Short Technical Reports

Increasing Proportions of Uracil in DNA Substrates Increases Inhibition of Restriction Enzyme Digests

ABSTRACT

Techniques that rely upon the incorporation of uracil into DNA are being published with increasing frequency, especially in PCR protocols. We report here the efficiency of 18 type II restriction enzymes to digest PCR amplicons synthesized with varying proportions of TTP to dUTP in the PCR mixture. We find that most enzymes with A:T/U bp in their recognition site digest the amplicons less efficiently as the percentage of dUTP in the reaction mixture is increased. This effect is most dramatic when the proportion of dUTP in the nucleotide mixture exceeds 50%. All but one of the enzymes which fail to digest amplicons that are synthesized with 100% dUTP digest some amplicons which are synthesized with 90% dUTP.

INTRODUCTION

The incorporation of uracil into DNA is used in a variety of techniques, such as (*i*) replacement of TTP with dUTP in polymerase chain reaction (PCR) nucleotide mixtures so that PCR amplicons are destroyable by use of uracil DNA glycosylase (UDG; Reference 6), (*ii*) oligonucleotide labeling (3) and (*iii*) synthesis of single-stranded phagemids in *ung*⁻ bacterial strains for pre-transformation selection of plasmid libraries (7). We are replacing TTP with dUTP in our PCR nucleotide mixtures to allow a UDG sub-sampling strategy to measure and control carryover contamination by PCR products in our studies of ancient DNA (5). For this strategy to be effective, we must incorporate uracil into all amplicons that may contaminate our ancient DNA amplifications.

Although producing DNA containing uracil (U-DNA) is attractive for a variety of purposes, U-DNA does not always have the same characteristics as "normal" DNA. For example, Carmody and Vary (2) found that some amplicons containing uracil did not hybridize to Southern blots as well as normal amplicons, and Bebee et al. (1) found that only 6 out of 13 restriction enzymes cut the multiple cloning site (MCS) of pSport 1 synthesized with uracil. We would like to use large amplicons from some individuals for restriction digest studies (cf Reference 4). However, this may not be successful because some restriction enzymes do not cut U-DNAs or do not cut them efficiently (1). Additionally, we are developing techniques that require U-DNA amplicons to be efficiently digested, ligated and replicated by a variety of enzymes. It is, therefore, desirable to understand the characteristics of U-DNA amplicons in a variety of common laboratory manipulations (e.g., modification with enzymes; cf References 1 and 8). In this paper, we present results of our experiments on the ability of 18 restriction enzymes to digest U-DNA amplicons synthesized with differing ratios of TTP to dUTP.

MATERIALS AND METHODS

The MCS of pBluescript® II KS+ (Stratagene, La Jolla, CA, USA) was amplified from 100 pg of the phagemid linearized with *Xmn*I. The M13 -20 universal (GTAAAACGACGGCCAGT) and M13 reverse universal (AACAGCTATGACCATG) primers were used, yielding a 223-bp amplicon. Reactions contained 1.5 mM $MgCl_2$; 1× Thermo buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0 at 25°C], 0.1% Triton® X-100 [Promega, Madison, WI, USA]); 2 U *Taq* DNA Polymerase [derived from *Thermus aquaticus*]; and 150 µM for each dATP, dCTP, dGTP and TTP/dUTP. The TTP and dUTP were combined so that the total concentration was equimolar to the other deoxyribonucleoside triphosphates (dNTPs) (i.e., TTP + dUTP = 150 µM). Nine dNTP mixtures were used to synthesize MCS amplicons. The percentages of dUTP in the dNTP mixtures tested were 100%, 90%, 80%, 70%, 60%, 50%, 25%, 10% and 0%. For each dUTP percentage, two or more independent 100-µL amplifications (using independent dNTP mixtures, *Taq* aliquots and a DNA Thermal Cycler 480 [Perkin-Elmer, Norwalk, CT, USA]) were combined to obtain about 20 µg of MCS with the desired proportion of dUTP. Thermal cycling parameters were 94°C for 2 min—1 cycle; 94°C for 1 min, 46°C for 30 s, 72°C for 30 s—30 cycles; and 72°C for 2 min.

