cursors of anti-sheep PFC in (C57BL/6 × DBA/2)$F_1$ mice, but differed from results obtained with marrow cells of other strains (*3, 4*). Class differentiation of potentially immunocompetent marrow cells is apparently controlled by genetic factors that are not determinant-specific. Another genetic control of this type was described for mouse serum levels of immunoglobulins and of agglutinins to sheep and pigeon erythrocytes (*11*).

It was desirable to subject the results obtained by transplanting fractionated marrow cells ($2.5 \times 10^5$ cells per mouse) to a more rigorous test. For this reason, twice as many cells of the fractions with 23 and 25 percent BSA were tested for anti-sheep PFC, and half as many cells for anti-burro PFC (Table 2). This change in the numbers of grafted marrow cells did not obscure the depletion of precursors of sheep specificity nor the enrichment of precursors of burro specificity. Results of similar variations of the numbers of transplanted marrow cells from the 31 percent BSA fraction strengthened the conclusion that in this region of the gradient precursors of anti-burro PFC were depleted instead of precursors of anti-sheep PFC.

The data are best explained by specificity differentiation of marrow cells responsible for PFC production, presumably of the precursor cells of PFC. Restriction for antibody specificity preceded that for immunoglobulin class in this mouse strain. Although differing density gradient profiles of specific splenic precursors of antibody-forming cells were resported (*12*), these data are the first direct demonstration of commitment to antibody specificity in less differentiated and mature cells of the immune system. The separation of restricted marrow cells could have been due to osmotic rather than to density gradients (*13*), but in either case it was not clear why physical parameters of precursors for SRBC and BRBC should differ. It is possible that prior exposure of the cells to immunogens cross-reacting with one or the other of the erythrocytes caused commitment. However, it would be unrealistic to expect density differences such as those reported here between marrow precursors for all specificities.

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7. The concentration of each bone marrow fraction was adjusted to $5 \times 10^5$ cell/ml. Thymocytes from 9-week-old, syngeneic female donors were suspended in Eagle's medium ($10^9$ cell/ml). Each mouse was injected with 1 ml of the cell mixture 2 to 4 hours after exposure to 950 to 1000 rads of $^{137}Cs$ gamma radiation.
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## Transcription of Nonrepeated DNA in Mouse Brain

Abstract. *Under normal conditions of DNA renaturation, about 60 percent of mouse DNA fragments renature at a rate consistent with their being present only once per sperm. These nonrepeated sequences (also called single-copy or unique) may be used in RNA-DNA hybridization experiments to provide quantitative estimates of RNA diversity. About 10 percent of the mouse single-copy sequences are transcribed in mouse brain tissue. Estimates of about 3 percent were obtained for mouse liver and kidney RNA's. If only one of the complementary DNA strands is transcribed, this hybridization value implies that the equivalent of at least 300,000 different sequences of 1000 nucleotides are expressed in mouse brain tissue. It is suggested that the large amount of DNA in mammals is functionally important, and that a substantial proportion of the genome is expressed in the brain.*

Estimates on the extent of transcription of RNA from DNA in various eukaryotic cells are usually based on experiments in which RNA is hybridized to denatured DNA immobilized in agar or on nitrocellulose filters (*1*). These data are interpreted in a qualitative manner because of experimental limitations imposed by most eukaryotic DNA's. Renaturation kinetics of single-strand, sheared DNA's are consistent with the suggestion that many eukaryotic genomes contain repeated (rapidly renaturing) and nonrepeated (also termed single-copy or unique) nucleotide sequences (*2*). In view of the low RNA concentrations and the relatively short time allowed for reaction, it is likely that many hybridization estimates of transcriptional diversity pertain to the repeated fraction of the eukaryotic genome. This latter inference is supported by physical characterization of such RNA-DNA hybrids. Thermal stability measurements indicate that incomplete base pairing occurs in contrast to the much more precise base pairing expected for RNA hybridized to nonrepeated DNA. Thus, while important conclusions can be drawn from this type of RNA-DNA hybridization, the quantitative aspects of transcription in eukaryotic cells are not well understood. This information is required for a better understanding of the control of cell and tissue differentiation, and for insight into the functional significance of the enormous sequence complexity of most eukaryotic genomes (*2, 3*).

