Complexity of transcriptional regulation within the Rag locus: identification of a second Nwc promoter region within the Rag2 intron

Agnieszka Laszkiewicz · Małgorzata Cebrat · Arkadiusz Miazek · Pawel Kisielow

Abstract Nwc represents a mysterious third evolutionarily conserved gene within the Rag locus. Here, we analyzed the phenotype of Nwctmpro1 mice, in which the Rag2 intragenic region containing the previously identified promoter responsible for initiating transcription of Nwc in all cells except lymphocytes was deleted by homologous recombination. Despite strong nonlymphocyte-specific inhibition of Nwc transcription which runs through the regulatory region of Rag genes, their expression remained suppressed, and no developmental, morphological, anatomical, functional, physiological, or cellular defects in Nwctmpro1 mice could be observed. However, careful analysis of the Rag2 intergenic region uncovered a second evolutionarily conserved Nwc promoter region from which a previously unknown Nwc transcript can be generated in nonlymphocytes of Nwctmpro1 and normal mice. The above results reveal an unexpected additional complexity of transcriptional regulation within the Rag/Nwc locus and show that strong inhibition of Nwc transcription in nonlymphoid cells is well tolerated. Complete inactivation of Nwc is necessary to get insight into its function at transcriptional and posttranscriptional levels.

Keywords Rag genes · Nwc gene · Transcriptional regulation

Introduction

Nwc is a third strongly evolutionarily conserved gene within the recombination activating gene (Rag) locus which overlaps the Rag2 gene (Cebrat et al. 2005). The function of Nwc, in contrast to the transposase encoding Rag1 and Rag2 genes (Hiom et al. 1998; Agrawal et al. 1998) enabling V(D)J recombination of antigen receptor genes, is unknown. Its elucidation may be important for better understanding the role of the Rag/Nwc locus in the evolution and ontogeny of the immune system of vertebrates. Unlike Rag genes, the expression of which is lymphocyte specific, Nwc is expressed ubiquitously. In lymphocytes, the transcription of Nwc is regulated by the Rag1 promoter, whereas the Nwc promoter located in the Rag2 intron, which activates Nwc transcription in all other cells, is inactivated by DNA methylation (Cebrat et al. 2008). As a result, in lymphocytes, hybrid Rag1/Nwc transcripts are generated (Cebrat et al. 2005). In the mouse, Nwc consists of 7 exons, which are interspersed by all known Rag enhancers. These observations led to the suggestion that Nwc transcription could interfere with Rag expression and thus be involved in their permanent suppression in nonlymphocytes and in their negative regulation in lymphocytes (Kisielow et al. 2008; Kuo and Schlissel 2009). With this idea in mind, we attempted to obtain genetically modified mice Nwctmpro1 unable to transcribe Nwc in nonlymphoid cells by deleting the Rag2 intragenic sequence containing the previously identified Nwc promoter (Cebrat et al. 2005, 2008).

The results and conclusions derived from analysis of these mice which showed very strong nonlymphocyte-specific but incomplete downregulation of Nwc are discussed.

Materials and methods

Mice

Nwctmpro1 mice were obtained by Drs. Mila Jankovic and Michel Nussenzweig (Rockefeller Institute, New York,
USA) by targeted deletion of the Rag2 intragenic region containing previously identified Nwc promoter sequence (Cebrat et al. 2005). A self-excising cassette (Bunting et al. 1999) was used to produce the targeting vector for homologous recombination in embryonic stem cells. Homology arms located 3' (5.8 kb) and 5' (2 kb) on either side of the region were produced by long-range PCR (Roche), and targeting was confirmed by Southern blot of embryonic stem cells and DNA obtained from tail tissue.

All mice were bred and housed under specific pathogen-free (SPF) conditions in the animal facilities at the Institute of Immunology and Experimental Therapy.

