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Mutagenesis by Neocarzinostatin in *Escherichia coli* and *Salmonella typhimurium*: Requirement for *umuC*$^+$ or Plasmid pKM101

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Neocarzinostatin, a protein with antibiotic activity, is a bacterial mutagen. We have investigated the mutagenicity of neocarzinostatin towards *Salmonella typhimurium* and discovered that, unlike the situation in *Escherichia coli*, neocarzinostatin will revert base pair substitution mutations (missense or nonsense). However, when the R46 factor derivative, plasmid pKM101, was introduced, the mutagenicity of neocarzinostatin towards base pair substitution-carrying mutants of *S. typhimurium* was readily detected. Neocarzinostatin had only modest activity in reverting a frameshift mutation in *S. typhimurium*, but that activity, too, required the presence of pKM101. Mutant pKM101 plasmids which no longer enhanced mutagenesis also lost their ability to promote neocarzinostatin-induced mutations. Finally, the *umuC36* mutation, which renders *E. coli* nonmutable by ultraviolet light, also rendered the bacteria nonmutable by neocarzinostatin. The effect of the *umuC36* mutation was suppressed by plasmid pKM101.

Neocarzinostatin (NCS) is an acidic protein (molecular weight, 10,700) of known amino acid sequence that has both antitumor and antibiotic activity (reviewed in I. H. Goldberg, T. Hatayama, L. S. Kappen, and M. A. Napier. Protein antibiotics as DNA damaging agents, *in* A. C. Sartorelli, J. R. Bertino, and J. S. Lazo (ed.), *Molecular Actions and Targets for Cancer Chemotherapeutic Agents*, Second annual Bristol-Myers Symposium in Cancer Research, Academic Press, Inc., N.Y., in press). The nonprotein chromophore associated with NCS has been isolated and found to possess the cytotoxic and in vitro activity of NCS (7). The chromophore (molecular weight, 661) consists, in part, of a 2,6-dideoxy-2-methylamino-galactose moiety and a naphthoic acid derivative (1). NCS causes single- and double-strand breaks in DNA both in vitro and in vivo, which probably accounts for its biological activity. Double-stranded DNA is a significantly better substrate than single-stranded DNA for this cutting activity. Scission of DNA by NCS is accompanied by the release of free thymine and, to a lesser extent, free adenine. By treating DNA restriction fragments of defined sequence with NCS, Hatayama et al. (4) and D'Andrea and Haseltine (2) directly demonstrated nucleotide specificity in DNA scission.

NCS is mutagenic for *Escherichia coli* B strain WP2. Tatsumi and Nishioka (12) demonstrated that treatment of strain WP2 for 30 min with very high concentrations of NCS (100 μg to 100 mg per ml) induced reversion of an ochre mutation in the *trp* operon. They further showed that the mutagenicity of NCS is *recA* dependent but *uvrA* independent. *recA* mutants, though nonmutable, were more sensitive to the lethal effects of NCS, whereas *uvrA* mutants were no more sensitive than parental strains to NCS.

We have investigated the mutagenicity of NCS further, and in this report we present evidence defining a need for either the *umuC*$^+$ allele (8) or an activity encoded by plasmid pKM101 (14-16) for NCS-induced mutations.

MATERIALS AND METHODS

Bacterial strains and phages. The bacterial strains used in this study are listed in Table 1. Phage P22 int3 HT12/4 was obtained by D. Botstein.

Media. The nutrient broth contained, per liter: 8 g of Difco nutrient broth and 5 g of NaCl. LB broth contained, per liter: 10 g of Difco tryptone, 5 g of Difco yeast extract, and 10 g of NaCl. Minimal plates for scoring Trp$^+$ revertants of *E. coli* WP2 were prepared as follows (per liter): 100 ml of 10× concentrated M9 salts (10), 50 ml of nutrient broth (as a source of tryptophan), 2 ml of 20% (wt/vol) $MgSO_4 \cdot 7H_2O$, 5 ml of 40% (wt/vol) glucose, 10 ml of 0.5 mM biotin, 833 ml of water, and 15 g of Difco agar. Supplemented minimal plates for determining reversion of mutations in *E. coli* K-12 and *Salmonella typhimurium* were prepared as follows (per liter): 20 ml of 50× concentrated Vogel-Bonner salts (13), 50 ml of 40% (wt/vol) glucose, 930 ml of water, and 15 g of Difco agar. Minimal plates were supplemented with growth-lim-

