Molecular Immunology 46 (2009) 2357–2362

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Antisense transcripts of V(D)J rearrangements; artifacts caused by false priming?

Yuan Zhao, Deborah K. Dunn-Walters, Francesca Barone, Jo Spencer*

Department of Immunobiology, King's College London, Guy's Hospital Campus, St. Thomas' St, London SE1 9RT, UK

ARTICLE INFO

Article history:
Received 23 January 2009
Received in revised form 21 March 2009
Accepted 27 March 2009
Available online 28 April 2009

Keywords:
Immunoglobulin
Somatic hypermutation
AID
Transcription
Antisense

ABSTRACT

Somatic hypermutation (SH) of V(D)J rearrangements at the IGH and IGL loci diversifies the IG repertoire during the germinal center response. SH is absolutely dependent on the enzyme activation induced cytidine deaminase (AID) that initiates the SH process by deaminating C nucleotides in ssDNA. Mutations from G and C are thought to occur as a result of strand symmetrical deamination of C by AID on the coding and non-coding strands respectively. Mutations from A and T are introduced in a strand biased way during error prone repair of the AID induced lesion. SH is linked to transcription and it has been proposed that bidirectional transcription across V(D)J rearrangements occurs in activated and quiescent B cells and that it is co-opted to facilitate the accessibility of the two DNA strands by regulating accessibility of single stranded DNA to AID. We have developed a quantitative method to study directional transcription. Our method controls for differences in efficiency and specificity of reverse transcription that are known to be able to generate false positive data. This method does not detect antisense transcripts in exonic or intronic regions within the hypermutation domain of the spontaneously hypermutating cell line Ramos, or in human blood B cells or tonsil cells, providing convincing evidence that antisense transcripts are rare or absent in human B cells.

1. Introduction

Somatic hypermutation (SH) diversifies the immunoglobulin variable region gene sequence (IGV) of mature activated B cells during the germinal center response, through introduction of predominantly point mutations (Jacob et al., 1991). SH is a multistep process that is initiated by the enzyme activation induced cytidine deaminase (AID), that catalyses the deamination of cytidine to uracil in DNA (Muramatsu et al., 2000; Petersen-Mahrt et al., 2002). There is a balance in most systems between the number of mutations from G (deamination of C on the coding strand) and C (deamination of C on the non-coding strand), and these mutations occur within their favored reverse complement hotspot motifs RGYW and WRCY respectively, consistent with their introduction by a mechanism that acts on both DNA strands equally (Milstein et al., 1998; Spencer et al., 1999). Mutations from A and T occur as a consequence of excision and error prone repair of the deamination event and, unlike most mutations from G and C, are not introduced in a strand-symmetrical way (Unniraman and Schatz, 2007).

There is a link between SH and transcription across IG genes. For example, the positions of the promoters and enhancers of transcription in the sense direction define the extent of hypermutation domain (Yélamos et al., 1995) and the frequency of SH has been linked directly to the levels of pre-mRNA transcripts in transgenic mice (Fukita et al., 1998). Functionally, RNA transcripts of IG sequences undergoing SH are thought to increase the accessibility of the opposite DNA strand to AID within the transcription bubble (Bransteitter et al., 2003). The strand symmetry in hypermutation of G and C nucleotides implies that transcription also occurs in both strand orientations. Studies investigating the spontaneously hypermutating human B cell line Ramos and murine B cells, suggest that bidirectional transcription of IGHV sequences occurs in B cells whether they are undergoing SH or not, with a ratio of sense to antisense of approximately 8:1 within exons (Ronai et al., 2007; Perlot et al., 2008). We were interested to know whether the ratio between sense and antisense transcription in primary transcripts reflects the relative amounts of G and C hypermutation. Sense transcription would theoretically increase the availability of the non-transcribed strand to deamination, which would be visualized as mutations from C in mutated gene sequences. Mutations from G, that are often in excess of mutations from C, according to current theory, would be facilitated by antisense transcription (Roa et al., 2008).

