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Detection of Genetic Variation with Radioactive Ligands. IV. X-Linked, Polymorphic Genetic Variation of Thyroxin-Binding Globulin (TBG)

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SUMMARY

A genetically determined, polymorphic electrophoretic variant of thyroxin-binding alpha-globulin (TBG) is found in sera from populations of African and Oceanian origin, although not in Caucasians nor Orientals. The TBG polymorphism is inherited in X-linked fashion, based on data from American blacks, and thus provides an X-chromosome marker with a relatively high gene frequency in this ethnic group (frequency of the slow allele, *TBG*^{S}, is 11%). This slow variant should prove valuable in expanding the map of the X chromosome and in linkage studies. An additional family exhibiting X-linked TBG deficiency is also described.

INTRODUCTION

Quantitative, sex-linked genetic variation of human TBG has been recognized for many years [1, 2]. Absence or substantial deficiency occurs in roughly 1/5,000 males, as detected in newborn screening programs for hypothyroidism [3]. Rare females with TBG deficiency have also been reported [4]. Autosomal dominant inheritance has been suggested in a few instances, in contrast to convincing evidence for X-linkage in most male-ascertained families. Sex-linked increase in TBG concentration has also been reported in a few families [5]. By use of these variants, TBG linkage has been tested with G6PD, Xg, and deutan-protan color blindness [6, 7]. Tight linkage of TBG with any of these markers has been excluded.

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In preliminary reports, we presented evidence for an electrophoretic, sex-linked variant of TBG with gene frequency greater than 10% in at least one population, American blacks [8]. The variant was detected by [^{14}C] thyroxin-labeling of serum samples followed by alkaline polyacrylamide gel electrophoresis (PAGE) and autoradiography. Here, we present family and population gene frequency data on the TBG variant. In addition to PAGE autoradiography, these data have been confirmed and extended by application of nonequilibrium isoelectric focusing (IEF) and autoradiography. Details of the intra- and interindividual heterogeneity of TBG detected by this new method are reported in [9]. Family data collected in the process of testing for linkage to X-chromosome markers will be presented later.

MATERIALS AND METHODS

Blood Samples

Population blood samples were from: (1) 811 African Pygmies [10], (2) 97 Alaskan Eskimos [11], (3) 108 Chinese and Japanese, (4) 103 Panamanian blacks [12], (5) 580 American blacks, and (6) 404 American Caucasians. Caucasians, Chinese, Japanese, and American blacks were from the San Francisco Bay region; Houston, Texas; St. Louis, Missouri; and Chapel Hill, North Carolina.

Radiolabeling

Blood samples were radiolabeled by (1) addition of 2 μl [^{14}C] T_4 (New England Nuclear, Boston, Mass.) in .1 N NaOH to 100 μl plasma, giving a final concentration of 7.6 μM T_4 (.23 μCi/ml), or (2) addition of 1 μl [^{125}I] T_4 (Amersham-Searle, Arlington Heights, Ill.) in 20% aqueous propylene glycol to 100 μl plasma, giving a final concentration of .05 μM T_4 (2 μCi/ml). Delay between labeling and initiation of electrophoresis does not influence results: samples labeled and run within 30 min produce patterns identical with those of samples labeled and stored 48 hrs at 3°C before electrophoresis. Likewise, there is no apparent distinction between plasma and serum.

PAGE and Autoradiography

PAGE was conducted on vertical slab gels at 3°C using a discontinuous Tris-HCl-glycine buffer system [8]. Separating gel was 10% total acrylamide (2.5% bisacrylamide), .4 M Tris, pH 9.3; stacking gel was 2.5% total acrylamide (2% bis), .4 M Tris, pH 7.4; upper buffer was .05 M Tris-glycine, pH 9.1. Running conditions were 19 V/cm for 4 hrs. Wells were loaded with 10 μl of sample.

After electrophoresis, the gel was dried on Whatman 3 M filter paper under vacuum and exposed to Kodak SB-5 X-ray film from 2 to 6 weeks for [^{14}C]thyroxin or from 24 to 48 hrs for [^{125}I]thyroxin.

IEF and Autoradiography

IEF was performed on prefocused, horizontal polyacrylamide slab gels. Gel was 5% total acrylamide (2.5% bis), 8% glycerol, 2% pH 4–6 Ampholines (LKB, Rockville, Md.) with .5 M NaOH at the cathode and 1 N H_3PO_4 at the anode. Running conditions were: (1) prefocusing for 3 hrs at 18 W constant power to a maximum of 1200 V at 3°C, and (2) application of 10 μl samples and isofocusing for an additional 1.5 hrs. Further details are in [9].

