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BROMODEOXYURIDINE SUBSTITUTION IN MAMMALIAN DNA CAN BOTH STIMULATE AND INHIBIT RESTRICTION CLEAVAGE

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SUMMARY

Total replacement of thymidine by 5-bromodeoxyuridine in mammalian DNA causes a 5-fold stimulation in the rate of DNA cleavage by Mbo I. This is the first report of the stimulation of restriction endonuclease activity by 5-bromodeoxyuridine and is in contrast to the inhibition found with other restriction enzymes. We propose a hypothesis to rationalize these results.

INTRODUCTION

We have explored the effect of 5-bromodeoxyuridine (BrdU) incorporation into DNA on the rate of DNA cleavage by restriction enzymes. It has already been shown in bacteriophage DNA that BrdU is capable of inhibiting the rate of cleavage (1,2). Here, we present evidence in a mammalian DNA that BrdU not only can exert an inhibitory effect on the cleavage rate but also can exert a stimulatory effect. These opposite effects are strikingly apparent in the enzymes Hpa I and Mbo I, respectively.

When bacteriophage λ DNA is cleaved by a restriction endonuclease, the rate of cleavage varies from site to site (3). This variation suggests that the nuclease can interact with DNA outside the site of sequence-specific recognition. Presumably these outside interactions are less specific but can significantly influence cleavage rate at the specific site. We have attempted to focus on the interaction within the specific site by looking at a DNA in which there is an extremely large number of cleavage sites, so that the outside interactions average out to be roughly the same for different nucleases.

Abbreviations: BrdU = 5-bromodeoxyuridine; B DNA = DNA with 100% of the thymidine residues replaced by BrdU; T DNA = DNA with 0% replacement of the thymidine residues by BrdU.

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Then, in comparing one nuclease to another, we can observe the change in effect of BrdU only within the specific recognition site.

MATERIALS AND METHODS

Cell Line and Growth of Cells. The DNA is prepared from a Syrian hamster melanoma line grown in Dulbecco's modified Eagles's medium in the presence of 10% fetal calf serum, 0.1 mM hypoxanthine, 0.4 mM aminopterin and either 0.01 mM BrdU or 0.01 mM thymidine for over twenty-five generations. Under these conditions, in the cells grown in the presence of BrdU, over 99.8% of the thymidine residues in the DNA are replaced by BrdU (4).

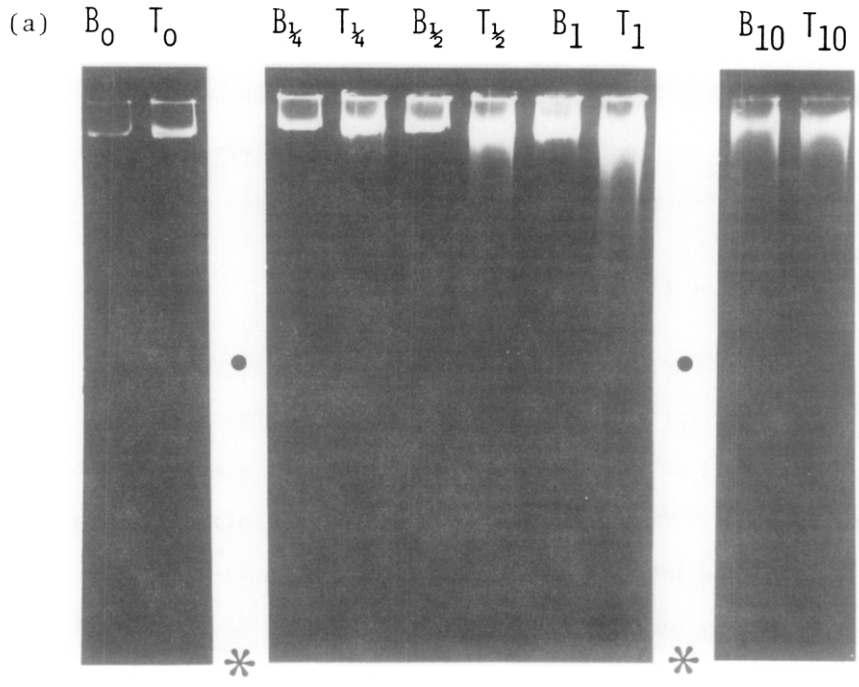
Isolation of DNA. The isolation of DNA is carried out in a manner designed to minimize degradation by endogenous nuclease (5). Equal numbers of cells are used to prepare DNA which contains BrdU or thymidine and the same DNA preparation is used to compare cleavage by both Hpa I and Mbo I. To avoid photodegradation, all manipulations with DNA are carried out under lighting conditions in which wavelengths below 550 nM are excluded. DNA concentration and purity are assayed by measuring absorbance at 260 and 280 nM (A_{260}/A_{280} = 1.81 and 1.69 for T and B DNA, respectively).

