Pairing properties of bromouracil and repair of bromouracil-containing DNA

Possible utilization of bromodeoxyuridine triphosphate for site-directed mutagenesis

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5-Bromo-2'-deoxyuridine triphosphate (Br-dUTP) and dTTP are used interchangeably for DNA synthesis *in vitro* by the Klenow fragment of *Escherichia coli* DNA polymerase I. When DNA containing Br-dUMP instead of dTMP at a few preselected sites is transfected into competent bacteria, no mutation occurs, indicating that *in vivo* *E. coli* DNA polymerase always places a dAMP residue in front of any unrepaired Br-dUMP residue. On the other hand, *in vitro* Br-dUTP can also replace dCTP, but only with difficulty: when dCTP is absent, Br-dUMP can be forced in front of a dGMP residue, but the Klenow polymerase pauses before and after addition of Br-dUMP. Transfection into *E. coli* of the substituted DNA leads to the expected G → A transitions. These mutations can easily be targeted by using a suitable primer and the correctly chosen mix of deoxynucleoside triphosphates containing Br-dUTP. When Br-dUMP has been placed in front of a dGMP residue, the mutation yield is not 100 %, showing a partial repair of the transfected DNA before it is replicated. Advantage can be taken of this partial repair to prepare a set of different mutations within a target region in a single experiment.

INTRODUCTION

Krych *et al.* (1979) observed a higher cytotoxicity and mutagenicity of 5-bromo-2'-deoxyuridine in *Escherichia coli* deficient in various DNA-repair enzymes. They proposed that, after dehalogenation of the abnormal base incorporated into DNA, the residual uracil is released by the uracil-DNA glycosylase in a first step of base-excision repair. In the present paper we show that bromouracil-containing DNA can indeed be repaired in transfected bacteria; the repair mechanism is, however, not very efficient, since a non-negligible fraction of the bromouracil is still in the DNA when it is replicated.

On the other hand, it was thought for a long time that bromouracil could pair with either adenine or guanine, and that this ambiguity was the basis of the mutagenic action of bromodeoxyuridine (Trautner *et al.*, 1962). This point of view has been challenged: according to Pietrzykowska *et al.* (1985), the mutagenic activity of bromouracil is due to apyrimidinic sites appearing in the course of the repair of bromouracil-containing DNA. In the present paper we provide evidence that, in *E. coli*, bromouracil always pairs with adenine, which contradicts the Trautner *et al.* (1962) hypothesis.

If *in vivo* bromouracil always pairs with adenine, we show that *in vitro* it can be forced in front of a guanine; transfection of the bromouracil-containing duplex DNA into bacteria then leads to the expected G → A transitions. Limiting DNA synthesis to a small region by omitting one or several nucleoside triphosphates, 5-bromo-2'-deoxyuridine monophosphate (Br-dUMP) can be incorporated instead of dCMP at specific locations. The method can thus be used for site-directed mutagenesis. Because of the partial repair of bromouracil-containing DNA, the transition yield is below 100 %; but this can be

used as an advantage, since it permits the preparation, in a single experiment, of several different mutants within the target region. In the present work we used the cDNA coding for human growth hormone (hGH) and the mutations were placed in the segment coding for the amino acid residues that are deleted in the 20 kDa variant of the hormone.

MATERIALS AND METHODS

Plasmids, bacteriophages and bacteria

Plasmid p-hGH-24 was a gift from Corine Lecomte of our laboratory. This recombinant plasmid contains a nearly full-length hGH cDNA (Lecomte *et al.*, 1987) inserted in the *EcoRI* site of plasmid pBR-322.

E. coli JM-103 and bacteriophage M13-mp10 were purchased from B.R.L. and Amersham respectively. Single-stranded and RF bacteriophage DNA were obtained as described by Messing (1983).

Enzymes and chemicals

E. coli DNA polymerase I large fragment (Klenow polymerase) and T4 DNA ligase were purchased from Boehringer, S1 nuclease was from P.L. Biochemicals and restriction enzymes were from Amersham, Boehringer and New England Biolabs.