Methods of assaying transcription of nonrepeated DNA have been developed and applied to *Xenopus* oocytes and fetal mice (*4*). We have examined the transcriptional representation of the nonrepeated DNA fraction in RNA's from mouse liver, kidney, and brain. Our results indicate that RNA from the brain hybridizes to a large fraction of the nonrepeated mouse DNA. The RNA's from mouse liver and kidney also represent extensive diversity of nucleotide sequence, although hybridization values for RNA's from these tissues are about one-third those obtained for brain RNA's. These data are in accord with the results of Smith (*5*) obtained by hybridizing labeled RNA to filter-bound, unique, mouse DNA.

For our experiments we used radio-

actively labeled DNA and unlabeled RNA (*6*). RNA-DNA hybrids are detected by their binding to hydroxyapatite. The labeled nonrepeated DNA, present at low concentrations, will not self-anneal during the time of reaction. High concentrations of RNA complementary to this DNA are expected to result in RNA-DNA hybrids that bind to the hydroxyapatite in 0.14*M* sodium phosphate buffer (PB), *p*H 6.8. The thermal stabilities of these complexes and the sensitivity of their formation to ribonuclease and alkaline hydrolysis support the conclusion that the reaction products are RNA-DNA hybrids.

Labeled DNA's were prepared from mouse L cells grown in Eagle's minimal essential medium containing 5 percent fetal calf serum and 5 $\mu$c of [methyl-$^3$H]thymidine per milliliter (specific activity 19.9 c/mmole). Cells were harvested after 72 hours, and nuclei were isolated (*7*). The protein was separated from the DNA by treatment with phenol before purification with ribonuclease and pronase (*8*). Labeled DNA, dissolved in buffered saline (0.1*M* NaCl, 0.02*M* tris-HCl, *p*H 72), was sheared in a pressure cell (*9*) at 30,000 psi (1 psi = 6.9 kN/m$^2$) to a single-strand molecular weight of about 120,000. After being precipitated with 2.5 volumes of 95 percent ethanol at −15°C, DNA's were dissolved in 0.14*M* PB, *p*H 6.8.

Sheared DNA was denatured at 102°C for 10 minutes and then allowed to renature at 2 mg/ml to a $C_0t$ value of 220 (*10*). The partially renatured DNA was then passed through a hydroxyapatite column (1.2 by 2.0 cm) (Clarkson, lot 6370) at 60°C in 0.14*M* PB. Unrenatured DNA was eluted first with 8 ml of 0.14*M* PB; renatured fragments were recovered by washing with 0.5*M* PB. About 60 percent of the DNA was eluted in the 0.14*M* fractions. Recovery of the DNA from the column was greater than 98 percent. For some DNA's, the 0.14*M* fractions were concentrated by filtration (Diaflo) and again passed through a hydroxyapatite column to remove reassociated DNA that failed to bind in the initial passage. About 96 percent of the DNA was collected in the 0.14*M* fraction. Portions of these unassociated DNA's, which were expected to be unique sequences, as defined by these reaction conditions, were renatured in the presence of unlabeled DNA from mouse liver and $^{14}$C-labeled bacterial DNA from *Bacillus subtilis*. The kinetics of renaturation, as measured by hydroxyapatite chromatography of fractions taken at different times during the reaction, are shown in Fig. 1. The mouse unique labeled DNA's exhibited second-order kinetics with a reaction rate about 1000 times slower than that of the *B. subtilis* DNA, as would be expected from the difference in unit genome size of these organisms (*3*). The unfractionated, mouse DNA renatured biphasically, with the fast reassociating component comprising about 40 percent of the total DNA. These experiments (Fig. 1) provide assurance that no significant proportion of the $^3$H-labeled, nonrepeated, mouse DNA is complementary to the fast renaturing fractions of mouse DNA, and that the rate constant of the expected second-order kinetics is consistent with that predicted for mouse DNA sequences present only once per sperm (*2*, *3*).

Nuclei from whole brain, liver, and kidney of Balb/C mice were prepared by homogenizing minced tissue at 2° to 4°C in 0.32*M* sucrose containing 1 m*M* $MgCl_2$, 0.001*M* PB, and 0.3 percent

Table 1. Hybridization values for nonrepeated mouse DNA and RNA's from different tissues. Treatment of RNA's with ribonuclease A was routinely included in parallel with nondigested RNA's. Reaction values for these controls for the RNA's shown here were equivalent to DNA alone or DNA with *Escherichia coli* RNA (see Fig. 2). Numbers after the source indicate different preparations of RNA. All mouse RNA's were isolated from nuclei.

