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ScienceDirect
Molecular Immunology 45 (2008) 1009–1017
Molecular Immunology
www.elsevier.com/locate/molimm

Identification of a new cis-regulatory element of the terminal deoxynucleotidyl transferase gene in the 5′ region of the murine locus

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Received 27 June 2007; received in revised form 26 July 2007; accepted 27 July 2007
Available online 12 September 2007

Abstract

Terminal deoxynucleotidyl transferase (TdT) expression is controlled at the transcriptional level, however, the *TdT* core promoter combining D, D′, an initiator (Inr) and downstream basal elements (DBE) does not recapitulate the whole complex regulation of TdT expression. We hypothesized that important cis-regulatory elements of the gene are located outside of the *TdT* promoter. In an attempt to identify these elements, we performed DNase I hypersensitivity assays over 24 kb including a 10 kb region located upstream of the transcription start site (+1) and a 14 kb region spanning exons and introns I to VI. Hypersensitive sites (HS) HS1 and HS2 were localized 8.5 and 8 kb upstream of the transcription start site, respectively, and were exclusively detected in TdT^+ cell types. HS3, HS4 and HS5 were mapped at positions −7, −3.4 and −3 kb, respectively, and detected in both TdT negative and positive cells. HS6, HS7 and HS8 were detected immediately upstream of the *TdT* promoter. HS10 and HS11 were localized in the first and third intron of the gene. Luciferase reporter assays revealed that HS1, HS2 and HS3 synergize with the *TdT* promoter to activate transcription in a TdT^+ pre-T cell line but not in a TdT^+ pro-B cell line. In summary novel cis-regulatory elements have been identified in the 5′ region of the *TdT* locus that synergize with the promoter to activate gene expression and our results suggest these elements may be more active in T cells.

Keywords: TdT; Gene regulation; cis-Regulatory element

1. Introduction

Terminal deoxynucleotidyl transferase (TdT) is responsible for non-templated addition of nucleotides (Bollum, 1974) at the coding ends of immunoglobulins (Igs) and T cell receptors (TCR) genes during V(D)J somatic recombination (Gilfillan et al., 1993; Kallenbach et al., 1992; Komori et al., 1993). These non-germline encoded nucleotides termed N-additions greatly contribute to the junctional diversity of B and T cell antigen receptors (Cabaniols et al., 2001; Gilfillan et al., 1995, 1993). In mice, TdT is expressed at a significantly lower level in fetal and neonatal primary lymphoid organs than in the adult (Chang, 1971; Grégoire et al., 1979). Consequently, T and B cell receptors that are generated during early stages of development are less diverse than their adult counterparts (Benedict et al., 2000; Bogue et al., 1991, 1992; Feeney, 1990, 1991, 1992, 1993). However, it has been shown that a low level of TdT expression is sufficient to generate N diversity in TCR α junctions derived from neonatal thymocytes (Cherrier et al., 2002).

After birth, TdT is expressed in bone marrow pro-B cells (Asarnow et al., 1993; Wasserman et al., 1997) and from the most immature $CD3^-CD4^-CD8^-$ [triple negative (TN)] stage to the $CD4^+CD8^+$ [double positive (DP)] stage where TCR-mediated positive selection occurs in the thymus (Bogue et al., 1992; Groves et al., 1995). TdT expression is regulated at the transcriptional level (Bogue et al., 1992; Smale and Baltimore, 1989; Trangas and Coleman, 1989). The murine *TdT* promoter (−111/+58) contains four major regulatory elements: D, D′, an initiator (Inr) and downstream basal elements (DBE). This "minimal" promoter supports lymphoid specific transcription (Garraway et al., 1996). Inr (−11/+6) and DBE (+33/+58)

Abbreviations: TdT, terminal deoxynucleotidyl transferase; Igs, immunoglobulins; TCR, T cell receptors; TN, $CD3^-CD4^-CD8^-$ [triple negative]; DP, $CD4^+CD8^+$ [double positive]; Inr, initiator; DBE, downstream basal elements; HS, hypersensitive sites; kb, kilobases; NT, non-treated controls

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doi:10.1016/j.molimm.2007.07.027

support basal transcription *in vitro* (Smale and Baltimore, 1989). The D′ element (−78/−51) binds transcription factors Elf-1 and Ikaros (Ernst et al., 1996; Garraway et al., 1996; Lo et al., 1991). Elf-1 binding accounts for the tissue-specificity of the promoter (Ernst et al., 1996), whereas Ikaros mediates repression of TdT expression through T cell differentiation (Trinh et al., 2001). Epigenetic factors such as the extent of CpG methylation and post-translational modifications of histone proteins are known to control chromatin conformation and accessibility of tissue-specific genes to the general transcription machinery. The CpG methylation status of a region located immediately upstream of the *TdT* promoter correlates with expression in lymphoid tissues and cell lines as well as during mouse ontogeny (Nourrit et al., 1999).

