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N-ACETYLTRANSFERASE POLYMORPHISM

COMPARISON OF PHENOTYPE AND GENOTYPE IN HUMANS

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Abstract—*N*-Acetyltransferase (NAT) isoenzymes are encoded at two loci. One locus encodes an NAT which is expressed widely in tissues, does not vary amongst human individuals and is termed monomorphic NAT (mNAT). The second locus encodes an NAT which is termed polymorphic NAT (pNAT), has a distinct tissue distribution and is responsible for the difference in ability between individuals in acetylating certain arylamine (e.g. sulphamethazine) and hydrazine (e.g. isoniazid) drugs which are polymorphic substrates. We describe a simple DNA based method for genotyping individuals for pNAT. The 'fast' NAT allele (F1) and the three 'slow' alleles (S1, S2 and S3) can be distinguished by using PCR with oligonucleotide primers specific for pNAT followed by restriction enzyme digestion of the amplified product. Heterozygotes are easily identified. The genotype of individual Caucasians compares well with the extent of acetylation of sulphamethazine. The allele distribution of the Caucasian population described here differs from that reported after Southern blot analysis of a Japanese population (Deguchi *et al.*, *J Biol Chem* **265**: 12757–12760, 1990). The most frequent allele at the polymorphic *nat* locus in Caucasians, S1, is absent in the Japanese population. This difference between the two populations is likely to be the basis of the known interethnic variation in acetylator phenotype frequencies.

N-Acetyltransferase (NAT†) catalyses the acetylation of arylamines and hydrazines [1]. The substrates of *N*-acetyltransferase include 2-aminofluorene and benzidine which are carcinogens, procainamide, an arylamine anti-arrythmic agent and hydralazine, an anti-hypertensive drug which is a substituted hydrazine. *N*-Acetyltransferase activity in humans has been identified as polymorphic [2] on the basis of the ability to acetylate certain arylamines and hydrazines which have been designated "polymorphic substrates" [3]. Acetylator phenotype has been associated with differing susceptibility to disease. Slow acetylators are at increased risk of benzidine-induced bladder cancer [4] and the slow acetylator phenotype has been identified as occurring at a greater frequency in bladder cancer patients from a low-risk population [5]. Colonic cancer, in contrast, may be associated with the fast acetylator phenotype [6]. Immunotoxic side effects associated with hydralazine are found almost exclusively amongst slow acetylators [7] and in procainamide-induced systemic lupus erythematosus slow acetylators are at increased risk [8]. Drug-induced immunotoxicity is likely to be a multi-factorial disorder but in the case of hydralazine-induced systemic lupus erythematosus it has been argued that the slow acetylator phenotype is the single most important contributory factor [9].

Human *N*-acetyltransferase isozymes [10] are encoded at two loci [11]. One locus encodes *N*-acetyltransferase associated with the enzyme which is expressed widely in tissues including the liver and the protein catalyses the acetylation of *p*-aminobenzoic acid and anisidine [12] as well as that of the carcinogenic arylamine, 2-aminofluorene. It appears that this gene does not vary amongst human individuals and has been termed monomorphic *N*-acetyltransferase. The other *nat* locus encodes a second intronless gene of the same size as the invariant or monomorphic *nat*. The second *nat* locus is the polymorphic locus and the allelic gene products of this locus are expressed in liver and probably also in intestinal epithelial tissue [10]. Sulphamethazine, a "polymorphic substrate", is one drug acetylated by the enzyme encoded at this locus [13]. Different alleles have been identified as corresponding to variants of the polymorphic *nat* gene [11, 13]. The identified variants of polymorphic *N*-acetyltransferase differ at specific point mutations which modify the restriction fragment sites present. We have identified the presence of particular alleles in humans using a combination of the polymerase chain reaction with oligonucleotide primers specific for polymorphic *N*-acetyltransferase together with restriction enzyme digestion of the amplified product. The distribution of particular alleles has been related to the ability of individuals to acetylate the polymorphic marker substrate sulphamethazine.

