

# Mutations in the general transcription factor TFIIH result in $\beta$ -thalassaemia in individuals with trichothiodystrophy

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**The transcription factor TFIIH is involved in both basal transcription and DNA repair. Mutations in the XPD helicase component of TFIIH can result in the diverse clinical features associated with xeroderma pigmentosum (XP) and trichothiodystrophy (TTD). It is generally believed that the multi-system abnormalities associated with TTD are the result of a subtle deficiency in basal transcription. However, to date, there has been no clear demonstration of a defect in expression of any specific gene in individuals with these syndromes. Here we show that the specific mutations in XPD that cause TTD result in reduced expression of the  $\beta$ -globin genes in these individuals. Eleven TTD patients with characterized mutations in the XPD gene have the haematological features of  $\beta$ -thalassaemia trait, and reduced levels of  $\beta$ -globin synthesis and  $\beta$ -globin mRNA. All these parameters were normal in three patients with XP. These findings provide the first evidence for reduced expression of a specific gene in TTD. They support the hypothesis that many of the clinical features of TTD result from inadequate expression of a diverse set of highly expressed genes.**

## INTRODUCTION

Trichothiodystrophy (TTD) is an autosomal recessive disorder, characterized by sulfur-deficient brittle hair, brittle nails, short stature, mental retardation, ichthyotic skin and in many cases, photosensitivity (1). Cells from photosensitive TTD patients are deficient in nucleotide excision repair (NER) of UV-induced

DNA damage. In this respect TTD cells resemble those from patients with xeroderma pigmentosum (XP), a quite different disorder characterized by extreme sensitivity of the skin to sunlight, sunlight-induced pigmentation changes in the skin and a very high frequency of skin cancers in sun-exposed areas (2,3). TTD patients in contrast have neither pigmentation changes nor skin cancers. The defect in NER in XP patients is associated with seven different complementation groups, and the majority of NER deficient TTD individuals have been assigned to the XP-D group (4,5). Clues to the paradox of mutations in a single gene resulting in these widely differing clinical conditions were provided by the discovery that the XPD protein was a subunit of the basal transcription factor TFIIH (4–6). Subsequent work revealed unequivocally that TFIIH has two distinct roles, first in basal transcription carried out by RNA polymerase II and secondly in NER of DNA damage. It was hypothesized that mutations in XPD that affected NER alone would result in the features of XP, whereas if the mutation affected the transcriptional role of TFIIH, the multi-system features of TTD would result. TTD was thus proposed to represent a 'transcription syndrome' (7). Consistent with this hypothesis, we showed that the sites of the mutations that resulted in XP and in TTD were different (8). Furthermore, one site that was mutated in several TTD patients (Arg722→Trp) was used to generate an XP-D mutant mouse (9). Remarkably, this mouse had many of the features characteristic of TTD patients (reduced size, hair loss, ichthyotic-like skin). These findings strongly supported the idea that the site of the mutation determines the clinical outcome.

TFIIH is made up of nine subunits, two of which are the products of the XP genes, XPD and XPB. Both XPB and XPD have ATP-dependent DNA helicase activities, of opposite polarity (5). The TFIIH holo-complex is comprised of a five-subunit core, which includes XPB, whereas XPD is less tightly

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**Table 1.** Clinical phenotypes in TTD with  $\beta$ -thalassaemia and XP-D patients

