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Title: **Effects of the multiple sclerosis associated -330 promoter polymorphism in *IL2* allelic expression**

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Abstract

The −330 IL2 gene promoter polymorphism has been associated with multiple sclerosis (MS) [J. Neuroimmunol. 119 (2001) 101], but the basis underlying this association remains unknown to date. In the present work, we have found that IL2 promoter-luciferase constructs, transfected in Jurkat cell line, showed twofold higher levels of gene expression in the −330 G allele. However, the transcriptional effect of this polymorphism in lymphocytes showed that the G allele was related to lower expression of IL2. This difference increased in the patient group. Divergence between in vivo and in vitro influence of the −330 IL2 promoter polymorphic site suggests the existence of additional unknown polymorphisms affecting gene regulation. Our data show an increased IL2 expression among GT and TT genotypes previously associated with susceptibility to MS.

Keywords: Interleukin-2; Polymorphism; Multiple sclerosis; Expression quantification; Transfection; Real-time PCR

1. Introduction

Multiple sclerosis (MS) is characterized by chronic inflammation and demyelination in the central nervous system (CNS) most likely arising from an autoimmune process (Brosnan and Raine, 1996). The precise molecular and cellular mechanisms that provoke the myelin damage have still to be elucidated although both genetic and environmental contributions to the pathogenesis are inferred from epidemiologic studies (Compston, 2000; Willer and Ebers, 2000). Genes may influence susceptibility to the development of MS and the subsequent course of the disease (Kantarci et al., 2002). To date, a gene within or close to the MHC II class locus is the most likely candidate determinant of susceptibility, although the form of this association is not considered to be as straightforward as previously thought (Herrera and Ebers, 2003). MS is believed to be a complex trait in which interactions between small effects of the several susceptibility genes and environment increase the probability of inflammatory pathways deregulation and demyelination in CNS (Kalman et al., 2002).

A number of genetic studies looked for associations between MS and polymorphic alleles of candidate genes which were selected mainly on the basis of their involvement in the autoimmune pathogenesis and include immunorelevant molecules such as cytokines, cytokine receptors, immunoglobulins, T cell receptor subunits and myelin antigens (Oksenberg et al., 1996; Epplen et al., 1997; Weinshenker et al., 1997; Vandenbroeck et al., 1997; Roth et al., 1995). Importance of IL-2/IL-2R system for the T cell homeostasis at the levels of repertoire selection, the generation of suppressive regulatory T cells, T cell homing and clonal contraction via activation induced cell death (reviewed in Schimpl et al., 2002) indicates its relevance in the development of autoimmune disease.

Studies in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, also give evidence for the implication of *IL2* locus in the susceptibility to this disease. The levels of IL-2 and *IL2* mRNA were found to be elevated in CNS during the induction and acute phase of EAE, and lowered during recovery (Kennedy et al., 1992). On the other hand, a genetic linkage analysis using a cross between susceptible and resistant mice revealed a locus affecting EAE severity which contains the *IL2* gene (Encinas et al., 1999). Vulnerability to EAE is markedly reduced in C57BL/ 6 mice lacking *IL2*, suggesting that this cytokine may play a critical role in autoimmune processes of the central nervous system (Petitto et al., 2000). In a previous study we reported that the genotypes (G/T and T/T) at –330 site (–384 from the ATG) in the human *IL2* promoter are associated with the susceptibility to MS (Matesanz et al., 2001). This single nucleotide polymorphism is located in the upstream region of the *IL2* gene promoter – enhancer domain (John et al., 1998). Recently, Hoffmann et al. (2001) reported differences in protein production between the *IL2* promoter polymorphic variants in healthy controls. In the present study we evaluate the influence of the –330 promoter polymorphism in the expression of the corresponding *IL2* alleles.

2. Materials and methods

2.1. Genotyping of healthy control subjects and patients with MS

Blood samples were obtained with the informed consent of unrelated Spanish Caucasoid patients with clinically definite multiple sclerosis who attended the Hospital Carlos Haya (Malaga, Spain) and healthy controls of similar age and genetic background. Patients had relapsing remitting or secondary progressive form of the disease (Lublin and Reingold, 1996). DNA was extracted by standard procedure (Ausubel et al., 1990). Genotyping of –330 and +114 SNPs was carried out by PCR-RFLP as previously described (Matesanz et al., 2001).

