*GENETIC POLYMORPHISM OF RHESUS THYROXINE-BINDING PREALBUMIN: EVIDENCE FOR TETRAMERIC STRUCTURE IN PRIMATES**

BY CHESTER A. ALPER, NOEL I. ROBIN, AND SAMUEL REFETOFF

BLOOD GROUPING LABORATORY AND DEPARTMENTS OF MEDICINE, CHILDREN'S HOSPITAL MEDICAL CENTER AND PETER BENT BRIGHAM HOSPITAL, BOSTON, MASSACHUSETTS

Communicated by Henry G. Kunkel, April 14, 1969

Abstract.—Polymorphism in primate thyroxine-binding prealbumin was investigated with agarose gel electrophoresis at pH 8.6. In the rhesus monkey (*Macaca mulatta*), three forms of this protein were found in random sera: a single rapidly migrating band similar to that in human and other primate sera, a single slowly migrating band cathodal to rhesus albumin, and a five-banded form, the most rapid and slowest bands of which corresponded to the other two forms. The frequencies of occurrence of these three forms were consistent with the hypotheses that rhesus prealbumin is under the control of two codominant autosomal alleles, PA^F and PA^S, and that the protein occurs naturally in serum as a tetramer composed of similar subunits.

It was possible, by simple mixing *in vitro*, to produce five-banded prealbumin patterns from rhesus PA SS serum and rhesus PA FF serum, *M. arctoides* serum, *P. hamadryas* serum, and human serum. Thyroxine was bound by all the hybrid molecules produced in this fashion.

Genetically controlled polymorphism of cellular and plasma proteins is common in all species of animals investigated, including man. In the rhesus monkey (*Macaca mulatta*), extensive polymorphism has been found in transferrin[1] and the immunoglobulins.[2] Variations in thyroxine-binding prealbumin (TBPA) in the serum of this species have also been described[1, 3] wherein this protein was found to form a single band which, on starch gel electrophoresis, either had the electrophoretic mobility of human thyroxine-binding prealbumin (type 1), migrated a little more slowly (type 3), or much more slowly (type 2) than human thyroxine-binding prealbumin. Radioautography of starch gel patterns of sera to which radioiodine-labeled thyroxine had been added confirmed that the observed variation resided in thyroxine-binding prealbumin[4] and not in alpha$_1$ acid glycoprotein, which also migrates as a prealbumin in this analytical system but does not bind thyroxine.

We have reinvestigated the problem of genetic polymorphism of thyroxine-binding prealbumin in the rhesus monkey and in man. Our results indicate that rhesus thyroxine-binding prealbumin is under the control of two autosomal codominant alleles. They further suggest that primate thyroxine-binding prealbumin, at least in the species investigated, occurs naturally as a tetramer, the four subunits of which are rather loosely linked to one another.

Materials and Methods.—Samples of peripheral blood were obtained (through the kind cooperation of Drs. Thomas C. Jones, Felix Garcia, and Bernard Trum) from animals housed at the New England Regional Primate Research Center, Southboro, Mass., from 36 *M. mulatta* (21 males and 15 females), 3 *M. arctoides*, and 3 *P. hamadryas*. Samples

were also collected from 227 randomly selected, unrelated persons. Serum was separated from cellular elements and clot by centrifugation and samples were stored at −20° or −80°C until examined.

Human thyroxine-binding prealbumin was purified by Thomas Boenisch of the Blood Grouping Laboratory by the following method. To 100 ml of serum was added 600 ml of 0.117 *M* sodium acetate. The pH was adjusted to 4.70 with glacial acetic acid and the mixture was submerged in a boiling water bath with stirring for 5 min. The mixture was filtered while hot and the precipitate was discarded. The filtrate, which contained chiefly thyroxine-binding prealbumin, $alpha_1$ acid glycoprotein, and hemopexin, was concentrated by ultrafiltration to a volume of approximately 5 ml. Neuraminidase (Sigma Chemical Co., St. Louis, Mo.) was added to reduce the charge of the $alpha_1$ acid glycoprotein by removal of sialic acid and the mixture was dialyzed at room temperature for 48 hr against 0.1 *M* acetate buffer pH 5.0 and then barbital buffer 0.05 *M* pH 8.6 prior to electrophoresis on Pevikon[5] in the latter buffer for 20 hr at 4°C at 12 v/cm. Protein concentrations in the eluates from 1-cm segments of supporting medium were analyzed by the Folin-Ciocalteu method.[6] Fractions containing the most anodal peak were concentrated by ultrafiltration. Examination in agarose electrophoresis[7] and immunoelectrophoresis[8] using a potent goat antiserum to whole human serum revealed the presence of TBPA but no other serum proteins. An aqueous solution of this preparation was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected into the dermis of a goat in multiple sites.[9] Animals received two injections of 100–200 μg of protein each at an interval of about 4 weeks. Antiserum was harvested after 2 weeks after the last injection. The antiserum was found to be monospecific for human thyroxine-binding prealbumin when tested against whole human serum in immunoelectrophoresis. It reacted with the thyroxine-binding prealbumin of the other primate sera tested in the same manner and no other arcs developed (Fig. 1).

