lacZ* cistron at the ninth codon from the NH_2-terminal end, in the correct translational frame was constructed by cutting the plasmid pJG100 (9) with *Bam*HI and ligating the *Bam*HI fragment containing the initiator to *Bam*HI-cleaved pMLB1031 vector (15). From previously published DNA sequence data (7, 8), half of the recombinant clones should have been in the correct reading frame with *lacZ*. Upon plating on McConkey lactose agar plates, half of the recombinants were colorless, whereas the other half produced pale red clones, indicating production of active β-galactosidase. One of the pale red clones was grown up to confirm that it contained the correct plasmid and also to determine the level of the hybrid protein produced, as measured by the specific activity of β-galactosidase. On the basis of these measurements (specific activity of crude hybrid protein, 4,100 units/mg), the clones produced ≈0.5–1% of the total cell protein as the hybrid.

Normally, in the R6K chromosome, the promoter is preceded by a sequence of eight 22-base-pair repeats (8), at which the initiator protein binds to the DNA (9) and apparently autoregulates its own synthesis. However, the pJG100 clone, from which the initiator moiety was derived, lacks seven of the eight repeats that are the DNA binding sites of the initiator protein.

Crude protein fractions prepared from the pJG10-containing bacterial cells were fractionated with $(NH_4)_2SO_4$, dialyzed, and further purified on the NH_2PhSGal-Sepharose affinity column (10) as described in a previous section. The composition and degree of purity of the crude protein, the 40% $(NH_4)_2SO_4$ fraction, and the affinity-purified fraction were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and are shown in Fig. 1, lanes A, B, and D, respectively. A densitometer trace of lane D showed that the protein was between 93% and 95% pure. The final yield was between 50% and 80% of the starting β-galactosidase activity (specific activity of pure protein, 240,000 units/mg).

Comparison of the mobility of the fused protein, that of β-galactosidase alone (Fig. 1, lane C), and molecular weight markers showed the expected increase in molecular mass of the hybrid protein that was predicted from the known size of the R6K initiator protein.

As an extension of these studies, we decided to construct an additional recombinant plasmid pJG11 that contained both the initiator–β-galactosidase-fused cistron and a separate tandem copy of the initiator cistron that was not fused to β-galactosidase. The nonfused initiator cistron is under the transcriptional control of the *lac* promoter and the natural promoter of the R6K initiator. On the other hand, the fused initiator cistron should be under the control of the *lac* promoter and two tandem copies of the R6K promoters (Fig. 2). The pJG11 plasmid containing cells produced deep red colonies on McConkey lactose agar plates and the specific activity of the β-galactosidase hybrid protein was 56,000 units/mg of total cellular proteins (10–20% of the total cell protein).

To investigate whether heteropolymers of both fused and nonfused initiator protein were produced in the cells that contained the pJG11 plasmid, proteins from these cells were prepared as described above and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. As shown in Fig. 2, lanes A–C, the gel profiles clearly show two proteins with the subunit molecular masses of 156 and 35 kDa isolated by affinity chromatography on NH_2PhSGal-Sepharose (Fig. 2, lane C). Both proteins are greatly overproduced in the cell, as indicated by the fact that, together, these constitute up to 10–20% of the total protein of the cell as estimated by densitometry (Fig. 2, lane A). Because the purified protein has β-galactosidase activity (250,000 units/mg), it is reasonable to conclude that the larger 156-kDa protomer is the fused initiator. Because the

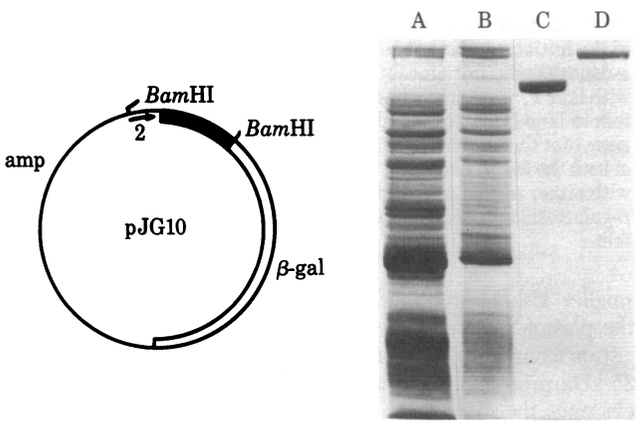
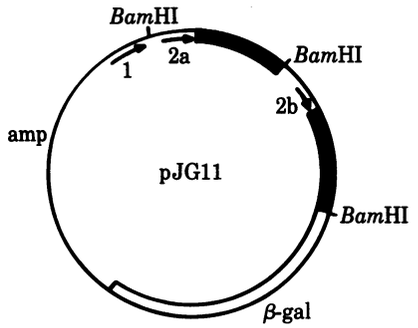