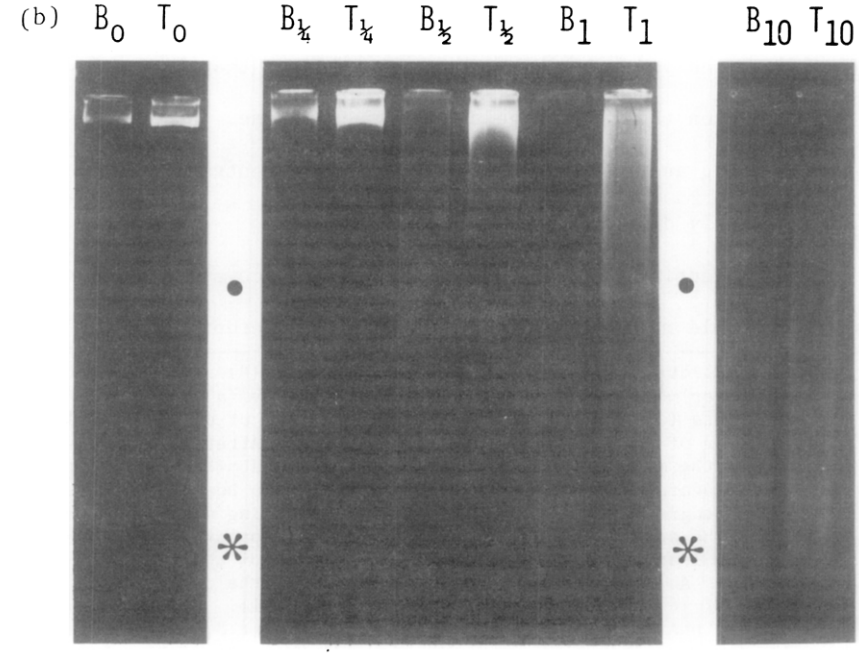
Enzyme Assays. The restriction nucleases Hpa I and Mbo I are obtained from New England Biolabs and assays performed under standard buffer conditions. After 1 hour of incubation at 37°C, the reaction is quenched by adding EDTA to a final concentration of 20 mM and the reaction mixture kept frozen until submitted to electrophoresis.

RESULTS AND DISCUSSION

The DNA chosen for the present study is obtained from a Syrian hamster melanoma cell line that allows virtually 100% of the thymidine residues in the DNA to be replaced by BrdU (4). As described in Materials and Methods, comparable samples of 100% substituted (B) and unsubstituted (T) DNA are isolated and incubated identically with each of the two enzymes, Hpa I and Mbo I. For each restriction enzyme, the degradation of DNA as a function of enzyme concentration is examined by gel electrophoresis and fluorescent staining (Fig. 1).

To measure the degradation, photographic negatives of the electrophoretic profiles are scanned by densitometry, along the direction of migration (from top to bottom). This provides a trace of fluorescence intensity vs. migration distance. The total intensity (I) is measured by integrating the total area under the trace, starting at the position of the sample well (near the top). The low molecular weight intensity (L) is measured by integrating only the area that appears below the leading (bottom) edge of the band of undegraded


(a)
B_0 T_0
$B_{1/4}$ $T_{1/4}$ $B_{1/2}$ $T_{1/2}$ B_1 T_1
B_{10} T_{10}


(b)
B_0 T_0
$B_{1/4}$ $T_{1/4}$ $B_{1/2}$ $T_{1/2}$ B_1 T_1
B_{10} T_{10}

DNA (B_0 or T_0). As seen in Table I, the values of I differ, even though the amount of DNA used in each case is the same (1 µg, based on UV absorption spectra). The I values indicate that the fluorescence efficiency of ethidium bromide is greater in T than in B DNA, and increases as the DNA distribution becomes broader with degradation. To minimize the error caused by the changes in I, the fraction degraded (f) is expressed in terms of L/I rather than L alone.

The value of f is determined as follows:

$$f = \frac{L/I - (L/I)_0}{1 - (L/I)_0}$$

where the subscript refers to 0 enzyme concentration.

