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Host Factor Titration by Chromosomal R-loops as a Mechanism for Runaway Plasmid Replication in Transcription Termination-defective Mutants of *Escherichia coli*

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Two *Escherichia coli* genes, *rnhA* and *recG*, encode products that disrupt R-loops by hydrolysis and unwinding, respectively. It is known that the propensity for R-loop formation *in vivo* is increased during growth at 21 °C. We have identified several links between *rnhA*, *recG*, and R-loop-dependent plasmid replication on the one hand, and genes *rho* and *nusG* involved in factor-dependent transcription termination on the other. A novel *nusG*-G146D mutation phenocopied a *rho*-A243E mutation in conferring global deficiency in transcription termination, and both mutants were killed at 21 °C following overexpression of *rnhA*$^+$. Mutant combinations *rnhA-nusG* or *recG-rho* were synthetically lethal at 21 °C, with the former being suppressed by *recG*$^+$ overexpression. *rho* and *nusG* mutants were killed following transformation with plasmids such as pACYC184 or pUC19 (which have R-loop replication intermediates) even at 30 °C or 37 °C, and the lethality was correlated with greatly increased content of supercoiled monomer species of these and other co-resident R-loop-dependent plasmids. Plasmid-mediated lethality in the mutants was suppressed by overexpression of *rnhA*$^+$ or *recG*$^+$. Two additional categories of *trans*-acting suppressors of the plasmid-mediated lethality were identified whose primary effects were, respectively, a reduction in plasmid copy number even in the wild-type strain, and a restoration of the proficiency of *in vivo* transcription termination in the *nusG* and *rho* mutant strains. The former category of suppressors included *rom*$^+$, and mutations in *rpoB*(Q513L), *pcnB*, and *polA*, whereas the latter included a mutation in *rho* (R221C) and several non-null mutations (E74K, L26P, and Δ64-137) in the gene encoding the nucleoid protein H-NS. We propose that an increased occurrence of chromosomal R-loops in the *rho* and *nusG* mutants leads to titration of a cyloplasmic host factor(s) that negatively modulates the stability of plasmid R-loop replication intermediates and consequently to runaway plasmid replication.

Keywords: R-loops; transcription termination; plasmid replication; *nusG*; *rho*

Introduction

An R-loop is a structure in which RNA is annealed to one strand of double-stranded DNA to form a persistent RNA–DNA hybrid, with consequent displacement of the complementary DNA strand. In *Escherichia coli*, two factors encoded by the genes *rnhA* (RNase H1) and *recG* (RecG helicase), have been identified that disrupt R-loops by distinct mechanisms of hydrolysis and unwinding, respectively.[1]

Indirect evidence for the occurrence of R-loops on the *E. coli* chromosome has come both from studies on the phenomenon called constitutive stable DNA replication that occurs in *rnhA* and *recG* strains, and from the demonstration of synthetic lethality of *rnhA-recG* double mutants.[1–3] It has been assumed from the latter that excessive

Abbreviation used: WT, wild-type.

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R-loops are detrimental to growth. Drolet and co-workers have shown that transcription-associated R-loops are generated in *topA* mutants (which are deficient in topoisomerase I),[4–10] and that their prevalence is increased when the mutants are cultured at 21 °C.[8]

R-loop formation is implicated in replication of the ColE1-like plasmids (which include the pMB9 and p15A replicons).[11–13] Briefly, a transcript RNA-II is initiated at a position approximately 550 bp upstream of, and is directed towards and beyond, the plasmid origin of DNA replication (taken as +1). In a certain proportion of cases, the transcript fails to be released from the DNA template in the vicinity of the replication origin but instead forms an R-loop. The transcript is then cleaved by RNase H1 at +1 to generate a replication primer for initiation of DNA strand synthesis by DNA polymerase I (which is encoded by *polA*). A second plasmid-encoded transcript RNA-I, whose intracellular concentration is proportional to plasmid copy number, is antisense to the 5′ end of RNA-II and acts *in trans* to complex with the elongating RNA-II transcript so as to prevent primer formation from the latter at the replication origin. The complex between RNA-I and RNA-II is stabilized by Rom, encoded by the plasmid-borne *rom* (or *rop*) gene. Plasmid copy number can be modulated by mutation in the chromosomal *pcnB* gene, encoding a poly(A) polymerase that polyadenylates RNA-I and reduces its half-life,[14,15] or by overexpression of the RecG helicase, which unwinds the R-loop prior to formation of the replication primer.[16,17]

In the present study, we have identified novel links between *rnhA*, *recG*, and replication of ColE1-plasmids on the one hand, and Rho-dependent (also called factor-dependent) transcription termination on the other. The latter is the process by which a protein Rho (encoded by the *rho* gene) binds a nascent transcript and interacts with the transcription elongation complex to mediate the release of RNA polymerase from the complex at appropriate sites on the DNA template.[18–20] *In vitro*, the efficiency of Rho-mediated transcription termination is inversely related to the rate of transcription elongation, suggestive of a kinetic component in the interaction.[21] *In vivo*, Rho-dependent termination sites are masked in situations where the transcript encodes a protein, presumably because transcription and translation are coupled and the ribosomes block loading of Rho on the transcript. Furthermore, *in vivo*, Rho-dependent termination requires a second factor NusG (encoded by *nusG*),[22] and the current model is that NusG serves as a bridge between Rho and RNA polymerase in the termination reaction.[18–20] The NusG protein also participates (i) in transcription termination mediated by the phage HK022-encoded Nun protein, and (ii) along with other Nus factors encoded by *nusA* and *nusB*, in an active process of transcription antitermination in the ribosomal RNA (*rrn*) operons as well as in phage λ (where the Nus factors act together with the phage encoded N protein). Both *rho* and *nusG* are essential for viability in *E. coli*.

Here, we provide the first description of a hypomorphic *nusG* mutation that is globally compromised for Rho-dependent transcription termination *in vivo*. We demonstrate that several plasmids of the ColE1 family such as pACYC184 (p15A-derived) or pUC4K (pMB9-derived) exhibit runaway replication in *nusG* and *rho* mutants, and that the mutant combinations *nusG-rnhA* or *rho-recG* are synthetically lethal. We propose that the transcription termination defect leads to increased R-loops on the chromosome and consequently to titration of a cytoplasmic factor that also modulates the stability of R-loops at the plasmid replication origin.

Results

Global deficiency of Rho-dependent transcription termination in a *nusG*-G146D mutant

A conditional lethal *E. coli* derivative (that failed to grow in low-osmolarity rich medium at 42 °C) had been isolated in this laboratory and the responsible mutation was mapped to lie in or near the *rpoB* locus.[23] The mutation was further characterized in this study, and shown to be in *nusG*, situated 4 kb away from *rpoB*. Thus, the mutation was complemented by plasmid pHYD547 and its subcloned derivative pHYD549; the latter carries a 0.95 kb fragment with all of *nusG* and the 3′end of the *secE* gene (which is situated upstream of *nusG* in a bicistronic operon). DNA sequence analysis of the entire *secE-nusG* operon from the isogenic wild-type (WT) and mutant strains (MC4100 and GJ1504, respectively) revealed a G to A substitution in codon 146 of *nusG*, predicted to cause an Asp substitution for the conserved Gly residue at the corresponding site in the NusG protein (*nusG*-G146D).

Four different *in vivo* assays (two previously described and two others identified in this study) were employed to demonstrate that the *nusG*-G146D mutant derivative is phenotypically similar to a *rho*-A243E (previously called *rho*-4) strain (Figure 1), indicative of a global deficiency in factor-dependent transcription termination in both mutants.

(i) The *galEp3* mutation is an IS2 insertion situated between the promoter and structural genes of the *gal* operon, which confers a Gal^- phenotype because of Rho-dependent transcriptional polarity exerted by IS2 on the structural genes.[24] The *rho* and the *nusG* mutations were each independently able to render the *galEp3* strain Gal^+, as determined both by color on D-galactose (Gal)-supplemented MacConkey medium and by growth on minimal-Gal medium (Figure 1(A), rows 1, 4 and 5).

