INTERACTION OF DNA POLYMERASE III γ AND β SUBUNITS *IN VIVO* IN *SALMONELLA TYPHIMURIUM*

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ABSTRACT

We show that temperature-sensitive mutations in *dnaZ*, the gene for the γ subunit of DNA polymerase III holoenzyme, can be suppressed by mutations in the *dnaN* gene, which encodes the β subunit. These results support a direct physical interaction of these two subunits during polymerase assembly or function. The suppressor phenotype is also sensitive to modulation by the *dnaA* genotype. Since *dnaA* is organized in an operon with *dnaN*, and *dnaA* is a regulatory gene of this operon, we propose that the *dnaA* effect on suppression can best be explained by modulation of suppressor *dnaN* levels.

IN *E. coli* and *Salmonella typhimurium*, DNA replication is carried out primarily by the DNA polymerase III (pol III) holoenzyme complex in cooperation with other proteins that mediate initiation at the chromosomal origin, formation of RNA primers for Okazaki fragments and topological changes in the DNA (helix winding and supercoiling) (KORNBERG 1980; MCHENRY 1985). An enduring question is the mechanism for coordination of the various aspects of the replication process. One approach to this question relies on the analysis of genetic interactions manifested by certain replication mutants. These genetic interactions potentially are based on physical or functional interactions at the protein level and, thus, can reveal the existence and structure of a complex protein machine for DNA replication (HARTMAN and ROTH 1973; BOTSTEIN and MAURER 1982; NOSSAL and ALBERTS 1983). Bacteria that escape the lethality of a temperature-sensitive mutation in a chosen DNA replication gene may carry suppressor mutations that are of interest for this type of analysis. Since pol III is likely to be at the heart of the replication complex, we have looked for genetic interactions involving genes for pol III subunits (MAURER, OSMOND and BOTSTEIN 1984).

Here we report the characterization of suppressor mutations generated in *Salmonella typhimurium* using *dnaZ*, the gene encoding the γ subunit of pol III (HÜBSCHER and KORNBERG 1980; KODAIRA, BISWAS and KORNBERG 1983; MULLIN *et al.* 1983) as the target gene. Several lines of investigation indicate that the γ subunit (or, more likely, a complex of two subunits, $\gamma \cdot \delta$) mediates the action of a key protein, the β subunit of pol III, in the replication process.

According to the early experiments of WICKNER (1976), the product of the γ-mediated reaction is a stable complex of template-primer with β. This complex can then bind a polymerase moiety, presumably the pol III core consisting of three subunits (α, ϵ and θ) (MCHENRY and CROW 1979) and rapidly initiate synthesis. Later work from the laboratories of KORNBERG and MCHENRY leads to the current, generally accepted view that γ, δ and β function as intrinsic subunit of the pol III holoenzyme. This picture was inferred from a comparison of the biochemical activity of pol III core, pol III holoenzyme and an intermediate assembly called pol III* that is deficient in β. These forms of pol III differ in processivity (FAY *et al.* 1981, 1982; LADUCA *et al.* 1983) and ability to copy long single-stranded regions, such as single-stranded virus DNA (MCHENRY and KORNBERG 1977). In particular, holoenzyme, or pol III* supplemented with β, can react with a primed viral template to form a stable "initiation complex" capable of rapid synthesis on addition of dNTPs. The importance of β is shown by sensitivity of complex formation to inhibition by anti-β antibody (JOHANSON and MCHENRY 1980, 1982) and to omission of β (unsupplemented pol III* reactions). MCHENRY'S model does not address the specific role of γ, but the $\gamma \cdot \delta$ complex and β are both required for polymerase activity on native templates (MCHENRY and KORNBERG 1977). Activity of β requires ATP or dATP (WICKNER 1976; WICKNER and KORNBERG 1973) unless β is present in excess (CRUTE *et al.* 1983).

A possible alternative or additional role for γ was proposed by WALKER on the basis of suppressor mutations occurring in the initiation gene, *dnaA*, directed against a temperature-sensitive mutation in *dnaZ* (WALKER, RAMSEY and HALDENWANG 1982). These findings were interpreted to mean that *dnaA* protein, in addition to its role in initiation, forms a structural matrix for the elongation complex containing pol III. The genetic interaction of *dnaA* and *dnaZ* would thus be a manifestation of a direct physical interaction at the protein level. To date, this proposal has not been critically tested in biochemical experiments.

The suppressor mutations we have derived also fall in the *dnaA* region; but in contrast to WALKER'S results, in our case the pertinent gene for suppression is clearly the adjacent gene, *dnaN*. Since *dnaN* codes for β (BURGERS, KORNBERG and SAKAKIBARA 1981), this finding supports a role for γ in mediating the activity of β. Certain additional findings lead us to suggest that the level of *dnaN* protein may play a critical role in suppression. On this basis we propose a unifying explanation for WALKER'S results and our own that does not require a *dnaA-dnaZ* protein interaction but, rather, depends on the ability of *dnaA* to influence the level of expression of *dnaN*.

MATERIALS AND METHODS

Strains: The following bacterial strains are described in detail in MAURER *et al.* (1984): DB4761, 4913, 4894, 4883, 4756 and 4884 *E. coli* replication mutants in *dnaZ*, *dnaX*, *dnaN*, *dnaA*, *dnaB* and *dnaG*, respectively; RB132, an *E. coli* strain used for transposition mutagenesis of phage λ with Tn*10*Δ; RM42, an *E. coli* host for selective growth of λ1059 recombinants; RM5 (=DB4673), RM6 (=DB4835) and RM10 (=DB9005) standard Salmonella strains in which temperature-sensitive mutations are

derived and analyzed. In addition, we used RM34, an *E. coli* strain for transposition mutagenesis of λ with Tn*10*Δkan^R. RM34 is identical to RB132, except that plasmid pNK289 (FOSTER *et al.* 1981) replaces pNK217. Salmonella *dnaZ* mutants are described below. Phage λ clones are derivatives of λ1059 (KARN *et al.* 1980), with the properties indicated in the tables and figures where they appear. Plasmids contain either a 7.3-kb *Bam*HI fragment or a 3.7-kb *Eco*RI fragment inserted in the *Bam*HI or *Eco*RI site, respectively, of pUC8 (VIEIRA and MESSING 1982). The orientation of the insert was determined by restriction enzyme mapping.