H.p.l.c.-purified 2'-deoxynucleoside 5'-triphosphates (dNTPs) and 2',3'-dideoxynucleosides 5'-triphosphates (ddNTPs) were purchased from P.L. Biochemicals, except 5-bromo-2'-deoxyuridine triphosphate (Br-dUTP), which was from Boehringer. The synthetic oligonucleotide sequencing primer was from Amersham, as were [α -³²P]dATP (400 Ci/mmol) and [α -³²P]dGTP (400 Ci/mmol).

Abbreviation used: hGH, human growth hormone (somatotropin).

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Fig. 1. Insertion of the (+) or (-) strand of hGH cDNA in M13-mp10 circular single-stranded DNA

p-hGH-24 is digested with *EcoRI* and *FnuDII*; the 660 bp fragment containing the boxed sequence is ligated into M13-mp10 RF DNA opened with *EcoRI* and *SmaI*. Bacteriophages are obtained from transfected bacteria and their single-stranded circular DNA, M13-mp10-hGH(+), is prepared. p-hGH-24 is digested with *EcoRI* and *RsaI*; the 440 bp fragment containing the boxed sequence is ligated into M13-mp10 RF DNA opened with *EcoRI* and *HincII*. Bacteriophages obtained from transfected bacteria are used to prepare M13-mp10-hGH(-) circular single-stranded DNA. The boxed sequence codes for the amino acid residues absent from the hGH 20 kDa variant.

Limited DNA synthesis

Recombinant M13-mp10-hGH circular single-stranded DNA (template; 25 fmol/ μ l), hGH double-stranded DNA restriction fragment (to generate primer; 250 fmol/ μ l), appropriate non-labelled dNTPs (1 mM each) and [α - 32 P]dATP or [α - 32 P]dGTP (0.5 μ Ci/ μ l) in 10–50 μ l of 63 mM-Tris/HCl buffer, pH 8.0, containing 6.3 mM-MgCl₂ and 6.3 mM-2-mercaptoethanol were heated for 5 min at 100 °C, then incubated for 30 min at 60 °C to allow hybridization. The Klenow polymerase (0.1 unit/ μ l final concentration) was then added and the reaction was carried out for 15 min at 11 °C. After addition of EDTA up to 5 mM, a small portion was used for analysis on 5% sequencing gel; the rest was phenol-extracted and ethanol-precipitated. In order to ensure perfect removal of the dNTPs, where indicated, an additional gel filtration on Sephadex G-50 was performed before precipitation.

Formation of complete closed duplexes and transfection in *E. coli*

The mixture containing the partial duplex DNA (400 fmol) in 13 mM-Tris/HCl buffer, pH 7.5, containing 20 mM-NaCl, 9 mM-magnesium acetate, 20 mM-2-mercaptoethanol, 0.5 mM of each of the four dNTPs, 1 mM-ATP, 2.5 units of Klenow polymerase and 1 unit of T4 DNA ligase was incubated overnight at 14 °C to produce complete circular closed duplexes. The reaction was stopped by the addition of EDTA up to 5 mM, the solution was extracted with phenol and the DNA was precipitated with ethanol.

The DNA was redissolved in 30 mM-sodium acetate/acetic acid buffer, pH 4.4, containing 280 mM-NaCl, 4.5 mM-ZnCl₂ and 0.1 mM-EDTA; after addition of S1

nuclease (1.7 units/ μ l), the mixture was incubated at 37 °C for 30 min in order to linearize any incomplete reaction product. The reaction was stopped by addition of 100 mM-Tris to reach pH 8. The remaining double-stranded closed circular DNA molecules were transfected into *E. coli* JM-103 (Messing, 1983).

RESULTS

Preparation of the template strands

p-hGH-24 DNA was completely digested by *EcoRI* and *FnuDII* restriction endonucleases, and the 660 bp fragment was purified by agarose-gel electrophoresis. The M13-mp10 RF DNA was completely digested by *EcoRI* and *SmaI* endonucleases and ligated to the *EcoRI*/*FnuDII* 660 bp fragment from p-hGH-24. The recombinant DNA was transfected into *E. coli* to grow bacteriophage virions. The single-stranded DNA of this recombinant phage, M13-mp10-hGH(+), contained the strand complementary to the mRNA coding for hGH (Fig. 1).

p-hGH-24 was also digested completely by *EcoRI* and *RsaI* endonucleases, and the 440 bp fragment was purified by agarose-gel electrophoresis. The M13-mp10 RF DNA was completely digested by *EcoRI* and *HincII* and ligated to the *EcoRI*/*RsaI* 440 bp fragment from p-hGH-24. After transfection of this DNA, bacteriophage virions were obtained. The single-stranded DNA of this recombinant bacteriophage, M13-mp10-hGH(-), contained the strand homologous to the mRNA coding for hGH (Fig. 1).

