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ORIGINAL PAPER

Novel and functional regulatory SNPs in the promoter region of *FOXP3* gene in a Gabonese population

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Abstract Parasites exert a selection pressure on their hosts and are accountable for driving diversity within gene families and immune gene polymorphisms in a host population. The overwhelming response of regulatory T cells during infectious challenges directs the host immune system to lose the ability to mount parasite specific T cell responses. The underlying idea of this study is that regulatory single nucleotide polymorphism (SNPs) can cause significant changes in gene expression in functional immune genes. We identified and investigated regulatory SNPs in the promoter region of the *FOXP3* gene in a group of Gabonese individuals exposed to a variety of parasitic infections. We identified two novel and one promoter variants in 40 individual subjects. We further validated these promoter variants for their allelic gene expression using transient transfection assays. Two promoter variants, −794 (C/G) and the other variant −734/−540 (C/T) revealed a significant higher expression of the reporter gene at basal level in comparison to the major allele. The identification of SNPs that modify function in the promoter regions could provide a basis for studying parasite susceptibility in association studies.

Keywords *FOXP3* · Polymorphism · Transfection · Tregs

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Introduction

In order to thrive successfully in a host, parasites strike a balance with the host immune system to increase their survival rate. This balance is accomplished by complex alteration of the innate and acquired immune response of the host. In this process, regulatory T (Tregs) cells play a central role (Belkaid et al. 2006). Tregs can suppress the function of conventional T cells and thus limit inappropriate or excessive immune responses (Workman et al. 2009). A few of them are induced during an infection episode and are so-called inducible Tregs. Another population exists prior to the pathogen exposure and are referred to as natural Tregs (Belkaid 2007). Natural Tregs play a vital role to avert autoimmunity. Parasites can ably manipulate natural Tregs by amending the T cell immune response at the infection site to an extent that could lessen the infection burden, thereby prevailing in the host for a longer time frame (Sakaguchi 2003). Immune system genes are predominantly polymorphic due to selection exerted by quickly varying pathogens (Maizels 2009). Promoter regions are potential candidates for the presence of functional single nucleotide polymorphism (SNPs), as they are involved in transcription initiation and many of the *cis*-acting elements that regulate gene expression possibly harbors functional polymorphisms (Hoogendoorn et al. 2003). Investigation of human polymorphisms in the promoter region of immuno-relevant genes may possibly reflect the level of Treg expression and thereby the extent of susceptibility to parasitic infection (Belkaid and Rouse 2005). One such gene of interest that plays a key role in the function of Tregs is the *FOXP3* gene locus.

FOXP3 belongs to a family of transcription factors that play a role in various cellular processes. The human *FOXP3* genes contain 11 coding exons. By genomic

sequence analysis, the *FOXP3* gene maps to the *p* arm of the X chromosome (specifically, X*p*11.23). It had been revealed that absence of functional *FOXP3* in mice and humans direct to autoimmune diseases, suggesting the pivotal role of *FOXP3* in regulation (Coffer and Burgering 2004). Mutations in the *FOXP3* gene in humans are known to be associated with immunedysregulation polyendocrinopathy and enteropathy X-linked syndrome disorder caused due to lack of natural Tregs (Sakaguchi et al. 2007a). Studies have demonstrated that absence of functional FOXP3 protein, ablate the efficiency of thymocytes to differentiate into mature T cells (Lin et al. 2007). *FOXP3* is described as a master regulator of natural Tregs development and function (Gavin et al. 2006; Kim and Leonard 2007; Sakaguchi et al. 2007b). Also, studies had revealed that defective and suppressive function of natural Tregs are known to be associated with type 1 diabetes or multiple sclerosis (Bacchetta et al. 2007). Recent studies have evaluated that promoter polymorphism in the *FOXP3* gene −2383C/T polymorphism is associated with systematic lupus erythematosus (SLE) and the −2383T allele is risk factor for SLE in the Chinese population (Lan et al. 2010). Also, three SNPS in the promoter region (−6054, −3279, and −924) and one in intron 9 (IVS9+459) of the *FOXP3* gene are associated with primary biliary cirrhosis (PBC) in females (Park et al. 2005).

In the current study, we aim to identify regulatory SNPs on *FOXP3* gene locus from 40 unrelated Gabonese individuals who had earlier infection episodes with various parasites. The SNPs were further validated for their allelic gene expression, which may possibly be correlated with various physiological responses.