Media and genetic procedures: Media and most genetic procedures were described previously (MAURER *et al.* 1984). Complementation and suppression mediated by phage λ clones were assayed using a slight modification of the red plaque assay described by MAURER *et al.* (1984). The bacterial strain used in the overlay (the tester strain) was grown overnight in broth containing 30 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 2 g of maltose, 1 mM $MgSO_4$, and 1 ml of 1 M NaOH per liter. Such an overnight culture could be used directly in the assay without prior dilution and growth in fresh broth. Once the tester strain was added, the plates were prewarmed on a 60° slide warmer for 3 min and were incubated at 30° for 35 min to permit gene expression. Then plates were rewarmed for 3–4 min and were incubated at the nonpermissive temperature. For complementation assays involving strains DB4761 and DB4894, the nonpermissive temperature used was 40°; for other strains, 42° was used. Suppression of *dnaZ*(Ts) mutations was assayed at 38°.

Complementation and suppression mediated by plasmid subclones were assessed by transforming appropriate mutants at 30° and then examining the growth of single transformant colonies restreaked at the nonpermissive temperature. In addition, in the case of the 3.7-kb *Eco*RI subclones, the relative efficiency of plating (e.o.p.) by the transformants (*i.e.*, colony formation at 38° relative to 30°) was used to verify the qualitative assessment of suppression. In this assay, the suppressor derived from λRM560 produced an improvement of 30- to 50-fold in relative e.o.p. of strain RM212 compared to derivatives harboring a wild-type 3.7-kb *Eco*RI subclone, or just pUC8. The effect of the same suppressor subclones on strain RM213, although detectable, was an order of magnitude weaker.

DNA preparation and treatment: Bacterial DNA was prepared by the method of EBEL-TSIPIS, BOTSTEIN and FOX (1972) as modified in MAURER *et al.* (1984). Plasmid DNA was prepared by the rapid isolation procedure described in DAVIS, BOTSTEIN and ROTH (1980) or by the amplification protocol of MANIATIS, FRITSCH and SAMBROOK (1982). Phage λ DNA was prepared as described in DAVIS, BOTSTEIN and ROTH (1980). Restriction digestions and ligations were performed under conditions recommended by the supplier or as described in DAVIS, BOTSTEIN and ROTH (1980). Gel electrophoresis was as described in MANIATIS, FRITSCH and SAMBROOK (1982). DNA fragments to be cloned were recovered from agarose gels by adsorption to glass beads (VOGELSTEIN and GILLESPIE 1979). The hybridization experiment shown in Figure 3 was conducted under "stringent" conditions, essentially as described in MANIATIS, FRITSCH and SAMBROOK (1982).

Localization of *dnaZ* in cloned DNA fragments: Seven λ*dnaZ*$^+$ clones isolated from a wild-type λ-Salmonella library have been reported previously (λRM154-λRM159 and λRM417; MAURER *et al.* 1984). All these clones complement *dnaX* as well as *dnaZ* mutants. Both *dnaZ* and *dnaX* were localized within the cloned fragments by restriction mapping to determine the limits of the DNA possessed in common by all seven phages. In addition, one phage (λRM417) was mutagenized with a transposon, Tn*10*Δ16Δ17 (or more simply, Tn*10*Δ), and the sites of *dnaZ*::Tn*10*Δ and *dnaX*::Tn*10*Δ insertions were determined by restriction mapping.

Isolation of Salmonella *dnaZ*(Ts) mutants: λRM417 was mutagenized with a hybrid kanamycin-resistance transposon, Tn*10*Δ16Δ17kan^R (FOSTER *et al.* 1981) by lytic passage through *E. coli* strain RM34. The transposition system used is completely analogous to the system used previously for Tn*10*Δ (MAURER *et al.* 1984). The passaged λ lysate

was used to transduce Salmonella strain RM5 to kanamycin resistance. In such transductants, the drug-resistance determinant is chromosomally located, closely linked to *dnaZ*; thus, it can be used to direct local mutagenesis (HONG and AMES 1971). A P22 phage lysate grown on one such strain, RM70, was subjected to hydroxylamine mutagenesis *in vitro* (MAURER *et al.* 1984). The mutagenized lysate was then used to transduce Salmonella strain RM6, selecting kanamycin resistance at 30° after a growth interval of 2 hr to allow expression of the drug resistance. Six temperature-sensitive mutants were recovered among the transductants, at least three of which must be of independent origin because they derive from different aliquots of the mutagenized P22 lysate. The independence of the remaining isolates is in doubt because the mutants could have divided during the expression period. However, it is known that, under similar conditions, transduced Salmonella cells do not resume cell division for 2 hr following addition of phage (EBEL-TSIPIS, FOX and BOTSTEIN 1972); therefore, the six mutations may well be independent.

As expected, the six mutations are linked to kanamycin resistance in transductional crosses. Red-plaque complementation assays using the λ*dnaZ*$^+$ phage and the Tn*10*Δ mutants thereof establish that all six mutants are temperature sensitive for *dnaZ* and apparently normal for *dnaX* (see Table 3). We designate these mutations *dnaZ730*, *731*, *732*, *735*, *737* and *738*, and the corresponding RM6 derivatives RM211, 212, 213, 239, 241 and 254.

