

The Consequence of Nucleotide Substitutions in the Triosephosphate Isomerase (TPI) Gene Promoter

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ABSTRACT: Mutations at $-5A \rightarrow G$, $-8G \rightarrow A$ within the cap proximal element (CPE), and $-24T \rightarrow G$ within the TATA box of the triosephosphate isomerase (TPI) gene promoter have been identified in populations with a wide geographical distribution. These mutations lie within, or in close proximity to, known *cis*-active elements in the TPI gene promoter. To determine the functional significance of mutation at these sites, which remains controversial, their effect on the expression of erythrocyte TPI enzyme activity was studied in 110 healthy unrelated subjects. The $-5G$ mutation did not alter erythrocyte TPI level, whereas the $-8A$ mutation was accompanied by a significant reduction in enzyme activity to around 90% and 76% of normal erythrocyte TPI activity in heterozygotes and homozygotes, respectively. The $-8A-24G$ genotype was associated with 75% of normal TPI activity in a heterozygote studied, implying that substitution of G at position -24 within the canonical TATA motif causes an additive decrease in TPI gene transcription in erythroid cells. A DNA-protein complex of 125kDa which was competitively blocked by specific unlabelled oligomers was demonstrated at the CPE and TATA box by electrophoretic mobility shift analysis. These findings provide direct evidence that TPI promoter mutations are linked to a reduction of TPI enzyme activity *in vivo*.

Keywords: erythrocyte, triosephosphate isomerase, TPI gene promoter, mutation, TATA binding protein, hexokinase, hemolytic anemia

INTRODUCTION

Erythrocyte enzymes have been used for the study of genetic diversity in human populations as they are accessible and readily analyzed on the basis of their biochemical and physical properties, each of which may be affected by quantitative or qualitative genetic variation. Triosephosphate isomerase (TPI), which catalyzes the isomerization of glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) in the anaerobic glycolytic pathway, has been utilized as a marker of genetic variation in human populations. An early study of TPI isozyme patterns in 2477 individuals from a variety of ethnic groups discovered only 3 individuals with altered TPI isozyme pattern, giving an estimated heterozygote frequency of 0.001 (1). Quantitation of erythrocyte TPI enzyme activity in a population from West Germany (2,3) revealed a frequency of 0.0037 for heterozygous TPI deficiency as defined by ~50% of normal enzyme activity. Other studies of TPI enzyme activity in Japanese (4), Caucasians and African-Americans (5,6)

revealed the frequency of TPI heterozygosity to be 0.0032 (1/310), 0.0048 (5/1048) and 0.048 (7/146), respectively. All ethnic groups studied show similar frequencies of TPI heterozygosity, with the exception of African-Americans among whom there is an almost 10-fold higher incidence of TPI heterozygosity. This predicts 1 in 2000 African-American newborns will inherit homozygous TPI deficiency. This is at odds with the rarity of clinical TPI deficiency, a severe disorder characterized by hemolytic anemia and progressive neuromuscular dysfunction, prompting speculation that the apparent discrepancy is explained by the presence of a null allele expected to be lethal in the homozygous state.

The higher prevalence of a TPI deficient allele in the African-American population was confirmed by a recent study which demonstrated TPI heterozygote frequencies of 0.0052 (9/1713) in Caucasians and 0.042 (7/168) in African Americans (7). In this study seven heterozygous African-American subjects identified by biochemical screening were found to