Following electrophoresis, gels were dried and autoradiographed as with PAGE. Because IEF gels rehydrate rapidly, we recommend drying under good vacuum and use of a thin plastic film to separate gel from X-ray.

TBG Quantitation

Serum or plasma TBG levels were measured by Laurell immunoelectrophoresis in 1% agarose gels, sodium barbital buffer, pH 8.4, with monospecific antihuman TBG (Atlantic Antibodies, Scarborough, Me.) or by radial immunodiffusion in similar gels [13]. Values are expressed as percent of a standard plasma sample from an adult male of type TBG-C.

RESULTS

Four thyroxin-binding proteins can be distinguished at this pH in 10% polyacrylamide gels with supersaturating amounts of label (fig. 1). These are the three major thyroxin-binding proteins (TBG, albumin, and prealbumin) and a fourth protein migrating in the post-albumin region [14]. This fourth binder is probably high-density lipoprotein [15]. At physiologic concentrations of T_4, the major transport protein for T_4-T_3 is TBG, with prealbumin playing a lesser role, as others have established (e.g., [16]).

Inherited Quantitative Variation in TBG

During routine screening with PAGE autoradiography, two individuals with diminished or absent TBG were detected. The first was an adult female Caucasian whose serum had been obtained at 2 days postpartum. Unfortunately, neither she nor her family were available for further testing.

The second individual was an apparently healthy, 21-year-old male of Jewish descent. His paternal grandmother, father, mother, and one brother were also tested (fig. 1). The proband's father and grandmother demonstrated normal TBG-binding capacities whereas the mother appeared to have decreased capacity. The proband and his brother showed an almost complete lack of T_4 binding by TBG. This pattern is consistent with an X-linked mode of inheritance. Autosomal reces-

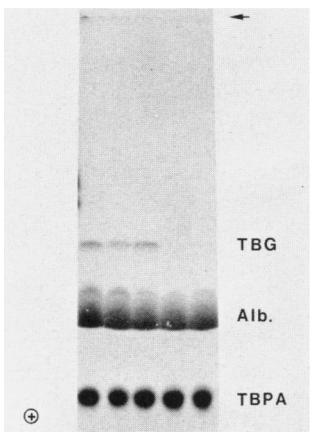

FIG. 1.—Autoradiograph of a 7.5% polyacrylamide gel showing [^{14}C]thyroxin-binding proteins in serum and absence of TBG in a Caucasian family. *From left to right*, family members are: paternal grandmother (normal TBG concentration), mother (half-normal), father (normal), brother (absent), and proband. See MATERIALS AND METHODS for details on radiolabeling and PAGE autoradiography. Film was exposed 65 days. *Alb*: albumin, *TBPA*: thyroxin-binding prealbumin, *arrow*: origin.

sive inheritance is not altogether excluded but is generally inconsistent with the levels of TBG observed in the parents.

By microdensitometry, the relative density of TBG-bound [^{14}C]thyroxin was 9:57:94:100 for proband (and brother):mother:father:paternal grandmother. Laurell immunoelectrophoresis and radialimmunodiffusion showed the amount of cross-reacting material in these samples to be of the same order. By the more sensitive technique of nonequilibrium IEF, this protein is present at a band density 10% of normal, with normal isofocusing pattern. This suggests the *TBG* allele in this case codes for a structurally normal protein in low concentration rather than for an abnormal protein with significantly reduced affinity for thyroxin.

Electrophoretic Mobility Variants

PAGE analysis of samples from seven populations of African origin (four American and three from Central Africa) and samples from Alaskan Eskimos showed the presence of one band (TBG-S) slower than the common band (TBG-C) in some individuals in each population, although not in Orientals nor in American Caucasians. Three phenotypes can be distinguished in females: C, S, and C-S (fig. 2), presumably the two homozygotes and one heterozygote. In males, only two phenotypes, C and S, are found and the presumed heterozygote is not observed. A mixture of C and S sera gives the same pattern as the female C-S phenotype.