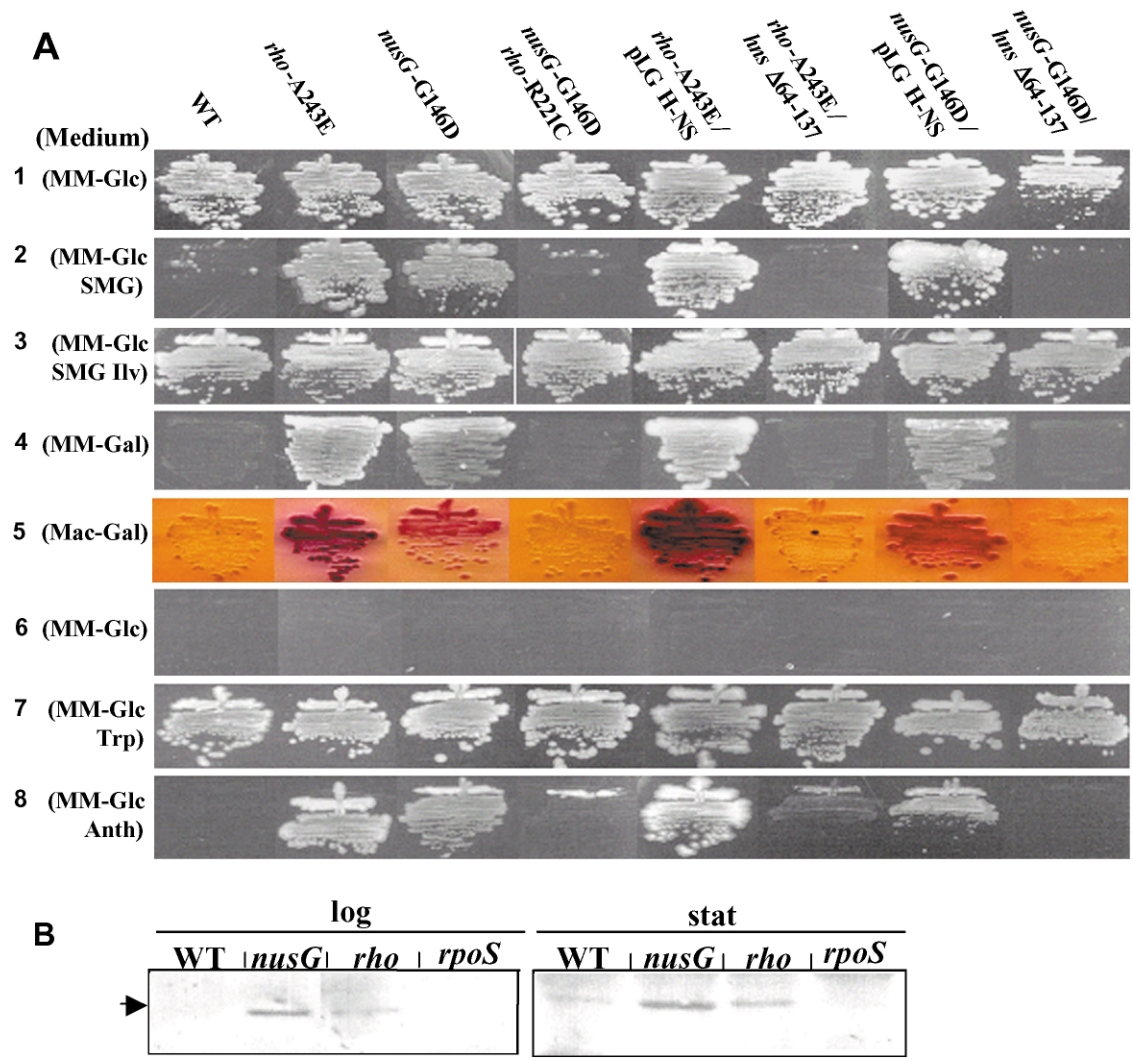

Figure 1. Relief of transcriptional polarity in *nusG*-G146D and *rho*-A243E mutants, and its suppression by *hns* Δ64-137 or *rho*-R221C. (A) An isogenic set of *galEp3 relA1* strains with genotypes at *rho*, *nusG* and *hns* loci as indicated above each column was assessed for transcriptional polarity at the *gal* and *ilvG* loci (rows 1–5) and an equivalent set of isogenic *trpE*(Oc) strains was assessed for transcriptional polarity at the *trp* locus (rows 6–8) as described in the text. MM, minimal A medium; Mac, MacConkey medium. Strains employed, respectively, for rows 1–5 and for rows 6–8 were: column 1, GJ3161, GJ3165; columns 2, 5, and 6, GJ3110, GJ3167; columns 3, 7, and 8, GJ3107, GJ3166; and column 4, GJ3181, GJ3183. Strain derivatives in columns 5 and 7 additionally carried plasmid pLG H-NS, and those in columns 6 and 8 carried plasmid pLG H-NS Δ64-137. (B) Immunoblot analysis with anti-σ^S antiserum of strains MC4100 (WT), GJ1504 (*nusG*), GJ863 (*rho*), and RH90 (*rpoS*) grown in LB to either mid-exponential phase (log) or stationary phase (stat). σ^S band is marked by the arrow.

(ii) Similarly, the *trpE9851*(Oc) mutation confers Rho-dependent transcriptional polarity on the downstream *trpCDBA* genes,[25] so that the mutant strain is unable to utilize anthranilate (Anth) to satisfy its Trp auxotrophy ($Anth^-$). Both the *rho* and the *nusG* derivatives of the *trpE*(Oc) mutant continued to be Trp^- but were now $Anth^+$ (Figure 1(A), compare rows 6–8).

(iii) *E. coli relA* mutants exhibit a SMG^S phenotype; that is, growth-sensitivity in the presence of Ser, Met and Gly at 1 mM each, and the growth inhibition is relieved by Ilv supplementation.[26] Lopes *et al*.[27] have shown that Rho-mediated transcriptional polarity, imposed by a frameshift mutation in the first gene *ilvG* on those further downstream in the biosynthetic *ilvGMEDA* operon of wild-type *E. coli* K-12, compromises expression of the enzymes involved in Ilv synthesis. We found in this study that both the *rho* and *nusG* mutations conferred an SMG^R phenotype in a *relA1* strain (Figure 1(A), compare rows 1–3), as well as in a Δ*relA*::Kan strain (data not shown), which we attribute to the relief from transcriptional polarity imposed by the frameshift mutation in *ilvG*.

(iv) Finally, we observed in immunoblotting

experiments that the *rpoS*-encoded stationary phase-specific sigma factor σ^S was not detectable (as expected) in cells of the wild-type strain during exponential growth,[28] but that it was present in cells of the *nusG* and *rho* mutants under the same conditions; furthermore, the σ^S level in each of the mutants in the stationary phase was higher than that in the wild-type strain (Figure 1(B)). We suggest that these results reflect increased transcriptional read-through, in the *rho* and the *nusG* mutants, into the *rpoS* gene from the *nlpD* promoters situated further upstream.[28]

Previous findings from *in vivo* NusG depletion experiments have established that the protein is required for efficient factor-dependent transcription termination and consequently for cell viability.[22] Our results therefore indicate that *nusG*-G146D is a "hypomorphic" yet viable *nusG* allele that (like *rho*-A243E) leads to defective Rho-dependent transcription termination at a variety of dispersed loci in *E. coli*. Some mutations in *rho* have been shown to confer resistance to phage P2,[24] and we found that the *nusG*-G146D as well as *rho*-A243E mutants are phage P2-resistant (data not shown).

RNase H1- and RecG-related phenotypes in *nusG* and *rho* mutants

As mentioned above, *topA* mutants exhibit an increased propensity for transcription-associated R-loop formation that is further aggravated upon growth at 21 °C; RNase H1 overproduction is detrimental to growth of the mutants at the low temperature presumably because of increased degradation of transcripts under these conditions.[8] A similar phenotype of toxicity associated specifically with RNase H1 overproduction at 21 °C (but not 37 °C) was observed in the *nusG* and *rho* strains when growth of the mutants carrying plasmids with deleted or WT versions of the *rnhA* gene was compared with that of the equivalent WT strain derivative (Figure 2(A)). Temperature-dependent synthetic lethality of *rnhA-nusG* was demonstrated when *rnhA*::Cm was transduced into the *nusG* mutant in presence of a *nusG*$^+$ isopropyl β-D-thiogalactopyranoside (IPTG)-dependent plasmid pHYD751, and the resultant strain was then tested (along with the *nusG rnhA*$^+$ derivative, as control) for growth in medium with, and without, IPTG supplementation; the *nusG-rnhA* derivative failed to grow in the absence of IPTG at 21 °C, and introduction of plasmid pHYD578 with multicopy *recG*$^+$ (but not of the control vector pBR329) suppressed the synthetic lethality (Figure 2(B)). Likewise, the *rho*-A243E and *recG*::Kan chromosomal mutations were synthetically lethal, since the double mutant strain carrying a *rho*$^+$ IPTG-dependent plasmid pHYD1201 was severely compromised for growth in the absence of IPTG at 21 °C (Figure 2(C)).

Greatly increased copy numbers of plasmids pACYC184 and pUC4K associated with lethality in *nusG* and *rho* mutants

Jayashree & Gowrishankar[23] have reported that when the p15A-derived plasmid pACYC184 is introduced into the mutant now identified as *nusG*-G146D, the transformants grow as transparent flattened colonies that are comprised largely of dying or inviable cells. As shown in Figure 3(A), whereas the optical absorbance of cultures of freshly constructed pACYC184 derivatives of a *nusG* or *rho* mutant increase with time, the colony-forming-unit numbers decrease more than 100-fold in the stationary phase of growth. The lethality was RecA-independent (data not shown), and was correlated with an increase of up to 30-fold in plasmid content in both mutants in the stationary phase (Figure 3(B); see also other panels in Figures 3–5). At 21 °C, no pACYC184 transformant colonies were at all recovered in either mutant, suggesting that the lethality phenotype is accentuated at the lower incubation temperature.

That the increase in pACYC184 content is the null phenotype was demonstrated in *nusG*::Kan or Δ*rho*::Kan strains with Ts and IPTG-dependent replicon plasmids carrying *nusG*$^+$ or *rho*$^+$, as appropriate. Plasmid pACYC184 transformants of the strains were grown initially under permissive conditions and then shifted to the restrictive condition. Plasmid pACYC184 content in the null mutants following this treatment was substantially higher than (i) that in similarly treated cultures of the WT strain (data not shown), as well as (ii) that in cultures of the mutant derivatives not exposed to the restrictive condition (Figure 3(C)). The increase in plasmid pACYC184 content in the *nusG* and *rho* strains was predominantly in the form of supercoiled monomers and was RecA-independent (Figure 3(D)).

The *nusG* and *rho* mutants were also killed with the very-high-copy-number pMB9-derived plasmids such as pUC19, pBluescript IIKS, or pUC4K. A specific increase in the pUC4K content was demonstrated when the *nusG* and *rho* derivatives carrying pUC4K and the IPTG-dependent *nusG*$^+$ or *rho*$^+$ plasmids were cultured in absence of IPTG (Figure 3(E)).

We observed that some other R-loop-dependent plasmids whose copy number is otherwise similar to that of pACYC184 did not confer lethality in the *nusG* and *rho* mutants, suggesting that *cis* determinants on the latter (as yet unidentified, but see below) contribute to the phenotype. In the *nusG* and *rho* strains, there was no increase in content of the ColE1-like plasmids that did not confer lethality, such as the vectors pACYC177 and pBR329, or two different pBR322 Rom$^-$ derivatives pHYD761 and pHYD762 (Figure 3(F) and (G)). However, the copy number of one of the latter (pHYD762) did increase substantially in *nusG* and *rho* derivatives that also carried pACYC184 (and

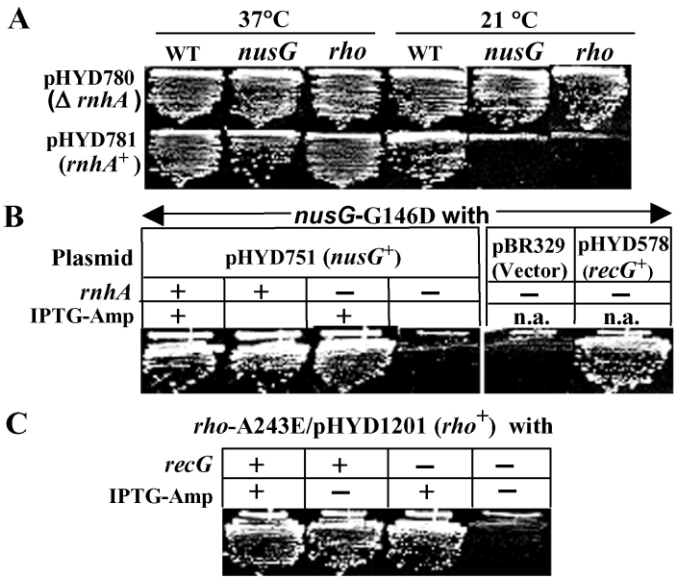

Figure 2. Effects of RNase H1 overexpression (A) or deficiency (B), and of RecG deficiency (C), in *nusG*-G146D or *rho*-A243E mutants. (A) Plasmid pHYD780 (Δ*rnhA*) or pHYD781 (*rnhA*$^+$) transformants of strains GJ3161 (WT), GJ3107 (*nusG*) and GJ3110 (*rho*) were scored for growth on LB-Amp plates at 37 °C or 21 °C, as indicated. (B) Derivatives of *nusG*-G146D strains GJ3107 (*rnhA*$^+$) and GJ3195 (*rnhA*::Cm) carrying plasmid pHYD751 (IPTG-dependent, *nusG*$^+$), pHYD578 (*recG*$^+$), or pBR329 (vector), were scored for growth at 21 °C on LB medium with appropriate antibiotic supplements; the pHYD751 derivatives were grown without, or with, Amp and IPTG supplementation as indicated. n.a., not applicable. (C) Plasmid pHYD1201 (IPTG-dependent, *rho*$^+$) derivatives of *rho*-A243E strains GJ3110 (*recG*$^+$) or GJ3196 (*recG*::Kan) were scored for growth at 21 °C on LB medium without, or with, Amp and IPTG supplementation as indicated.

that were consequently killed in the stationary phase) (Figure 3(G)).