The published methodology used to demonstrate bidirectional transcription at IG loci has been based on strand specific reverse transcription (RT) and PCR. Our initial studies of human cells using comparable methods also supported the concept of bidirectional transcription, corroborating all of the findings above. However, we became concerned that cDNA generated from 'specific' RT reactions may not always be specific and the sensitivity and specificity of the subsequent PCR could propagate false positive data (Beiter

* Corresponding author. Tel.: +44 207 188 1614; fax: +44 207 188 3385.
E-mail address: jo.spencer@kcl.ac.uk (J. Spencer).

0161-5890/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2009.03.020

Table 1
Sequences of primers used for reverse transcription.

	IGHV	IGH-intron	IGL-intron
S1[+]	AAGAACCACAGGTGTTTCAT (46)[^]	CGACGGTGACCGTGGTCCCTT (60)	GGTCAGCTKGGTYCCTCCKCC (54)
S2	GWGCCCWACTCCTGCAGCTGCACC (63)	CAAAGGCCCTAGAGTGGCCATTC (54)	AGGACGGTSASCTKGGT (43)
S3	CTGCTGTAGCTGCACCTG (47)	CCCCACAGGCAGTAGCAG (50)	CAGGGAGAAAGGCTGACC (47)
S4	GCGTTCACAGAGCTCAACTTC (49)	GGACCAACCTGCAATGCTCAGG (53)	TAAAGTCTCCCAGGAGGT (43)
S5	CCCTGAGGAGACGGTGAC (51)	CCCAGATCCTCAAGGCACCCC (55)	GGCTCAGGGCTCAGTCCC (54)
S6	CGACGGTGACCGTGGTCCCTT (62)	CCCTCCTGGCTGGATTCACCC (55)	CAGGGGATATGTTATGAA (41)
S7	ACCTGAGGAGACGGTGACC (51)	CATCAAGACCGGGGCTACGCG (55)	GATCGGAGAGCCTGCCTG (50)
S8		CTACCCCTGCCCCCAACCCTGC (59)	CTCCCCTGCACAGCGAAC ((50)
S9		CCTGGAGCCTCCCTAAGCCCC (57)	CGGGGAGCTGTTTTACCT (45)
AS1	ATGAAACACCTGTGGTTCTT (46)	GGCAAGGGACCACGGTCACCGTCG (60)	CTCCTCASYCTCCTCACT (59)
AS2	GGTGCAGCTGCAGGAGTSGGGCSC (63)	GAATGGCCACTCTAGGGCCTTTG (54)	CAGTCTGCCCTGACTCAGCC (54)
AS3	CAGGTGCAGCTACAGCAG (47)	CTGCTACTGCCTGTGGGG (50)	TTCGGCGGAGGGACCAAG (50)
AS4	GAAGTTGAGCTCTGTGAACGC (49)	CCTGAGCATTGCAGGTTGGTCC (53)	GGGTCTCTAAGCCTTGTT (43)
AS5	CGCGGACACGGCTGTGTATTAC (53)	GGGGTGCCTTGAGGATCTGGG (55)	GAAGGACCCAGTGCCCTC (50)
AS6	GTCACCGTCTCCTCAGGG (62)	GGGTGAATCCAGCCAGGAGGG (55)	AGCCTGAGACTCAGGAAA (43)
AS7	GGCAAGGGACCACGGTCACCGTCG (51)	CGCGTAGCCCCGGTCTTGATG (55)	TGCCCAGGGGGATCAGAG (50)
AS8		GCAGGGTTGGGGGCAGGGGTAG (50)	CCCCCAGGCCCTTGCCCC (57)
AS9		GGGGCTTAGGGAGGCTCCAGG (57)	ACACAGAGCTCTCTTTAT (41)

[+]Sense (S) and antisense (AS) primer sequences are as coded in Figs. 1, 3 and 5. [^]Temperatures used for specific RT in °C.

et al., 2007). We therefore developed a method to analyze RNA transcripts, based on 'specific' RT, but using a range of quantitative PCR (qPCR) and a range of RT primers that flanked the PCR assays to control for differences in efficiency in each of these reactions. This eliminates the possibility of false positive data by the non-specific RT that always accompanies the specific reactions. This approach was sufficiently sensitive to detect spliced and unspliced RNA transcripts and to observe different timing of polyadenylation and splicing in IGH and IGL encoding RNAs in Ramos. Antisense transcripts were either absent or present at vanishingly low levels in all assays. We also studied direction of transcription across the intronic sequence immediately 3′ of IGHJ6 in human blood B cells and tonsil cells and similarly saw no evidence of antisense transcription when controls for false priming were included. We conclude that as yet there is no convincing proof that bidirectional transcription occurs at IG loci in human B cells that could facilitate strand balanced hypermutation of G and C nucleotides.

