JOURNAL OF BACTERIOLOGY, May 1997, p. 2817–2822 Vol. 179, No. 9
0021-9193/97/$04.00+0

Cnr Protein, the Negative Regulator of Bacteriophage P4 Replication, Stimulates Specific DNA Binding of Its Initiator Protein α

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Received 3 October 1996/Accepted 15 February 1997

Bacteriophage P4 DNA replication depends upon the phage-encoded α protein, which has DNA helicase and DNA primase activity and can specifically bind to the replication origin (*ori*) and to the *cis* replicating region (*crr*). The P4 Cnr protein functions as a negative regulator of P4 replication, and P4 does not replicate in cells that overexpress *cnr*. We searched for P4 mutants that suppressed this phenotype (Cnr resistant [αcr]). Eight independent mutants that grew in the presence of high levels of Cnr were obtained. None of these can establish the plasmid state. Each of these mutations lies in the DNA binding domain of gpα that occupies the C terminus of the protein. Five different sequence changes were found: T675M, G732V (three times), G732W (twice), L733V, and L737V. A TrxA-Cnr fusion protein does not bind DNA by itself but stimulates the *ori* and *crr* binding abilities of α protein in vitro. The αcr mutant proteins were still able to bind specifically to *ori* or *crr*, but specific DNA binding was less stimulated by the TrxA-Cnr protein. We present evidence that Cnr protein interacts with the gpα domain that binds specifically to DNA and that gpαcr mutations impair this interaction. We hypothesize that gpα-Cnr interaction is essential for the control of P4 DNA replication.

Satellite phage P4 can replicate bidirectionally *via* the θ-mode under the control of its own initiator protein (6, 15). This protein, called gpα (777 amino acids), has DNA binding, helicase, and primase activity (31). The targets for DNA binding are the origin (*ori*) and the *cis*-replication region (*crr*), which are both required for replication. The latter region lies 4.5 kb from *ori* and contains the same repeating octamer (type I repeat), TGTTCACC, that is found in *ori* (8). Another repeated sequence of *ori* is YCACYTAAAG (type II repeats). gpα recognizes and binds exclusively to type I repeats, not to type II iterons (31). Looping between *ori* and *crr* was detected in vitro in the presence of gpα in supercoiled or linear P4 DNA (29). The DNA binding activity of α protein lies near its C terminus (30), while the primase activity is near the N terminus (26). Both domains containing these activities can function independently from each other: the N-terminal truncation containing the primase domain complements a P4 primase-null phage and retains primase activity in vitro (26). The C-terminal 150 amino acids of gpα have specific DNA binding activity in vitro. The helicase domain, probably spanning the middle and the C-terminal third of gpα, requires the integrity of a large fraction of the protein (28).

P4 requires the late gene products of a helper phage from the P2 family to produce progeny (18). In the absence of a helper, it can replicate as a plasmid (5, 9, 21). The copy number of P4 is negatively regulated by the P4 *cnr* gene, which lies upstream of the α gene in the same transcription unit (27). In the absence of Cnr, P4 overreplicates, and this leads to cell killing. As a consequence, phage production in the lytic cycle is decreased, and propagation of P4 in the plasmid state is impaired. Overexpression of *cnr* from a plasmid inhibits P4 DNA replication (27). The target of action of the Cnr protein was not previously known. To define this target, we have isolated P4 α mutants resistant to the inhibitory action of Cnr. We conclude that Cnr protein interacts with gpα to limit DNA replication by a novel mechanism.

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MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. The *Escherichia coli* strains and P4 phages used are listed in Table 1. P4 coordinates are from the updated DNA sequence (GenBank accession no. X51522 [10, 32]). High-titer stocks of P4 were prepared on P2-lysogenic host strains as described before (14). The plasmids used are listed in Table 2. The media used for P4 growth are described by Kahn et al. (14). Cells harboring plasmids were grown in YT medium (20) buffered with 25 mM 3-(*N*-morpholino)propanesulfonic acid (sodium salt; pH 8.0) and supplemented with 0.1% glucose and 25 μg of thiamine-HCl per ml. When appropriate, 100 μg of ampicillin (sodium salt) per ml or 10 μg of chloramphenicol per ml was added. GI724(pTZ106) was grown in IMC broth (16) with 100 μg of ampicillin (sodium salt) per ml.

Isolation of P4 phages resistant to Cnr. The P4 mutants resistant to Cnr were isolated starting from different genetic backgrounds: P4, P4 *vir1*, P4 *del51*, P4 *del51 vir1*, and P4 *vir1* δφR73. This variety was used to enlarge the probability of obtaining a diversity of mutants. About 10^7 phage were plated on C-2422 (pST106), giving rise to two to five plaques in each experiment. The efficiency of plating of the mutant phages on C-2422 and C-2422(pST106) was measured. After the mutations were mapped, P4 *vir1* derivatives carrying the different mutations were constructed by in vitro recombination. The mutant phages are listed in Table 1.

