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Products of Partial Digestion with *Hae* III. Part 1. Characterization, Casework Experience and Confirmation of the Theory of Three-, Four- and Five-Banded RFLP Pattern Origins Using Partial Digestion

REFERENCE: Benzinger EA, Emerek E, Grigsby N, Duewer D, Lovekamp ML, Deadman H, Thompson J, Sallee PJ, Riech AK. Products of partial digestion with *Hae* III. Part 1. Characterization, casework experience and confirmation of the theory of three-, four- and five-banded RFLP pattern origins using partial digestion. J Forensic Sci 1997;42(5):850–863.

ABSTRACT: The sizes of *Hae* III partial digestion products at D1S7, D2S44, D4S139, D5S110, D10S28, and D17S26 were evaluated in experimentally generated partial digestions of liquid blood DNA. The partial digestion products were highly predictable, suggesting a very high level of sequence conservation in regions flanking variable number tandem repeat (VNTR) blocks. Partial digestion bands associated with three-or-more-banded patterns were also characterized. Partial digestion of three-banded patterns can be used to determine whether the extra bands arise due to internal *Hae* III sites in the VNTR block and to identify hidden three-banded patterns. Partial digestion products from forensic casework also conformed to size expectations. Presumed partial digestion bands from 27 forensic samples were compared to the experimentally generated data. The causes of partial digestion are examined and recommendations for interpreting forensic DNA evidence exhibiting partial digestion products are given.

KEYWORDS: forensic science, band shift, band sizing, DNA typing, gel electrophoresis, restriction fragment length polymorphism, variable number tandem repeat, partial digestion

Forensic DNA profiling has become a widely used and widely accepted method of determining the potential source of forensic body fluid or tissue samples. Unknown samples can be associated with a known individual with a high degree of confidence. Its widest application has been in the resolution of sexual assault and other violent crimes in which the transfer of body fluids is common. The method based on restriction length fragment polymorphism (RFLP) analysis of variable number tandem repeat (VNTR) regions is capable of identifying DNA profiles with an estimated frequency of occurrence in the population of one in one million or less when four or more loci are studied. The use of such a method of identification capable of virtual individualization results in the near certainty that, when identity is the issue, all wrongfully accused individuals will be exonerated.

Forensic RFLP analysis has been standardized in most local, state, and federal crime laboratories in the United States around a protocol developed at the FBI which uses the restriction enzyme *Hae* III. In forensic RFLP analysis, DNA is extracted from human body fluid or tissue samples, digested with *Hae* III, subjected to electrophoresis, southern blotted and probed sequentially with several single locus probes (1,2). The vast majority of individuals are heterozygous at forensically useful loci and thus two RFLP bands are observed for each probe. These bands represent the *Hae* III restriction fragments which contain the VNTR region complimentary to the probe. Match/no match decisions and population frequency estimates are ultimately based on the relative electrophoretic mobility of these bands (3). The VNTR repeat sequences at those loci which are suitable for use with *Hae* III do not contain regular internal *Hae* III sites (5′GGCC). If a *Hae* III site did occur regularly within the VNTR repeat sequence, the fragment would be cut into many small fragments. These fragments might appear as a ladder on an autoradiograph where each fragment represents incremental increases in the number of repeats (4).

Hae III has been shown to be a particularly robust restriction enzyme (5). Although a number of the factors which affect *Hae* III performance are well understood (6), limit digest (where no uncut DNA is detectable) of some forensic samples is not possible. Failure of the enzyme to cut has been associated with partial degradation and sample deposition on substrates such as leather and heavily dyed denim fabrics (7–10). Occasionally, a significant portion of the *Hae* III sites are not cut during the digestion step and artifactual bands are observed in addition to the normal allelic bands. These bands represent the VNTR-containing restriction fragment plus the adjoining 5′ and/or 3′ restriction fragments (Fig. 1a). Bands corresponding to additional contiguous restriction fragments beyond those shown in Fig. 1a may also be observed. Because the probes are complimentary to the VNTR repeat sequence, only fragments containing the VNTR region will be detected.

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*This study was supported in part by a grant from the Midwestern Association of Forensic Scientists.

Received 9 Aug. 1996; and in revised form 27 Dec. 1996; accepted 24 Jan. 1997.

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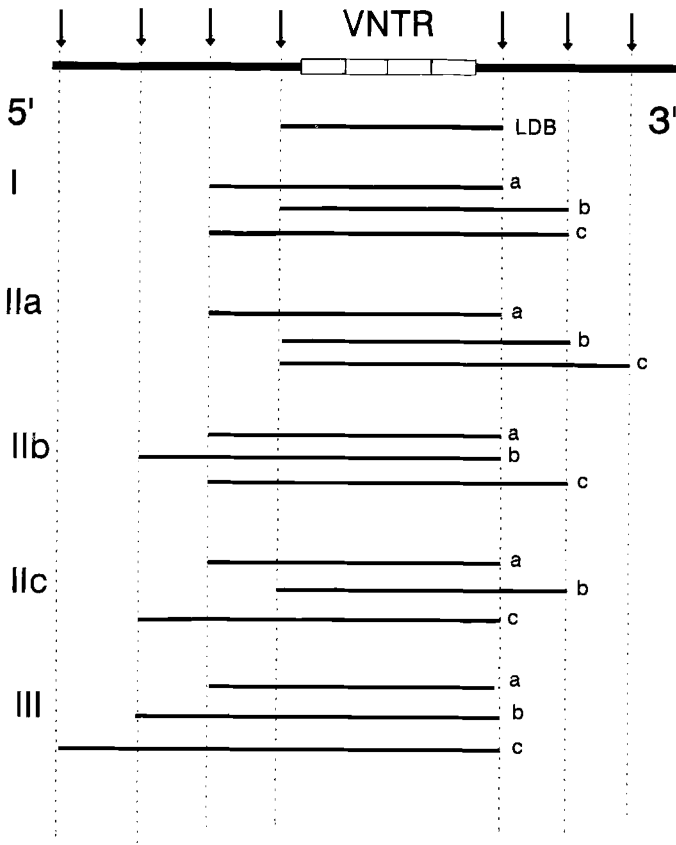


FIG. 1a—*Extra bands produced by incomplete digestion with* Hae *III. The arrows indicate the location of* Hae *III sites. After relatively complete digestion, only the limit digest band (LDB) is detected by a probe complimentary to the variable number tandem repeat (VNTR) sequence. When significant undigested DNA remains, bands representing the VNTR-containing fragment plus the 5' and/or 3' flanking fragments (a, b, and c in order of increasing size) will be detected by the probe. Several different geometric arrangements of the uncut* Hae *III sites (I–III) are possible. LDB = limit digest band.*

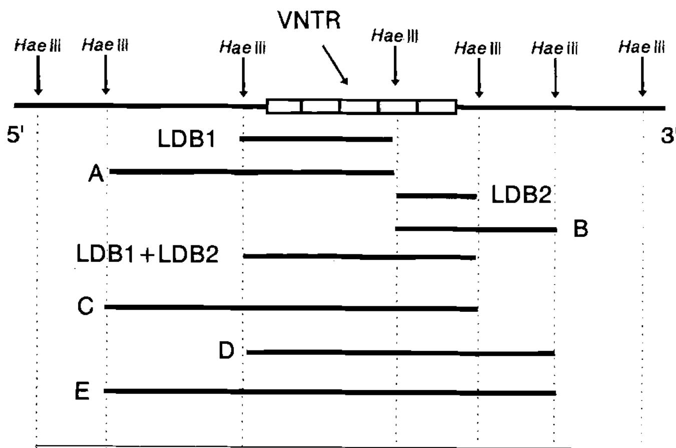


FIG. 1b—*Extra bands predicted by incomplete digestion of a three-banded RFLP pattern. If two of the three bands (LDB1 and LDB2) of a three-banded pattern are generated by the occurrence of a* Hae *III site inside a VNTR block, a fourth band (LDB1 + LDB2) would result from incomplete digestion with* Hae *III. Additional bands representing LDB1 plus the 5' flanking fragment (A), LDB2 plus the 3' flanking fragment (B), and LDB1 + LDB2 plus the 5' (C), 3' (D) and 5' plus 3' (E) flanking regions might also be observed.*

Single-banded patterns are sometimes observed in limit digests. The single-banded patterns can signal a true homozygote, but most often are artifacts of the agarose gel system. Single-banded patterns may arise from two different restriction fragments with insufficient size difference to be resolved in the gel. Alternately, one of the restriction fragments may be so small as to be undetectable or to have run off of the gel during electrophoresis. These single-banded patterns are commonly referred to as "apparent homozygotes" to denote the fact that they may not be true homozygotes. Steinberger et al. and Chakraborty et al. have demonstrated that both phenomena cause single-banded RFLP patterns in true heterozygotes: (11,12) Three- (and sometimes four- and five-) banded single-contributor patterns are observed as well. The occurrence of such banding patterns in forensic data was reported as early as 1990 (13). In limit digests, these "extra" bands are thought to be due to point mutations resulting in a *Hae* III site within the VNTR region. Other explanations, such as somatic mosaicism are possible but unlikely (14). Duplication of the VNTR region cannot, however, be ruled out.

