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BRIEF COMMUNICATION

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

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Abstract *Nwc* represents a mysterious third evolutionarily conserved gene within the *Rag* locus. Here, we analyzed the phenotype of Nwc^{tmpro1} 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 Nwc^{tmpro1} 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 Nwc^{tmpro1} 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 Nwc^{tmpro1} 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

Nwc^{tmpro1} mice were obtained by Drs. Mila Jankovic and Michel Nussenzweig (Rockeffeler Institute, New York,

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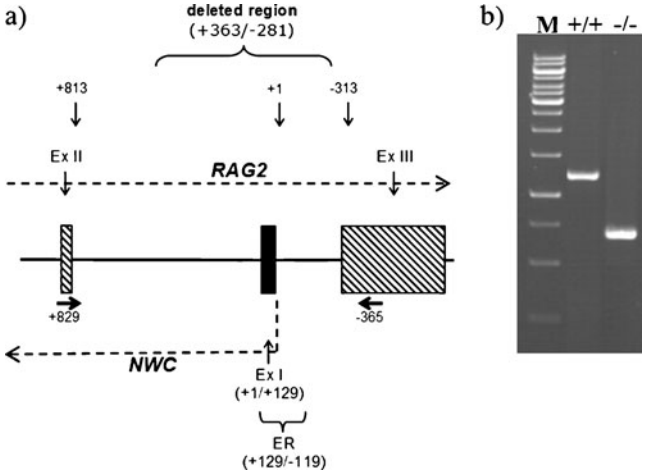

Fig. 1 Nwc^{tmpro1} mice lack the known *Nwc* promoter region. **a** schematic representation of the *Rag2* intragenic region containing the known *Nwc* exon1/promoter (*black box*). The region deleted by homologous recombination and the primers (*thick arrows*) used for screening the generated mutant mice are indicated. **b** The result of PCR using the indicated primers confirming that homozygous NWC^{tm1pro} mice lack the above indicated region (+/+ wild type, −/− Nwc^{tmpro1}). DNA ladder: GeneRuler™ 1 kb (Fermentas)

Table 1 Proportions of indicated T- and B-lymphocyte subsets in indicated lymphoid organs of wild-type (WT) and homozygous mutant (Nwc^{tmpro1}) littermates, 5–6 weeks old

Percent of the total monocyte number (±S.D.) in a given organ[a]		
	WT	Nwc^{tmpro1}
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 marrow[b]		
Pro-B (CD19+117+)	0.3 (±0.09)	0.27 (±0.1)
Pre-B (CD19+25+)	12.2 (±4.9)	10.0 (±2.9)