TABLE 1. *Bacterial strains used in this study*

Strain	Relevant genotype or phenotype[a]	Source or reference
E. coli B		
WP2	*trp*(Oc)	E. Witkin
E. coli K-12		
AB1157	*argE3*(Oc)	G. Walker
TK603	*his-4*(Oc) *uvrA-umuC*$^+$	G. Walker
TK610	*his-4*(Oc) *uvrA-umuC36*	G. Walker
TK603(pKM101)	as TK603 plus pKM101	G. Walker
TK610(pKM101)	as TK610 plus pKM101	G. Walker
S. typhimurium		
LT7	*hisC117*(Oc)	B. Ames (3)
LT2	*hisC342*(Oc)	B. Ames (3)
LT2	*hisC354*(Oc)	B. Ames (3)
LT2	*hisC502*(Oc)	B. Ames (3)
LT2	*hisC50*(Am)	B. Ames (3)
LT2	*hisC340*(Am)	B. Ames (3)
LT2	*hisC434*(Am)	B. Ames (3)
LT2	*hisC501*(Am)	B. Ames (3)
LT2	*hisC508*(Am)	B. Ames (3)
LT2	*hisC527*(Am)	B. Ames (3)
LT2	*hisG46*(Ms)	B. Ames (3)
LT2	*hisC3076*(Fs)	B. Ames (3)
NK30	Δ*his-712*	D. Botstein
DB7000	*leuA414*(Am)	D. Botstein
DB7000(pKM101)	as DB7000 plus pKM101	This study
NK30-117	*hisC117*	This study
NK30-342	*hisC342*	This study
NK30-354	*hisC354*	This study
NK30-502	*hisC502*	This study
NK30-50	*hisC50*	This study
NK30-340	*hisC340*	This study
NK30-434	*hisC434*	This study
NK30-501	*hisC501*	This study
NK30-508	*hisC508*	This study

[a] Abbreviations: Oc, ochre; Am, amber; Ms, missense; Fs, frameshift; Δ, deletion.

iting amounts of histidine or arginine (100 or 200 nmol per plate, respectively) and excess amounts of other amino acids or vitamins required for growth (100 μg/ml for amino acids and 1 to 2 μg/ml for vitamins). The tryptophan, histidine, or arginine content of supplemented minimal plates allows several generations of bacterial growth before selection sets in for revertants and thus permits expression of mutations.

NCS. NCS was provided by S. Shepartz of the National Cancer Institute (lot no. 780222). NCS was kept frozen in 0.015 M sodium acetate (pH 5.0) at −20°C in foil-wrapped tubes. It could be frozen and thawed up to eight times with no loss of activity.

Mutagenesis experiments. Bacteria were grown overnight in nutrient or LB broth at 37°C with shaking. The bacteria was washed and concentrated by centrifugation and suspended in M9 buffer (M9 salts plus 1.67 mM $MgSO_4$) to various optical densities at 660 nm. An optical density at 660 nm of 1.0 corresponds to approximately 10^9 cells per ml. Cells were generally treated in suspension with NCS and then plated in duplicate on supplemented minimal plates to determine revertants or diluted and plated in duplicate on supplemented minimal or nutrient broth plates to determine the surviving fraction. Sometimes untreated cells were spread in the presence of added NCS. Minimal plates were incubated for 2 days at 37°C and then counted. Broth plates were counted after 1 day at 37°C.