The theory that three- four- and five-banded RFLP patterns are produced by the occurrence of *Hae* III sites within the VNTR block can be tested by predicting the unique set of partial digestion products which would be expected for a three-banded pattern. Incomplete or partial digestion with *Hae* III results in the production of fusion products of *Hae* III fragments. Figure 1b illustrates the bands which would be produced by partial digestion of a VNTR region interrupted by a *Hae* III site.

Partial digestion bands do not normally interfere with the interpretation of single-donor DNA samples. However, forensic RFLP analysis is often called upon to resolve mixtures of body fluids from multiple donors (as in the case of sexual assault evidence). Extra bands caused by partial digestion may confound the interpretation of mixed or potentially mixed forensic RFLP profiles. Bands due to partial digestion may be misinterpreted as evidence of additional contributors. As a result, some analysts may choose not to interpret partially digested samples. Those who do draw conclusions from such specimens must be able to rule out the possibility that the bands arose as a result of an additional contributor.

Partial digestion bands were identified as such early in the history of forensic DNA profiling in the FBI laboratory (H. Deadman, unpublished data). By 1989, the size relationships of partial digestion bands to the limit digest bands were understood as well as the ability to experimentally create similar partial digestion bands in pristine standards. Court testimony concerning the origin of partial digestion bands first occurred in October, 1990 (Arizona vs. Despain). This study is the first extensive documentation and analysis of experimentally induced partial digestion products.

Methods and Materials

DNA Sources and Extraction

Liquid blood was obtained from anonymous donors. To facilitate the analysis of partial digestion bands at the locus D4S139, a subset of samples were chosen which, during previous analysis, had been found to possess comparatively small alleles at D4S139. Additional samples giving three-banded RFLP patterns at D4S139 or D5S110 were analyzed. DNA analysis methods were adapted from Budowle and Baechtel (1). Five to seven hundred μL of liquid blood was lysed by freezing at $-20°C$. The liquid blood was thawed, mixed with 1X SSC (20X SSC = 3 M NaCl and 0.3 M Na_3 Citrate) and centrifuged at 16,000 $\times$ g for 3 min to pellet white blood cells. The supernatant was decanted and the pellet washed once with 1X SSC and centrifuged as before.

The pellet was then suspended in a lysis buffer consisting of 10 mM Tris-HCl pH 7.5, 2% SDS, 100 mM NaCl, 10 mM EDTA, and 4 μg/mL proteinase K and incubated 1 h to overnight at 56°C. Following incubation, the lysis mixture was extracted once with 25:24:1 phenol:chloroform:isoamyl alcohol equilibrated with 100 mM Tris-HCl pH 7.5. DNA was filtered from the aqueous phase in Microcon 100 spin filters (Amicon, Inc., Beverly, MA) and washed once with 50 μL TE (10 mM Tris-HCl; 1 mM EDTA, pH 7.5). The DNA was then recovered from the Microcon 100 filter and resuspended in 1.0 mL TE. DNA yield estimates were made by electrophoresis in 1% agarose next to a standard dilution series.

Generation of Partially Digested DNA

Hae III and 10X reaction buffer (1X reaction buffer is 50 mM NaCL, 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT) was obtained from New England Biolabs (Beverly, MA). Partially digested DNA was generated by incubating DNA samples with *Hae* III and terminating the reaction in aliquots removed at several time points. Five μg of DNA were equilibrated to 37°C in 600 μL of 1X reaction buffer. Approximately ten units of *Hae* III was then added to the DNA sample. Aliquots of 120 μL of the reaction were removed at 15, 22, 30, 45, and 60 min intervals and added to a tube containing 50 μL of 50 mM EDTA to stop the reaction. The DNA samples were then collected in Microcon 100 spin filters and resuspended in 18 μL TE. The degree of digestion of the samples was assessed by electrophoresis of a small aliquot of each time point on a 1% agarose minigel.

Electrophoresis and Southern Blotting

The partially digested DNA samples were subsequently electrophoresed in an 11 by 20 cm 1% LE agarose gel (IDNA agarose, FMC, Inc., Rockland, ME) and transferred to either Biodyne A or Biodyne B membrane (Life Technologies, Inc., Gaithersburg, MD). For transfer to Biodyne A, gels were denatured for 20 min in 500 mM NaOH; 1.5 M NaCl, neutralized for 20 min in 1.0 M Tris HCl pH 8; 1.5 M NaCl and transferred for 2 h to overnight in 10X SSC. Following transfer, the membranes were rinsed in 2X SSC and DNA was fixed to the membranes by UV fixation (254 nm; 120 micro joules/cm^2) in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). For transfer to Biodyne B, gels were denatured for 30 min and transferred in 400 mM NaOH for 2 h to overnight. Following transfer the Biodyne B membranes were neutralized in 100 mM Tris-HCl pH 7.5.

Hybridization, Autoradiography, and Interpretation

The membranes were probed at D1S7 (15, 16; Cellmark Diagnostics, Gaithersburg, MD), D2S44 (17; Promega Corp., Madison, WI or Cellmark Diagnostics), D4S139 (18; Life Technologies, Inc., Gaithersburg, MD), D5S110 (19; Life Technologies, Inc., Gaithersburg, MD), D10S28 (20; Promega Corp. or Cellmark Diagnostics) and D17S26 (21; Promega Corp. or Cellmark Diagnostics) using either ^{32}P (Biodyne A or B; 1) or chemiluminescent (Biodyne A; 22 23) detection.

Membranes hybridized with ^{32}P-labeled probes were imaged on Kodak XAR film. Membranes hybridized with alkaline phosphatase-labeled probes were imaged on Kodak Clinic Select High Speed Blue X-ray film after an overnight ramp. Hybridized probe was stripped from Biodyne A membranes by two 5 min soaks in boiling stripping solution (10 mM Tris HCl pH 7.6, 1 mM EDTA and 0.5% (v/v) Tween 20). Hybridized probe was stripped from Biodyne B membranes by briefly rinsing in 400 mM NaOH at room temperature followed by neutralization in 100 mM Tris-HCl pH 7.5. Computer-assisted band size determinations were made with software provided by the Federal Bureau of Investigation (24). Approximately 120 partially digested samples were prepared.

Casework Samples

Examples of actual forensic samples exhibiting partial digest bands were obtained from casework conducted at the Illinois State Police Forensic Sciences Command Research and Development Laboratory during the period 1992–1995. Twenty-seven samples from 14 different cases were examined. To protect confidential information, all identification markings were removed from the data prior to analysis. The samples were evaluated for source, condition of DNA, number of digestion reactions performed, appearance of DNA on post-restriction test gels, loci exhibiting partial digestion bands and conformity to partial digestion band size expectations. An attempt was made to determine whether the partialed condition was due to unremediable sample characteristics or to procedural characteristics.

Mathematical Analysis

The expected base pair size differences between the partial digestion bands and the limit digest band were estimated as weighted means. Approximate 95% confidence bounds for the evaluation of putative partial digestion bands were estimated as functions of the limit digest band size. These uncertainty estimates were established using knowledge of the RFLP measurement process calibrated with K562 cell line control data obtained by the Illinois State Police Forensic Science Command during routine casework, offender, and population studies. The partial digestion products at each locus were evaluated for fit to the possible restriction site geometries given in Fig. 1. A detailed description of these operations is given in Part 2 of this series (25).

Results

Efficacy of Controlled Partial Digestion

Figure 2 depicts a typical post-restriction minigel of the type used to assess the degree of completeness of *Hae* III digestion time points during production of partially digested DNA. Lanes 1–5 were digested for 15, 22, 30, 45, and 60 min, respectively. Only lane 5 is considered to be a limit digest. In casework, complete digestion is accomplished sooner because the ratio of *Hae* III units to ng DNA is greater (see 6).

Partial Digestion Product Assessment and Geometry of Restriction Sites

Partial digestion bands were observed for all probes. Table 1a summarizes the number of alleles observed at each locus, the weighted mean (±standard deviation of the mean) of the size difference between the partial and limit digest bands for the first 3 partial digestion products at each locus and the probable restriction site geometries. Details of the mathematical analysis are given in Part 2 of this series (25).