Flow cytometry

Cell suspensions were prepared by standard procedures and, after staining with antibodies, were analyzed as described (Miazek et al. 2009). Bone marrow cell

![Fig. 1 Nwctmpro1 mice lack the known Nwc promoter region. a schematic representation of the Rag2 intragenic region containing the known Nwc exon1/promoter (black box). The region deleted by homologous recombination and the primers (thick arrows) used for screening the generated mutant mice are indicated. b The result of PCR using the indicated primers confirming that homozygous NWCtm1pro mice lack the above indicated region (+/+ wild type, −/− Nwctmpro1). DNA ladder: GeneRuler™ 1 kb (Fermentas)]

![Fig. 2 Tissue expression pattern of Rag1 and Rag2 genes in Nwctmpro1 mice](#)

Table 1

| Percent of the total monocyte number (±S.D.) in a given organa | WT | Nwctmpro1 |
|---|---|---|
| Thymus | | |
| CD4+8- | 7.9 (±1.2) | 6.7 (±0.5) |
| CD4-8+ | 3.5 (±1.6) | 2.4 (±0.4) |
| DP (CD4+8+) | 83.8 (±2.9) | 86.0 (±3.6) |
| DN (CD4-8-) | 4.8 (±0.3) | 4.9 (±0.8) |
| DN1 (CD25-44+) | 0.51 (±0.13) | 0.59 (±0.02) |
| DN2 (CD25+44+) | 0.15 (±0.02) | 0.23 (±0.06) |
| DN3 (CD25+44-) | 1.39 (±0.88) | 1.86 (±0.57) |
| DN4 (CD25-44-) | 2.75 (±0.51) | 2.22 (±0.49) |
| Spleen | | |
| TCRαβ | 12.1 (±8.0) | 12.6 (±6.7) |
| CD4+(CD4+3+) | 8.5 (±5.4) | 8.6 (±3.3) |
| CD8+(CD8+3+) | 3.6 (±2.7) | 4.0 (±2.7) |
| TCRγδ | 0.37 (±0.06) | 0.37 (±0.08) |
| NK (DX5+) | 1.7 (±0.07) | 1.9 (±0.42) |
| B cells (CD19+) | 45.6 (±13.1) | 40.6 (±16.1) |
| Other monocytes | 40.2 (±12.5) | 44.5 (±19.4) |
| Bone marrowb | | |
| Pro-B (CD19+117+) | 0.3 (±0.09) | 0.27 (±0.1) |
| Pre-B (CD19+25+) | 12.2 (±4.9) | 10.0 (±2.9) |

- At least three mice of each genotype were analyzed
- Mature B cells were depleted
suspension was depleted of mature B cells with dynabeads coupled with anti-mouse Ig (Dynal). Except for anti-CD49b (DX5)-allophycocyanin conjugates that were from eBioscience, all other monoclonal antibodies (Mab) were from BD Pharmingen. The following PE, FITC, or biotin Mab conjugates were used: anti-CD3e (145-2C11), anti-CD4 (RM4-5), anti-CD8a (53-6-7), anti-CD19 (ID3), anti-CD25 (7D4), anti-CD44 (IM7), anti-TCR-γδ (GL3), anti-TCR-αβ (H57). For indirect staining, biotinylated Mabs were visualized with streptavidin–PE conjugate (BD Pharmingen).

RT-PCR

Total RNA was extracted from homogenized tissues using the Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. About 4 μg of RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) at 50°C using random hexamers (Amersham Pharmacia). PCR reactions were performed under the following conditions: preheat denaturing at 95°C for 3 min, followed by 35 cycles (Fig. 2, 30 cycles) of denaturing at 94°C for 30 s, primer annealing at 52°C for 30 s, and extension at 72°C for 1 min. Final extension was performed at 72°C for 7 min. To control for genomic DNA contamination, primers were designed to be intron spanning, and an equal amount of total RNA was amplified without previous reverse transcription (RT-control). PCR products were separated by agarose-gel electrophoresis and visualized with ethidium bromide. Real-time RT-PCR was performed on a DNA Engine Opticon 2 apparatus (MJ Research) using a Quantitect SYBR Green PCR Kit (Qiagen). The thermal-cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 cycles of three-step PCR, including 15 s of denaturation at 95°C, 30 s of annealing at 55°C, and 30 s of elongation at 72°C. Expression values were normalized to HPRT.