Table 1. Scale Used to Score Restriction Enzyme Cutting Efficiency

Rating	% MCS Cut
0	0
1	1–24
2	25–49
3	50–74
4	75–99
5	100

About 500 ng of amplicon from each dNTP mixture were digested with each of 18 type II restriction enzymes with 6-bp recognition sites. These restriction enzymes can be placed into three groups based upon the percentage of A:T/U base pairs within the restriction enzyme recognition sequence: 0% (no A:T/U bp; *Apa*I, *Bst*ZI, *Eco*O109I, *Not*I, *Sac*II and *Sma*I); 33% (2 A:T/U bp; *Acc*I, *Bam*HI, *Bst*XI, *Hin*cII, *Kpn*I and *Pst*I) and 67% (4 A:T/U bp; *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sst*I and *Xba*I). Restriction enzymes were obtained from the following manufacturers: New England Biolabs, Beverly, MA, USA (*Apa*I, *Eco*O109I, *Sma*I, *Acc*I, *Bam*HI, *Bst*XI, *Hin*cIII, *Pst*I, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I); Promega (*Bst*ZI and *Not*I); Life Technologies, Gaithersburg, MD, USA

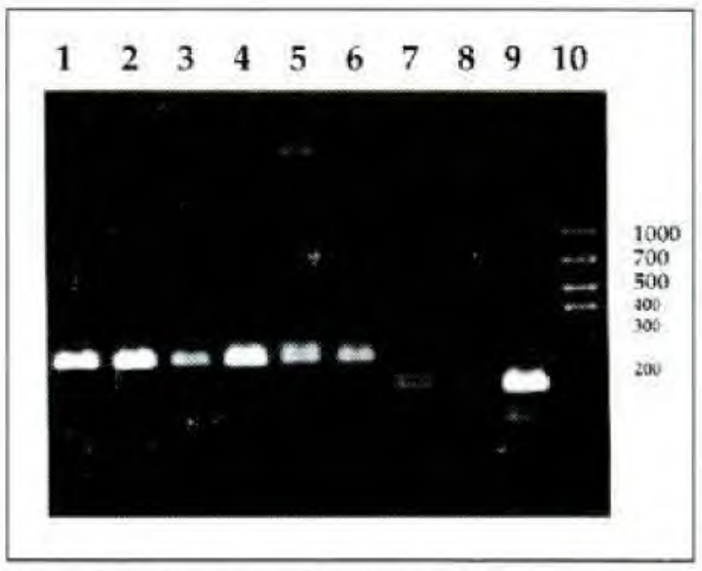

Figure 1. Results of restriction digests with *Xba*I (see text). Lane 1, 100% dUTP; lane 2, 90% dUTP; lane 3, 80% dUTP; lane 4, 70% dUTP; lane 5, 60% dUTP; lane 6, 50% dUTP; lane 7, 25% dUTP; lane 8, 10% dUTP; lane 9, 0% dUTP; and lane 10, Gel Marker I (Research Genetics, Huntsville, AL, USA).

(*Sst*I); Sigma Chemical, St. Louis MO, USA (*Kpn*I); and United States Biochemical, Cleveland, OH, USA (*Sac*II). To ensure a complete digestion of the 0% dUTP amplicons, a 20-fold excess of restriction enzyme (i.e., 10 units) was used. Manufacturers' recommended optimal reaction conditions were used in all experiments. Digestions were allowed to proceed for 2 h. Each 20-µL digestion was loaded onto a 2% agarose minigel containing ethidium bromide. Enzyme cutting efficiency was scored on a scale from 0 to 5 by visual inspection of the relative intensity of fluorescence of cut and uncut bands examined under UV light (Table 1).

RESULTS AND DISCUSSION

As expected, all enzymes cut the 0% U amplicon to completion. All enzymes with 0% A:T/U bp in the recognition site cut all amplicons completely (except *Bst*ZI, which failed to cut approximately 10% of the amplicons with 90% and 100% dUTP). Enzymes with 33% or 67% A:T/U bp in the recognition site varied greatly in their ability to cut amplicons with uracil (Figures 1, 2 and 3). Some enzymes, such as *Hin*cIII (33%) and *Xba*I (67%), were dramatically inhibited by increasing levels of dUTP. Others, such as *Bst*XI (33%) and *Cla*I (67%) showed little effect. Most 33% and 67% enzymes showed some inhibition when levels of dUTP were 50% or greater. The enzymes with 0% A:T/U bp in the recognition site cut much more efficiently than either the 33% or 67% enzymes. However, on average, most 67% enzymes were only slightly more sensitive to inhibition by uracil than the 33% enzymes.