Despite recent advances made in the description of mechanisms underlying the inhibition of TdT expression after the DP stage (Su et al., 2004; Trinh et al., 2001), many gaps remain to be filled in the understanding of the complex transcriptional regulation of the *TdT* gene. Indeed, the *TdT* promoter fails to support substantial expression levels in transient transfection assays (Lo et al., 1991). Reporter gene assays showed that transcription drops by 80% when the 5 kb located upstream of the gene are replaced by a minimal *TdT* promoter (−111, +58) (Lo et al., 1991) strongly suggesting that additional cis-regulatory elements are required to fully activate transcription in adult life and remain to be identified.

In order to identify new regulatory elements of the gene lying outside of the *TdT* promoter, we choose to localize regions binding factors that are specifically expressed in lymphoid tissues and cell lines using DNase I hypersensitivity assays. Ten lymphoid specific hypersensitive sites (HS) could be detected. Functional analysis of hypersensitive sites was subsequently performed using a luciferase reporter gene assay in TdT positive B and T cell lines. Briefly, putative regulatory elements were introduced in a proximal position upstream of the promoter driving luciferase expression. Three different promoters were used, the minimal *TdT* promoter (−111/+58) containing D, D′, Inr and DBE elements; a larger *TdT* promoter (−548/+58) including G, F and EE′ upstream elements (Nourrit et al., 1999); the ubiquitous SV40 promoter (PSV40) as control.

2. Material and methods

2.1. Isolation of nuclei from tissues and cell lines and treatment with DNase I

Nuclei from thymocytes, RL12, 18.8 (Nourrit et al., 1999) and BASP (Kallenbach et al., 1992) cells were prepared as previously described (Forrester et al., 1990). The thymus was removed from 4-week-old C57/BL6 mice and dissociated through a 70 μm cell strainer in phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum (FCS). 1–2 × 10^8 cells were wash in cold PBS and centrifuged at 1000 rpm for 5 min, resuspended in 1–2 mL of reticulocyte standard buffer (RSB) containing 10 mM Tris–HCl pH 8, 10 mM NaCl, 10 mM $MgCl_2$ and 0.1% Nonidet P-40. Samples were kept on ice for 5 min, centrifuged at 4000 rpm for 4 min and washed in 1–2 mL RSB. Nuclei were resuspended in RSB at the concentration of 10^8 nuclei per mL. Hepatocyte nuclei were isolated from mouse liver homogenate using a sucrose density barrier (as described in Blobel and Potter, 1966). Tissue was homogenized in an iso-osmotic medium containing 0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$ and 10 mM Tris HCl, pH 7.4. Homogenate was filtered and centrifuged at 800 × *g* for 10 min. The pellet was then resuspended in ∼1.6 M sucrose and layered over 2.3 M sucrose. After centrifugation at 10,000 × *g* for 1 h, nuclei were washed and resuspended in RSB at the concentration of 10^8 nuclei per mL. Nuclei suspensions derived from thymocytes, RL12, BASP and 18.8 cells were then treated with DNase I (Worthington, DPFT) at the concentration of 3 μg/mL at 37 °C for 20 min, 100 μL aliquots were collected every 2 or 4 min. Non-treated controls (NT) were set up as follows: a 100 μL aliquot of nuclei suspension was incubated without DNase I at 37 °C for 20 min.

Hepatocyte nuclei were treated with DNase I concentrations ranging from 0.5 to 6 μg/mL at 25 °C for 4 min. Non-treated controls (NT) were set up as follows: a 100 μL aliquot of nuclei suspension was incubated without DNase I at 25 °C for 4 min. In each case, the reaction was immediately stopped with 100 μL lysis buffer containing 50 mM EDTA, 2% SDS. Lysates were treated with RNAse A 50 μg/mL and proteinase K 30 μg/mL overnight at 37 °C, DNA was isolated by phenol chloroform extraction.

2.2. Southern blot analysis

Thirty micrograms of DNA were digested with 200 U of restriction enzyme (100 U/μL), precipitated and fractionated by electrophoresis on a 0.8% agarose gel. Acid depurination was performed in 0.25N HCl and DNA was denatured in 0.4N NaOH. DNA was transferred to a positively charged nylon membrane Hybond-N^+ Amersham in 0.4N NaOH, membrane was neutralized in 2× SSC. Probes a–h (Table 2) were labeled with [α-^{32}P] dCTP using Megaprime DNA labeling system from Amersham Biosciences according to manufacturer's instructions, specific activity was around (5 × 10^8 cpm/μg). Membranes were prehybridized in 0.25 M sodium phosphate buffer, 1 mM EDTA, BSA 1%, SDS 7% for 1 h at 65 °C. Radio-labeled probe (50–100 ng) was denatured 5 min at 95 °C in the presence of salmon sperm DNA prior to hybridization. Hybridization was performed in the same buffer at 65 °C overnight. Membranes were washed in 2× SSC 0.1% SDS, 1× SSC 0.1% SDS and 0.5× SSC 0.1% SDS, successively. Hybridization signals were detected by autoradiography.

2.3. Construction of plasmids

Fragments of the *TdT* gene were isolated from an I129 mouse genomic DNA library in two distinct Lambda Dash II vectors (sequence in DNA contig **AC121873.2.86332.208188**), one containing the whole 5′ region of the gene and exon I, the other containing exons I to VI. In order to prepare pGL2 luciferase reporter vectors (Promega) containing the minimal (DD′InrDBE) or the large (GFEE′DD′InrDBE) *TdT* promoter,