2.2. Cell cultures

Human Jurkat leukemia cell line was maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum (Gibco), 2.0 mM glutamine, 50 μM 2-mercaptoethanol, and 50 mg/l gentamycin (Sigma)(complete medium).

2.3. Plasmid construction

Two variants of human *IL2* promoter –enhancer fragment from −500 to + 1 containing G or T at −330 site were prepared by PCR from −330 *IL2* heterozygous individual genomic DNA as template and CGGGATCCCAGGAAACCAATAAC TTCC (forward) and GAAGATCTTGTGGCAGGAGTTGAGGTTA (reverse) oligonucleotides, creating a *BamH*I and a *Bgl*II restriction sites. The amplification products were cloned into the pGEM-T vector (Promega). The insert sequences of the recombinants were confirmed by sequencing. Recombinants containing either the G or the T allele were prepared and the inserts were excised using restriction endonucleases *BamH*I and *Bgl*II. Then the fragments were subcloned into luciferase vector pXP2 (Nordeen, 1988). The

final plasmids pXP2-330IL-2/ G-Luc and pXP2-330IL-2/T-Luc were sequenced to verify that no unwanted mutations were introduced in the process of cloning. Large-scale plasmid DNA was prepared using plasmid purification kit (Qiagen), following the recommended protocols. Experiments were performed with three independent plasmid preparations.

2.4. Transient transfection

Jurkat cells were transfected by electroporation. Briefly, plasmid DNA (40 μg) was mixed with exponentially growing Jurkat cells (20 x 10^6) in complete medium, and the cells were electroporated in an electrocell manipulator 600 (BTX, San Diego, CA) at 250 V and a capacitance of 975 μF. The transfected cells were cultured for 24 h, purified in a Histopaque-1077 gradient (Sigma), washed twice in complete medium and activated with phorbol-myristate acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml), both from Sigma. After various period of time (4, 14 and 20 h), activated cells from an independent flask and control transfected cells without activation were harvested (300 x g, 5 min, 4 ºC) and washed twice in phosphate-buffered saline (4 ºC).

2.5. Luciferase activity analysis

Luciferase activity was evaluated using reagents from the Luciferase Assay System (Promega). Washed pellets of transfected cells were treated with 80 μl of 1 x Reporter Lysis Buffer for 5 min on ice. Cells were repipetted five times to ensure lysis of all the cells, lysates were spun down for 1 min and stored at −80 ºC. For analysis, lysates were thawed on ice and the protein concentration was measured (Bradford method). Then 40 μg of protein (in a volume of less than 20 μl) from all supernatants were analyzed with Luciferase Assay Reagent (100 μl) and measured as quadriplicate for 30 s in luminometer F12 (Berthold Detection Systems). Control samples (transfected cells that had not been

activated and activated cells that had been transfected with ‘‘empty’’ plasmid pXP2-Luc) contained equal minimal levels of luciferase activity (< 500 RLU). The level of luciferase activity is represented as RLU/40 μg of protein.

2.6. Relative allelic expression

Total RNA from + 114 G/T heterozygous individual PBL, stimulated for 4 h with PMA (50 ng/ml) and ionomycin (1 μg/ml), were extracted with Trizol and checked for accidental degradation. Total RNA (200 ng) were used for cDNA synthesis with MMLV reverse transcriptase (Invitrogen), RNAguard (Amersham Biosciencies) and oligonucleotide IP41 (GTTTCAGATCCCTTTAGTTC), a bridge between the 3rd and 4th exons. Five microliters of cDNA was used to amplify a 372-bp fragment of the *IL2* (+ 1 to + 372). PCR was performed in a 30-μl volume (forward primer ATGTACAGGATGCAACTCCT) under the following conditions: 95 ºC for 5 min followed by 25 cycles of 20 s at 94 ºC, 40 s at 54 ºC, and 20 s at 72 ºC and a final prolongation step of 10 min at 72 ºC. During the last cycle, 1 pmol of ^{32}P oligonucleotide IP 41 labeled with [gamma-^{32}P] ATP plus 0.5 U of fresh Taq polymerase was added to the PCR tubes. Ten microliters of the final PCR product was used for restriction reaction with *Mwo*I (cuts the G allele) and electrophoresed on 10% PAGE. Bands were quantified by phosphoimager (Image Eraser, Amersham Bioscienses).

