Base substitution mutations induced in the *cl* gene of lambda phage by neocarzinostatin chromophore: correlation with depyrimidination hotspots at the sequence AGC

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ABSTRACT

Treatment of intact lambda phage with the nonprotein chromophore of neocarzinostatin resulted in efficient phage inactivation and generation of clear-plaque mutants. Both effects required a preincubation at low pH to allow diffusion of chromophore into the phage head. Chromophore activation was then effected by addition of a sulfhydryl cofactor, followed by a shift to neutral pH. Sequence analysis of mutations mapped to the DNA-binding region of the *cl* gene revealed that nearly all were single base substitutions. Significant numbers of all possible base changes were found, with A:T to G:C transitions being the most frequent events. Of 11 G:C to A:T transitions, 7 were found at C residues in the trinucleotide sequence AGC, which has previously been shown to be a hotspot for chromophore-induced depyrimidination. This result, as well as the SOS dependence of mutagenesis and the overall distribution of various types of base substitutions, is consistent with the hypothesis that apurinic/apyrimidinic sites are important mutagenic lesions.

INTRODUCTION

Neocarzinostatin (NCS) is an antitumor antibiotic (1) and a mutagen of unique specificity (2,3). Although the predominant lesions induced in DNA by NCS are strand breaks occurring at T and A residues (4,5), a high proportion of NCS-induced nonsense mutations in the *lacI* gene of *E. coli* were found to occur at G:C base pairs (3). These results suggest that either in vitro studies of DNA damage do not reflect the damage occurring in the cell, or that mutagenesis is due primarily to some relatively minor NCS-induced lesion which occurs with significant frequency at G or C residues. In order to examine more closely the relation between NCS-induced DNA damage and mutagenesis we have developed a procedure for mutagenesis of intact lambda phage and have determined the forward mutational spectrum of NCS chromophore in the *cl* gene, using methodology developed by Hutchinson and coworkers (6).

MATERIALS AND METHODS

Phage Mutagenesis. The phage lambda cl857 ind1 Oam amp (7) was maintained as a lysogen in A15 sup E44 cells. Following heat induction of an exponentially growing culture, phage were isolated by precipitation with polyethylene glycol, followed by banding in CsCl gradients (8). For treatments with NCS chromophore, the phage stock was diluted to a titer of 10^9 /ml, in 10 mM sodium acetate - 10 mM magnesium chloride, pH 4. NCS chromophore, prepared as described previously (9), was diluted to 10 μ M in the same buffer immediately before use, and added to the phage suspension. This mixture (0.38 ml) was kept on ice for 1 hr. Ten microliters of concentrated sulphhydryl were added, and the mixture was kept on ice for 30 min. Ten microliters of 2.5 M Tris-HCl were added and the mixture was incubated at 22°C for 10 min and then at 37°C for 20 min. Suitable dilutions were immediately plated on freshly prepared E. coli A15 cells. All operations prior to phage dilution were performed under dim light to avoid chromophore photolysis.

For preparation of host cells, a culture of A15 sup E44 was grown in lambdaT broth (containing 10 g/l Bactotryptone, 5 g/l NaCl, 2.5 g/l MgSO_4 , 2 g/l maltose, 1 g/l glucose and 50 mM Tris-HCl, pH 7.5) to an OD_{600} of 0.3. The cells were pelleted, and then washed and resuspended in 6 mM Tris-HCl - 10 mM MgSO_4 , pH 7.2 at an OD_{600} of 2. In some cases, 1.5-ml aliquots of cell suspension were spread on a 100-mm diameter tissue culture dish and irradiated with light from an inverted 254-nm transilluminator at a dose rate of 3.3 J/m²-sec. For plating, 0.13 ml of diluted phage was added to 0.13 ml of cell suspension and incubated at 37°C for 20 min. 0.2 ml of this mixture was added to 3 ml of soft agar at 47°C, and spread on a lambda plate (10). Plaque-forming units and the fraction of clear-plaque mutants were determined after 40 hr at 30°C.