Construction of a "Z minus" genomic library: DNA isolated from the Salmonella *dnaZ*(Ts) strain RM262 (=RM10 *dnaZ731*, kan^R) was partially digested with *Sau*3AI, and 15- to 20-kb fragments were gel purified. The fragments were cloned into *Bam*HI-digested λ1059 and were packaged *in vitro* (KARN *et al.*, 1980; HOHN 1979). An aliquot of the packaged phage containing about 5000 distinct recombinants was amplified selectively by two successive passages in strain RM42.

RESULTS

The *dnaZ* locus in *E. coli* and Salmonella: Our results have been interpreted in the light of an underlying similarity of the *dnaZ* gene and, presumably, its product in *E. coli* and *S. typhimurium*. In addition to the functional interchangeability of the wild-type genes (ROWEN, KOBORI and SCHERER 1982; MAURER *et al.* 1984) and homology at the DNA level (ROWEN, KOBORI and SCHERER 1982), the genetic properties of mutants and the organization of the gene locus are similar in the two organisms, as described below.

In *E. coli*, *dnaZ* is adjacent to another replication locus, *dnaX*. By the classical criteria of genetic complementation, *dnaZ* and *dnaX* were shown to be distinct genes (HENSON *et al.* 1979). They were initially thought to encode distinct proteins of 52 and 32 kD, respectively (HÜBSCHER and KORNBERG 1979, 1980). More recently, experiments have identified a 78 kD subunit of pol III (the τ subunit) as a *dnaZ-dnaX* fusion product (KODAIRA, BISWAS and KORNBERG 1983). It is unclear at the present time just how these three proteins are generated from two "genes." Peptide mapping showed the 52 kD protein to be contained within the larger 78 kD protein, suggesting that the *dnaZ* gene coding sequence determines the amino acid sequence of two proteins (at least in part). Yet *in vitro*, extracts of *dnaZ* mutant bacteria are functionally deficient in the 52 kD protein. Therefore, it is believed that the essential product of the *dnaZ* gene *in vivo* is the 52 kD protein (the γ subunit of pol III), with the role and requirement for τ *in vivo* remaining uncertain.

The functional organization of the *dnaZ* locus in Salmonella was determined

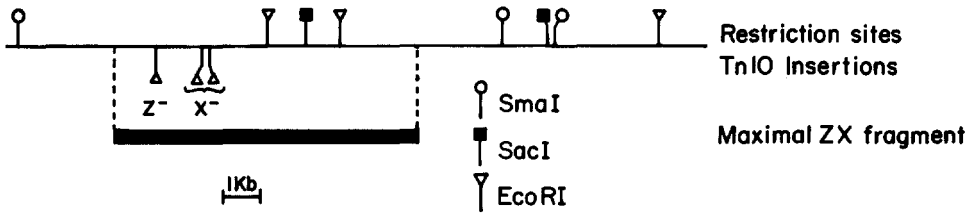

FIGURE 1.—Restriction map of the *dnaZ-dnaX* region. The map of the insert in phage λRM417 is shown on the top line (*upper*). The sites of Tn*10* insertions discussed in the text are shown on the top line (*lower*). The approximate position of *dnaZ-dnaX*, defined as the DNA possessed in common by seven overlapping *dnaZ-dnaX* clones, is shown by the bar. The leftmost boundary of this region is established by phage λRM158 and the rightmost boundary is established by phage λRM159. The λ arms are not shown.

TABLE 1

Complementation patterns of Tn*10*Δ insertion mutants of λ*dnaZ*⁺

Phage clones	Bacterial strains: *E. coli* *dnaZ*(Ts)	Bacterial strains: *E. coli* *dnaX*(Ts)	Deduced phage genotype
λRM417	+	+	*dnaZ*⁺*dnaX*⁺
λRM114	−	−	*dnaZ*::Tn*10*Δ (polar on X)
λRM118, λRM119, λRM120	+	−	*dnaX*::Tn*10*Δ

λRM417 is the parent of the other phage, which were derived by mutagenesis with Tn*10*Δ. +, a red plaque was formed in the indicated complementation test; −, the plaque was not red.

by cloning and insertion mutagenesis of this gene. For each clone or its mutant derivative, the presence or absence of a functional *dnaZ* or *dnaX* gene in the clone was determined by genetic complementation of an appropriate temperature-sensitive *E. coli* mutant. Each of seven independent γ clones selected from a Salmonella genomic library as *dnaZ*⁺, was also found to be *dnaX*⁺. Restriction maps were determined for all seven phage DNAs. As expected, the inserts present on these phages overlap one another, and all share a common 8.0-kb segment shown in Figure 1. Therefore, in Salmonella, *dnaZ* and *dnaX* must lie within 8 kb of one another.