2.7. Real-time quantitative transcript analysis

Total RNA (200 ng) from 4 h activated PBL were converted into cDNA using MMLV RT enzyme and hexanucleotides. Three microliters was PCR amplified in a final volume of 25 μl with 75 mM Tris –HCl, pH 9, 4 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1 mM dNTP, 0.1 x SYBR Green I (Molecular Probes), 20 pmol of specific oligonucleotides and 0.6 U *Taq* polymerase (Biotools). The oligonucleotides used were

loop incorporated primers to overcome the dimers and nonspecific products formation (Ailenberg and Silverman, 2003). The sequences were as follows: *IL2* forward (CAAGAAGCAGTTCTGTGGCCTTCTTG), reverse (GTAGAACGCACCTACTTCAAGTTCTAC); *UbcH5b*, forward (CAATTCCGAAGAGAATCCACAAGGAATTG), reverse (GTGTTCCAACAGGACCTGCTGAACAC). The realtime PCR consisted (for the *UbcH5b* as well as for the *IL2*) of one cycle 94 ºC, 3 min, followed by 45 cycles of 94 ºC 20s, 60 ºC 20s, 72 ºC 20s. The amplification and the detection of the product was performed with the iCycler iQ apparatus (BioRad). Every sample was analyzed in triplicate. No dimer formation was observed in any PCR product by the melting curve analysis. Reverse transcription real-time PCR efficiencies were calculated from the given slopes in the iCycler iQ software obtained from RT-PCR amplification of 6 twofold serial dilutions of RNA (200 –6.25 ng). The mean normalized expression values were calculated from the obtained *ct* of the *IL2* and *UbcH5b*, and their respective standard curve slopes with the qgene software (Muller et al., 2002) http://www.wzw.tum.de/gene-quantification/).

2.8. Statistical analysis

Transfection data were analyzed by Student's t-test. Results are presented as means ± S.E.M. (n = 4) for representative experiments. Significant differences in values were similar in all experiments, although actual values for experimental and control samples varied between experiments. Differences between allele ratios in groups and results of *IL2* RNA quantification were compared by Mann –Whitney test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. The –330 IL2 promoter polymorphism affects gene transcription

Our previous results showed that the –330 genotypes (G/T and T/T) were associated with susceptibility to MS. In order to examine the role of the –330 G/T polymorphism in transcriptional regulation of the human *IL2* gene, we performed transient transfection of Jurkat cells with reporter constructs pXP2-330IL-2/G-Luc and pXP2-330IL-2/T-Luc containing either nucleotide G or T at –330. Cells were stimulated with PMA–ionomycin after 24 h of transfection and the luciferase activity was quantified at different times after activation. Fig. 1 shows that reporter constructs containing the fragment of human *IL2* promoter with nucleotide G at –330 have twofold increased ($P < 0.0001$) activity upon transfection compared with reporter constructs containing nucleotide T at –330. This difference was sustained at all studied time intervals after stimulation.

3.2. The –330 promoter polymorphism associates with a differential IL2 allele expression in lymphocytes from multiple sclerosis patients

To determine the influence of the –330 G/T *IL2* promoter polymorphism in human lymphocytes, we measured the relative expression of G/T alleles by means of a +114 G/T SNP located in the first exon of the *IL2*. This SNP is silent, it does not change the IL-2 protein sequence and it can be used as a marker to quantify expression of maternal and paternal alleles in heterozygous cells by RT-PCR-RFLP method (Matesanz et al., 2000).

As we described (Matesanz et al., 2001), linkage disequilibrium exists between –330 and + 114 *IL2* polymorphisms, there is no –330 G/G genotype in + 114 G/T and + 114 T/T individuals, therefore, the –330 G/+114 T haplotype does not exist. The + 114 G allele is always found on the same haplotype as –330 G allele. In this way when we

quantify the expression of the + 114 G or T allele we can ascribe each one to a −330 G or T allele.

We performed PCR with a final cycle-labeling methodology (Kaijzel et al., 2001) using a ^{32}P-antisence primer that labeled the PCR product of one DNA chain. This method avoids the quantifications of heteroduplex which cannot be cut by the restriction enzyme. We validated the procedure of quantification by measuring the intensities of the fragments resulting from PCR-RFLP of DNA mixtures with a known distribution of + 114 G and + 114 T alleles. Fig. 2 shows that the ratios of band intensity in DNA mixtures after PCR-RFLP equals the ratios in the DNA input ($R2$ = 0.9999).