Table 1b presents approximate 95% confidence intervals for use in the evaluation of putative partial digestion bands, calculated using RFLP measurement characteristics appropriate for interlaboratory comparison data (26,27). Because *inter*laboratory measurements are expected to be more variable than *intra*laboratory

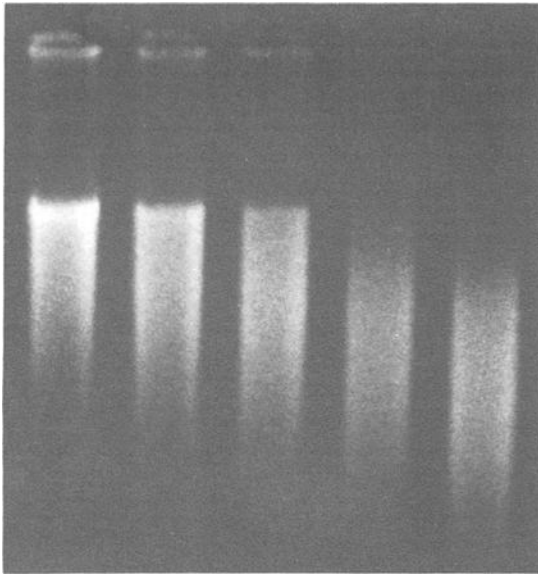


FIG. 2—*Post-restriction minigel of partial digestion time points. Human DNA extracted from liquid blood and digested with* Hae *III for 15, 22, 30, 45, and 60 min, respectively, as described in Methods. Electrophoresis was in 1% agarose with 0.5 ng/mL ethidium bromide. Only lane 5 is a limit digest. The band at the top of the streak in lane 1 coelectrophoresis with the lambda/*Hind *III 23, 130 bp band. The top of the smear of DNA in the limit digest (lane 5) is at approximately 4300 bp.*

measurements, the values in Table 1b are wider than those calculated specifically for measurements performed by the ISP. Calculation details are provided in Part 2 of this series.

Figures 3a–f show the appearance of partial digestion bands at each of the loci examined. Three evenly spaced partial digestion bands (205, 398, and 612 bp greater than the limit digest band, respectively) were routinely observed for D1S7 (Fig. 3a). At this locus, no more than three partial digestion bands were ever observed for each limit digest band. D2S44 produced numerous partial digestion bands (Fig. 3b). Only the first three at 577, 1771, and 2333 bp larger than the limit digest band were analyzed. The great number of partial digestion bands produced at D2S44 frequently caused partial digestion bands from the smaller limit

TABLE 1a—*Weighted mean of partial band sizes observed for the first three most commonly observed partial digestion products at each locus. The band sizes represent the difference between the size of the limit digest band and that of the partial digestion product. The possible restriction site geometries are indicated (refer to Fig. 1a). Detailed explanation is given in references 25.*

	Partial 1*		Partial 2*		Partial 3*		
Locus	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	Patterns†
D1S7	115	205 (1)	114	398 (1)	107	612 (1)	I,II
D2S44	135	577 (1)	165	1771 (1)	132	2333 (2)	I,II
D4S139	35	269 (4)	17	1065 (10)	27	1561 (8)	II,III
D5S110	48	191 (2)	43	305 (2)	20	497 (3)	I,II
D10S28	83	267 (1)	70	1399 (2)	41	1675 (3)	I,II
D17S26	42	239 (3)	75	980 (2)	28	1289 (3)	II,III

*Number of partial bands, weighted mean of the observed base pair size differences between the partial digestion bands and the limit digestion band, and the standard deviation of the weighted mean.

†Possible restriction site geometries.

TABLE 1b—*95% confidence limits on difference between partial digest band and limit digest band (LDB) size based on interlaboratory measurement characteristics. Forensic casework samples fell within the upper, but not always the lower bounds. Detailed explanation is given in reference 25.*

	D1S7						D2S44					
	Partial 1		Partial 2		Partial 3		Partial 1		Partial 2		Partial 3	
LDB	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
1000	193	217	385	411	598	626	563	591	1751	1791	2309	2358
1500	190	220	383	413	596	629	561	593	1748	1795	2305	2362
2000	188	222	380	416	593	631	558	596	1744	1798	2300	2366
2500	185	225	377	419	590	635	555	599	1739	1803	2295	2372
3000	181	229	373	423	586	638	551	603	1734	1808	2289	2377
3500	177	233	369	427	582	643	547	607	1728	1814	2283	2384
4000	173	237	364	432	577	648	542	612	1722	1820	2275	2392
4500	168	242	359	437	571	653	536	618	1714	1828	2267	2400
5000	162	248	353	443	565	660	530	624	1706	1836	2257	2410
5500	155	255	346	450	558	667	523	631	1697	1845	2246	2421
6000	148	262	338	458	549	675	515	639	1686	1856	2234	2433
6500	140	270	330	466	540	684	506	648	1674	1868	2221	2446
7000	130	280	320	476	530	694	496	658	1661	1881	2206	2461
7500	120	290	309	487	519	706	484	670	1646	1896	2189	2478
8000	108	302	297	499	506	718	472	682	1630	1912	2171	2496
8500	95	315	283	513	492	733	457	696	1612	1930	2150	2517
9000	80	330	268	528	476	749	442	712	1592	1950	2127	2539
9500	64	347	251	545	458	766	424	730	1569	1973	2102	2565
10000	45	365	232	564	439	786	405	749	1545	1997	2075	2592
10500	25	385	211	585	417	807	383	771	1518	2024	2044	2623
11000	3	407	188	608	393	831	359	795	1488	2054	2010	2656
11500		432	163	633	366	858	333	821	1455	2087	1974	2693
12000		459	135	661	337	887	304	850	1418	2124	1933	2734
	D4S139						D5S110					
1000	255	284	1040	1090	1537	1586	178	203	292	318	483	512
1500	253	286	1038	1092	1534	1588	176	205	290	320	481	514
2000	251	288	1036	1094	1531	1591	173	208	287	323	478	517
2500	248	291	1033	1097	1527	1595	170	211	284	326	475	520
3000	244	294	1030	1100	1523	1599	167	214	281	329	471	523
3500	241	298	1026	1105	1518	1604	163	218	277	333	467	527
4000	236	303	1021	1110	1512	1610	159	223	272	338	462	532
4500	231	308	1015	1115	1506	1617	154	228	267	343	457	538
5000	225	314	1008	1122	1498	1624	148	234	261	349	451	544
5500	219	320	1001	1129	1489	1633	141	240	254	356	444	551
6000	211	328	992	1138	1479	1643	134	248	247	363	436	559
6500	203	336	983	1148	1468	1654	125	256	238	372	427	567
7000	193	346	972	1159	1456	1666	116	265	229	382	417	577
7500	183	356	959	1171	1442	1680	105	276	218	392	406	588
8000	171	368	945	1185	1427	1696	94	288	206	404	394	601
8500	157	382	930	1200	1409	1713	81	301	192	418	380	615
9000	142	396	913	1217	1390	1732	66	315	177	433	364	630
9500	126	413	864	1237	1369	1753	50	332	161	449	347	648
10000	108	431	872	1258	1345	1777	31	350	142	468	328	667
10500	87	452	849	1281	1319	1803	11	370	122	488	307	688
11000	65	474	823	1307	1291	1832		392	99	511	283	711
11500	40	499	794	1336	1259	1863		417	74	536	257	737
12000	12	527	763	1368	1224	1898		444	46	564	229	766
	D10S28						D17S26					
1000	254	279	1381	1417	1655	1695	226	253	964	995	1271	1308
1500	252	281	1378	1420	1652	1699	224	255	961	998	1268	1310
2000	249	284	1375	1424	1648	1702	221	258	958	1001	1265	1314
2500	246	287	1371	1428	1644	1707	218	260	954	1005	1261	1318
3000	243	291	1366	1432	1639	1712	215	264	950	1009	1257	1322
3500	239	295	1361	1438	1633	1717	211	268	946	1013	1252	1327
4000	234	299	1355	1444	1627	1723	207	272	940	1019	1246	1333
4500	229	304	1348	1450	1620	1731	202	277	934	1025	1240	1339
5000	223	310	1341	1458	1612	1739	196	283	927	1032	1232	1346
5500	216	317	1332	1466	1603	1748	189	290	920	1040	1224	1355
6000	209	324	1322	1476	1592	1758	182	297	911	1048	1215	1364
6500	200	333	1312	1487	1581	1770	173	306	901	1058	1204	1375
7000	191	342	1300	1499	1568	1783	164	315	890	1069	1192	1386
7500	180	353	1286	1512	1554	1797	153	326	878	1082	1179	1400
8000	168	365	1271	1528	1538	1813	141	337	864	1095	1164	1414
8500	155	378	1254	1544	1520	1831	128	351	848	1111	1148	1431

TABLE 1b—*Continued.*

	D10S28						D17S26					
	Partial 1		Partial 2		Partial 3		Partial 1		Partial 2		Partial 3	
LDB	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
9000	140	393	1236	1563	1500	1850	113	366	831	1128	1130	1449
9500	123	410	1215	1584	1478	1872	97	382	812	1147	1110	1469
10000	105	428	1192	1606	1545	1896	79	400	791	1168	1087	1491
10500	85	449	1167	1632	1427	1923	58	421	768	1191	1063	1516
11000	62	471	1139	1660	1398	1953	36	443	742	1217	1035	1543
11500	37	496	1108	1690	1366	1985	11	468	714	1245	1005	1573
12000	10	523	1075	1724	1330	2021		495	683	1276	972	1606

digest band to have a higher molecular weight than the larger limit digest band, thus complicating the analysis.