MATERIALS AND METHODS

Determination of acetylation of sulphamethazine in urine. In order to determine the acetylator phenotype, sulphamethazine was used as the test drug. Volunteers (all Caucasians aged between 22 and 56) emptied their bladders, took 500 mg of sulphamethazine (sulphadimidine; CP Pharma-

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† Abbreviations: NAT, *N*-acetyltransferase; SMZ, sulphamethazine; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

ceuticals Ltd, Wrexham, U.K.) and collected urine either 3 or 6 hr later. It was found that there was a less than 5% difference between the percentage acetylation determined for individuals who were tested with SMZ on separate occasions or when urine was collected after either 3 or 6 hr. Urine was either used immediately or was stored at −20°. No caffeine-containing drinks were consumed for 6 hr prior to taking sulphamethazine and for the duration of the test period. Urine was centrifuged (14,000 rpm, 5 min in a microfuge), the supernatant was diluted 1:10 with HPLC-grade water and 10 μL of the diluted sample was applied to a Waters C18 Novapak column. Sulphamethazine and *N*-acetylsulphamethazine were separated using a Waters HPLC (600E solvent delivery system and 484 absorbance detector) with a mobile phase of acetonitrile:water (15:85), after the method of Baty *et al.* [14], and a flow rate of 1 mL/min. Sulphamethazine as standard was purchased from the Sigma Chemical Co. (Poole, U.K.). *N*-Acetylsulphamethazine as standard was synthesized and recrystallized from ethanol as described [14]. All HPLC-grade water was generated with a Purite reverse osmosis system linked to deionizer and organic product removal cartridges (Stillplus, Purite, Thame, U.K.). Sulphamethazine and *N*-acetylsulphamethazine in urine were determined from the areas under the peaks using a Waters Data module integrater. The percentage acetylation was determined from the area of the acetyl sulphamethazine peak expressed as a percentage of the combined sulphamethazine and acetylsulphamethazine peaks in urine. In this test, fast acetylators were taken to include all individuals with more than 88% acetylation.

Polymerase chain reaction. Genomic DNA from cultured cells or from the buffy coat of whole blood from volunteers was prepared by proteinase K digestion and phenol–chloroform extraction [15]. Amplification of genomic DNA (approximately 200 ng) was carried out by the polymerase chain reaction (PCR) in 100 μL (total volume) of 0.01 M Tris–HCl (pH 8.3), 0.05 M KCl, 1.5 mM $MgCl_2$, containing 200 μM of each dNTP (Boehringer-Mannheim, Lewis, U.K.), 100 μg/mL gelatin, 50 pmol of each primer and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus). Two sets of oligonucleotide primers have been used which amplify polymorphic *N*-acetyltransferase. Using Nat-Hu 7 and Nat-Hu 8, the amplification conditions were 30 cycles: annealing at 58°, 1 min; extension at 72°, 2 min and denaturation at 94°, 0.5 min; and using Nat-Hu 14 and Nat-Hu 16, the amplification conditions were 30 cycles: annealing 56°, 1 min; extension 72°, 2 min and denaturation 94°, 0.5 min. Oligonucleotide primers (see Table 1) were synthesized by Dr A. J. Day, MRC Immunochemistry Unit, Oxford. PCR was carried out using a Prem III automated temperature cycling device (LEP Scientific Ltd., U.K.). After amplification, the DNA product (10 μL) was used directly from the aqueous phase or was precipitated with ethanol [16] and resuspended in 10 μL of water.

Restriction enzyme digestion. Digestion of DNA (approximately 1 μg in 10 μL) was carried out in a total volume of 20 μL using the appropriate SuRE cut buffer (Boehringer-Mannheim). For digestion of DNA with *Kpn*I, 9 units of enzyme were used in a buffer containing 100 μg/mL BSA at 37° for 3 hr. For digestion with *Bam*HI, 11 units of enzyme were used at 37° for 3 hr and for digestion with *Taq*I, 11 units of enzyme were used at 65° for 3 hr. In cases where some amplification of monomorphic *nat* had occurred using Nat-Hu 14 and 16 (less than 3% of the total product), *Hin*dIII (6 units) was added to

