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IGH Switch Breakpoints in Burkitt Lymphoma: Exclusive Involvement of Noncanonical Class Switch Recombination

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Most chromosomal t(8;14) translocations in sporadic Burkitt lymphomas (BL) are mediated by immunoglobulin class switch recombination (CSR), yet all tumors express IgM, suggesting an incomplete or exclusively monoallelic CSR event. We studied the exact configuration of both the nontranslocated *IGH* allele and the *MYC/IGH* breakpoint by applying a combination of low- and high-resolution methods (interphase FISH, DNA fiber FISH, long-distance PCR, and Southern blotting) on 16 BL. *IGH* class switch events involving the nontranslocated *IGH* allele were not observed. Thirteen cases had *MYC/IGH* breakpoints in or nearby *IGH* switch (S) sites, including five at Sμ, three at Sγ and five at Sα. All eight translocations with a breakpoint at Sγ or Sα were perfectly reciprocal, without deletion of Cμ-Cδ or other CH elements. Internal Sμ deletions claimed to be a marker for CSR activity and implicated in stabilization of IgM expression were found in BL but did not correlate with downstream translocation events. This study shows that switch breakpoints in sporadic BL are exclusively resolved by a noncanonical recombination mechanism involving only one switch region.

INTRODUCTION

Burkitt lymphoma (BL) is characterized by a specific chromosomal translocation involving the *MYC* locus located on 8q24 and one of the three immunoglobulin (Ig) loci (*IGH*, *IGK*, *IGL*), resulting in the juxtaposition of the *MYC* gene to the immunoglobulin enhancers. Several studies reported the mapping of the *MYC* and *IGH* breakpoints involved in the t(8;14), association of breakpoints with the geographical origin of the patient, and *MYC* expression levels (Pelicci et al., 1986; Neri et al., 1988; Gutierrez et al., 1992; Wilda et al., 2004). In general, the exact position of the *IGH* breakpoints provides important information on the origin of the translocation, since the breakpoints likely are mediated by mechanisms that are also active during the physiological *IG* gene recombinations/mutations, i.e., V-D-J rearrangement, Ig somatic hypermutation (SHM), and *IGH* class switch recombination (CSR) (for review, see Küppers and Dalla-Favera, 2001; some experimental evidence for the role of SHM in translocations can be found in Bemark and Neuberger, 2003).

In BL, breakpoints in *IGH* are located either in the VDJ-region, the switch μ region (Sμ) or in the downstream switch regions (Sγ or Sα). Breakpoints in the VDJ-region are postulated to be generated by the SHM process in early germinal center B cells. Breakpoints in the switch regions are most probably initiated by an erroneous CSR process. In a previous long distance PCR study on 25 sporadic type pediatric BLs, all breakpoints in the constant gene region were at switch sites: 12 at Sα, 7 at Sγ, and 6 at Sμ (Wilda et al., 2004). However, these PCR-based studies have a limited resolution and do not allow the assessment of the configuration of the entire *IGH* locus.

Other important characteristics of germinal center derived lymphomas are (ongoing) SHM, CSR-related events like internal Sμ deletions (ΔSμ) and downstream class switch recombinations, as recently described by us and others (Zhang et al., 1995; Vaandrager et al., 1998; Nardini et al., 2000; Dudley et al., 2002). In IgM-expressing follicular lymphomas downstream CSR events were associated with ΔSμ, suggesting that the internal deletion

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TABLE 1. Summary of Interphase FISH, Southern Blotting, and LD-PCR Results

Patients & cell lines	Origin	Sex & age (yrs)	Localization	Interphase FISH	Southern blotting switch regions	Sμ LD-PCR	*IGH*/*MYC* LD-PCR	Conclusion
94-738	sporadic	F 8	abdomen	JH/Sμ	R Sμ	G	MYC/Cμ 6.5 kb	Sμ breakpoint
98-5735	sporadic	M 7	abdomen	JH/Sμ	R Sμ; ΔSμ	Δ 1.9 kb	N.A.	Sμ breakpoint
99-375	sporadic	M 6	abdomen	JH/Sμ	R Sμ	G	N.A.	Sμ breakpoint
94-5883	sporadic	M 4	abdomen	JH/Sμ	R Sμ	G	MYC/Cμ 5.8 kb	Sμ breakpoint
01-7243	sporadic	M 5	urinary bladder	Sγ/Sα	R Sγ; ΔSμ	Δ 3.0 kb	MYC/Cγ 4.8 kb	Sγ breakpoint
02-5814	sporadic	M 12	urinary bladder	Sγ/Sα	R Sγ; ΔSμ ΔSμ	Δ 2.8 + 3.0 kb	MYC/Cγ 7.0 kb	Sγ breakpoint
98-3815	sporadic	M 13	abdomen	Sγ/Sα	R Sα; ΔSμ ΔSμ	Δ 1.8 kb	N.A.	Sα breakpoint
98-4847	sporadic	M 12	abdomen	Sγ/Sα	R Sα; ΔSγ2-Sγ4	G	N.A.	Sα breakpoint
98-15878	sporadic	M 9	ileocecum	Sγ/Sα	R Sα	G	N.A.	Sα breakpoint
96-13428	sporadic	M 6	abdomen	Sγ/Sα	R Sα	G	N.A.	Sα breakpoint
94-9283	sporadic	M 17	abdomen	JH/Sμ	ΔSμ	Δ 0.2 kb	N.A.	JH breakpoint
99-12033	sporadic	M 9	neck	JH/Sμ	G	G	N.A.	JH breakpoint
Jiyoye	endemic	–	–	JH/Sμ	G; ΔSμ	Δ 0.8 kb	N.A.	JH breakpoint
BL-65	endemic	–	–	JH/Sμ	R Sμ	G	N.A.	Sμ breakpoint
Raji	endemic	–	–	Sγ/Sα	N.D.	Δ 3.0 kb	MYC/Cγ 7.6 kb	Sγ breakpoint
CA-46	sporadic	–	–	Sγ/Sα	N.D.	G	MYC/Cα 4.2 kb	Sα breakpoint

R: illegitimate rearrangement; G: germline configuration; Δ: internal deletion; N.D.: Not determined; N.A.: Not assessable.

renders Sμ unavailable for CSR, thereby favoring downstream recombinations (Vaandrager et al., 1998).