The distribution of phenotypes among the sexes strongly suggests X-linkage. Table 1 shows expected genotype frequencies computed on this hypothesis, which is in good agreement with observations. The three American black populations do not show significant differences of gene frequencies (see footnote *, table 1), nor do they differ significantly from Panamanian blacks. The suggestion of X-linkage from population data has been confirmed by family studies. Some pedigrees collected by

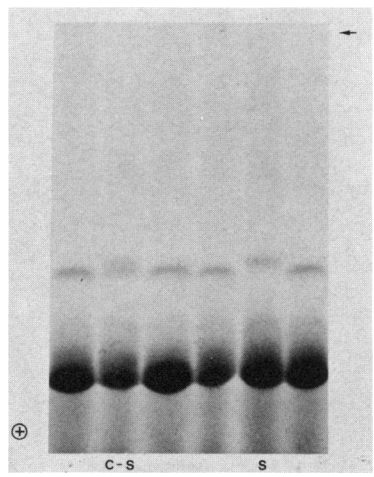

FIG. 2.—Autoradiograph of a 10% polyacrylamide gel showing genetic variants of TBG labeled with [^{14}C]T_4. *From left to right*, TBG phenotypes are: TBG-C, TBG C-S, TBG-C, TBG-C, TBG-S, and TBG-C. Film was exposed 60 days.

TABLE 1

POPULATION DATA ON THE ELECTROPHORETIC POLYMORPHISM FOR TBG

POPULATION	NO. TOTAL	FEMALES				MALES			GENE FREQUENCY OF *S* ALLELE
		TBG-C	TBG-CS	TBG-S	Total	TBG-C	TBG-S	Total	
American blacks*:									
San Francisco, Ca.	340	207	46	3	256	73	11	84	.1057
St. Louis, Mo.	154	72	13	1	86	63	5	68	.0833
Chapel Hill, N.C.	86	30	13	2	45	37	4	41	.1603
Total observed.....	580	309	72	6	387	173	20	193	.1075
Expected†.....		308.27	74.26	4.47	387	172.25	20.75	193	...
Panamanian blacks.....	103	50	9	1	60	39	4	43	.0920
Expected‡		49.47	10.02	.51	60	39.04	3.96	43	...
African Pygmies§:									
Cameroon	45	23	3	1	27	17	1	18	.0833
Central African Republic	704	315	25	3	343	341	20	361	.0487
Zaire	62	28	0	0	28	34	0	34	.00
Total‖	811	366	28	4	398	392	21	413	.0471
Orientals (San Francisco Bay area)	108	57	...	...	57	51	...	51	.00
Alaskan Eskimos.....	97	40	...	...	40	56	1	57	.0073
American Caucasians#...	404	235	...	...	235	169	...	169	.00

NOTE: All χ^2 computed using the likelihood ratio (G) formula.

* Heterogeneity between populations of three geographic origins: $\chi^2 = 4.96$, 2 df, $10 > P > 5\%$.

† $\chi^2 = 0.57$ for Hardy-Weinberg fit, with 2 df, $80 > P > 70\%$.

‡ $\chi^2 = 0.08$ for Hardy-Weinberg fit, with 2 df, $98 > P > 95\%$.

§ Heterogeneity between populations of three geographic origins: $\chi^2 = 10.47$, 2 df, $P < 1\%$.

‖ Because of heterogeneity of the three Pygmy groups, the χ^2 test for Hardy-Weinberg equilibrium was done only on the second group (Central African Republic), the sample size of which is not too small. The χ^2 for Hardy-Weinberg for 2 df is 5.42; $10 > P > 5\%$.

\# Three additional individuals deficient for TBG not included; see text.

us in the Bay Area are reported in figure 3, and they all agree with X-linkage. Other pedigrees that also confirm the hypothesis and are being tested for linkage with other X-chromosome markers will be reported later.

TBG C-S heterozygotes and mixtures of C and S hemizygotes show two distinct bands, but the distance between the C and S bands in PAGE is at most 3% and separation is not always sufficient. Use of [^{125}I]T_4 with PAGE is not satisfactory because the C and S bands are even less clearly resolved with ^{125}I than with ^{14}C, apparently because the radiation involved is more penetrating and produces broader bands. As summarized below, IEF provides increased resolution, permitting use of ^{125}I with greatly reduced exposure times. All C-S and S individuals, and about 10% of C individuals listed in table 1, were retested, and there were no inconsistencies between IEF and PAGE.