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harbor mutations within the TPI promoter region at positions -5 (A \rightarrow G) and -8 (G \rightarrow A), combined in three cases with an additional mutation at -24 (T \rightarrow G) (Fig. 1). It was proposed that these mutations were responsible for functional hemizyosity at the TPI locus. Two of these mutations lie within known regulatory elements, -8 G \rightarrow A in the Cap proximal element (CPE) and -24 T \rightarrow G within the TATATAA box (8,9). Schneider et al. discovered four subjects without clinical evidence of TPI deficiency who were homozygous for the -5 G and -8 A mutations (10). This genotype was associated with only a modest reduction in erythrocyte TPI enzyme activity when compared to homozygotes for the common haplotype (-5 A -8 G). It was concluded that these mutations were responsible for only a subtle decrease in TPI enzyme activity. The high prevalence of TPI promoter mutations in African-Americans and their apparent absence in Caucasians led to the suggestion that these are polymorphisms specific to the African lineage. We similarly identified several healthy subjects of African origin homozygous for the -5 G -8 A haplotype, but found these mutations to be widely distributed in human populations, including those of Northern European, Mediterranean, Middle Eastern, Asian Indian and Oriental origin (11). To assess the functional effect of these mutations we studied the significance of the -5 A/G, -8 G/A and -24 T/G genotypes on the expression of erythrocyte TPI enzyme activity in unrelated subjects. The -5 G polymorphism had no detectable effect on erythrocyte TPI activity,

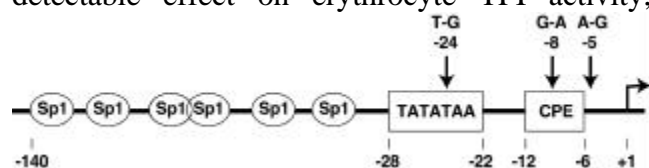


Figure 1. TPI gene promoter region. Schematic diagram from nucleotide -140 to $+1$ of the TPI gene encompassing six Sp1 sites, the TATATAA box and cap proximal element (CPE). Numbers indicate position relative to the transcription initiation site ($+1$). Position of the nucleotide substitutions studied is indicated.

whereas the -8 A variant resulted in a reduction in TPI activity to around 76% of normal in homozygotes. Electrophoretic mobility shift analysis demonstrated that in variant haplotypes the CPE and TATA box maintained their ability to bind cognate proteins consistent with their subtle functional effects *in vivo*.

MATERIALS AND METHODS

Blood Samples

Blood samples were collected in EDTA after informed consent from healthy unrelated adult subjects. Two subjects heterozygous for the Glu104Asp TPI gene mutation were also studied.

TPI Promoter Genotype Analysis

DNA was extracted from whole blood. PCR was used to generate DNA fragments corresponding to the TPI promoter (-188 to $+74$) and containing the -5 , -8 , and -24 nucleotide sites (Fig. 1) which were subsequently sequenced and haplotypes assigned in compound heterozygotes by cloning as previously described (11).

Triosephosphate Isomerase (TPI) and Hexokinase (HK) Assays

White cells and platelets were removed from whole blood by separation through microcrystalline cellulose- α -cellulose and hemolysates were prepared using methods recommended by the International Committee for Standardization in Haematology (ICSH) (12) and stored at -70 C until assayed. Hemolysate hemoglobin concentration was measured by the cyanmethemoglobin method (13) using an ICSH standard (BDH Clinical Reagents, Poole, UK). TPI and Hexokinase (HK) assays were carried out on hemolysates at 30 C using standard protocols (13).

Electrophoretic Mobility Shift Analysis

All reagents were supplied by Promega Ltd., UK unless otherwise stated. Double stranded DNA oligonucleotide probes (Table 1) corresponding to the four CPE haplotypes *c*, the common haplotype (–5A–8G), –5G, –8A, –5G–8A (TPI gene positions –16 to +1) and the two TATA box haplotypes, the common haplotype (–24T) and variant –24G (TPI gene positions –35 to –18) were constructed from commercially manufactured single stranded DNA fragments (Life Technologies, UK) by annealing complementary strands. Probes were 5' end-labeled with [γ^{32} P]ATP (DuPont NEN, UK) using T4 polynucleotide kinase. Binding reactions (10 μ l) for the CPE haplotypes were carried out under conditions previously described (9) using 0.2ng labeled CPE oligonucleotide with 10 μ g HeLa nuclear extract and 0 \times , 25 \times and 50 \times of specific (common CPE oligonucleotide) or non-specific competitor (NSE oligonucleotide, which contains the nuclease sensitive element of the *c-myc* promoter). A 14 C-labelled protein molecular weight marker (Amersham Pharmacia Biotech, UK) was used to determine the approximate size of the retarded band. For the TATA box haplotypes, 10 μ l binding reactions contained 0.2ng 32 P-labelled oligonucleotide, 10mM Tris-HCl (pH 7.5), 1mM MgCl₂, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 0.05 μ g/ μ l polydI:dC, 4% glycerol. The common TATA oligonucleotide was used as the specific competitor, and the NSE