Antigen-antibody crossed electrophoresis was performed as described by Laurell,[10] except that the initial agarose electrophoresis was carried out on a modified apparatus permitting greater separation.[11] Immunofixation electrophoresis was performed as described previously.[12]

Selected sera were enriched with 0.01–0.02 μg of ^{125}I-labeled tetraiodothyronine (T4) or ^{125}I-labeled triiodothyronine (T3) per ml (Abbott Laboratories, Chicago, Ill.). The labeled preparations were at least 90% pure as judged by descending paper chromatography using hexane:tertiary amyl alcohol:2 *M* ammonia (1:5:6).[13] Enriched sera were subjected to agarose electrophoresis in 0.05 *M* Tris-maleate buffer pH 8.6.[14] After electrophoresis the gels were rapidly dried in a current of warm air without prior fixation of proteins or staining and exposed to Kodak No-screen X-ray film for appropriate intervals prior to hand development. After radioautography, the proteins were fixed with aqueous methanol-acetic acid and stained with amido black.

Results.—On immunofixation agarose gel electrophoresis, three types of patterns were found in rhesus thyroxine-binding prealbumin. The most common pattern was a single band with approximately the same electrophoretic mobility

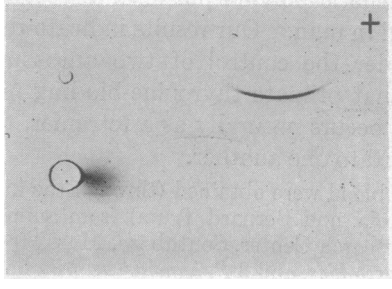

FIG. 1.—Immunoelectrophoretic pattern of human serum (*top antigen well*) and rhesus serum (*bottom antigen well*) developed with goat antihuman prealbumin antiserum (cf. *Materials and Methods*) in the trough.

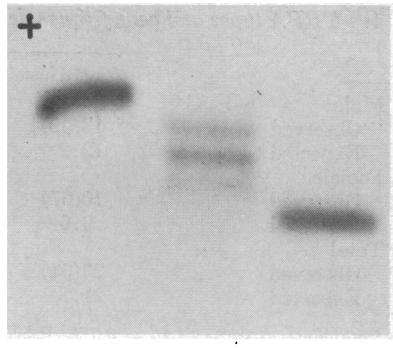

FIG. 2.—Immunofixation electrophoresis in agarose gel of fast rhesus TBPA (*left*), five-banded rhesus TBPA (*middle*), and slow rhesus TBPA (*right*). The patterns were developed with goat antihuman TBPA.

as human thyroxine-binding prealbumin, and the least common was a single band with an electrophoretic mobility slightly slower than that of rhesus albumin (Fig. 2). Of intermediate frequency was a multiple-banded pattern, the darkest component of which migrated exactly midway between the fast and slow single bands of the adjacent rhesus sera. Two less dense bands of equal intensity were situated midway between the central band and the single bands of the adjacent sera. Finally, two bands with mobilities identical to those of the rhesus fast and slow bands were faintly discernible. The relationships between these bands and the other forms of rhesus thyroxine-binding prealbumin are more clearly seen in the antigen-antibody crossed electrophoresis patterns shown in Figure 3. It was also possible to determine the relative concentrations of the five components by estimating the areas under the peaks in this pattern. When this was done, the ratio was found to be 1:6:12:6:1.

Examination of 227 human serum samples and three samples each from *M. arctoides* and *P. hamadryas* in immunofixation electrophoresis revealed only single-banded thyroxine-binding prealbumin.