Measurements of the + 114 G/T allele ratio were performed in total RNA of activated PBL from 32 healthy and 23 MS patients with −330 GT or TT genotypes. In the healthy control group, expression of allele + 114 G was significantly lower than expression of allele + 114 T (ratio 0.88) for −330 G/T individuals in comparison with −330 T/T individuals (ratio 0.96, P= 0.0021). This difference increased in the MS patient group (ratio 0.69 for −330 G/T group, ratio 0.87 for −330 T/T group, P= 0.0009). Significant differences were observed comparing + 114 G/T ratios for the −330TT or GT genotypes between patients and healthy controls (Table 1).

3.3. The –330 G/G genotype promoter is linked to a lower level of IL2 RNA

We determined the *IL2* expression level in stimulated PBL of 23 healthy individuals bearing different genotypes for the −330 SNP by reverse transcription real-time PCR using SYBR Green I. Relative quantification method was applied to each sample using the *UbcH5B* housekeeping gene as reference (Hamalainen et al., 2001). The mean normalized expression (n = 3) between the *IL2* and *UbcH5B* was calculated according to Eq. (3) of Muller et al. (2002) by the *qgene96* software. The slopes of the

standard curve graph performed to determine the PCR efficiency were −3.421 for the *IL2* and −3.693 for the *UbcH5b* housekeeping gene. Out of 23 RNA analyzed, 9 were of T/T, 9 were T/G and 5 were G/G genotype at −330position. The mean normalized expression values for each genotype is represented in Table 2. The expression of the IL2 is significantly lower for samples with the G/G than with the T/T and T/G genotype.

4. Discussion

Our previous work showed that the IL2 −330 G/T and T/T genotypes were associated with susceptibility to MS. In this study, we examined the influence of the −330 IL2 promoter polymorphism on the gene expression in healthy and MS individuals. This was carried out by transfection of Jurkat cells with constructions containing two allelic variants upstream a reporter gene, measurement of the relative expression of the −330 G allele against the −330 T, and quantification of the total *IL2* expression in individuals carrying the three different −330 genotypes.

The transfection study in Jurkat cells demonstrated differential promoter activity between the G and the T alleles. The construction carrying G was twice more active than the one with T. Conversely, quantification of allelic expression in lymphocytes showed that the −330 T allele was associated with a higher level of transcription than the −330 G. These data are in concordance with our real-time quantification results, since a higher expression level of *IL2* mRNA was observed in the samples of individuals with −330 T/T and G/T genotypes compared with the ones bearing the −330 G/G genotype. These results are in disagreement with those of Hoffman et al. which described an enhancement in *IL-2* production in individuals genotyped as −330 G/G.

The divergence between our results obtained in transfected Jurkat cells and the ones obtained in peripheral lymphocytes suggests that the −330 promoter polymorphism is influenced by factors linked to the −330 polymorphism. The transfection study in Jurkat cells showed the effect on the luciferase gene expression of an *IL2* promoter fragment isolated from other regulatory elements. They could be located at the far upstream promoter or in the gene where several RNA stability regulatory elements are defined

(Ragheb et al., 1999). This regulation could be modified by polymorphisms or methylation pattern.

In the relative allelic quantification study of healthy controls, the ratio + 114 G/T in −330 T/T genotype was close to 1 (0.96) as theoretically expected. However, in MS patients bearing the same promoter genotype, this value was significantly lower than 1 (0.87). Since we compared the −330 T/ + 114 G with the −330 T/ + 114 T haplotype, the difference cannot be attributed to sequence polymorphism at −330. The mechanism involved in this process is unknown but it seems to be associated with the + 114 polymorphism.

In the data of + 114 G/T relative allelic quantification for −330 G/T genotype, the ratio difference between healthy controls and MS patients is augmented (0.88 vs. 0.69, P= 0.0057). This indicated that both haplotypes were differently affected by regulatory factors and this phenomenon ought to be distinct in cells from patients and healthy controls. The existence of a linkage disequilibrium with other unknown polymorphisms can influence an allelic transcription and this may be more evident for MS patients.