Chromosomal breakpoints in the *IGH* switch regions are solely found in tumors arising from (post-) germinal center B cells. For instance, the majority of multiple myelomas have such translocations (Bergsagel and Kuehl, 2001; Kuehl and Bergsagel, 2002). Several recurrent partner chromosomes may be involved in these breakpoints. DNA sequence analysis of the breakpoints and flanking regions reveal that, in many instances, the breakpoints are located in Sγ or Sα. The breakpoints on the derivative chromosomes in these cases involved the Sμ region or the 5′-end of Sγ or Sα, implicating that these breakpoints are mediated by a canonical CSR, accompanied by deletion of the Sμ-Sγ or Sμ-Sα regions, or a noncanonical CSR without deletion of *IGH* constant regions.

Although independently reported (Showe et al., 1985; Chesi et al., 1997; McKeithan et al., 1997; Gilles et al., 2000; Schmidt et al., 2004; Guikema et al., 2005), it is unknown whether chromosomal translocations with breakpoints in downstream *IGH* switch regions but not in Sμ are regularly encountered in certain lymphomas.

In this report, we present a comprehensive analysis of the *IGH* alleles in BL and consistently show that the switch breakpoints are perfectly reciprocal, without deletion of intervening DNA between Sμ and any of the downstream switch regions. These data suggest that switch breakpoints in BL are exclusively mediated by a noncanonical CSR involving only one switch region.

MATERIALS AND METHODS

Patient Material

Frozen tissue samples from 12 sporadic Burkitt lymphoma (BL) patients were retrieved from the tissue banks of the Department of Pathology of the University Medical Center Groningen and the Department of Pathology of the Academic Medical Center, Amsterdam. All BL cases were diagnosed using standard histology and immunohistochemistry for IgM, CD10, Ki67, and a FISH segregation assay for *MYC* as previously described (Haralambieva et al., 2004). BL cases were reviewed by two pathologists (E.H. and P.K.). Frozen sections used for FISH and DNA isolation contained at least 80% tumor cells in all studied cases. FISH and immunohistochemistry data from the BL cases from the University Medical Center Groningen has been published elsewhere (Haralambieva et al., 2004). Patient characteristics are listed in Table 1. This study was approved by the ethical committee of the University Medical Center Groningen.

BL Cell Lines

The endemic BL cell lines Jiyoye and Raji were obtained from the American Type Culture Collection (Manassas, VA); the sporadic BL cell line CA-46 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); the endemic BL-65 cell line was a kind gift from Dr. Lenoir (IARC, Lyon, France) (Lenoir et al., 1985). All cell lines were maintained in supplemented RPMI 1640 (Cambrex Bio

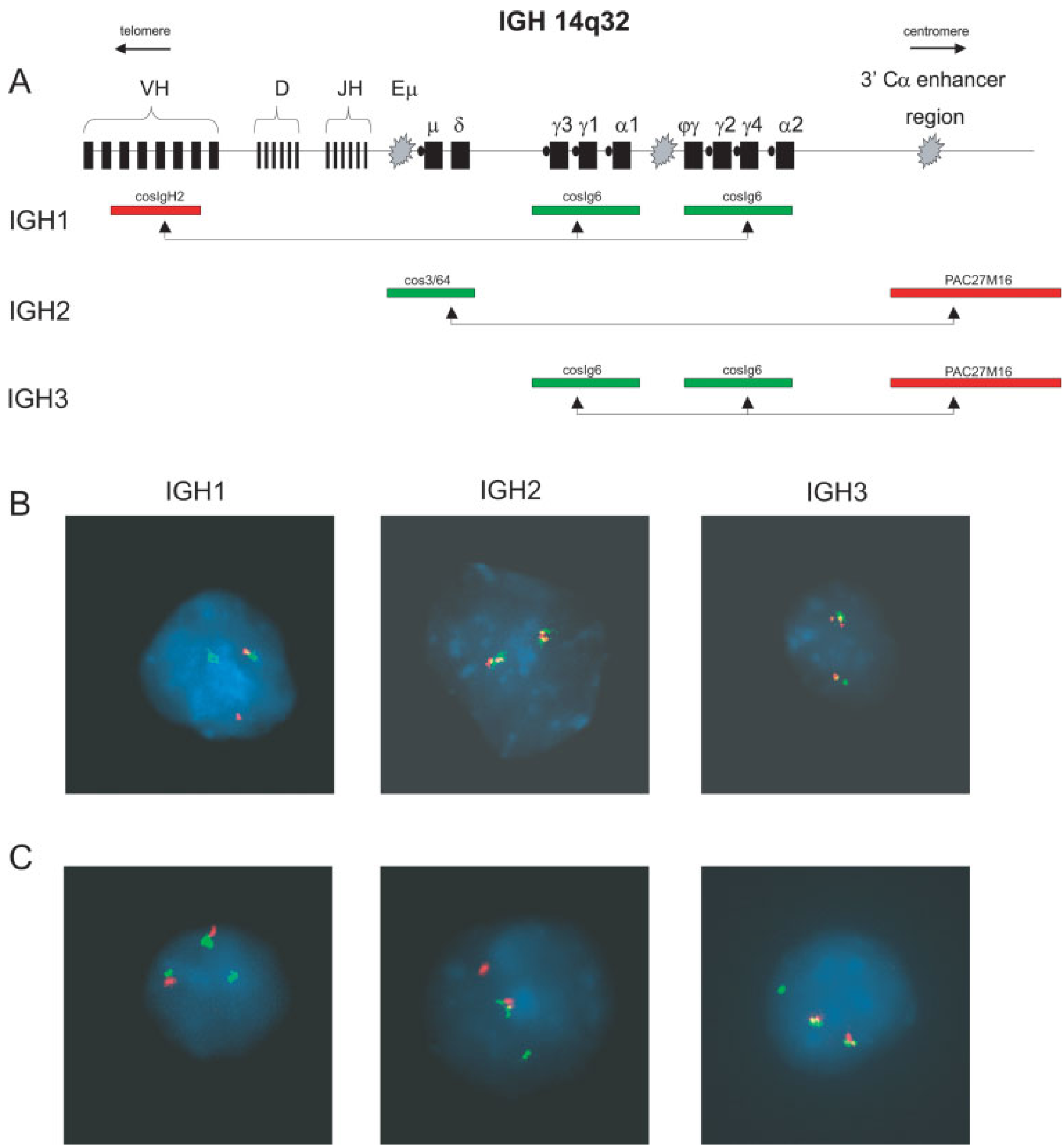

Figure 1. Interphase FISH segregation assay for *IGH*/14q32. A: Schematic overview of the *IGH* locus and interphase FISH probes used for detection of *IGH*/14q32 breakpoints. B: BL tumor with an *IGH* breakpoint located in the JH/Sμ region showing a segregation pattern using probe set IGH1 (cosIgH2 and cosIg6) and colocalization patterns using probe sets IGH2 (cos3/64 and PAC27M16) and IGH3 (cosIg6 and PAC27M16). C: BL tumor with a breakpoint in a Sγ or Sα region, showing a segregation pattern with probe set IGH2 and a colocalization pattern with an extra cosIg6 signal using probe sets IGH1 and IGH3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Science, Walkersville, Maryland) with 10% FCS (Cambrex).