Mapping and sequencing of the cr mutations. The DNA fragment exchange procedure was described by Ziegelin et al. (30). DNA fragments exchanged to map the mutations and to construct P4 *vir1* derivatives were sequenced.

DNA techniques. Standard techniques for plasmid DNA isolation and molecular cloning were used (22). To amplify *cnr* and to introduce a *Nde*I recognition site as part of the ATG start codon, the following oligodeoxynucleotides served as primers (P4 sequences underlined): AACAT**ATG**AAAACACCCTTACCGC CCG-TCTTAC (5′ end of *cnr*) and TTGTCGACGTTGTTCTCCT**TCA**GTGC AGTACCGG (3′ end of *cnr*). For PCR, we supplied pMS4 (Table 2) as a template and *deep vent* DNA polymerase, which has proofreading activity. Following PCR, the reaction mixture was phenol extracted and the DNA was ethanol precipitated. After cleavage with *Nde*I, the amplified fragments were separated from primers and template DNA by agarose gel electrophoresis. DNA was sequenced by the dideoxy chain termination method (23) with T7 DNA polymerase (Pharmacia). The amplified *cnr* gene was sequenced and found to be identical to the published nucleotide sequence (10). αcr-overexpressing plasmids were constructed by exchanging the P4 *Bam*HI-*Asc*I fragment containing the cr

TABLE 1. Bacterial strains and P4 phages used in this study

Strain or phage	Description or relevant genotype	Reference or source
E. coli		
C-1a	Prototroph	24
C-2422	*recA*::Cm (P2 *lg del-1*)	26
SCS1	*hsdR hsdM*$^+$ *recA1*; highly transformable variant of DH1	11
GI724	*lac*PL8, *ampC*::P_{trp}*c*I	16
XL1-Blue	Δ*lac*/F-*lacI*^{q} *Z*Δ*M15*, Tn*10* (Tc)	3
Bacteriophage P4		
P4		25
P4 *vir1*	Immunity insensitive	19
P4 *del51*	Deletion of *cnr*	27
P4 *vir1* δφR73	δ gene of φR73	13
P4 *vir1 del51*	Deletion of *cnr*	27
P4 *vir1* αT675M	C4946T	This work
P4 *vir1* αG732V	G4775T	This work
P4 *vir1* αG732W	G4776T	This work
P4 *vir1* αL733V	C4773G	This work
P4 *vir1* αL737V	C4761G	This work

mutation into pMS4Δ1 to result in pMS4Δ0.5cr. pGZ4Δ1cr was generated by insertion of the *Eco*RI-*Eco*RV fragment of pMS4Δ0.5cr into pGZ119EH.

Partial purification of gpαcr proteins. The procedure described by Ziegelin et al. (31) was slightly modified. The cells were lysed in the absence of high NaCl concentrations to keep α protein precipitated. Following centrifugation, the pellet containing α protein was washed in lysis buffer without salt to remove soluble cellular polypeptides. After solubilization of gpαcr with buffer containing 1 M NaCl and centrifugation, the dialyzed supernatant was fractionated on heparin-Sepharose CL-6B as described previously (31). The protein was approximately 90% pure.

Construction of plasmids overexpressing *trxA-cnr* fusion genes. The *cnr* gene of pST106 (*Nde*I-*Bam*HI fragment) was fused to the *trxA* gene of pTRXFUS (16) to give pTZ106. pTRXFUS contains *trxA* under the transcriptional control of λ p_L and the translational control of the λ *c*II ribosome binding site. *E. coli* GI724 delivers chromosomally encoded λ repressor controlled by p_{trp}. Hence, tryptophan-free medium is required to maintain the repression of the *trx*-fusion. To adapt the production of TrxA-Cnr to our convenient p_{tac}/*lacI*-regulated system in a *recA* background (30), the *trxA-cnr* fusion including the λ *c*II ribosome binding site (*Nsi*I-*Bam*HI fragment) was inserted into pMS119HE prepared with *Pst*I and *Bam*HI to result in pTZ1106. To facilitate the purification of TrxA-Cnr, we inserted a $(His)_6$ tag into the active-site loop of the thioredoxin portion of *trxA-cnr* by using the unique *Rsr*II site of pTZ1106 to give pTZ1106His.