oligonucleotide as the non-specific competitor at 20 \times , 60 \times , 100 \times and 140 \times concentrations of the labeled TATA oligonucleotide. Binding reactions were initiated by the addition of 10 μ g of HeLa nuclear extract and allowed to proceed for 15 minutes at room temperature. Antibody supershift assays were performed under the described conditions but with the addition of 3 μ g of anti-TFIID mAb (Transcription Factor II D, alternative term for TATA binding protein). For all assays, 1 μ l of gel loading buffer was added to control reactions (without HeLa extract) and 2 μ l of reaction mixture was electrophoresed through a native 4% polyacrylamide gel for 30 minutes at 4°C. After electrophoresis the gel was dried under vacuum and bands visualized by autoradiography.

Statistical Methods

Results are expressed as mean \pm standard deviation (SD) and differences between groups were examined by one-way analysis of variance (ANOVA) or unpaired two-tailed t-test. P-values \leq 0.05 were considered significant.

RESULTS

Genotype Analysis

The distribution of genotypes defined in relation to deviation from the common (*c*) promoter sequence (–5A–8G–24T) were, *c/c* (n=73), *c/–5G* (n=17), *c/–5G–8A* (n=13), *–5G/–*

Table 1. Sequence of Oligonucleotide Probes for EMSA

Probe name	Sense sequence
Common CPE	5'CAGTGGCCGCGACTGCGC 3'
–5G	5'CAGTGGCCGCGGCTGCGC 3'
–8A	5'CAGTGGCCACGACTGCGC 3'
–5G–8A	5'CAGTGGCCACGG
Common TATA	5'GGCGCTCTATATAAGTGG 3'
–24G	5'GGCGCTCTATAGAAGTGG 3'

Bases in bold indicate position of TPI promoter variants

5G-8A (n=3), -5G-8A/-5G-8A (n=3) and -5G/-5G-8A-24G (n=1).

Controlling Enzyme Activity for Red Cell Age

Subjects with reticulocytosis display an increase in detectable levels of several erythrocyte enzymes including TPI and HK (14). HK was found to be the enzyme most sensitive to high reticulocyte levels (13,14) and was therefore used as a marker for variation due to red cell age. Twelve samples (7 with c/c, 3 with c/-5G, one with c/-5G-8A and 1 with -5G/-5G-8A genotypes) were removed from further data analysis on the basis that their HK levels (range 1.86-4.48 U/gHb) were greater than 3 SD above the mean (1.06 ± 0.25 U/gHb). TPI activity in these 12 samples (1227 ± 219 U/gHb) was significantly higher ($p < 0.0001$) than in samples with the c/c genotype (950 ± 145 U/gHb).

Analysis of TPI Activity

TPI activity in groups of different genotype are shown in figure 2. Table 2 shows a comparison of individual groups with variant genotypes and the c/c genotype group. Overall a significant difference was observed by ANOVA ($p = 0.017$). However, no significant difference between TPI activity in subjects with the c/c genotype and subjects with the c/-5G was observed. Since variation at position -5 appears to have no effect on TPI activity, the chromosomes -5A-8G-24T (c) and -5G-8G-24T were re-designated as "a", -5G-8A as -8A, and -5G-8A-24G as -8A-24G, to remove nucleotide sequence variation at -5 from further analysis.