Materials and methods

Genomic DNA isolation

A total of 40 DNA samples obtained from unrelated Gabonese individuals were used to identify SNPs in the promoter region of *FOXP3* genes by direct sequencing. Blood samples were collected from adult male patients with uncomplicated malaria at the Medical Research Unit of the Albert Schweitzer Hospital, Lambaréné, Gabon, between August and November 2004. The study was approved by the local ethics committee of the International Foundation of the Albert Schweitzer Hospital. DNA was isolated using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany).

Sequencing and SNP identification

For sequencing analysis, gene and genomic sequences (NM014009) of the *FOXP3* gene was obtained from the SNPper database (http://snpper.chip.org/). PCR primers were designed to amplify the promoter region of the gene using the PRIMER3 Software [(Rozen and Skaletsky 2000); http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi]. Promoter regions were amplified by PCR from genomic DNA isolated from the 40 subjects using the forward primer FOXP3_F 5′- ATGATGGCGGATATTTG-GAA - 3′ and the reverse primer FOXP3_R 5′-CACATCTGGTAGGGGAGA - 3′ (MWG Operon, Ebersberg, Germany). PCR amplifications were carried out in 50 μl reaction volumes with 5 ng of genomic DNA, 10 μl of 5x HotStar HiFidelity PCR buffer (contains dNTPs and 7.5 mM $MgSO_4$), 1 μM of each primer and 2.5 U HotStar HiFidelity DNA-Polymerase on a PTC-200 thermal cycler (MJ Research, USA). Thermal cycling parameters were: initial denaturation at 94°C for 5 min, followed by 40 cycles of 15 s at 94°C denaturation, 1 min at 61°C annealing temperature, 1 min at 72°C extension, followed by a final additional extension of 10 min at 72°C. The amplified PCR products were analyzed by electrophoresis in 1% agarose gels, with a 100 bp DNA ladder molecular size marker (Invitrogen, Karlsruhe, Germany). PCR products were cleaned up using Exo-SAP-IT (USB, Affymetrix, USA) and 1 μl of the purified product was directly used as templates for sequencing, using the BigDye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI 3100 DNA sequencer, according to the manufacturer's instructions. The primers employed for sequencing are the FOXP3_forward and FOXP3_reverse. DNA polymorphisms were identified when assembled with reference sequence of the *FOXP3* gene (NM014009) in the chromosome (position: chrX 48,994,354-49,008,232) obtained from SNPper database (http://snpper.chip.org/) using the BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Seqscape V2.5 (Applied Biosystems, USA) program.

Cloning and construct preparation

To confirm that the single nucleotide polymorphisms are without PCR errors induced by Taq polymerase, the respective genomic DNA identified to have SNPs was amplified with primers containing a 15 bp homology to the linearised vector and were cloned into a pGL3 basic vector. In brief, PCR amplifications were carried out in 50 μl reaction volumes with same program conditions as mentioned above. The amplified PCR products were analyzed by electrophoresis in 1.5% agarose gels with a 100 bp DNA ladder molecular size marker (Invitrogen, Karlsruhe, Germany). PCR products were eluted from the gel and purified using a Nucleospin kit (Macherey-Nagel, Düren, Germany). Cloning was done into the pGL3 basic

vector using infusion advantage PCR cloning kit (Clonetech, Mountain View, CA). Plasmids were isolated using illustra™ plasmid Prep Mini Spin kit (GE Healthcare, Freiburg, Germany). The plasmids carrying the promoter fragment were identified by restriction enzyme digest with *Bgl*II. Since one of the investigated plasmid DNA carried two SNPs, a site-directed mutagenesis was carried out using the QuikChange® Lightning Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) to analyze the effect of both SNPs independently. To ensure accuracy of the sequenced promoter regions, several independent plasmids containing inserts were sequenced in both directions using appropriate primer pairs. The plasmid that exhibits true polymorphism was transformed into one shot *Escherichia coli* (Invitrogen, Karlsruhe, Germany). Two independent colonies were picked from these transformations and DNA was prepared using Endofree plasmid maxi kit (Qiagen, Hilden, Germany).