Sequencing of Mutants. Chromophore-induced clear-plaque mutants were plaque-purified twice and tested for a cl⁻ genotype by a complementation assay (11). cl⁻ mutants were subjected to deletion mapping (6) using the phage KH54 Nam and KH70 Nam. Mutants mapping to the first third of the cl gene were sequenced by forced cloning (12) into M13, a method adapted to cl mutants by K. Tindall and F. Hutchinson (unpublished). Briefly, DNA from the cl mutant phage was digested with EcoRI, HindIII and XmnI, and the single fragment with EcoRI and HindIII ends was selectively ligated into EcoRI/HindIII-cut M13mp18. The resulting recombinant phage, containing bases 1 to 352 of the coding strand of the mutant cl gene, was grown on

Figure 1. Inactivation of lambda phage by NCS chromophore. A. Phage were treated with chromophore in the presence of 1 mM glutathione (Δ), 10 mM 2-mercaptoethanol ($\blacksquare$) or no sulfhydryl ($\bullet$) and plated on cells irradiated with 20 J/m² of ultraviolet light. B. Phage were treated with chromophore in the presence of glutathione and plated on cells irradiated with 0 ($\blacktriangle$) or 20 (Δ) J/m² of ultraviolet light.

JM103 host cells, and viral DNA was prepared. The cI insert was sequenced by the dideoxy method (13,14), using [³⁵S](α -thio)dATP and a primer consisting of bases 264 through 250 of the noncoding strand of cI . A few sequences with mutations between bases 220 and 243 were determined using a universal M13 primer (17-mer, New England Biolabs).

RESULTS

Effects of NCS Chromophore on Intact Phage. In order to effect damage to DNA in intact phage, chromophore was preincubated with phage at pH 4 to allow diffusion of chromophore into the phage head. Following addition of a sulfhydryl cofactor, chromophore activation was initiated by a shift to neutral pH (15). A final incubation at 37°C was included to degrade any chromophore not activated by sulfhydryls.

With this protocol, phage inactivation was evident at less than 1 μM chromophore, and inactivation was strongly dependent on the presence of a sulfhydryl cofactor (Fig. 1A). Little or no inactivation occurred if the preincubation of phage and chromophore was omitted or if sulfhydryls were

Figure 2. Effect of SOS induction of host cells on chromophore-induced mutagenesis. Phage were treated with various concentrations of chromophore in the presence of glutathione and plated on cells irradiated with 0 (●) or 20 (Δ) J/m² of ultraviolet light. The fraction of clear-plaque mutants was determined and plotted as a function of the loss in plaque-forming ability. Each point represents a single determination and error bars represent the Poisson error in the plaque counts ($N^{1/2}$, where N is the number of clear plaques counted).

added after the incubation at neutral pH. Sulfhydryls alone likewise had no effect (not shown).

When phage were treated with chromophore plus glutathione and then plated on cells which had been ultraviolet-irradiated to induce SOS functions, efficient generation of clear-plaque mutants was observed. Because the concentration of chromophore required to produce a given level of phage survival and mutagenesis varied between experiments by as much as a factor of 2, it was difficult to obtain an accurate dose-response curve for mutagenesis. However, the relation between survival and mutagenesis was reproducible (Fig. 2); a maximum mutation frequency of 0.4% was obtained at survival levels of a few per cent or less. When treated phage were plated on unirradiated cells, the mutation frequency was at least an order of magnitude lower, although irradiation of cells had very little effect on phage inactivation (Fig. 1B). The failure of chromophore-treated phage to exhibit Weigle reactivation (16) suggests that phage inactivation may have been due to some nonrepairable lesion, such as a double-strand break. When 2-mercaptoethanol was used in place of glutathione, the maximum mutation frequency was likewise approximately 0.4%, although somewhat

higher chromophore concentrations were required to effect both phage inactivation (Fig. 1) and mutagenesis.

In order to assess the role of chromophore-DNA adducts in mutagenesis, similar experiments were performed under anoxia using dithiothreitol as a cofactor, conditions known to maximize adduct formation while minimizing strand breakage and base release (17, 18). Anoxia was found to decrease the slope of the survival curve by a factor of two, while decreasing the mutation frequency by a factor of approximately five (data not shown). These results suggest that lesions other than adducts are primarily responsible for mutagenesis.

