Use of a rapid DNA sequencing system to demonstrate the induction of frameshift mutations by bleomycin

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The rapid DNA sequencing system based on the single-stranded bacteriophage M13 and the chain-terminator method has been used to look directly for mutational alterations. A small DNA fragment that primes DNA synthesis through the N-terminal 200 base pairs of the β-galactosidase gene was prepared, and used to detect changes in base sequence among phages that give white plaques after treatment of the host cells with bleomycin. Bleomycin treatment of *E. coli* in which M13 mp2 was growing gave an increase in white plaque frequency. DNA sequence analysis of phage from 7 independent mutant plaques showed them all to have a frameshift mutation.

DNA sequencing M13 *Mutagenesis screen* *Bleomycin* *Frameshift* *Carcinogenesis*

1. INTRODUCTION

There is a high degree of correlation between the carcinogenicity and mutagenicity of many chemicals [1], and this provides the main support for the somatic mutation theory of carcinogenesis. A number of systems using bacterial [2] or mammalian cells [3,4] have been developed to measure the mutagenicity of chemicals in order to give an indication of their potential role as environmental or experimental carcinogens. A useful addition to such systems would be a system which allowed the nature of the mutational alteration to be shown directly by DNA sequence analysis. A prototype for such a system is described here, and is used to investigate the results of exposing growing *Escherichia coli* cells to bleomycin.

The single-stranded bacteriophage M13 does not kill its host, allowing one to induce mutations in it in vivo with ease simply by exposing the growing infected host bacteria to the chemical under investigation. M13 has been developed into a useful cloning vector by insertion of a portion of the lactose operon of *Escherichia coli* into the silent region [5], the production of a recognition site for the restriction endonuclease *Eco*RI near the N-terminal coding end of the *lacZ* gene [6] and subsequent insertion into the *Eco*RI site of DNA fragments carrying other restriction endonuclease recognition sites [7]. The mature virion of M13 contains a single circular single-stranded DNA molecule which is easily obtained pure. Thus M13 DNA, and any other DNA that has been cloned into it, is in a convenient form to act as the template in the primed DNA synthesis reaction that forms the basis of the chain terminator DNA sequencing method [8]. Therefore the changes in DNA sequence that mutations in a particular region represent can be investigated simply and quickly by chain terminator DNA sequencing if a convenient small DNA fragment is available to act as a primer in the DNA synthesis reaction in the region of interest. The other requirements for a direct DNA sequence analysis system for the effects of mutagens are that a screening or selection method for mutations is available and that the mutations do not affect the growth of the phage. In the prototype system described here we have used the N-terminal region of the β galactosidase gene in M13 mp2, which fulfills both criteria, as the target.

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Bleomycin [9] is a glycopeptide with antibiotic and antineoplastic activities, which has been used chemically for the treatment of human cancers [10]. Bleomycin induces single-strand and double-strand breaks in vitro [10,11], and this is believed to be related to its antitumour activity. Bleomycin itself induces chromosome breaks in vivo [10,12], and has both recombinogenic and mutatgenic activity [13–15]. Here, we show directly by DNA sequence analysis of mutations induced by bleomycin that this compound is capable of inducing frameshift mutations in target DNA molecules.

2. MATERIALS AND METHODS

2.1. *DNA sequencing*

Preparation of M13 mp2 DNA was carried out as in [16], except that phage were precipitated by the addition of polyethylene glycol [17]. Chain terminator sequencing reactions and analyses of the products were performed as in [8].

2.2. *Primer preparation*

M13 mp2 replicative form was prepared by a modification of the plasmid preparation method in [18], cells being harvested 6 h after infection with M13 mp2 at a multiplicity of infection of 10. Aliquots (50 μg) of M13 mp2 were digested with *Sau*3A (New England Biolabs) in 7 mM Tris–HCl (pH 7.4), 7 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, a 1 μg portion end-labelled by filling in with DNA polymerase I (Klenow fragment) [19] and the primer fragment purified on an 8% thin polyacrylamide gel [20] using end-labelled *Sau*3A digests of pBR322 as size markers. The primer fragment was cut out of gel, soaked out of the intact gel in 1 ml 0.15 M NaCl, 10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA for 24 h at 37°C, ethanol precipitated and used directly in primed synthesis reactions.