M13-mp10-hGH(+) and M13-mp10-hGH(-) were checked by dideoxy sequencing by the method of Sanger

Fig. 2. Part of nucleotide sequence of hGH cDNA containing the codons for the amino acid residues absent from the 20 kDa variant of the hormone

The broken box contains the 45 nucleotide pairs coding for the missing 15 amino acid residues of the hGH 20 kDa variant. The restriction sites used to prepare the primers are indicated. The (-) strand (top arrow) of the *PvuII*/*BstNI* fragment is annealed to the (+) strand, or the (+) strand (lower arrow) of the *PstI*/*HinfI* fragment to the (-) strand of the hGH cDNA to synthesize DNA in the boxed region.

Fig. 3. One-step primer extension on M13-mp10-hGH(+) or M13-mp10-hGH(-) template

(a) *PvuII*/*BstNI* (-) strand is used as primer for the Klenow polymerase with different dNTP mixtures always containing [α - 32 P]dATP. (b) *PstI*/*HinfI* (+) strand is used as primer for the Klenow polymerase with different dNTP mixtures always containing [α - 32 P]dGTP. The products are analysed by electrophoresis on denaturing polyacrylamide gels that are subsequently autoradiographed. Lanes 1, dATP, dGTP and dTTP mixture; lanes 2, dATP, dGTP and Br-dUTP mixture. The sequence synthesized in the presence of Br-dUTP is presented; B indicates the incorporation of Br-dUMP.

et al. (1977). Both contained the region spanning from codon 58 to codon 72 of hGH (box in Fig. 1) that is deleted in the mRNA coding for the 20 kDa protein variant.

Preparation of the primers

As shown in Fig. 2, the *PvuII*/*BstNI* fragment of p-hGH-24 contains a strand of 19 nucleotide residues that, after hybridization with M13-mp10-hGH(+), is able to initiate the (-) strand synthesis in the region of interest (codons 58 to 72 of hGH).

Similarly, the *PstI*/*HinfI* fragment of p-hGH-24 contains a strand of 36 nucleotide residues that, after hybridization with M13-mp10-hGH(-), is able to prime the (+) strand synthesis in the region of interest.

Primer extension and mutant production

The *PvuII*/*BstNI* fragment was heat-denatured and the (-) strand was annealed to the single-stranded circular (+) template. In parallel, the *PstI*/*HinfI* fragment was heat-denatured and the (+) strand was annealed to the single-stranded circular (-) template. The partial duplexes were used as substrates for the Klenow polymerase with different mixtures of dNTPs always containing the first nucleotide to be incorporated as a radioactive precursor {[α - 32 P]dATP for the (+) template and [α - 32 P]dGTP for the (-) template}, the products of the reactions were analysed by electrophoresis on denaturing 5% polyacrylamide gels, and the labelled strands were detected by autoradiography.

On the (+) template, in the presence of dATP, dGTP

Fig. 4. Dideoxythymidine analysis of clones obtained after primer extension in the presence of Br-dUTP on M13-mp10-hGH(+) template

Sequencing reactions were performed with the universal primer. The gel shows the ddTTP pattern obtained in the region shown in Fig. 2. Lane 1 presents the analysis of M13-mp10-hGH(+) used as template in the reaction carried out *in vitro*. Lanes 2–25 correspond to clones obtained after primer extension in the presence of Br-dUTP (Fig. 3a, lane 2) and transfection into bacteria. Lane 10 is clearly anomalous; it is likely to be due to deletion of part of the hGH insert. Lane 8 (mutant 2-0431) shows two mutations.

and dTTP (Fig. 3a, lane 1), extension stopped after the addition of 15 nucleotide residues, just before the missing dCTP was needed. When Br-dUTP was substituted for dTTP (Fig. 3a, lane 2), synthesis proceeded further; however, some of the elongating molecules were interrupted every time Br-dUMP had to be placed in front of a dGMP, either before or after the addition of Br-dUMP, whereas no such interruption was seen when Br-dUMP had to be placed in front of a dAMP residue.