The proximity and functional relationship of the two genes was defined more explicitly by examining the properties of four mutant derivatives of λRM417 carrying a Tn*10* insertion in the Salmonella *dnaZ-dnaX* segment (Table 1). Two pertinent types of insertion were distinguished by complementation assays. In three mutants, the *dnaX* gene is inactivated but the *dnaZ* gene is functional. In the other mutant, both *dnaZ* and *dnaX* are inactivated. Each mutant DNA was restriction mapped and revealed a simple insertion without accompanying derangement of target DNA. All four insertions are located

TABLE 2

Complementation analysis of temperature-sensitive Salmonella mutations in the vicinity of *dnaZ*

Bacterial strains	Tester phage genotype (phenotype)		
	dnaZ$^+$*dnaX*$^+$ (Z^-X^+)	*dnaZ*::Tn*10*Δ (Z^-X^-)	*dnaX*::Tn*10*Δ (Z^-X^-)
RM211, RM212, RM213, RM239, RM241, RM254	+	−	+
E. coli dnaZ2016	+	−	+
E. coli dnaX(Ts)	+	−	−

The Salmonella strains are temperature-sensitive, kanamycin-resistant derivatives of RM6. The *E. coli* strains are DB4761 and DB4913 (MAURER *et al.* 1984). +, a red plaque was formed in the indicated complementation test; −, the plaque was not red.

within the 8-kb segment and within 1.3 kb of one another. As shown in Figure 1, the three *dnaX*$^-$ insertions occupy two closely adjacent sites, whereas the *dnaZ*$^-$*dnaX*$^-$ insertion is located some distance apart. Since the *E. coli dnaZ-dnaX* region is 2.2 kb in length, the two types of insertions can be most easily accounted for as insertions directly in the *dnaZ* or *dnaX* genes. According to this interpretation, the insertion in *dnaZ* is polar on *dnaX*, whereas the three insertions in *dnaX* are not polar on *dnaZ*. This interpretation suggests the gene order promoter-*dnaZ-dnaX* in Salmonella, a conclusion that is also consistent with *dnaZ* forming the promoter-proximal portion of the *dnaX* gene as in *E. coli* (HENSON *et al.* 1979; KODAIRA, BISWAS and KORNBERG 1983; MULLIN *et al.* 1983).

Temperature-sensitive mutations of *dnaZ*: Temperature-sensitive mutations in the vicinity of *dnaZ* were isolated by local mutagenesis (MATERIALS AND METHODS). These mutations were further characterized by genetic complementation tests using the Z^+X^+ λ clones and their Tn*10* insertion derivatives described above (Table 2). All six temperature-sensitive mutants could be complemented by the Z^+X^+ and Z^+X^- test phage, but not by the Z^-X^- phage. Thus, the Salmonella temperature-sensitive mutants behave identically to the standard *E. coli dnaZ*(Ts) mutant, and therefore we consider them equivalent *dnaZ* mutants of Salmonella. The complementation response of these Salmonella mutants with the Z^+X^- tester phage suggests that the *dnaX* gene is intact in these temperature-sensitive mutants. Further confirmation of this point is contained in the next section.

Suppressors of *dnaZ*(Ts): The strategy for isolating *dnaZ*(Ts) suppressors permits the suppressor genes to be retrieved already cloned on phage λ (MAURER, OSMOND and BOTSTEIN 1984). A genomic library containing 5000 distinct clones was created in λ with DNA from one of the *dnaZ*(Ts) mutants. The representation of various replication genes in this library was determined by screening an amplified aliquot of the library for complementation of temperature-sensitive mutants in six unlinked replication genes. As expected,

clones with *dnaZ*$^+$ activity were not found (0 of 250,000 recombinant plaques), whereas the other five genes (*dnaN*, *dnaB*, *dnaG*, *dnaE* and, significantly, *dnaX*) were all represented at a frequency of 1 of 400 to 1 of 1000 recombinant plaques. Presumably, then, any gene capable of mutating to a form that can suppress *dnaZ*(Ts) was also well represented in this library. An important conclusion from the *dnaX*$^+$ clones (which were tested and found to be *dnaZ*$^-$) is that the *dnaZ*(Ts) mutation, unlike *dnaZ*::Tn*10*, is not detectably polar on *dnaX*. Nor is the functional integrity of the *dnaZ* gene needed for the adjacent (or, perhaps, overlapping) *dnaX* gene to function. These points substantiate the close similarity of the *dnaZ*(Ts) mutations of *E. coli* and Salmonella.

To obtain suppressors, the "Z$^-$" library was mutagenized with hydroxylamine and was then screened using the red-plaque assay for phage which, as prophage, restored growth ability to a Salmonella *dnaZ*(Ts) mutant at the nonpermissive temperature. Five candidates (λRM560–λRM564) were found among ~3.6 × 10^6 plaques.

The initial analysis of these phages was aimed at identifying the fragment of Salmonella DNA they carried. We reasoned that suppressor mutations might arise in a DNA replication gene; therefore, we tested the ability of the candidate phages to complement standard *dna*(Ts) mutants other than *dnaZ*. In this test, the five phages gave positive results with *dnaA* and *dnaN* tester mutants and negative results with *dnaX* and every other mutant tested. This outcome indicated that the phages did not arise by reversion of the *dnaZ* mutation present in a parental λ*dnaZ*(Ts)*dnaX*$^+$ clone and, instead, pointed to genuine suppressor mutations arising in the *dnaA-dnaN* region. This preliminary assignment was confirmed by the experiments described in the next section. In the subsequent section, experiments probing the contributions of *dnaA* and *dnaN* to the suppressor phenotype are presented.

***dnaZ* suppressor region is *dnaA-dnaN*:** The identity of the genomic DNA carried by the *dnaZ* suppressor phages was established by comparison of their restriction maps with that of a phage carrying the wild-type Salmonella *dnaA-dnaN* region (isolated from a genomic library made with wild-type DNA; MAURER *et al.* 1984). The suppressor phage maps are largely, although not entirely, congruent with the wild-type map of the *dnaA-dnaN* region (Figure 2). In particular, each suppressor phage carries all or most of a 7.3-kb *Bam*HI fragment that is shown in subcloning experiments described below to contain *dnaA* and *dnaN*. Moreover, the presumptive *dnaA-dnaN* region of each suppressor phage DNA hybridizes to a wild-type *dnaA-dnaN* probe. This is shown in Figure 3, in which the suppressor phage DNAs, digested with *Eco*RI, are probed with the wild-type 7.3-*Bam*HI fragment. Each track shows hybridization in bands of 3.7 and 1.0 kb, which are internal to the *Bam*HI fragment, and in two other large fragments that extend beyond the *Bam*HI site a variable distance to the end of the phage DNA in one direction, or to the next *Eco*RI site in the other direction. Phage λRM562 (lane 4) is an exception because only one variable band is seen. This difference may reflect substitution of heterologous DNA near one end of the *Bam*HI 7.3-kb region in λRM562, as is also suggested by an anomalous *Sma*I site in this phage. (This is one of

