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Suppression of *dnaE* Nonsense Mutations by *pcbA1*

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DNA polymerase III has been recognized as the required replication enzyme in *Escherichia coli*. The synthesis subunit of DNA polymerase III holoenzyme (α subunit) is encoded by the *dnaE* gene. We have reported that *E. coli* cells can survive and grow in the absence of a functional *dnaE* gene product if DNA polymerase I and the *pcbA1* mutation are present. Existing mutations in the *dnaE* gene have been conditionally defective thermolabile mutations. We report here construction of nonsense mutations in the *dnaE* gene by use of a temperature-sensitive suppressor mutation to permit survival at the permissive temperature (32°C). Introduction of the *pcbA1* mutation eliminated the temperature-sensitive phenotype. We confirmed by immunoblotting the lack of detectable α subunit at 43°C.

The *dnaE* gene codes for the α subunit (130 kilodaltons) of DNA polymerase III holoenzyme, the synthetic subunit (11, 12, 17). Temperature-sensitive mutations in the *dnaE* gene make cells conditionally lethal, with a defect in DNA replication at the elevated temperature (43°C). These observations have defined the *dnaE* gene product as the required replication synthesis activity in *Escherichia coli*. On the other hand, mutants deficient in the synthesis activity of DNA polymerase I are viable (4, 8).

We have described polymerase I-dependent replication in which the synthetic activity of DNA polymerase I can substitute for the α-subunit activity of DNA polymerase III (3, 15, 16). DNA polymerase I-dependent replication requires the *pcbA1* mutation. This mutation lies in the *gyrB* region (2, 3).

The observation that DNA replication could occur in *E. coli* strains in the absence of a functional DNA polymerase III α subunit if DNA polymerase I and the *pcbA1* mutation were present prompted us to investigate further inactivation of the *dnaE* gene. It is possible, for example, that a strain which contains a *dnaE*(Ts) gene may retain significant activity in vivo at a restrictive temperature. It is also possible that the *dnaE* gene product supplies additional functions in DNA replication that are not yet recognized. Therefore, whereas it might be possible to dispense with the synthesis function, other functions might be strictly required and might be preserved in the existing temperature-sensitive mutations of the gene. Further mutagenesis of the gene could help to define such contributions. To determine whether the DNA polymerase I replication pathway is independent of any functional *dnaE* gene product, we undertook additional mutagenesis of the *dnaE* gene.

We report here that we have derived nonsense mutations in the *dnaE* gene. The nonsense mutations could produce a mutator phenotype and were phenotypically suppressed in the presence of DNA polymerase I and the *pcbA1* allele. By immunoblot analysis, we show that the α subunit of DNA polymerase III was absent in holoenzyme preparations when cells were grown at 43°C.

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MATERIALS AND METHODS

Strains, bacteriophages, and plasmids. Bacterial strains and plasmids used are listed in Table 1. CSM61 and RM552 have been described previously (3). HMS83 is wild type for *dnaE*, and JW353 has Tn*10* linked to *dnaE* 55% by P1. Phage φ80 *supF*(Ts) was from M. Imai, λ i^{21} pSu1$^+$ was from H. Ozeki, λ *h* pSu2$^+$ was from H. Inokuchi, and λpapa was from D. Kaiser.

Materials. Growth media were purchased from Difco Laboratories (Detroit, Mich.). ^{3}H-labeled thymidine triphosphate was from ICN Pharmaceuticals Inc. (Irvine, Calif.). Unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Methyl methanesulfonate was from Eastman Organic Chemicals. Polyclonal antibody against DNA polymerase III holoenzyme was a gift from C. McHenry (University of Colorado Health Science Center, Denver). ^{125}I-containing Bolton Hunter reagent used to make goat ^{125}I-labeled anti-rabbit antibody was obtained from Amersham Corp. (Arlington Heights, Ill.). Polyacrylamide gel electrophoresis reagents and molecular weight standards were from Bio-Rad Laboratories (Richmond, Calif.).