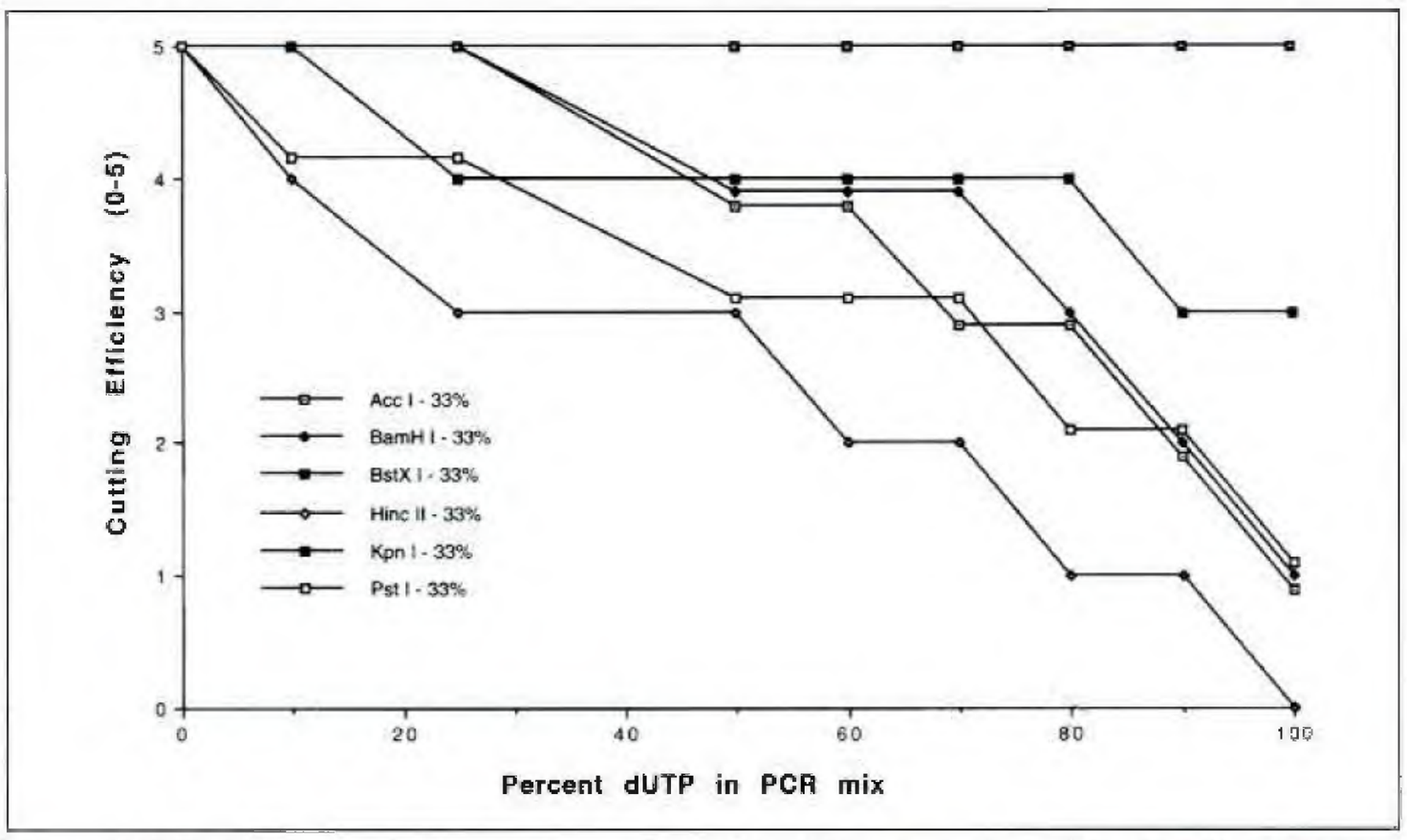

Figure 2. Effect of increasing proportions of uracil in DNA substrate on the digestion efficiency of restriction enzymes with recognition sites containing 33% A:T/U bp. Substrates are the MCS of pBluescript II KS+ amplified with varying proportions of dUTP/TTP in the PCR mixture (see text).