| RNA's | | DNA | | Hybridization (%) |
|---|---|---|---|---|
| Source | Amount (mg/ml) | Prep. No. | Amount ($\mu$g/ml) | |
| Brain 1 | 9 | 1 | 12 | 12.4 |
| Brain 1 | 9 | 1 | 12 | 11.6 |
| *E. coli* | 9 | 1 | 12 | 1.6 |
| Brain 2 | 11 | 2 | 10 | 10.8 |
| Liver 1 | 11 | 2 | 10 | 4.1 |
| Kidney 1 | 11 | 2 | 10 | 4.0 |
| *E. coli* | 11 | 2 | 10 | 0.2 |
| None | | 2 | 10 | 0.8 |
| Brain 2 | 12 | 1 | 12 | 13.0 |
| Liver 1 | 12 | 1 | 12 | 5.2 |
| Kidney 1 | 12 | 1 | 12 | 4.8 |
| *E. coli* | 12 | 1 | 12 | 1.2 |
| Brain 4 | 12 | 2 | 12 | 8.6 |
| Liver 3 | 12 | 2 | 12 | 3.0 |
| Kidney 3 | 12 | 2 | 12 | 2.7 |
| *E. coli* | 12 | 2 | 12 | 0.2 |

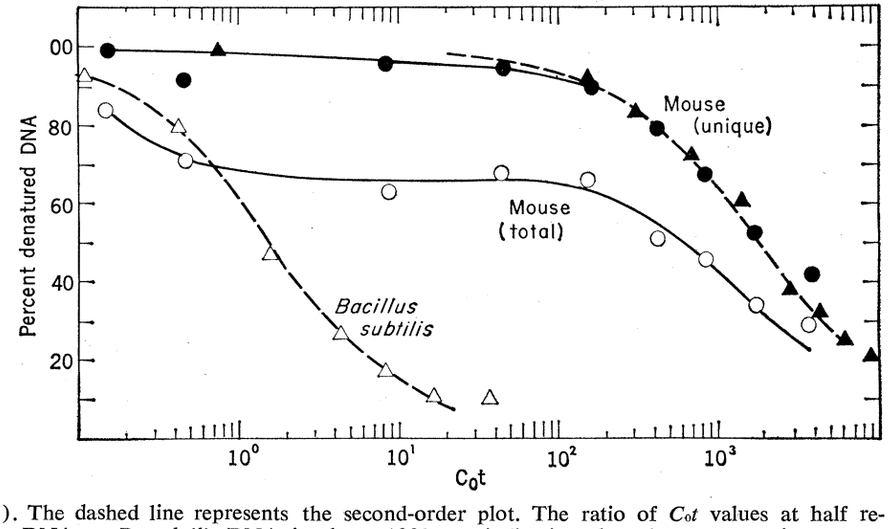


Fig. 1. Unlabeled DNA from mouse liver (2 mg/ml) was mixed in 0.12*M* PB with a low concentration of $^3$H-labeled unique (nonrepeated) mouse L-cell DNA (DNA 1 in Table 1; 420,000 count/min per microgram, 10 $\mu$g/ml). $^{14}$C-Labeled *Bacillus subtilis* DNA (800 count/min per microgram, 12 $\mu$g/ml) was included as an internal renaturation standard. All DNA's were sheared to a single-strand molecular weight of about 120,000. The mixture was heat-denatured (98°C, 10 minutes) and incubated at 62°C. Portions containing 100 $\mu$g of DNA were fractionated on hydroxyapatite columns to determine the percentage of denatured DNA remaining at various times. The $C_0t$ values for mouse DNA's (total and unique) are based on the concentration of unlabeled mouse DNA. $C_0t$ values for *B. subtilis* DNA were calculated from the concentration of this DNA, since its renaturation is unaffected by the heterologous mouse DNA (*3*). The dashed line represents the second-order plot. The ratio of $C_0t$ values at half renaturation ($C_0t_{1/2} = 1/k_2$) of mouse unique DNA to *B. subtilis* DNA is about 1000, an indication that the mouse unique DNA fraction represents sequences present once per sperm (*3*). The mouse unique DNA shows no tendency to reassociate with the fast-renaturing mouse sequences, verifying that little repeated DNA contaminates the $^3$H-labeled unique sequences. Similar results were obtained with a second preparation of mouse unique DNA, ▲, (DNA 2 in Table 1).