Interphase and DNA Fiber FISH

An interphase FISH segregation analysis was set up to determine the relative location of breakpoints within the *IGH* locus on 14q32. Using three different probe combinations, spanning the entire *IGH* locus 5′ from the VH-genes through the 3′ Cα enhancer region (Fig. 1A), breakpoints were detected in all patient samples and cell lines. Probes for *IGH* and *MYC* used in this study were partly

TABLE 2. Primer Sequences

Primer	Sequence 5′ to 3′	Target	Reference
JH	ACCTGAGGAGACGGTGACCAGGGT	IGH/MYC	Basso et al. (1999)
Cα/01	TCGTGTAGTGCTTCACGTGGCATGTCACGGACTTG	IGH/MYC	Basso et al. (1999)
Cγ/02	AGGGCACGGTCACCACGCTGCTGAGGGAGTAGAGT	IGH/MYC	Basso et al. (1999)
Cμ/03	TGCTGCTGATGTCAGAGTTGTTCTTGTATTTCCAG	IGH/MYC	Basso et al. (1999)
MYC/04	ACAGTCCTGGATGATGATGTTTTTGATGAAGGTCT	IGH/MYC	Basso et al. (1999)
5M1	AGCCCTTGTTAATGGACTTGGAGG	Sμ	Fenton et al. (2003)
3M	CGTTCTGAGTGCCCTCACTACTTGC	Sμ	Fenton et al. (2003)
5′σμ	CAGATCTGAAAGTGCTCTACTG	Sμ	Nardini et al. (2002)
3MRB	GTGATGGGAACGCAGTGTAGA	Sμ	Nardini et al. (2002)
5MFB	GGCAATGAGATGGCTTTAGCTGA	Sμ	Nardini et al. (2002)
CmR1	ACACGTGTCAGCCCGGTGCC	Sμ	Nardini et al. (2002)

described elsewhere (Vaandrager et al., 1998; Haralambieva et al., 2004). In addition, the PAC27M16 (kind gift from Dr. D. Cox, Hospital for Sick Children, Toronto, Canada) mapping to the most 3′ located *IGH* Cα enhancer region and the cosmid IgH2 (kind gift from Dr. T. Rabbitts, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) mapping to the telomeric part of the *IGH* locus encompassing the distal VH genes were used for interphase FISH and/or DNA fiber FISH analysis.

Preparation of interphase nuclei and DNA fibers was performed as described previously (Vaandrager et al., 1998). For dual color FISH, probes were labeled with digoxigenin-11-dUTP (Roche, Basel, Switzerland) or biotin-16-dUTP (Invitrogen, Carlsbad, California) by standard nick-translation. The hybridization solution contained 50% formamide (for interphase FISH) or 30% formamide (for DNA fibers), 10% dextran sulfate, 50 mM sodium phosphate, pH = 7, 2× SSC, 3 ng/μl of each probe, and a 50-fold excess of human C0t-1 DNA (Invitrogen). Immunodetection was performed as described earlier (Haralambieva et al., 2004). Images were captured using a Leica DMRA2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DC 350F charge-coupled device camera. Digital images were processed with Leica CW4000 and Adobe Photoshop software, version 7.0.

Southern Blotting

High molecular weight genomic DNA was extracted from frozen tissue sections (3 × 45 μm) by the 'salting out' procedure. Approximately 5 μg of genomic DNA was digested with *Hin*dIII or *Sph*I (all purchased from Invitrogen). Digested DNA was run overnight on 0.7%, ×0.5 Tris-Boric acid-EDTA (TBE) agarose gels. Subsequently, DNA was transferred to positively charged nylon membranes (Roche) by capillary blotting in ×20 Standard Saline Citrate (SSC). Digoxigenin-labeled probes, which flank the immunoglobulin switch regions both 5′ and 3′, were generated by PCR using the primers described previously (Chesi et al., 1996). Alkali-labile digoxigenin-11-dUTP (Roche) was added to each PCR reaction at a final concentration of 7 μM. Labeled probes were purified by PCR-clean-up (Qiagen, Hilden, Germany). Hybridizations were performed in the Dig-Easy Hyb™ hybridization mixture (Roche) at 42°C. Stringency washes were performed with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) twice at room temperature, followed by 0.1× SSC, 0.1% SDS twice at 68°C. Membranes were developed by alkaline phosphatase conjugated rabbit-anti-dig F(ab′) fragments and the chemoluminescent substrate CDP-Star™ (Roche). Kodak XAR films (Eastman Kodak, Rochester, New York) were exposed for a maximum of 2 hr to developed membranes. Membranes were stripped in 0.2 M NaOH, 0.1% SDS for 15 min at 37°C.

Long-Distance PCR

Breakpoints within *MYC* and *IGH* were determined by long-distance polymerase chain reaction (LD-PCR) using the Elongase™ PCR system (Invitrogen). Primers were described previously (Basso et al., 1999). Integrity of Sμ was assessed by LD-PCR using three overlapping primer sets, which span from the intronic enhancer (Eμ) to the coding region of Cμ. The Sμ region was amplified using the 5M1-3M primer set (Fenton et al., 2003), whenever this yielded a band of the expected size (germline Sμ); genomic DNA was subsequently subjected to LD-PCR with the 5′σμ-3MRB primer set, located 5′ from the 5M1-3M primer set, and the 5MFB-CmR1 primer set (Nardini et al., 2002), located 3′ from the 5M1-3M primer set. Sequences of primers are listed in Table 2.