There was no alteration in the copy number of co-resident R-loop-independent plasmids (pSC101-derived) such as pCL1920 or pLG339 in either the *nusG* or the *rho* mutants, even in the presence of the lethality-conferring plasmids pACYC184 (Figure 3(B) and (G)) or pUC4K (Figure 3(E)).

Cell lysis in cultures of pACYC184 transformants of *nusG* and *rho* mutants

Freshly obtained pACYC184 transformants of isogenic WT, *nusG*, and *rho* strains were grown to stationary phase in medium supplemented with IPTG, so that the *lac* operon was fully induced in each of the cultures. Measurements of β-galactosidase activity in the cultures and in the culture filtrates indicated that the enzyme had been induced in all the strains but that it had been released into the medium only in the case of the *nusG* and *rho* mutants; no enzyme activity was detected in culture filtrates of the mutant strain derivatives not transformed with pACYC184 (Table 1). We interpret these findings as evidence for concurrent cell lysis during growth of cultures of the pACYC184 transformants of *nusG* and *rho* strains. The fraction of total enzyme activity that was detected in the culture filtrate may underestimate the true extent of cell lysis, because it is uncertain whether the enzyme is stable following its release into the medium. Consistent with the hypothesis that cell lysis occurs in cultures of the mutants transformed with pACYC184, high molecular mass RNA was detectable in these culture filtrates (data not shown).

Suppression of plasmid-associated lethality by RNase H1 or RecG overexpression

Of a pair of Rom$^-$ ColE1-derived plasmids carrying either Δ*rnhA* (pHYD780) or *rnhA*$^+$ (pHYD781), only the latter was able to suppress both pACYC184-mediated lethality and increased pACYC184 content in the *nusG* and *rho* mutants at 30 °C (Figure 4(A)) or 37 °C (data not shown). The copy number of the non-suppressing Δ*rnhA* plasmid was gratuitously elevated along with that of pACYC184 in either mutant (Figure 4(A)).

That *recG*$^+$ overexpression suppresses plasmid-associated lethality was first established when multicopy suppressors were identified from an *E. coli* genomic library that had been constructed by an *in vivo* cloning approach;[29] four plasmids so obtained (one of which was pHYD758) carried varying lengths of chromosomal DNA that included *recG*$^+$ as a common gene (data not shown). A plasmid (pHYD578) with a 3.2 kb *recG*$^+$ fragment subcloned from pHYD758 suppressed the lethality in both *nusG* and *rho* derivatives (Figure 4(B)). Another plasmid (pHYD583) with an *araBADp-recG*$^+$ construct conferred L-arabinose (Ara)-dependent restoration of pACYC184 copy number and suppression of lethality in the *nusG* mutant (Figure 4(C)); in the glucose (Glc)-supplemented control culture, the content of the *ara-recG*$^+$ plasmid was elevated substantially along with that of pACYC184 in the mutant (Figure 4(C)). Consistent with earlier reports,[16,17] plasmid pACYC184 content was reduced even in

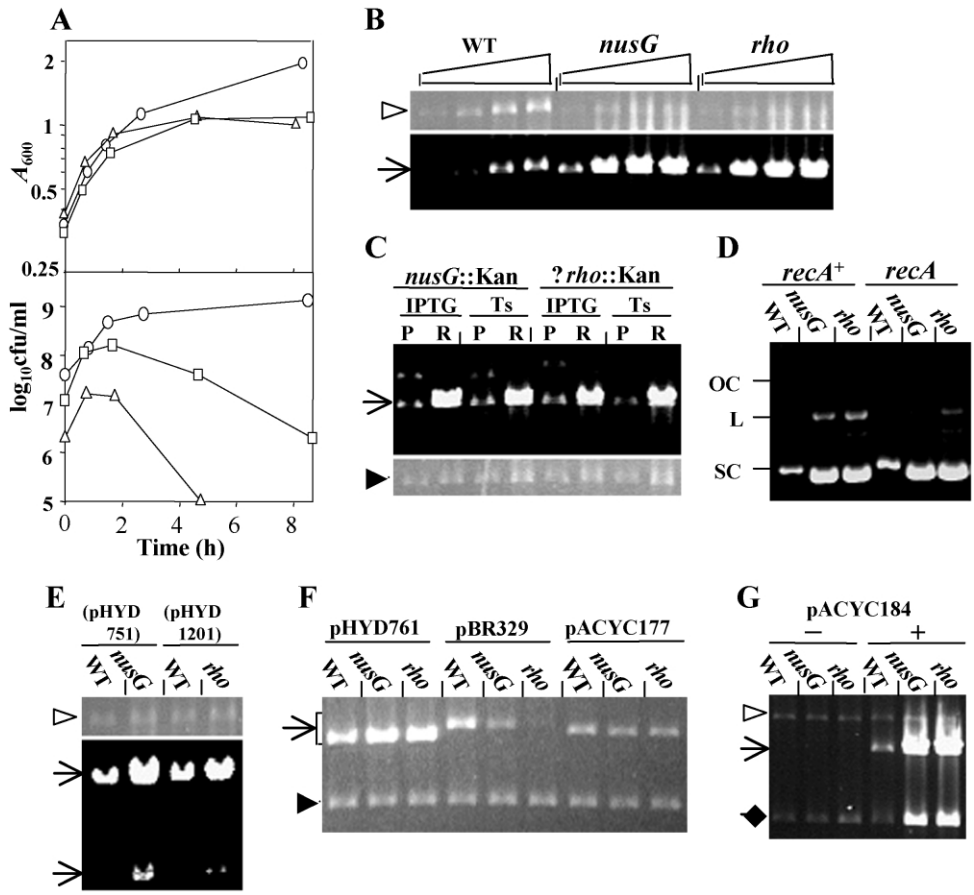

Figure 3. Correlation of increased plasmid content with lethality in *nusG*-G146D and *rho*-A243E mutants. Unless otherwise indicated, (i) the WT, *nusG*, and *rho* strains were GJ3161, GJ3107, and GJ3110, respectively; and (ii) the test, internal control, and external control (pHYD762) plasmid bands are denoted by arrow, open arrowhead and solid arrowhead, respectively. In each of (B), (C), and (E), test and control plasmid images were recorded from the same gel at different exposure settings. (A) Growth curves (A_{600} and colony-forming-units (cfu)/ml) for pACYC184 transformants of WT (○), *nusG* (△), and *rho* (□) strains in LB-Cm medium. (B) Content of test plasmid pACYC184 and internal control pLG339, determined after *Eco* RI digestion, in WT, *nusG*, and *rho* strains with increasing A_{600} (C) Test plasmid pACYC184 content (*Eco* RI-linearized) in derivatives of *nusG*∷Kan strain GJ3191 with the conditional replicon plasmids pHYD751 (IPTG-dependent) or pHYD763 (Ts) carrying *nusG*$^+$, and of Δ*rho*∷Kan strain GJ3192 with the conditional replicon plasmids pHYD1201 (IPTG-dependent) or pPMrhoCam (Ts) carrying *rho*$^+$, following growth under permissive conditions (P) or after shift to the restrictive condition (R), as marked. (D) Plasmid pACYC184 preparations (undigested) from cultures of the WT, *nusG*, and *rho* strains (all *recA*$^+$), as well as the *recA* derivatives GJ3162 (WT), GJ3163 (*nusG*), and GJ3164 (*rho*); SC, OC, and L, supercoiled, open circular, and linear monomers, respectively. (E) Content of test plasmid pUC4K and internal control pCL1920 following Amp and IPTG-withdrawal from cultures of WT and *nusG* derivatives carrying the *nusG*$^+$ IPTG-dependent replicon pHYD751, and of WT and *rho* derivatives carrying the *rho*$^+$ IPTG-dependent replicon pHYD1201, determined following digestion with *Eco* RI (which cleaves pUC4K into two fragments). (F) Content of R-loop-dependent but non-lethal test plasmids (*Eco* RI-linearized) pHYD761, pBR329, or pACYC177 in WT, *nusG*, and *rho* strain derivatives. (G) Content of co-resident plasmids pHYD762 (pMB9-derived, filled diamond) and pLG339 (pSC101-derived, open arrowhead) that are R-loop-dependent and -independent, respectively, in WT, *nusG*, and *rho* strains with and without pACYC184 (p15A-derived, arrow), determined following digestion with *Eco* RI.

the WT strain following RecG overexpression (compare lanes 1 and 4 in Figure 4(B), or lanes 1 and 2 in Figure 4(C)).

Suppressors of plasmid-associated lethality with primary effect on plasmid replication: *rom*$^+$, and mutations in *pcnB*, *polA* or *rpoB*

Additional suppressors of pACYC184-associated lethality in *nusG* and *rho* strains were identified by candidate gene approaches as well as by selection. The suppressors were classified into two categories. One category had a primary effect on plasmid copy number even in the WT strain, and is described in this section. This category of suppressors included *rom*$^+$ and mutations in chromosomal loci *pcnB*, *polA*, and *rpoB*.

The initial suggestion that the Rom protein suppresses plasmid-associated lethality came from the observation that *nusG* or *rho* strains are protected by the compatible plasmid pBR322, but not its Δ*rom* derivative pHYD761, from being killed by

Table 1. β-Galactosidase activity in cultures and culture filtrates of *nusG* and *rho* strains

Strain (Genotype)	pACYC184	β-Galactosidase activity (U/ml) in Culture (A)	Culture filtrate (B)	B/A (%)
GJ3169 (*nusG*$^+$ *rho*$^+$)	−	987	<1	<0.2
	+	840	<1	<0.2
GJ3168 (*nusG*-G146D)	−	1255	<1	<0.2
	+	736	27	3.7
GJ3171 (*rho*-A243E)	−	1096	<1	<0.2
	+	461	13	2.8

The occurrence of spontaneous cell lysis in *nusG* or *rho* derivatives transformed with pACYC184 was assessed by measurements of the total β-galactosidase activity (A) in IPTG-induced cultures (following lysis of cells with sodium dodecyl sulfate and chloroform), and of the activity (B) present in the culture filtrates (prepared by forcing culture supernatants through 0.45 μm filters). For this purpose, the indicated strain derivatives without (−) or with (+) plasmid pACYC184 were grown to stationary phase in LB medium supplemented with IPTG. The unit (U) of enzyme activity is that defined by Miller.[69]