The position of this polymorphism is not at any known transcription factor consensus sequence. However, deletion studies of the human *IL2* promoter region, containing this polymorphism have shown that the elimination of the −361/−292 (from the transcriptional start site) sequence reduces the reporter gene activity in Jurkat cells to 40% (Williams et al., 1988). On the other hand, *DNase* I footprinting studies of the mouse distal −600/−300 bp *IL2* region revealed multiple tissue-specific hypersensitive sites (Ward et al., 1998). The *IL2* promoter sequences up to 600 bp are highly conserved between mouse and human (Novak et al., 1990). It has been postulated that the region outside the established *IL2* minimal enhancer may serve as a stable nucleation site for

tissue-specific factors and as a potential initiation site for activation-dependent chromatin remodeling (Ward et al., 1998).

Our results reflect the complex regulatory mechanism of *IL2* gene expression and indicate that the influence of the −330 polymorphism is modulated by other elements. The higher expression of *IL2* −330 GT and TT genotypes, associated with susceptibility to MS, and the altered relative expression between alleles in MS patients supports the importance of *IL2* in the MS. On the other hand, the contribution of different *IL2* expression levels to the MS pathogenesis should be seen in the context of a multifactorial disease, considering interactions with other susceptibility genes.

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References

Ailenberg, M., Silverman, M., 2003. Differential effects of trichostatin A on gelatinase A expression in 3T3 fibroblasts and HT-1080 fibrosarcoma cells: implications for use of TSA in cancer therapy. Biochem.Biophys. Res. Commun. 302, 181– 185.

Ausubel, F.A.R., Brent, R.E., Kingston, D.D., Moore, J.G., Seidman, J.A., Smith, J.A., Struhl, K., 1990. Current Protocols in Molecular Biology.Wiley Interscience, New York.

Brosnan, C.F., Raine, C.S., 1996. Mechanisms of immune injury in multiple sclerosis. Brain Pathol. 6 (3), 243– 257.

Compston, A., 2000. The genetics of multiple sclerosis. J. Neurovirology 6 (Suppl. 2), S5– S9.

Encinas, J.A.,Wicker, L.S., Peterson, L.B., Mukasa, A., Teuscher, C., Sobel, R., Weiner, H.L., Seidman, C.E., Seidman, J.G., Kuchroo, V.K., 1999. QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing IL-2. Nat. Genet. 21, 158– 160.

Epplen, C., Jackel, S., Santos, E.J., D'Souza, M., Poehlau, D., Dotzauer, B., Sindern, E., Haupts, M., Rude, K.P., Weber, F., Stover, J., Poser, S., Gehler, W., Malin, J.P., Przuntek, H., Epplen, J.T., 1997. Genetic predisposition to multiple sclerosis as revealed by immunoprinting. Ann. Neurol. 41 (3), 341–352.

Hamalainen, H.K., Tubman, J.C., Vikman, S., Kyrola, T., Ylikoski, E.,Warrington, J.A., Lahesmaa, R., 2001. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal. Biochem. 299,63–70.

Herrera, B.M., Ebers, G.C., 2003. Progress in deciphering the genetics of multiple sclerosis. Curr. Opin. Neurol. 16 (3), 253– 258.

Hoffmann, S.C., Stanley, E.M., Cox, D.E., Craighead, N., DiMercurio, B.S., Koziol, D.E., Harlan, D.M., Kirk, A.D., Blair, P.J., 2001. Association of cytokine polymorphic inheritance and in vitro cytokine production in anti-CD3/CD28-stimulated peripheral blood lymphocytes. Transplantation 72 (8), 1444– 1450.

John, S., Turner, D., Donn, R., Sinnott, P., Worthington, J., Ollier, W.E.R.,Hutchinson, I.V., Hajeer, A.H., 1998. Two novel biallelic polymorphisms in the IL-2 gene. Eur. J. Immunogenet. 25, 419– 420.

Kaijzel, E.L., Bayley, J.P., van Krugten, M.V., Smith, L., van de Linde, P., Bakker, A.M., Breedveld, F.C., Huizinga, T.W., Verweij, C.L., 2001. Allele-specific quantification of tumor necrosis factor alpha (TNF) transcription and the role of promoter polymorphisms in rheumatoid arthritis patients and healthy individuals. Genes Immun. 2 (3), 135– 144.

Kalman, B., Albert, R.H., Leist, T.P., 2002. Genetics of multiple sclerosis: determinants of autoimmunity and neurodegeneration. Autoimmunity 35 (4), 225– 234.

Kantarci, O.H., de Andrade, M., Weinshenker, B.G., 2002. Identifying disease modifying genes in multiple sclerosis. J. Neuroimmunol. 123 (1–2), 144–159.