2.3. *Mutagenesis and identification of mutants*

Preparations of bleomycin that were currently in clinical use were kindly provided by Bristol Hellas (Athens). Bleomycin was dissolved in distilled water 2 mg/ml shortly before use. A fresh stock of M13 mp2 that was isolated from a single blue plaque on IPTG-X gal plates (see below) was used in these experiments; its DNA sequence was determined and found to agree with the sequence of this portion of the *E. coli lac* operon (A. Maxam et al., personal communication). 10^9 M13 mp2 bacteriophage particles (1 μl of a suspension at 10^{12}/ml) were mixed with 10^8 log-phase JM101 cells in 0.1 ml dYT medium (16 g Difco tryptone, 10 g yeast extract, 5 g NaCl/l). After 10 min at room temperature the bacteria were diluted with 5 ml warm (37°C) dYT medium and incubated with shaking for 5 min. Unadsorbed M13 mp2 phage were removed by washing 5 × with ice-cold TM buffer (20 mM Tris–HCl (pH 7.5), 85 mM NaCl, 0.001 M $MgSO_4$). The pellet from the last wash was taken up in 1 ml cold TM and divided into 2 × 0.5 ml aliquots. Bleomycin was added to one aliquot to give final conc. 10 μg/ml, a concentration which had been shown to induce an increase in mutation frequency in *Aspergillus nidulans* (N.D., C.S., in preparation). The other aliquot served as a control. After 30 min incubation at 37°C, the infective centres were spun down, washed 3× in TM buffer, resuspended in 2.5 ml dTY medium, and 0.1 ml aliquots of this distributed to sterile 1.5 ml conical microcentrifuge tubes containing 1 ml dYT at 37°C. After 1 h agitation at 37°C 2 drops of chloroform were added, and after 10 min the cell debris was removed by centrifugation. The phage titre in the supernatant was determined, and then phage were plated at 10^3 plaques/plate in 3 ml soft agar mixed with 0.3 ml an overnight culture of JM101, 20 μl 20 mg/ml IPTG (isopropyl-thio-β-D-galactoside) and 30 μl 20 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Sigma). The frequency of mutant phage in the supernatants with and without bleomycin treatment was determined by counting the number of white (*lacZ*$^-$ mutant) plaques among the total. Only one white plaque from any one subculture was analysed further.

3. RESULTS

In the DNA cloning system on M13 mp2, clones are identified because the insertion of foreign DNA at the *Eco*RI site leads to inactivation of the α-fragment of β-galactosidase. In host strains as JM101, β-galactosidase activity is produced by the association of the α-fragment from M13 mp2 and the ω-fragment from the *lac* operon in the F′ carried by the host bacteria. Thus in the presence of

the gratuitous inducer IPTG, β-galactosidase activity is produced in the plaques where the host bacteria are infected by M13 mp2, and if the colourless X-gal is present this compound will be split and the bacteriophage plaques will be blue. Inactivation of the portion of β-galactosidase carried by M13 mp2 by insertion of foreign DNA or by alterations in DNA sequence induced by mutagens will lead to the phage making colourless plaques, or light blue plaques if the mutation is leaky. Thus M13 mp2 has a built-in system for detecting mutations.

The frequency of white plaques among the M13 mp2 population after bleomycin treatment was 35 in 10^6 (in 2×10^5 plaques) whereas no white plaques occurred among 2×10^5 plaques from the untreated cultures. Therefore at least a 10-fold stimulation of the mutation rate in phage DNA molecules replicating within *E. coli* is produced by

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+      ATGACCATGATTACGAATTCACTGGCC
-      TACTGGTACTAATGCTTAAGTGACCGG

+   GTCGTTTTACAACGTCGTGACTGGGAAAAC
-   CAGCAAAATGTTGCAGCACTGACCCTTTTG

+   CCTGGCGTTACCCAACTTAATCGCCTTGCA
-   GGACCGCAATGGGTTGAATTAGCGGAACGT

+   GCACATCCCCCTTTCGCCAGCTGGCGTAAT
-   CGTGTAGGGGGAAAGCGGTGGACCGCATTA
            *

+   AGCGAAGAGGCCCGCACCGATC-92bp-GATC
-   TCGCTTCTCCGGGCGTGGCTAG <------
                              Sau3A primer
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Fig.1. DNA sequence of a portion of the *E. coli lac* operon insert in M13 mp2. The sequence shown is that of the N-terminus of β-galactosidase up to the 96 bp *Sau*3A primer which was used in these experiments. The template strand is the plus-strand, and in the sequencing reaction minus-strand DNA is synthesised from the left end of the *Sau*3A primer from right-to-left and bottom-to-top of this figure. Thus bands at the bottom of the autoradiograph in fig.2 correspond to oligonucleotides ending a short distance to the left of the primer. The position of the *Sau*A3 primer is shown by a heavy line ending in an arrow showing the direction of synthesis. The run of G–C basepairs to which an extra basepair is added in all 7 bleomycin-induced mutants that were investigated is boxed and labelled with a star.