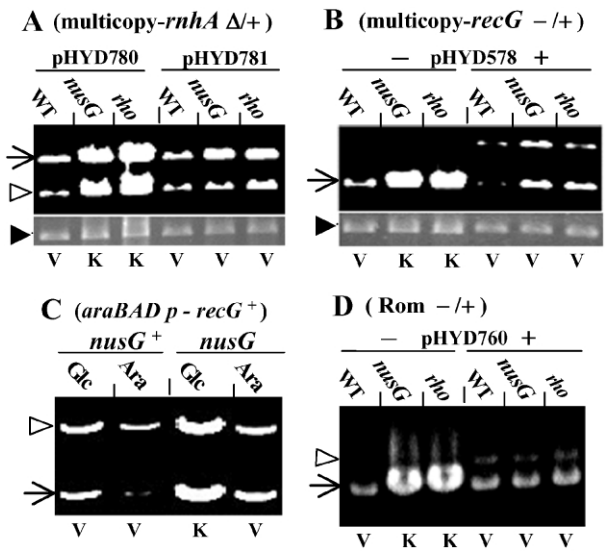

Figure 4. Plasmid content associated with suppression of pACYC184-mediated lethality in *nusG*-G146D or *rho*-A243E mutants by overexpression of RNase H1 (A) or RecG (B) and (C), or by expression of Rom (D). Unless otherwise indicated, the WT, *nusG*, and *rho* strains were GJ3161, GJ3107, and GJ3110, respectively. Plasmid preparations were linearized with *Eco*RI (A)–(C) or *Hin*dIII (D). pACYC184 bands are denoted by arrows, external control bands (pHYD762; recorded with a longer exposure) by filled arrowheads, and bands corresponding to *rom*$^+$, *recG*$^+$, or *rnhA* plasmids by open arrowheads; symbols K and V beneath the lanes identify derivatives that, respectively, were killed and were viable following pACYC184 transformation. (A) pACYC184 content in WT, *nusG*, and *rho* derivatives carrying plasmid pHYD780 (Δ*rnhA*) or pHYD781 (*rnhA*$^+$). (B) pACYC184 content in WT, *nusG*, and *rho* derivatives, without (−) or with (+) plasmid pHYD578 carrying *recG*$^+$. (C) pACYC184 content in GJ3169 (WT) and GJ3168 (*nusG*), each carrying pHYD583 (*araBADp-recG*$^+$), and grown in LB supplemented with Glc or Ara as indicated. (D) pACYC184 content in WT, *nusG*, and *rho* derivatives without (−) or with (+) pSC101-derived plasmid pHYD760 carrying *rom*$^+$.

pACYC184 (data not shown). A pSC101-derived plasmid pHYD760 carrying *rom*$^+$ was able to suppress increase in pACYC184 content and associated lethality in the *nusG* and *rho* mutants (Figure 4(D)). Plasmid pUC*rop*$^+$ (a pUC19 derivative carrying *rom*$^+$),[30] unlike pUC19 itself, yielded viable transformants in the *nusG* and *rho* strains (data not shown).

Several mutations in *pcnB* (encoding poly(A) polymerase), including some that were newly isolated and others previously characterized, suppressed the pACYC184-mediated lethality phenotype. The data for one of the latter (*pcnB1*)[31] are presented in Figure 5. The mutation reduced pACYC184 content drastically both in the WT strain, as expected (Figure 5(A)), and in the *nusG* (Figure 5(B)) and *rho* (Figure 5(C)) derivatives.

The *polA12* mutation (in the gene for DNA polymerase I) fails to support replication of ColE1-like plasmids at 42 °C.[32] That *polA12* is a suppressor of plasmid-associated lethality in *nusG* and *rho* mutants was demonstrated with the aid of a plasmid (pHYD573) bearing a dual origin of replication (that is, of both pACYC184 and pSC101). When transformed into isogenic pairs of *polA*$^+$ and *polA12* derivatives of the *nusG* (GJ1560 and GJ1561, respectively) and *rho* (GJ3189 and GJ3190, respectively) mutants, the plasmid conferred inviability in the *polA*$^+$ but not *polA12* strains, even at 30 °C (data not shown).

An *rpoB* suppressor was identified from an experiment in which several spontaneous Rif^R mutants of the *nusG* strain were each tested for viability following pACYC184 transformation. The suppressor mutation was also associated with reversal of increased plasmid content in the mutant (Figure 5(B)). A segment of the *rpoB* gene encompassing the region where Rif^R mutations are clustered[33] was PCR-amplified from chromosomal DNA of the mutant and sequenced. The mutation was an A to T transversion in codon 513 of *rpoB*, predicted to cause a Gln to Leu substitution (Q513L) in the protein. The same alteration has earlier been characterized as *rpoB101*,[33] which had been shown by Das *et al.*[24] to suppress the transcription termination defect in one *rho* mutant

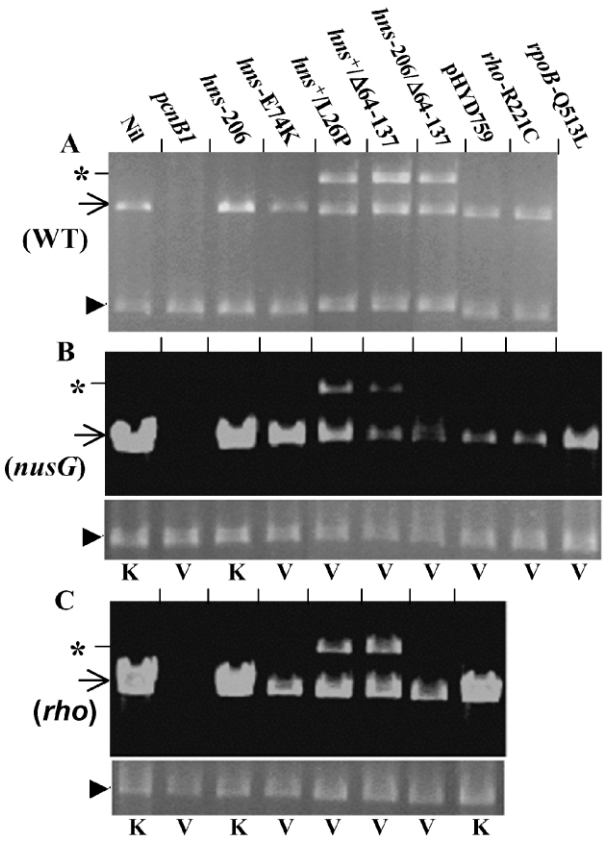

Figure 5. Test plasmid content in WT (A), *nusG* (B), and *rho* (C) derivatives without (Nil) or with *pcnB1*, *rpoB*-Q513L, *rho*-R221C, *hns*, or *cis* suppressor mutations. *Eco* RI-linearized bands of test plasmid (pHYD759 (the *cis* suppressor variant of pACYC184) in column 8 of each panel, or pACYC184 itself in other columns) and of external control plasmid pHYD762 are denoted by arrows and filled arrowheads, respectively. In (B) and (C), test and control plasmid band images were recorded at different exposure settings, and symbols K and V beneath the lanes identify derivatives that, respectively, were killed and were viable following transformation with the test plasmids. Strains for the indicated columns in (A), (B), and (C), respectively, were: nil, GJ3161, GJ3107, GJ3110; *pcnB1*, GJ3185, GJ3186, GJ3187; *hns*-E74K, GJ3178, GJ3180, GJ3179; and *hns-206*, PD32, GJ3176, GJ3177 For lanes corresponding to columns marked pHYD759, *hns*$^+$/pLG H-NS L26P, and *hns*$^+$/pLG H-NS Δ64-137, the said plasmids were transformed into GJ3161, GJ3107 and GJ3110 for (A), (B), and (C), respectively; likewise, for lanes corresponding to column marked *hns-206*/pLG H-NS Δ64-137, the said plasmid was transformed into PD32, GJ3176, and GJ3177, respectively. Additional strains employed were GJ1565 (*nusG rpoB*-Q513L), GJ3182 (*rho*-R221C), and GJ3181 (*nusG rho*-R221C). Asterisks (∗) denote the pLG plasmid bands in the appropriate lanes.

(*rho-15*) but not another (*rho-112*). We found that the mutation did not suppress the termination defect of the *nusG* mutant at the *galEp3*, *trpE*(Oc), and *ilvG* loci, nor its phage P2-resistance phenotype. On the other hand, Yang & Polisky[34] have shown that the *rpoB*-Q513L mutation leads to reduced copy number of the plasmid pUC19 in an otherwise WT strain. The latter result allows us to include this mutation in this category of suppressors.

Lethality suppression associated with reversal of transcription termination defect: mutations in *rho* and *hns*

The other category of *trans*-acting suppressors of pACYC184-mediated lethality was comprised of mutations that led to a reduced plasmid content only in the *nusG* and *rho* mutants, and not in the *nusG*$^+$ *rho*$^+$ strains (Figure 5). This category included several mutations in *hns*, and one in *rho*; and as described below, their ability to suppress two phenotypes in the mutants, namely plasmid-associated lethality and the transcription termination deficiency, was well correlated.

A spontaneous suppressor mutation (subsequently mapped to *rho*) was identified in one of the surviving papillae on a dying colony following pACYC184 transformation of the *nusG* strain, and was associated with reduction of plasmid content in the mutant (Figure 5(B)) but not the WT strain (Figure 5(A)). After transductional mapping, the *in vivo* cloning strategy[29] was employed to clone the mutant allele in linkage with the selectable Δ*rep*::Cm marker (data not shown), and the mutation was shown to be a C to T transition in codon 221 of *rho*, predicted to cause an Arg to Cys substitution in the protein (R221C). Plasmid pHYD564, with a subcloned 3.3 kb *Nsi* I fragment including *rho*-R221C, exhibited dominant suppression of pACYC184-associated lethality in the *nusG*-G146D strain, whereas the equivalent 3.3 kb *Nsi* I fragment with *rho*$^+$ (on pHYD567) was ineffective (data not shown). *rho*-R221C restored transcriptional polarity in the *nusG* mutant at the *galEp3*, *ilvG*, and *trpE*(Oc) loci (Figure 1(A)), and partially reversed its phage P2-resistance phenotype. Both plasmids pHYD564 and pHYD567 (carrying *rho*-R221C and *rho*$^+$ respectively) complemented the *rho*-A243E chromosomal mutant for transcriptional polarity relief as well as pACYC184-mediated lethality (data not shown).

The *hns* gene in *E. coli* encodes the high-abundance DNA-binding protein H-NS, which is a structural constituent of the nucleoid in the cell.[35,36] Jayashree & Gowrishankar[23] had identified the *hns-200* allele as a spontaneous suppressor of pACYC184-mediated lethality in the strain now shown to carry the *nusG*-G146D mutation. Further characterization of *hns-200* in this study revealed that (i) it is a G to A transition in codon 74 of *hns*, predicted to cause a Glu to Lys substitution (E74K), and (ii) it suppresses pACYC184-*rho* lethality. (We have followed the practice of Williams *et al.*[37] to count the N-terminal Met of H-NS as residue 1, although it is post-translationally removed from the mature protein.[38,39])

Upon testing a set of previously described plasmid-borne dominant-negative *hns* mutations,[37] we discovered that two of them (L26P and Δ64-137)

were very effective dominant suppressors of pACYC184-mediated lethality in the *nusG* and *rho* strains; remarkably, both were effective in suppressing the relief of transcriptional polarity at the *galEp3*, *trpE*(Oc), and *ilvG* loci in the mutants. Figure 1 depicts, as an example, the ability of *hns* allele Δ64-137 to dominantly suppress the relief of transcriptional polarity in the *nusG* and *rho* strains (whereas the control plasmid carrying hns^+ was unable to do so). The *hns-200* (that is, E74K) mutation partially suppressed polarity relief in the *nusG* and *rho* mutants, whereas a null *hns-206*::Amp allele[40] was ineffective for suppression of either pACYC184-mediated lethality or polarity relief at *galEp3* in the *nusG* or *rho* strains (data not shown).