P1 transduction. P1 transduction was done as described previously (14). Selection was on L-broth plates containing tetracycline (10 μg/ml) plus or minus NaCl.

Transformation. Transformation of bacteria by plasmid DNA was performed by the method of Morrison (13). Selection was on L-broth plates containing ampicillin (50 μg/ml) plus or minus NaCl.

Cell growth. All cell growth was carried out as previously described (16) except that the *dnaE*(Am) strains were grown on L-broth agar or in liquid L broth without NaCl. In experiments in which growth at 43°C is indicated, the *dnaE*(Am) strains were grown at 32°C for 2 h and then shifted to 43°C for 5 h.

Mutagenesis. Mutagenesis trials were performed as previously described (5). Spontaneous mutation frequency was determined as described elsewhere (9).

Enzyme isolation and assay conditions. DNA polymerase III holoenzyme was isolated and assayed by a gapped-template assay according to the method of McHenry and Kornberg (12) as previously described (14). DNA polymer-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Source
Strains		
CSM61	*polA1 polB100 polC1026* Leu⁻ His⁻ Thy⁻ *pcbA1 supE* Tr	This laboratory (3)
HMS83	*polA1 polB100* Leu⁻ Cys⁻ Lys⁻ Thy⁻	C. C. Richardson
JW353	*thr-1 leuB6 thyA6 metB89 thi-1 deoC1 lacY1 rpsL67 tonA21* λ⁻ *supE44 zae-502*::Tn*10 Tr*	*E. coli* Genetic Stock Center
MK329	HfrC *metB1 proA3*	This report
MK385	HfrC *metB1 proA3 metD88 lac-3 tsx-76 relA6 sup-126* (*sup*D[Ts])	This report
MK390	HfrC *metB1 proA3 metD88 lac*(Am) *sup-126* φ80 *supF*(Ts)	This report
MK437	HfrC *metB1 proA3 metD88 lac-3 metA38 tsx-76, relA1*, φ80 *supF*(Ts)	This report
MK503	*dnaE52*(Am) derivative of MK385	This report
MK505	*dnaE94*(Am) derivative of MK385	This report
MK513	*dnaE245*(Am) derivative of MK385	This report
MK519	*dnaE73*(Am) derivative of MK437	This report
MK531	*dnaE245*(Am) derivative of MK437	This report
RM552	*dnaE511 pcbA1* Leu⁻ His⁻ Thy⁻ *zic-1*::Tn*10* Tr	This laboratory (3)
W3110	IN(*rrnD-rrnE*)*1* λ⁻ F⁻ Thy⁻ Tr	J. Cairns
Plasmids		
pDS4-26	*dnaE* in pBR322	C. McHenry
pSB5	*dnaE1026* in pBR322	This laboratory
pMM1	*dnaE* in pBR322	M. Maruyama (10)

[a] Tr, Temperature resistant; Ts, temperature sensitive.

ase I was extracted by the Brij-lysozyme-EDTA method and assayed as previously described (14).

Immunoblotting. Preparations of DNA polymerase III holoenzyme FxII, from various strains grown at 32 and 43°C, were run on a 7.5% polyacrylamide gel under denaturing conditions for protein transfer or Western blotting (immunoblotting) (1, 18). The protein bands were transferred to nitrocellulose paper by using a Hoeffer Scientific Instruments apparatus at 1 mA for 1.5 h. The nitrocellulose paper was incubated in Tris-buffered saline (0.05 M Tris, 0.15 M NaCl [pH 8.4]) plus 5% Carnation nonfat dry milk with a 1:1,600 dilution of purified rabbit immunoglobulin G antiholoenzyme polyclonal antibody overnight at room temperature. After the primary antibody was washed off, the nitrocellulose was incubated in an excess of goat ^{125}I-labeled anti-rabbit immunoglobulin G antibody (10^6 cpm/ml or 10^8 cpm/μg) in Tris-buffered saline with 5% milk for 3 h. After extensive washing, the nitrocellulose was dried and an autoradiograph was performed with Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.).