FIG. 1. Physical structure of the plasmid pJG10 and NaDodSO$_4$/polyacrylamide gel analysis of proteins encoded in pJG10. (*Left*) Map of the plasmid pJG10. ▬, Initiator cistron of R6K as a *Bam*HI insert; 2, promoter of the initiator protein cistron; amp, β-lactamase; β-gal, β-galactosidase. (*Right*) Lanes: A, total proteins from MC1000 (pJG10); B, 40% $(NH_4)_2SO_4$ pellet; C, purified β-galactosidase marker; D, purified initiator–β-galactosidase hybrid protein eluted from NH_2PhSGal-Sepharose column with 0.1 M sodium borate (pH 10.00).



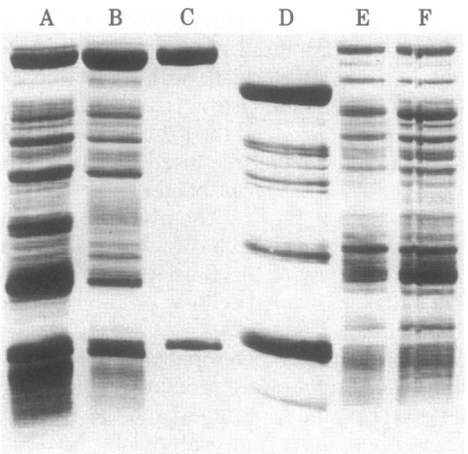


FIG. 2. Physical structure of the plasmid pJG11 and the NaDodSO$_4$/polyacrylamide gel profile of proteins encoded by pJG11. (*Upper*) Map of pJG11 that consists of two tandem copies of the initiator cistron (■), one of which is fused to β-galactosidase. amp, β-Lactamase; β-gal, β-galactosidase; 1, promoter of *lac*; 2a and 2b, promoters of the initiator cistrons. (*Lower*) NaDodSO$_4$/polyacrylamide gel profiles of protein fractions. Lanes: A, total proteins from MC1000 (pJG11); B, 40% $(NH_4)_2SO_4$ pellet of the fraction shown in *Upper*; C, protein purified by affinity column chromatography through NH_2PhSGal-Sepharose; D, α-complemented hybrid protein from pJG100 (see ref. 9); E, protein from MC1000 cells with resident pUC9 plasmid; F, protein from MC1000 cells containing α-donor-tagged initiator cistron. Note the overproduction of the nonfused (35 kDa) and fused (165 kDA) subunits of the initiator in lane A (10% of total protein) and the enrichment of α-donor-tagged initiator (40 kDa) in lane D. When comparing lane E with lane F, no enrichment of a band (corresponding to the tagged protein in lane D) can be found in lane F. This observation strongly suggests that the initiator is not stable, even under transcriptional control of both the *lac* and initiator tandem promoters. In contrast, association with either α-acceptor peptide of β-galactosidase or fusion to a larger β-galactosidase peptide apparently stabilizes the hybrid initiator protein.

smaller 35-kDa protomer is only present in the cells harboring the plasmid having both the fused and the nonfused initiator cistron (compare Fig. 1, lane D, with Fig. 2, lane C), the smaller 35-kDa protomer was the nonfused initiator. More definitive evidence that this was the case was obtained by NH_2-terminal amino acid sequence analysis of the heteropolymeric hybrid protein as described below.