TABLE 3. Southern Blot Analysis of Sμ, Sγ, and Sα Regions in BL Tumors

	Rearranged fragments (kb)							
	*Hind*III		*Sph*I		*Hind*III		*Hind*III	
BL	5′ Sμ	3′ Sμ	5′ Sμ	3′ Sμ	5′ Sγ	3′ Sγ	5′ Sα	3′ Sα
94-738	3.6	20.8	5.6	11.6	G	G	G	G
98-5735	9.0*	9.0*	6.3*	6.3*	G	G	G	G
	7.8		6.5					
99-375	4.2	G	5.6	G	G	G	G	G
			7.1					
94-5883	5.3	24.6	6.9	15.3	G	G	G	G
01-7243	8.0*	8.0*	5.5*	5.5*	5.8	4.4	G	G
02-5814	8.1*	8.1*	5.5*	5.5*	3.4	2.1	G	G
	8.3*	8.3*	5.7*	5.7*				
98-3815	8.8*	8.8*	6.4*	6.4*	G	G	14.8	18.8
			7.3*	7.3*				
98-4847	G	G	G	G	ΔSγ2-Sγ4	ΔSγ2-Sγ4	14.3	18.8
98-15878	G	G	G	G	G	G	26.3	19.5
96-13428	G	G	G	G	G	G	15.7	19.0
94-9283	G	G	G	G	G	G	G	G
99-12033	G	G	G	G	G	G	G	G

Genomic DNA was digested with *Hind*III or *Sph*I and hybridized with switch region flanking probes (5′ and 3′). Sizes (kb) of the fragments are shown in the table. G: only germline bands detected.
*Internal Sμ deletion; Δ: deletion.

RESULTS

Interphase FISH Analysis for IGH and MYC Breakpoints

In all 16 BLs (12 patient samples and 4 cell lines), *MYC* breakpoints could be confirmed by interphase FISH segregation assays using two probe combinations spanning a region of ~1,000 kb (700 kb centromeric till 300 kb telomeric of *MYC*).

The *IGH* breakpoints in the 16 BL samples were mapped by interphase FISH, Southern blotting, and LD-PCR (results are summarized in Tables 1 and 3). Using DNA fiber FISH, we could obtain a more comprehensive overview of both the translocated and nontranslocated *IGH* allele for nine BL tumors (five patient samples and four cell lines). The hybridization patterns representing the translocated as well as the nontranslocated *IGH* allele were identified for all the studied cases (results summarized in Table 4).

BL Patients and Cell Lines with IGH Breakpoints Located in VDJ or Sμ

By use of interphase FISH with different probe sets (IGH1, 2 and 3), we determined the breakpoint position in the *IGH* locus (Fig. 1). Eight BL tumors had a breakpoint in the VDJ/Sμ region (Table 1). This was shown by the segregation for probe set IGH1 and a colocalization pattern for the other probe sets (IGH2, IGH3) (Fig. 1B). Using Southern blotting with Sμ flanking probes, in five out of these eight BLs, illegitimate switch recombination fragments were detected, whereas the Sγ- and Sα-flanking probes showed germline configurations (Table 3, Fig. 2A). In patient samples 9283 and 12033 and in the Jiyoye cell line, the Sμ flanking probes only detected germline bands, which in conjunction with the *IGH* interphase FISH results, suggest a breakpoint in or nearby VDJ but not in Sμ. Long-distance PCR confirmed a breakpoint in Sμ for two patient samples (736 and 5883) (Table 1). Legitimate switch recombinations could not be detected by Southern blotting in any of these eight BLs.

IGH Breakpoints Involving Sγ and Sα are Perfectly Reciprocal in BL

In the remaining eight BLs, the interphase FISH showed a segregation pattern with probe set IGH2, suggesting a breakpoint in Sγ or Sα. Most essentially, both interphase and DNA fiber FISH showed that the Cμ-Cδ region was preserved on the der(8) chromosome, indicating that no interstitial Sμ-Sγ/α deletion had occurred. To further prove that the Sμ region was not involved in these breakpoints, Southern blotting was performed with switch region flanking probes. No illegitimate Sμ fragments, but only germline fragments or internal deletions (see later results section) were found in these tumors (Tables 1 and 3, Figs. 2B and 2C). DNA fiber FISH analysis was successful for seven of these BLs and consistently showed that the non-

TABLE 4. DNA Fiber FISH Results

Patients and cell lines	*IGH* breakpoint*	Fiber FISH results der(14)	Fiber FISH results der(8)	Fiber FISH results 14q32
01-7243	Sγ	MYC-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	DJH-μ-δ-8q24	D-JH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
02-5814	Sγ	MYC-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	D-JH-μ-δ-γ3-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
98-3815	Sα	MYC-α2-Ψγ-γ2-γ4-ε-α2-3′Cα	D-JH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
98-4847	Sα	MYC-α1-3′Cα	DJH-μ-δ-γ3-γ1-8q24	3′Cα
98-15878	Sα	MYC-α2-3′Cα	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
Jiyoye	JH	MYC-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	?-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
BL-65	Sμ	MYC-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	JH-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
Raji	Sγ	MYC-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	D-JH-μ-δ-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα
CA-46	Sα	MYC-α1-Ψγ-γ2-γ4-ε-α2-3′Cα	DJH-μ-δ-γ3-γ1-8q24	DJH-μ-δ-γ3-γ1-α1-Ψγ-γ2-γ4-ε-α2-3′Cα

**IGH* Breakpoint position as determined by interphase FISH and Southern blotting. The pattern of constant regions and *MYC*/8q24 hybridization signals is described. Unrearranged D and JH regions are designated as D-JH and could be identified by an unrearranged U2-2 probe signal. Rearranged D and JH regions are represented by DJH. When probe signals for the partner chromosome were not found it was represented by a "?".

translocated *IGH* allele did not undergo any class switch recombination event (Table 4).

In three BLs with Sγ or Sα breakpoints (7243, 5814, and 3815), interphase FISH revealed three hybridization signals for the cosIg6 probe instead of two. Two cosIg6 signals colocalized with either the VH-flanking probe (IGH1) or the 3′ Cα region probe (IGH3) (Fig. 1C). The cosIg6 probe was originally cloned from the Cγ3 region but hybridizes at two adjacent positions within the *IGH* locus (Fig. 1A, and was shown earlier by DNA fiber FISH (Vaandrager et al., 1998), which is due to an evolutionary duplication. A breakpoint within the area covered by this probe, therefore, yields an extra interphase FISH hybridization signal of comparable intensity in most instances. Two other BLs (15878 and 13428) showed a colocalization pattern with the IGH1 probe set and a segregation pattern without the extra cosIg6 signal with the IGH3 probe set. This pattern suggests an *IGH* breakpoint at the far 3′ end of the region covered by cosIg6, probably in Sγ4 or Sα2.