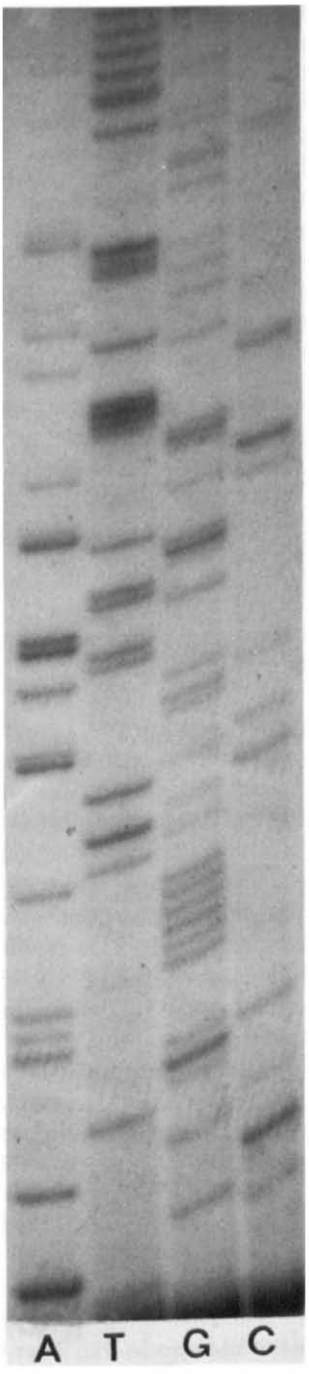

Fig.2. Autoradiograph of chain terminator DNA sequencing analysis of M13 mp2 b1, one of the bleomycin-induced β-galactosidase defective mutants. The position of the G run containing an extra base in the mutant sequence is indicated by a bracket and a star.

treatment with the available bleomycin preparation at 10 μg/ml. White plaques were isolated from among the progeny of 7 separate bleomycin-treated cell populations, and purified. Each mutant phage was grown from a well-isolated single-plaque and single-stranded DNA template for DNA sequence analyses was prepared from each of them as in section 2.

Fig.1 shows the location of the 96 basepair *Sau*3A DNA fragment that was used as a primer in the sequencing reactions. This was prepared as in section 2 from the double-stranded replicative form of M13 mp2. Each of the 7 mutant phage DNA templates gave the same DNA sequence which differed from the M13 mp2 sequence in having an extra G in the minus-strand sequence that was synthesised in a position corresponding to the run of 5 Cs in the the plus-strand from 104–108 inclusive in fig.1, which shows the DNA sequence of the portion of the β-galactosidase gene that was used. Fig.2 shows the position of the insertion mutation in an autoradiograph of a chain terminator sequencing experiment for one of the mutant DNAs, using the 96 bp *Sau*3A fragment as a primer.

4. DISCUSSION

The results clearly show that direct DNA sequence analysis of the base sequence alterations induced by mutagens is possible in this system. The results also show that bleomycin is capable of inducing frameshift mutations in a prokaryotic system. Bleomycin has previously been shown to act as a mutagen in the fungi *Saccharomyces cerevisiae* and *Aspergillus nidulans* (N.D., C.S., in preparation) [14,15]. Runs of the same base, and particularly G–C pairs, are usually hot-spots for frameshift mutations. One of the likely chemical modes of action of bleomycin on DNA is via intercalation of the bithiazole moiety [23–25]. All 7 independent frameshift mutations occurred at the same place, and comprised the insertion of an extra G–C basepair (in the RF) in or at the end of a run of G–C basepairs with all the Gs in the minus-strand and all the Cs in the plus-strand. This fits well with standard hypotheses [22] of the origin of frameshift mutations. The M13 mp2 system is, however, not ideal for the detection and study of mutagenic effects of chemical compounds because parts of the α-region of β-galactosidase can tolerate base sequence changes or even insertions and deletions that do not change the reading frame without loss of function sufficient to alter the colour phenotype of the plaque (B. Müller-Hill, personal communication). Frameshift mutations result in the total loss of information from a large stretch of the protein, and are therefore more likely to be detected than single base changes that lead to missense mutations, or even small deletions and insertions that maintain the reading frame. Nonsense mutations should be detectable in this system, and there are 9 sites in the region which we have studied where a single base-change would give rise to a nonsense mutation. Therefore, although we cannot conclude from these experiments that bleomycin only causes frameshift mutations, the induction of 7 frameshift but no nonsense mutations indicates that frameshift mutations are a major product of mutagenesis by bleomycin. This provides strong support for the hypothesis that at least part of the action of bleomycin on DNA is due to intercalation.

These experiments have also established the feasibility of direct DNA sequence screening of the products of mutagenesis. A further advantage of a system such as this is that it relies on the production of any kind of 'forward' (loss of function) mutation, rather than on the more usual reversion to wild-type of a single-site mutation, which must be differentially sensitive to different types of mutagen. We feel that this approach will be useful in the study of the action of mutagens, and potentially in screening environmental carcinogens for their mutagenic activity, and we are engaged in adapting the system to ensure that a wide range of mutational changes can be detected.

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