Partial digestion bands were observed more rarely at D4S139 (Fig. 3c). This locus possesses the largest alleles of all the loci studied. Many D4S139 partial digestion bands are too close to the limit digest allele to be measured because of the compression in the upper portion of the gel where most D4S139 limit digest bands occur. Because migration in the gel is inversely proportional to the log of the molecular weight of the fragment, resolution of closely spaced bands is progressively poorer towards the upper region of the analytical range. Frequently, partial digestion bands will appear as a "hat" or a smear on top of the limit digest bands. Thus, D4S139 may not clearly exhibit partial bands when all other loci have them. Some of the D4S139 data presented here were

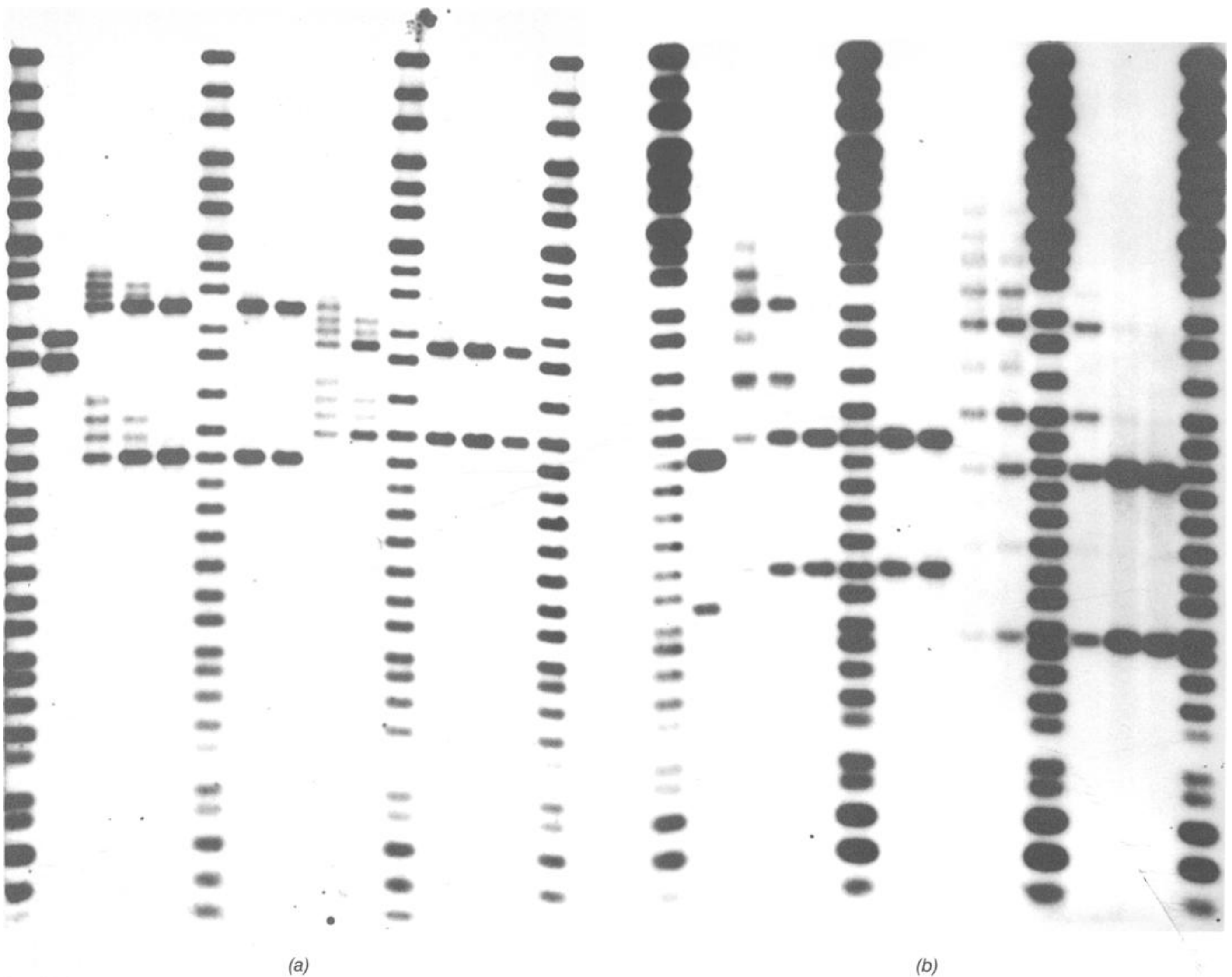


FIG. 3—*Appearance of partially digested DNA at each locus studied. The marker lane is the Life Technologies 30-band Molecular Weight Marker. In a, b, d, and f, the left most allelic lane is cell line K562. The partial digestion series corresponds to the lanes shown in Fig. 2 and runs from least digested on the left to the limit digest on the rightmost sample: a) D1S7, b) D2S44, c) D4S139, d) D5S110, e) D10S28, and f) D17S26. Two different genomic DNAs are pictured in a, b, d, and f. Partial digestion bands at D4S139 are frequently not distinguishable from the limit digest bands because of the relatively large band size of D4S139 alleles and the compression that occurs in the upper region of the gel. Partial digestion bands at D5S110 frequently appear as a smear above the limit digest bands.*