Clinical symptoms	TTD with $\beta$ -thalassaemia								XP-D		
	TTD2BR	TTD5BR	TTD7BR	TTD2GL	TTD1BI	TTD1ED	TTD2LO	TTD3LO	XP31BR	XPJCLO	XP107LO
Photosensitivity	+/-	+	NA	+	+	+	-	+	+++	+++	+++
Ichthyosis	+	+	+	+	+	+	+	+	-	-	-
Brittle hair and nails	+	+	+	+	+	+	+	+	-	-	-
Mental retardation	+++	-	+++	+++	+++	++	+++	+++	-	+	++
Accelerated neurodegeneration	+	-	+	+ <sup>b</sup>	+	ND	+	+	-	+	-
Growth failure	+	+	+	+	+	+	+	+	-	-	-
Delay pubertal development	ND	+	ND	ND	+	ND	ND	ND	ND	ND	-
Ataxia	+	+	NA	+	+	ND	-	ND	-	-	+
Recurrent infection	+	-	NA	NA	NA	-	+	-	-	-	-
Cataract	+	+	+	+	+	-	-	-	-	-	-
Dental caries	+	-	NA	NA	+	-	-	-	-	-	-
Abnormal skin pigmentation <sup>a</sup>	-	-	-	-	-	-	-	-	+	+	+
Skin cancer <sup>a</sup>	-	-	-	-	-	-	-	-	+	+	+
Result/outcome	Alive 11 years	Alive 16 years	Dead, 2.8 years	Dead, 3.5 years	Alive 18 years	Alive 1.5 years	Alive 4 years	Alive 2.5 years	Alive 6 years	Alive 11 years	Alive 23 years

Summary of the clinical phenotypes in eight of 11 patients with TTD (full clinical details were not available in three patients studied by S. Marinoni) and three with XP-D. ND, not determined; NA, not available. Clinical feature present (+) or absent (-).

<sup>a</sup>Specific features of XP.

<sup>b</sup>Diminished myelination of the central nervous system was demonstrated at post mortem.

associated and attaches the three-subunit 'CAK sub-complex' to the core (10,11). Although the helicase activity of XPD is required for NER, it is dispensable for transcription (12). Thus, it appears that XPD needs to be present to maintain the stability of the TFIIH complex for transcription initiation, but that its transcriptional function is quite tolerant of mutational changes. This may explain why many *XPD* mutations are compatible with human life.

Most patients with TTD who have mutations in the *XPD* gene are either homozygotes or compound heterozygotes for an allele that perturbs, but does not abolish XPD function. In many compound heterozygotes, the second allele is a null (8). *XPD* mutations found in TTD patients alter the conformation of XPD, reducing the stability of the TFIIH complex (M. Stefanini, unpublished data). In patients with one specific mutation (Arg658→Cys) this results in temperature sensitive instability of the TFIIH complex, which is manifested both in the patients and in their cultured cells (13).

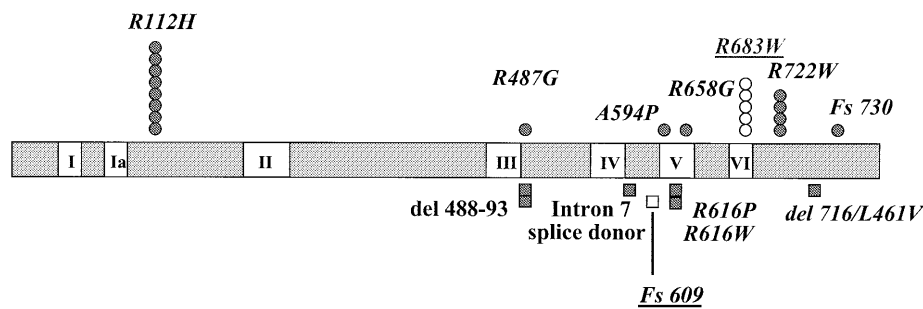
It might be anticipated that a deficiency in basal transcription resulting from a subtle change in TFIIH would result in rather general abnormalities in patients or mutant mice. In fact, the deficiencies, for example in Cys-rich matrix proteins (CRPs) of the hairshafts, are very specific. In most cells, *de novo* synthesis of the components of TFIIH is thought to compensate for the deficiency, allowing survival. However, de Boer *et al.* (9) proposed that in terminally differentiating cells (e.g. skin, hair and neurons) TFIIH may become limiting before the transcriptional programmes are complete. This may particularly affect the synthesis of highly expressed genes such as those

encoding CRPs in hair follicles, small proline-rich proteins in skin keratinocytes and myelin basic protein in neurons (9).