Additional Southern blotting experiments on the BL cases with Sγ or Sα breakpoints showed Sγ breakpoints in two patient samples (7243 and 5814; Fig. 2B). *IGH*/*MYC* LD-PCR confirmed Sγ breakpoints in these cases and in the Raji cell line (Table 1). Four patient samples were shown to have a Sα breakpoint by Southern blotting (3815, 4847, 15878, and 13428) (Fig. 2C and Table 3). Additionally, the CA-46 cell line was shown to have a Sα breakpoint, using LD-PCR (Table 1). No (il)legitimate recombinations involving any other switch region than the one affected by the translocation was found in any of the cases. These results consistently show that Sμ is not involved in the BL cases with Sγ or Sα breakpoints.

For BL 4847, an abnormal *IGH* interphase FISH pattern was found in all experiments. In general, only one hybridization signal was observed with the Cμ-Cδ (3/64), the VH-flanking probe, and the Cγ-Cα probe, but the 3′Cα enhancer probe yielded two signals. This suggested a large monoallelic telomeric deletion at 14q32. Using the probe set IGH2, the Cμ-Cδ probe did not colocalize with the 3′Cα enhancer probe, indicating that Cμ was present on the translocated allele. Using the probe set combinations IGH1 and IGH3, no colocalization of the remaining signals was seen (Fig. 3A). Southern blot analysis with the Sγ flanking probes demonstrated the absence of any Sγ2 and Sγ4 region, whereas the Sμ flanking probes revealed only the germline fragment (Tables 1 and 3). The DNA fiber FISH hybridization pattern representing the nontranslocated *IGH* allele indeed showed loss of the telomeric part of chromosome 14. The translocated *IGH* allele had a breakpoint at Sα, and harbored only a Cγ3-Cγ1-Cα1 constant region cluster (Fig. 3B). These results imply that the Cγ2-Cγ4-Cα2 constant gene cluster was absent on the translocated and the nontranslocated IGH allele. Southern blotting experiments showed no evidence for recombinations between downstream switch regions. An Ig constant region polymorphism could possibly explain the configuration in this BL tumor, but unfortunately, this could not be confirmed because

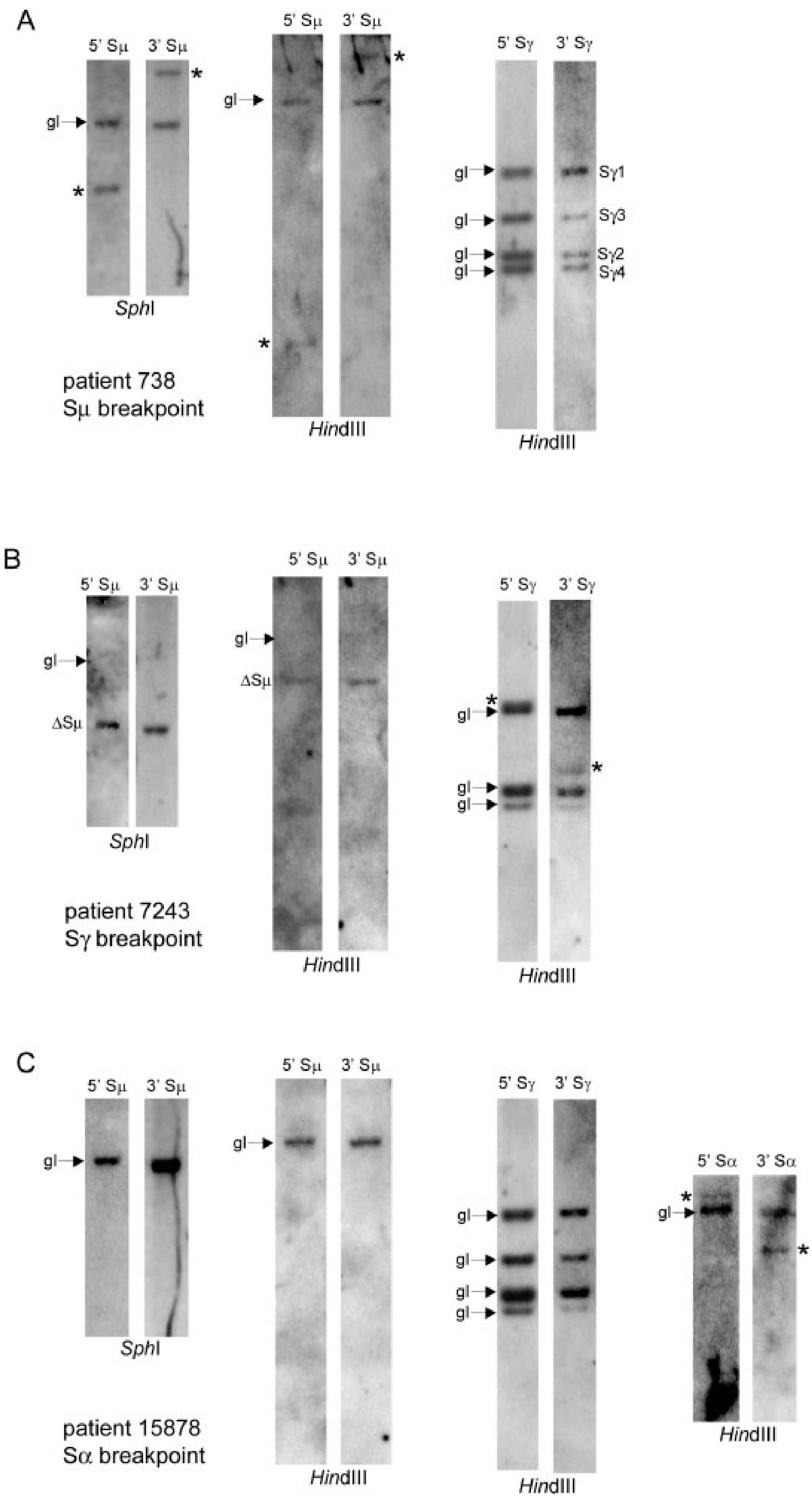

Figure 2. Southern blotting with immunoglobulin switch region flanking probes. A: Patient 738 Southern blotting results, illegitimate Sμ recombination. B: Patient 7243 Southern blotting results, illegitimate Sγ recombination and internal Sμ deletion. C: Patient 15878 Southern blotting results, illegitimate Sα recombination. *Hin*dIII digested genomic DNA fragments were hybridized to 5′ Sμ, 3′ Sμ, 5′ Sγ, 3′ Sγ, 5′ Sα and 3′ Sα probes. *Sph*I digested DNA fragments were hybridized to 5′ Sμ and 3′ Sμ probes. The switch regions are in germline configuration when the tumor clone has not undergone isotype switch recombination. Under these circumstances the switch region flanking probes will cohybridize with DNA fragments of the same size. Legitimate (physiological) switch recombination will result in the cohybridization of the 5′Sμ probe with the 3′ switch probe of the involved isotype. If any of the 5′ switch region flanking probes hybridizes with a band of a different size than the germline fragment and does not cohybridize with any of the 3′ switch probes the recombination is illegitimate and represents a switch breakpoint. Internal Sμ deletions can be discerned by the cohybridization of both the 5′Sμ and the 3′Sμ probe with a fragment smaller than the expected germline fragment. gl, germline; ∗, illegitimate recombinations; Δ, deletion.