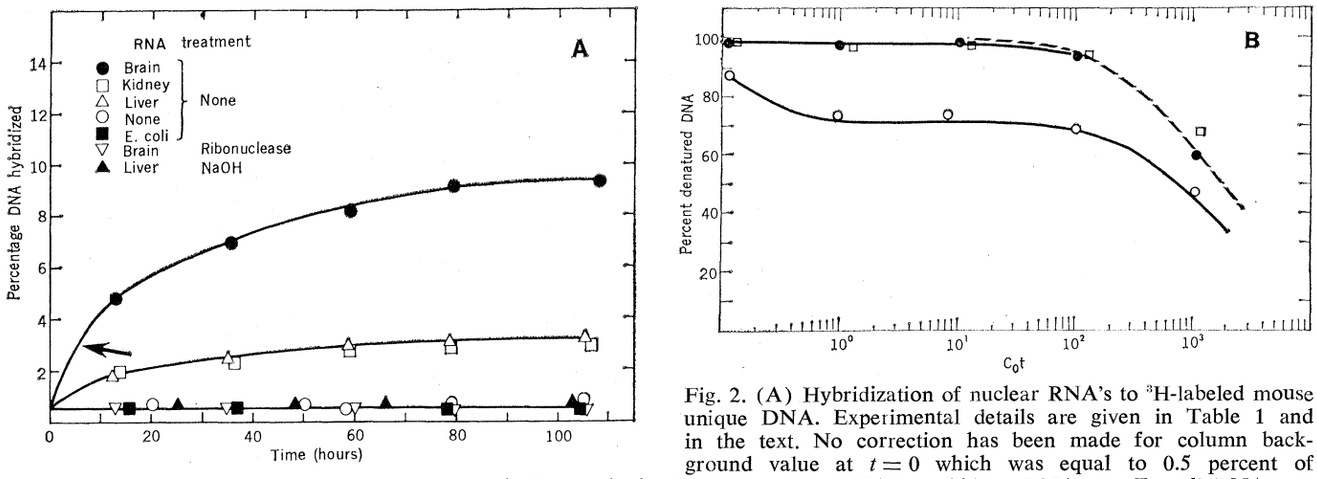


Fig. 2. (A) Hybridization of nuclear RNA's to $^3$H-labeled mouse unique DNA. Experimental details are given in Table 1 and in the text. No correction has been made for column background value at $t = 0$ which was equal to 0.5 percent of the input DNA. Symbols: ○, DNA only (12 μg/ml); ●, brain RNA; △, liver RNA; □, kidney RNA; ■, *E. coli* RNA; ▽, ribonuclease-treated brain RNA (20 μg of ribonuclease A, 30 minutes at 37°C); ▲, liver RNA first treated with 0.2*M* NaOH (10 hours at 37°C). Ribonuclease-treated liver and kidney RNA's, not shown, were equivalent to ribonuclease-treated brain RNA. Concentrations of all RNA's were 12 mg/ml. (B) Renaturation of labeled DNA isolated from DNA-RNA hybrids. DNA-RNA hybrids formed during the early part of the reaction (see arrow in A) and after 90.5 hours of incubation with brain RNA were isolated with hydroxyapatite columns. The labeled hybrids were then mixed with 4.2 mg or 0.1 mg total mouse DNA per milliliter. Each reaction mixture contained approximately 100 μg of sheared total DNA and approximately 0.03 μg (13,000 count/min) of labeled DNA. The samples were denatured by heat and allowed to reanneal at 60°C in 0.12*M* phosphate buffer. Because the reaction is driven by the high concentration of unlabeled DNA, it was unnecessary to remove the small amount of RNA. Samples were removed at various times, diluted in 0.12*M* PB, and fractionated on hydroxyapatite columns. The optical density and the radioactivity were determined for the denatured and renatured fractions. Labeled DNA (●) recovered from DNA-RNA hybrids formed early in the reaction renatured at a rate expected for nonrepeated DNA. Similar renaturation kinetics were observed with DNA recovered from the hybrids formed after long-term incubation (□). The dashed line is a curve of the expected second-order kinetics for nonrepeated, mouse DNA (see legend, Fig. 1). Total unlabeled DNA renatured biphasically as expected for a mixture of repeated and nonrepeated sequences (○).