NH_2-Terminal Amino Acid Sequence of the Purified Heteropolymer. If the 156- and the 35-kDa subunits are indeed the fused and nonfused initiator proteins, their NH_2-terminal sequence should be identical to each other. To test this prediction we analyzed the NH_2-terminal sequence of a mixture of the 156- and 35-kDa subunits (present in a molar ratio of 1:2) by automated Edman degradation. The sequence assigned, Met-Arg-Leu-Lys-Val-Met-Met-Asp-Val-Asn, agreed fully with the sequence predicted from the nucleic acid sequence data (7, 8) and placed the correct start point of the protein at the first of the three methionine codons found in the NH_2-terminal seven residues of the coding frame (data not shown). In addition, only a single sequence was detected in the initial cycles of degradation and in the correct yield expected from the total amount of protein in the applied sample. Thus, the results confirm that the 35- and 156-kDa subunits have the same NH_2-terminal sequence and that the 35-kDa protein that was isolated by its apparent association with the 156-kDa fused protein is indeed the monomer of the R6K initiator protein.

The Tagged Initiator Protein Is Biologically Active. We have performed the following experiments to determine if the tagged initiator protein initiates R6K DNA replication *in vivo* and *in vitro*.

We have constructed a recombinant DNA clone, pJG160, that contained the replication origin γ of R6K, inserted into the pJG10 clone (Fig. 1) at the single *Eco*RI site. Therefore, the pJG160 recombinant plasmid contained two replication origins, the *ColE1*-type origin of the vector and the R6K origin. We transformed a *pol*Ats mutant of *E. coli* with the pJG160 DNA with the following objective. Because *ColE1*-type origins have an obligatory requirement for functional DNA polymerase I (16), the vector origin will be "silent" in the *pol*Ats (*pol*A12) host. The R6K origin should be functional because of its nondependence on active DNA polymerase I, provided that the β-galactosidase-tagged initiator protein were biologically active. We consistently observed that the pJG160 plasmid replicated in the *pol*A12 (*pol*Ats) host yielding ampicillin-resistant transformants (data not shown). Therefore, the β-galactosidase-tagged initiator appears to be biologically active *in vivo*.

We also examined the ability of the purified β-galactosidase-tagged initiator to trigger DNA replication *in vitro* as described below. We prepared cell extracts from *E. coli* K12 *pol*A$^-$ (strain D110) that did not harbor a resident R6K plasmid. We purified supercoiled DNA of a recombinant plasmid, pJG14, that contained the region of ori γ of R6K cloned in the vector pUC9 (17). The exogenously added DNA was incubated with or without the addition of the purified initiator, in cell extracts as described (14). The replication, as measured by the template-dependent incorporation of [α-^{32}P]dATP into acid-precipitable material, was greatly stimulated by the added initiator (Fig. 3). The incorporation was rifampicin sensitive and novobiocin sensitive, thus suggesting the possibility of a RNA polymerase-mediated primer and the participation of DNA gyrase in the synthesis (Fig. 3). The pUC9 DNA without the R6K insert was only slightly stimulated in the *in vitro* reaction described above, thus suggesting the requirement of the R6K specific DNA sequence in the *in vitro* reaction (data not shown).

Does the *in vitro* reaction produce Cairns-type replication intermediates that are characteristic of *in vivo* replication of R6K (3) and also of replication in crude cell extracts containing endogenous initiator (18)? To investigate this point, we purified the products of the *in vitro* reaction by phenol extraction and gel filtration and subjected the labeled DNA to two-dimensional neutral-neutral (Fig. 4 *Upper*) and neutral-alkaline (Fig. 4 *Lower*) agarose gel electrophoresis, as described by Sundin and Varshavsky (13). In the two-dimensional neutral-neutral gel, the Cairns-type intermediates that migrated between the supercoiled band and past the open circular band to a position further above in the first dimension formed an arc upon electrophoresis in the second dimension. In addition to the Cairns arc, various topoisomers were also resolved. To make certain that the newly synthesized daughter strands were not cova-