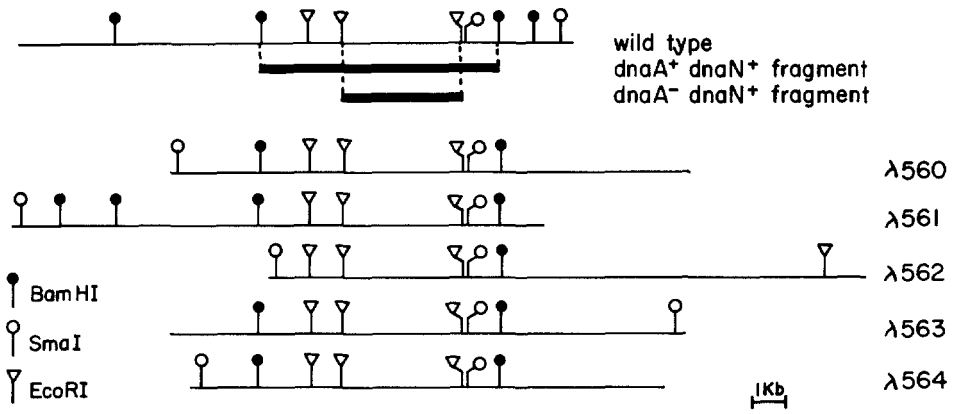

FIGURE 2.—Restriction map of the *dnaA-dnaN* region compared to the maps of the five suppressor phages. The map of the insert in the wild-type phage, λRM187, is shown on the top line, and specific fragments that were subcloned are indicated (see Table 4). The various suppressor phages are aligned below. *DnaN* is contained wholly within the 3.7-kb *Eco*RI fragment, whereas *dnaA* is not. The λ arms are not shown.

several unexplained restriction site anomalies noted in the regions flanking *dnaA-dnaN*.)

From these data we conclude that the suppressor mutations arose in λ*dnaA-dnaN* phages. Thus, the observed complementation of *dnaA* and *dnaN* mutations by the suppressor phages need not be considered a new property conferred by the suppressor mutations. This information gives the approximate location of the suppressor gene, but does not identify it.

The suppressor gene is *dnaN*: Our approach to identifying the suppressor gene involved the derivation of $dnaA^-$ or $dnaN^-$ versions of the suppressor phages by hydroxylamine mutagenesis, followed by analysis of suppressor function in the mutants (Table 3). For example, if *dnaN* were the suppressor gene, then $dnaN^-$ mutants of the suppressor phages should be deficient in both the *dnaN* complementation assay and the *dnaZ* suppression assay. In fact, this was precisely the result when $dnaN^-$ mutations were examined, suggesting that the suppressor gene is *dnaN*. To sustain the contrary hypothesis, that the suppressor gene is not *dnaN*, it is necessary to suppose that each *dnaN* mutation was accompanied by a second mutation affecting the suppressor gene. This appears unlikely because ten out of ten independently derived *dnaN* mutations, distributed among four of the five suppressors, resulted in loss of suppression. More particularly, none of the $dnaN^-$ phages was $dnaA^-$ in complementation tests.

When the contribution of *dnaA* was independently examined in a similar fashion, surprising variability in the effect of *dnaA* mutations was noted. Some *dnaA* mutations eliminated suppression, suggesting that *dnaA* is the suppressor gene; other *dnaA* mutations had no effect, paradoxically suggesting the opposite conclusion. The $dnaA^-$ phages were still $dnaN^+$ in complementation tests, ruling out the trivial explanation that the loss of suppression was the result of $dnaA^-dnaN^-$ double mutations in some of the phages. In addition, the *dnaN* complementation behavior ruled out the possibility that some of the $dnaA^-$

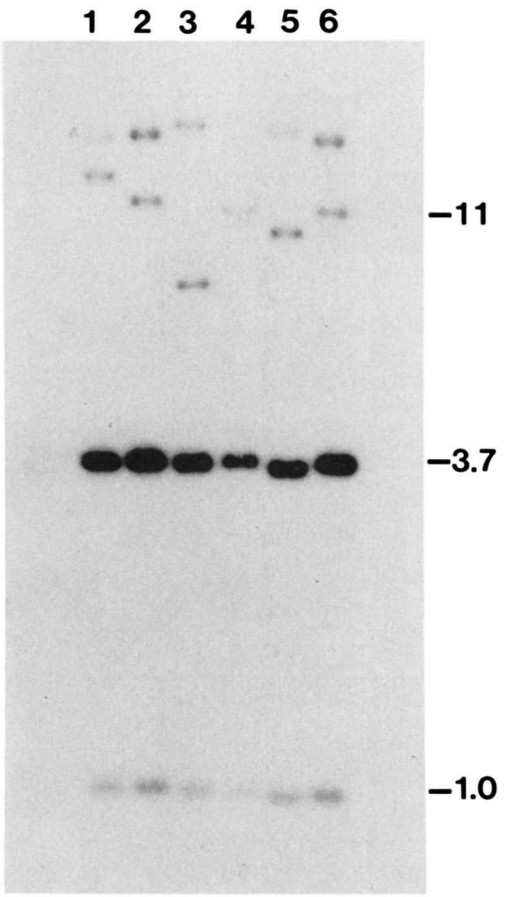

FIGURE 3.—Southern blot of λRM187 and the five suppressor phage DNAs digested with *Eco*RI and probed with the 7.3-kb *Bam*HI fragment cloned from λRM187. Lane 1, λRM187 (wild type); lane 2, λRM560; lane 3, λRM561; lane 4, λRM562; lane 5, λRM563; lane 6, λRM564.

mutations were dominant lethals. Had this been the case, *dnaZ* suppression and *dnaN* complementation would both have been blocked. Together, these mutational studies suggested that *dnaN* contributes in an indispensable way to suppression, whereas the contribution of *dnaA* is more subtle.