Despite the widespread acceptance of the idea that TTD is a transcription syndrome, there is surprisingly little direct evidence for deficient transcription in TTD (4). Reduced levels of mRNA of the *SPRR2* gene were found in the skin of the 'TTD' mouse, whereas two other genes were expressed at near-normal levels (9). This is the only example to date of a specific gene that has been shown to be affected in TTD. We now present the first example of a human gene that is specifically affected in TTD. The process of erythropoiesis is a further example of terminal differentiation, in which there are high levels of gene expression—in this case, of the globin genes (14). We show in this paper, that synthesis of  $\beta$ -globin is specifically reduced in TTD patients, but not in XP individuals from the XP-D complementation group.

## RESULTS

A report in the literature described  $\beta$ -thalassaemia trait in a TTD individual (15) and a second case became apparent in routine analysis of patient TTD7BR by P. Lunt. Therefore, we decided to evaluate the haematological status of a population of British and Italian TTD patients, to which we had access. We have analysed 11 TTD patients, all of whom were examined clinically by V. Viprakasit, R.J. Gibbons and D.R. Higgs. All the patients had the classical clinical features of TTD, including brittle hair and nails, ichthyosis, reduced growth,



**Figure 1.** Mutations in the XPD protein. Missense (circles) and null (squares) mutations in the XPD protein, in patients described here with TTD (closed circles, closed squares) and XP (open circles, open squares). The helicase motifs are labelled (I, Ia–VI). Mutations in XP patients are also underlined.

**Table 2.** Haematological data in patients with TTD and XP

Cases	Hb (g/dl) (11–18)	MCV (fl) (80–99)	MCH (pg) (27–31)	HbA <sub>2</sub> (%) (2.5–3.5)	NER <sup>b</sup> (%)	XPD mutation
<b>TTD</b>						
TTD2BR	10.1	59	19.5	4.3	30	Fs730 [del 488–93]
TTD5BR <sup>a</sup>	13.8	82	26.7	4.8	16	A594P [splicing intron7]
TTD7BR <sup>a</sup>	12.3	71	22.0	5.8	27	R487G [R616P]
TTD2GL	10.3	66	20.2	4.4	10	R112H [del 488–93]
TTD1BI	13.2	77	24.0	5.1	50	Fs 730 [not expressed]
TTD1ED <sup>a</sup>	11.7	78	23.4	5.1	nd	R658G [R616W]
TTD2LO <sup>a</sup>	12.5	69	20.7	4.7	17	R722W [del 716–30/L416V]
TTD3LO <sup>a</sup>	12.8	66	22.3	4.4	27	R722W R722W
TTD1PV	13.3	74	ND	4.9	12	R112H R112H
TTD2PV	14.3	81	ND	4.4	12	R112H R112H
TTD3PV	13.2	76	ND	5.1	12	R112H R112H
<b>XP</b>						
XP107LO	15	83	27.4	2.8	15	R683W R683W
XPJCLO	13	82	27.1	2.5	30	R683W R683W
XP30BR <sup>a</sup>	12.5	86	28.0	3.3	30	R683W [Fs609]

Mutations in both alleles are shown for each individual. Mutations thought to be nulls (8) are indicated between square brackets.