[a] At least three mice of each genotype were analyzed

[b] Mature B cells were depleted

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

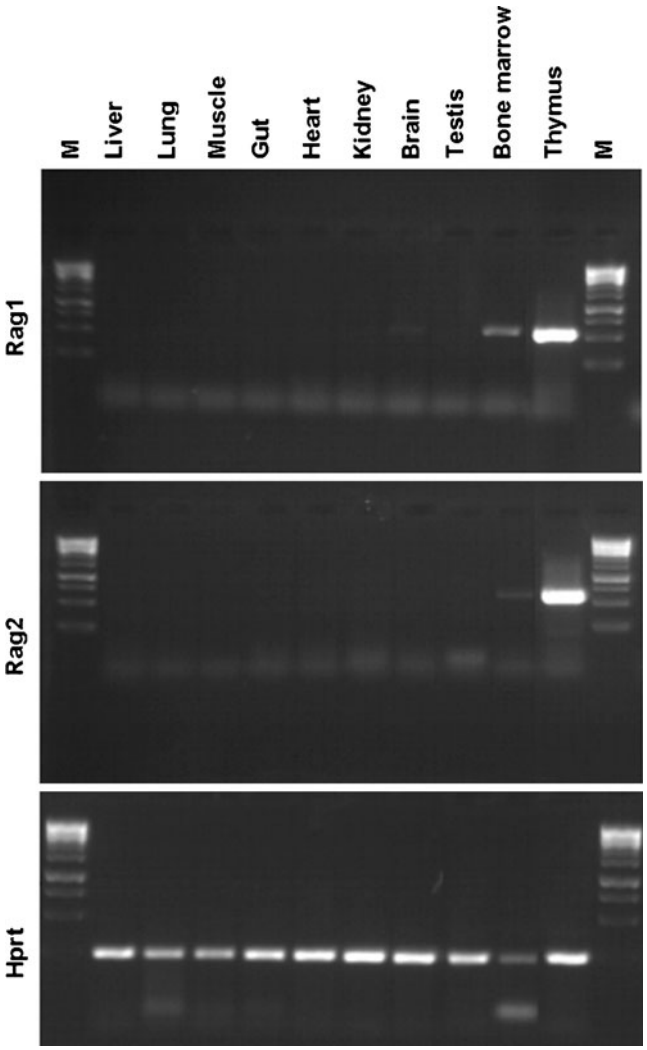

Fig. 2 Tissue expression pattern of *Rag1* and *Rag2* genes in Nwc^{tmpro1} mice

suspension was depleted of mature B cells with dynabeads coupled with anti-mouse Ig (Dynal). Except for anti-CD49b (DX5)-allophycoyanin conjugates that were from eBioscience, all other monoclonal anibodies (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
NwcExIIF gaaatgcttgctcaaataccg
NwcExIIR gtttgctcatggcagttgac
NwcExIVR caccttgaaaggaactccca

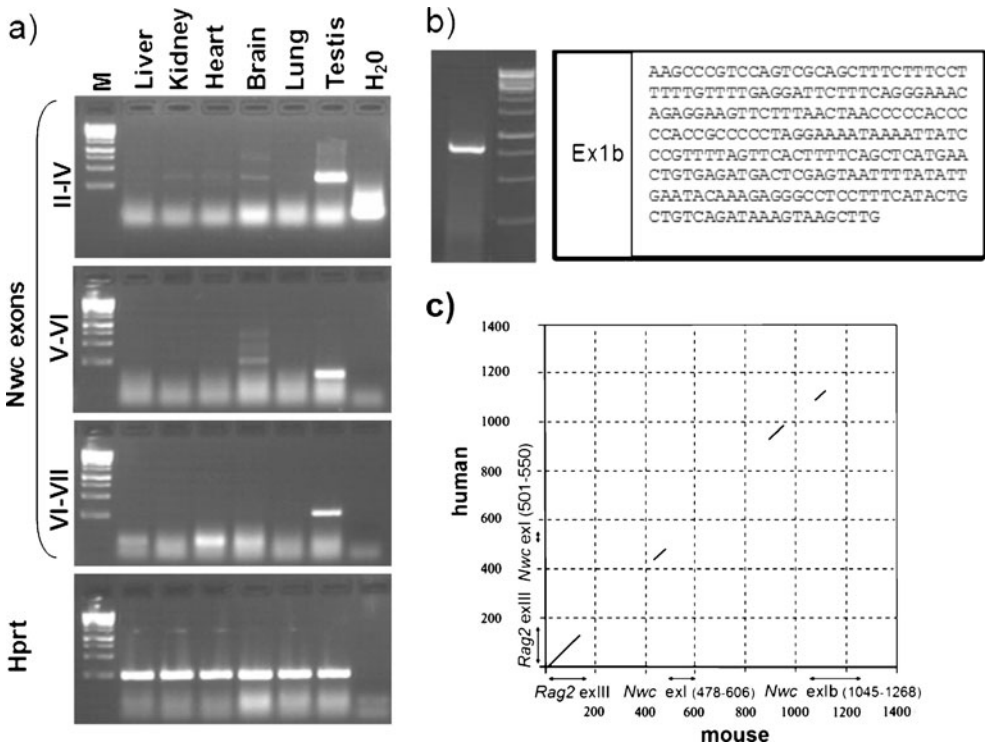

Fig. 3 Expression of *Nwc* in Nwc^{tmpro1} mice: identification of a new (alternative) evolutionarily conserved *Rag2* intergenic *Nwc* exon1 (ex1b). **a** Results of RT-PCR performed on mRNA from indicated tissues using primers identifying indicated *Nwc* exons. **b** Amplification product of 5′ RACE 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* exIb)