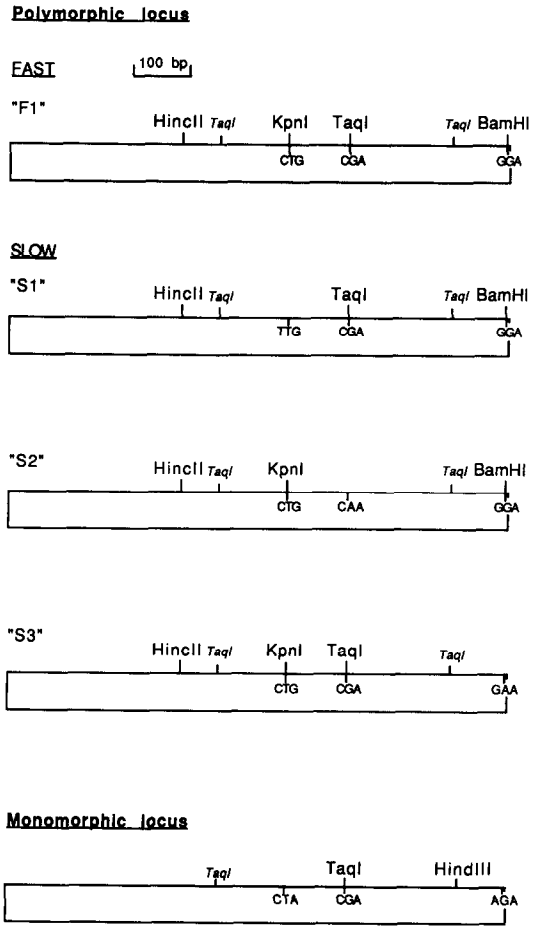


Fig. 1. Summary of the human *N*-acetyltransferase genes encoded at the monomorphic and polymorphic loci. The coding region of all the genes is 870 base pairs. The *Hinc*II site is present in all the polymorphic genes but absent in the monomorphic gene while the *Hin*dIII site is absent from the polymorphic genes but is present in the monomorphic gene. The monomorphic gene has been described as D-24 [13] and NAT1 [11]. Four distinct alleles have been identified at the polymorphic locus. F1 encodes NAT which corresponds to the fast phenotype and has previously been described as gene 1 [17], O-7 [13] and NAT2 [11]. S1, S2 and S3 encode *N*-acetyltransferase which corresponds to the slow phenotype. S1 has been described [15, 19] and S2 has previously been called Gene 3 [17]. S3 has previously been described as Gene 2 [17] and D-14 [13]. The *Kpn*I site (480 bp) is absent from the S1 allele though present in the other polymorphic alleles. Two *Taq*I sites (363 and 759 bp in *italics*) are present in all the polymorphic alleles. The S2 allele lacks a third *Taq*I site at 588 bp (large type). The *Bam*HI site (857 bp) is absent from the S3 allele but present in the other polymorphic alleles.

Table 1. Oligonucletides complementary to the polymorphic *nat* gene used to prime PCR

Name	Nucleotide sequence
Nat-Hu7	tgtcgacGGCTATAAGAACTCTAGGAAC (sense)
Nat-Hu8	tgtcgacAATAGTAAGGGATCCATCACC (antisense)
Nat-Hu14	GACATTGAAGCATATTTTGAAAG (sense)
Nat-Hu16	GATGAAAGTATTTGATGTTTAGG (antisense)

Triangles (△) show nucleotides not complementary to the monomorphic *nat* locus allele. Lower case letters refer to nucleotides added to the *nat* sequence in order to incorporate a *Sal*I restriction site.

the *Bam*HI digest (above) at 1½ hr to remove residual monomorphic *nat* (Fig. 1). *Hinc*II digestion (37°, 3 hr) was also carried out using 3 units of enzyme to confirm that polymorphic *nat* had been amplified. Digested DNA (10 μL) was separated by agarose gel electrophoresis (1.5%, 10 V/cm) along with DNA molecular weight markers (Gibco, BRL, Uxbridge, U.K.) for comparison. DNA was visualized by ethidium bromide and UV trans-illumination. All restriction enzymes were purchased from Boehringer-Mannheim.

Calculations. The distribution of genotypes expected assuming a Hardy–Weinberg equilibrium was calculated from the allele frequencies, using the equation $p^2+2pq+2pr+2ps+q^2+2qr+2qs+r^2+2rs+s^2$ where p is S1, q is S2, r is S3 and s is F1, corresponding to the 10 possible NAT genotypes under the classification in Fig. 1. For the Japanese population a truncated equation, $q^2+2qr+2qs+r^2+2rs+s^2$ was used since the S1 allele (p) was not identified [17].

