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Hae III—A Suitable Restriction Endonuclease for Restriction Fragment Length Polymorphism Analysis of Biological Evidence Samples

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ABSTRACT: Hae III has been selected by our laboratories as the restriction endonuclease of choice for restriction fragment length polymorphism analysis of forensic science samples. The enzyme is compatible with the D2S44 probe system and generates relatively small DNA fragments for that marker system. Similarly, Hae III is compatible with several other independent polymorphic loci, including D1S7, D4S139, D16S85, D17S74, D17S79, D14S13, and D20S15. Hae III is functional under a variety of adverse conditions for DNA digestion and is not affected by the methylation pattern in mammals. Finally, Hae III is a relatively inexpensive restriction endonuclease.

KEYWORDS: biology and pathology, deoxyribonucleic acid (DNA), enzymes, restriction fragment length polymorphism, restriction endonuclease, Hae III, D2S44 (YNH24), methylation, variable number of tandem repeats

Restriction endonucleases (RE) are enzymes that recognize specific sequences within deoxyribonucleic acid (DNA) molecules. Class II REs, in particular, are most useful for molecular biology and genetics since they cleave DNA at or near their particular recognition sequences. Shortly after the discovery of REs, it was noted that these enzymes could be used to detect DNA-based polymorphisms, and the term restriction fragment length polymorphism (RFLP) was introduced to describe polymorphisms resulting from the creation or elimination of an RE recognition site or variation of the distance separating two RE recognition sites [*1*]. The most polymorphic loci described to date are those which are based on a variable number of tandem repeats (VNTR) located between "fixed" RE recognition sites [*2*]. Because these RFLPs are not due to variation in RE recognition sites, they may be detected using any RE which cleaves outside of the tandem array.

Although any number of REs can be used to detect RFLPs associated with VNTR loci, several factors should be considered with respect to forensic applications. The RE

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should (1) cleave in the flanking sequences of several independent and highly polymorphic VNTR loci, generating restriction fragment lengths which are easily resolved by conventional agarose electrophoresis, (2) function faithfully under a wide range of conditions, (3) be unaffected by mammalian methylation patterns, and (4) be inexpensive. This technical note describes data supporting the use of the RE Hae III for RFLP analysis of forensic biological specimens.

Materials and Methods

Blood samples were drawn by fingerprick, placed onto washed cotton sheeting, and air-dried. Bloodstains/cm^2 in size were cut into small pieces and placed into 1.5-mL microfuge tubes. A 400-μL solution containing 0.01*M* tris, 0.01*M* disodium ethylenediaminetetraacetic acid (EDTA), 0.10*M* sodium chloride (NaCl), and 0.039*M* dithiothreitol was added to each tube. Subsequently, 10 μL of proteinase K (20 mg/mL) were added to each tube. The contents were mixed and then centrifuged in a microcentrifuge 235C (Fisher Scientific) for 2 s to force the cuttings into the solution. The extracts were incubated at 56°C overnight. The next day, a hole was punched in each microfuge cap and the cuttings were placed in the caps. The contents were centrifuged for 5 min. The cutting pieces and the microfuge tube caps were removed. New caps were placed on the microfuge tubes. Five hundred microlitres of phenol/chloroform/isoamyl alcohol (25:24:1) were added to each microfuge tube. The microfuge tubes were shaken vigorously and centrifuged for 2 min. The aqueous phase (top layer) was transferred to a new microfuge tube. One millilitre of cold absolute ethanol (4°C) was added to the aqueous layer. The tube was mixed by hand and placed at −20°C for 30 min. The microfuge tubes were allowed to warm to ambient temperature and then centrifuged for 15 min. The alcohol was removed by decantation. The pellets were washed with 1 mL of 70% ethanol and centrifuged for 5 min. The alcohol was removed by decantation and the microfuge tubes were placed in a Speedvac concentrator centrifuge (Savant) for 5 min to remove residual alcohol. The DNA pellet was resolubilized in a solution containing 0.01*M* tris and 0.0001*M* EDTA at 56°C for 2 h.