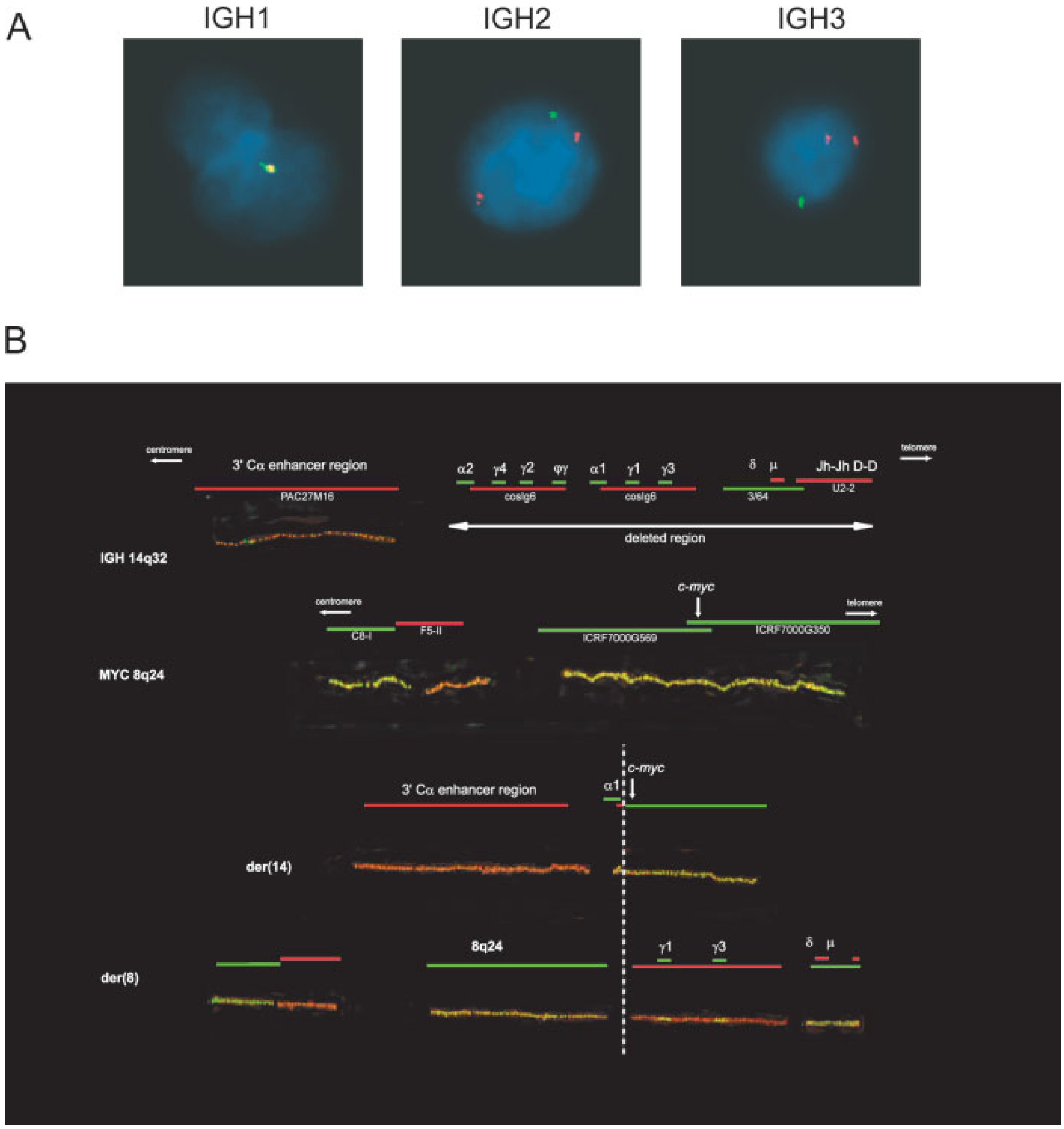

Figure 3. *IGH* interphase FISH, Southern blotting and DNA fiber FISH results for patient 4847. A: *IGH*/14q32 segregation interphase FISH assay showing loss of one *IGH* allele. Single cosIgH2 and cosIg6 hybridization signals were observed using probe set IGH1. Probe set IGH2 showed two PAC27M16 hybridization signals but only one for cos3/64, which did not colocalize with either PAC27M16 signal. Similarly, a loss of one cosIg6 signal was observed using probe set IGH3. B: Stretched DNA fibers were hybridized with pooled differentially labeled (red and green) cosmids and P1-derived artificial chromosomes (PACs) covering ~400 kb of the *IGH* locus and 200 kb of *MYC* at 8q24. The top half depicts the hybridization patterns of the nontranslocated *IGH* allele showing a large telomeric deletion, and the nontranslocated *MYC* allele. The lower half shows the hybridization patterns of the der(14) and the der(8) involved in the t(8;14) translocation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

normal tissue from this patient was not available. Earlier, we described a polymorphism in a hairy cell leukemia patient, who lacked the Cγ2-Cγ4-Cα2 cluster (Vaandrager et al., 1998).

DNA fiber FISH confirmed that the Cμ-Cδ probe was juxtaposed to the der(8), also showing a recombined DJH probe signal. Therefore, the part of the *IGH* locus that is linked to the der(8) chromosome is responsible for IgM expression in this BL.