The *hns* suppressor genotypes, E74K, +/L26P, and +/Δ64-137, but not the non-suppressing null allele *hns-206*::Amp, were associated with reversal of the increased plasmid pACYC184 content in *nusG* and *rho* mutant derivatives (Figure 5(B) and (C)). Even in the *hns-206*::Amp strain, plasmid-borne *hns* Δ64-137 suppressed the phenotypes of both polarity relief (data not shown) and increased pACYC184 content correlated with lethality (Figure 5(B) and (C)), in the *rho* or *nusG* derivatives.

Cis-acting suppressors of *rho*- or *nusG*-pACYC184 lethality

In an attempt to identify *cis* determinants on pACYC184 that contribute to its ability to kill the *nusG* and *rho* derivatives, strain MC4100/pACYC184 was subjected to nitrosoguanidine mutagenesis and a pACYC184 variant (pHYD759) was obtained that had an A-base insertion situated two nucleotides downstream of the −35 hexamer sequence of the *tet* promoter. The plasmid was lethal in the *rho* but not the *nusG* strain, and its content in the latter but not the former was restored to that in the WT (Figure 5). Another pACYC184 derivative (pHYD753), obtained by destruction of the *Hin*dIII site overlapping the −10 region of the *tet* promoter, was non-lethal in both the *nusG* and *rho* strains, and its content in either mutant was similar to that in the WT strain (data not shown). Both plasmids pHYD753 and pHYD759 conferred a reduced level of Tet^R (to 5 but not 15 μg/ml), suggesting that *tet* promoter strength may be one of the factors that contribute to pACYC184's ability to kill the mutants.

Discussion

NusG role in global Rho-dependent transcription termination

Because *nusG* is essential for cell viability, most *in vivo* studies that had been undertaken earlier were in cells transiently depleted of NusG by incubation of a conditional lethal mutant at the restrictive temperature.[22,41,42] The recessive *nusG*-G146D mutation is the first example of a viable *nusG* allele that confers a transcription termination-defective phenotype. The mutation phenocopies a defective *rho* allele (*rho*-A243E) in conferring phage P2 resistance and relief of transcriptional polarity at each of four widely separated genetic loci (Figure 1), implying a global deficiency of Rho-dependent transcription termination in the *nusG* mutant. This conclusion is further supported by our finding that *nusG*-G146D is synthetically lethal with *rho*-A243E (data not shown). The G146D substitution is expected to affect the structure of a conserved KOW domain in NusG.[43] Interestingly, the vicinal N145D or F144Y variants of NusG are not defective for Rho-dependent transcription termination.[44]

We have obtained evidence that the *nusG*-G146D mutant may be compromised for transcription antitermination in both the *rrn* operons and phage λ, since the *nusG* mutation (i) was synthetically lethal with *nusB5*, and (ii) conferred a λ plaquing defect even at 30 °C in a *nusA1* mutant background (data not shown).

Evidence for chromosomal R-loops in *nusG* and *rho* mutants

Direct evidence exists for transcription-associated R-loops on plasmid templates in *topA* mutants,[4,6,7] but on the other hand all the evidence in support of R-loops on the chromosome (in *topA*, *rnhA*, *recG*, or *rpoB* strains) has necessarily been indirect.[1–3,5,10] For example, *rnhA-recG* synthetic lethality has been explained on the premise that the corresponding proteins alone have the ability to disrupt pre-existing R-loops. It is therefore highly significant that *nusG* and *rho* mutants exhibited several RNase H1- and RecG-associated phenotypes, including sensitivity to RNase H1 overexpression at 21 °C (very similar to that described earlier in *topA* mutants[8]); *nusG-rnhA* and *rho-recG* synthetic lethalities, with suppression of the former by RecG overexpression; and suppression of pACYC184-associated killing by overexpression of either RNase H1 or RecG. We propose that these findings can be explained on the hypothesis that the *nusG* and *rho* mutants suffer increased R-loops on the chromosome.

On the basis of their studies with the *topA* mutants, Drolet's group[7–9] had suggested an increased propensity for chromosomal R-loops from untranslated transcripts. The prevalence of untranslated transcripts is expected to be high in the *nusG* and *rho* mutants for several reasons. First, failure of normal termination at the ends of operons would generate transcript extensions that are not translated. Second, one important function of Rho *in vivo* may be to mediate the premature termination of coding-region transcripts on which, for stochastic reasons, ribosomes have failed to initiate translation; therefore, the absence of this function in the *rho* and *nusG* mutants would increase the

abundance of untranslated transcripts in the cell. Third, in situations where an elongating mRNA molecule is subjected to spontaneous endonucleolytic cleavage downstream of the initiation codon, the untranslated region 3′ to the cut would signal premature transcription termination in a *rho*$^+$ *nusG*$^+$ cell but not in either mutant.

A link between inefficient transcription termination and R-loop formation was suggested earlier,[5,45] on the basis of studies with "fast" and "slow" RNA polymerase mutants exhibiting, decreased and increased efficiencies of Rho-dependent termination, respectively. Our model explains the increased tendency for R-loops to occur during low-temperature growth,[8] given that transcription termination may be less efficient under these conditions.[46,47] The process of protein secretion across the cytoplasmic membrane in *E. coli* shares the feature of cold-sensitivity,[48] which may perhaps provide a basis for the genes *secE* and *nusG* (otherwise functionally unrelated) to be in a single operon. Finally, Kogoma has suggested that an increased occurrence of chromosomal R-loops is associated with chronic SOS induction,[1,45] and we have found that cultures of both the *nusG*-G146D and *rho*-A243E strains are moderately SOS induced (data not shown).

The mechanism of lethality following the postulated accumulation of chromosomal R-loops (for example, in the *nusG-rnhA*, *rho-recG*, or *recG-rnhA* double mutants) is not known. The R-loops may serve as aberrant sites for initiation of DNA replication,[1] or as blocks for the progression of DNA or RNA polymerases. Excessive degradation of transcripts associated with the R-loops may be responsible for the lethality.

Runaway replication of plasmids pACYC184 and pUC19 in *nusG* and *rho* mutants

The fact that both the *rho* and *nusG* mutants exhibited plasmid-mediated lethality, together with the identification of *rho*-R221C and *hns* mutations as suppressors that reversed the transcription termination defect in the mutants, indicates that deficient transcription termination is responsible for lethality. Furthermore, the identification of *rom*$^+$ and multicopy *recG*$^+$ as well as of mutations in *rpoB*, *pcnB*, and *polA* (which act by distinct mechanisms to reduce plasmid copy number in WT strains) as suppressors suggests that increased plasmid content is causal to lethality. In work to be described elsewhere, we have identified a mutation in *dnaC* that suppressed pACYC184-mediated killing in the *nusG* or *rho* mutants by down-regulating plasmid replication.

It has been shown that the intracellular content of plasmids is substantially elevated in *recBC* or *recD* mutant strains, and that this increase is RecA-dependent and occurs as linear multimers.[49,50] On the other hand, our findings indicate that the plasmid content increase in *rho* or *nusG* strains is as supercoiled monomers, and that it is both RecA-independent and responsive to the factors modulating normal ColE1 replication, suggesting that the two phenomena are mechanistically very different.

Taken together, therefore, the data suggest that deficiency in Rho-dependent transcription termination leads to runaway replication of plasmids pACYC184 or pUC4K and, in turn, to cell death. A similar phenotype of transparent inviable colonies following transformation with ColE1 plasmids that exhibit runaway replication has been described.[51] There were earlier reports of the inability to obtain transformants with pBR322 and related plasmids in certain other *rho* mutant strains,[52–54] but the phenomena and underlying mechanisms appear to be different from that described here. We could demonstrate the phenotype of increased plasmid content also in cells depleted of Rho or NusG; that is, in the null mutants (Figure 3(C)).

The magnitude of plasmid content increase in the *nusG* and *rho* mutants is likely to be much higher than that suggested from the band intensities in Figures 3–5. Cultures of the *nusG* and *rho* transformants exhibited cell lysis and were populated with varying proportions of suppressor mutants, both of which would be expected to result in reductions in plasmid recovery. Cell death associated with plasmid runaway replication has been reported to occur when the increased DNA load approaches that of a few additional chromosome equivalents.[55]

A titration mechanism for runaway plasmid replication

The number of plasmid replication primers generated from RNA-II is determined as the product of two fractions; namely, the proportion of RNA-II transcripts that escapes being complexed with the antisense inhibitor RNA-I, and the proportion of uncomplexed RNA-II molecules that go on to form R-loops at the replication origin. Runaway plasmid replication will occur if the number of productive replication primers generated per plasmid per generation is consistently greater than unity.[56]

To explain runaway replication of plasmids pACYC184 and pUC19 in the *nusG* and *rho* mutants, one may consider mechanisms in which the mutations directly affect the transcription termination efficiency of plasmid-encoded RNA-I or RNA-II, but these are unlikely given that (i) termination of RNA-I transcription in all ColEI-like plasmids, as well as termination of RNA-II transcription in pACYC184, is Rho-independent;[57,58] and (ii) the nature or extent of RNA-II transcription beyond around +9 in any case has no bearing on its ability to form replication primer in *rnhA*$^+$ cells.[59] We have obtained evidence that regulation of the RNA-I and RNA-II promoters is not altered in the *nusG* and *rho* derivatives (data not shown).

With regard to indirect mechanisms, one possibility is that the intracellular concentration of

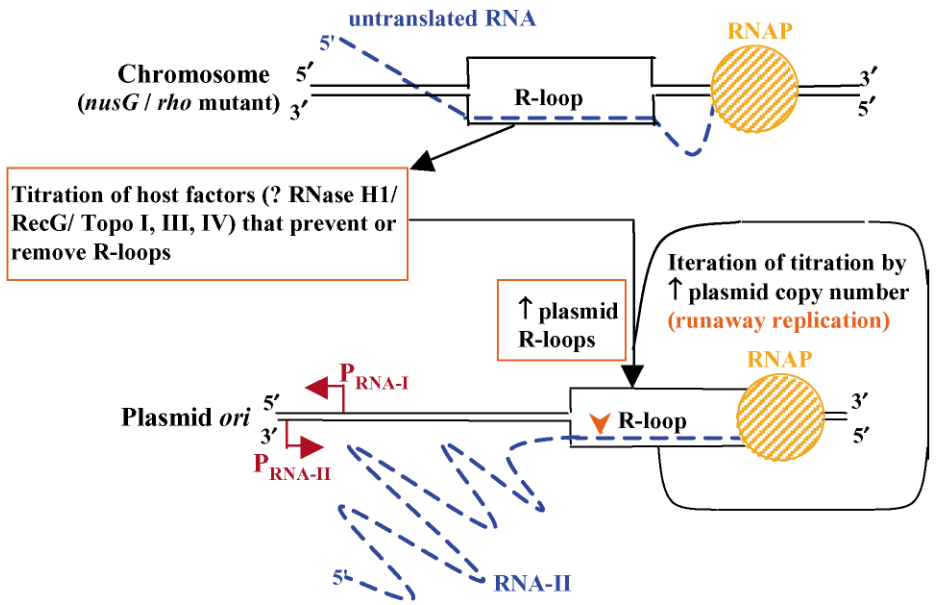

Figure 6. Model for activation of replication of ColE1-like plasmids in *nusG* and *rho* mutants following host factor titration by chromosomal R-loops. DNA and RNA strands (not to scale) are represented by continuous and interrupted lines, respectively. RNAP, RNA polymerase; Topo, topoisomerase. The arrowhead denotes the site of cleavage of RNA-II in the plasmid R-loop by RNase H1. Iteration of titration by plasmid R-loops is depicted.

a factor that modulates RNA-I stability is altered in the *rho* and *nusG* mutants, but the expected outcome of such an event would have been a stable change in plasmid copy number rather than a runaway-replication phenotype.[56] Instead, we argue below that our findings support the model outlined in Figure 6, in which the increased occurrence of chromosomal R-loops in strains with global deficiency in transcription termination leads to a titration effect that incidentally affects plasmid replication.