The Chi Squared test [18] was used to compare the observed genotype incidence $(O)_n$ with the expected genotype incidence $(E)_n$ calculated above using the equation:

$$\chi^2 = \Sigma (O_n - E_n)^2 / E_n$$

where, for the Caucasian population, the number of possible genotypes (n) is 10. For the Japanese population the number of possible genotypes (n) is 6. The number of degrees of freedom (df) is defined as one less than the number of possible genotypes. The smaller the value of χ^2 the less significant the difference between the observed and expected genotype frequencies.

RESULTS

Amplification of polymorphic nat

The primers used are complementary to the sequence of the polymorphic *nat* gene [11, 13]. Table 1 illustrates where the primers are not complementary to the monomorphic *nat*. The size of the product obtained with Nat-Hu 7 and Nat-Hu 8 as primers was 840 base pairs (bp) [19] and with Nat-Hu 14 and Nat-Hu 16 was 1000 base pairs (Figs 2 and 3c, track 6). The predicted size [11, 13] of these products was 839 and 999 base pairs, respectively. The DNA which was amplified with Nat-Hu 7 and Nat-Hu 8 as a pair of primers was digested with *Hinc*II into fragments of 560 and 280 bp and the DNA which was amplified with Nat-Hu 14 and Nat-Hu 16 as a pair of primers was digested with *Hinc*II into fragments of 700 and 300 bp (Table 2). The monomorphic and polymorphic *nat* genes differ by the presence of a *Hinc*II restriction site which is

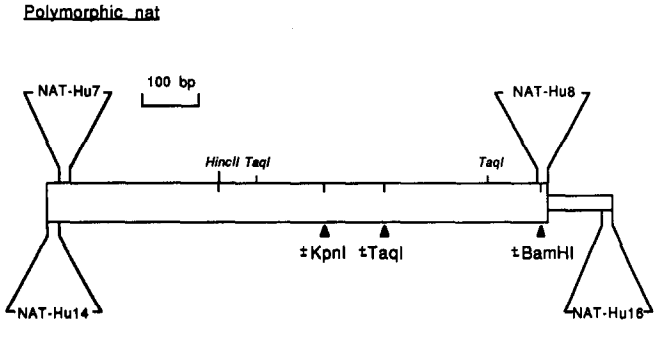
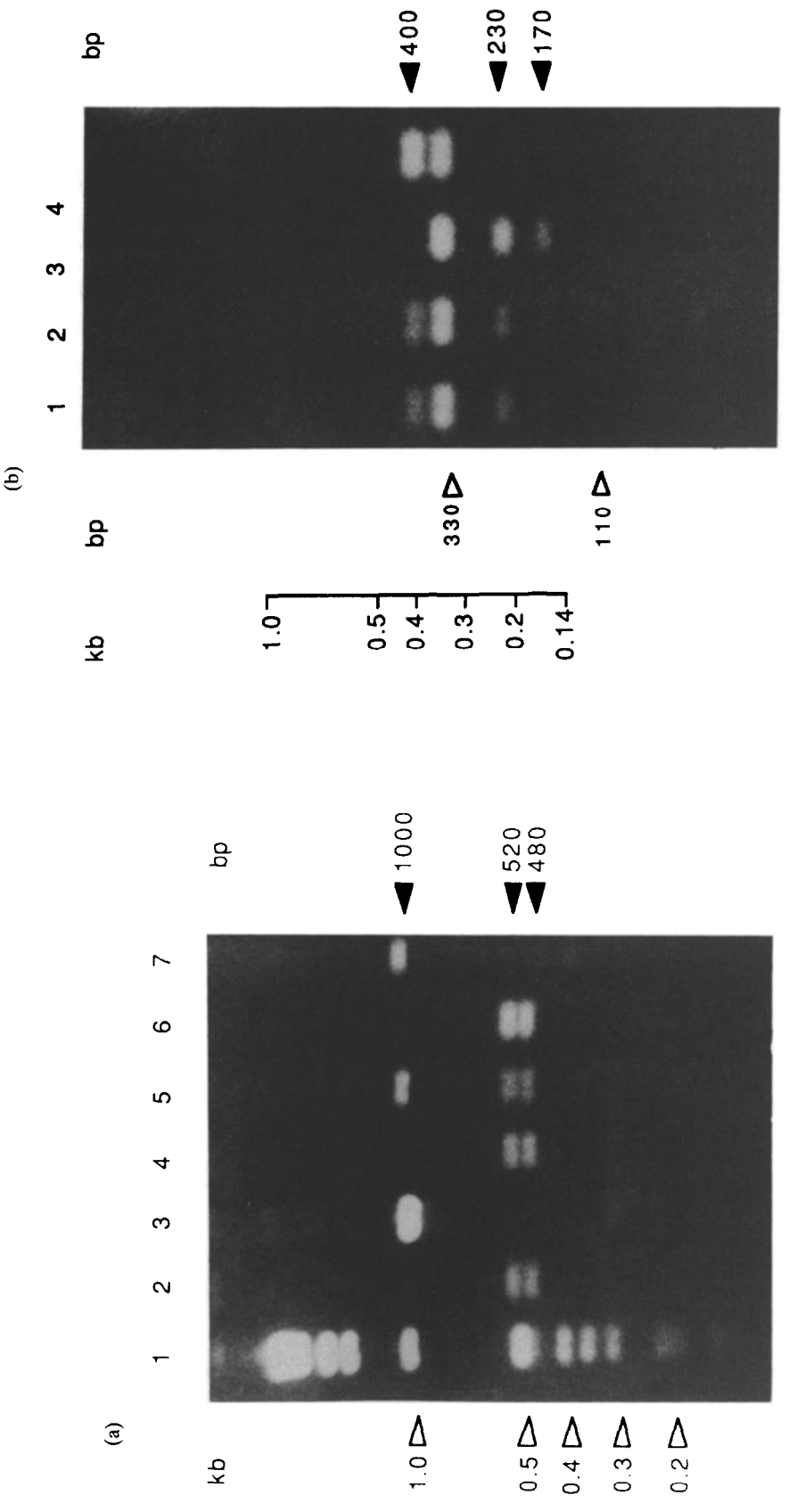