Internal Sμ Deletions Burkitt Lymphomas and Cell Lines

Large internal Sμ deletions (ΔSμ) result from intra switch region recombination and are related to AID expression and class switch recombination (CSR) activity (Dudley et al., 2002). Furthermore, it has been suggested that ΔSμ is involved in IgM isotype stabilization by rendering Sμ unavailable for further CSR (Zhang et al., 1995). Previously, we showed that downstream CSR events in IgM-expressing FL correlated with ΔSμ (Vaandrager et al., 1998). We, therefore, assessed ΔSμ in BL by Southern blotting and LD-PCR. Restriction enzyme digested DNA fragments (*Hin*dIII, *Sph*I) that were smaller than the expected Sμ germline band, but hybridized with both the 5′ flanking and the 3′ flanking Sμ probe, represent a ΔSμ. For LD-PCR, three primer sets that span the region between the intronic enhancer (Eμ) and Cμ were used. ΔSμ were found in seven out of 16 BLs (Table 3). The size of the deleted region ranged from 0.2 to 3 kb. For all but one case (3815), Southern blotting and LD-PCR yielded similar results. Southern blotting identified two different ΔSμ (0.2 and 1.8 kb) in 3815, whereas only the 1.8 kb deletion was found by LD-PCR. This could be due to deletion or mutation of (part of) one of the Sμ primer sites. Two different deletions were also identified in patient 5814; this could be caused by a biallelic event or the presence of two subclones with different deletion on one allele. The ΔSμ did not correlate with a translocation in Sγ or Sα, making it unlikely that ΔSμ is of importance for breakpoints involving downstream switch regions.

DISCUSSION

We have characterized the t(8;14) chromosomal translocation in 16 Burkitt lymphomas and show that cases with a Sγ or Sα breakpoint have two unusual features. (1) No Sμ-Sγ/Sα recombinations were present but only breakpoints directly involving the downstream switch regions. (2) Illegitimate switch region recombination was not accompanied by class switch recombination on the nontranslocated *IGH* allele.

Normally, CSR is resolved by ligation of DNA double stranded breaks in Sμ and a downstream switch region, whereby the intervening DNA between Sμ and the downstream switch region is excised and circularized (Fig. 4A). In case of a breakpoint involving a downstream switch region, this process would result in the juxtaposition of a putative oncogene to the downstream switch region at the der(14) chromosome and the juxtaposition of Sμ to the derivative chromosome that originally harbored the oncogene (Fig. 4B). However, while this configuration is encountered in mouse plasmacytomas and human myelomas, it was not observed in BL with Sγ or Sα breakpoints, as those breakpoints exclusively involved the downstream switch region. Furthermore, secondary CSR events in which a switch breakpoint was followed by a Sμ-Sγ/Sα recombination (Figs. 4C and 4D) were not found in BL. Moreover, in most multiple myelomas and mouse plasmacytomas, both *IGH* alleles have undergone CSR events, which is clearly not the case in BL. The perfect reciprocal nature of the switch breakpoints is not unique to BL, but has been described also for other leukemias and lymphomas (Showe et al., 1985; Ohno et al., 1993; Muller et al., 1995; Chesi et al., 1997, 1998a,b; McKeithan et al., 1997; Schmidt et al., 2004). Importantly, however, we show that this switch breakpoint configuration is the only one encountered in BL.

We previously described abnormal downstream CSR events in follicular lymphoma. In these lymphomas, all cases with a downstream CSR events also had large internal Sμ deletions (Vaandrager et al., 1998), suggesting that internal Sμ deletion is a primary event rendering Sμ physically unavailable for normal CSR. In consequence, CSR could only start at downstream Sγ or Sα sites. A similar hypothesis has been proposed for the role of ΔSμ in 'IgM-isotype stabilization' in normal mouse B cells (Zhang et al., 1995). Internal Sμ deletions, which are closely related to physiological CSR, are restricted to germinal center or post-germinal center B-cell derived malignancies like BL, follicular lymphoma, B-cell chronic lymphocytic leukemia (Nardini et al., 2002), and hairy cell leukemia (own unpublished observations), and are not found in lymphomas derived from pregerminal center B cells such as mantle cell lymphoma (data not shown). In this study, we show that not all BL cases with downstream switch breakpoints have a ΔSμ, and opposite not all BL cases with ΔSμ show downstream switch breakpoints. Our observations, therefore, argue against a model in which the downstream switch translocations are just the favorable outcome of an erroneous CSR process wherein Sμ cannot take part in the recombination process. This is in agreement with the results of a recent mouse gene-targeting study showing that deletion of Sμ and large parts of its flanking sequences did impair but not completely abolish normal CSR (Khamlichi et al., 2004).

Germline transcription through unrearranged constant regions directs CSR. Although germline transcription from Iμ through Sμ is independent of cytokines, germline transcription of the individual-γ, -α, or -ϵ switch regions is directed by specific cytokines (Stavnezer-Nordgren and Sirlin, 1986; Coffman et al., 1993; Stavnezer, 1996). One could reason that defective/abnormal germline transcription is involved in misguided CSR and switch translocations. In this line of thinking, downstream switch breakpoints might result from improper activation or timing of germline transcription through