TPI activities in these groups are shown in Table 3. Subjects with a/-8A and -8A/-8A genotypes have significantly lower ($P = 0.023$ and 0.005) erythrocyte TPI activity than a/a genotypes with 90% and 76% of normal mean TPI activity, respectively. The TPI activity (442 ± 57 U/gHb) of heterozygotes for Glu104Asp, the common causative mutation in TPI deficiency,

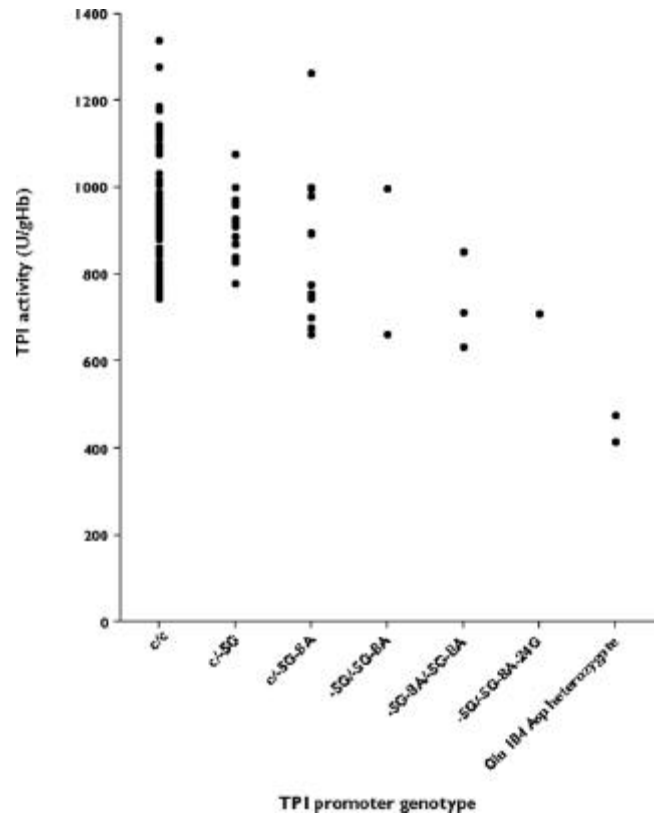


Figure 2. Erythrocyte TPI activity in groups with different TPI gene promoter genotypes, and heterozygotes for the Glu104Asp mutation. Data points represent mean TPI activity of samples in triplicate. Number of samples for individual groups are c/c (n=66), c/-5G (n=14), c/-5G-8A (n=12), -5G/-5G-8A (n=2), -5G-8A/-5G-8A (n=3), -5G/-5G-8A-24G (n=1) and Glu104Asp heterozygotes (n=2).

was approximately 46% of the normal mean TPI activity (950 ± 145 U/gHb).

Electrophoretic Mobility Shift Analysis

The affinity of protein binding to the three mutation sites within the TPI promoter region was assessed by electrophoretic mobility shift analysis. A similar band shift was observed for oligonucleotides containing either the TATA box (encompassing -24 nucleotide position) or the CPE site (encompassing -5 and -8 nucleotide positions) when incubated with HeLa nuclear extract (Fig 3). Protein binding to the TATA oligonucleotides was shown to be dependent on TATA binding protein by antibody supershift

Table 2. Comparison of Erythrocyte TPI Activity in Variant TPI Promoter Genotypes with the c/c Genotype

Genotype	P-value*	
c/-5G	0.300	NS
c/-5G-8A	0.047	
-5G/-5G-8A	0.203	NS
c/-5G-8A + -5G/-5G-8A	0.026	
-5G-8A/-5G-8A	0.006	

*By ANOVA

Abbreviation: NS, not significant

assays. Addition of antibody raised against TATA binding protein prevented formation of the protein-DNA complex so that no shift could be seen (Fig. 3c). Protein binding specificity for CPE oligonucleotides was confirmed using the common CPE and NSE oligonucleotides as specific and non-specific competitors, respectively, at concentrations of 12.5×, 25× and 50× the amount of labeled CPE oligonucleotide (Fig. 3a). At all concentrations non-specific competitor had little effect on protein binding whereas increasing concentrations of specific competitor correlated with reduction in band intensity. A protein molecular weight marker showed the approximate size of the protein-DNA complex (Fig. 3d) to be 125kDa. Common and variant haplotypes for both the TATA and CPE oligonucleotides have the ability to bind protein in HeLa nuclear extract. The intensity of the retarded band decreased as the amount of specific competitor was increased, but no clear-cut differences in binding affinity between common and variant haplotypes could be demonstrated (Figs. 3a and 3d).