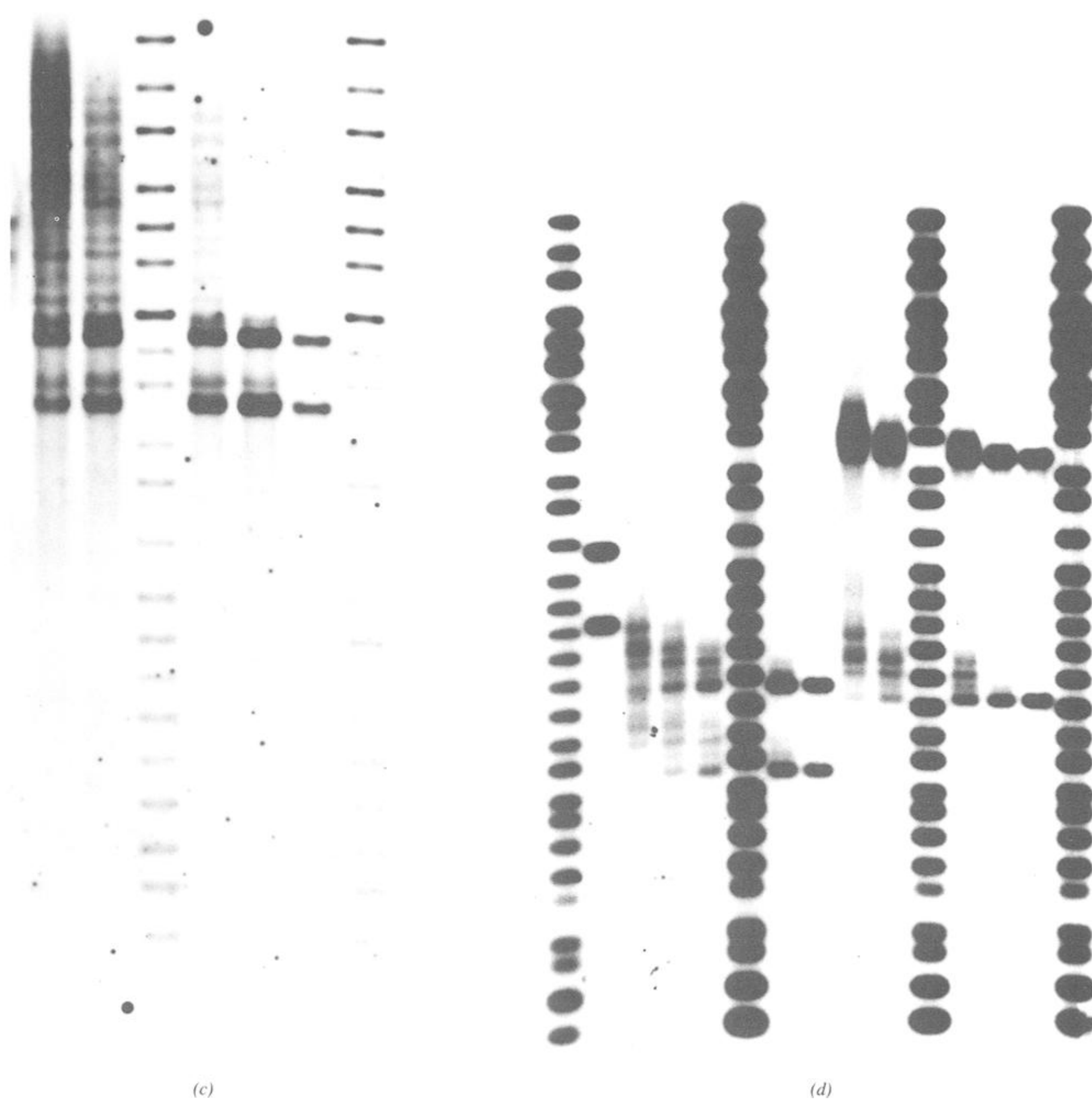


FIG. 3—*Continued*

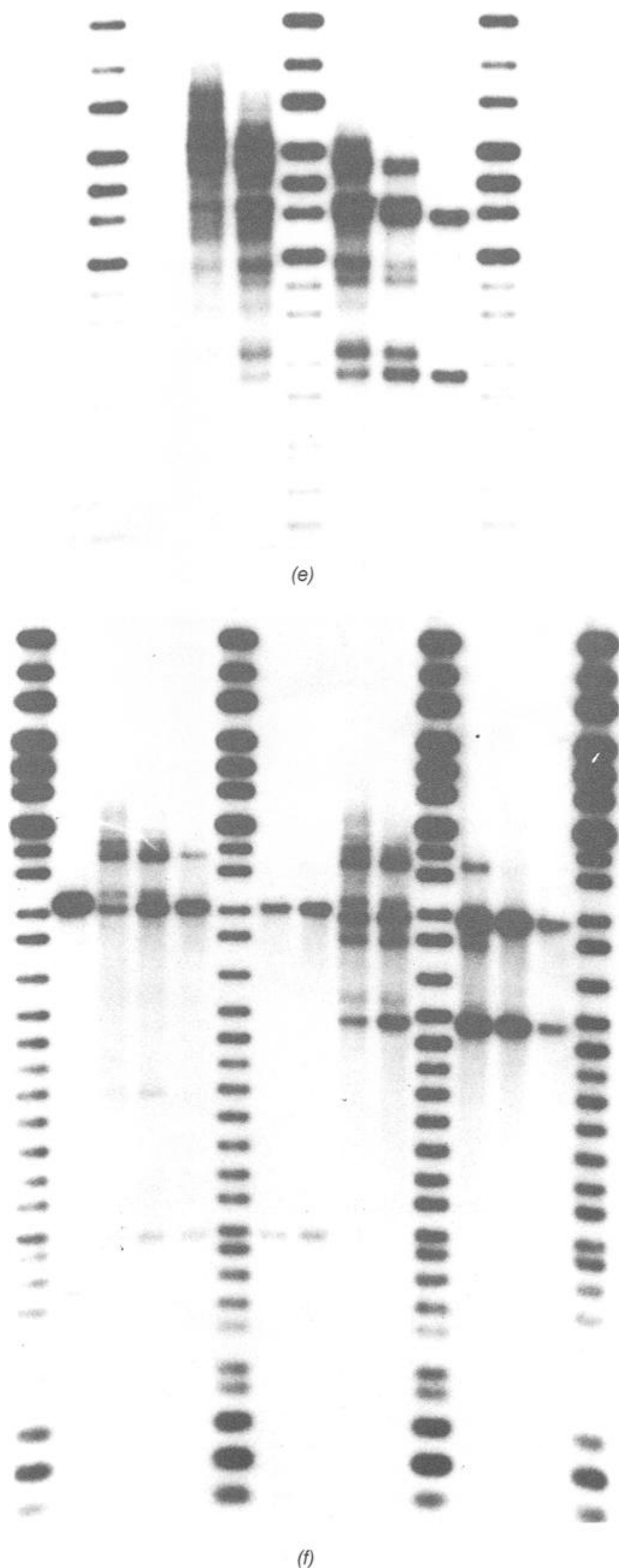
collected by analyzing previously profiled samples which were observed to have relatively small D4S139 limit digest bands. The limit digest bands in Fig. 3c are at 6110 and 5188 bp. In these samples, a great number of partial digestion bands were observed. Only the first three bands at 269, 1065, and 1561 bp greater than the limit digest bands were studied.

Locus D5S110 partial digestion products tended to produce smears above the limit digest bands (Fig. 3d). Even samples with relatively small limit digest bands were likely to appear smeared. Partial digestion bands were measured at 191, 305, and 497 bp greater than the limit digest bands. An additional band at approximately 100 bp greater than the limit digest bands was observed in a few samples with limit digest bands sufficiently small enough to permit resolution of the 100 bp band. The first three partial digestion bands for D10S28 were measured at 267, 1399, and 1675 bp greater than the limit digest bands (Fig. 3e) and for D17S26 at 239, 980, and 1289 bp greater than the limit digest bands (Fig. 3e).

Although not all test samples gave interpretable partial digestion bands at all loci, no partial digestion banding patterns were observed in any of the true one- or two-banded test samples which were completely uncharacteristic of the locus. Eight bands which were smeary and difficult to size gave values outside of the bounds established in Table 1b.

Three-, Four- and Five-banded RFLP Patterns

Additional partial digestion products are observed in three- and four-banded samples. These patterns are thought to arise from point mutations which generate *Hae* III sites within VNTR blocks.



(e)

(f)

FIG. 3—*Continued*

Hence, in a three-banded RFLP pattern, one of the bands represents the normal VNTR fragment and the other two bands each represent sides of a "broken" VNTR fragment (Fig. 1b). If this model is correct, partial digestion of three-banded DNA samples should generate a fourth fragment which represents the fusion of the "broken" fragments. DNA from four individuals possessing three-banded patterns at D4S139, and two individuals possessing three-banded patterns at D5S110 was studied by partial digestion. In all six cases, a fourth band was produced upon partial digestion that was equal to the sum of two of the three limit digest bands (Fig. 4a; Table 2). Two apparent two-banded D4S139 pattern behaved like three-banded patterns upon partial digestion. The samples, which gave only two bands after a limit digest, produced a fusion band after partial digestion. These samples could either be homozygous for the split allele, have an allele that migrates off the gel, or have an allele which comigrates with one of the sides of the split allele. In Fig. 4b, the two limit digest bands (6560 and 1441 bp) give rise to a fusion product of 8010 bp.

While additional partial digestion products corresponding to fusion products containing 5′ and 3′ flanking fragments were visible in several of the three-banded patterns, only one set was sufficiently distinct to allow imaging. In this one D5S110 sample, additional partial digestion products were resolvable and supported the model from Fig. 1b (see Fig. 5 and Table 2). The normal allele (2310 bp) displayed partial digestion fragments of 2522, 2629, and 2832 bp (differences of 212, 319, and 522). The broken allele (3331 and 3843 bp) produced a fused fragment of 7273 bp (3331 + 3843 = 7174). The smaller fragment of the broken allele displayed only one partial digestion fragment at 3651 bp (a difference of 320) and the larger fragment of the broken allele (3843 bp) displayed only one partial digestion fragment of 4049 bp (a difference of 206). Partial digestion fragments were present above the fusion fragment but were unresolvable.

Experimentally Induced Partial Digestion Products are Similar to "Naturally" Occurring Ones

Twenty-seven forensic casework samples which displayed "extra" bands which could not be explained by incomplete stripping of previous probes or other causes were examined (Table 3). All putative partial digestion products derived from casework examples fell within the upper but not the lower norms established for the experimentally produced partial digestion products. The means established for evidentiary material fell as low as 3% below the means for pristine samples (see 25).

The Causes of Partial Digestion in Forensic Casework

After an examination of the correlation of post-restriction test gel appearance and occurrence of partial digestion products in controlled partial digestions, an analysis of the causes of partial digestion bands in forensic casework samples was possible. On the post-restriction minigel (refer to Fig. 2), undigested DNA appears as a high molecular weight (HMW) band which coelectrophoreses with the 23, 130 bp band of a lambda/*Hind* III digest. As digestion proceeds, the band is converted to a relatively HMW streak. In the final stage of digestion, the HMW band is missing and the streak is lower, with the largest visible portion being at approximately 4300 bp. This is considered to be a limit digest and partial digest bands are rarely seen. Samples containing a very faint HMW band or streak may or may not give partial digestion bands.