DNA was isolated from liquid blood samples as previously described [*3*]. RFLP analysis was performed as previously described [*3*]. The probe used for this study was YNH24 (for locus D2S44).[3]

Results and Discussion

In the past, several different REs, most notably Hinf I [*4*] and Pst I [*5*], have been used in forensic and paternity applications. Although substantial population allele frequency data exist for several loci typed with Pst I [*5*], in our opinion this, in itself, is not a sufficient criterion to adopt an existing RE system. A systematic evaluation of REs with respect to several features deemed desirable for use in forensic analysis was undertaken. As a result of this evaluation, we present data which support the use of Hae III for forensic applications.

Table 1 displays several REs and their respective restriction sites. Hae III recognizes the nucleotide sequence G-G-C-C and forms a blunt end cut between the G and C [*6*]. In contrast, Pst I recognizes a six-base restriction site. Since a specified array of six bases is less likely to occur than four bases along a sequence of DNA, Hae III recognition sites, in general, will occur more frequently than Pst I sites. Thus, the Hae III-generated DNA fragments will be smaller than Pst I-generated DNA fragments. For the D2S44

[3]The probe was kindly provided by Dr. R. White and his colleagues at the Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT.

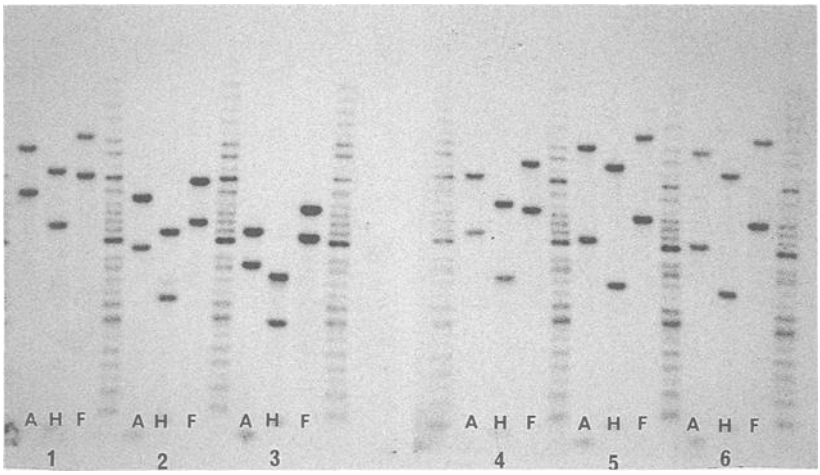

FIG. 1—*Autoradiograph displaying D2S44 RFLP profiles. DNA from each individual (1–6) was digested with Alu I (A), Hae III (H), and Hinf I (F), respectively.*

TABLE 1—*Restriction sites for several restriction endonucleases.*

Restriction Endonuclease	Restriction Site
Hae III	G-G-C-C
Alu I	A-G-C-T
Hpa II	C-C-G-G
Msp I	C-C-G-G
Hinf I	G-A-N-T-C
Pst I	C-T-G-C-A-G
Eco RI	G-A-A-T-T-C

system, Hae III-digested DNA yields RFLPs ranging from 700 base pairs (bp) to 7000 bp,[4] while Pst I-digested DNA produces fragments in the range of 6600 bp to 16 400 bp [5]. Because of the nature of electrophoresis, the smaller the DNA fragments, the more likely it is that small size differences in fragments can be resolved. This has the effect of increasing the discrimination power of a particular variable number of tandem repeats system. Thus, in our judgment, a four-base cutting RE is more desirable compared with REs recognizing larger restriction sites.

The RE should be compatible with the best probe system available to the laboratory. YNH24 (for locus D2S44) is common to many laboratories and is commercially available. It is extremely sensitive [*3,7*], highly polymorphic [*2,5,8*], and works well under a variety of protocols [*2,3,5*]. Hae III, Hinf I, and Alu I are compatible with D2S44. A comparison was made among these REs to determine which generated the smallest fragments for D2S44 RFLPs. Figure 1 demonstrates that Hae III yields the smallest fragments which are best resolved by electrophoresis. Table 2 gives the bp sizes of the different fragments. It is apparent that Hae III is a desirable RE for generating RFLPs at the D2S44 locus.

In addition, Hae III is similarly compatible with several other independent and highly

[4]Unpublished data.