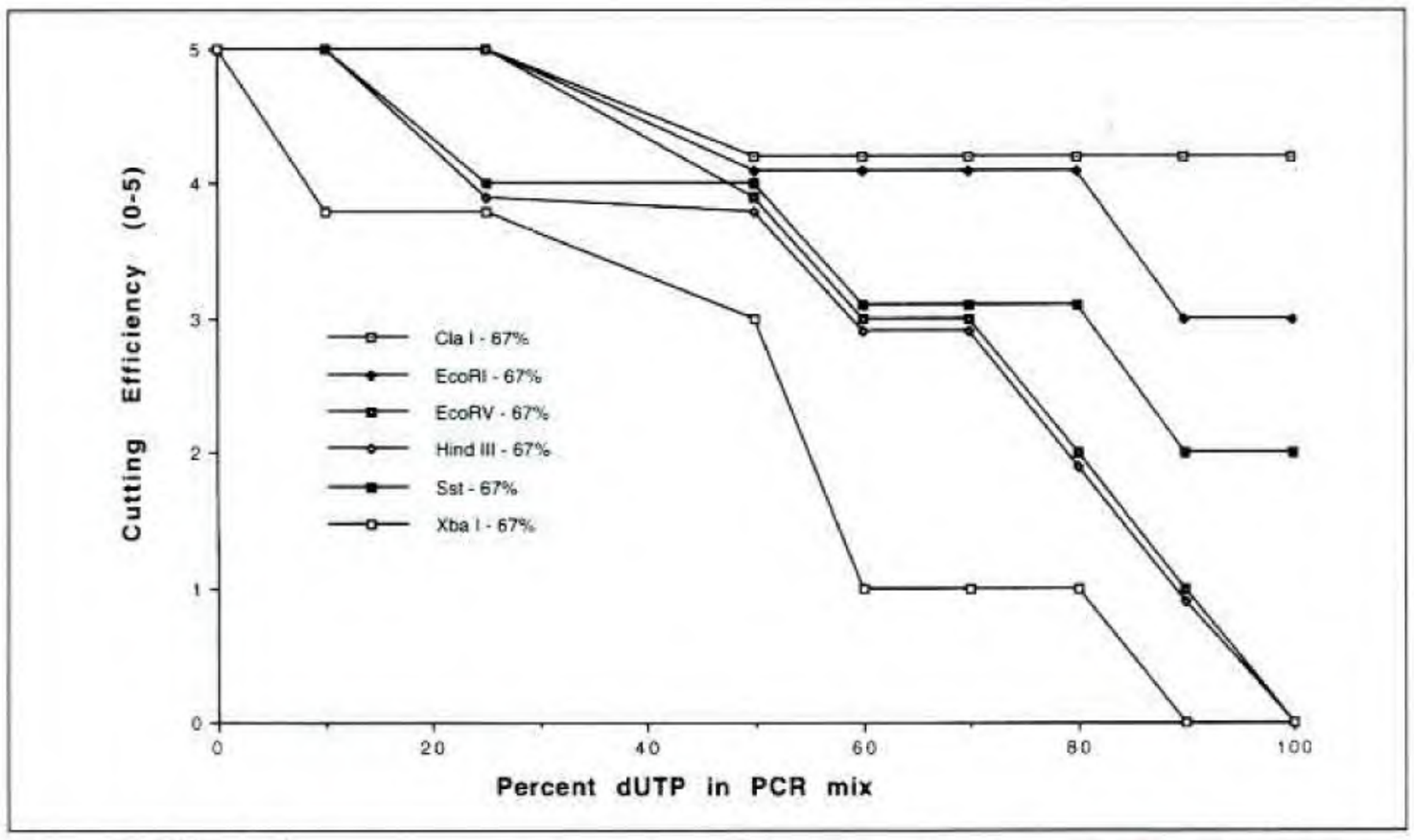

Figure 3. Effect of increasing proportions of uracil in DNA substrate on the digestion efficiency of restriction enzymes with recognition sites containing 67% A:T/U bp. Substrates are the MCS of pBluescript II KS+ amplified with varying proportions of dUTP/TTP in the PCR mixture (see text).