An analysis of the f values is carried out by a simple graphical procedure (Fig. 2). This allows us to linearly extrapolate our data to obtain the ratio of the initial rates of cleavage, $\frac{k_B}{k_T}$. A 40-fold difference is found in the $\frac{k_B}{k_T}$ values for the two enzymes Hpa I and Mbo I. A value of $\frac{k_B}{k_T}$ is found to be 0.12 ± .02 for Hpa I and 5 ± 1 for Mbo I. We interpret this to mean that BrdU substitution causes an 8-fold inhibition in the initial rate of cleavage by Hpa I and a 5-fold stimulation in the initial rate of cleavage by Mbo I. At the present time we have no evidence that BrdU substitution has had any effect on the limit digests of either enzyme. As seen in Table I, the values of f_B and f_T at the highest enzyme concentration (10 units) are not significantly different.

Upon considering the palindromic sequences recognized by the enzymes, we see a possible reason for the dramatically different responses of Hpa I

Figure 1. Electrophoretic profiles of substituted (B) and unsubstituted (T) Syrian hamster melanoma DNA subjected to cleavage by (a) Hpa I, (b) Mbo I. The subscripts (0, ¼, ½, 1, 10) indicate the number of units of nuclease added to 1 µg of DNA in an assay volume of 20 µℓ. After incubation at 37°C for 1 hour, the mixture containing 1 µg of DNA is submitted to electrophoresis on 1.4% agarose (for 16 hours at 2 volts/cm in a horizontal gel apparatus) and photographed with ultraviolet light following ethidium bromide staining. The original uncleaved DNA (B_0 and T_0) appears as an intense band, with a sharp leading edge, a short distance away from the sample well (fainter band above). As cleavage proceeds (left to right) the degraded DNA appears as a smear of increasing intensity away from the well. The leading edge of the original DNA corresponds to fragments of at least 8,000 b.p., based on a comparison with restriction fragments of φX174 DNA. The dye markers, xylene cyanole and bromophenol blue, indicated by a dot (•) and asterisk (*), correspond to fragments of about 1,800 and 400 b.p., respectively.

Table I. Evaluation of the degradation of substituted (B) and unsubstituted (T) DNA at each enzyme concentration.

Enzyme Concentration (Units)	Total Intensity (I)		Low M. W. Intensity (L)		Fraction Degraded (f)*	
	B	T	B	T	B	T
(a) Hpa I						
0	2.90	4.80	0.10	0.15	0.000	0.000
1/8	3.00	5.45	0.15	0.75	0.016	0.110
1/4	3.70	7.60	0.30	2.00	0.048	0.240
1/2	6.15	12.50	1.00	6.85	0.132	0.534
1	9.90	19.35	3.20	12.95	0.299	0.659
5	11.65	18.70	8.05	13.30	0.680	0.702
10	12.45	18.70	9.20	13.85	0.730	0.732
(b) Mbo I						
0	2.50	4.40	0.15	0.25	0.000	0.000
1/16	2.70	4.35	0.55	0.40	0.153	0.037
1/8	3.95	4.40	1.30	0.55	0.286	0.072
1/4	5.00	6.95	2.95	1.85	0.564	0.222
1/2	9.40	10.10	7.95	5.35	0.836	0.501
1	10.60	18.50	9.90	16.00	0.930	0.857
10	13.25	18.75	13.00	18.35	0.980	0.977

$$*f = \frac{(L/I) - (L/I)_0}{1 - (L/I)_0}$$

and Mbo I. In the Hpa I sequence, $(\overset{\downarrow}{GTT}AAC / CAA\overset{\uparrow}{T}TG)$, the T's are as close as possible to the points of cleavage (arrows). In the Mbo I sequence, $(\overset{\downarrow}{}GATC / CTAG\overset{\uparrow}{})$, on the other hand, the T's are as far as possible from the points of cleavage.

We postulate that, when T is replaced by BrdU, there is a net inhibitory effect of BrdU when T is close to the point of catalytic cleavage and a net stimulatory effect when it is farther away from the point of cleavage. We