TABLE 2—*D2S44 fragment lengths generated by digestion with Alu I, Hae III, and Hinf I.*[a]

Individual[b]	Fragment Size in Base Pairs		
	Alu I	Hae III	Hinf I
1	4394,3259 (11 mm)[c]	3824,2607 (14 mm)[c]	4906,3708 (9 mm)[c]
2	3165,2230 (12 mm)	2492,1576 (17 mm)	3591,2681 (10 mm)
3	2507,1983 (8 mm)	1838,1334 (11 mm)	2959,2419 (6 mm)
4	3729,2481 (15 mm)	3044,1824 (19 mm)	4124,2942 (11 mm)
5	4795,2411 (24 mm)	4121,1757 (31 mm)	5424,2874 (21 mm)
6	4705,2396 (24 mm)	4089.1726 (31 mm)	5136,2849 (21 mm)

[a]Values derived from Fig. 1.
[b]Each individual is a heterozygote. Therefore, two fragment sizes are provided for each restriction endonuclease digestion.
[c]The value in parentheses is the distance between two bands in a sample lane.

polymorphic VNTR loci. These include D1S7 [*9,19*], D4S139 [*11*], D14S13 [*12*], D16S85 [*13*], D17S79 [*5*], D17S74 [*14*], and D20S15 [*15*]. Thus, Hae III may be incorporated into systems which involve the simultaneous or sequential analysis of RFLPs associated with multiple independent VNTR loci.

Some REs are unable to cleave DNA when methylation of one of the bases in the restriction site has occurred. Additionally, methylation can take place in some tissues of an individual but not in other tissues [*16–22*]. Thus, methylation-sensitive REs potentially could generate different RFLP profiles for different tissues derived from the same individual. Because RFLP analysis of biological evidence materials often will consist of a comparison of DNA profiles derived from different tissues (for example, blood and semen), the RE used for cleavage of human DNA should be unaffected by recognition site methylation. In mammals, the most common methylation pattern is the methylation of a cytosine residue that is immediately followed by a guanine residue [*18–22*]. The methylation-sensitive dinucleotide CpG is not a component of the recognition site for Hae III (5′-GGCC-3′), and methylation of the 3′cytosine in the recognition site does not affect Hae III catalytic activity.

In contrast, Hinf I catalytic activity potentially can be affected adversely by methylation. If the 3′ cytosine in the Hinf I recognition site (5′-GANTC-3′) is methylated (this would occur most often when the Hinf I site is followed by a guanine residue), the activity of the enzyme toward such DNA could be substantially reduced [*23–26*]. This may make it difficult to achieve complete digestion with Hinf I (see following). Our investigations have found no differences in the Hae III-generated RFLP profiles obtained from blood, saliva, hair root sheaths, and semen or vaginal secretions derived from the same individual. An intense investigation is underway to evaluate DNA profiles from other sources, such as organs, bones, and muscle tissues.

Since the samples encountered at crime scenes are not of pristine quality, it was deemed necessary to use a RE whose activity remains unchanged over a wide range of conditions. Blakesley et al. [*27*] demonstrated, using phi X174 DNA, that under less than optimum conditions, such as temperatures as high as 70°C, ionic strengths varying from 25 to 150 mM, and a pH range of 6 to 9, Hae III cleaves at its recognition site with no observable difficulties. Our data support the findings of Blakesley et al. [*27*], but for human genomic DNA. Figure 2 shows that Hae III can properly digest human genomic DNA under low, medium, and high ionic strength conditions[5] at 56°C (digestion is routinely performed at

[5]The ionic strength buffers were supplied by Bethesda Research Laboratories, Gaithersburg, MD.