Transfection assays

We tested the activities of the observed polymorphic promoters using a Jurkat T cell line (DSMZ, Braunschweig, Germany) at basal level. Four independent transfection experiments for each construct in duplicates were performed with Jurkat cells. The procedure was performed in 24 well plates. Cells were incubated until the time of transfection. Jurkat cells (0.8×10^6 cells/μl) were grown in RPMI-1640 medium (Sigma-Aldrich, Hamburg, Germany) supplemented with 10% FBS (SAFC Biosciences, Portland, USA), 2 mM L-glutamine (Sigma-Aldrich, Steinheim, Germany), 1% streptomycin–penicillin substrate (Invitrogen, Karlsruhe, Germany), and 5 μg/ml plasmocin (InvivoGen, San Diego, USA). Jurkat cells (0.8×10^6 cells/μl) were transfected with TransIT reagent (Mirus Bio, Madison, USA) as recommended by the manufacturer. In brief, 120 μl of TransIT reagent was added to 3 ml of RPMI 1640 serum-free medium (Sigma-Aldrich, Hamburg Germany) and was incubated for 20 min at room temperature. Each of the 24 well plates was then seeded with 0.5 ml (0.8×10^6 cells/μl) of Jurkat T cells along with 2.5 μg of plasmid DNA constructs in addition to 20 ng of Renilla and was allowed to incubate for 20 min. After incubation, 52 μl of TransIT + RPMI serum-free medium mix were suspended across each well. The whole procedure was performed in 2×24 well plate formats. After 48 h of incubation, cells were harvested by centrifugation, washed twice with phosphate-buffered saline, and lysed in 100 μl of 1x passive lysis buffer (Promega, Mannheim, Germany). After incubation of 20 min at room temperature on a rocking platform, 10 μl of the lysate was used for the measurement of luciferase activity in the SIRIUS luminometer (Berthold detection system, Pforzheim, Germany). Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Mannheim, Germany). For each experiment, a plasmid expressing constitutively Renilla luciferase in low amounts was used as transfection control (Juliger et al. 2003), while a promoterless plasmid (pGL3-Basic) was integrated as a negative control. Each transfection experiment was performed four times in duplicates with two different DNA preparations. Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity.

Transcription factor-binding search

An extensive search for transcription factor-binding sites for the observed SNPs in the promoter region was performed using TF-Search online tool (http://www.cbrc.jp/research/db/TFSEARCH.html).

Statistical analysis

Data have been analyzed by StatView (http://www.statview.com). Comparison of the activity of the *FOXP3* promoter variants among each other and to the activity of major allele was analyzed by their luciferase activities. *P* values are calculated by ANOVA and corrected by Bonferroni/Dunn. The statistically significant level was set as 0.05. Haplotype analysis was performed using the Haploview v4.2 software (Barrett et al. 2005).

Results

We constructed five plasmids: construct 1 carried SNP −794 C/G; construct 2 contained two SNP variants at positions −738 C/T and −540 C/T; construct 3 possessed SNP variant −738 C/T; construct 4 contained SNP variant −540 C/T; and construct 5, a plasmid with the most frequent alleles. Comparison of the activity of the *FOXP3* promoter variants analysed by luciferase activities revealed significant differences between constructs (Fig. 1). Promoter variant −794 C/G showed a significantly increased activity in comparison to variant −738 C/T ($P=0.0003$), to variant −540 C/T (P value<0.0001) and to the major allele ($P=0.0002$). The promoter variant with two SNPs −738 C/T and −540 C/T displayed a significantly higher activity compared with variant −540 C/T ($P<0.0001$) and the major allele ($P=0.0031$). A detailed search for putative transcription factor-binding sites indicated that the new SNP −794 C/G was positioned one base next to the binding site of the transcription factor acute myeloid leukemia-1a (AML-1a). This transcription factor binds in the sequence either in presence or absence of the SNP. At position −738, a