Sequence Analysis of Chromophore-induced Mutants. All mutants chosen for sequencing were obtained in a single experiment, in which phage were treated with 0.17 μ M chromophore in the presence of 1 mM glutathione. Phage survival was 13% and the mutation frequency with irradiated cells was 0.23%. The same treated phage plated on unirradiated cells gave 0.01% clear-plaque mutants, while phage treated identically but with chromophore omitted gave 0.006% clear-plaque mutants. Thus, virtually all the mutants sequenced were chromophore-induced and SOS-dependent. Since treated phage were plated directly on SOS-induced lawn cells, each mutant must have been independently generated.

About half the mutants were unambiguously cl⁻. Of these, 60% formed turbid recombinants with KH70 Nam but not with KH54 Nam, suggesting a sequence change in the first 250 bp of the gene (6). Sequences of 58 such mutants were determined (Table 1). Nearly all were single-base substitutions, with approximately equal numbers of transitions and transversions. One frameshift, two double nontandem base substitutions and one base substitution with a nearby frameshift were also observed. The proportion of substitutions occurring at A:T base pairs was approximately equal to the A:T content of the target sequence (60%), indicating that A:T and G:C base pairs were equally mutable. For mutations at A:T base pairs, no consensus sequence was evident.

However, 10 of the 23 substitutions at G:C base pairs and 7 of the 11 G:C to A:T transitions (including one which was part of a double substitution) occurred at C residues in the trinucleotide sequence AGC. This specificity appears to be significant, since there are 51 C residues in the target sequence at which G:C to A:T transitions have been detected (using a variety of mutagens) and only 5 of these are in an AGC sequence (F. Hutchinson, personal communication). Furthermore, most of

Table 1. NCS Chromophore-induced Mutations in the cl Gene

Single-base Substitutions:		
Change	Occurrences	Positions in Genes
A:T to G:C	16	2,2,7,89,95,101,134,152,157,173,173,175,175,185,185,242
A:T to C:G	6	2,60,95,110,185,185
A:T to T:A	10	58,136,153,163,166,166,167,168,173,182
G:C to A:T	10	5,34*,34*,37*,46,92*,92*,148*,149,214
G:C to T:A	8	78,92*,92*,149,161,218,235,235
G:C to C:G	4	3,61,92*,214
Other:		
-1 frameshift at base 129-132		
A:T to G:C at base 25 plus G:C to A:T at base 36*		
A:T to C:G at base 11 and at base 76		
A:T to G:C at base 136 plus -1 frameshift at base 139-140		

* C residue in an AGC sequence.

§ For the complete cl sequence see ref. 19.

the available AGC sites were mutated by NCS; there are only two AGC sites not present in the NCS spectrum (bases 62 and 178) at which G:C to A:T transitions would have produced amino acid changes in lambda repressor, and neither of these amino acid changes has been shown to produce a clear-plaque phenotype.

Most of the NCS-induced base transitions have also been observed in studies of mutagenesis in the cl gene by other mutagens. However, 19 of the 26 different NCS-induced transversions have not been detected with any other agent (F. Hutchinson, personal communication).

DISCUSSION

In our studies with lambda phage, NCS chromophore was found to be a potent mutagen, with induced mutation frequencies comparable to those obtained with ultraviolet light (6). Mutagenesis is apparently due to DNA damage induced by activation of chromophore within the phage head by exogenous sulfhydryl cofactors (Fig. 1). In generating a collection of mutants for sequence analysis, glutathione was used as a cofactor, in order to most closely approximate conditions of activation within the cell.

For the most part the spectrum of forward mutations induced by NCS in the cl gene approximates the previously reported spectrum in the lacI gene (3), which also showed substantial numbers of all types of base

substitutions. Notably, however, the base substitution most frequently induced in cI was the A:T to G:C transition; the lacI system cannot detect such transitions, since they never generate amber or ochre codons. Also, the lacI spectrum showed a predominance of G:C to A:T transitions (even when normalized to the number of available sites), which was not evident in the cI spectrum.

Nevertheless, both spectra indicate that, on average, G:C base pairs are at least as mutable as A:T base pairs. In the cI gene, this is true even for G:C base pairs flanked on both sides by other G:C base pairs. However, both strand breaks (4, 5), which are the predominant NCS-induced DNA lesions, and covalent NCS chromophore-deoxyribose adducts (17, 18), which are minor lesions, have been found to occur almost exclusively at T and A residues. These results suggest that many of the NCS-induced base substitutions either are untargeted (or only regionally targeted) or are produced by other DNA lesions which occur with significant frequency at G or C residues.