We suggest that the fraction of (RNA-I-uncomplexed) RNA-II transcripts that become productive replication primers is determined by a balance between factors that stabilize R-loops and those that disrupt them; and that in the *nusG* and *rho* mutants, a factor(s) that would otherwise act to disrupt R-loops on the plasmid is titrated by the R-loops on the chromosome. The resultant increase in plasmid copy number would lead to a vicious cycle of titration and hence to runaway replication (Figure 6). Our identification of a gratuitous increase in copy numbers of several otherwise non-lethal R-loop-dependent plasmids only if any of them is co-resident with pACYC184 (Figure 3(G) (pHYD762), Figure 4(A) (pHYD780), and Figure 4(C) (pHYD583-Glc)), provides strong support to a titration mechanism in the mutants and indicates that the mechanism of copy number dysregulation cannot be based solely on plasmid-borne determinants. The *tet* promoter on pACYC184 may influence the balance between R-loop formation and disruption by modulating local DNA supercoiling in the *ori* region;[7,8] and the correlation of lethality with growth phase (Figure 3(A)) might simply reflect the increased propensity for R-loops in the stationary phase.[8,60,61] Furthermore, the well known phenomenon of amplification of ColE1-family plasmids by chloramphenicol or spectinomycin[62,63] may itself entail runaway replication consequent to titration by chromosomal R-loops following the uncoupling of transcription from translation.

The possible candidates for titration by chromosomal R-loops in the *nusG* and *rho* strains include RNase H1 and RecG. The topoisomerases I, III, and IV may also be involved in precluding the occurrence of transcription-associated chromosomal R-loops,[5,7–9] and whether any of them is titrated remains to be determined. In the case of RNase H1, the possibility that it may play a dual role in both cleaving RNA-II to generate the primer needed for ColE1-like plasmids to replicate and destroying the R-loops required for primer generation may be an important, if confusing, factor in interpreting how its overexpression results in the suppression of plasmid-associated lethality; indeed, earlier work has suggested that the cytoplasmic level of RNase H1 in the WT strain is in 40-fold excess over that needed for cleavage of RNA-II within the plasmid R-loop.[64]

Finally, we suggest that persistent RNA–DNA hybrid formation at the plasmid *ori* region during the process of RNA-II transcription may represent a special case of a more general phenomenon that occurs at various chromosomal loci in bacterial cells. Its mechanism remains to be investigated, since the current structural model for RNA polymerase during transcription elongation[65] does not explain its occurrence adequately.

H-NS role in factor-dependent transcription termination

Our studies indicate that certain (but not all) mutations affecting H-NS, which until now has been implicated mainly in determination of nucleoid architecture and the repression of transcription initiation,[35,36] suppress the relief of transcriptional polarity in the *rho* and *nusG* strains. At present, however, our data are merely descriptive of a novel phenomenon. Although the concerned *hns* mutations are indistinguishable from a null allele with respect to derepression of gene expression, for example, in the *proU* operon,[23,37,40] the null *hns* allele itself did not suppress the *rho* or *nusG* mutant phenotypes. Based on the facts, therefore, that the suppression is a non-null phenotype and that it is observed in both *rho* and *nusG* strains, we suggest that the mutant H-NS proteins may exert a direct influence on the molecular complexes involved in transcription elongation and Rho-dependent termination.

The following features previously described for H-NS may be relevant for further investigation of this phenomenon: (i) unlike wild-type H-NS, which undergoes high-order self-association, the Δ64-137 mutant protein exists uniquely as stable dimers; furthermore, the latter inhibits the formation of higher-order oligomers by full-length H-NS.[38,39] We observed that the cognate allele is the most prominent in exhibiting the suppressor phenotype, which it did equally in cells that contained or were devoid of wild-type H-NS protein. (ii) H-NS was identified in a putative "antiterminating" transcription complex downstream of a Rho-dependent terminator during *rrn* transcription *in vitro*, but its role in the complex was not analyzed further.[66] (iii) StpA, an *E. coli* RNA-binding protein, is closely related to H-NS both structurally and functionally, and forms hetero-oligomeric complexes with H-NS, suggesting that H-NS might be involved in some RNA-associated reactions.[67] This is further supported by a report that H-NS co-purifies with another RNA-binding protein, Hfq.[68] However, a null mutation in *stpA* had no effect on any of the phenotypes reported here (data not shown).

Materials and Methods

Bacterial strains, plasmids, and primers

Genotypes of *E. coli* K-12 strains are listed in Table 2, and routine growth media were Luria-Bertani (LB) and Glc-minimal A, as described.[69] Unless otherwise indicated, the growth temperature was 30 °C.

Plasmids previously described include (salient features in parentheses): (i) p15A-derived: pACYC177 (Amp^R Kan^R) and pACYC184 (Cm^R Tet^R);[62] (ii) pMB9-derived: pBR322 (Amp^R Tet^R),[70] pUC19 (Amp^R),[71] pBR329 (Amp^R Cm^R Tet^R),[72] pUC4K (Amp^R Kan^R) (Amersham Pharmacia), pAM34 (Amp^R, IPTG-dependent replicon),[73] pBAD18 (Amp^R, for Ara-induced

Table 2. List of *E. coli* K-12 strains

Strain[a]	Genotype[b]
DH5α	Δ(*argF-lac*)*U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (ø80*lacZ*ΔM15)
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*
MG1655	WT
PD32	MC4100 *hns-206*::Amp
RH90	MC4100 *rpoS359*::Tn*10*
GJ863	MC4100 *rho*-A243E[c]
GJ1504	MC4100 *nusG*-G146D[d]
GJ1560	MC4100 *nusG*-G146D *zih-3166*::Tn*10*Kan
GJ1561	MC4100 *nusG*-G146D *polA12 zih-3166*::Tn*10*Kan
GJ1565	MC4100 *nusG*-G146D *rpoB*-Q513L *zja-900*::Tn*10*
GJ3107	MC4100 *nusG*-G146D *galEp3*
GJ3110	MC4100 *rho*-A243E *galEp3*
GJ3161	MC4100 *galEp3*
GJ3162	GJ3161 *recA*::Kan
GJ3163	GJ3107 *galEp3 recA*::Kan
GJ3164	GJ3110 *galEp3 recA*::Kan
GJ3165	MC4100 *galEp3 trpE9851*(Oc) *zci-506*::Tn*10*
GJ3166	GJ3165 *nusG*-G146D
GJ3167	GJ3165 *rho*-A243E
GJ3168	MG1655 *nusG*-G146D *argE86*::Tn*10*
GJ3169	MG1655 *argE86*::Tn*10*
GJ3170	GJ3168 *recA*::Kan
GJ3171	MG1655 *rho*-A243E
GJ3172	GJ3171 *recA*::Kan
GJ3173	MG1655 *recA*::Kan
GJ3176	GJ3107 *hns-206*::Amp *nadA57*::Tn*10*
GJ3177	GJ3110 *hns-206*::Amp *nadA57*::Tn*10*
GJ3178	MC4100 *hns*-E74K[d] *zch-900*::Tn*10*dKan
GJ3179	GJ3110 *hns*-E74K *zch-900*::Tn*10*dKan
GJ3180	GJ3107 *hns*-E74K *zch-900*::Tn*10*dKan
GJ3181	GJ3107 *rho*-R221C
GJ3182	GJ3161 *rho*-R221C
GJ3183	GJ3165 *nusG*-G146D *rho*-R221C
GJ3185	GJ3161 *pcnB1 zad-981*::Tn*10*dKan
GJ3186	GJ3107 *pcnB1 zad-981*::Tn*10*dKan
GJ3187	GJ3110 *pcnB1 zad-981*::Tn*10*dKan
GJ3189	GJ3110 *zih-3166*::Tn*10*Kan
GJ3190	GJ3110 *polA12 zih-3166*::Tn*10*Kan
GJ3191[e]	GJ3161 *nusG*::Kan
GJ3192[e]	GJ3161 Δ*rho*::Kan
GJ3195[e]	GJ3107 *rnhA339*::Cm
GJ3196[e]	GJ3110 *recG258*::Kan

[a] The following strains have been described: DH5α;[63] MC4100;[78] MG1655;[80] PD32;[40] and RH90 and GJ863.[79] All other strains were obtained or constructed in this study.

[b] Genotype designations are as described.[81] All strains are F^-. Allele numbers are indicated where they are known. References or sources for the mutations that were introduced by transduction into the strains were as follows: *argE86*::Tn*10*, *nadA57*::Tn*10*, *zci-506*::Tn*10*, and *zih-3166*::Tn*10*Kan;[80,81] *zch-900*::Tn*10*dKan, *zja-900*::Tn*10*;[23] *pcnB1* and *zad-981*::Tn*10*Kan;[31] *recA*::Kan (R. Jayaraman); *polA12* (*E. coli* Genetic Stock Center); *recG258*::Kan;[16] *rnhA339*::Cm;[17] *nusG*::Kan;[22] Δ*rho*::Kan;[77] *galEp3*;[24] and *trpE9851*(Oc).[25]

[c] The *rho*-A243E allele was previously called *rho-4*(Am),[25] but was later shown by Ratner[82] to be a missense mutation. We sequenced the mutation in this study and demonstrated it to be a C to A transversion in codon 243 of *rho*, predicted to cause an Ala to Glu substitution at the cognate position in the encoded protein (*rho*-A243E).

[d] The *nusG*-G146D and *hns*-E74K alleles were previously called *rpoB364* and *hns-200*, respectively.[23] See the text for details.

[e] Strains GJ3191 and GJ3195 were routinely recovered and maintained as transformant derivatives with plasmids carrying *nusG*$^+$, as were strains GJ3192 and GJ3196 with plasmids carrying *rho*$^+$.