Strain constructions. The plasmid pKM101 was transferred from DB7000(pKM101) to recipient cells by the method of McCann et al. (9). The isogenic set of *hisC* amber and ochre mutants was constructed by transduction of the *his* deletion mutant Δ*his-712* with P22 lysates. Bacteria able to express the *hisD* function can grow on minimal medium supplemented with histidinol even if they are defective in any other *his* function. Thus, Δ*his-712*, carrying a deletion through *hisD*, was treated with P22 lysates prepared from the different *hisC* mutants, and transductants capable of growth on histidinol were selected and subsequently screened for histidine auxotrophy.

RESULTS

Reversion by NCS of point mutations in the *his* operon of *S. typhimurium*—requirement for plasmid pKM101. We compared the ability of NCS to induce reversion of some well-characterized nonsense mutations in *E. coli* and *S. typhimurium* (Table 2). These results confirmed those of Tatsumi and Nishioka (12) showing that NCS treatment of bacteria induced reversion of the *trp* ochre mutation in *E. coli* strain WP2, an *E. coli* B derivative. NCS also had mutagenic activity against an *E. coli* K-12 strain, AB1157. However, there was no muta-

TABLE 2. *NCS-induced reversion of ochre mutations in E. coli and S. typhimurium*[a]

Strain	Ochre allele or mutation	NCS (μg/ml)	No. of revertants per plate
E. coli WP2	*trp*	0	23
	trp	60	214
E. coli AB1157	*argE3*	0	7
	argE3	51	93
S. typhimurium LT2	*hisC342*	0	3
	hisC342	60	4
S. typhimurium LT7	*hisC117*	0	4
	hisC117	60	39
	hisC117	180	82
	hisC117	300	108

[a] Bacteria were resuspended in M9 buffer to an optical density at 660 nm of 4.4 (for *E. coli* strains; 4 × 10^9 to 6 × 10^9 cells per ml) or 3.0 (for *Salmonella* strains; 3 × 10^9 to 5 × 10^9 cells per ml). Cells were treated with NCS at the indicated concentrations for 30 min at 37°C in the dark. Survival varied from 25 to almost 100% but did not correlate with mutability.

genic activity against three *S. typhimurium* LT2 strains carrying the *his* ochre alleles *hisC342, hisC354,* and *hisC502* (3) (Table 2 and data not shown). Similarly, NCS did not show mutagenic activity against LT2 strains carrying any of six different amber mutations in *hisC* (data not shown). Higher concentrations were also not mutagenic for these strains.

When *S. typhimurium* strain LT7, carrying the *hisC117* ochre mutation, was treated with NCS, weak but concentration-dependent mutagenic activity was seen (Table 2). As the difference in response to NCS between the *hisC117* allele and the other *hisC* nonsense mutations seemed attributable to differences in genetic background between LT2 and LT7 strains (see below), an isogenic set of strains carrying these alleles in the LT2 background was constructed. Furthermore, since plasmid R46 derivative pKM101 had been shown to enhance mutagenesis in *S. typhimurium* (9), we also constructed pKM101-bearing derivatives of the isogenic strains carrying the nonsense mutations. The results of treating these strains with NCS are summarized in Table 3.

Without the plasmid, none of the ochre or amber mutations, including the *hisC117* allele, was reverted as a consequence of exposure to NCS. However, when plasmid-bearing derivatives of these strains were treated with NCS, reversion of all nonsense mutations was readily detected. The reversion incidence induced by NCS appeared to be similar for all alleles. Average survival of strains without pKM101 in 10 experiments was 52 ± 11%, and average survival for strains with pKM101 was 38 ± 23%. Thus, plasmid pKM101 did not protect the bacteria from the lethal effects of NCS.

Nonsense mutations are revertible by external suppressor mutations as well as by intragenic reversion events. To determine whether the NCS-induced His^+ revertants were structural gene revertants, we picked 20 NCS-induced His^+ revertants of the *hisC527* allele and screened them for their ability to serve as donors in P22-mediated transduction of the *his* deletion mutant, Δ*his-712,* to His^+ phenotype. Transduction of the deletion mutant indicates that reversion occurred in the structural gene; 15 of 20 spontaneous and 13 of 20 NCS-induced colonies gave rise to P22 lysates with His^+ transducing activity for Δ*his-712.* The seven lysates without His^+ transducing activity which were prepared from NCS-induced revertants had comparable transducing activity for other markers and were also capable of suppressing the *hisC527* allele. Thus, we conclude that both structural gene and external suppressor mutations were induced by NCS, but that structural gene reversions predominated.