Purification of TrxA-$(His)_6$-Cnr protein (Cnr-th). A 500-ml culture of SCS1(pTZ1106His) was grown at 37°C with shaking. At an absorbance at 600 nm (A_{600}) of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM. Shaking was continued for 5 h. Cells were centrifuged at 4,000 × *g* for 10 min, resuspended in 1 mM spermidine Tris HCl–200 mM NaCl–2 mM EDTA (pH 7.5) (1 g of wet cells in 5 ml), and frozen in liquid nitrogen. All the following steps were performed at 0 to 4°C. Frozen cells were thawed (3 g in 15 ml) and adjusted to 40 mM Tris-HCl (pH 7.6)–4% sucrose–0.13% Brij 58–1 M NaCl–2.5 mM dithiothreitol (DTT)–0.3 mg of lysozyme per ml. Following incubation for 1 h, the highly viscous lysate was centrifuged at 100,000 × *g* for 60 min. To remove soluble cellular proteins, the pellet was washed twice with 1 M NaCl in buffer A (20 mM Tris-HCl [pH 7.6], 2.5 mM DTT, 1 mM EDTA) by rigid homogenization. Then the pellet was resuspended twice in 6 M urea–1 M NaCl in buffer A to dissolve Cnr-th protein. The supernatants of both urea steps were combined (fraction I; 43 ml), and the proteins were precipitated with ammonium sulfate at 60% saturation. The pellet was solubilized in buffer B (6 M urea, 50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl) containing 20 mM imidazole-HCl (pH 8.0) and dialyzed against buffer B–20 mM imidazole-HCl (pH 8.0) (fraction II; 42 ml). A 21-ml volume of fraction II was applied at 5 ml/h to a Ni^{2+}-nitrilotriacetic acid column (0.9 by 7.5 cm) equilibrated with buffer B–20 mM imidazole-HCl (pH 8.0). The column was washed with 20 ml of buffer B–20 mM imidazole-HCl (pH 8.0). The proteins were eluted with 250 mM imidazole-HCl (pH 8.0) in buffer B. Peak fractions containing Cnr-th were pooled (13 ml) and dialyzed four times against 20 ml of buffer C (20 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM DTT, 1 mM EDTA) to renature the protein and then twice against a 10-fold volume of buffer C to get rid of residual urea. The fraction was concentrated by dialysis against 20% polyethylene glycol 20000, dialyzed against 50% glycerol in buffer C (fraction III; 1.4 ml), and stored at −20°C.

Fragment retention assay. DNA fragments containing *ori* or *crr* were incubated with gpα and Cnr protein for 30 min at 37°C in a total volume of 20 μl of buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, and 10 μg of bovine serum albumin per ml and electrophoresed on nondenaturing 3.5% polyacrylamide gels as described before (31). The DNA bands were stained with ethidium bromide, visualized and documented with a FluorImager 575 (Molecular Dynamics), and analyzed with ImageQuant software version 3.3 (Molecular Dynamics). Since both *ori* and *crr* fragments were complexed specifically by gpα forming large aggregates that do not enter polyacrylamide or agarose gels (31), the decreasing intensity of the uncomplexed *ori* or *crr* band was measured. The sources of P4 *ori* or P4 *crr* fragments were the double-stranded M13mp18 derivatives mMSP4ori or mMSP4crr containing the 361-bp *ori* or the 335-bp *crr* fragment inserted into the *Hin*cII site of the multiple-cloning site (Table 2). Digestion with *Eco*RI, *Hin*dIII, and *Bgl*II yielded *ori*- or *crr*-containing fragments of 412 or 386 bp, respectively and additional fragments of 653 and 6,597 bp. The 653- and 6,597-bp fragments served as competitor DNA.

RESULTS

Isolation of P4 mutants resistant to the action of Cnr. Expression of *cnr* from a plasmid inhibits P4 lytic growth (27). To define the target of action of the Cnr protein, we have sought

TABLE 2. Plasmids and phage M13 derivatives used in this study

Plasmid or derivative	Description	P4 coordinates (bp)	Reference
Plasmids			
pGZ119EH	Vector plasmid, ColD replicon, p_{tac}/*lacI*, Cm^r		17
pGZ4Δ1T675M	pGZ119EH Ω[pMS4Δ0.5 T675M *Eco*RI-*Eco*RV, 2,462 bp]	7045–4596	This work
pGZ4Δ1G732W	pGZ119EH Ω[pMS4Δ0.5 G732W *Eco*RI-*Eco*RV, 2,462 bp]	7045–4596	This work
pMS119HE	Vector plasmid, pMB1 replicon, p_{tac}/*lacI*, Ap^r		2
pMS4	pMS119EH Ω[P4 *vir1 Bam*HI-*Dra*I, 3,391 bp]	7652–4261	26
pMS4Δ1	pMS119EH Ω[pMS4Δ0.5 *Eco*RI-*Eco*RV, 2,462 bp]	7045–4596	26
pST106	pMS119HE Ω[T7 gene *10* SD, *cnr*]	7304–6973	This work
pTRXFUS	Vector plasmid, pMB1 replicon, λp_L, λ*c*II SD, *trxA*, Ap^r		16
pTZ106	pTRXFUS Ω[*Kpn*I-*Nde*I adapter,[a] pST106 *Nde*I-*Bam*HI, 342 bp]	7304–6973	This work
pTZ1106	pMS119HE Ω[pTZ106 *Nsi*I-*Bam*HI, 761 bp]	7304–6973	This work
pTZ1106His	pTZ1106 Ω[*Rsr*II $(His)_6$-encoding fragment[a]]	7304–6973	This work
M13 derivatives			
M13mMSP4ori	M13mp18 Ω[P4 *Ssp*I-*Bst*NI, 361 bp]	9105–9465	This work
M13mMSP4crr	M13mp18 Ω[P4 *Eco*RV-*Bam*HI, 335 bp]	4261–4595	This work