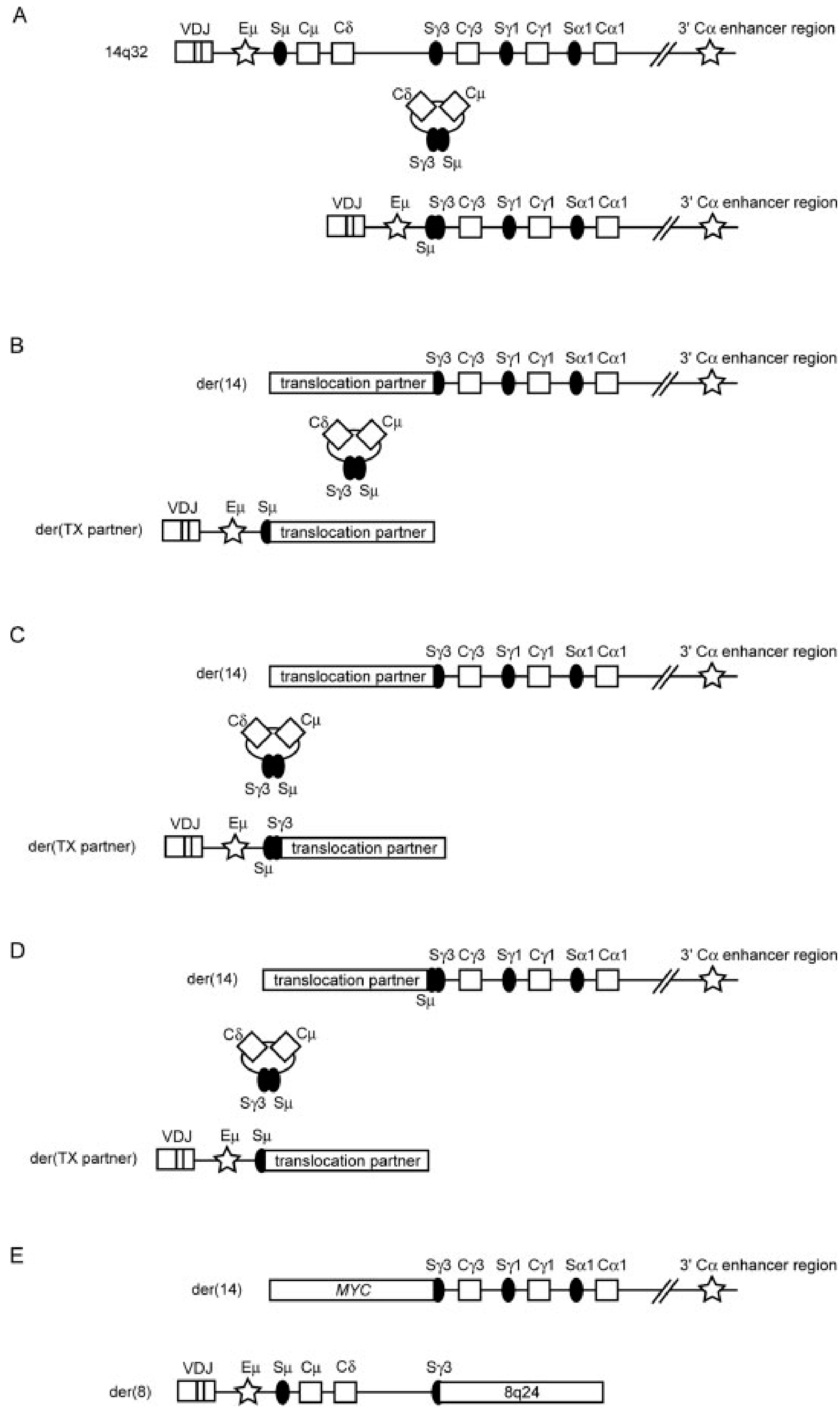

Figure 4. Schematic representation of CSR and different switch translocations. A: Legitimate μ-γ3 CSR. B: Typical switch translocation, the der(14) chromosome harbors a breakpoint in Sγ3, whereas Sμ is linked to the derivative partner chromosome. Intervening DNA is looped out and excised as during normal CSR. C: Translocation involving a switch–switch junction that is linked to the derivative of the partner chromosome. Either the translocation was preceded by a legitimate switch μ-γ3 recombination, or the translocation event was followed by the switch recombination. D: Translocation involving a switch–switch junction that is linked to der(14). This particular structure suggests that the translocation event was preceded by a legitimate CSR. E: Example of a downstream switch translocation found in BL. Sμ is not involved, no looping out and excision of DNA intervening Sμ and a downstream switch region.

Cμ and the downstream constant region to which *MYC* is eventually juxtaposed. Nonsimultaneous activation/targeting of the switch regions would then result in a ΔSμ and a downstream switch breakpoint structure as observed in BL. BL cell lines such as Ramos, DG-75, and DND39 have low levels of baseline germline transcription of other isotypes than IgM, but germline transcription can be stimulated by cytokines (Ichiki et al., 1992, 1993; Ford et al., 1998; Ikizawa and Yanagihara, 2000; Basaki et al., 2002). Thus, although germline transcription can occur in BL under in vitro conditions, it is not known whether at the moment of the generation of the translocation, upstream and downstream germline transcription was properly executed. This question is also relevant, since BL generally express IgM and not IgG or IgA, and our analysis showed a complete germline status of the CH region of the non-translocated allele. Furthermore, in only two cases the results suggested a biallelic ΔSμ (5814, 3815). This lack of any normal CSR might indicate that BL cells or its predecessors in which the translocation took place simply represent B cells that are not exposed to the appropriate microenvironment and extracellular signals necessary for CSR, whereas other intracellular signals that are independent of cytokines are present.

An important feature of SHM, CSR, and ΔSμ is that they all depend on the expression and activity of the activation-induced cytidine deaminase (AID) protein (Muramatsu et al., 2000; Revy et al., 2000; Dudley et al., 2002). In a previous study, we determined AID expression in this group of BL patients and showed that AID is expressed in all cases, albeit at a heterogeneous level (Smit et al., 2003). No apparent correlation was found between the AID expression level and the position of the breakpoint in the *IGH* locus or the presence of ΔSμ (data not shown). However, also here it should be taken in to account that the breakpoint is initiated in a precursor cell, while we can study only the end-stage tumor cells.

The role of AID in SHM and CSR has unequivocally been demonstrated. However, the involvement of AID in the generation of *IGH/MYC* translocation is still controversial; although AID in combination with down-regulation of TP53 certainly favors the occurrence of Myc-IgH recombinations in the mouse (Ramiro et al., 2006), other studies suggested that these translocations can also occur in an AID independent manner and might be favored by the intrinsic fragility of switch regions in B cells (Unniraman et al., 2004). Fragility of switch regions is enhanced by germline transcription and hyperacetylation of histones (Nambu et al., 2003), resulting in the formation of stable R-loops (Yu et al., 2003). These features are considered as crucial factors for CSR, and therefore, the intrinsic fragility of switch regions can be regarded as an important functional component of CSR. Since an interswitch recombination, the hallmark of bonafide AID mediated CSR, was absent in our cases, we cannot rule out that the breakpoints described in this study were independent of AID. Analysis of t(8;14) breakpoint DNA sequences (few patients from this series and retrieved from the NCBI database; Wilda et al., 2004) showed the presence of somatic mutations adjacent to the breakpoint (data not shown), which are considered a hallmark feature of AID activity. Although this does not formally prove the involvement of AID in the generation of the translocation itself, it shows that the involved switch regions in BL were targeted by AID.

Finally, a functional consequence of downstream switch translocation is that the VDJ-Cμ transcription that is present on der(8) is not structurally disrupted and therefore could still be responsible for IgM expression. Previously, we have demonstrated that this is actually the case for the IgM-expressing Z-138 cell line (Guikema et al., 2005). In this cell line, both *IGH* alleles are involved in chromosomal translocations, and IgM transcripts are derived from one allele which harbors a t(8;14) breakpoint involving a Sγ region. In the present study, at least one patient (4847) showed this phenomenon as well.

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