Of the 27 partially digested casework samples studied (Table 3), eight were attributed to unremediable sample characteristics, ten were attributed to procedural characteristics and nine were

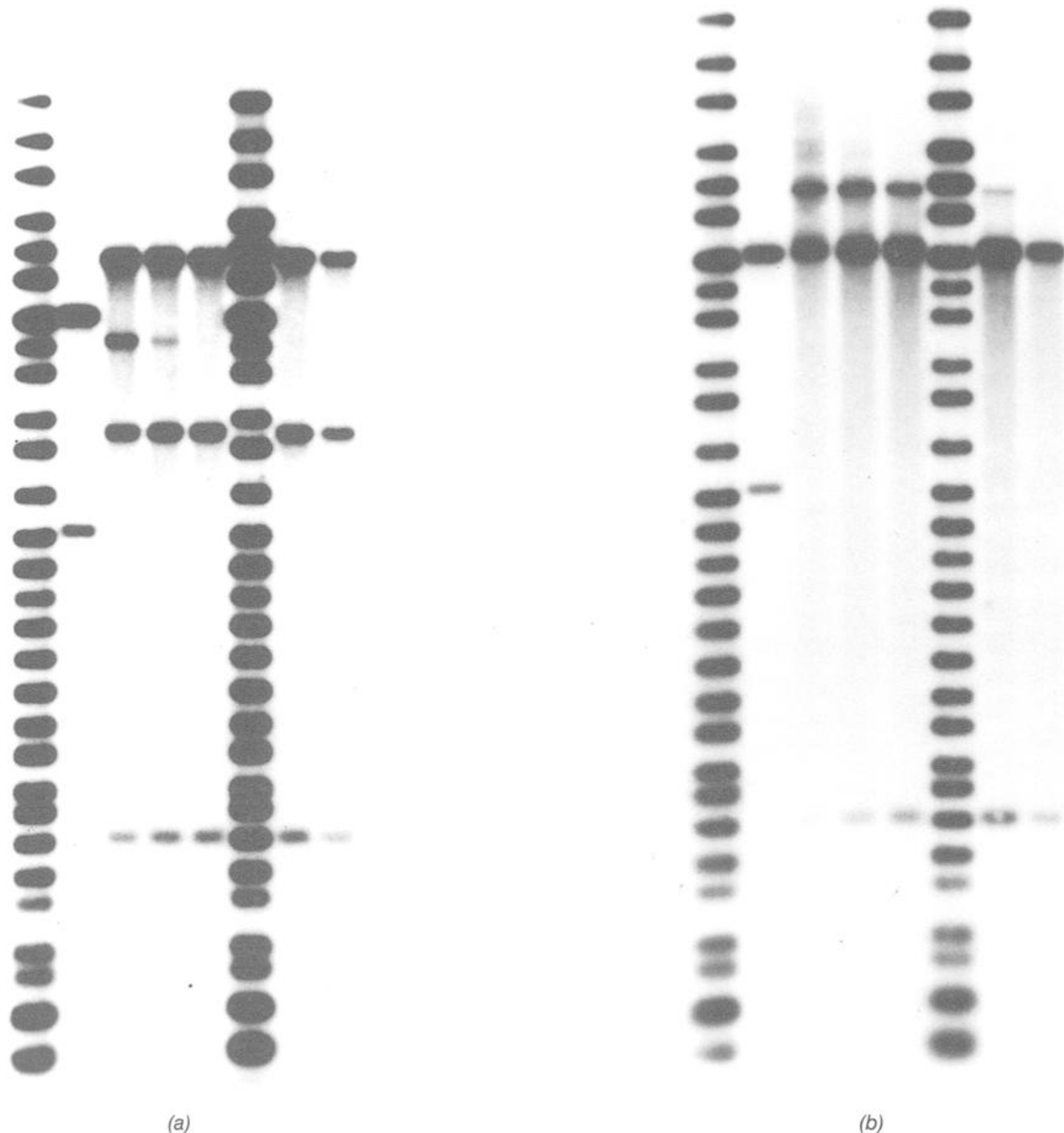


FIG. 4—*Partial digestion of three-banded RFLP patterns at D4S139. The left most allelic lane in each figure is cell line K562. The partial digestion series runs from least digested on the left to the limit digest on the rightmost sample: a) the lower two D4S139 bands (1449 and 4550 bp) of this three-banded sample fuse into a fourth (5967 bp) upon partial digestion; b) apparent 2-banded D4S139 behaves as a three-banded pattern when partially digested.*

attributed to either sample or procedural characteristics. "Unremediable sample characteristics" refers to samples that either appeared cut on the test gel or remained partially digested even after several digestion attempts and/or re-extractions. "Procedural characteristics" was defined as the acceptance of samples still containing traces of HMW bands or partially digested HMW DNA as "cut" where additional *Hae* III incubations or clean-up steps may have resulted in a limit digestion. In a few cases, heavily loaded quantitation or post-restriction gels, or very light ethidium staining of the post-restriction gel reduced the analyst's ability to detect undigested DNA. Analytical gels where >>400 ng human DNA is loaded are likely to exhibit partial digestion products even when the DNA appeared cut on the post-restriction minigel.

Discussion

The most widely used protocol for forensic DNA profiling provides a means to assure complete digestion with *Hae* III (1). Several-fold excess enzyme units (40 U per approximately 500 ng DNA) are used. A small portion of the reaction is evaluated on a minigel prior to electrophoresis so that the digestion can be repeated if a limit digest has not been achieved. Most forensic DNA specimens are readily digested by *Hae* III. However, some samples which are degraded or deposited on materials such as blue jeans are resistant to digestion. Repeated purification and digestion attempts are often unsuccessful. Therefore, a method for interpreting samples which display partial digestion products is needed.

Predictability/Reproducibility of Partial Digest Bands

The data presented here supports the model in which partial digestion bands consist of the VNTR-containing *Hae* III fragment plus various combinations of flanking *Hae* III fragments. The size of the expected partial digestion bands can be described as the sum of the sizes of the limit digest band plus the 5′ and/or 3′

TABLE 2—*Fragment sizes of limit digest bands and fusion bands in three-banded patterns at D4S139 and D5S110. Specimens 4 and 6 are hidden three-banded patterns. The limit digest bands which contribute to the fusion fragment are bolded. The fragment predicted by the fusion of those two limit digest bands is given in parenthesis. The % difference between the predicted and observed fragment sizes never exceeded the laboratory match criteria (2$^1/_2$%).*

Specimen (Figure #)	Locus	Limit Digest Band Sizes	Observed Fusion Fragment Sizes (Theoretical)	% Difference
1 (Fig. 4a)	D4S139	8041 4550 1449	5967 (4550 + 449 = 5999)	0.05
2	D4S139	6838 5323 1505	8331 (6838 + 1505 = 8343)	0.14
3	D5S110	5664 3572 3370	9226 (5664 + 3572 = 9236)	0.11
4 (Fig. 4b)	D4S139	6560 1441	8010 (6560 + 1441 = 8001)	0.11
5 (Fig. 5)	D5S110	3843 3331 2310	7273 (3843 + 3331 = 7174)	1.36
6	D4S139	5440 1509	6994 (5440 + 1509 = 6949)	0.64
7	D4S139	9441 5502 1502	7035 (5502 + 1502 = 7004)	0.44
8	D4S139	6249 4869 1503	7743 (6249 + 1503 = 7752)	0.12

flanking fragments. The products of partial digestion with *Hae* III were found to be highly predictable and no uncharacteristic partial digestion patterns were observed in any of the sizeable experimentally produced or casework samples. This apparent sequence conservation of VNTR flanking regions is not surprising because the human genome is estimated to differ by no more than 0.3% (28). Four samples among the experimentally produced data gave smeared bands that did not size within the bounds established in Table 1b. Smeary or difficult to size bands must be interpreted in light of the quality of the data.

The data collected do not permit absolute mapping of restriction fragments around the VNTR region. However, differences in restriction site geometries were noted among the loci tested. Other differences between loci in partial digestion fragment patterns are unexplained. D1S7 produces only three bands whereas D2S44 produces numerous bands. D5S110 partial digestion products frequently smear.

Resolution of Three-Banded RFLP Patterns

Data presented here supports the model wherein three- (and four- and five-) banded limit digest RFLP patterns are attributable to the presence of *Hae* III sites within the VNTR regions (Fig. 1b). In all cases of three-banded individuals studied here, partial digestion was able to fuse two of the three bands into a fourth band that is equal in size to the sum of two of the original bands (Fig. 4a). In two cases, a two-banded pattern (possibly with one additional unobserved allele) behaved like a three-banded pattern upon partial digestion (Fig. 4b).