We have also determined the revertibility by NCS of a missense mutation and frameshift mutation in *S. typhimurium* (Table 4). As with the nonsense mutations, no His^+ revertants were induced by NCS in the absence of plasmid pKM101. In the presence of pKM101, the *hisG46* mutation was reverted by NCS at a frequency comparable to that of the nonsense mutants. The frameshift mutation, *hisC3076,* was also reverted by NCS when tested in its plasmid-bearing derivative. This is an interesting result in view of the known specificity of NCS for cutting DNA primarily at deoxythymidylic and deoxyadenylic acid residues (4) and the genetic evidence that the *hisC3076* frameshift mutation is an added G·C base pair result-

TABLE 3. *Plasmid pKM101 dependence of NCS mutagenesis in isogenic S. typhimurium his nonsense mutants*[a]

hisC allele in NK30 background	No. of His^+ revertants per plate			
	Without pKM101		With pKM101	
	Untreated	NCS treated	Untreated	NCS treated
hisC117(Oc)	1.5	1.0	6	452
hisC342(Oc)	1.5	3.0	5.5	410
hisC354(Oc)	0.5	4.0	8	723
hisC502(Oc)	2.0	3.5	6	405
hisC50(Am)	4	6.5	17	896
hisC340(Am)	4.5	2	19	445
hisC434(Am)	19.5	15	15	710
hisC501(Am)	5.5	5.5	31	555
hisC508(Am)	12	9.5	25	648
hisC527(Am)	6.5	3.5	16	595

[a] Bacteria were suspended in M9 buffer to an optical density at 660 nm of 4.5 and treated with 120 μg of NCS per ml for 30 min before plating. Abbreviations: Oc, ochre; Am, amber.

TABLE 4. *Plasmid pKM101 dependence of NCS-induced reversion of missense and frameshift mutations in S. typhimurium*[a]

his allele	No. of His^+ revertants per plate			
	Without pKM101		With pKM101	
	Spontaneous	NCS treated	Spontaneous	NCS treated
hisG46 (missense)	5	2	57	501
hisC3076 (frameshift)	15	9	15	171

[a] Bacteria were resuspended in M9 buffer to approximately 1.2×10^{10} cells per ml and diluted 1:1 with a solution of NCS (600 μg/ml); 0.1 ml was spread immediately onto plates of minimal medium.

ing in a GGGG/CCCC sequence (discussed in reference 1a).

Effect of *umuC* allele on NCS mutagenesis in *E. coli*. The *umuC* mutation renders *E. coli* nonmutable by UV light while only modestly increasing the sensitivity of the bacteria to the lethal effects of UV irradiation (8). Walker and Dobson (16) demonstrated that the pKM101 plasmid suppresses the *umuC* mutation. In view of the requirement of pKM101 for NCS mutagenesis in *Salmonella* but not *E. coli*, we determined the mutagenicity of NCS for *umuC* derivatives of *E. coli* in the absence or presence of pKM101 (Table 5). Clearly, the *umuC* mutation abolished the mutagenic effect of NCS treatment, and pKM101 suppressed the effect of the *umuC* mutation.