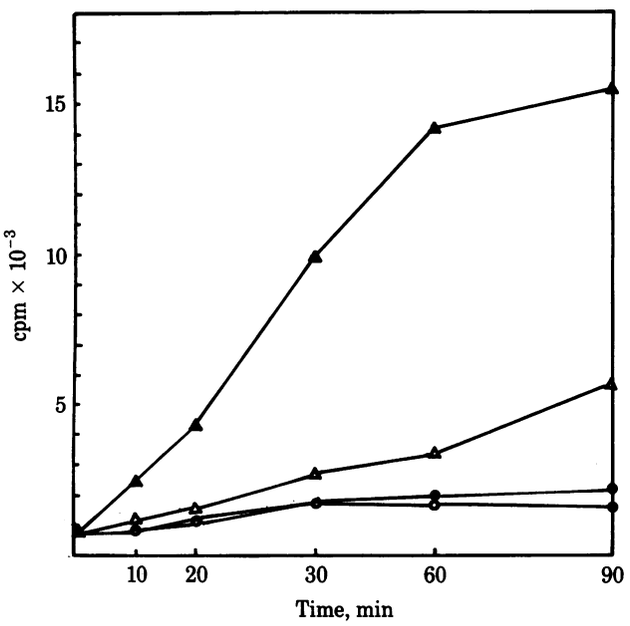


FIG. 3. Activity of the β-galactosidase-tagged replication initiator protein *in vitro*. Lysates were prepared from *E. coli*, D110 (polA) strain, by chloramphenicol treatment and $(NH_4)_2SO_4$ and streptomycin precipitation as described by Conrad and Campbell (see ref. 14). The 50-μl reaction mixture contained [α-^{32}P]dATP, 100 μM (each) dCTP, dGTP, and TTP, 500 μM (each) GTP, CTP, and UTP, 2 mM ATP, 40 mM Hepes (pH 7.6), 11 mM $(CH_3COO)_2Mg$, 100 μg of creatine kinase per ml, 21.6 mM creatine phosphate, 50 μg of bovine serum albumin per ml, 6% polyethylene glycol 6000, 200 μg of prepared lysate per 50 μl, 8.6 μg of pJG14 DNA, and 420 μg of β-galactosidase-tagged initiator protein per ml. Reactions were carried out at 30°C for up to 90 min. Acid-precipitable radioactivity was counted on GF/C filters (Whatman). ▲, pJG14 DNA with the initiator; △, pJG14 DNA without the initiator; ●, effect of 10 μg of rifampicin per ml; ○, effect of 200 μg of novobiocin per ml. The reaction was partially dependent on exogenous ATP, suggesting the presence of some endogenous ATP.