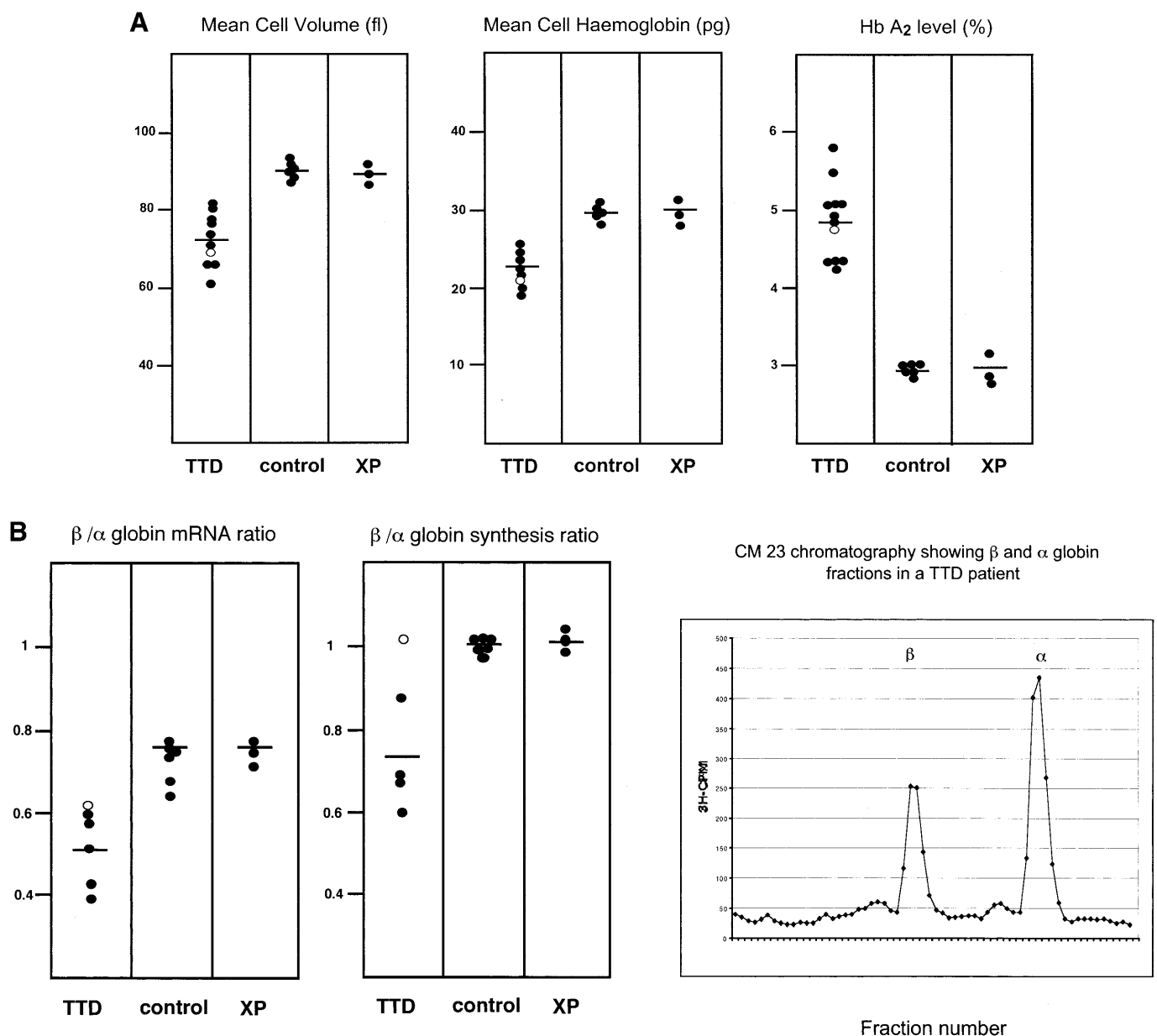
<sup>a</sup>Cases in which the mutations have not been reported previously. Others were described by Taylor *et al.* (8), Broughton *et al.* (25) and Botta *et al.* (29).

<sup>b</sup>NER measured as unscheduled DNA synthesis expressed as a percentage of value in normal cells.

and, with one exception, mental retardation (Table 1). We also included in our study three patients who had classical XP features of acute photosensitivity, abnormal skin pigmentation and skin cancers (Table 1). All patients were assigned to the XP-D complementation group using standard procedures. The NER levels of the patients, as measured by unscheduled DNA synthesis in cultured fibroblasts, were reduced to 10–50% of that of normal individuals (Table 2), as is characteristic for XP and TTD cells in the XP-D complementation group. The mutations in the *XPD* gene in all the patients are presented in Table 2 and Figure 1.