Effect of plasmid pKM101 mutations on NCS-induced mutagenesis. Walker (15) has isolated and characterized mutants of plasmid pKM101 which are altered in their ability to enhance methyl methanesulfonate-induced reversion of the *hisG46* missense mutation. These plasmid mutations also affected the ability of the plasmid to enhance survival of UV-irradiated bacteria and to enhance spontaneous reversion of the *hisG46* allele. One of these mutant plasmids, pGW21, has completely lost its ability to enhance the reversion of the *hisG46* allele by methyl methanesulfonate. Another mutant plasmid, pGW16, was slightly more effective in enhancing methyl methanesulfonate-induced reversion of the *hisG46* allele, but was much more effective than the pKM101 plasmid in enhancing the spontaneous reversion of *hisG46*. We determined the effect of these two mutant plasmids on the NCS-induced reversion of *hisG46* (Table 6). Plasmid pGW21 had totally lost its ability to enhance both spontaneous and NCS-induced reversion of *hisG46*, whereas pGW16 increased the spontaneous but not the NCS-induced reversion of *hisG46* compared with the pKM101 plasmid.

TABLE 5. *Plasmid pKM101 dependence of NCS mutagenesis in E. coli with umuC36*[a]

NCS (μg/plate)	No. of induced His⁺ revertants per plate in:			
	umuC⁺	*umuC36*	*umuC36*⁺ (pKM101)	*umuC36* (pKM101)
0	21	35	49	74
3	41	26	59	253
6	61	19	102	355
12	83	19	135	458
30	169	9	209	544

[a] Bacteria were grown in LB broth and suspended in M9 buffer to an optical density at 660 nm of 7.5. Cells (0.1 ml) were then plated onto plates containing minimal medium in the presence of indicated amounts of NCS.

TABLE 6. *Effect of pKM101 and pKM101 mutants on NCS-induced reversion of hisG46 allele*[a]

hisG46 (plasmid)	No. of His⁺ revertants per plate	
	Spontaneous	With NCS
hisG46	7	5
hisG46 (pKM101)	34	266
hisG46 (pGW21)	10	3
hisG46 (pGW16)	276	501

[a] Bacteria were grown overnight in LB and suspended after centrifugation at twice the concentration in M9 buffer. 0.1 ml of each culture were then spread on plates containing minimal medium in the presence of 12 μg of NCS.

Thus, the effect of the pKM101 mutations on NCS mutagenesis is analogous to the effects described by Walker for methyl methanesulfonate mutagenesis.

DISCUSSION

It was initially surprising to find that *E. coli* and *S. typhimurium* differed with respect to induction of mutations by NCS. The evidence presented here demonstrates that a function coded for by either the *umuC*⁺ allele or by plasmid pKM101 is required for detection of NCS-induced reversion of certain base pair substitutions and at least one class of frameshift mutation. The pKM101 function is presumably that supplied by the *muc* locus on pKM101 (Shanabruch and Walker, Mol. Gen. Genet., in press; P. Langer, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1980). The only exception to pKM101 dependency was the behavior of *hisC117* in the genetic background of *S. typhimurium* strain LT7, which is known to harbor a colicinogenic factor (K. Mortelmans, personal communication). Colicinogenic factors such as colI have been shown to enhance both mutagenesis and survival after UV irradiation (5, 6). It is possible, then, that the mutability of the *hisC117* allele in LT7 is attributable to the presence of a plasmid since this allele was not mutated by NCS in the LT2 background except in the presence of pKM101.

The dependency of NCS mutagenesis on genes whose functions affect mutation response in cells treated with DNA-damaging agents implies that the NCS-induced lesions in DNA (and perhaps other lesions created by other agents) are not mutagenic per se, but rather are substrates for processes that generate mutational events at the site of lesions. The molecular nature of the *hisG46* mutation has been determined recently (W. Barnes, personal communication). The wild-type codon CTC (Leu at amino acid residue 69 [11] has been mutated to CCC

(Pro) in *hisG46*. Thus, reversion to the wild-type sequence at this site would involve a GC → AT transition. It would be of interest to know the molecular nature of the pKM101-dependent reversion events induced by NCS and other mutagenic agents.

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ADDENDUM

We have now demonstrated that chromophore extracted from NCS (1, 7), but not the apo-protein, is directly mutagenic for *S. typhimurium* carrying the *hisG46* mutation and pKM101. Thus, the bacterial mutagenicity of NCS is entirely attributable to the relatively low-molecular-weight chromophore associated with it.

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