RNA ligase-mediated 5′ RACE

For determining the transcription initiation site, RNA ligase-mediated 5′ rapid amplification of cDNA ends (RACE) was performed with the FirstChoice RLM-RACE Kit (Ambion) according to the manufacturer's protocol. After ligation of an oligonucleotide 5′ RACE adapter, RNA was reverse-transcribed with an NWCEx7R primer. Nested PCR was performed using gene-specific primers NWCEx7R i NWCEx6R in conjunction with primers complementary to the 5′ RACE adapter. PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced.

Primer sequences

NwcExIb cgccccctaggaaaataaaa
NwcExIIIF gaaatgcttgctcaaataccg
NwcExIIR gtgtgctcatgcagtgtgac
NwcExIVR cacttgaaaggaactccca

Fig. 3 Expression of Nwc in Nwctmpro1 mice: identification of a new (alternative) evolutionarily conserved Rag2 intergenic Nwc exon 1 (ex1b). a Results of RT-PCR performed on mRNA from indicated tissues using primers identifying indicated Nwc exons. b Amplification product of 5′ RACE using testis mRNA was separated by gel electrophoresis and sequenced. c LAST homology plot (http://mafft.cbrc.jp/alignment/server/index.html) of mouse vs human DNA genomic sequences spanning Rag2 exonIII-Nwc promoter region. Double-headed arrows indicate the positions of a fragment of the Rag2 coding exon (Rag2 exIII), Nwc exon I (Nwc ex1), and alternative exon 1 (Nwc ex1b)
**Results and discussion**

Phenotype of Nwctmpro1 mice

Figure 1 shows the Rag2 intragenic region deleted by homologous recombination, containing the previously identified Nwc promoter sequence (Cebrat et al. 2008). It also shows the result of the PCR, confirming that Nwctmpro1 mice lack the indicated region.

Macroscopic observations and comparison with wild-type littermates revealed no difference in embryonic and postnatal developments, body size and weight, longevity, fertility, behavior, morphology, or anatomy. Flow cytometric analysis of primary and secondary lymphoid organs showed normal cellular composition (Table 1), and in the SPF environment, the mice did not show increased susceptibility to infectious or noninfectious diseases.

Expression of Nwc and Rag genes in Nwctmpro1 mice: identification of a second Nwc promoter region within Rag2 intron

The initial screening of the thymus and nonlymphoid tissues for expression of Nwc by RT-PCR using one set of primers suggested complete, nonlymphocyte-specific inhibition of Nwc transcription. Analysis of Rag1 and Rag2 genes by RT-PCR in different tissues of Nwctmpro1 mice revealed a normal pattern of expression (Fig. 2). Expression of Rag1 and Rag2 in Nwctmpro1 mice was detected only in the thymus and in the bone marrow but not in the nonlymphoid tissues, while Rag1 was also detected in the brain (Chun et al. 1991). In some experiments, very weak expression of both Rag genes could be also detected in the

---

**Primers used**

- NwcExVF gatgtggacatggaggaagagagta
- NwcExVIF ataagcaccagcgttccatct
- NwcExVIR cctcagaggtgagcggtaggt
- NwcExVIIR gacatcagtcatggagttcg
- Nwc 829 cacttcatacctctctaaggt
- Nwc-384 tgacccactgttaccatctgcaggga
- HprtF gctggtgaaaa ggacctct
- HprtR cacaggactagaacacctgc
- Rag1F ccaagctgcagacattctagcactc
- Rag1R tcaacatctgccttcacgtcgatcc
- Rag2F ttcagagagggataagcagc
- Rag2R gtagaaggcatgtatgaacgtc

---

**Fig. 4** The second (alternative) Nwc promoter is active in normal mice. Results of RT-PCR performed on mRNA from indicated tissues of wild-type mice using primers identifying indicated Nwc exons are shown.

**Fig. 5** Current picture of the indicated region of Rag/Nwc locus. The gray box represents new Nwc exon1 (ex1b)
spleen (not shown), in agreement with published observation (Yu et al. 1999).

In view of the strong evolutionary conservation of Nwc, the lack of an observable effect on the phenotype was surprising and prompted more detailed analysis of Nwc transcription in nonlymphoid tissues of Nwcmpro1 mice.