2. Materials and methods

2.1. Preparation of cDNA

Cell line Ramos was obtained from the European Collection of Cell Cultures and grown as recommended. Human blood B cells were isolated by FACS from buffy coats stained with mouse CD79b-RPE (BD, UK) mouse anti-human IgD and goat anti-mouse -FITC (Dako, UK). Tonsils were taken from a stock of snap frozen control tissues, and tissue sampled using a cryostat. Total RNA from each sample was isolated using an RNAeasy minikit (Qiagen). RNA isolates were treated with DNase (Qiagen) for 40 min at 25 °C. RNA was reverse transcribed using RT primers relevant to the target sequence purchased from Invitrogen (Table 1) and M-MLV reverse transcriptase (Promega). RT enzyme was omitted from a control group.

2.2. Quantitative PCR assays

Quantitative PCRs were designed by applied biosciences using their Custom Taqman Gene Expression Assay Service using the imgt database for identification of target sequences (http://imgt.cines.fr). Three assays were designed within the heavy-chain sequence of Ramos, one within the intronic sequence downstream of IGHJ6 and two within the intronic sequence between IGLJ2 or 3 and IGLC2 or 3 (Table 2). The sequence chosen between IGLJ2 or 3 and IGLC2 or 3 is identical in both of these regions. RT values from the quantitative PCR were converted into numeric values and standardised so that oligo-dT in the sense direction for each qPCR was 1. The qPCR reactions were used according to the manufacturer's protocols using and ABI Prism 7900T sequence. Although these PCR reactions differ in their efficiency, this was not a problem in this assay, where the same assay was used to compare the products of multiple RT reactions. Likewise, the RT primers are likely to differ in their efficiency even after optimization, so to compensate for this, multiple RT primers were used.

Experiments were rejected when the triplicates were not consistent (if more than one cycle difference between replicates). All experiments were repeated at least 3 times and gave the same outcome. Representative experiments are illustrated in the figures.

3. Results

3.1. Investigation of sense and antisense transcription in Ramos IGH and IGL exons.

Ramos has a single IgHVDJ rearrangement using IGHV4-34 and IGHJ6 (Sale and Neuberger, 1998). RNA was isolated from Ramos cells and cDNA prepared in multiple independent RT reactions primed with either oligo-dT or each of a series of oligonucleotide RT primers designed to target sense and antisense RNA transcripts along the mutated VH sequence of Ramos (Fig. 1). cDNAs were

Table 2
Primers and probes used for quantitative PCR.

Target sequence	Real time forward primer	Reporter	Real time reverse primer
Ramos IGHV FR2	AGTGGTTACTACTGGAGCTGGAT	CTCCAGCCCCTTCCC	TGGTGCTTCCACTATGATTGATTTCC
Ramos IGHV FR3	AGAGTTATTACTAGGGCGAGTCCTG	CCGTACCTCCCGTCTGTT	TGGCCCCAGACGTCCATA
Ramos IGHV CDR2	AATCAATCATAGTGGAAGCACCAACT	CCGTCCCTCAAGAGTC	GGACGTGTCTACTGATATGGTGACT
IGH intron	AGGAGCGGTGTCTGTAGGA	CTGTGCAGCGATCTTG	ACAGCGCACCTCATAATTCTAAAGA
IGL intron 1	GGAGAAGGGAGAAGCAGACTCT	CCTTCCCCTGGCTTTC	CCGTCACATCCACTCCTTTTGT
IGL intron 2	CCCCACTGCTATGACCAGGTA	ATCCACCCCACGTCCC	GCTCTCTCTTATTCCGTGGAGTCT