Fig. 2. Partial restriction map of the human polymorphic *N*-acetyltransferase genes showing the position of the oligonucleotides (Table 1) used to amplify specifically the polymorphic alleles using the polymerase chain reaction. The arrow heads represent informative restriction sites (Fig. 1). Restriction enzyme sites which have been used and which are present in all known polymorphic alleles are indicated in *italics*.


(b)
bp
400
230
170
1 2 3 4
bp
330
110
kb
1.0
0.5
0.4
0.3
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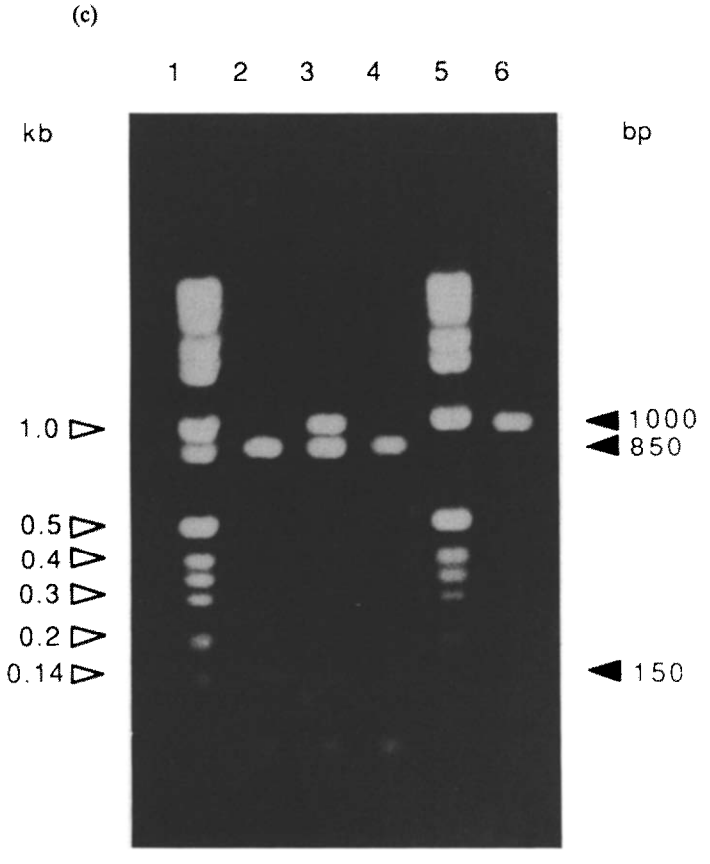