[a] Nucleotide sequences: *Kpn*I-*Nde*I adapter: 5′-CCGGGCA
3′-CATGGGCCCGTAT
$(His)_6$-encoding fragment: 5′-GTCGACATCACCATCACCATCACG
3′-CTGTAGTGGTAGTGGTAGTGCCAC

TABLE 3. Amino acid exchanges in αcr proteins and corresponding base exchanges

Name of mutation	Codon and base exchange[a]	P4 coordinate of exchanged base[b]	Amino acid exchange	Amino acid position	No. of mutants
αT675M	A**C**G→A**T**G	4946	Thr→Met	675	1
αG732V	G**G**G→G**T**G	4775	Gly→Val	732	3
αG732W	**G**GG→**T**GG	4776	Gly→Trp	732	2
αL733V	**C**TG→**G**TG	4773	Leu→Val	733	1
αL737V	**C**TG→**G**TG	4761	Leu→Val	737	1

[a] Bases exchanged are given in boldface type.
[b] The P4 position given refers to the 11,624-bp sequence (10).

TABLE 4. Effect of the αcr mutations on P4 plasmid propagation

Infecting phage[a]	Surviving clones (%)[b]	Plasmid P4 carriers/ survivors (%)[c]
P4 *vir1*	100	100
P4 *vir1* αT675M	3	<0.1
P4 *vir1* αG732V	2	<0.1
P4 *vir1* αG732W	3.4	<0.1
P4 *vir1* αL733V	1.5	<0.3
P4 *vir1* αL737V	10	<0.1

[a] Strain C-1a was infected with the phages indicated at a multiplicity of 10.
[b] The cells surviving the infection were assayed 20 min after infection.
[c] Plasmid P4-carrying clones were identified by their colony morphology as described by Alano et al. (1).

P4 mutants resistant to Cnr. *E. coli* C-2422(pST106), which produces the Cnr protein and blocks P4 plaque formation, was used as a selective host. P4 phages with different genetic backgrounds were plated on this strain. With each of the phages, P4 plaques arose with a frequency of 2×10^{-7} to 5×10^{-7}. The phages of these plaques plated on C-2422(pST106) with an efficiency of 1. We isolated eight independent P4 mutants of this type. These Cnr-resistant mutants were called cr.

The cr mutations map in the 3′ half of the α gene. The eight mutations were mapped by fragment exchange as described in Materials and Methods. All were located within P4 coordinates 4261 to 5653. This part of the P4 genome contains the 3′ end of the α gene and the *cis* replication region (*crr*). To demonstrate that the mutations did not lie in *crr*, we exchanged the *Eco*RV fragment (positions 3970 to 4596) containing *crr* from the mutants into P4 *vir1* and found the phage to be inhibited by Cnr protein encoded by pST106. Since we used genetically different P4 phages to search for Cnr resistant mutants but obtained mutations only in the 3′ end of the α gene, we surmise that Cnr acts only at the C-terminal half of gpα.

cr mutations are clustered within the DNA binding domain of gpα. The *Dra*III-*Bam*HI segments (positions 5653 to 4261) derived from the eight mutants were inserted into pGZ119EH and sequenced to determine the exact position of the mutation. All mutants contained a single base exchange leading to a single amino acid exchange. We found that the first and second bases of codons were affected. The positions, the exchanges, and the number of respective mutants are summarized in Table 3. Except for T675M, all the mutations were clustered between gpα amino acids 732 and 737. Most of the exchanges increase the local hydrophobicity of gpα. This indicates that these positions may be important in the interaction with Cnr either by direct protein-protein contact or by stabilization of a certain conformation required for gpα-Cnr interaction. However, it could not be ruled out that Cnr might control P4 replication indirectly, for instance by activating a cellular protein that interacts with gpα.

The αcr mutations impair P4 propagation in the plasmid state. The Cnr protein is essential for the control of P4 copy number when P4 propagates as a plasmid. A phage carrying a deletion of the *cnr* gene overreplicates and causes death of the host cell (27). We tested whether the P4 αcr mutants were able to establish the plasmid state after infection of *E. coli*. Infection of strain C-1a with each of the P4 *vir1* αcr mutants caused severe cell killing, and no plasmid P4 carriers could be found among the survivors (Table 4). Thus, all the αcr mutations impair P4 plasmid propagation, suggesting that the mutations compromise the negative control of Cnr on DNA replication.