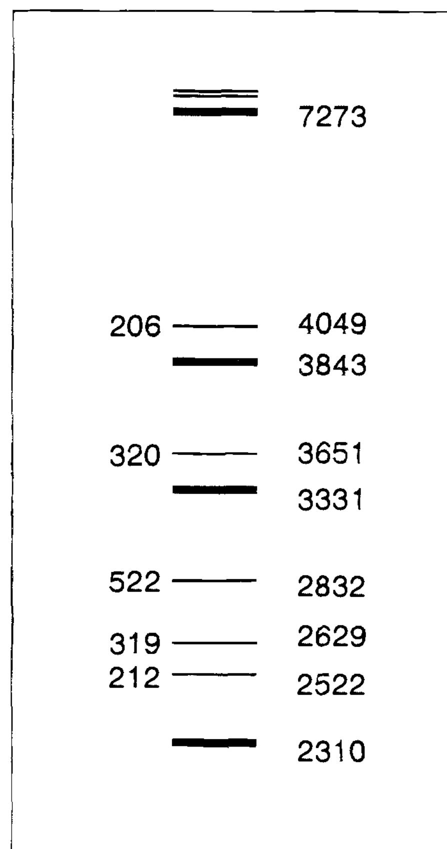


FIG. 5—*Diagram of partial digestion bands for three-banded pattern at D5S110. The diagram is a composite of measurements from several different lanes. The numbers on the right are size determinations in base pairs and the numbers on the left are the differences between the partial digestion band and its corresponding limit digest band. The lower three heavy bands (2310, 3331, and 3843) represent the limit digest bands. The upper heavy band represents the fusion fragment of the split allele (the 3331 and 3843 bands). All three partial bands are present for the lowest limit digest band, 2310, which is not part of the split allele. As predicted by the model, the split allele fragments produce only the 5′ or 3′ (or vice versa) partial band. All three D5S110 partial digestion bands are expected for the fusion fragment, but were unresolvable due to their position in the gel.*

Three- or more-banded patterns have been reported previously for D2S44, D4S139, D10S28, D14S13, and D17S26 (29), but are rare, except for D4S139 in certain American Indian populations. Three-, four-, or five-banded patterns at D4S139 were found in 21% of Apaches and 13% of Navajos but only 2.2% of Caucasians, 2.8% of Hispanics and 0% of Blacks (30). Waye and Fourney (13) found three-banded patterns at D4S139 in 1% of Canadian Caucasians. Based on the observations from this study, three or more-banded patterns are more common than previously thought due to the existence of hidden three-banded patterns. The relative frequency of three-, four- or five-banded patterns at different loci may be related to the VNTR sequence and the ease with which point mutations give rise to the 5′-GGCC *Hae* III recognition site.

Bever et al. reported in a paternity trio where both D10S28 bands observed in the alleged father's sample were transmitted to the child (29). The authors speculate that the father is homozygous for the split allele. Alternately, he may possess a hidden three-banded pattern.

Others have used different restriction enzymes or double digestions to resolve three banded patterns. Waye and Fourney (13)

TABLE 3—*Partial digestion products observed for 27 samples from 14 forensic cases. Each letter is a different case. Partial digestion products are most often observed at D2S44, then D10S28. Partial digestion products at D1S7, D5S110, and D17S26 may be underrepresented due to the less frequent use of probes to these loci in the casework laboratory. The shaded values fell below experimentally determined norms based on pristine material.*

Sample	LD Band	D1	D1	D1	LD Band	D2	D2	D2	LD Band	D4	LD Band	D5	D5	D5	LD Band	D10	D10	D10	LD Band	D17	D17	D17
A1					1707	550	1776	2310			1642	200			3537	253	1376					
					1412	579	1758	2320							3321		1395					
B1	3598	201	411	606	1701	571	1789												2526	224	977	
					1287	574	1776								5352	256	1411	1590	3931	195	967	
C1					4190	602	1768															
D1					3424	563	1715															
					4240		1798															
E1					2924	585	1717								3953	249						
					2861	569	1731								1726	272						
E2					2961	531	1733					smear			3961	247						
					2849	562	1761					smear			1735	261						
F1					1660	559	1747	2301							4731	224	1342		6145		935	
					1530	569	1777	2339							2654	272	1407	1660	1741		978	
G1					2912		1749	2308							3412	283	1395	1630			smear	
					1801	572	1719	2301							2010	270						
H1					5686	569	1709								3892	233	1354					
					1511		1739															
H2					5702	567	1706	2280	3416	255					3882	270	1372	1658				
					1509		1752															
H3					5706	605	1750	2468							3900	273	1319					
					1508		1774															
H4					5726	534	1706								3910		1326					
					1510		1765															
I1	4012	208	386	643	1947	568	1785	2300							2877	236	1379		1496		980	
					1210	551	1756								2100	268	1384					
I2					1607	578	1771	2315							2714	270	1385	1659				
					1209	637	1752								2109	263	1393	1681				
J1					3075	578	1756	2328							3950	292	1376	1642				
					1319	569	1756	2334							3447	283	1425	1626				
J2					3071	576	1758															
J3					3085	557	1743	2299							3950	292						
					1322	566									3424	280						
J4					3100	564	1757															
J5					3101	570	1746	2311							4001	241						
					1320	563									3451	272	1370					
J6					1322			2348														
K1					2562		1749				5522	223			5039	306	1362	1755				
					2005		1763				3245	204	329									
L1					2378		1765									305						
					1521		1783															
M1					1698	523	1711															
					1570	564	1715															
M2					1698	543	1751															
					1567	553	1703															
M3					1701	563	1750															
					1567	559	1768															
M4					2435	561	1763															
					1567	585	1752	2316														
N1					3736	549	1744															

used other restriction enzymes (*Hinf* I, *Mbo* I, *Alu* I, and *Rsa* I) which cut outside, but not within the VNTR block to demonstrate that three-banded patterns at D4S139 were caused by an internal *Hae* III site in the VNTR block. Digestion with one of these enzymes produced only two bands, whereas digestion with *Hae* III alone or in combination with one of the other enzymes produced three bands. Allen and Sanford-Sharp (31) reported a five-banded *Pvu* II pattern at D10S28 which was resolved into a two-banded pattern when digested with *Hae* III. Furthermore, four of the five *Pvu* II fragments were transmitted to an offspring. Because the other restriction enzymes cut at sites in the flanking DNA different from *Hae* III, the fusion band produced with an alternate enzyme will not be the same size as the fused *Hae* III allele. The partial digestion method of resolving multiple-banded patterns produces a fusion band which is more representative of the actual split allele.

One example of a three-banded *Pst* I pattern identified by probe pR365-1 which was not resolved by cutting with *Hae* III or *Hinf* I was reported by Schanfield (32). This three-banded pattern may be attributable to a duplication of the VNTR region. Other mutations would then be required to produce the third fragment.

The Experimental Data is Consistent with the Field Collected Data

Twenty-seven partially digested samples from 14 forensic cases were examined (Table 3). D2S44 was most likely to display partial digestion products. All of the partial digestion products measured in the casework examples were within the upper bounds of the experimentally determined norms. The small decrease (up to 3%; see 25) in means observed for casework samples relative to pristine samples is consistent with observations of anodal shifts in degraded DNA (8).

The Causes of Partial Digestion in Forensic Casework

For a portion of the forensic samples examined, it is possible that further optimization of the post-restriction evaluation step may have alerted the examiner to the existence of small amounts of undigested DNA (see Fig. 2 and Table 4). Based on experience, a second digestion attempt might have resulted in a more complete digestion for at least some of these samples. If incomplete digestion is due to residual contaminants or extraction reagents (see 6), the

TABLE 3—*Continued*

Sample	LD Band	D1	D1	D1	LD Band	D2	D2	D2	LD Band	D4	LD Band	D5	D5	D5	LD Band	D10	D10	D10	LD Band	D17	D17	D17
A1					1707	550	1776	2310			1642	200			3537	253	1376					
					1412	579	1758	2320							3321		1395					
B1	3598	201	411	606	1701	571	1789												2526	224	977	
					1287	574	1776								5352	256	1411	1590	3931	195	967	
C1					4190	602	1768															
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E1					2924	585	1717								3953	249						
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					1530	569	1777	2339							2654	272	1407	1660	1741		978	
G1					2912		1749	2308							3412	283	1395	1630			smear	
					1801	572	1719	2301							2010	270						
H1					5686	569	1709								3892	233	1354					
					1511		1739															
H2					5702	567	1706	2280	3416	255					3882	270	1372	1658				
					1509		1752															
H3					5706	605	1750	2468							3900	273	1319					
					1508		1774															
H4					5726	534	1706								3910		1326					
					1510		1765															
I1	4012	208	386	643	1947	568	1785	2300							2877	236	1379		1496		980	
					1210	551	1756								2100	268	1384					
I2					1607	578	1771	2315							2714	270	1385	1659				
					1209	637	1752								2109	263	1393	1681				
J1					3075	578	1756	2328							3950	292	1376	1642				
					1319	569	1756	2334							3447	283	1425	1626				
J2					3071	576	1758															
J3					3085	557	1743	2299							3950	292						
					1322	566									3424	280						
J4					3100	564	1757															
J5					3101	570	1746	2311							4001	241						
					1320	563									3451	272	1370					
J6					1322			2348														
K1					2562		1749				5522	223			5039	306	1362	1755				
					2005		1763				3245	204	329									
L1					2378		1765									305						
					1521		1783															
M1					1698	523	1711															
					1570	564	1715															
M2					1698	543	1751															
					1567	553	1703															
M3					1701	563	1750															
					1567	559	1768															
M4					2435	561	1763															
					1567	585	1752	2316														
N1					3736	549	1744															

ethanol precipitation or Microcon filtration step that occurs between digestion reactions may serve to resolve the problem.