Some clarification of this picture was obtained by an alternative approach to identifying the suppressor gene. DNA fragments from one suppressor phage, λRM560, were subcloned in plasmids and assayed for *dnaZ* suppression activity as well as *dnaA* and *dnaN* complementation activity (Table 4). The 7.3-kb *Bam*HI fragment derived from λRM560 carried all three activities. In contrast, the corresponding fragment from a wild-type phage complemented *dnaA* and *dnaN* but did not suppress *dnaZ*. This result showed that the suppressor function could be detected specifically in plasmid subclones derived from the suppressor phage; it also confirmed *dnaA* or *dnaN* as possible candidates for the suppressor gene. Smaller subclones containing the 3.7-kb *Eco*RI fragment (Fig-

TABLE 3

Properties of suppressor phage and mutant derivatives

Parental phage	Derivatives	Phenotype	Suppression assays				
			212	213	239	241	254
λRM560		A^+N^+	+	+	+	+	+
	λ692, 693, 694	A^+N^-	−	−	−	−	−
	λ684, 689	A^-N^+	−	−	−	−	−
	λ690	A^-N^+	−	±	−	−	−
	λ688	A^-N^+	−	±	±	+	−
	λ685	A^-N^+	+	+	+	+	±
	λ686, 687	A^-N^+	+	+	+	+	+
λRM561		A^+N^+	+	+	+	+	+
	λ695, 698, 699	A^+N^-	−	−	−	−	−
λRM562		A^+N^+	+	+	+	+	+
	λ696, 697	A^+N^-	−	−	−	−	−
λRM563		A^+N^+	+	+	+	+	+
λRM564		A^+N^+	+	+	+	+	+
	λ716, 718	A^+N^-	−	−	NT	NT	NT
	λ709, 712, 713	A^-N^+	+	NT	NT	NT	NT
λRM187	Nonsuppressor	A^+N^+	−	−	−	−	−

The results of red plaque assays are shown. +, a red plaque is formed; −, the plaque is not red; ±, very weak or inconsistent response; NT, not tested. The phenotypes indicated are based on red plaque complementation tests using *E. coli* strains DB4883 and DB4894. The designation A^+N^+ should not be taken to indicate true wild-type genotype, as a true wild-type phage carrying *dnaA* and *dnaN* does not give a red plaque with *dnaZ*(Ts) tester strains (last line).

ure 2) showed that the suppressor gene of λRM560 is not *dnaA*. These subclones complement *dnaN* and, if derived from the suppressor phage, suppress *dnaZ*, but they do not complement *dnaA* in either case (Table 4). Thus, the *dnaA* deletion mutation, like the *dnaA* point mutations found in λRM685, λRM686 and λRM687, does not interfere with suppression of *dnaZ* mutations. Apparently, *dnaA* function provided by the suppressor template is dispensable for suppression by λRM560.

In light of the unambiguous involvement of *dnaN* in suppression, a plausible explanation for the *dnaA* mutations that interfere with suppression (*e.g.*, in λRM684, λRM688, λRM689 and λRM690) is that they do so by altering the synthesis or activity of suppressor *dnaN* protein. In support of this suggestion, we note that when the 3.7-kb *Eco*RI fragments from λRM684 and λRM688 are subcloned in plasmid pUC8, the plasmids exhibit suppression. Thus, deletion of the *dnaA* gene of these mutants, and possibly of λRM689 as well, unmasks the suppressor phenotype (Table 4). This result shows that at least some *dnaA* mutations that block suppression do so indirectly. Specific mechanisms by which this might occur are considered in the DISCUSSION.

DISCUSSION

***DnaZ-dnaN* interaction:** Our results show an interaction in Salmonella between two DNA replication genes, *dnaZ* and *dnaN*. The interaction consists of

TABLE 4

Properties of plasmid subclones of wild-type and suppressor *dnaA-dnaN* region

Cloned fragment	Phenotype of bacterial transformants		
	dnaA(Ts)	*dnaN*(Ts)	*dnaZ*(Ts)
Wild-type *Bam*HI 7.3 kb	+	+	−
λRM560 *Bam*HI 7.3 kb	+	+	+
Wild-type *Eco*RI 3.7 kb	−	+	−
λRM560 *Eco*RI 3.7 kb	−	+	+
λRM684 *Eco*RI 3.7 kb	−	+	+
λRM688 *Eco*RI 3.7 kb	−	+	+
λRM689 *Eco*RI 3.7 kb	−	+	−/+

Fragments were subcloned from λRM187 (wild type), λRM560 (suppressor) and *dnaA*⁻ derivatives of λRM560 that are nonsuppressing (λRM684, λRM689) or weakly suppressing (λRM688). Suppression assays are described in MATERIALS AND METHODS. The strains used were DB4883, DB4894, RM212 and RM213. The results for the latter two strains were in agreement except for the λRM689 subclone, which behaved as nonsuppressor in RM212 and as a suppressor in RM213. Plasmids were chosen having similar orientation of the cloned fragment. For most of the fragments, plasmids in the opposite orientation were also obtained, but their instability in some of the hosts used here prevented their systematic evaluation. Where their properties could be examined, the orientation was found to have no effect. +, the transformants grew at the nonpermissive temperature; −, the transformants did not grow at the nonpermissive temperature. Cells transformed with vector only (pUC8) served as a negative control.