Overexpression of Cnr proteins. *cnr* could not be expressed efficiently under the control of the native *cnr* ribosome binding site in the *lacI* regulated expression plasmid pMS4 (data not shown). Therefore, we used pST106 in which an amplified P4 DNA fragment encoding Cnr was fused to the efficient ribosome binding site of T7 gene *10* retaining the original *cnr* nucleotide sequence. This plasmid was suitable for producing Cnr in large quantities (Fig. 1, lane b). A polyclonal Cnr-th-specific antiserum was prepared from the purified Cnr-th protein [(TrxA-$(His)_6$-Cnr); see below]. In both SCS1(pMS4) and SCS1(pST106) cell extracts, the cross-reacting product corresponded in size to the overproduced Cnr protein (data not shown), and this product was not detectable in cells harboring the vector plasmid. This suggests that the protein overproduced from SCS1(pST106) containing a *cnr* gene genetically manipulated at the 5′ end is identical to the molecule encoded by pMS4. The overproduced Cnr protein was highly insoluble in nondenaturing buffers. Following solubilization in 6 M urea, the protein was precipitated during dialysis against physiolog-

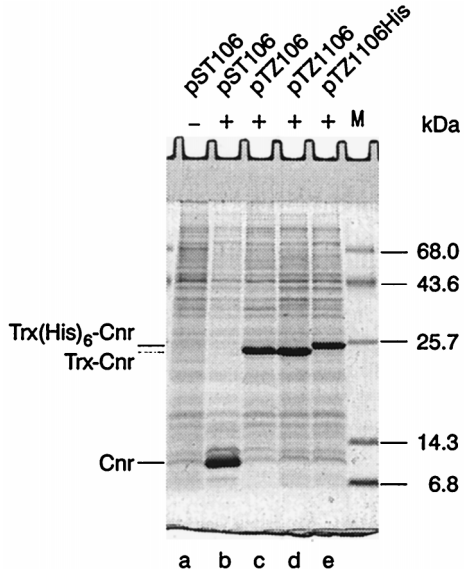

FIG. 1. Overproduction of Cnr proteins. Cells containing various *cnr*-overexpressing plasmids were grown at 37°C [GI724(pTZ106) at 30°C] to an A_{600} of 0.5. IPTG was added to 1 mM. For GI724(pTZ106), tryptophan was added to 100 μg/ml. Shaking was continued for 4 h. Cell extracts from cells with an A_{600} of 0.08 were electrophoresed on denaturing 17.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrophoresis, proteins were stained with 0.25% Coomassie brilliant blue R-250. Cnr-th denotes the TrxA-$(His)_6$-Cnr fusion protein encoded by pTZ1106His. Lanes: +, extracts of induced cells, −, extracts of noninduced cells; M, marker proteins. The *cnr*-overexpressing plasmids are indicated above the lanes. The marker proteins were bovine serum albumin (68 kDa), ovalbumin (43.6 kDa), chymotrypsinogen (21.5 kDa), lysozyme (14.3 kDa), and aprotinin (6.8 kDa).

TABLE 5. Effects of Cnr proteins on P4 yield[a]

Plasmid	Protein	P4 yield (no. of phage/cell)	Efficiency of plating
Vector		275	1
pST106	Cnr	4.6	1×10^{-7}
pTZ1106	TrxA-Cnr	26	2×10^{-7}
pTZ1106His	Cnr-th	55	0.22

[a] *E. coli* C-2422 carrying the indicated plasmids was grown at 37°C in Luria-Bertani broth without IPTG inducer. The cells were infected with P4 *vir1*, and the burst size was measured after 2 h as described by Ziegelin et al. (30). Cnr-th denotes the TrxA-$(His)_6$-Cnr fusion polypeptide.

ical buffers. Since thioredoxin A fusions circumvent inclusion body formation of certain proteins in the *E. coli* cytoplasm (16), we fused the N terminus of Cnr to thioredoxin A by using different vectors to give pTZ106 and pTZ1106 (see Materials and Methods). Both constructs encode the TrxA-Cnr fusion. Following induction, GI724(pTZ106) and SCS1(pTZ1106) overproduced a protein with the expected molecular mass (lanes c and d). The fusion protein of either strain remained soluble under physiological conditions. Although the hexahistidine-tagged TrxA-$(His)_6$-Cnr derivative (Cnr-th) encoded by pTZ1106His was produced as efficiently as TrxA-Cnr (lane e), the His-tagged fusion polypeptide was insoluble. However, after solubilization in 6 M urea and renaturation by dialysis against physiological buffers, Cnr-th remained in solution.