Two enzymes exhibited star activity when digesting U-DNA amplicons. *Cla*I completely cut the amplicons synthesized with 25%, 10% and 0% dUTP. Additionally, however, *Cla*I exhibited strong star and/or endonuclease activity for the amplicons synthesized with more than 25% dUTP. This effect was strongest for the 50%–80% dUTP amplicons. *Sst*I produced similar results, although the effect was not as drastic.

It is clear that different restriction enzymes are affected differently by varying levels of uracil in the substrate DNA. One would expect enzymes without A:T/U bp in their recognition site to be more or less unaffected by the incorporation of uracil into the amplicon, and this expectation is realized. One might also expect that enzymes with greater percentages of A:T/U bp in their recognition site would be affected more by incorporation of uracil than enzymes with a lower percentage of A:T/U bp in their recognition site. However, there is only a slight decrease in the extent of digestion for most enzymes with restriction sites containing 67% A:T/U sites instead of 33% A:T/U sites (Figures 2 and 3). Therefore, incorporation of uracil into enzyme recognition sites does affect the extent of digestion, but the relationship does not appear to be directly proportional.

The inhibitory effects of uracil-substituted recognition sites on restriction digestions could derive from several mechanisms. We considered two of these. First, uracil-substituted sites might be unrecognizable or uncleavable by restriction enzymes. Effectively, then, uracil-substituted sites are not substrates for the enzymes, and the observed inhibition by increasing uracil