Fig. 3. (a) Identification of the S1 allele by *Kpn*I digestion. Tracks 2–7 show DNA from different individuals after amplification with the Nat-Hu 14 and Nat-Hu 16 primer pair which have been subsequently digested with *Kpn*I. The open arrow heads denote the DNA molecular weight markers (track 1). The filled arrow heads show the sizes of the DNA lacking a *Kpn*I site (1000 bp, tracks 3, 5 and 7) and possessing a *Kpn*I site (520 and 480 bp; tracks 2, 4, 5 and 6). The 1000 bp band corresponds to the S1 allele. (b) Identification of the S2 allele by *Taq*I digestion. Tracks 1–4 show DNA from different individuals after amplification with the Nat-Hu 7 and Nat-Hu 8 primer pair which have been subsequently digested with *Taq*I. The open arrow heads show the sizes of DNA fragments present in all *Taq*I digests. The filled arrow heads show the sizes of the DNA fragments which provide information on the allele composition of the individuals. DNA lacking a *Taq*I site at 588 bp (see Fig. 1) gives a 400 bp band (tracks 1, 2 and 4) and possessing a *Taq*I site gives 230 and 170 bp bands (tracks 1, 2 and 3). The 400 bp band corresponds to the S2 allele. (c) Identification of the S3 allele by *Bam*HI digestion. Tracks 1–4 show DNA from different individuals after amplification with the Nat-Hu 14 and Nat-Hu 16 primer pair which have been subsequently digested with *Bam*HI. The open arrow heads denote the DNA molecular weight markers (tracks 1 and 5). The filled arrow heads show the size of the DNA lacking a *Bam*HI site (1000 bp, track 3) and possessing a *Bam*HI site (850 and 150 bp; tracks 1, 2, 3 and 4). The 1000 bp band corresponds to the S3 allele. Track 6 shows undigested DNA.

Table 2. Digestion of amplified polymorphic *nat* with *Hinc*II and *Hin*dIII

Primers	Fragment size (bp)		
	Undigested	*Hinc*II	*Hin*dIII
Nat-Hu 7, 8	840	280,560	840
Nat-Hu 14, 16	1000	300,700	1000

The amplification and digestion conditions are described in Materials and Methods.

present in all the polymorphic alleles which have been identified [11, 13, 19] but is absent from the monomorphic *nat* locus allele which has been described [11, 13]. The sizes of the *Hinc*II fragments which were generated were as predicted from the positions of the primers and the *Hinc*II site (Table 2). It has been demonstrated previously by direct sequencing of the DNA product of the polymerase chain reaction with Nat-Hu 7 and Nat-Hu 8 as primers that the polymorphic *nat* gene is amplified [19]. In some cases less than 3% of the total staining intensity of the PCR products remained undigested with *Hinc*II. This is likely to have been due to monomorphic *nat* since the *Hinc*II resistant material, unlike the major product, was cleaved by *Hin*dIII.

Determination of N-*acetyltransferase genotype*

The different alleles encoding the polymorphic *N*-acetyltransferase are summarized in Figs 1 and 2.

The S1 allele lacks a *Kpn*I site. Figure 3a shows the identification of the S1 allele by *Kpn*I digestion of the PCR product amplified in the presence of primers Nat-Hu 14 and Nat-Hu 16 (1000 bp). The absence of digestion in tracks 3 (*EW*) and 7 (*JW*) indicates that both polymorphic alleles are the S1 type (i.e. S1S1). Track 5 (*TD*) shows incomplete digestion with *Kpn*I, indicating one S1 allele (more information on the second allele is gained by *Taq*I and *Bam*HI digestions). Complete digestion to fragments of 520 and 480 bp demonstrates the absence of an S1 allele in subjects *HB*, *SP* and *CM* (tracks 2, 4 and 6).