On the (–) template, in the presence of dATP, dGTP and dTTP (Fig. 3b, lane 1), synthesis stopped after the addition of ten nucleotide residues, again before dCTP was needed. When Br-dUTP was substituted for dTTP, synthesis proceeded further, up to at least 34 nucleotide residues, where a prominent band can be seen after the addition of Br-dUMP in front of a dGMP residue (Fig. 3b, lane 2). Again pauses occurred every time Br-dUMP had to be placed in front of dGMP, but not when it had to be placed in front of dAMP.

The interruptions are likely to be the consequence of two successive pauses of the DNA polymerase because Br-dUTP substitutes for dCTP only with difficulty; on the other hand, it seems that the enzyme uses Br-dUTP and dTTP without discrimination. Two controls, one without primer, the other using M13-mp10 without hGH insert, showed no incorporation of radioactivity (results not shown).

After elimination of the mixtures containing Br-dUTP by ethanol precipitation, the products of the reactions were converted into complete duplexes by using the Klenow polymerase and the four usual dNTPs: after sealing with T4 DNA ligase and nuclease S1 digestion,

the DNA was used to transfect bacteria for a plaque assay. Individual plaques were picked up and used to prepare single-stranded bacteriophage DNA. With the use of M13 universal primer, ddTTP reactions were performed (Fig. 4); 24 clones were examined in each case. From the synthesis on the (+) template, only one clone (mutant 2-0431) contained two G → A transitions in the hGH (+) strand, exactly at positions where BrU had been paired to G. From the synthesis on the (–) strand, one clone (3-0835) contained three G → A transitions in the hGH (–) strand exactly at positions where BrU had been paired to G; another one contained a C → T transition in the hGH (–) strand in a different location where no Br-dUMP could have been incorporated. The complete sequence of these mutants (2-0431 and 3-0835) in the region that was synthesized *in vitro* in the presence of Br-dUTP is compared in Fig. 5 with that of the corresponding wild-type (wt); the transitions are bracketed.

A two-step limited primer extension on M13-mp10-hGH(–) template, and mutant production

It is possible, with the same primer, to study Br-dUMP incorporation and mutagenesis in different regions of the target DNA. Limited extension is first allowed with a part of the normal nucleotides before use of the mixtures containing Br-dUTP.

M13-mp10-hGH(–) was annealed with the (+) strand of the *Pst*I/*Hinf*I fragment. In a first step, with the use of the Klenow polymerase with [α - 32 P]dGTP, dATP and dTTP, the expected ten-nucleotide extension was observed (Fig. 6, lane A). This first mixture of dNTPs

wt	(-) AGGAGTTTGAAGAAGCCTATATCCCAA
	(+) TCCTCAAACTTCTTCGATATAGGTTT
2-0431	(-) AGGAGTTTGAAGAAGCCTATATCTCAA
	(+) TCCTCAAACTTCTTCGATATAGGTTT
wt	(+) GGAATGAATACTTCTGTTCCCTTGGGATATAGGCTTCTTC
	(-) CCTTACTTATGAAGACAAGGAAACCTATATCCGAAGAAG
3-0835	(+) GGAATGAATACTTCTGTTCTTGGGATATAGGTTTCTTC
	(-) CCTTACTTATGAAGACAAGGAAACCTATATCCGAAGAAG

Fig. 5. Primer extension in the presence of Br-dUTP and mutant production

The sequences of mutants 2-0431 and 3-0835, in the regions synthesized *in vitro* in the presence of Br-dUTP, are compared with the corresponding regions of the wild-type (wt) recombinant. The transitions are bracketed.