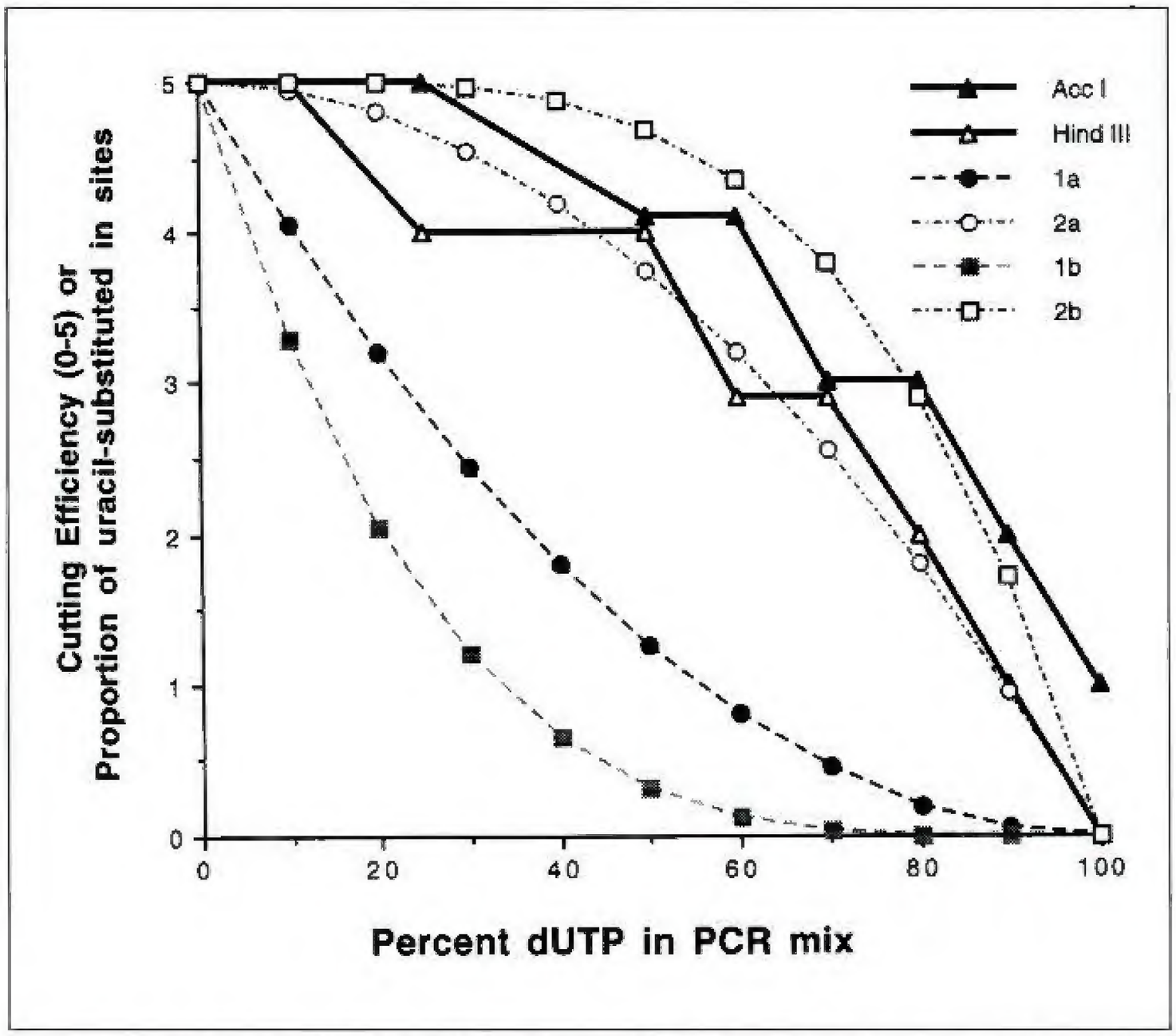

Figure 4. Comparison of the observed digestion profiles to the predicted proportion of amplicons susceptible to cleavage under two models. Curves 1a and 1b represent the proportion of cleavable amplicons in 33% A:T/U sites and 67% A:T/U sites, respectively, under the single-substitution model (see text). Curves 2a and 2b represent the proportion of cleavable amplicons in 33% A:T/U sites and 67% A:T/U sites, respectively, under the multiple-substitution model. *Acc*I and *Hin*dIII are representative plots for 33% A:T/U and 67% A:T/U sites, respectively (from Figures 2 and 3).

is tantamount to lowering the concentration of substrate in the reaction. Under this mechanism, neither increasing enzyme concentration nor increasing incubation time would result in increased extent of digestion of amplicons. Secondly, the enzymes might have reduced V_{max} or increased K_m for uracil-substituted sites. Either of these possibilities would reduce the reaction rate, but not the final extent of digestion. Under this mechanism, increasing enyzme concentration or incubation time would be expected to yield a proportional increase in the extent of digestion.

To test the inhibitory effects on one restriction enzyme, two 30-µL digestions were made for *Hin*dIII (4× and 60×) for each of the nine dUTP concentrations. Ten-microliter aliquots were removed from the digests after 1 h, 2 h and 16 h. Each 10-µL aliquot was visualized as above. The amount of amplicon digested increased slightly with time for both enzyme concentrations (data not shown). The amount of substrate DNA digested was also slightly higher in the 60× reaction than in the 4× reaction (data not shown). The increase in digestion, however, was not proportional to the increase in enzyme concentration or incubation time. The slight increases observed may have simply been due to more complete digestion of unsubstituted sites. Thus, the major inhibitory effect seems to be due to the enzyme's inability to digest the uracil-substituted sites.

To estimate the number of positions that must be substituted with uracil before inhibition is observed, we compared the observed plots with simple models of the relationship between inhibition and uracil incorporation. We assumed that the frequency of incorporation of uracil at all A:T/U positions is equal to its relative concentration in the nucleotide mixtures. Additionally, we considered only two extreme cases: 1) uracil substitution in a given site causes complete inhibition, and 2) all thymines must be replaced by uracil to completely inhibit digestion. Thus, we calculated the proportion of amplicons with restriction sites containing at least one uracil substitution (model 1) or all thymines substituted by uracils (model 2) at various dUTP/TTP ratios for both 33% A:T/U sites and 67% A:T/U sites. By subtracting that proportion from one (to determine the cleavable proportion of amplicons) and multiplying that value by five (to make a comparable scale with Figures 2 and 3), a direct comparision of the proportions of digestable amplicons predicted under each model with the proportions observed in this study may be made (Figure 4). The plots of observed restriction digestions are more similar to the curves expected under the multiple-substitution model than those expected under the single-substitution model (Figure 4). Therefore, we conclude that multiple uracil substitutions within a restriction site are necessary to inhibit most restriction enzymes.