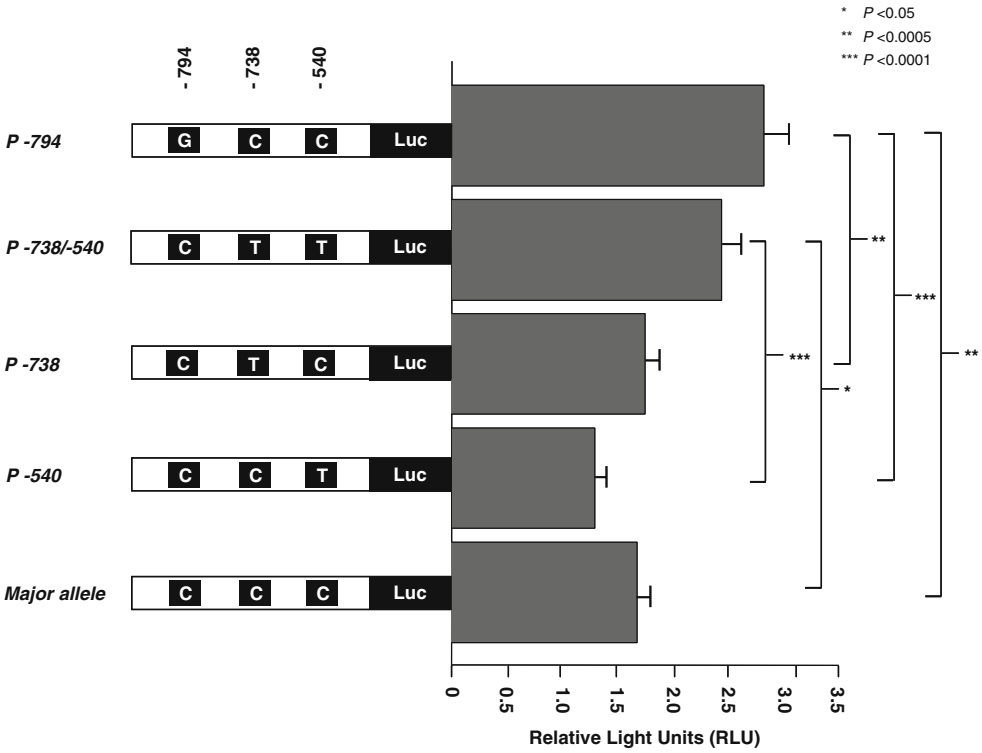

Fig. 1 Comparison of the activity of the four different *FOXP3* promoter variants analyzed by luciferase activities. Given is the ratio of the relative light units (firefly/renilla). *P* values are calculated by ANOVA and corrected by Bonferroni/Dunn from four different experiments done in duplicates with two different DNA preparations

transcription factor nuclear factor kappa B (NF-κB) and c-Rel binding was located. At position −540 the transcription, factor MZF-1 (myeloid zinc finger-1) binds in the sequence either in presence or absence of the SNP. An additional transcription factor p300 was found binding to the SNP variant −540.

All 40 subjects were sequenced for the promoter region of the *FOXP3* gene and were analyzed for SNPs. Two novel SNPs were identified at positions −794 C/G and −540 C/T and also an SNP that was described earlier in the SNPper database was identified at position −738 C/T rs11091253 (Table 1). The number of haplotypes observed and their corresponding frequencies is presented in Table 2. Since only males were investigated, all three SNPs were hemizygous. All positions are provided relative to the *FOXP3* transcription start site. DNA sequence of the promoter of the *FOXP3* gene, the primer pairs used for PCR amplification, and the observed mutation sites are presented in Fig. 2. The comparison of linkage disequilibrium between three loci in the promoter regions of the *FOXP3* gene locus as inferred from haploview revealed that the SNP variants were not linked.

Discussion

The analysis of regulatory SNPs in populations that are naturally exposed to a variety of parasites contributes to a

Table 1 Genetic variants identified in the promoter region of the *FOXP3* gene locus

SNP	Polymorphism	Flanking sequences	Analysed Individuals	Allele	Frequency
# ss270137548	−794 C>G	GGCCT [C/G] TGGGG	39	C	0.975
			1	G	0.025
# rs11091253	−738 C>T	CCTTC [C/T] CAGTG	29	C	0.725
			11	T	0.275
# ss270137549	−540 C>T	CTCAC [C/T] CTCTG	38	C	0.950
			2	T	0.050