DISCUSSION

To resolve the functional effect of polymorphism within the TPI gene promoter, erythrocyte TPI activity in unrelated subjects was studied. We previously proposed that the TPI promoter -5A/G polymorphism is unlikely to exert an independent effect on transcriptional

Table 3. Variation in Erythrocyte TPI Activity in Subjects with -8 and -24 Nucleotide Substitutions

Promoter genotype	N	TPI activity (U/gHb)	
		Mean	SD
a/a*	80	943	137
a/-8A	14	847	193
-8A/-8A	3	712	71
a/-8A-24G	1	708	-

*a denotes -5A/G-8G-24T haplotypes

control of the TPI gene (11). Nucleotide position -5 lies outside of any known regulatory elements within the TPI promoter. In addition, the high frequency of the -5A/G polymorphism, particularly in African and Oriental subjects, and the presence of G at position -5 in Chimpanzees support the notion that -5G may denote the ancestral haplotype (11). Our data support this hypothesis as no significant difference in erythrocyte TPI enzyme activity could be found between subjects with the c/c and c/-5G genotypes. The study clearly demonstrates the -8A mutation is linked to reduced erythrocyte TPI enzyme activity *in vivo*. This would be expected as -8 lies within the CPE, an essential regulatory element required for full expression of the TPI gene. Heterozygotes and homozygotes for the -8A allele have a significant reduction in erythrocyte TPI enzyme activity of around 10% and 24%, respectively, demonstrating a dosage effect for this mutation. This is consistent with a previous study (10) which found a higher proportion of -5G-8A chromosomes in the lower quartile range of TPI activity.

Heterozygotes for the common causative mutation in TPI deficiency have approximately 50% erythrocyte TPI enzyme activity and are phenotypically normal. In the present study, erythrocyte TPI enzyme activity in heterozygotes was 46% (414-477 U/gHb) of the normal mean, significantly lower than that observed in subjects with the variant promoter genotypes (Fig. 2). These observations make it unlikely that