In standard haematological assays, we discovered that the group of 11 TTD patients all had the haematological

phenotype of  $\beta$ -thalassaemia trait. As shown in Table 2 and Figure 2, the mean red cell volume (MCV) was reduced (59–82 fl in TTDs, compared to 86–92 fl in normals and XPs), as was the mean cell haemoglobin (MCH) (19–26 pg in TTD compared to 27–31 pg in normals and XPs). The levels of HbA<sub>2</sub> were substantially elevated (4.3–5.8% in TTD, 2.7–3.1% in normals and XPs). Such changes are usually caused by mutations in the  $\beta$ -globin gene (16), and in particular the raised level of HbA<sub>2</sub> is the hallmark of  $\beta$ -thalassaemia. However, in the eight families from whom we were able to obtain data we ascertained that none of the parents of the affected individuals had  $\beta$ -thalassaemia. Haplotype analysis in four families confirmed that the affected individual had



**Figure 2.**  $\beta$ -Thalassaemia in TTD patients. (A) The MCVs, MCHs and levels of HbA<sub>2</sub> in TTD patients were clearly different from those in normal individuals and patients with XP ( $P < 0.005$ ). (B) The  $\beta/\alpha$ -globin RNA ratios in TTD patients determined by RNase protection assay were significantly different from controls and XP patients ( $P < 0.05$ ). Similarly, the  $\beta/\alpha$ -globin chain synthesis ratios in TTD patients were significantly different from controls ( $P < 0.05$ ). The open circle denotes TTD2LO who coincidentally inherited  $\alpha$ -thalassaemia ( $-\alpha/\alpha$ ). On the right, the profile of  $^3\text{H}$ -leucine incorporated into nascent  $\beta$ - and  $\alpha$ -globin chains during a 1 h incubation as described by Weatherall *et al.* (27). In this case the  $\beta/\alpha$ -globin synthesis ratio was 0.61 (control 0.99).

inherited two intact copies of the  $\beta$ -globin locus. Finally, we sequenced the  $\beta$ -globin gene in eight out of the 11 TTD patients but did not find any mutations. Therefore, these data rule out  $\beta$ -globin mutations as a cause of  $\beta$ -thalassaemia in our TTD patients.

We next examined reticulocytes from these individuals for their ability to synthesize  $\alpha$ - and  $\beta$ -globin (Fig. 2B). With one exception, all TTD patients had decreased levels of  $\beta$ -globin chain synthesis, when compared with corresponding levels of  $\alpha$ -globin. Furthermore the levels of  $\beta$ -globin mRNA were

reduced relative to  $\alpha$ -globin mRNA. (The one exception was TTD2LO. Interpretation of results with this individual was confounded because he coincidentally inherited  $\alpha$ -thalassaemia.) These findings are consistent with a reduced level of transcription of the  $\beta$ -globin gene, and therefore the simplest conclusion is that reduced  $\beta$ -globin gene expression resulted from the mutations in TFIIF. In order to show that these haematological deficiencies were specific for TTD individuals, we have also examined three XP-D patients who had clinical features of XP rather than TTD. All three were haematologically normal

(Table 1), with normal levels of  $\beta$ -globin mRNA and globin synthesis (Table 2; Fig. 2). We also analysed two patients with TTD who were not deficient in NER and presumably not mutated in the *XPD* gene. We found no abnormalities in globin synthesis in these patients (data not shown).

## DISCUSSION

Our data provide the first evidence of a specific human gene ( $\beta$ -globin), whose synthesis is affected by the postulated transcription abnormality in TTD. They provide strong support for the model that only those *XPD* mutations that result in TTD affect transcription, whereas those causing XP do not, thus supporting the repair/transcription model set out above.