The incidence of the three types of rhesus-thyroxine-binding prealbumin in the 36 samples examined is given in Table 1. There was no significant difference

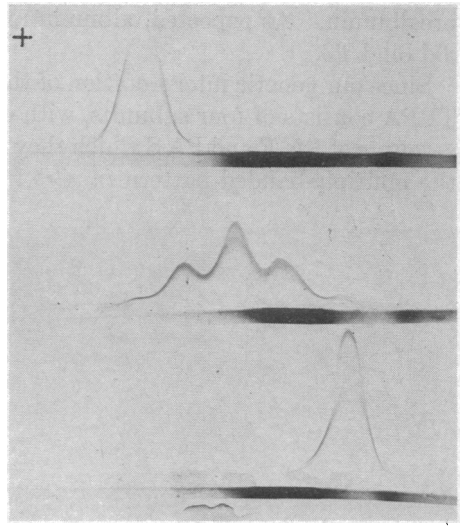

FIG. 3.—Antigen-antibody crossed electrophoresis pattern of fast rhesus TBPA (*top*), five-banded rhesus TBPA (*middle*), and slow rhesus TBPA (*bottom*). A portion of the agarose gel from the initial electrophoretic separation has been stained and placed under the antigen-antibody peaks. The dark stained area in each strip is rhesus albumin.

TABLE 1. *TBPA types and gene frequencies in the rhesus monkey (M. mulatta).*

	TBPA Types		
	Single fast	Five-banded	Single slow
Male			
Observed	13(62)†	6(29)	2(9)
Expected*	12.2	7.6	1.2
Female			
Observed	10(67)	4(27)	1(7)
Expected	9.6	4.8	0.6
Total			
Observed	23(64)	10(28)	3(8)
Expected	21.8	12.4	1.8

Gene frequencies (total): $PA^F = 0.778$, $PA^S = 0.222$.
* Calculated from the Hardy-Weinberg equilibrium assuming two autosomal alleles.
† The numbers in parentheses represent percentage of population of type listed in column headings

between the incidences in males and females. Although inheritance data are not yet available, it seems most reasonable to assume that the single fast band is the product of a gene which we shall call rhesus PA^F and, similarly, that the slow band is the product of an allelic gene, PA^S. Animals with only one band would thus be homozygous, either PA^{FF} or PA^{SS}, whereas those with five-banded patterns would be heterozygous PA^{FS}. Observed and expected gene frequencies calculated on the basis of these assumptions agree reasonably well, in view of the small number of samples (Table 1).

To establish further that the observed polymorphism involved thyroxine-binding prealbumin, experiments with ^{125}I-labeled T3 and ^{125}I-labeled T4 were carried out. A radioautograph of electrophoretic patterns of sera enriched with ^{125}I-labeled T4 is shown in Figure 4. It is evident that similar patterns are produced in this fashion as were produced by immunofixation, although the fastest and slowest bands in the multiple-banded pattern are not clearly discernible. Thus, all forms of rhesus thyroxine-binding prealbumin bind T4. Experiments with ^{125}I-labeled T3 failed to show binding to rhesus thyroxine-binding prealbumin by any of the variants, as is also the case for human thyroxine-binding prealbumin. As expected, albumin and inter-alpha thyroxine-binding globulin did bind T3.

Since our genetic interpretation of the five-banded patterns implies that rhesus TBPA consists of four subunits, with each of the bands differing by the relative amounts of PA F and PA S which they contain (Fig. 4), we attempted to produce the multiple-banded pattern *in vitro* from mixtures of PA FF and PA SS sera.

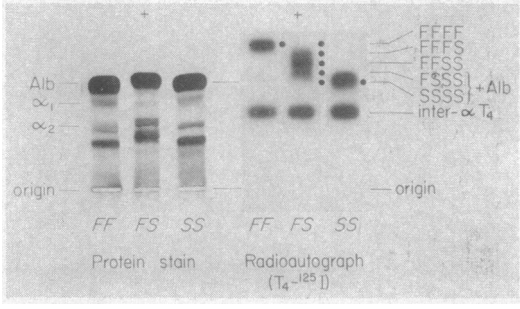

FIG. 4.—Protein stain and radioautograph of agarose electrophoretic patterns of rhesus sera of three types enriched with T4-^{125}I (cf. *Materials and Methods*). The presumed composition of each of the five bands of rhesus TBPA are given at the right of the figure, and the presumed genotypes are given below the patterns (cf. *Results*).