Localized mutagenesis. By hydroxylamine treatment of phage P1 particles, localized mutagenesis was performed as described previously (6). A high-titer stock (about 10^{10} PFU/ml) of P1 *vir* grown on MK329 was concentrated 100-fold by high-speed centrifugation (50,000 × *g* for 1 h). The phage particles were treated with a mutagen solution containing 0.45 M hydroxylamine hydrochloride, 2 mM EDTA, and 10 mM $CaCl_2$ (pH 6, adjusted with NaOH) at 37°C for 18 h. By the end of this treatment, plaque-forming activity had decreased to 10^7/ml. Transduction was done at 30°C with hydroxylamine-treated phage P1. The recipient strain used was MK390, and *metD*$^+$ transductants were selected.

RESULTS

Isolation of *dnaE* nonsense mutants. Since the *dnaE* gene has been located at approximately 4.0 min on the standard genetic map, we chose *metD*, located at 5.2 min, as a marker for selecting transductants (produced by infection of hydroxylamine-treated phage P1 *vir*) to isolate mutants whose conditionally lethal mutations were in the vicinity of *dnaE*. Of 3,552 *metD*$^+$ transductants of MK390 tested, 69 were found to be temperature sensitive for growth. Because the parental strain of these mutants (MK390) carried temperature-sensitive amber suppressors, some of the temperature-sensitive mutants were expected to be amber mutants of essential genes. Therefore, all of the temperature-sensitive mutants were screened to identify those with an amber mutation by cross-streaking mutant cell suspensions against phage λ *i*^{21} pSu1$^+$ and λ *h* pSu2$^+$, suspensions on nutrient agar plates, which were incubated overnight at 43°C. A suspension of λpapa served as a control. Mutants that grew at the cross areas with the amber suppressor-transducing phage but not with λpapa were judged to be amber mutants. Of the 69 temperature-sensitive mutants, 22 were found to be amber mutants.

Amber mutations in these mutants were analyzed by complementation tests with plasmid pMM1, which carries the *dnaE*$^+$ gene (10). Upon introduction of the plasmid, 10 amber mutants were complemented for growth at 43°C. Phage P1 stocks were prepared with these candidates for *dnaE*(Am) mutants (designated MK398, MK400, etc., in Table 2), and *metD*$^+$ transductions were performed with P1. The recipients were MK385 and MK437, which harbor *sup-126* and *supF*(Ts), respectively. Each MetD$^+$ transductant was tested for the temperature-sensitive phenotype (Table 2). When P1 grown on MK398, MK402, or MK414 was used, the temperature-sensitive phenotype cotransduced into either recipient with a frequency about 50%. However, phage P1 grown on other mutants could cotransduce the temperature-sensitive phenotype into both or only one of the recipients at less than 50% linkage. This finding suggested that some amber mutations could not be suppressed well by one or the other suppressor but had survived selection, because all of the primary strains of the mutants carried both *sup-126* and *supF*(Ts). From the complementation tests and P1 mapping, these 10 amber mutants were designated *dnaE*(Am) mutants.

TABLE 2. P1 transduction of *dnaE*(Am) mutations

Donor[a]	Allele	Cotransduction frequency (% Ts/MetD$^+$) with given recipient	
		MK385 (*sup-126*)	MK437 (*supF* [Ts])
MK398	*dnaE52*	45	55
MK400	*dnaE73*	0	40
MK402	*dnaE94*	55	65
MK404	*dnaE108*	0	35
MK406	*dnaE143*	0	35
MK409	*dnaE154*	45	30
MK410	*dnaE163*	55	45
MK412	*dnaE170*	45	0
MK414	*dnaE245*	45	70
MK416	*dnaE281*	35	30

[a] The *dnaE*(Am) mutation candidates were isolated as described in the text. Each was grown and used for P1 transduction; 20 colonies were selected for *metD*$^+$ on the indicated recipients and tested for the temperature-resistant or -sensitive phenotype.