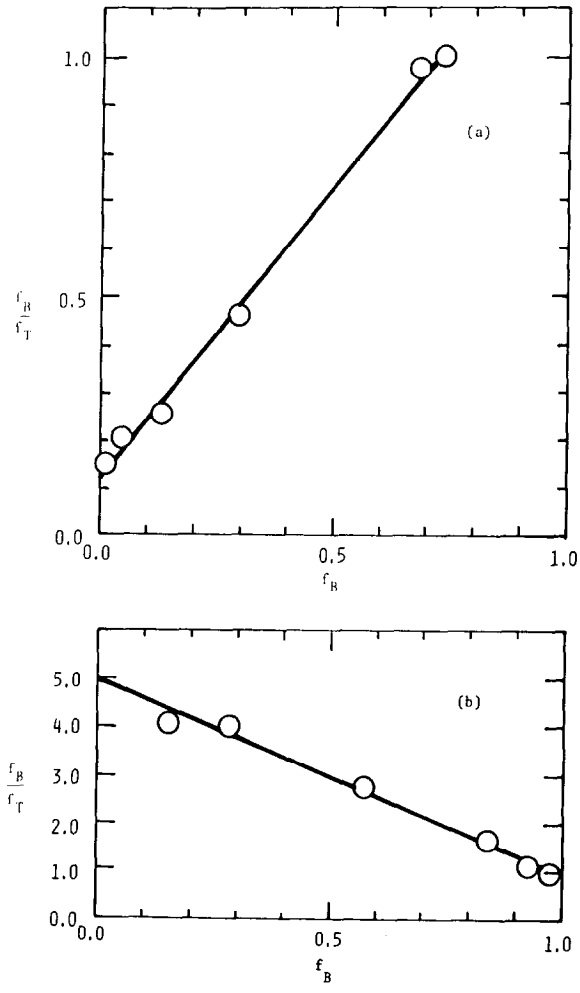


Figure 2. Graphical analysis of the relative amounts of degradation in substituted (B) and unsubstituted (T) Syrian hamster melanoma DNA as observed with (a) Hpa I, (b) Mbo I. For each enzyme concentration, the f values for B and T DNA are determined (Table I) and their ratio, f_B/f_T, is plotted against the value of f_B. The points are fitted by a straight line which is extrapolated to $f_B = 0$ to find the initial ratio of cleavage rates, k_B/k_T. By this procedure it is found that $k_B/k_T = 0.12 \pm 0.02$ for Hpa I and 5.0 ± 0.5 for Mbo I, using the data in Table I. In another experiment with different preparations of DNA (data not shown), k_B/k_T is found to be 0.12 ± 0.02 and 0.45 ± 1.0 for Hpa I and Mbo I, respectively (11).

suggest that the inhibitory effect is a consequence of the bromine atom (in BrdU) being larger than the methyl group (in T) and therefore tending to hold the nuclease away from the site of cleavage on the DNA. The stimulatory effect, on the other hand, may be a consequence of nuclease binding more tightly to bromine than to the replaced methyl group. Several examples of BrdU causing a tighter binding of protein to DNA are known (6-10).

If the hypothesis presented here is correct, we would expect to see a systematic variation in the effect of BrdU on restriction cleavage in terms of the number and placement of T's in relation to the points of cleavage. Data from studies on Hind III and Eco RI cleavage of λ DNA are consistent with this idea. Using an end labeling procedure to measure initial rates of cleavage, Berkner and Folk (2) have obtained data which show that $\frac{k_B}{k_T} = 0.58$ and 0.33 for Eco RI and Hind III, respectively. Combining these findings with ours, we see that the order of $\frac{k_B}{k_T}$ values is:

$$\text{Mbo I} > \text{Eco RI} > \text{Hind III} > \text{Hpa I}$$

This trend is explained by postulating that the inhibitory effect of T substitution is maximal when T lies within one base pair of the point of cleavage. Also, when T is located on the same side (α) as the cleavage point, the effect is expected to be greater than when T is on the opposite side (β). In the corresponding recognition sequences, the net effect of T substitution in base pairs adjacent to each cleavage point increases as follows:

$$\begin{array}{ccccccc} \overset{\downarrow}{\text{G}}\text{ATC} & < & \text{G}\overset{\downarrow}{\text{A}}\text{ATTC} & < & \text{A}\overset{\downarrow}{\text{A}}\text{GCTT} & < & \text{GTT}\overset{\downarrow}{\text{A}}\text{AC} \\ \text{CTAG}_{\uparrow} & & \text{CTTA}\underset{\uparrow}{\text{A}}\text{G} & & \text{TTCGA}\underset{\uparrow}{\text{A}} & & \text{CAA}\underset{\uparrow}{\text{T}}\text{TG} \\ (0) & & (1\beta) & & (1\beta + 1\beta) & & (1\alpha + 1\beta) \end{array}$$

Thus we see that inhibition should be minimal (0) for Mbo I but maximal ($1\alpha + 1\beta$) for Hpa I. Of course, the fact that Mbo I recognizes only 4 base pairs and cuts on the border of its recognition sequence should also be considered in attempting to explain the observed stimulation. Additional studies will be required to account for the magnitude of the observed result.

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