Fig. 6. Two-step primer extension on M13-mp10-hGH(-) template

PstI/HinI (+) strand is used as primer for the Klenow polymerase. During the first step of elongation, [α - 32 P]-dGTP, dATP and dTTP are used (lane A). The second step is carried out with Br-dUTP alone (lane 1) or Br-dUTP and dGTP (lane 2) or Br-dUTP, dGTP and dTTP (lane 3). After each step the products are analysed by electrophoresis on denaturing polyacrylamide gels that are subsequently autoradiographed. The synthesized sequences are presented; when synthesis occurred in the presence of Br-dUTP a B indicates the incorporation of Br-dUMP.

was eliminated by filtration through a Sephadex G-50 column, followed by ethanol precipitation, and the DNA synthesis was continued with a different mixture. Addition of Br-dUTP alone allowed the incorporation of

wt	CTTCTGTTCCCTTGGGATATA GAAGACAAGGAAACCTATAT
3-1628	CTTTTGTTCCTTGGGATATA GAAACAAGGAAACCTATAT
3-1629	TTTGTTCCTTGGGATATA AAAACAAGGAAACCTATAT
3-1640	TTTGTTCCTTGGGATATA AAGACAAGGAAACCTATAT
3-1703	CTTTGTTCCTTGGGATATA GAAACAAGGAAACCTATAT
3-1712	CTTCTGTTCTTGGGATATA GAAGACAAGGAAACCTATAT
3-1830	CTTTGTTCCTTGGGATATA GAAACAAGGAAACCTATAT
3-1840	TTTGTTCCTTGGGATATA AAGACAAGGAAACCTATAT

Fig. 7. Two-step primer extension, the second in the presence of Br-dUTP, and mutant production

The sequences of the seven mutants obtained are compared to the wild-type (wt) recombinant in the region that was synthesized *in vitro* in the presence of Br-dUTP. The transitions are bracketed.

five additional nucleotide residues, three in front of dAMP residues and two in front of dGMP residues (Fig. 6, lane 1). Addition of Br-dUTP and dGTP (Fig. 6, lane 2) or Br-dUTP, dGTP and dTTP (Fig. 6, lane 3) yielded the same band patterns, confirming that Br-dUTP and dTTP are interchangeable. In the maximally extended primer, among the 16 additional nucleotide residues, four were Br-dUMP paired with dGMP residues.

The DNA types analysed in Fig. 6, lanes 1, 2 and 3, were converted into continuous duplexes and transfected into bacteria. Single-stranded bacteriophage DNA was isolated from individual clones (24 for each reaction product) and submitted to ddTTP sequencing. Of the 72 clones examined, seven were mutants (3-1628, 3-1629, 3-1640, 3-1703, 3-1712, 3-1820 and 3-1840) that contained ten transitions G $\rightarrow$ A in the hGH (-) strand at positions where BrU had been paired to G, and one was a transition T $\rightarrow$ C outside the region where Br-dUMP had been incorporated. The sequences of these mutants are compared in Fig. 7 with that of the wild-type (wt) in the region that was synthesized during the second step of elongation in the presence of Br-dUTP; the transitions are bracketed.

DISCUSSION

In this work, the pairing properties of bromouracil and its mutagenic potential have been studied in detail.

During DNA synthesis, bromouracil easily pairs with adenine; Klenow polymerase appears to use Br-dUTP and dTTP interchangeably. *In vitro*, in absence of dCTP, Br-dUMP can also be forced in front of dGMP in the template, but the enzyme pauses before and after placing the anomalous nucleotide, indicating that the formation

	33	34	35	36	37	38	39	40	41	42	43	44	45
wt	Ala	Tyr	Ile	Pro	Lys	Glu	Gln	Lys	Tyr	Ser	Phe	Leu	Gln
	GCC	TAT	ATC	CCA	AAG	GAA	CAG	AAG	TAT	TCA	TTC	CTG	CAG
2-0431	Ala	Tyr	Ile	<u>Ser</u>	Lys	Glu	Gln	Lys	Tyr	Ser	Phe	Leu	Gln
	GCT	TAT	ATC	TCA	AAG	GAA	CAG	AAG	TAT	TCA	TTC	CTG	CAG
3-0835	<u>Thr</u>	Tyr	Ile	Pro	Lys	Glu	Gln	Lys	Tyr	Ser	Phe	Leu	Gln
	ACC	TAT	ATC	CCA	AAA	GAA	CAA	AAG	TAT	TCA	TTC	CTG	CAG
3-1703	Ala	Tyr	Ile	Pro	Lys	<u>Lys</u>	Gln	Lys	Tyr	Ser	Phe	Leu	Gln
	GCC	TAT	ATC	CCA	AAG	AAA	CAA	AAG	TAT	TCA	TTC	CTG	CAG