This proved to be extraordinarily simple to do, since mixing and incubation at 4°C for three days or more without special treatment sufficed. Dissociation and reassociation appeared to be independent of temperature, freezing and thawing, and treatment with 6 *M* urea with or without 0.1 *M* 2-mercaptoethanol. Figure 5 shows the immunofixation electrophoresis patterns of PA FF, PA SS, and a reassociation experiment resulting from simple mixture and incubation of equal parts of PA FF and PA SS serum. When human serum, the thyroxine-binding prealbumin of which is similar in mobility to rhesus PA F, was mixed with rhesus PA SS serum, multiple component patterns resulted, as shown in Figure 6. It can be seen that dissociation and reassociation were incomplete since material with the mobilities of the original reactants was relatively abundant. Five-banded patterns were also produced on mixing and incubating *M. arctoides* or *P. hamadryas* with rhesus PA SS serum.

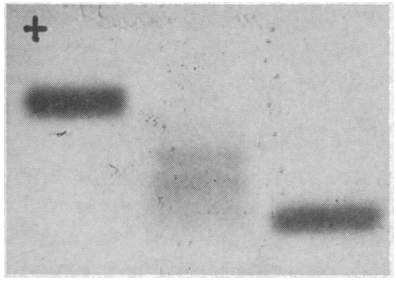

FIG. 5.—Immunofixation electrophoresis in agarose gel of sera with fast rhesus TBPA (*left*), slow rhesus TBPA (*right*), and a mixture of equal parts of these sera (*middle*).

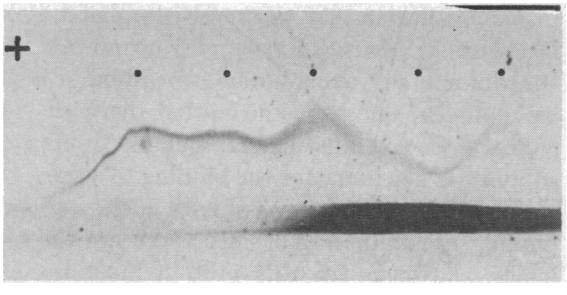

FIG. 6. — Antigen - antibody crossed electrophoresis pattern of a mixture of equal volumes of human (fast) and rhesus slow TBPA sera. Although the pattern is irregular, five components are recognizable, as indicated by the dots above the pattern (cf. *Results*). The dark material in the strip below the pattern is rhesus albumin, as in Fig. 3.

When ^{125}I-labeled T4 was added to mixtures of sera, radioautographs of agarose electrophoretic separations revealed that all bands in the hybrid thyroxine-binding prealbumin patterns bound T4, as seen in Figure 7.

Discussion.—The type of genetic polymorphism exhibited by rhesus thyroxine-binding prealbumin has many precedents among the isoenzymes, most notably lactic acid dehydrogenase,[15] in which tetrameric structure as the basis for the five-component electrophoretic pattern was first demonstrated.[16] An important contrast with polymorphism in this and other similarly constituted isoenzymes is that their multiple components usually result from association of the products of nonallelic genes, whereas rhesus FS-type thyroxine-binding prealbumin appears to consist of associated products of allelic genes.

Since dissociation and reassociation of primate thyroxine-binding prealbumin subunits occur under physiological conditions of ionic strength and pH, the forces binding the subunits of the tetrameric complete molecule appear to be rather

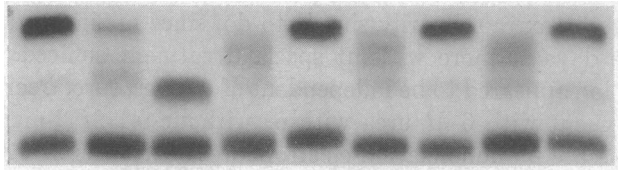

FIG. 7.—Radioautograph of agarose electrophoretic patterns of various T4-^{125}I enriched primate sera and mixtures of primate sera (cf. Fig. 4). From left to right, the samples are (*a*) rhesus PA FF, (*b*) rhesus PA FF plus rhesus PA SS, (*c*) rhesus PA SS, (*d*) rhesus PA SS plus *P. hamadryas*, (*e*) *P. hamadryas*, (*f*) rhesus PA SS plus *M. arctoides*, (*g*) *M. arctoides*, (*h*) rhesus PA SS plus human, and (*i*) human.