suppressor mutations, located in *dnaN* or affecting this gene, that restore growth to temperature-sensitive *dnaZ* mutants. The suppressor in λRM560, which was characterized in most detail, is representative of this group of suppressors. This suppressor maps in the *dnaA-dnaN* region, as shown by the physical map of the clone in which it was isolated. λRM560 suppresses the six available Salmonella *dnaZ* mutants, and it loses its ability to suppress any of the *dnaZ* mutants when the cloned *dnaN* gene is inactivated by mutation. Suppression does not depend on functional *dnaA* product made from the suppressor template, because plasmid subclones lacking *dnaA* still exhibit suppression; however, some *dnaA* point mutations derived in λRM560 interfere with suppression.

Ample evidence indicates that the gene products of *dnaZ* and *dnaN* in Salmonella are similar to the corresponding proteins of *E. coli*. The evidence includes the functional interchangeability of these genes (ROWEN, KOBORI and SCHERER 1982; MAURER *et al.* 1984), parallel genetic behavior of *dnaZ* and *dnaX* mutants and similarities in the organization of the *dnaZ-dnaX* gene cluster (Figure 1; Table 2). Since, in *E. coli*, *dnaZ* and *dnaN* code for the γ and β subunits of pol III, respectively, our data support the idea of an interaction of these subunits *in vivo* during polymerase assembly or action. In all likelihood, this interaction is a direct physical interaction at the protein level, but our data do not rule out other alternatives. An understanding of the *dnaZ-dnaN* interaction at the molecular level must await biochemical investigation. We note

that a physical interaction between β and $\gamma \cdot \delta$ is explicit in the model for pol III proposed by WICKNER (1976) and is also compatible with the proposals of MCHENRY and his colleagues (MCHENRY and KORNBERG 1977).

Role of *dnaA* in suppression: In view of the subcloning results showing that intact *dnaA* is dispensable for suppression, and of the mutational studies showing that *dnaN* is required, it was surprising to find that some *dnaA* point mutations interfere with suppression. Two trivial explanations (double mutations and dominant lethality) were easily ruled out.

Our preferred explanation for these observations about *dnaA* is based on current understanding of the organization and control of the *dnaA-dnaN* region. We propose that the *dnaA* mutations affect suppression by altering the level of suppressor *dnaN* protein. In *E. coli*, *dnaA* and *dnaN* are organized in an operon, with *dnaA* closest to the promoter (SAKO and SAKAKIBARA 1980; SAKAKIBARA, TSUKANO and SAKO 1981). There is also a weak, *dnaN*-specific promoter located near the beginning of *dnaN* (SAKO and SAKAKIBARA 1980). The operon is under negative transcriptional control by *dnaA* protein *in vivo* (BRAUN, O'DAY and WRIGHT 1985; ATLUNG, CLAUSEN and HANSEN 1985). Although the comparable facts about Salmonella are mostly unknown, all observations to date suggest that the situation is essentially the same. The pertinent observations include adjacent map position of *dnaA* and *dnaN*, polarity of Tn*10* insertions in *dnaA* on *dnaN*, expression of *dnaN* from plasmid clones presumably lacking the functional *dnaA* promoter, and functional interchangeability of *dnaA* between *E. coli* and Salmonella (MAURER *et al.* 1984 and our unpublished results).

These facts suggest two ways that *dnaA* mutations can affect the level of *dnaN* protein and, hence, interfere with suppression: by polarity or by altering the regulatory activity of *dnaA*. It is important to note that the expected effects of *dnaA* mutations would be subtle, because in all cases the *dnaA* mutations that interfere with suppression of *dnaZ*(Ts) mutations do not affect complementation of *dnaN*(Ts) mutations in a *dnaZ*$^+$ background. We envision that in a *dnaZ*(Ts) cell, the level of suppressor *dnaN* protein is just sufficient for replication at the nonpermissive temperature because this protein is assembled inefficiently into the polymerase complex or is held there loosely. Under these conditions, a small reduction in suppressor *dnaN* protein abolishes suppression. In contrast, in a *dnaZ*$^+$ cell (*i.e.*, in a *dnaN* complementation test), suppressor *dnaN* protein is assembled more efficiently or held more tightly in the polymerase complex; here, minor variations in suppressor *dnaN* levels would be inconsequential. These ideas are summarized in Table 5.

We have also considered the possibility that *dnaA* plays a direct role in the suppression mechanism. If so, it is necessary to postulate further that the chromosomal wild-type *dnaA* gene can play this role, since suppression can be observed in the absence of any alternative source of *dnaA* (*e.g.*, suppression mediated by the 3.7-kb *Eco*RI subclones). According to this view, the λ-borne *dnaA* mutations that interfere with suppression produce altered *dnaA* proteins that block the suppression activity of chromosomally encoded *dnaA*$^+$ protein. Thus, in such a *dnaA*$^+$/*dnaA*$^-$ partial diploid involving one of these special

TABLE 5

Interpretation of suppressor *dnaN* behavior in suppression and complementation tests