Kennedy, M.K., Torrance, D.S., Picha, K.S., Mohler, K.M., 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. J. Immunol. 149, 2496–2505.

Lublin, F.D., Reingold, S.C., 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 46 (4), 907–911.

Matesanz, F., Delgado, C., Fresno, M., Alcina, A., 2000. Allelic selection of human interleukin-2 gene. Eur. J. Immunol. 30, 3516– 3521.

Matesanz, F., Fedetz, M., Collado-Romero, M., Fernandez, O., Guerrero, M., Delgado, C., Alcina, A., 2001. Allelic expression and interleukin-2 polymorphisms in multiple sclerosis. J. Neuroimmunol. 119, 101–105.

Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Bio-Techniques. 32, 1372-1374, 1376, 1378– 9.

Nordeen, S.K., 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques 6 (5), 454–457.

Novak, T.J., White, P.M., Rothenberg, E.V., 1990. Regulatory anatomy of the murine interleukin-2 gene. Nucleic Acids Res. 18 (15), 4523–4533.

Oksenberg, J.R., Seboun, E., Hauser, S.L., 1996. Genetics of demyelinating diseases. Brain Pathol. 6 (3), 289– 302.

Petitto, J.M., Streit, W.J., Huang, Z., Butfiloski, E., Schiffenbauer, J., 2000. Interleukin-2 gene deletion produces a robust reduction in susceptibility to experimental autoimmune encephalomyelitis in C57BL/6 mice. Neurosci.Lett. 285, 66– 70.

Ragheb, J.A., Deen, M., Schwartz, R.H., 1999. CD28-mediated regulation of mRNA stability requires sequences within the coding region of the IL-2 mRNA. J. Immunol. 163, 120–129.

Roth, M.P., Dolbois, L., Borot, N., Pontarotti, P., Clanet, M., Coppin, H.,1995. Myelin oligodendrocyte glycoprotein (MOG) gene polymorphisms and multiple sclerosis: no evidence of disease association with MOG. J. Neuroimmunol. 61 (2), 117– 122.

Schimpl, A., Berberich, I., Kneitz, B., Kra¨mer, S., Santner-Nanan, B., Wagner, S., Wolf, M., Hu¨nig, T., 2002. IL-2 and autoimmune disease. Cytokine Growth Factor Rev. 13, 369– 378.

Vandenbroeck, K., Martino, G., Marrosu, M., Consiglio, A., Zaffaroni, M., Vaccargiu, S., Franciotta, D., Ruggeri, M., Comi, G., Grimaldi, L.M., 1997. Occurrence and clinical relevance of an interleukin-4 gene polymorphism in patients with multiple sclerosis. J. Neuroimmunol. 76 (1– 2), 189– 192.

Ward, S.B., Hernandez-Hoyos, G., Chen, F., Waterman, M., Reeves, R., Rothenberg, E.V., 1998. Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions. Nucleic Acids Res. 26, 2923–2934.

Weinshenker, B.G., Wingerchuk, D.M., Liu, Q., Bissonet, A.S., Schaid, D.J., Sommer, S.S., 1997. Genetic variation in the tumor necrosis factor alpha gene and the outcome of multiple sclerosis. Neurology 49 (2), 378–385.

Willer, C.J., Ebers, G.C., 2000. Susceptibility to multiple sclerosis: interplay between genes and environment. Curr. Opin. Neurol. 13 (3), 241– 247.

Williams, T.M., Eisenberg, L., Burlein, J.E., Norris, C.A., Pancer, S., Yao, D., Burger, S., Kamoun, M., Kant, J.A., 1988. Two regions within the human IL-2 gene promoter are important for inducible IL-2 expression. J. Immunol. 141, 662–666.

Table 1.

Relative allelic expression of IL2[a]

Genotype –330	+ 114 G/ + 114 T ratio
TT	–330T +114G/ –330T +114T[b]
Healthy controls *(n*= 15)	0.96 ± 0.11[c]
MS patients (*n*= 14)	0.87 ± 0.08[d]
GT	–330G +114G/ –330T +114T[b]
Healthy controls *(n*= 17)	0.88 ± 0.12[e]
MS patients (*n*= 9)	0.69 ± 0.12[f]

Mann– Whitney two-sided test.

[a] Mean ratio ± S.E.M.