IEF

Application of nonequilibrium IEF to [^{125}I]T_4-labeled samples produced patterns showing at least three major bands and one or more anodal bands in the pH range 4.2 to 4.5 in all individuals tested (fig. 4 and [9]). This labeled protein was deficient in samples of known TBG deficiency and absent from samples immunoprecipitated by antihuman TBG. Samples of TBG-S phenotype also show three or more bands, but these bands are displaced by .5 pH units from the TBG-C pattern. TBG S-C heterozygotes produce patterns with 6^+ bands. Heterozygote patterns are identical with patterns produced by mixing samples of TBG-C and TBG-S phenotypes. The pattern produced by TBG-S samples from American blacks, African Pygmies, and Alaskan Eskimos are identical by isofocusing. Also, there were no age-related changes observed in these patterns.

Mild neuraminidase treatment of TBG-C samples results in a stepwise shift of the isoelectric pattern toward the cathode, generating several bands in addition to the original 3^+. Treatment of the TBG-S variant also produces shifted patterns, but these remain exactly out of phase with band patterns produced by TBG-C samples treated in an identical manner [9]. We conclude that the 3^+ band pattern seen in all homozygous individuals is the result of differing degrees of TBG sialylation but that the difference between the TBG-common and TBG-slow types is *not* the result of

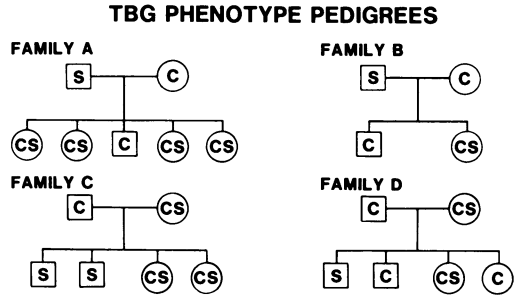

FIG. 3.—Pedigrees of American black families demonstrating X-linked inheritance of the TBG variant.

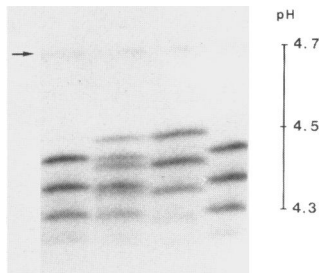

FIG. 4.—Autoradiograph of an IEF gel showing genetic variants of TBG labeled with [^{125}I]T_4. *From left to right*, TBG phenotypes are: TBG-C, TBG C-S, TBG-S, and TBG-C. See MATERIALS AND METHODS for details on radiolabeling and IEF autoradiography. Film was exposed 7 days.

sialic acid differences. The X-linked nature of this genetic polymorphism is strong evidence that the difference is at the TBG structural locus.

IEF reveals an additional type of variation: anodal bands are more intense in samples from pregnant women, giving the impression of a shift of the pattern toward the anode [9]. Although this acquired difference is often observed in population samples, it is easily distinguished from the heritable TBG-S type because it differs not only in relative band density but also in overall isoelectric point.

DISCUSSION

We report the existence of a codominant, sex-linked allele of TBG in several human populations. The variant is a thyroxin-binding protein with electrophoretic mobility slower than that of "common" TBG and a gene frequency of 11% in American blacks. A similar genetic variant occurs in other populations of African and American origin but not in Caucasians nor Orientals.

Our tests provide adequate evidence that the protein under study is TBG, and that the variation observed is a true genetic polymorphism, not due to physiologic effects, such as desialylation. In particular, there seems to be no connection between our slow variant and STBG [17–19], a TBG variant with mobility slower than that of normal TBG, found in patients with advanced hepatic cirrhosis, in obese subjects, and rarely in normal individuals. STBG has the same molecular weight and antigenic determinants as TBG, but its affinity for T_4 is one-tenth that of TBG. STBG is apparently composed of TBG desialylated to varying degrees [18].

There are various indications that STBG differs from our slow polymorphic variants. Both proteins have a mobility slower than TBG, but the separation between TBG and STBG appears to be much greater than that between TBG and the genetic variant based on published data [17]. Normal TBG and STBG differ significantly in affinity for thyroxin, whereas the slow and common types seen in our system appear similar in their affinities for the radiolabel—as determined by autoradiography and microdensitometry. Finally, STBG is not inherited.

There is no evidence for or against the hypothesis that the quantitative variation of TBG is due to the same gene determining structural variation of TBG that we report here, or, alternatively, to a closely linked gene. The evidence is insufficient for

determining whether the mutation is structural or regulatory. However, the fact that there is a quantitative decrease of TBG in the deficiency mutant, which is approximately the same by immunologic and binding assays with no detectable alteration in isoelectric pattern, suggests that the quantitative mutation is regulatory.