Figure 3b shows the identification of the S2 allele by *Taq*I digestion of the PCR product amplified in the presence of primers Nat-Hu 7 and Nat-Hu 8 (840 bp). There are *Taq*I sites common to all polymorphic alleles (Figs 1 and 2) which generate bands of 330 and 110 bp in all tracks on digestion with *Taq*I. There is one *Taq*I site which is absent from the S2 allele (Fig. 3). Informative *Taq*I digestion products are bands of 400, 230 and 170 bp. The presence of a band at 400 bp indicates the absence of the central *Taq* site at 588 bp (Fig. 1, large print) which characterizes the S2 allele. Track 4 (Fig. 3b) (*GS*) illustrates an S2 homozygote since there is no digestion of the 400 bp fragment. Where the 400 bp fragment is split into two smaller products (230 and 170 bp) one of the other alleles (S1, S3 or F1) is indicated. Tracks 1 (*FM*) and 2 (*SK*) illustrate S2 heterozygotes while track 3 (*RS*) demonstrates the absence of an S2 allele.

The S3 allele lacks a *Bam*HI site and Fig. 3c shows the identification of the S3 allele by *Bam*HI digestion of the PCR product amplified in the presence of primers Nat-Hu 14 and Nat-Hu 16 (1000 bp). An S3 heterozygote (*KN*) is shown in Fig. 3c, track 3. The upper band co-migrates with the PCR product undigested by *Bam*HI in track 6 indicating an S3 allele; the lower bands at 850 and 150 bp indicate a second distinct allele. The band at 150 bp is very faint. Tracks 2 (*CM*) and 4 (*AC*) (Fig. 3c) show complete digestion of the 1 kb PCR product with *Bam*HI indicating the absence of an S3 allele.

The genotype of each individual was determined by carrying out three separate restriction enzyme digests with *Kpn*I, *Taq*I and *Bam*HI. If, after digestion with *Kpn*I, *Taq*I, and *Bam*HI, alleles could not be identified as either S1, S2 or S3, the remaining allele(s) were assigned as F1 since the F1 polymorphic allele possesses all of these restriction sites (Fig. 1).

So far, genotyping of 31 individuals has been carried out in this way. All 31 individuals could be assigned a genotype consisting of two alleles at the polymorphic locus compatible with the scheme shown in Fig. 1.

In the heterozygote individual *KN*, Fig. 3c, track 3, who has been assigned the genotype S2S3, digestion of the PCR amplification product using *Bam*HI produced two bands of approximately equal intensity, the 1000 bp band derived from the S3 allele lacking the *Bam*HI site and the 850 bp band derived from the S2 allele which contains the *Bam*HI site (Fig. 1). This illustrates that each polymorphic *nat* allele in any given individual is amplified to the same extent. The relative intensity of the bands in any individual track of Fig. 3a and b (*Kpn*I and *Taq*I digestion, respectively) also suggests equal amplification of distinct alleles at the polymorphic locus.

Altogether, 10 genotypes are possible under the existing classification scheme. So far only seven of these genotypes have been identified. The S1S3, S3S3 and S3F1 genotypes have not been identified due to the low frequency of occurrence of the S3 allele (Table 3).

Comparison of phenotype and genotype

The phenotype of NAT was determined after ingestion of SMZ by 22 of the 31 genotyped individuals. The relationship between genotype and phenotype is illustrated in Fig. 4.

The S1, S2 and S3 alleles are clearly associated with the slow phenotype when present without the F1 allele. The F1 allele in heterozygotes and homozygotes produces the fast phenotype, predominantly (Fig. 4). There appears to be no difference in the S1, S2 or S3 polymorphic alleles in

Table 3. Comparison of polymorphic *nat* allelic frequencies of two different ethnic populations

Origin	Allelic frequencies (1%)			
	S1	S2	S3	F1
Caucasian	45.2	27.4	1.6	25.8
Japanese	—	25	7	68

The data for the Japanese population was deduced from Fig. 3 of Ref. 17.

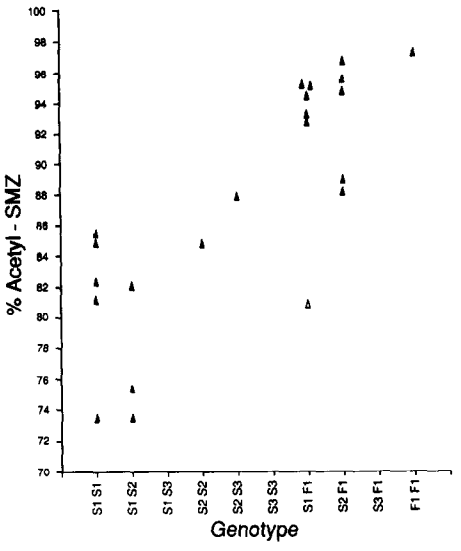

Fig. 4. Comparison of acetylator phenotype and genotype of human polymorphic *N*-acetyltransferase. The percentage conversion of sulphamethazine (SMZ) to acetyl-SMZ was determined for 22 unrelated Caucasian individuals as described in Materials and Methods. The open symbol (*EC*) does not fit its phenotypic classification and is referred to in the discussion.