As shown in Fig. 3a, using primers identifying different Nwc exons, strong products of expected sizes were obtained in the testis and much weaker products by some primers, in other tissues (upper panel). In view of the fact that the known promoter region was deleted in Nwcmpro1 mice, rapid amplification of cDNA 5’ ends (5’ RACE) beginning from the seventh Nwc exon, using as a template mRNA from the testis, was performed to identify the transcription start site of this new transcript. The result is shown in Fig. 3b. Sequencing of the amplification product identified a new Nwc transcription start site within the Rag2 intron between the second and third Rag2 exons downstream of the deleted region. The comparison of the mouse and the human DNA genomic sequences spanning the Nwc promoter region (Fig. 3c) provides evidence that the alternative Nwc promoter region is evolutionarily conserved.

Hierarchy of Nwc promoters in normal mice Identification of the second Nwc promoter in Nwcmpro1 mice raised the question about its status in normal mice. The RT-PCR using specific primers demonstrated that the second promoter is also active in normal mice: again, a strong product of RT-PCR reaction was obtained in the testis and much weaker products by some primers, in some other tissues (Fig. 4). A comparison of the expression level of the new alternative exon 1b by real-time RT-PCR in the testis of normal and Nwcmpro1 mice showed 10× reduction in the latter (not shown); a similar difference in the level of expression of exon 1 exists between the testis and other tissues in normal mice (Cebrat et al. 2005). In thymocytes, expression of Nwc exon 1b was not detected, similarly as Nwc exon 1 (Cebrat et al. 2005).

The present report adds new observation concerning the structure of the Rag/Nwc locus (Fig. 5). So far, our attempts to get insight into the function of Nwc have failed partly because of the unforeseen complexity of transcriptional regulation within the Rag/Nwc locus. The biological importance of such a complex regulation of Nwc transcription is unclear, but intimate physical and functional association of Nwc with Rag genes—which lasts for hundreds of millions of years—as well as evolutionary conservation of Nwc structure (Cebrat et al. 2005), seems to justify further study. One explanation for the existence of the second Nwc promoter could be the necessity to back up the first one because of the possible important physiological role of Nwc, which is able to perform its function at the extremely low level of expression. Complete inactivation of Nwc is necessary to get insight into its function at transcriptional and post-transcriptional levels.

Acknowledgments We would like to thank Drs. Mila Jankovic and Michel Nussenzweig (Rockefeller Institute, New York, USA) for the generous gift of Nwcmpro1 mice and Monika Kasztura for the discussion. This work was supported by the Ministry of Science and Higher Education Grant N N401 049138.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

Agrawal A, Eastman QM, Schatz DG (1998) Implications of transposition mediated by V(DJ) recombination proteins RAG1 and RAG2 for origins of antigen-specific immunity. Nature 394:744–751

Bunting M, Bernstein KE, Greer JM, Capecci MR, Thomas KR (1999) Targeting genes for self-excision in the germ line. Genes Dev 13:1524–1528

Cebrat M, Milezak A, Kisielow P (2005) Identification of a third evolutionarily conserved gene within the RAG locus and its RAG1-dependent and -independent regulation. Eur J Immunol 35:2230–2238

Cebrat M, Cebula A, Laszkiewicz A, Kasztura M, Milezak A, Kisielow P (2008) Mechanism of lymphocyte-specific inactivation of RAG-2 intragenic promoter of NWC: implications for epigenetic control of RAG locus. Mol Immunol 45:2297–2306

Chun JJ, Schatz DG, Oettinger MA, Jaenisch R, Baltimore D (1991) The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell 64:189–200

Hiom K, Melek M, Gellert M (1998) DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. Cell 94:463–470

Kisielow P, Milezak A, Cebrat M (2008) NWC, a new gene within RAG locus: could it keep GOD under control? Int J Immunogenet 33:395–399

Kuo TC, Schlissel MS (2009) Mechanisms controlling expression of the RAG locus during lymphocyte development. Curr Opin Immunol 21:1–6

Milezak A, Macha K, Laszkiewicz A, Kissenpfennig A, Malissen B, Kisielow P (2009) Peripheral Thy1+ lymphocytes rearranging TCR-gamma delta genes in LAT-deficient mice. Eur J Immunol 39:2596–2605

Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, Melchers F, Meffre E, Nussenzweig MC (1999) Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature 400:682–687