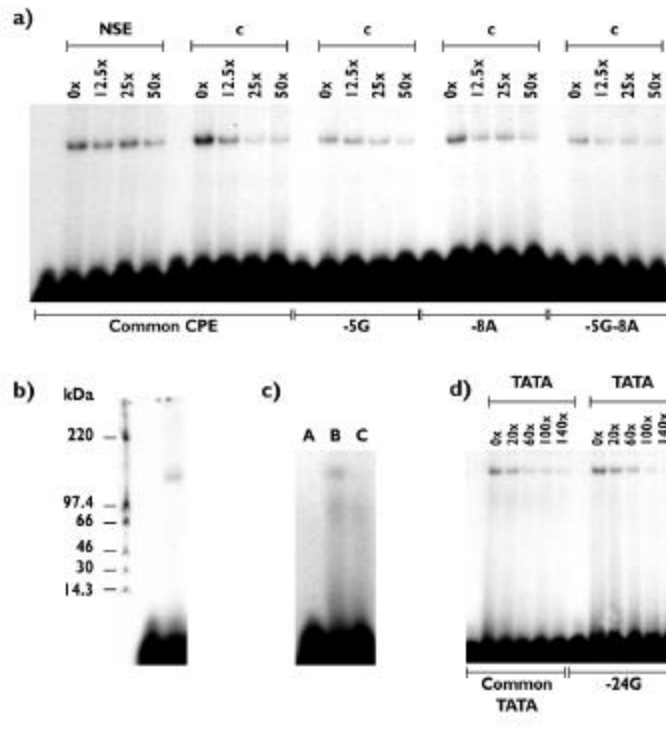


Figure 3. Electrophoretic mobility shift assays. (a) Competition assays for CPE oligonucleotides. Lanes 1-5 show $\gamma^{32}\text{P}$ [ATP] labeled common CPE oligonucleotide. Lanes 2-5 include HeLa nuclear extract and increasing concentrations of cold NSE non-specific competitor oligonucleotide. Lanes 6-10 show the effect of specific competition with oligonucleotide corresponding to the common CPE sequence. Lanes 11-15, 16-20 and 21-25 show conditions as for lanes 6-10 but include $\gamma^{32}\text{P}$ [ATP] labeled -5G, -8A or -5G-8A CPE oligonucleotides, respectively. (b) Protein molecular weight size marker demonstrating size of protein-DNA complex. Molecular weight of each band is indicated on the left. (c) Antibody supershift assays for common TATA oligonucleotide. Lane A shows $\gamma^{32}\text{P}$ [ATP] labeled common TATA oligonucleotide, lane B is as lane A but with the addition of HeLa nuclear extract and lane C is as lane B but with the addition of anti-TFIID mAb. (d) Competition assays for TATA oligonucleotides. Lanes 1-6 show $\gamma^{32}\text{P}$ [ATP] labeled common TATA oligonucleotide. Lanes 2-6 include HeLa nuclear extract with increasing concentrations of cold common TATA specific competitor oligonucleotide as indicated at the top of the figure. Lanes 7-12 are as lanes 1-6 but instead include $\gamma^{32}\text{P}$ [ATP] labeled -24G TATA oligonucleotide.

the -5, -8 and -24 promoter mutations contribute independently to clinical TPI deficiency.

The recognized consensus sequence for a TATA box is T-A-T-A^A/T-A^A/T-N, where ^A/T is A or T and N is any base. Observation on the effect of variation in the canonical motif TATAAA on gene expression in HeLa cells reveal that TATATAA (identical to the wild type TPI gene TATA box), TATATA and TATAGA have relative *in vitro* activity of 172%, 72% and 5%, respectively (15). It is therefore likely that the -24G allele, which creates a TATA box with the sequence TATAGAA, is associated with reduced TPI gene transcription. The level of erythrocyte TPI enzyme activity (708U/gHb) in a subject with

the -5G/-5G-8A-24G genotype was within the same range as homozygotes for the -8A allele (662-794U/gHb) and below the normal range (732-1391U/gHb), suggesting that this mutation has an additive effect on the level of TPI activity on the background of -8A heterozygosity. This is supported by findings in 4 subjects with the c/-5G-8A-24G genotype, all of whom showed TPI activity within the lower quartile of the normal range (10).

The present study is the first to demonstrate protein binding to the TATA box of the TPI gene promoter. Taken in conjunction with previous evidence of protein binding to the CPE site (8,9), this highlights the importance of these regulatory

regions for TPI gene regulation. The affinity of protein binding to both common and variant haplotypes appears similar and consistent with the relatively modest reduction in erythrocyte TPI enzyme activity associated with TPI promoter variation. The lack of differential protein binding *in vitro* may reflect the greater sensitivity of TPI enzyme assay relative to electrophoretic mobility shift analysis. The approximate size of the retarded band for the CPE site was 125kDa, which is close to that (110kDa) reported by Boyer and Maquat (9). Despite absence of overlap in sequence, the CPE and TATA oligonucleotides bind a protein of the same size. It is possible that a single complex binds to both the CPE and TATA oligonucleotides, and that this interaction can be facilitated through either the TATA box or the CPE element. Regulatory or “initiator” elements located around the transcriptional start or CAP site have been shown to be involved in expression from TATA-less RNA polymerase II promoters in which TATA binding protein is known to play a role (16).

TPI is functionally a housekeeping enzyme. Differences have been noted between the regulatory regions of housekeeping genes and differentially expressed or tissue specific genes. Housekeeping gene promoters are often associated with a high GC content (17) and lack diversity in protein binding motifs in comparison to facultative genes (18). In this respect, regulation of TPI gene expression is of interest as the TPI gene has a TATATAA box common to facultative gene promoters. Furthermore, TPI gene expression has been shown to be responsive to external stimuli such as serum (19), plasma (20) and sodium butyrate (21). Study of the effect of variation within the TPI promoter sequence may contribute to a more comprehensive understanding of the control of constitutively expressed genes.

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