weak. The lack of influence of temperature or urea concentration suggests that such forces may be hydrophobic rather than electrostatic. Further evidence that the subunits are bound through hydrophobic linkages has been obtained in preliminary experiments with purified human thyroxine-binding prealbumin labeled covalently with ^{131}I by the chloramine T technique of Hunter and Greenwood.[17] On gel filtration through columns of Sephadex G-100 equilibrated with various solutions, a portion of the human thyroxine-binding prealbumin dissociated into a peak of approximately 15,000–20,000 mol wt in 1 per cent sodium dodecyl sulfate, whereas in 1 *M* NaCl, 6 *M* urea, and 0.5 *M* acetic acid the molecular weight of the human TBPA was approximately 60,000–70,000, as reported by others.[18] These experiments suggest dissociation into monomer under conditions that weaken hydrophobic linkages but not those that weaken electrostatic bonds. Since it may be inferred from the hybridization experiments that human thyroxine-binding prealbumin is a tetramer of similar or identical subunits and since it is known that there is a single binding site for T4 per molecule of thyroxine-binding prealbumin,[18] it appears that the association of the four subunits is necessary for the binding to occur.

Since there is expression of both alleles in monkeys heterozygous for thyroxine-binding prealbumin and since there are the expected number of heterozygous males, the locus for this protein must reside on an autosomal chromosome. From the symmetry of the antigen-antibody crossed electrophoresis patterns of FS-type rhesus thyroxine-binding prealbumin, it is clear that protein synthesis by the two alleles occurs at closely similar rates in the individual heterozygous monkeys we have examined. The ratio of bands predicted by random reassociation of four subunits of two different electrophoretic mobilities is $1:4:6:4:1$[16] rather than the ratio of $1:6:12:6:1$ that we observed in heterozygous monkeys. It may therefore be that a stronger bond is formed when the tetramer consists of two pairs of dissimilar subunits than when it is composed mostly or entirely of one kind of subunit.

It is not entirely clear what relationship the polymorphism described in this report bears to the previously described variations in this protein.[1, 4] Most reasonably, type 1 is the same as PA FF, type 2 is PA SS, and type 3 is PA FS, since the middle component of the five-banded FS pattern is most prominent and the remaining four bands might not have been seen.

Note added in proof: Published X-ray crystallographic evidence for tetrameric structure of human thyroxine-binding prealbumin (Rérat, C., and H. G. Schwick, *Acta Cryst.*, **22**, 441 (1967)) has recently come to our attention.

* This work was supported in part by grants from the USPHS (HD 02723) and the John A. Hartford Foundation. Miss Lillian Watson provided expert technical assistance. N. I. R. is the recipient of a fellowship (1-F3-CA-39,761) from USPHS.

[1] Blumberg, B. S., *Proc. Soc. Exptl. Biol. Med.*, **104**, 25 (1960).

[2] Litwin, S. D., in *Symposium on Experimental Medicine and Surgery in Primates*, Sept. 27, 1967.

[3] Miller, J. B., *Nature*, **200**, 810 (1963).

[4] Blumberg, B. S., and J. Robbins, *Endocrinology*, **67**, 368 (1960).

[5] Müller-Eberhard, H. J., *Scand. J. Clin. Lab. Invest.*, **12**, 33 (1960).

[6] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[7] Laurell, C.-B., and J.-E. Nilehn, *J. Clin. Invest.*, **45**, 1935 (1966).

[8] Scheidegger, J. J., *Intern. Arch. Allergy Appl. Immunol.*, **7**, 103 (1955).

[9] Dr. John Robbins of the Albert Einstein Medical College performed the immunization and harvesting of antiserum.

[10] Laurell, C.-B., *Anal. Biochem.*, **10**, 358 (1965).

[11] Alper, C. A., and R. P. Propp, *J. Clin. Invest.*, **47**, 2181 (1968).

[12] Alper, C. A., and A. M. Johnson, manuscript in preparation.

[13] Bellabarba, D., R. E. Peterson, and K. Sterling, *J. Clin. Endocrinol. Metab.*, **28**, 305 (1968).

[14] Di Giulio, W., Z. Michalak, P. A. Weinhold, J. R. Hamilton, and G. E. Thoma, *J. Lab. Clin. Med.*, **64**, 349 (1964).

[15] Weiland, T., and G. Pfleiderer, *Biochem. Z.*, **329**, 112 (1957).

[16] Appella, E., and C. L. Markert, *Biochem. Biophys. Res. Commun.*, **6**, 171 (1961).

[17] Hunter, W. M., and F. C. Greenwood, *Biochem. J.*, **91**, 43 (1964).

[18] Oppenheimer, J. H., M. I. Surks, J. C. Smith, and R. Squef, *J. Biol. Chem.*, **240**, 173 (1965).