N-terminal modifications of Cnr reduce activity. To decide whether these fusions are suitable for in vitro studies, we wished to test whether the proteins retained Cnr activity in vivo. Thus, we infected C-2422 and three of its plasmid-carrying derivatives with P4 *vir1* and measured the burst sizes. Table 5 shows that wild-type Cnr reduced the burst of P4 progeny by 47-fold whereas TrxA-Cnr reduced the P4 burst by only 8-fold and Cnr-th reduced the P4 burst by only 4-fold. Thus, the fusion proteins are less active than wild-type Cnr, but they still have measurable activities. This conclusion was confirmed by the efficiencies of plating of P4 *vir1* on the different strains summarized in Table 5.

Purification of Cnr-th. The $(His)_6$ tag of Cnr-th allows isolation of the protein by a single column purification step, since it binds selectively to an Ni^{2+}-chelating matrix from which it was easily eluted. The Cnr-th protein obtained by the procedure described in Materials and Methods was approximately 95% pure.

Cnr-th stimulates the binding of gpα to *ori* or *crr*. In vivo, *cnr* overexpression inhibits P4 DNA replication. This inhibition is suppressed by the concurrent overexpression of α (27). Therefore, we assumed that Cnr either binds to the origin to prevent access of gpα or interacts with gpα to alter one or several of its replication activities. Cnr-th did not bind specifically or nonspecifically by itself to double-stranded or single-stranded DNA (data not shown). To analyze the effects of Cnr on gpα-DNA interaction, *ori* or *crr* fragments in combination with competitor DNA were incubated with gpα at a subsaturating concentration and with increasing amounts of Cnr-th. The amount of gpα used in the assay was such that a portion of the *ori* fragment was bound. The amount of unbound fragment in the absence of Cnr-th was set 100%. Protein-DNA complex formation was analyzed on nondenaturing polyacrylamide gels (see Materials and Methods). Increasing amounts of Cnr-th decreased the fraction of unbound *ori* or *crr* DNA fragments (Fig. 2A and B). At a 25-fold molar excess of Cnr-th over gpα, the fraction of unbound *ori* or *crr* was reduced by approximately 10-fold without binding to competitor DNA (Fig. 2A and B). At lower ratios, this stimulation of binding was weaker. Cnr-th enhanced the binding of α protein to *ori* and *cnr* equally well, indicating interactions between gpα and Cnr-th. The excess of Cnr-th required may be due to weak protein-protein interactions, to steric hindrance of the TrxA portion resulting in a less active Cnr protein, or to partially active Cnr-th protein that was not completely renatured.

The stimulatory effect of Cnr-th on αcr proteins is reduced. Since Cnr-th enhances the specific DNA binding capability of gpα, the Cnr-resistant phenotype may be due to gpα molecules that either interact more weakly with *ori*/*crr* or are not stimulated by Cnr-th to bind *ori*/*crr*. To test these hypotheses, two different αcr proteins were partially purified by chromatography on heparin-Sepharose (see Materials and Methods) and tested for complex formation with *ori* or *crr* in the presence and absence of Cnr-th. We chose gpαcrT675M and gpαcrG732W to analyze the effect of mutations in separated regions of the protein. Specific binding of both mutant α proteins to *ori* or *crr* was not significantly different from that of the wild-type protein. However, in the presence of Cnr-th, both gpαcr were less stimulated in complexing *ori* than was the wild type protein (Fig. 2C). At a 25-fold molar excess of Cnr-th over the αcr molecules, binding was only weakly enhanced. No significant differences between gpαcrT675M and gpαcrG732W could be observed. Similar observations were made with *crr* instead of *ori* (data not shown). These results demonstrate that amino acids T675 and G732 were not essential for the DNA binding specificity but are involved in interacting with Cnr-th or in stabilizing a structure important for gpα-Cnr interactions.

DISCUSSION

The P4 Cnr protein is a small molecule of 106 amino acid residues (11,802 Da) with four cysteines and a calculated pI of 7.4. The overall character of Cnr is hydrophilic according to the prediction method of Engelman et al. (7). From the amino acid composition or its sequence, it is not clear why this protein is so insoluble. Derivatization of the N terminus of Cnr by thioredoxin only partially moderated the problem of solubility, and this increase in solubility was achieved at the cost of losing in vivo activity. The lower activity of TrxA-Cnr and TrxA-$(His)_6$-Cnr (Cnr-th) compared to wild-type Cnr might indicate the importance of a freely available N terminus for efficient Cnr function. Thus, reduction of activity might be due to steric hindrance by the TrxA moiety in the interaction between Cnr and gpα. On the other hand, the Cnr fusion proteins might be adequate to control P4 replication. In our experiments, Cnr proteins were delivered in *trans* by plasmids. The slight leakiness of the p_{tac}/*lacI* system causes background level expression of the Cnr polypeptides. Since the 5′ end of each *cnr* gene contributes to the ribosome binding site and therefore to the efficiency of translation initiation, the cellular concentration of each Cnr protein would be different. This could also contribute to the reduced in vivo activity of Trx-Cnr and Cnr-th.