Limit digestion was not achieved for a portion of the forensic samples even after repeated purification and digestion attempts. The fact that significant digestion did occur in these samples argues against the presence of soluble inhibitors as the sole cause of incomplete digestion (the enzyme was able to cut a portion of the samples). Rather, random modification of *Hae* III sites may have occurred. The similarity of patterns in experimentally induced and "naturally" occurring partial digests suggests a random mechanism of *Hae* III recognition site modification in actual forensic samples. The tendency for casework partial digestion bands to fall below the norms for experimentally generated partial digestion bands is consistent with their being caused in part by DNA degradation.

In casework experience, the same samples which demonstrate DNA degradation upon minigel quantitation are more likely than undegraded samples to give partial digestion bands, resist PCR-attempts and in extreme cases, display modified electrophoretic mobility. In ancient DNA, PCR success has been linked to DNA condition (33,34). In forensic samples, changes in electrophoretic mobility, usually anodal shifts, have been linked to DNA degradation (8,35). Single-strand nicks, caused by endogenous endonucleases (i.e., during apoptosis; 36) or exogenous factors, may inhibit *Hae* III cutting when at or near the recognition site. These same single-strand nicks may increase electrophoretic mobility via relaxation of sequence-directed secondary structure.

Enzymatic inhibition might result from covalent or noncovalent DNA modifications which either prevent or inhibit enzyme binding to the recognition sequence or interfere with active site chemistry. For example, phosphorothioates of deoxynucleotides, which carry a sulfur instead of an oxygen on the α-phosphorus, inhibit restriction enzyme cleavage to various degrees when the modification is at the cleavage site. *Hae* III is strongly inhibited by this modification (37). DNA modifications which affect restriction enzyme cleavage would be expected to alter also molecular weight or conformation and hence electrophoretic mobilities. The relative prominence of first, second, third, or subsequent partial digestion bands may vary depending on the type of modification present. For instance, if thymine residues flanked the 5′ *Hae* III site but not the 3′ site, a bulky thymine modification might only hinder digestion at the 5′ site.

Not All Undigestible DNA Is of Human Origin

A few samples have been noted in casework which exhibited significant undigestible DNA on the post-restriction minigel, yet did not give partial digestion bands. These samples are likely to contain significant amounts of bacterial DNA. An indication of

TABLE 4—*Examination of forensic cases containing partially digested samples. Eight partially digested samples were considered unavoidable, or due to sample characteristics. Complete digestion may have been possible in another 10 samples given tighter post restriction test gel interpretation criteria, and for 9 samples, partial digestion may have been due to either unavoidable sample characteristics or procedural characteristics.*

Sample Number	Sample Type	Total Probes	Partialed Probes	Number of Digestions	Appearance of DNA on Test Gel	Probable Cause
A1	blood stain from cap	2,4,5,10	2,5,10	3	partially digested, gel heavily loaded	sample/procedure
B1	blood stain on shorts	1,2,4,10,17	1,2,10,17	1	cut, ethidium stain light	procedure
C1	female fraction-panties	1,2,4,10	2	1	cut, degraded	sample
D1	vag swab female fraction	1,2,4,10	2	1	partially digested, heavily loaded	procedure
E1	blood stain from trunk	2,4,5,10	2,10	1	partially digested, degraded	sample/procedure
E2	blood stain from trunk	2,4,5,10	2,10	1	partially digested, degraded	sample/procedure
F1	oral swab female fraction	2,4,10,17	2,10,17	1	partially digested, heavily loaded	procedure
G1	carpet blood stain	1,2,4,10,17	2,10,17	3	partially digested, (DNA re-extracted)	sample
H1	sweatpants blood stain	1,2,4,10	2,10	2	partially digested, degraded	sample
H2	sweater blood stain	1,2,4,10	2,4,10	3	partially digested, degraded	sample
H3	sweater blood stain	1,2,4,10	2,10	3	cut, sample limited	sample
H4	carpet blood stain	1,2,4,10	2,10	1	cut, sample limited	sample
I1	post mortem blood	1,2,4,10,17	1,2,10,17	1	partially digested, heavily loaded, streaky	procedure
I2	sheet blood stain	1,2,4,10,17	2,10	3	partially digested	sample
J1	blood stain on stairs	1,2,4,10,17	2,10,17	1	partially digested	procedure
J2	blood from nail clippings	2,4	2	1	partially digested	procedure
J3	blood stain on shoe	1,2,4,10,17	1,2,10,17	1	partially digested	procedure
J4	blue jeans blood stain	1,2,4,10,17	2,10	2	partially digested	sample/procedure
J5	blue jeans blood stain	1,2,4,10,17	2,10,17	2	partially digested	sample/procedure
J6	t-shirt blood stain	1,2,4,10,17	2	2	partially digested	sample
K1	victim blood standard	2,4,5,10	2,5,10	1	partially digested, ethidium stain light	procedure
L1	vag swab female fraction	1,2,4,10,17	2,10	2	partially digested	sample/procedure
M1	blood stain on doll	1,2,4,10	2	1	partially digested? degraded, very short migration distance	sample/procedure
M2	post mortem blood std	1,2,4,10	2	1	cut? degraded, very short migration distance	sample/procedure
M3	blood stain from sheet	2	2	1	partially digested, degraded, heavily loaded	procedure
M4	blood stain from sheet	2	2	1	partially digested, degraded, heavily loaded	procedure
N1	condom, female fraction	1,2,4,10	2	2	partially digested, band, heavily loaded	sample/procedure

the presence of bacterial DNA is lower molecular weight bands on the yield gel. Non-human DNA may also be identified through the use of human DNA-specific hybridization systems. When present, bacterial DNA may be methylated at the inner cytosine of the *Hae* III site, thus preventing digestion. Therefore, samples which contain undigestible DNA of bacterial origin will appear to have a large undigested component on the post-restriction test gel even when the human DNA component is fully digested.

Recommendations/Conclusions

The first defense against partial digestion is careful extraction and quantitation. Attention to removing ethanol precipitation supernatants by pipetting or decantation rather than vacuum desiccation or the addition of a TE wash to microconcentrator protocols will minimize the retention of extraction reagents and low molecular weight inhibitors (6). Those inhibitors which cannot be removed by microconcentrators or ethanol precipitation might be removed by ion exchange chromatography (38). DNA samples should be resolubilized in TE volumes sufficient to allow accurate yield gel quantitation (normally <60 ng/μL) to avoid subsequent overloading of analytical gels. Minigels which contain sufficient ethidium bromide, are not overloaded and which run a sufficient length are most likely to demonstrate the presence of undigested DNA. Samples which display residual HMW bands or partially digested HMW DNA are likely to produce partial digestion bands. However,

the analyst may choose not to re-digest samples containing a small fraction of uncut DNA if the amount of DNA is limiting. Because each digestion/test gel cycle consumes a fraction of the DNA sample, it might be better to accept the prospect of partial digestion bands rather than to consume the DNA further. In samples which contain bacterial DNA, the uncut fraction may consist of *only* bacterial DNA. Some samples which appear completely digested on the post-restriction test gel will exhibit partial digestion bands if grossly overloaded (>> 400 ng/lane) on the analytical gel. More partial digestion bands may be observed due to the increased sensitivity of new alkaline phosphatase conjugated DNA probes (22,23,38).

The second defense against partial digestion bands is to understand them as an unavoidable, but well characterized artifact. The data presented here may be used to support the useful interpretation of forensic DNA samples exhibiting partial digestion products. A conclusion of partial digestion should be based on: a) a post-restriction minigel indicative of incomplete digestion (with some exceptions); b) consideration of all loci probed; and 3) agreement of the extra bands with the size ranges given in Table 1b. However, deviations from those size ranges (particularly the lower bounds) should be expected due to the nature of some DNA samples which are resistant to digestion. Fusion bands may occur in partially digested samples containing three- or four-banded patterns (or apparent two-banded patterns such as the one in Fig. 4b) at one or more loci. An additional option, which is used in one of the author's laboratories (Deadman) is to induce experimentally partial digestion in the DNA standard which matches the sample in question.

Acknowledgments

This study was made possible in part by a grant through the Illinois Criminal Justice Information Authority and a grant to E.A.B. from the Midwestern Association of Forensic Scientists. The authors would like to thank Taylor C. Scott of the Illinois State Police and Eric Buel of the Vermont Department of Public Safety for critical readings of the manuscript and Virginia Dahms of Bement Technologies (Bement, IL) for photography.

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