Triton X-100. Pellets of crude nuclear material obtained after centrifugation at 1200*g* were resuspended with a Dounce homogenizer in 2.0*M* sucrose (ribonuclease-free). The suspension was layered over 2.2*M* sucrose and centrifuged at 75,000*g* for 35 minutes at 0°C in an SW-41 rotor (Beckman). RNA was extracted from the nuclei with a 1 : 1 mixture of 0.5 percent sodium dodecyl sulfate in tris-buffered 0.1*M* NaCl and phenol at 65°C. The interfaces between the layers were reextracted twice. RNA was precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol; this procedure was repeated three times to remove phenol. RNA, to which mouse DNA labeled with [2-$^{14}$C]-thymidine (5 μg/ml, 22,000 count/min per microgram) was added to monitor deoxyribonuclease activity, was further purified with deoxyribonuclease (200 μg/ml, 1.5 hours at 37°C, 0.001*M* $MgCl_2$, 0.025*M* tris, *p*H 6.8) and self-digested pronase (50 μg/ml, 30 minutes at 37°C). RNA preparations were passed through G-100 Sephadex (Pharmacia) and twice precipitated with 2.5 volumes of ethanol. The final RNA preparations contained only background amounts of $^{14}$C, an indication that the deoxyribonuclease hydrolysis and subsequent filtration on Sephadex effectively removed the DNA.

Unlabeled RNA's from brain, liver, kidney, and *Escherichia coli* were mixed with $^3$H-labeled, unique DNA from mouse L cells. The RNA concentrations were adjusted in 0.14*M* PB to about 12 mg/ml in the presence of 12 μg of $^3$H-labeled DNA per milliliter. The specific activities of the DNA's were 3.9 to $4.2 \times 10^5$ count/min per microgram (30 percent counting efficiency). Portions (5 or 10 μl) of these reaction mixtures were sealed in 20-μl, glass capillary pipettes to eliminate evaporation and to facilitate sampling. Reaction mixtures were held at 102°C for 8 minutes, and annealing was then conducted at 62°C. Samples were taken periodically, and reactions were stopped by dilution with 0.5 ml of 0.14*M* PB at 4°C.

Hybridized $^3$H-labeled DNA was separated from nonhybridized DNA on hydroxyapatite columns (1 by 0.8 cm) at 62°C. Samples were eluted stepwise with 0.14*M* and 0.5*M* PB (usually 8 ml at each buffer concentration) until no further radioactivity was removed. Portions of the fractions eluted at 0.14 and 0.5*M* PB, adjusted to equal salt concentrations, were counted in toluene-based scintillation fluid (Liquifluor) containing 10 percent (by volume) Bio-Solv BBS-3 (Beckman).

Nuclear RNA's from mouse brain, liver, and kidney react with about 9, 3, and 3 percent of mouse unique DNA (Fig. 2A). The control experiments show that these reactions are RNA-dependent. Prior treatments of RNA preparations with ribonuclease A or NaOH, or incubation of DNA without RNA, reduce DNA binding to that observed for single-strand DNA, that is, 0.4 to 0.8 percent. The ribonuclease-treatment control was included with all RNA preparations and gave similar results.

Additional evidence that the observed hybrids are due to RNA and not to the possible presence of DNA in the RNA preparations was obtained by treatment of the hybrids with ribonuclease (50 μg/ml) at 50° to 55°C, which reduced the binding of the hybrid fraction to hydroxyapatite to approximately 10 percent of the control value. At high temperatures ribonuclease has no effect on the binding of DNA-DNA duplexes. Hence this experiment supports the conclusion that the hybridization values are due to RNA and not the presence of contaminating DNA. The reaction is species specific, since *Escherichia coli* RNA does not increase DNA binding (Fig. 2A).

Further evidence that it is nonrepeated DNA that hybridizes with brain RNA is shown in Fig. 2B. DNA

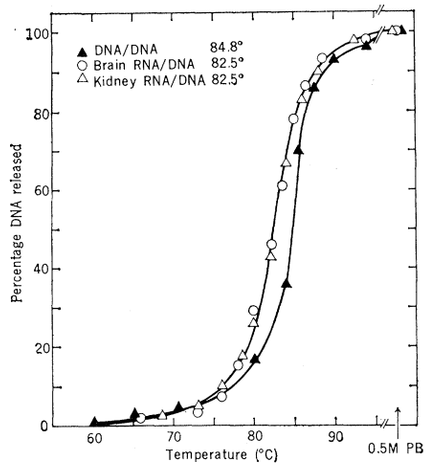


Fig. 3. Thermal denaturation of brain and kidney RNA-DNA hybrids and DNA-DNA duplexes. Melting points were obtained in 0.14*M* PB by means of stepwise temperature increases on a jacketed hydroxyapatite column. Prior to the temperature increases the column containing hybrids or duplexes was washed at 60°C with 0.14*M* sodium phosphate to free the column of nonhybridized DNA. After elution up to 94°C, the remaining 2 to 3 percent of the DNA was removed by washing with 0.5*M* PB. ▲, DNA-DNA duplexes formed with nonrepeated DNA fraction, renatured to a $C_0t$ of 800 mole sec/liter; ○, brain RNA-DNA hybrids; △, kidney RNA-DNA hybrids. Denaturation of 100 percent corresponds to 127,000, 28,000, and 9,000 count/min of $^3$H-labeled DNA for the respective hybrids and duplexes.