Test	Host genotype	Phage genotype	Sensitivity of test to *dnaA* genotype of phage	Explanation
Complementation of *dnaN*(Ts)	*dnaN*(Ts) *dnaZ*$^+$	*dnaN*(Sp)	No	*dnaZ*$^+$ protein recruits and/or holds *dnaN*(Sp) protein efficiently
Suppression of *dnaZ*(Ts)	*dnaN*$^+$ *dnaZ*(Ts)	*dnaN*(Sp)	Yes	*dnaZ*(Ts) protein recruits and/or holds *dnaN*(Sp) protein inefficiently; *dnaN*$^+$ protein plays no role

dnaA mutations, a *dnaZ*(Ts) mutation cannot be suppressed. At the same time, the mutant *dnaA* protein cannot interfere with the "standard" function of *dnaA*$^+$, because *dnaN*(Ts) mutations can be complemented in a *dnaA*$^+$/*dnaA*$^-$ partial diploid. This highly specialized set of circumstances would seem to dictate rare occurrence of such mutations; in contrast, they were quite common in a small sample (four of seven *dnaA* mutations derived in λRM560; Table 3). A further weakness of this proposal is that it implies a complicated tripartite suppression mechanism involving *dnaA* protein, *dnaN* protein and *dnaZ* protein acting in concert. Nonetheless, this possibility cannot be rigorously excluded with the available data.

Effect of varying wild-type *dnaN* levels: The preceding discussion raises the question whether alteration of the level of wild-type *dnaN* protein could cause suppression of *dnaZ*(Ts) mutations. This is a pertinent question because we have not shown that any of our suppressor mutations produce altered *dnaN* protein, nor have the suppressor mutations been mapped to the *dnaN* coding sequence. In the best-characterized case, λRM560, we have shown that the suppressor mutation is contained within a 3.7-kb *Eco*RI fragment that includes *dnaN* and adjacent material (Figure 2). Subcloning analysis establishes the same conclusion for the suppressor in λRM564 (data not shown). In principle, though, any of our suppressor mutations could lie outside the *dnaN* structural gene and act by increasing the level of *dnaN* expression.

Two types of evidence argue against overproduction of *dnaN*$^+$ as a suppression mechanism. First, multicopy plasmids carrying *dnaN*$^+$ do not confer suppression of *dnaZ*(Ts). Although we do not know the *dnaN* level in such plasmid-bearing strains, it would be surprising if it were not somewhat elevated. Second, we have examined proteins synthesized by λ*dnaN*$^+$ and several of the suppressor phages in heavily UV-irradiated cells. In these preliminary experiments, the *dnaN* product was identified on Laemmli gels as a band (apparent molecular mass = 42–44 kD) which was absent when λ*dnaN*::Tn*10* control phages were used. No apparent overproduction of *dnaN* was seen under these conditions (data not shown). Thus, a situation that should have caused some overproduction of *dnaN* did not result in suppression, and genuine suppressors were not associated with overproduction of *dnaN*. Therefore, we favor the

idea that the suppressor mutations alter the structure of *dnaN* protein, not its synthesis.

Comparison with previous studies: Our results clearly differ from those obtained by WALKER and his colleagues (WALKER, RAMSEY and HALDENWANG 1982; BLINKOWA *et al.* 1983). They isolated extragenic suppressors of a *dnaZ*(Ts) mutation in *E. coli* that simultaneously confer a cold-sensitive (Cs) phenotype on the cell. The mutations responsible for the cold-sensitivity map in the *dnaA* gene, not in *dnaN*. Conceivably, this striking difference with our result could reflect profound differences in the function of *dnaA*, *dnaN* and *dnaZ* in *E. coli* and Salmonella, but the functional interchangeability of these genes *in vivo* renders this explanation most unlikely (MAURER *et al.* 1984). It is also possible that the target *dnaZ*(Ts) mutations being suppressed differ in the two studies in ways that lead to the isolation of distinct classes of suppressor mutations. This possibility is consistent with the observation that the Salmonella suppressors are unable to suppress the *E. coli dnaZ2016* mutation used in WALKER'S studies.

The results reported here showing that *dnaA* mutations can influence the expression or activity of a *dnaN* suppressor gene suggest another possible interpretation of the *E. coli dnaZ* suppression studies. Perhaps the *dnaA*(Cs) mutations confer altered regulation on *dnaN* in these cells, and it is really the altered expression of *dnaN* (which itself may be mutated or wild type) that accounts for the suppression of *dnaZ*(Ts). This idea was considered previously, but was not favored (WALKER, RAMSEY and HALDENWANG 1982). In connection with this proposal it is noteworthy that *dnaA*(Ts) mutants exhibit elevated expression of the *dnaA-dnaN* operon, even at permissive temperatures (BRAUN, O'DAY and WRIGHT 1985; ATLUNG, CLAUSEN and HANSEN 1985). This suggestion has the appealing feature of placing all the reported observations about *dnaZ* suppressors in a single conceptual framework.

The *dnaN* gene has appeared once before as the locus of suppressor mutations. In *E. coli*, mutations that suppress *dnaE*(Ts) mutations map in *dnaN* (KUWABARA and UCHIDA 1981). *DnaE* codes for the α subunit of pol III (WELCH and MCHENRY 1982), which contains the polymerization activity of the enzyme complex (MAKI and KORNBERG 1985; MAKI, HORIUCHI and KORNBERG 1985). These results were interpreted to support a physical interaction between the α and β subunits of pol III. There is nothing inherently contradictory about *dnaN* appearing as a suppressor of *dnaE* and *dnaZ* mutations in different experiments. Indeed, it seems likely that, in a multisubunit assembly, each subunit may be bound to the whole through interactions with several of its neighbors. Each of these interactions may be revealed independently by the appropriate experiments. The assembly pathway of a ribosome provides ample precedent for these ideas (JASKUNAS, NOMURA and DAVIES 1974; GEYL, BÖCK and WITTMANN 1977; NOMURA, MORGAN and JASKUNAS 1977). Therefore, we envision that β makes contacts with both α and γ, and we can be hopeful that the study of suppressor mutations, such as we have described, will help to identify the parts of β involved in each set of interactions.

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