The position of each SNP was determined as regard to transcription start site

Table 2 Observed haplotypes in the promoter regions of the *FOXP3* gene locus

Haplotypes	Expected numbers	Frequency
CCC	56	0.700
CTC	18	0.225
CTT	4	0.050
GCC	2	0.025

better understanding on how the immune system and its genes respond to parasites. Parasites represent a major selective force by influencing the genetic predisposition determined by immune system gene variants (Maizels 2009). SNPs in the promoter region may potentially alter gene expression by changing the binding specificity of transcription factors to their binding sites and by modifying the kinetics of transcription initiation. In our current study, we identified two novel promoter variants of *FOXP3*, namely −794 C/G and −540 C/T. We also found one predescribed variant −738 C/T. When compared to the NCBI HapMap database, the observed SNP variant #rs11091253 and the two novel SNP variants remained not polymorphic for the Yoruba population of Nigeria that represents a sub-Saharan African group. The Gabonese individuals belong to different ethnic groups but most of the individuals in the current study represent Bantu population. A very large cohort will remain obligatory to associate any disease severity with these identified mutations. One interesting part in our study was that the identified promoter variant construct −794 C/G showed a significant increased activity in comparison to the constructs with SNPs at positions −738 C/T, −540 C/T, and the major allele. Upregulation of *FOXP3* was shown to be correlated with rapid parasite growth during a malarial infection (Walther et al. 2005). A putative binding site for the transcription factor AML-1a situated in proximity to position −794. The protein AML-1a is produced by the AML1 gene by alternative splicing. Studies had indicated that the production of IL-2 and IFN-gamma is suppressed by the interaction of *FOXP3* with AML1 in natural Tregs, whereby molecules associated with Tregs are upregulated and thereby suppressive activity is exercised (Ono et al. 2007). This effectively signifies that *FOXP3* controls regulatory T cell function by interacting with AML1, whereby this interaction in turn could possibly increase *FOXP3* promoter activity. SNP variant −738 is located in a putative binding site for the transcription factors NF-κB and c-Rel. The pathway that is activated by NF-κB plays a vital role in activating essential pathways to regulate the *FOXP3* expression. It is also shown that increased activity of NF-κB results in a raised number of $FOXP3^{+}$ cells in the thymus (Long et al. 2009). The other transcription factor c-Rel, a member of NF-κB family is known to be essential for the regulation of the transcription of *FOXP3* (Hori 2010). This substantiates the lower activity of the *FOXP3* promoter in constructs carrying the SNP −738 C/T in comparison to variant −794 C/G. SNP variant −540 C/T is located in the transcription factor site MZF-1. MZF-1 is a krupple class zinc finger genes encoding a putative transcriptional regulator of myeloid differentiation. MZF-1 is expressed in nonhematopoietic cells as a transcriptional repressor, whereas in hematopoietic cells, it acts as a transcriptional activator (Morris et al. 1995). However, a separate study concluded that there was no expression of MZF-1 expression levels in Jurkat T Cells (Hromas et al. 1991). This explains the reason that −540 C/T SNP variant exhibited a lowest activity in comparison to other constructs. At the very instance, the SNP variant −540 C/T also alter the transcription factor p300, a coactivator with histone acetyltransferase activity. This transcription factor protects *FOXP3* from polyubiquitination and degradation in the proteasome by acetylation (van Loosdregt et al. 2010) leading to the regulation of Tregs by stabilizing the FOXP3 protein.

Several studies have revealed *FOXP3* act as target genes for development and maturation of Tregs (Marson et al. 2007; Zheng et al. 2007). A recent study showed that promoter polymorphism −3279 A/C relative to the translation start site is associated with an increased risk of psoriasis in a Chinese population (Gao et al. 2010). Variants in the promoter of *FOXP3* are also associated with type 1 diabetes in Japanese patients (Bassuny et al. 2003). Three SNPS in the promoter region (−6054, −3279, and −924) and one in intron 9 (IVS9+459) of the *FOXP3* gene are associated with PBC in females (Park et al. 2005). The lack or reduced activity of certain Tregs owing to mutations in *FOXP3* should be considered critical in autoimmune and inflammatory diseases. All these autoimmune and allergic events could be unintended evolutionary consequences of alleles that evolve to increase fitness for resistance to parasitic infections.

The identified regulatory SNPs in this current study will afford useful information for understanding the relevance of sequence polymorphisms in populations of different ethnic background and may serve as a basis to study parasite susceptibility in association studies. These regulatory polymorphisms in the promoter region of *FOXP3* may aid to identify potential targets for the therapeutic control in gene expression. The detection and analysis of such human immune gene polymorphisms contribute to a better understanding of the interaction between host and parasite and will remain crucial in a genetic makeup of the patient population to level of infection and to that of expression of Treg activity.