Additional factors are probably involved in confounding the relationships observed. First, although the percentage of dUTP is known in all dNTP mixtures, the percentage of uracil actually incorporated into the amplicons is unknown (except for the 100% and 0% mixtures). Second, all amplicons from a PCR with both TTP and dUTP will not have a single amount of uracil incorporated into them. The PCRs will, instead, result in amplicons representing a distribution of uracil incorporation particular to those reaction conditions. For example, the PCR mixture with 50% dUTP and 50% TTP may yield restriction sites with 0%–100% uracil incorporation. Thus, amplicons cleaved by restriction enzymes may be only those amplicons with the lowest amount of dUTP incorporation in the restriction site. Third, we suspect some of the differences observed between enzymes (e.g., *Bam*HI and *Pst*I, Figure 2) are likely to be due to differences in the enzyme activity (i.e., 1 unit of *Bam*HI may not be equivalent to 1 unit of *Pst*I due to differences in manufacturers, age of the enzymes, etc.). Fourth, we tested 20× overdigestions of the amplicons because lower enyzme concentrations in the initial test sometimes gave erratic results (e.g., 70%

amplicons being more efficiently digested than 60% or 80% amplicons). Fifth, some enzymes were clearly capable of cutting 100% uracil-substituted amplicons but did not do so to completion under the conditions we tested (e.g., *Sst*I; Figure 2). Thus, we recognize that additional experiments utilizing more reaction conditions, synthetically prepared duplex oligonucleotides as substrates and better quantification techniques would be desirable to further explore inhibitory mechanisms. However, the results we present are of immediate practical use for molecular biology laboratory applications and provide a foundation for those additional experiments.

In conclusion, it is clear that using a dNTP mixture with a high ratio of dUTP to TTP rather than 100% dUTP has the advantage of producing amplicons that are more easily cut by most restriction enzymes but are still probably destroyable by use of UDG. Initial results from amplicons synthesized with 100%, 90%, 50%, 10% and 0% dUTP in the PCR nucleotide mixture indicate that the amplicons from dNTP mixtures with 90% dUTP are usually destroyable by standard UDG protocols, whereas amplicons with 50% or less dUTP are not. Thus, a high dUTP to TTP ratio may be extremely useful in some situations, but additional experiments to determine the characteristics of U-DNA amplicons in a variety of common laboratory manipulations are still needed.

REFERENCES

1. **Bebee, R.L., C.G. Thornton, J.L. Hartley and A. Rashtchian.** 1992. Contamination-free polymerase chain reaction: endonuclease cleavage and cloning of dU-PCR products. Focus *14*:53-56.
2. **Carmody, M.W. and C.P.H. Vary.** 1993. Inhibition of DNA hybridization following partial dUTP substitution. BioTechniques *15*:692-699.
3. **Craig, A.G., D. Nizetic and H. Lehrach.** 1989. Labeling oligonucleotide to high specific activity. Nucleic Acids Res. *17*:4605-4610.
4. **Cronin, M.A., W.J. Spearman, R.L. Wilmot, J.C. Patton and J.W. Bickham.** 1993. Mitochondrial DNA variation in chinook salmon (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Can. J. Fish Aquatic Sci. *50*:708-715.
5. **Glenn, T.C. and M.J. Braun.** 1992. Toward the elimination of contamination from ancient DNA amplifications: use of 2′–deoxyuridine–5′–triphosphate and uracil DNA glycosylase to eliminate carryover PCR products. Ancient DNA Newslett. *1(2)*:28-31.
6. **Longo, M.C., M.S. Berninger and J.L. Harley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene *93*:125-128.
7. **Ostrander, E.A., P.M. Jong, J. Rine and G. Duyk.** 1992. Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proc. Natl. Acad. Sci. USA *89*:3419-3423.
8. **Thornton, C.G., J.L. Hartley and A. Rashtchian.** 1992. Utilizing uracil DNA glycosylase to control carryover contamination in PCR: characterization of residual UDG activity following thermal cycling. BioTechniques *13*:180-184.

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