recovered from RNA-DNA hybrids formed after short-term incubation (arrow, Fig. 2A) was renatured with unlabeled, mouse DNA. No apparent reaction with the rapidly renaturing sequences was observed. Instead, this recovered DNA renatures in accordance with the kinetics expected for nonrepeated, mouse DNA. DNA recovered from hybrids formed after long-term incubation (90.5 hours) with brain RNA (Fig. 2A) also renatured with total DNA in the same manner (Fig. 2B). Therefore, we conclude that the results shown in Fig. 2A are due to reactions between RNA and nonrepeated DNA.

The stabilities of these RNA-DNA hybrids and of unique DNA-DNA duplexes were determined by thermal elution from hydroxyapatite. As the DNA becomes single stranded, it no longer binds in 0.14*M* PB (*11*). The $T_m$'s (temperature at which 50 percent is eluted) of RNA-DNA hybrids formed with kidney or brain RNA's were 83°C, while that of DNA-DNA duplexes was 85°C (Fig. 3). Under these conditions, the $T_m$ of native mouse DNA is about 87°C (not shown) (*12*). These high thermal stabilities indicate that extensive base pairing is present in the hybrids, as expected for association products of unique-sequence polynucleotides. These high stabilities are in contrast to those observed with most RNA-DNA hybrids involving repeated sequences. Such hybrids exhibit $T_m$'s ranging from 68° to 75°C, depending on the reaction conditions (*8*).

Hybridization values obtained with preparations of DNA and nuclear RNA (Table 1) indicate that RNA's complementary to at least 8 to 12 percent of mouse unique DNA are present in mouse brain. If only one of the complementary DNA strands is transcribed, this represents about 20 percent of the potential information in nonrepeated DNA, or 12 percent of that in the total genome. This preliminary estimate of gene activity in mouse brain represents the equivalent of more than 300,000 different sequences of 1,000 nucleotides each. While it is likely that these sequences are functionally as well as structurally diverse, we wish to emphasize that nonrepeatedness (or uniqueness), as defined by our experimental conditions, does not imply absolute lack of relationship of such sequences. However, based on the temperature and salt restrictions, we estimate that these "unique" polynucleotides differ from each other sequentially in at *least* 20 to 30 percent of their nucleotides (*12*).

The brain may be considered to be several organs in one. Considering this complexity on the basis of the variety of neuronal and glial cell populations in various areas, it is perhaps not surprising that the range of genetic activity in the entire brain is much greater than in organs such as the liver and kidney.

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## Sex and Population Differences in the Incidence of a Plasma Cholinesterase Variant

Abstract. *Accumulating knowledge of polymorphic enzyme systems poses intriguing possibilities of anthropologic genetics. Development of an automated procedure for determination of heterozygosity or homozygosity of the atypical plasma cholinesterase allele ($E_1^a$) permitted screening of 2317 individuals during a national Preschool Nutrition Survey and several smaller population studies. Frequencies of the allele ($E_1^a$) closely parallel those previously reported. Caucasians manifested a heterozygote male preponderance of 1.85 : 1.*

The plasma cholinesterase enzyme system is a polymorphic one with multiple alleles and isoenzymes (*1*). At this time, three alleles have been described based upon inhibition studies: the usual enzymatic form ($E_1^u$), the atypical allele ($E_1^a$), and the fluoride-resistant allele ($E_1^f$). When the enzyme has been subjected to electrophoresis, four isoenzymes ($C_1$, $C_2$, $C_3$, $C_4$) are usually observed. Recent investigators report successful demonstration of one to three additional isoenzymes ($C_5$, $C_6$, $C_7$) (*2*), and one investigator has demonstrated a total of 12 ($C_1$–$C_{12}$) in human serums (*3*). The atypical allele is inherited as an autosomal recessive. The homozygous atypical condition predisposes to prolonged apneic periods and muscular paralysis following ad-