[b] Haplotype frequencies: –330 T/114 T (MS = 0.294; Controls =0.296); –330 G/ 114 G (MS = 0.313; Controls = 0.317); –330 T/114 G (MS = 0.391; Controls = 0.385).

[c] *P* (TT healthy controls/GT healthy controls) = 0.0021.

[d] *P* (TT healthy controls/TT MS patients) = 0.05.

[e] *P* (GT healthy controls/GT MS patients) = 0.0057.

[f] *P* (TT MS patients/GT MS patients) = 0.0009.

Table 2.

Mean normalized expression (MNE) of *IL2* in activated PBL[a]

Genotype −330	MNE *IL2* ± S.E.M.
GG *(n*= 5)	577 ± 458[b]
GT *(n*= 9)	1142 ± 610[c]
TT (*n*= 9)	936 ± 471[d]

[a] *P* analyzed by Mann– Whitney one-sided test.

[b] *P* (GG vs. GT) = 0.03.

[c] *P* (GT vs. TT) = 0.33.

[d] *P* (TT vs. GG) = 0.03.

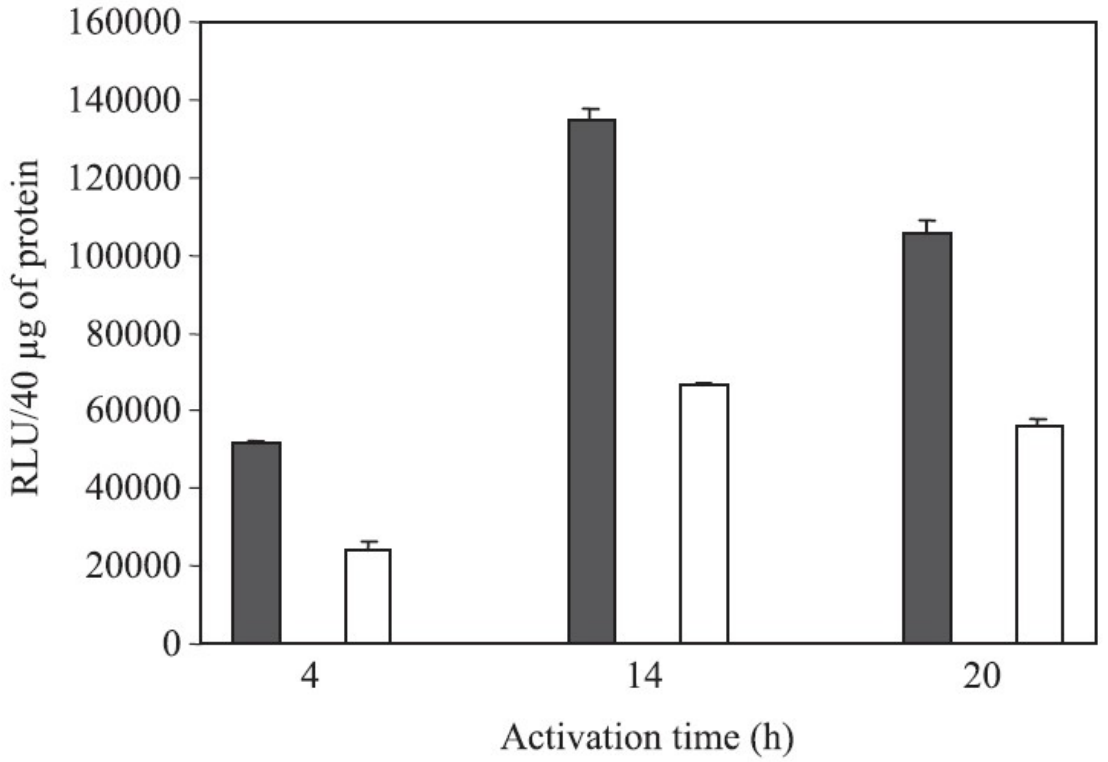

Fig. 1. Transcriptional activity of the −330 *IL2* G and T alleles in Jurkat cells. Cells were transiently transfected with a plasmid pXP2 containing a luciferase reporter gene controlled by the two −330 variants (G/T) of the *IL2* promoter– enhancer region (−501 to + 1), and activated with PMA–ionomycin during 4, 14 and 20 h. Promoter activity was measured by conducting the luciferase assay as described in Materials and methods. Luciferase activity is expressed in relative light units/40 µg of protein. Results are represented as means ± S.E.M. (n = 4) for representative experiments. White bars, the promoter activities of reporter constructs containing nucleotide T at −330; black bars, the promoter activities of reporter constructs containing nucleotide G at −330. RLU= relative light units.