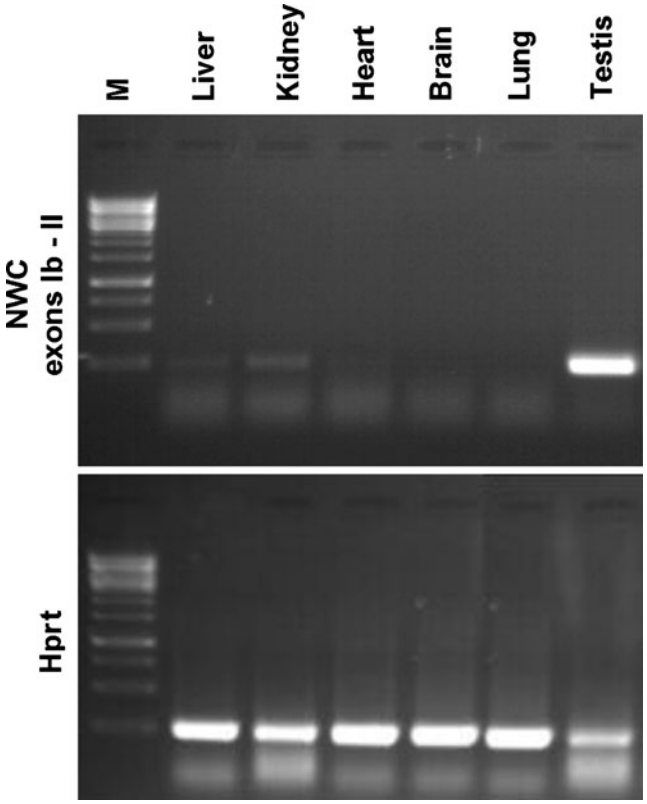

Fig. 4 The second (alternative) *Nwc* promoter is active in normal mice. Results of RT-PCR performed on mRNA from indicated tissues of wild-type mice using primers identifying indicated *Nwc* exons are shown

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

Results and discussion

Phenotype of Nwc^{tmpro1} 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 Nwc^{tmpro1} 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 Nwc^{tmpro1} 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 Nwc^{tmpro1} mice revealed a normal pattern of expression (Fig. 2). Expression of *Rag1* and *Rag2* in Nwc^{tmpro1} 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

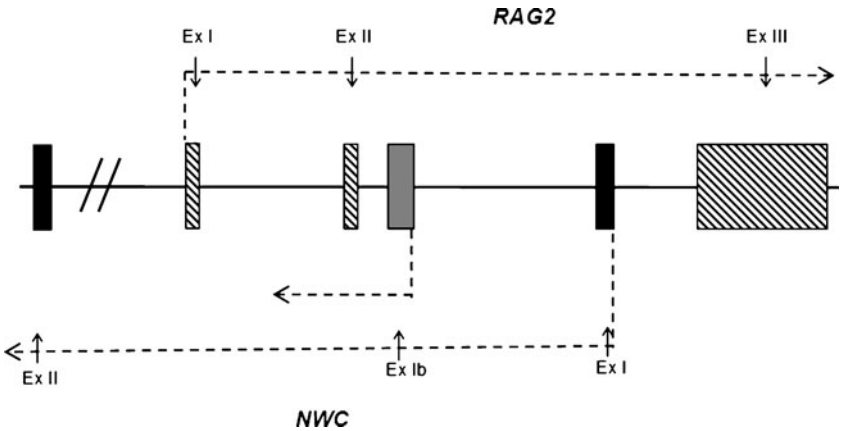

Fig. 5 Current picture of the indicated region of *Rag/Nwc* locus. The *gray box* represents new *Nwc* exon1 (ex1b)

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 Nwc^{tmpro1} 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 Nwc^{tmpro1} 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 Nwc^{tmpro1} 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, 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 Nwc^{tmpro1} 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.

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