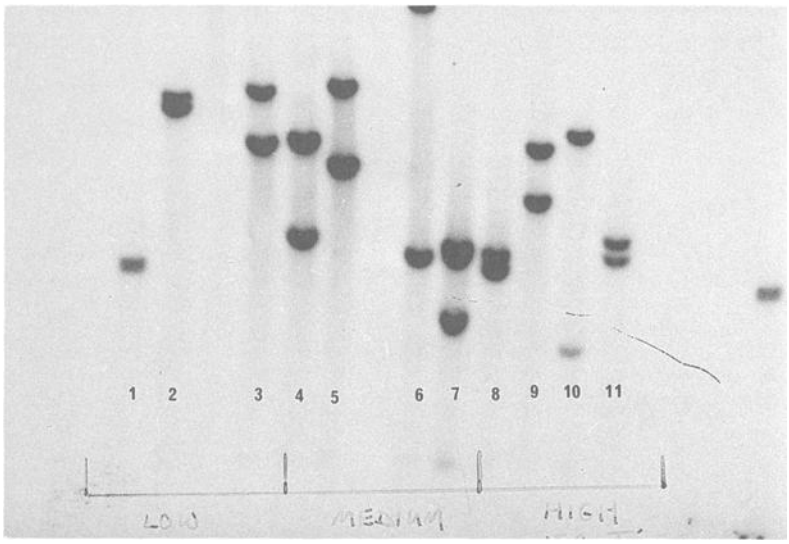

FIG. 2—*D2S44 RFLP profiles generated by digestion with Hae III. Digest carried out at 56°C. Specimens 1–3 were digested in a low-salt environment, Specimens 4–7 in a medium-salt environment, Specimens 8–11 in a high-salt environment.*

37°C). Thus, it should be anticipated that Hae III, compared with more fastidious REs, should be able to properly digest human genomic DNA that contains cellular and environmental contaminants.

Figure 3 demonstrates the ability of Hae III to digest genomic DNAs of varying degrees

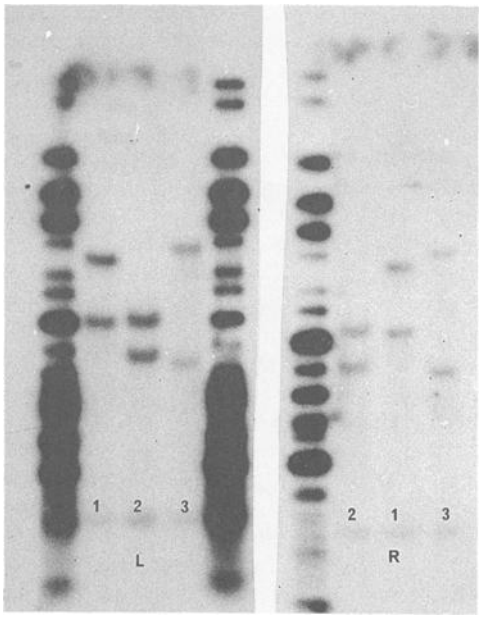

FIG. 3—*Figure on left was generated using DNA obtained from the standard DNA extraction protocol; figure on right was generated using DNA obtained from additional organic extractions.*

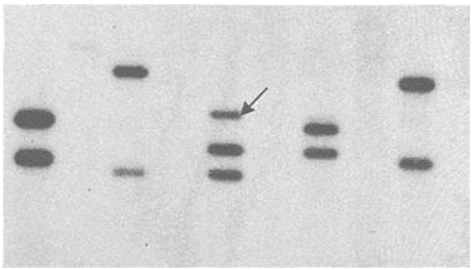

FIG. 4—*Enzyme to DNA ratio was held constant (4:1); however, volume of the reaction solution was increased ten-fold compared with the standard protocol. An additional band* (arrow) *can appear in a normal, two-banded pattern by alteration of the overall concentration of DNA and Hinf I.*

of purity. When DNA was isolated from these particular bloodstains, the initial pellets contained a brown flocculent material that obscured the DNA. Additional organic extractions with phenol and chloroform were used to remove the contaminating material. The RFLP typing results of Hae III-digested samples of the contaminated DNA and the DNA obtained after additional organic extractions are shown in Fig. 3. Both yielded identical and correct RFLP patterns, indicating the Hae III activity was not affected by the contaminating material.

Previously, we reported that Hinf I was found to partially digest DNA [*28*]. Not only is methylation a consideration for partial digestion by Hinf I, but the generated profile can be affected by altering the concentration of the interacting reagents. Figure 4 shows that one additional band could be generated for a profile by changing the concentration of DNA and Hinf I, although the ratio of enzyme units to DNA was maintained. Thus, a normal two-banded pattern could be induced to become a three-banded pattern. This phenomenon could make RFLP pattern interpretation difficult for forensic materials. This type of partial digestion was not observed for Hae III.

Finally, the cost of an RE should be considered. Although prices of REs are subject to change, Hae III is currently inexpensive relative to most REs.

Conclusion

Several factors must be considered when selecting an RE for RFLP analysis of evidentiary material. The RE should be an inexpensive four-base cutter, provide small DNA fragments for the D2S44 locus, be insensitive to the methylation patterns that occur in mammals, and be sufficiently resilient to cleave potentially contaminated DNA samples. Based on these criteria, Hae III is an appropriate RE. An additional paper on the validity of typing results of Hae III-digested contaminated samples will follow.

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