Characteristics of *dnaE*(Am) strains. Either transduction by *dnaE* (linked to Tn*10* from strain JW353) or transformation with a plasmid DNA carrying *dnaE* (pDS4-26) suppressed *dnaE*(Am) temperature-sensitive growth on L-broth plates without NaCl. We concluded that the amber mutations were alleles of the *dnaE* gene. The *dnaE*(Am) strains showed temperature-sensitive growth on salt-free L-broth plates at 43°C but grew well at 32°C. At 40°C, they produced very small pinpoint colonies. Addition of 5 g of NaCl per liter to the medium allowed growth at 43°C.

The *dnaE*(Am) strains showed a higher than normal frequency of mutation from Rif^s to Rif^r (MK503, 58-fold; MK505, 96-fold; MK513, 106-fold; and MK519, 26-fold). Selection was at 30°C on L-broth plates containing no NaCl and 100 μg of rifampin per ml.

Production by *pcbA1* of a temperature-resistant phenotype in *dnaE*(Am) strains. We introduced *pcbA1* into the *dnaE* (Am) mutant strains by P1 transduction from RM552 (which contains *pcbA1* linked to Tn*10*). *pcbA1* phenotypically suppressed the temperature sensitivity of the *dnaE*(Am) mutant MK519, as did the *dnaE* plasmid pDS4-26 (Fig. 1). The *pcbA1* mutation phenotypically suppressed the temperature sensitivity of the other amber mutations as well (data not shown). Thus, *pcbA1* suppresses not only multiple *dnaE*(Ts) alleles (2) but also the *dnaE*(Am) mutations.

UV mutagenesis. We compared the effect of UV on mutagenesis in the *dnaE*(Am) strains with the same strains carrying *pcbA1*. MK519 had a mutagenesis profile at 32°C (Fig. 2A) similar to those of other *dnaE*(Ts) and *dnaE*(Am) strains; however, the background mutation rate in this strain was at least 25 times higher than normal. This result is not surprising, since *dnaE* mutations can have mutator phenotypes (7). By transducing *pcbA1* into this strain, the background mutation rate was lowered to normal levels. Without *pcbA1*, the mutator effect caused frequent reversion to *dnaE*$^+$, and we lost several strains because of reversion at the *dnaE* locus before we introduced *pcbA1*. With the introduction of *pcbA1*, we were also able to follow mutagenesis at 43°C in MK519 (Fig. 2B). We observed no mutagenesis in response to DNA damage at 43°C, as we reported for *dnaE*(Ts) strains with DNA polymerase I and *pcbA1* (5).

Immunoblot analysis. Our observations led us to isolate DNA polymerase holoenzyme from the *dnaE*(Am) strains to confirm the absence of the α subunit at 43°C. We analyzed the holoenzyme fractions from cells at 43°C on a 7.5% polyacrylamide gel and blotted against antiholoenzyme antibody. The holoenzyme isolated from HMS83 had about the same amount of binding to α subunit by the antiholoenzyme antibody at both temperatures (Fig. 3, lanes A and B). There was much less binding with the *dnaE*(Am) α subunit at 32°C even though the same amount of protein (150 μg) was loaded (note band at 140 kilodaltons). This confirmed enzymatic data indicating less than normal DNA polymerase III activity at 32°C in *dnaE*(Am) strains. There seemed to be two bands near the molecular weight of DNA polymerase III α subunit at 32°C, the smaller of which might have been the amber fragment. If this were the case, the amber mutation would be at the COOH-terminal end of the α subunit of DNA polymerase III. The top band is probably the readthrough of the normal-length polypeptide (Fig. 3, lane C). There was no reaction of the holoenzyme antibody with α subunit at 43°C in the *dnaE*(Am) strain MK519 (Fig. 3, lane D) even when the autoradiograph was overexposed.