The amino acid sequence does not offer any hint to the possible function of the protein as a negative regulator of the copy number in the P4 plasmid state (27). To unravel the mechanism of Cnr action, we have followed a combined in vivo and in vitro approach.

P4 mutants able to plate on a *cnr* overexpressing strain, which is not permissive for wild-type P4, have mutations that map in the α gene, specifying the initiator protein of P4 DNA replication. The mutations are clustered in a 60-amino-acid subdomain of the C-terminal 150-amino-acid-residue domain of gpα that directs specific origin recognition and binding. The αcr mutations do not alter P4 lytic growth but impair propa-

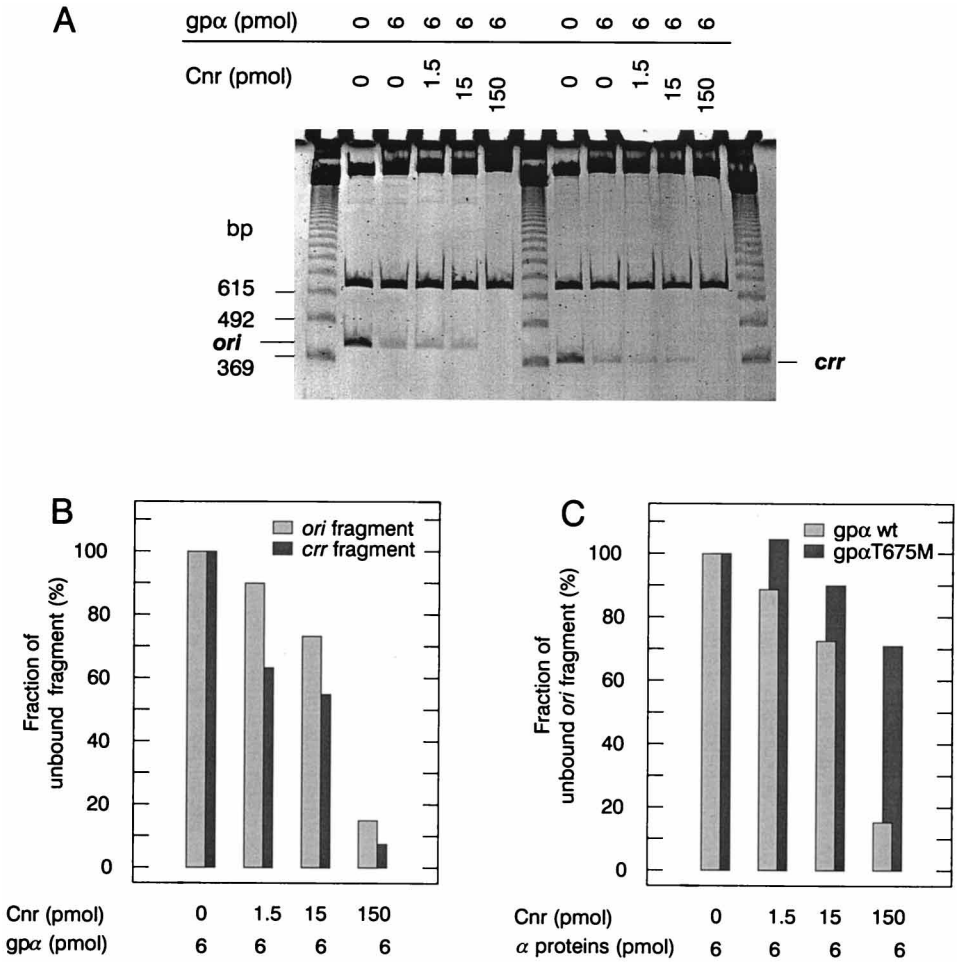

FIG. 2. Cnr-th-stimulated specific DNA binding of α proteins. A mixture of restriction fragments containing either *ori* (170 fmol each) or *crr* (140 fmol each) was incubated with the indicated amount of proteins, the complexes formed were electrophoresed on polyacrylamide gels, and the fraction of unbound *ori* (*crr*) fragments was quantified (see Materials and Methods). (A and B) Cnr-th-stimulated binding of wild-type gpα to *ori* or *crr*. (A) Printout of an electronic image of a gel stained with ethidium bromide. (B) The fraction of unbound *ori* (*crr*) fragment in the presence of 6 pmol of wild-type gpα but in the absence of Cnr-th was set to 100%. (C) Binding of wild-type gpα or gpαcrT675M to *ori* in the presence of Cnr-th. Since the results obtained with gpαcrT675M and gpαcrG732W were very similar, only data obtained with gpαcrT675M are shown.

gation in the plasmid state. This suggests that the DNA replication ability of the mutated gpα is not affected but, rather, that the proteins are insensitive to the negative control of Cnr, which is essential in the plasmid state.