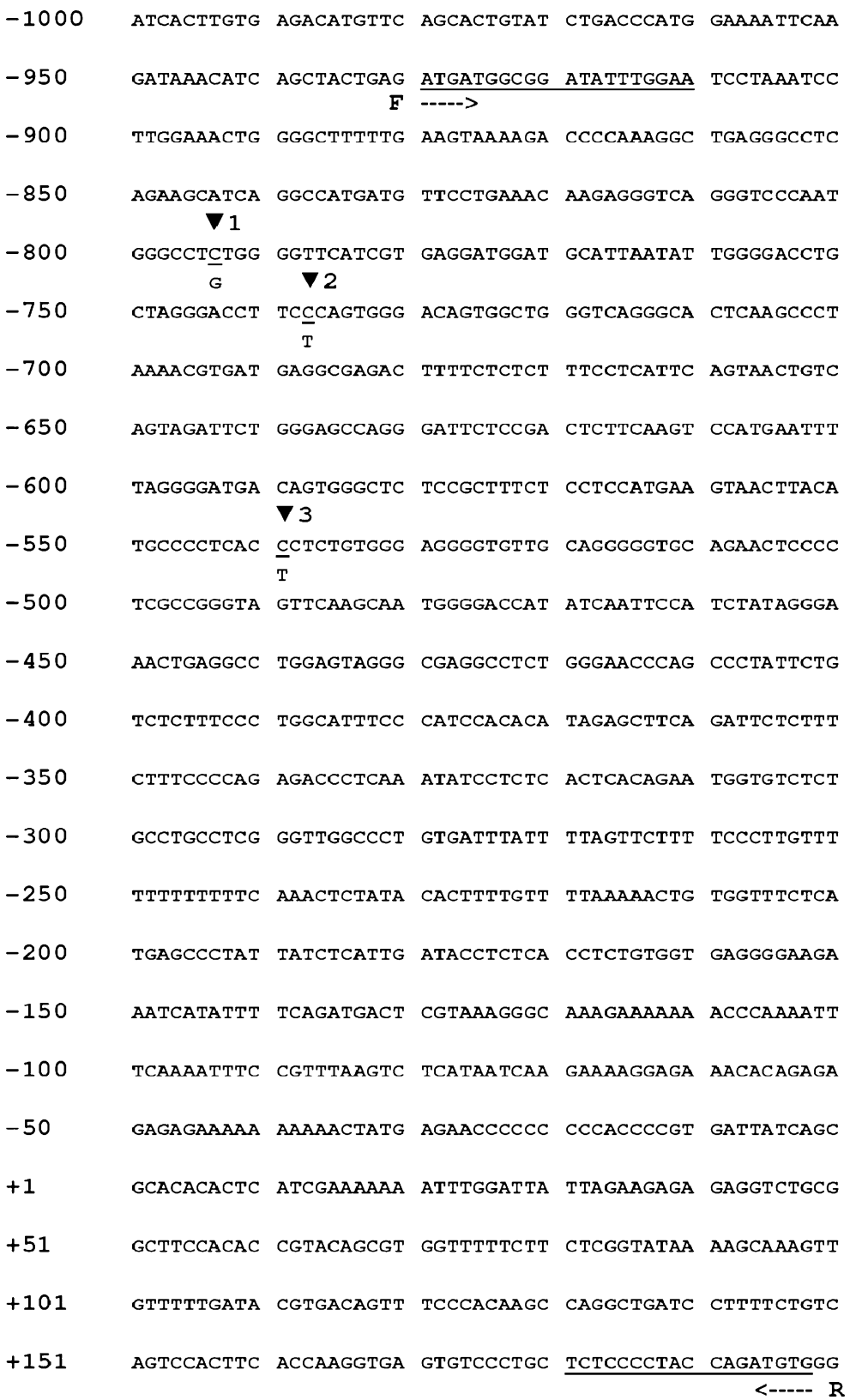

Fig. 2 DNA sequence of the promoter of the *FOXP3* gene. Primer sequences used for amplification of the promoter sequence are *underlined* (*F* forward, *R* reverse). The SNP positions relative to transcription start site in the promoter region are marked as *inverted triangle 1* (−794 C/G), *inverted triangle 2* (−738 C/T rs 11091253), and *inverted triangle 3* (−540 C/T). The start site of transcription is initiated by +1

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