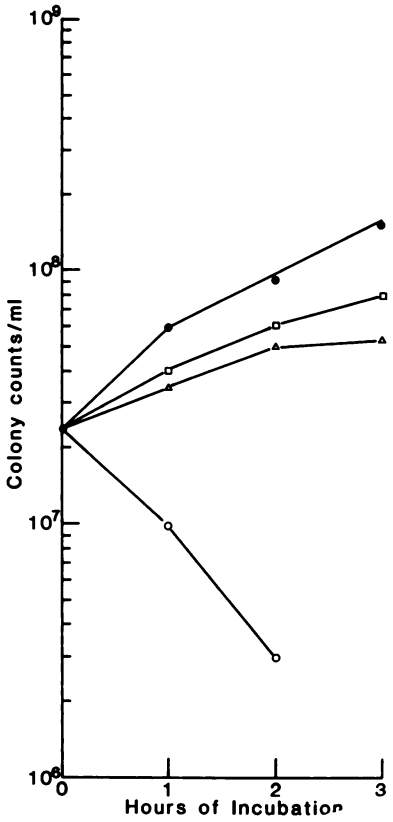

FIG. 1. Growth of *dnaE*(Am) strain MK519. Shown are colony counts of liquid cultures growing in L broth. Symbols: ●, MK519, 32°C; ○, MK519, 43°C (shifted from 32 to 43°C at time zero); □, MK519(pDS4-26), 43°C; △, MK519 (*pcbA1*), 43°C.

***dnaE*(Am) in other backgrounds.** Tn*10* was introduced into MK519 from JW353. Cells that were Tc^r and still temperature sensitive were identified as having Tn*10* linked to the *dnaE*(Am) gene. These cells were used as a source to transduce Tn*10* into W3110, a strain without suppressor mutations and containing pSB5, a plasmid with *dnaE1026*, a temperature-sensitive allele. Thirty percent of the transductants became temperature sensitive, which indicates that replication was dependent on the plasmid-encoded *dnaE1026*.