The sites of mutation in gpα indicated that Cnr might interact with gpα and influence the DNA binding affinity. Indeed, the presence of Cnr-th in the DNA binding assay increased the affinity of gpα to *ori* or *crr*. In contrast, Cnr-th has a much smaller effect on the specific DNA binding affinity of the αcr mutant proteins, probably due to their amino acid exchanges. These mutations suppress the phenotype of P4 growth inhibition at high cellular Cnr concentrations. Since Cnr-th does not bind to DNA by itself, we concluded that Cnr-th interacts with α protein directly either by complex formation or by alteration of the conformation of the DNA binding domain. This raises the question whether Cnr is present in the complex of gpα and DNA. We also concluded that the interaction of Cnr-th and gpα must be rather weak, because Cnr-th is needed in a 25-fold excess over gpα to detect the Cnr-induced stimulation in DNA binding. Cnr-th might have a reduced activity due to conformational changes triggered by the Trx moiety. Taking into account the weaker inhibitory effect of Cnr-th on phage propagation compared to that of Cnr, it could be expected that Cnr itself interacts more strongly with gpα than does Cnr-th. Since increased cellular concentrations of Cnr inhibit P4 DNA replication, the complexes formed between α-Cnr and DNA are probably unable to allow for initiation of replication.

The initiation of DNA replication is usually regulated by temporarily inactivating origins. Examples are the antisense RNA model and the iteron model. The copy number of *E. coli* plasmids of the ColE1 family is regulated at the level of the formation of an RNA molecule that serves as a primer for DNA polymerase I. Negative regulation is obtained by the interaction between the precursor of the primer and RNA I, a plasmid-encoded small antisense RNA. The RNA-RNA interaction between the two regulatory molecules is modulated by the Rop protein (4). Currently, there is no indication that the control of P4 DNA replication initiation involves a regulatory RNA species. Certain plasmids, such as P1 and RK2/RP4, are thought to control their replication by dimerization (12). A replicator protein binds to several copies, or iterons, of a specific nucleotide sequence. When the copy number is too high, the multiple molecules of replicator protein bound to the iterons cause the DNA-protein complexes to dimerize and be-

come inactive. The formation of gpα-P4 DNA aggregates might be interpreted as an equivalent to those dimerized inactive complexes. However, P4 seems to use a novel mechanism to control replication: interaction of gpα with Cnr increases the binding affinity for *ori*, yielding protein-DNA complexes inactive in initiation of replication.

The α protein has three distinct replication functions: *ori*/*crr* recognition and binding, DNA helicase, and DNA primase. Of these activities, only the DNA binding ability is influenced in the presence of Cnr-th. Under the conditions used, Cnr-th apparently does not affect ATPase or reduce or increase the helicase or primase activity (data not shown). These findings suggest that blocking the initiation of DNA replication occurs already in the initial step of replication, the formation of an initiation complex between the origin and the initiator protein.

Support for this conclusion comes from the location of mutations that render P4 DNA replication insensitive to increased Cnr concentrations. The eight single amino acid exchanges localize to the DNA binding domain at the C terminus of α, and seven of them are clustered within a stretch of six residues, 732 to 737. These residues might not be involved in DNA binding directly, but Cnr-gpα interaction could change the conformation of gpα. In addition, the mutated residues do not appear to be involved in DNA binding, because the DNA binding ability of the αcr proteins remained unchanged compared with wild-type gpα.

Since Cnr-th neither stimulates nor inhibits the primase or helicase activity of gpα, some other event in initiation should be affected. Hence, we assume that replication initiation is inhibited at an early stage. Taking into account the stimulatory effect of Cnr-th on the specific DNA binding ability of gpα, we speculate that Cnr arrests the initiator protein at the origin to prevent the formation of an initiation complex that is ready for local unwinding.

ACKNOWLEDGMENTS

The expert technical assistance of Marianne Schlicht is greatly appreciated. We thank Simona Türk for the construction of the pTZ plasmids. We thank Gianni Dehò for helpful suggestions and critical reading of the manuscript.

Work in our laboratories was supported by grant La 672/3-2 and by Sonderforschungsbereich grant 344/B2 of the Deutsche Forschungsgemeinschaft to E.L., grant AI-08722 from the National Institute of Allergy and Infectious Diseases to R.C., and the Target Project on Genetic Engineering from the Consiglio Nazionale delle Ricerche and by MURST, Rome, Italy, to D.G. S.T. was a recipient of a Fondazione Buzzati Traverso fellowship, offered by Lepetit Research Center, Gerenzano, Italy.

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