Table 3. Plasmids constructed in this study

Plasmid	Description (replicon; antibiotic markers)
pHYD547	3.8 kb chromosomal *Sma* I fragment carrying *nusG*$^+$ cloned into *Sma* I site of pCL1920 (pSC101; Sp, Sm)
pHYD549	0.95 kb *Pvu* II-*Hpa* I fragment carrying *nusG*$^+$ subcloned from pHYD547 into *Sma* I site of pCL1920 (pSC101; Sp, Sm)
pHYD562	17 kb fragment including Δ*rep*::Cm and *rho*-R221C obtained in mini-Mu vector by *in vivo* cloning (pMB9; Kan, Cm)
pHYD564	3.3 kb *Nsi* I fragment carrying *rho*-R221C subcloned from pHYD562 into *Pst* I site of pCL1920 (pSC101; Sp, Sm)
pHYD566	Derivative of pHYD564 obtained after partial digestion with *Apa* LI and re-circularization, carrying 1.3 kb *Apa* LI-*Nsi* I fragment with *rho*-R221C (pSC101; Sp, Sm)
pHYD567	3.3 kb *Nsi* I fragment carrying *rho*$^+$ cloned from λ phage 556 of Kohara *et al.*[83] into *Pst* I site of pCL1920 (pSC101; Sp, Sm)
pHYD573	Cointegrate of plasmids pACYC184 and pCL1920 obtained following ligation of the *Bam* HI-linearized molecules (pSC101 and p15A; Cm, Sp, Sm)
pHYD578, −583	3.2 kb *Sph* I-*Eco* RV chromosomal fragment carrying *recG*$^+$ subcloned from pHYD758 *via* another plasmid (providing an *Sph* I site after *Eco* RV) into, respectively, *Sph* I site of pBR329 (pMB9; Amp, Cm) and *Sph* I site of pBAD18 (pMB9; Amp)
pHYD751	2.1 kb *Eco* RI-*Sal* I fragment carrying *nusG*$^+$ subcloned from pHYD547 into *Eco* RI-*Sal* I sites of pAM34 (pMB9; Amp)
pHYD753	pACYC184 derivative obtained following destruction of its *Hin*dIII site (p15A; Tet, Cm)
pHYD758	14 kb fragment including *recG*$^+$ obtained in mini-Mu vector by *in vivo* cloning (pMB9; Kan)
pHYD759	pACYC184 derivative with single base-insertion in spacer region of *tet* promoter (p15A; Tet, Cm)
pHYD760	0.2 kb *Pst* I-*Bam* HI fragment carrying *rom*$^+$ subcloned from pUC*rop*$^+$ into *Pst* I-*Bam* HI sites of pCL1920 (pSC101; Sp, Sm)
pHYD761	Derivative of pBR322 with 0.64 kb *Pvu* II-*Ava* I deletion encompassing *rom* (pMB9; Amp, Tet)
pHYD762	Derivative of pBR322 with 2 kb *Hin*dIII-*Pvu* II deletion encompassing *tet* and *rom* (pMB9; Amp)
pHYD763	3.8 kb *Bam* HI-*Sac* I fragment carrying *nusG*$^+$ subcloned from pHYD547 into *Bam* HI-*Sac* I sites of pMAK705 (pSC101; Cm)
pHYD780, −781	*rom*$^-$ derivatives, obtained by deletion of 1.5 kb *Sph* I-*Pvu* II fragment from, respectively, Δ*rnhA* plasmid pSK762c (pMB9; Amp) and *rnhA*$^+$ plasmid pSK760 (pMB9; Amp)
pHYD1201	3.3 kb *Hin*dIII-*Sal* I fragment carrying *rho*$^+$ subcloned from pHYD567 into *Hin*dIII-*Sal* I sites of pAM34 (pMB9; Amp)

expression of target genes),[74] pUC*rop*$^+$ (Amp^R),[30] and pSK760 and pSK762c (Amp^r, carrying full-length and and deleted versions of *rnhA*, respectively);[5] and (iii) pSC101-derived: pCL1920 (Sm^R Sp^R),[75] pMAK705 (Cm^R, Ts replicon),[76] pPMrhoCam (Cm^R, Ts replicon with *rho*$^+$),[77] and pLG339, and its derivatives with *hns*$^+$ (pLG H-NS) or mutant *hns* genes as described (Kan^R).[37] Plasmids that were constructed in this study are described in Table 3.

The following primers were employed for PCR amplification and sequencing of the indicated chromosomal loci: (All primers are given in 5′–3′ polarity, and were used for DNA sequencing; those employed also as forward and reverse primers in PCR are identified by the letters F and R, respectively, in parentheses after the sequence, and the size of the PCR product obtained is given after each of the genes.) (i) *secE-nusG*, 1.04 kb: SECEFP, TTGCCTCGCGATCGCGGG (F); NUSGRP, TCCAATCTCACGCCTTGTGCA (R); SECERP, CCTGA ACGACGTACCAG; and NUSGFP, TGGTATTCTGGTT CGCCTGG. (ii) *rho*, 1.7 kb: RHOFP, TCCTCGACGCTAA CCTGGC (F); RHORP, ACATCGCCAGCGCGGCAT (R); RHOIF, GGGGCTGGAAAACCTGGC; and RHOIR, AGGCTGCCGCCCTCTTCC. (iii) *rpoB* fragment, 1.1 kb: SEQB2, CTGCTGGCTAAGCTGAGCC (F); and SEQB3, CGCAGAGTCGGAACGGCC (R). (iv) *hns*, 0.61 kb: HNSFP, GCTATATGCCGCGTCTTTTCTG (F); and HNSRP, GGCGGGATTTTAAGCAAGTGCAAT (R).

Genetic and DNA methods

All strain constructions were by P1 transduction.[78] Procedures for *in vivo* cloning[29] and nitrosoguanidine mutagenesis[69] were as described. Standard protocols were followed for experiments involving recombinant DNA.[63] Intracellular content of test plasmids (normalized to A_{600} of the culture) was estimated following agarose gel electrophoresis, and the strains often carried a second pSC101-derived plasmid (pLG339 or pCL1920) as internal control; in some experiments, prior to the plasmid isolation procedure, a fixed volume of culture of DH5α carrying a pBR322Δ*rom* derivative (pHYD762) was added as external control in order to normalize for efficiency of plasmid recovery.

Experiments with conditional replicon plasmids

In experiments that demanded conditional loss of Ts or IPTG-dependent plasmid replicons, the plasmid-bearing derivatives were grown overnight to stationary phase under permissive conditions (30 °C or with 1 mM IPTG as appropriate) and then diluted 10^3 to 10^4-fold into fresh medium for further growth under the restrictive condition (42 °C or without IPTG, as appropriate). The cultures grew to an A_{600} of around 0.6–1.0 before the conditional replicon and its encoded products had been depleted from the cells.

Other techniques

Immunoblot analysis with anti-σ^S Ab[79] and β-galactosidase activity measurements[69] were undertaken as described. Ara-induction experiments[74] were performed by supplementation of LB medium with 0.2% (w/v) Ara (or 0.2% (w/v) Glc, as a control).

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References

1. Kogoma, T. (1997). Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. *Microbiol. Mol. Biol. Rev.* **61**, 212–238.
2. Asai, T. & Kogoma, T. (1994). D-loops and R-loops: alternative mechanisms for the initiation of chromosome replication in *Escherichia coli*. *J. Bacteriol.* **176**, 1807–1812.
3. Hong, X., Cadwell, G. W. & Kogoma, T. (1995). *Escherichia coli* RecG and RecA proteins in R-loop formation. *EMBO J.* **24**, 2385–2392.
4. Drolet, M., Bi, X. & Liu, L. F. (1994). Hypernegative supercoiling of the DNA template during transcription elongation *in vitro*. *J. Biol. Chem.* **269**, 2068–2074.
5. Drolet, M., Phoenix, P., Menzel, R., Masse, E., Liu, L. F. & Crouch, R. J. (1995). Overexpression of RNase H partially complements the growth defect of an *Escherichia coli* Δ*topA* mutant: R-loop formation is a major problem in the absence of DNA topoisomerase I. *Proc. Natl Acad. Sci. USA*, **92**, 3526–3530.
6. Masse, E., Phoenix, P. & Drolet, M. (1997). DNA topoisomerases regulate R-loop formation during transcription of the *rrnB* operon in *Escherichia coli*. *J. Biol. Chem.* **272**, 12816–12823.
7. Masse, E. & Drolet, M. (1999). *Escherichia coli* DNA topoisomerase I inhibits R-loop formation by relaxing transcription-induced negative supercoiling. *J. Biol. Chem.* **274**, 16659–16664.
8. Masse, E. & Drolet, M. (1999). R-loop-dependent hypernegative supercoiling in *Escherichia coli topA* mutants preferentially occurs at low temperatures and correlates with growth inhibition. *J. Mol. Biol.* **294**, 321–332.
9. Broccoli, S., Phoenix, P. & Drolet, M. (2000). Isolation of the *topB* gene encoding DNA topoisomerase III as a multicopy suppressor of *topA* null mutations in *Escherichia coli*. *Mol. Microbiol.* **35**, 58–68.
10. Hraiky, C., Raymond, M. A. & Drolet, M. (2000). Rnase H overproduction corrects a defect at the level of transcription elongation during rRNA synthesis in the absence of DNA topoisomerase I in *Escherichia coli*. *J. Biol. Chem.* **275**, 11257–11263.
11. Polisky, B. (1988). ColE1 replication control circuitry: sense from antisense. *Cell*, **55**, 929–932.
12. Cesareni, G., Helmer-Citterich, M. & Castagnoli, L. (1991). Control of ColE1 plasmid replication by antisense RNA. *Trends Genet.* **7**, 230–235.
13. Eguchi, Y., Itoh, T. & Tomizawa, J. (1991). Antisense RNA. *Annu. Rev. Biochem.* **60**, 631–652.
14. Lopilato, J., Bortner, S. & Beckwith, J. (1986). Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen. Genet.* **205**, 285–290.
15. Xu, F., Lin-Chao, S. & Cohen, S. N. (1993). The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *Proc. Natl Acad. Sci. USA*, **90**, 6756–6760.
16. Vincent, S. D., Mahdi, A. A. & Lloyd, R. G. (1996). The RecG branch migration protein of *Escherichia coli* dissociates R-loops. *J. Mol. Biol.* **264**, 713–721.
17. Fukuoh, A., Iwasaki, H., Ishioka, K. & Shinagawa, H. (1997). ATP-dependent resolution of R-loops at the ColE1 replication origin by *Escherichia coli* RecG protein, a Holliday junction-specific helicase. *EMBO J.* **16**, 203–209.
18. von Hippel, P. H. (1998). An integrated model of the transcription complex in elongation, termination, and editing. *Science*, **281**, 660–665.
19. Nudler, E. & Gottesman, M. E. (2002). Transcription termination and anti-termination in *E. coli*. *Genes Cells*, **7**, 755–768.
20. Richardson, J. P. (2002). Rho-dependent termination and ATPases in transcription termination. *Biochim. Biophys. Acta*, **1577**, 251–260.
21. Jin, D. J., Burgess, R. R., Richardson, J. P. & Gross, C. A. (1992). Termination efficiency at rho-dependent terminators depends on kinetic coupling between RNA polymerase and rho. *Proc. Natl Acad. Sci. USA*, **89**, 1453–1457.
22. Sullivan, S. L. & Gottesman, M. E. (1992). Requirement for *E. coli* NusG protein in factor-dependent transcription termination. *Cell*, **68**, 989–994.
23. Jayashree, P. & Gowrishankar, J. (1995). An unusual suicidal interaction in *Escherichia coli* involving nucleoid protein H-NS. *J. Genet.* **74**, 1–17.
24. Das, A., Merril, C. & Adhya, S. (1978). Interaction of RNA polymerase and rho in transcription termination: coupled ATPase. *Proc. Natl Acad. Sci. USA*, **75**, 4828–4832.
25. Morse, D. E. & Guertin, M. (1972). Amber suA mutations which relieve polarity. *J. Mol. Biol.* **63**, 605–608.
26. Uzan, M. & Danchin, A. (1978). Correlation between the serine sensitivity and the derepressibility of the *ilv* genes in *Escherichia coli* relA$^-$ mutants. *Mol. Gen. Genet.* **165**, 21–30.
27. Lopes, J. M., Soliman, N., Smith, P. K. & Lawther, R. P. (1989). Transcriptional polarity enhances the contribution of the internal promoter, *ilvEp*, in the expression of the *ilvGMEDA* operon in wild-type *Escherichia coli* K12. *Mol. Microbiol.* **3**, 1039–1051.
28. Hengge-Aronis, R. (2002). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**, 373–395.
29. Groisman, E. A. & Casadaban, M. J. (1986). Mini-Mu bacteriophage with plasmid replicons for *in vivo* cloning and *lac* gene fusing. *J. Bacteriol.* **168**, 357–364.
30. Sozhamannan, S. & Stitt, B. L. (1997). Effects on mRNA degradation by *Escherichia coli* transcription