Fig. 8. Mutations within the 45 bp region that is deleted from the cDNA for the 20 kDa variant of hGH

The sequence of nucleotides, in the target region, of mutants 2-0431, 3-0835 and 3-1703, and the corresponding amino acids, are compared with those of the wild-type (wt) hGH. Numbers refer to amino acid residues in mature hGH.

of the two phosphodiester bonds on either side of Br-dUMP is difficult.

In the experiments reported here many Br-dUMP residues have been placed *in vitro* in front of dAMP and dGMP residues in the template strand. However, after transfection of the DNA into competent bacteria and a few cycles of replication, the only observed mutations were G → A transitions in the strand corresponding to the template *in vitro*, and these transitions were located where Br-dUMP residues had been placed in front of dGMP residues. The absence of observed mutation when Br-dUMP had been placed *in vitro* in front of dAMP means that *in vivo* the DNA polymerase always places dAMP in front of Br-dUMP. This is in agreement with the published work of Pietrzykowska *et al.* (1985), who showed that the mutagenic action of bromouracil is not the result of pairing ambiguity but is due to apyrimidinic sites appearing transiently during the repair of DNA.

If it is indeed true that in *E. coli* BrU always pairs with A, it would seem that every Br-dUMP placed *in vitro* in front of dGMP should lead to a mutation. In the 120 examined clones that came from bacteria transfected with DNA synthesized *in vitro* with Br-dUTP replacing dCTP, only ten mutants were observed with G → A transitions in the strand corresponding to the template *in vitro*. A part of the non-mutated clones can derive from molecules whose elongation had been stopped before the addition of the first Br-dUMP in front of a dGMP residue. But this does not explain cases like the mutant 2-0431: the region used as template for the Klenow polymerase contained at least four dGMP residues in front of which Br-dUMP residues were placed; of these only two, the second and the fourth, led to mutations, whereas the first and the third were silent. The most obvious explanation is the replacement of Br-dUMP by dCMP *in vivo* before the transfected DNA could replicate.

Our results indicate that the repair of bromouracil-containing DNA is, however, not very efficient in *E. coli*, since we have observed the expected mutations; the limiting step of the repair is probably the dehalogenation (Krych *et al.*, 1979), since the uracil is very efficiently removed from DNA. We have indeed shown (M. Muller, J. Martial & W. G. Verly, unpublished work) that dUMP incorporated *in vitro* in front of dGMP does not lead to a mutation.

In this work Br-dUTP has been used for site-directed mutagenesis. It is as straightforward as the use of a synthetic oligonucleotide, and it does not need expensive equipment. The low yield of mutagenesis observed in our experiments has presumably two causes: interruption of DNA synthesis *in vitro* before insertion of the first Br-dUMP residue in front of a dGMP residue, and repair of the bromouracil-containing DNA *in vivo*. This second cause could be eliminated by transfecting the DNA synthesized *in vitro* into ung⁻ bacteria. Partial repair is, however, sometimes useful to obtain a set of different mutations within the target region from the same primer in a single experiment.

The mutations are located in the region that has been synthesized *in vitro* in the presence of Br-dUTP. By choosing the correct mixture of dNTPs it is possible to affect a large or a small region, and even a single nucleotide pair. With the same primer it is possible to change the target region by extending the primer in the presence of one, two or three usual precursors, in one or several steps, before incorporating Br-dUMP to mutagenize the chosen region. By inserting, in M13-mp10, the (+) or (-) DNA strand, any G·C pair can be substituted by an A·T pair.

In this work the target chosen for mutagenesis was the 45 bp of hGH cDNA containing codons 58–72 and corresponding to the missing amino acid residues of the hormone 20 kDa variant. All the mutants obtained here were fully sequenced by the dideoxy method of Sanger *et al.* (1977); as shown in Fig. 8, three of them are expected to lead to amino acid substitutions.

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