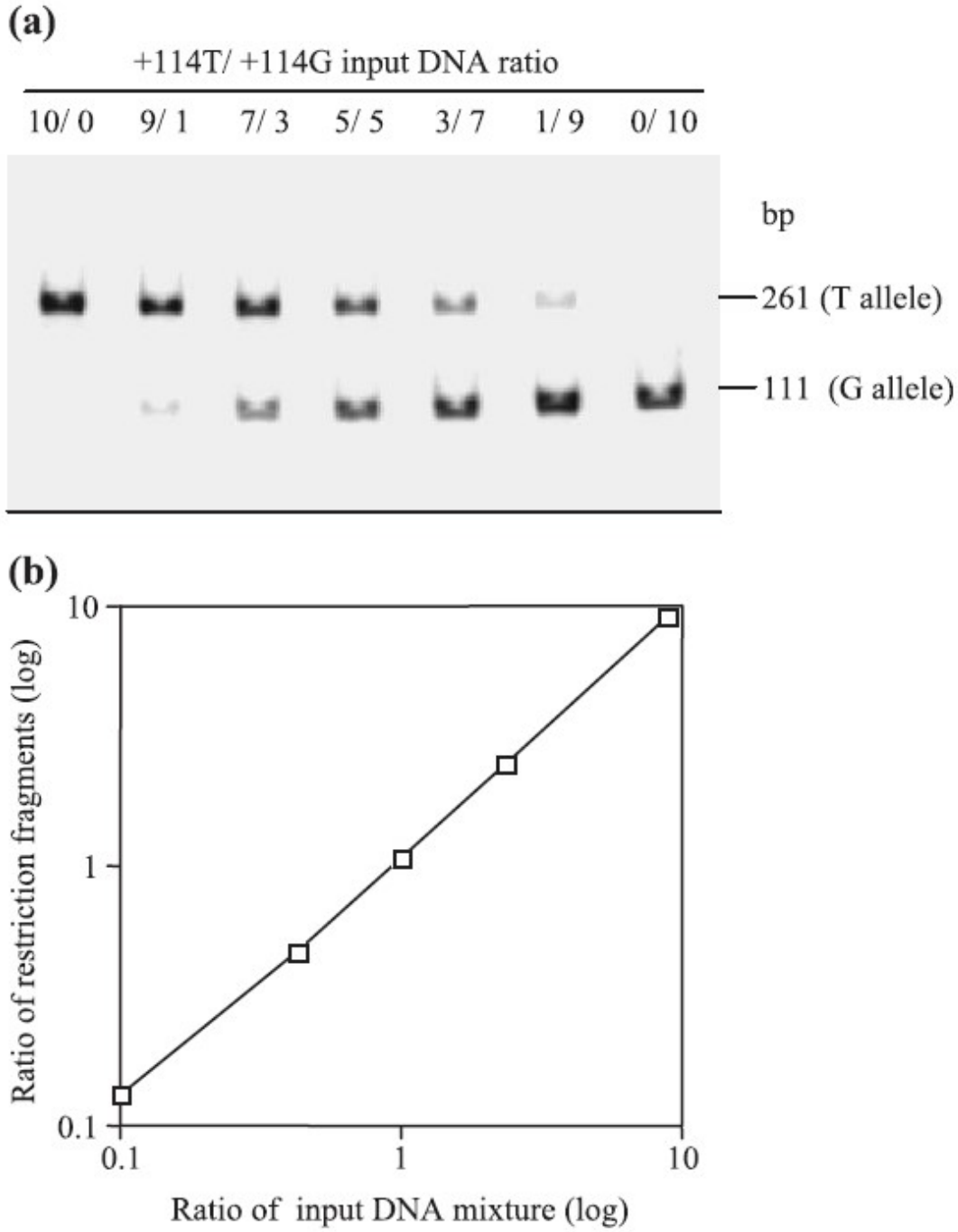

Fig. 2. Validation of relative allelic quantification procedure. (a) DNA mixtures containing different ratios of + 114 T and + 114 G DNA were used for PCR with final cycle radiolabelling then were digested with *Mwo*I and subjected to gel electrophoresis. (b) Results of gel phosphorimaging presented in graphic form demonstrate relationship between the input DNA template ratio and the ratio of restriction fragments.