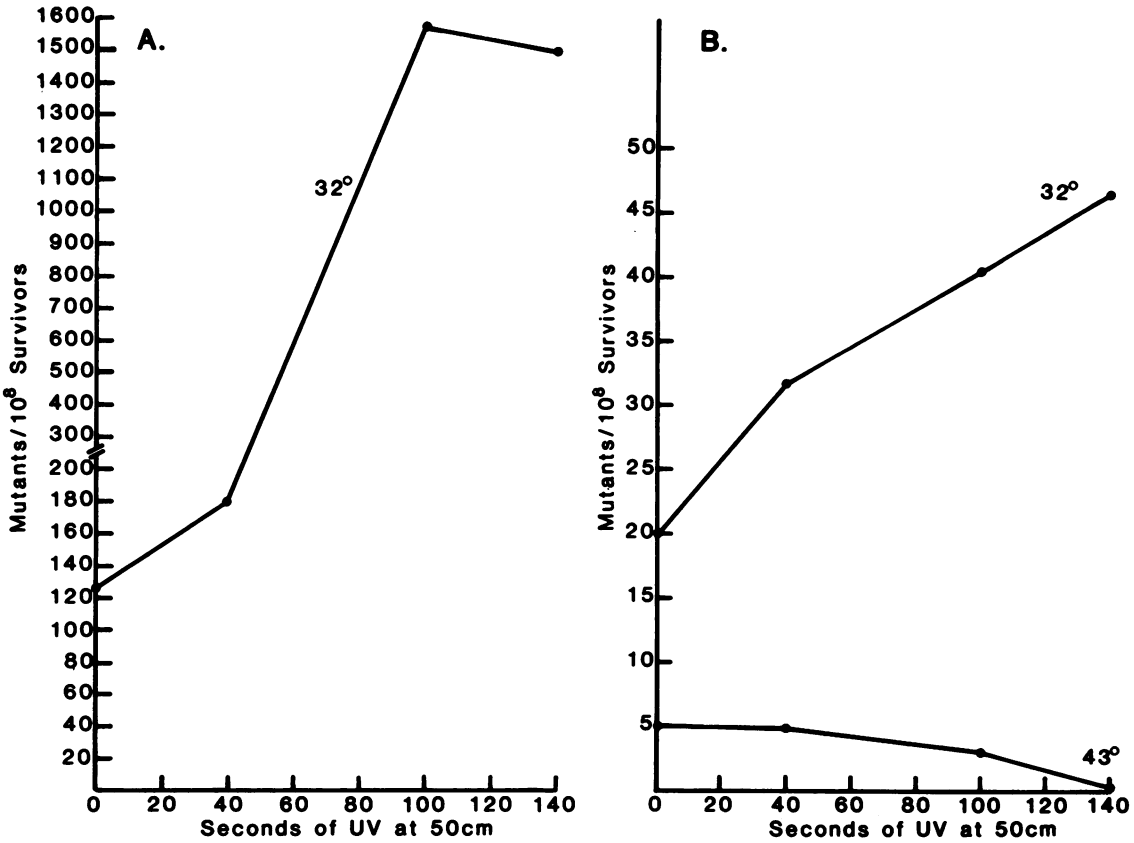

FIG. 2. UV mutagenesis of *dnaE*(Am) strain MK519. (A) MK519; (B) MK519 (*pcbA1*). Cells were grown to mid-log phase and then exposed to a UV flux of 1 J/m^2 per s for various times as described in Materials and Methods. Samples were withdrawn, diluted for cell survival, and plated on L-broth plates containing 100 μg of rifampin per ml to score for mutants. Cells were grown and plated at 32 and 43°C. D37 (the dose to kill to 37% survivors) values were 22 to 28 s at both temperatures. Survivors were 0.4% (A) and 0.35% (B) at 140 s.

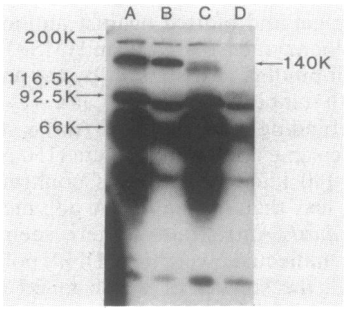

FIG. 3. Immunoblot of DNA polymerase III holoenzyme FxII against antiholoenzyme antibody. DNA polymerase III holoenzyme FxII was prepared from HMS83 and MK519 *dnaE*(Am) cells grown at 32 and 43°C and run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel; the protein was then transferred to nitrocellulose paper for immunoblotting with antiholoenzyme antibody. Arrow at 140 kilodaltons (140K) indicates the α subunit of DNA polymerase III holoenzyme. Lanes: A, holoenzyme from HMS83 grown at 32°C (150 μg); B, holoenzyme from HMS83 grown at 43°C (150 μg); C, holoenzyme from MK519 grown at 32°C (150 μg); D, holoenzyme from MK519 grown at 43°C (140 μg).

DISCUSSION

The results presented here demonstrate that nonsense mutations can be introduced into the *dnaE* gene. Thus, we describe a new category of *dnaE* mutations, in addition to the temperature-sensitive mutations that have been more extensively studied.

The *pcbA1* allele phenotypically suppressed the deficiency in the *dnaE* nonsense mutations if DNA polymerase I activity was present. Therefore, the effect of the *pcbA1* mutation is not limited to reversal of the phenotype resulting from temperature-sensitive mutations but also bypasses the defect in early-termination mutations. This finding argues that the *pcbA1*-polymerase I replication pathway is not limited to suppression of a certain category of mutations in the *dnaE* gene.

We have presented data indicating that DNA polymerase I is strictly required for replication in the presence of the *pcbA1* mutation and in the absence of a functional *dnaE* gene product (15, 16). There are reports of DNA polymerase I being associated with other proteins (2). However, it appears that there are two definite states of the replicative complex: one in which DNA polymerase I, the product of the *polA* gene, is present and functions in replication, and one in which the α subunit, the product of the *dnaE* gene, is present and functions in replication. We suggest that these two alternate functional forms of the replicative complex be termed REP-A and REP-E. REP-A indicates the holoenzyme complex with the *polA* gene product functionally included, and REP-E indicates the replicative complex with the *dnaE* gene product functionally included. The conditions under which either of these exists remain to be determined.

The data presented in this paper do not rule out a continuing contribution by a fragmentary polypeptide product of the *dnaE* gene in a nonsynthesis role in DNA replication.

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