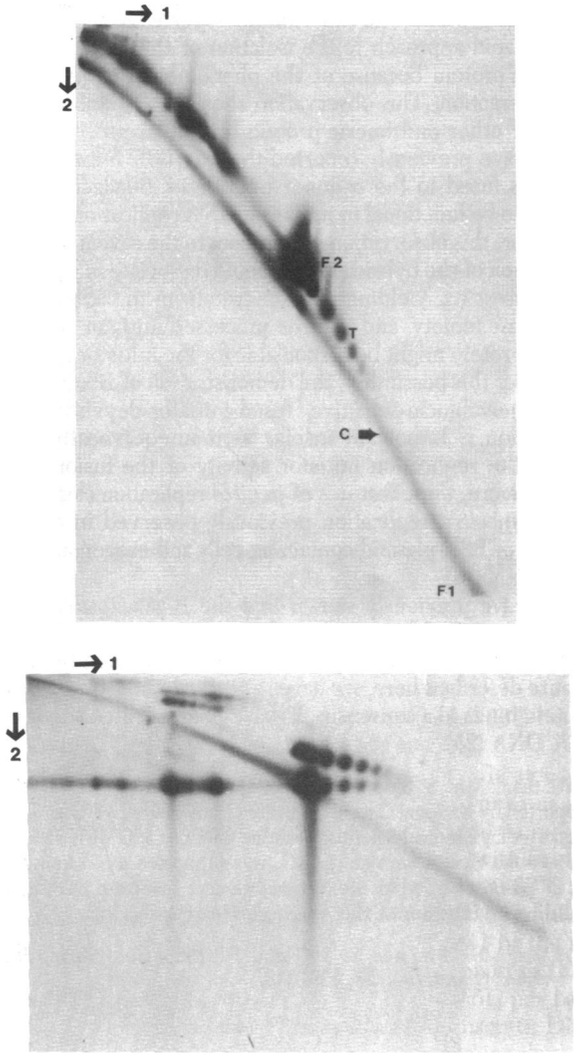


FIG. 4. (*Upper*) Autoradiograms of two-dimensional neutral-neutral agarose gel electrophoresis of the reaction products of pJG14 DNA replicated in the presence of added initiator. Note the Cairns-type intermediates that form an arc (C). The positions of supercoiled (F1), open circular DNA (F2), and topoisomers (T) of finished molecules are indicated. (*Lower*) Two-dimensional neutral (1)-alkaline (2) gel electrophoresis of the *in vitro* replication products. Note the arc formed by the dissociation of newly replicated daughter DNA from the parental DNA. The topoisomers run in a region that is off the diagonal arc of daughter DNA strands. The replicative intermediates were prepared as described in the legend to Fig. 3.

lently attached to the parental DNA, neutral-alkaline gels were run (Fig. 4 *Lower*). Under these conditions of electrophoresis, the daughter strands dissociated from the parental DNA and formed an arc, whereas the topoisomers were resolved in positions that were clearly located off the arc of daughter molecules. The results of the two-dimensional gel analysis strongly suggested that Cairns-type intermediates were indeed produced in the *in vitro* replication reaction that required the purified β-galactosidase-tagged initiator. A more detailed analysis of the *in vitro* replication reaction will be published elsewhere.

Partial Purification of the α-Complemented Hybrid Initiator. It was previously shown by Zabin and co-workers that some α-complemented forms of β-galactosidase bind to the NH_2-PhSGal-Sepharose columns (19). Therefore, we attempted to purify the α-complemented fused initiator by the affinity chromatographic procedure and found that the α-complemented protein can be purified in this manner, although the protein is less pure than the initiator that was fused to the larger β-galactosidase polypeptide (Fig. 2, lane D). We also attempted to purify the α-donor-tagged initiator by the same procedure but found that the 89-residue-long α-donor peptide apparently did not by itself bind to the NH_2PhSGal-Sepharose. Furthermore, the α-donor-tagged peptide was much less stable and did not accumulate in the cell without the presence of the α-acceptor protein (compare Fig. 2, lanes E and F). Therefore, the larger β-galactosidase protein appears to stabilize the initiator in the cell.

DISCUSSION

We have described a procedure for the rapid purification to near homogeneity of the highly labile replication initiator protein of the plasmid R6K. Previous attempts to purify this protein by more conventional approaches had failed. Attempts to overproduce the protein by positioning a strong promoter upstream from the cistron of the protein failed to yield overproducer of the protein. Fusion to the α-donor peptide of β-galactosidase yielded detectable levels of active protein but the hybrid was not sufficiently stable to be purified to homogeneity (9). Therefore, association with a large β-galactosidase moiety appears to be crucial for obtaining a stable protein.

There are reports of purification of β-galactosidase-fusion peptides in the literature (20–22) but not of a biologically active bifunctional protein. However, it has been reported (23, 24) that fusion of a protein with β-galactosidase can yield a bi-

functional protein. The experiments reported in this paper provide a rapid approach to the isolation of the native, nonfused initiator protein because of the phenomenon of protein–protein interaction. This observation should be useful for the isolation of other multimeric protein by similar procedures.

We have previously reported that the R6K initiator protein that was fused to the α-donor peptide of β-galactosidase appeared to be functional in initiating DNA replication *in vivo* (9). However, this observation was subject to the caveat that a small proportion of the hybrid protein could dissociate *in vivo* by protein processing, yielding initiator free from the attached β-galactosidase moiety, and that the processed initiator and not the hybrid protein might be responsible for the initiator activity (9). In view of this possibility, the demonstration of rifampicin-sensitive, novobiocin-sensitive fused-initiator-dependent DNA replication is important insofar as it unequivocally demonstrates the replication initiator activity of the fusion protein. Furthermore, these features of *in vitro* replication closely mimic that of *in vitro* replication previously observed in crude cell extracts of R6K plasmid-containing cells and exogenously added R6K DNA (18).

We have previously shown that the β-galactosidase-tagged partially purified protein specifically bound to two regions of R6K DNA (9). Using the purified protein obtained by the procedure described here, we have recently demonstrated that the protein binds to a consensus 22-base-pair repeated sequence of R6K DNA (25).

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