We found no certain evidence by this technique of inherited increase in TBG production. The major difficulty is that of hormonal influences (e.g., pregnancy and oral contraceptives) on TBG level. Clearly, family studies are required for demonstrating such variants.

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REFERENCES

1. Köbberling J, Emrich D: The genetic polymorphism of the thyroxine-binding globulin (TBG). *Humangenetik* 14:85–94, 1972
2. Refetoff S, Robin NI, Alper CA: Study of four new kindreds with inherited thyroxine-binding globulin abnormalities. Possible mutation of a single gene locus. *J Clin Invest* 51:848–867, 1972
3. Fisher DA, Dussault JH, Foley TP Jr, et al.: Screening for congenital hypothyroidism: results of screening one million North American infants. *J Pediatr* 94:700–705, 1979
4. Nusynowitz ML, Clark RF, Strader WJ III, Estrin HM, Seal US: Thyroxine-binding globulin deficiency in three families, and total deficiency in a normal woman. *Am J Med* 50:458–464, 1971
5. Fialkow PJ, Giblett ER, Musa B: Increased serum thyroxine-binding globulin capacity: inheritance and linkage relationships. *J Clin Endocrinol Metab* 30:66–70, 1970
6. Keats BJB, Morton NE, Rao DC, Williams WR: *A Source Book for Linkage in Man.* Baltimore, Johns Hopkins Univ. Press, 1979
7. Leiba S, Landau B, Ber A, Adam A, Sterling K: Thyroxine-binding-globulin (TBG) deficiency and glucose-6-phosphatate dehydrogenase (G-6-PD) deficiency in the same family. *J Clin Endocrinol Metab* 38:569–573, 1974
8. Cavalli-Sforza LL, Daiger SP, Rummel DP: Detection of genetic variation with radioactive ligands. I. Electrophoretic screening of plasma proteins with a selected panel of compounds. *Am J Hum Genet* 29:581–592, 1977
9. Daiger SP, Wildin RS: Human thyroxin-binding globulin (TBG): heterogeneity within individuals and among individuals demonstrated by isoelectric focusing. *Biochem Genet.* In press, 1981
10. Cavalli-Sforza LL: Pygmies, an example of hunters-gatherers, and genetic consequences for man of domestication of plants and animals. *Proceedings 4th International Congress of Human Genetics*, Paris, 1971, pp 79–95
11. Ferrell RE, Chakraborty R, Lauhlin W, Schull WJ: The Eskimos of St. Lawrence Island: genetic variation and genetic affinities. *Am J Phys Anthropol.* In press, 1981
12. Ferrell RE, Nunez A, Bertin T, Labarthe D, Schull WJ: The blacks of Panama: their genetic diversity as assessed by 15 inherited biochemical systems. *Am J Phys Anthropol* 48:269–275, 1978
13. Verbruggen R: Quantitative immunoelectrophoretic methods: a literature survey. *Clin Chem* 21:5–43, 1975

14. HOCMAN G: Human thyroxine-binding globulin. Isolation and chemical properties—I, II, III. *Int J Biochem* 9:9–12, 295–298, 639–640, 1978
15. FREEMAN T, PEARSON JD: The use of quantitative immunoelectrophoresis to investigate thyroxine-binding human serum proteins. *Clin Chim Acta* 26:365–368, 1969
16. DAVIS PJ, GREGERMAN RI: Separation of thyroxine-binding proteins in human serum at pH 7.4. II. Effect of pH and temperature on the binding capacities of thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA). *J Clin Endocrinol Metab* 33:699–708, 1971
17. MARSHALL JS, PENSKY J, GREEN AM: Studies on human thyroxine-binding globulin. VI. The nature of slow thyroxine-binding globulin. *J Clin Invest* 51:3173–3181, 1972
18. PREMACHANDRA BN, PERLSTEIN IB, BLUMENTHAL HT: Studies on obesity. II. Slow-moving thyroxine-binding globulin in the sera of normal and obese subjects. *J Clin Endocrinol Metab* 30:752–762, 1970
19. REFETOFF S, FANG VS, MARSHALL JS: Studies on human thyroxine-binding globulin (TBG). IX. Some physical, chemical and biological properties of radioiodinated TBG and partially desialylated TBG. *J Clin Invest* 56:177–187, 1975