termination factor Rho and pBR322 copy number control protein Rop. *J. Mol. Biol.* **268**, 689–703.
31. Liu, J. D. & Parkinson, J. S. (1989). Genetics and sequence analysis of the *pcnB* locus, an *Escherichia coli* gene involved in plasmid copy number control. *J. Bacteriol.* **171**, 1254–1261.
32. Grindley, N. D. & Kelley, W. S. (1976). Effects of different alleles of the *E. coli* K-12 *polA* gene on the replication of non-transferring plasmids. *Mol. Gen. Genet.* **143**, 311–318.
33. Jin, D. J. & Gross, C. A. (1988). Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J. Mol. Biol.* **202**, 45–58.
34. Yang, Y. L. & Polisky, B. (1999). Allele-specific suppression of ColE1 high-copy-number mutants by a *rpoB* mutation of *Escherichia coli*. *Plasmid*, **41**, 55–62.
35. Ussery, D. W., Hinton, J. C., Jordi, B. J., Granum, P. E., Seirafi, A., Stephen, R. J. *et al.* (1994). The chromatin-associated protein H-NS. *Biochimie*, **76**, 968–980.
36. Williams, R. M. & Rimsky, S. (1997). Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. *FEMS Microbiol. Letters*, **156**, 175–185.
37. Williams, R. M., Rimsky, S. & Buc, H. (1996). Probing the structure, function, and interactions of the *Escherichia coli* H-NS and StpA proteins by using dominant negative derivatives. *J. Bacteriol.* **178**, 4335–4343.
38. Smyth, C. P., Lundback, T., Renzoni, D., Siligardi, G., Beavil, R., Layton, M. *et al.* (2000). Oligomerization of the chromatin-structuring protein H-NS. *Mol. Microbiol.* **36**, 962–972.
39. Esposito, D., Petrovic, A., Harris, R., Ono, S., Eccleston, J. F., Mbabaali, A. *et al.* (2002). H-NS oligomerization domain structure reveals the mechanism for high order self-association of the intact protein. *J. Mol. Biol.* **324**, 841–850.
40. Dersch, P., Kneip, S. & Bremer, E. (1994). The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol. Gen. Genet.* **245**, 255–259.
41. Zellars, M. & Squires, C. L. (1999). Antiterminator-dependent modulation of transcription elongation rates by NusB and NusG. *Mol. Microbiol.* **32**, 1296–1304.
42. Zhou, Y., Filter, J. J., Court, D. L., Gottesman, M. E. & Friedman, D. I. (2002). Requirement for NusG for transcription antitermination *in vivo* by the λ N protein. *J. Bacteriol.* **184**, 3416–3418.
43. Steiner, T., Kaiser, J. T., Marinkovic, S., Huber, R. & Wahl, M. C. (2002). Crystal structures of transcription factor NusG in light of its nucleic acid- and protein-binding activities. *EMBO J.* **21**, 4641–4653.
44. Burova, E., Hung, S. C., Chen, J., Court, D. L., Zhou, J.-G., Mogilnitskiy, G. & Gottesman, M. E. (1999). *Escherichia coli nusG* mutations that block transcription termination by coliphage HK022 Nun protein. *Mol. Microbiol.* **31**, 1783–1793.
45. Kogoma, T. (1994). *Escherichia coli* RNA polymerase mutants that enhance or diminish the SOS response constitutively expressed in the absence of RNase HI activity. *J. Bacteriol.* **176**, 1521–1523.
46. Bae, W., Xia, B., Inouye, M. & Severinov, K. (2000). *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. *Proc. Natl Acad. Sci. USA*, **97**, 7784–7789.
47. Rajkumari, K. & Gowrishankar, J. (2001). *In vivo* expression from the RpoS-dependent P1 promoter of the osmotically regulated *proU* operon in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium: activation by *rho* and *hns* mutations and by cold stress. *J. Bacteriol.* **183**, 6543–6550.
48. Pogliano, K. J. & Beckwith, J. (1993). The Cs *sec* mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself. *Genetics*, **133**, 763–773.
49. Cohen, A. & Clark, A. J. (1986). Synthesis of linear plasmid multimers in *Escherichia coli* K-12. *J. Bacteriol.* **167**, 327–335.
50. Biek, D. P. & Cohen, S. N. (1986). Identification and characterization of *recD*, a gene affecting plasmid maintenance and recombination in *Escherichia coli*. *J. Bacteriol.* **167**, 594–603.
51. Masukata, H. & Tomizawa, J. (1986). Control of primer formation for ColE1 plasmid replication: conformational change of the primer transcript. *Cell*, **44**, 125–136.
52. Arnold, G. F. & Tessman, I. (1988). Regulation of DNA superhelicity by *rpoB* mutations that suppress defective Rho-mediated transcription termination in *Escherichia coli*. *J. Bacteriol.* **170**, 4266–4271.
53. Li, T. K., Panchenko, Y. A., Drolet, M. & Liu, L. F. (1997). Incompatibility of *Escherichia coli rho* mutants with plasmids is mediated by plasmid-specific transcription. *J. Bacteriol.* **179**, 5789–5794.
54. Sozhamannan, S., Morris, J. G., Jr & Stitt, B. L. (1999). Instability of pUC19 in *Escherichia coli* transcription termination factor mutant, *rho026*. *Plasmid*, **41**, 63–69.
55. Nordstrom, K. & Uhlin, B. E. (1992). Runaway-replication plasmids as tools to produce large quantities of proteins from cloned genes in bacteria. *Bio/Technology*, **10**, 661–666.
56. Brenner, M. & Tomizawa, J. (1991). Quantitation of ColE1-encoded replication elements. *Proc. Natl Acad. Sci. USA*, **88**, 405–409.
57. Itoh, T. & Tomizawa, J. (1980). Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc. Natl Acad. Sci. USA*, **77**, 2450–2454.
58. Selzer, G. & Tomizawa, J. (1982). Specific cleavage of the p15A primer precursor by ribonuclease H at the origin of DNA replication. *Proc. Natl Acad. Sci. USA*, **79**, 7082–7086.
59. Tomizawa, J. & Masukata, H. (1987). Factor-independent termination of transcription in a stretch of deoxyadenosine residues in the template DNA. *Cell*, **51**, 623–630.
60. Fitzwater, T., Zhang, X. Y., Elble, R. & Polisky, B. (1988). Conditional high copy number ColE1 mutants: resistance to RNA1 inhibition *in vivo* and *in vitro*. *EMBO J.* **7**, 3289–3297.
61. Hong, X., Cadwell, G. W. & Kogoma, T. (1996). Activation of stable DNA replication in rapidly growing *Escherichia coli* at the time of entry to stationary phase. *Mol. Microbiol.* **21**, 953–961.
62. Chang, A. C. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**, 1141–1156.
63. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
64. Dasgupta, S., Masukata, H. & Tomizawa, J. (1987). Multiple mechanisms for initiation of ColE1 DNA replication: DNA synthesis in the presence and absence of ribonuclease H. *Cell*, **51**, 1113–1122.
65. Korzheva, N., Mustaev, A., Kozlov, M., Malhotra, A.,

Nikiforov, V., Goldfarb, A. & Darst, S. A. (2000). A structural model of transcription elongation. *Science*, **289**, 619–625.
66. Torres, M., Condon, C., Balada, J. M., Squires, C. & Squires, C. L. (2001). Ribosomal protein S4 is a transcription factor with properties remarkably similar to NusA, a protein involved in both non-ribosomal and ribosomal RNA antitermination. *EMBO J.* **20**, 3811–3820.
67. Zhang, A., Rimsky, S., Reaban, M. E., Buc, H. & Belfort, M. (1996). *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. *EMBO J.* **15**, 1340–1349.
68. Kajitani, M. & Ishihama, A. (1991). Identification and sequence determination of the host factor gene for bacteriophage Q_β. *Nucl. Acids Res.* **29**, 1063–1066.
69. Miller, J. H. (1992). *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for* Escherichia coli *and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
70. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene*, **2**, 95–113.
71. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.
72. Covarrubias, L. & Bolivar, F. (1982). Construction and characterization of new cloning vehicles. VI. Plasmid pBR329, a new derivative of pBR328 lacking the 482-base-pair inverted duplication. *Gene*, **17**, 79–89.
73. Gil, D. & Bouche, J. P. (1991). ColE1-type vectors with fully repressible replication. *Gene*, **105**, 17–22.
74. Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121–4130.
75. Lerner, C. G. & Inouye, M. (1990). Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. *Nucl. Acids Res.* **18**, 4631.
76. Hamilton, C. M., Aldea, M., Washburn, B. K., Babitzke, P. & Kushner, S. R. (1989). New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**, 4617–4622.
77. Martinez, A., Opperman, T. & Richardson, J. P. (1996). Mutational analysis and secondary structure model of the RNP1-like sequence motif of transcription termination factor Rho. *J. Mol. Biol.* **257**, 895–908.
78. Gowrishankar, J. (1985). Identification of osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. *J. Bacteriol.* **164**, 434–445.
79. Rajkumari, K. & Gowrishankar, J. (2002). An N-terminally truncated RpoS (σ^S) protein in *Escherichia coli* is active *in vivo* and exhibits normal environmental regulation even in the absence of *rpoS* transcriptional and translational control signals. *J. Bacteriol.* **184**, 3167–3175.
80. Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W. *et al.* (1989). A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**, 1–24.
81. Berlyn, M. K. B., Low, K. B. & Rudd, K. E. (1996). Linkage map of *Escherichia coli* K-12, Edition 9. Escherichia coli *and* Salmonella*: Cellular and Molecular Biology* (Neidhardt, F. C., *et al.*), pp. 1715–1902, American Society for Microbiology, Washington, DC.
82. Ratner, D. (1976). Evidence that mutations in the suA polarity suppressing gene directly affect termination factor rho. *Nature*, **259**, 151–153.
83. Kohara, Y., Akiyama, K. & Isono, K. (1987). The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell*, **50**, 495–508.

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