Recently, we characterized another lesion formed by NCS chromophore in the presence of glutathione (20), which occurred at certain C residues, and which could be converted to a strand break by treatment with either alkali or endonuclease IV, an apurinic/apyrimidinic (AP) endonuclease from E. coli. These and other results suggested that this lesion was a modified apyrimidinic site, in which the cytosine base had been released and the deoxyribose sugar had been modified, probably by oxidation. These modified apyrimidinic sites, while cleaved by endonuclease IV, were not substrates for two other AP endonucleases of E. coli, endonuclease III and exonuclease III (endonuclease VI).

Studies with several DNA restriction fragments (20) indicated that NCS-induced apyrimidinic sites occurred most frequently at C residues in the sequence AGC. This specificity appears to be reflected in the cI mutational spectrum (Table I), the most striking feature being that 7 of 11 G:C to A:T transitions occurred at AGC sequences. Overall, AGC sites were eightfold more mutable than other G:C base pairs.

Kunkel (21) has determined the specificity of the SOS-dependent mutagenesis seen after transfection of bacteria with heat-depurinated single-stranded ϕ X174. The data were consistent with the proposal that nucleotides were inserted opposite apurinic sites during replication with the specificity A > T > G > C. Following this specificity, apyrimidinic sites at C residues would be expected to generate mutations with relative frequency

(G:C to A:T) > (G:C to T:A) > (G:C to C:G). This specificity is seen in the cl data for all mutations at G:C base pairs (Table I), and is more pronounced for those mutations occurring at AGC sequences. Thus, a consideration of all the cl data suggests that the modified apyrimidinic site is an important lesion in NCS-induced mutagenesis, at least at G:C base pairs. This hypothesis was also supported by studies of NCS-induced mutagenesis in the lacI gene, in that (i) the single AGC site at which G:C to A:T transitions are monitorable was the most prominent mutational hotspot in the gene and (ii) a correlation was found between the frequency of AP site formation in vitro and the frequency of G:C to A:T transitions in vivo, at individual C residues in the gene (3, 20).

At A:T base pairs, the implication of AP sites in mutagenesis is less clear. Because of the predominance of strand breaks, the frequency of NCS-induced AP sites at T and A residues is difficult to assess on sequencing gels, although at least some endonuclease IV-susceptible and alkali-labile sites have been detected (22). One study with supercoiled DNA indicated that there were nearly as many AP sites as strand breaks (23), far more than can be accounted for by the relatively infrequent modified apyrimidinic sites which we have observed at C residues. If AP sites, like strand breaks, occur mainly at T residues, they may frequently be mutationally silent, due to selective incorporation of A (the correct base) opposite the AP site (21, 24). Somewhat less frequent incorporation of G and T residues opposite these sites, combined with incorporation of A and G residues opposite less frequent AP sites at A residues, could generate the observed spectrum. The fact that AP sites at C residues more frequently result in mutations may compensate for the fact that they are formed less frequently than AP sites at T residues.

The apparently strong mutagenicity of the modified apyrimidinic site induced at C residues by NCS may be due in part to its resistance to repair AP endonucleases (20). The chemical structure of this NCS-induced lesion is unknown, but a bleomycin-induced AP site involving oxidation of C-4' to a ketone (25), and X-ray-induced AP sites involving oxidation of C-2' to an aldehyde or of C-1' to a carboxylic acid (26), have been described. The X-ray-induced sites are resistant to endonuclease III (27), and may be likely candidates for mutagenic lesions. However, the base substitutions generated in cl by treatment of phage with gamma rays are remarkable in that the new base pair is virtually always an A:T (K. Tindall, personal communication); this asymmetry is clearly not seen with NCS.

The single frameshift observed with NCS, occurring in a run of four identical bases, is typical of untargeted mutations (28) and may have been spontaneous in origin. Alternatively, it may have resulted from an intercalated NCS chromophore-DNA adduct (9, 17).

NCS is strongly mutagenic, and its specificity, particularly the high proportion of transversions, is different from that of other convenient mutagens. Thus, it could be a useful reagent for generation of base substitution mutations. Since NCS is specific for double-stranded DNA (4), *in vitro* mutagenesis could in principle be localized to a small double-stranded region of an otherwise single-stranded vector.

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