relation to acetylator phenotype, however, more individuals are required before firm conclusions can be drawn on the relative contribution of these different alleles to acetylator phenotype. The slow acetylation phenotype has been related to a reduced amount of the NAT protein both in the liver cytosol of human surgical patients [20] and in an *in vitro* transient expression system [13] but further studies are required before the molecular relationship between these mutations and NAT phenotype can be defined. Results from all 31 genotyped individuals predict 52% slow (6 S1S1; 7 S1S2; 2 S2S2 and 1 S2S3) and 48% fast (9 S1F1; 5 S2F1 and 1 F1F1). This distribution is in agreement with phenotypic studies on Caucasian populations which have been performed over the past 30 years [21, 22].

Table 3 shows the allelic frequency in 31 Caucasian volunteers who were genotyped as described here. The expected genotype frequency of this Caucasian population, assuming a Hardy–Weinberg equilibrium, has been shown to be not significantly different from that observed using the Chi Squared test ($\chi^2 = 12.4$, df = 9 and $P > 0.1$). The NAT genotypes of individuals from a Japanese population have been described [17] using restriction enzyme digestion of genomic DNA in conjunction with Southern blotting. The expected genotype frequency of this Japanese population, assuming a Hardy–Weinberg equilibrium, has also been shown to be not significantly different from that observed using the Chi Squared test ($\chi^2 = 1.5$, df = 5 and $P > 0.1$). There was no suggestion of an S1 allele in the Japanese population using the method of Deguchi *et al.* [17] which would have identified the S1 allele had it been present. In the Caucasian population described in the present study the S1 allele accounts for 45% of all alleles at the polymorphic *nat* locus. This difference alone is enough to account for the striking difference in the distribution of phenotypes in these two populations, approximately 50% fast in Caucasians but 90% fast in Japanese [17, 21].

DISCUSSION

Determination of the *N*-acetyltransferase phenotype has been reported regularly for 30 years (see Ref. 23 for review). It has often been difficult to distinguish homozygote and heterozygote fast acetylators. Disease and possibly age may also affect the results of phenotypic studies involving the use of probe drugs [23, 24] or make the use of probe drugs impracticable. The genotype of an individual, in contrast to the phenotype which is determined, will not vary with age or disease state and requires only a small blood sample. The PCR method which is described here allows identification of RFLPs within the coding region of the polymorphic *nat* and shows a clear relationship to phenotype in the populations which have been investigated (above and Ref. 17). This also allows a genetic explanation for the inter-ethnic difference in polymorphic N-acetylation which have been described [21, 23].

Using this rapid, simple assay it will be possible to study the relationship between NAT genotype and diseases such as bladder cancer, colonic cancer and breast cancer where phenotypic studies have implicated that differences in NAT phenotype may be important (see Ref. 23 for review). This test can also be applied to populations of hydralazine and procainamide-induced systemic lupus erythematosus patients where a clear association with the slow phenotype has been demonstrated [7, 8, 25]. Investigation of an allele specific association rather than a phenotypic association might provide a more precise method of risk assessment in humans.

The method described, of genotyping individuals for NAT in conjunction with phenotyping, may also lend itself to the identification of as yet unidentified alleles of the polymorphic *nat* by sequence analysis of the *nat* genes of those individuals who do not exhibit the fast phenotype but are apparently fast under the existing genotyping regime (see Fig. 4, open symbol). Subsequently identified alleles could be added to the existing scheme of digestions. Indeed the existence of additional alleles at the polymorphic locus has been suggested by Blum *et al.* [26]. Should new alleles not contain mutations which modify restriction sites within the open reading frame of this gene, genotyping should still be possible by other methods currently under development in this laboratory for NAT such as allele specific PCR (ASPCR), which can differentiate alleles by selective amplification of DNA, which differs in only one base at the 3′ end of the oligonucleotide primer [27, 28].

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