Our results also give some insight into how specificity can result from alterations in TFIID, which a priori might be expected to have similar effects on different genes. Our results demonstrate that the reduced activity of this general transcription factor has different effects on the  $\alpha$ - and  $\beta$ -globin genes, which are both expressed at high levels in terminal erythroid differentiation. TFIID is thought to create an 'open' promoter complex, melting DNA via its helicase activity. This facilitates access of the transcription machinery, synthesis of the first phosphodiester bond and promoter clearance (17). However, promoters may differ in their requirement for TFIID. For example, some promoters may be premelted topologically rather than via helicases. This would reduce the requirement for TFIID in transcription initiation (18). Furthermore, the activity of TFIID may be modified via its interaction with different activators and repressors (19). Therefore, the  $\alpha$  and  $\beta$  genes may differ in their requirement for TFIID or use a different repertoire of activators and repressors that interact with TFIID. By implication, initiation of transcription from the human  $\alpha$ - and  $\beta$ -globin promoters may occur via different mechanisms. This would be consistent with many previously described structural and functional differences between the  $\alpha$ - and  $\beta$ -globin genes (20). For example, although both promoters have classical TATA boxes, the  $\alpha$  gene is associated with a CpG island whereas the  $\beta$  gene is not. The  $\beta$  gene shows replication/transcription coupling, the  $\alpha$  gene does not (21). Expression of the  $\alpha$  gene is downregulated by mutations of the chromatin associated SNF2-like factor (ATR) whereas the  $\beta$  gene is unaffected (22). In a variety of expression assays,  $\alpha$  globin is consistently induced more rapidly and easily than  $\beta$ -globin (reviewed in 20,23) as if it were already 'poised' for expression.

In summary, we have provided the first demonstration of a specific gene ( $\beta$ -globin) whose expression is affected in man by a mutation in the general transcription factor TFIID. We found reduced  $\beta$ -globin synthesis in all TTD patients examined, but in no XP patients. Our results provide strong support for the idea that TTD is a transcription syndrome and provide further insights into the mechanism whereby a defect in a general transcription factor can affect specific genes. Furthermore, the  $\alpha$ - and  $\beta$ -globin genes provide a clear example of co-ordinately expressed genes with apparently different requirements for, or regulation by, TFIID *in vivo*. Our findings also provide an important additional simple diagnostic tool for differential diagnosis of TTD.

## MATERIALS AND METHODS

### DNA repair and mutation analysis

For DNA repair and mutation analysis, fibroblast cultures were established from skin biopsies of all patients. NER was measured as unscheduled DNA synthesis, using autoradiography or liquid scintillation counting of  $^3\text{H}$ -thymidine incorporated into DNA of non-dividing cells following UV irradiation. Assignment to the XP-D complementation group has been described by Stefanini *et al.* (24). Mutations in the *XPD* gene were identified by RT-PCR of total cellular RNA, followed by direct sequencing (25).

### Globin gene analysis

$\beta$ -Globin haplotypes were determined by PCR amplification across seven restriction sites along the  $\beta$ -globin cluster and subsequent digestion with appropriate enzymes ( $\epsilon$ , *Hind*II;  $\gamma$ , *Hind*III;  $\delta$ , *Hind*III;  $5'$  and  $3'\psi\beta$ , *Hind*II;  $5'\beta$ , *Ava*II;  $3'\beta$ , *Hinf*I) extending from upstream of the  $\epsilon$  gene to downstream of the  $\beta$  gene. In some patients the  $\beta$ -globin gene was sequenced from  $-124$  bp upstream of the cap site to  $+163$  bp downstream of the poly(A) site. The  $\alpha$ -globin genotypes were analysed as described previously (26).

### Globin synthesis

Peripheral blood samples (10–15 ml) were washed four times with low salt buffer (0.13 M NaCl, 0.005 M KCl, 0.0074 M  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) to remove plasma and enrich for reticulocytes which were subsequently incubated with  $^3\text{H}$ -leucine,  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  and incubation media without leucine for 1 h. The globin chains were precipitated by 2% acid-acetone solution and separated by CM-23 cellulose column chromatography using a urea phosphate buffer gradient. The fractions were collected and the incorporated  $^3\text{H}$  measured by scintillation counting (27). The  $\beta/\alpha$ -globin RNA ratios in the reticulocytes were determined by the RNase protection assay (28).

### Statistical analysis

All differences between TTD donors on the one hand and normal and XP donors on the other hand were analysed using two-tailed Student *t*-tests.

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