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AID binds to transcription-induced structures in c-*MYC* that map to regions associated with translocation and hypermutation

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Translocation and aberrant hypermutation of c-*MYC* are common in B-cell lymphomas. Activation-induced Cytidine Deaminase (AID) initiates switch recombination and somatic hypermutation in B cells by targeted deamination of transcribed genes. We show that transcription of the immunoglobulin S regions and c-*MYC* results in formation of similar DNA structures, 'G-loops', which contain a cotranscriptional RNA: DNA hybrid on the C-rich strand and single-stranded regions and G4 DNA on the G-rich strand. AID binds specifically to G-loops within transcribed S regions and c-*MYC*, and G-loops in c-*MYC* map to the regions associated with translocation breakpoints and aberrant hypermutation in B-cell lymphomas. Aberrant targeting of AID to DNA structures formed upon c-*MYC* transcription may therefore contribute to the genetic instability of c-*MYC* in B-cell malignancies.
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Introduction

Recombination and mutagenesis of the immunoglobulin loci are essential to normal B-cell development. Many B-cell malignancies carry evidence of genomic instability affecting other loci (Bergsagel and Kuehl, 2001; Kuppers and Dalla-Favera, 2001; Fenton *et al*., 2002; Potter, 2003). A common target is the c-*MYC* gene, which encodes a key regulator of cell proliferation (Boxer and Dang, 2001). In tumors of early stages of B-cell development, c-*MYC* typically translocates to the V(D)J region of the immunoglobulin heavy or light chain loci, and these translocations depend on the RAG1/2 gene products, which recombine V, D, and J segments to generate the variable regions of the antigen receptor (Mills *et al*., 2003). In tumors of human mature B cells, both translocation and hypermutation of c-*MYC* are common. Translocations of c-*MYC* to the immunoglobulin heavy chain switch regions are typical of sporadic Burkitt's lymphomas (Neri *et al*., 1988; Apel *et al*., 1992; Muller *et al*., 1995) and are also found in multiple myelomas (Bergsagel *et al*., 1996; Shou *et al*., 2000; Avet-Loiseau *et al*., 2001; Bergsagel and Kuehl, 2001). Aberrant hypermutation of c-*MYC* has been documented in Burkitt's lymphoma (Cesarman *et al*., 1987; Bhatia *et al*., 1993; Bemark and Neuberger, 2000), diffuse large B-cell lymphoma (DLBCL) (Pasqualucci *et al*., 2001), AIDS-associated non-Hodgkin's lymphoma (Gaidano *et al*., 2003), and primary central nervous system lymphomas (Montesinos-Rongen *et al*., 2004). It is not known whether special features of the c-*MYC* locus make it a particular target for factors normally involved in class switch recombination and hypermutation in activated B cells, and thus contribute to genetic instability of this potent oncogene in human B cells.

Two processes alter genomic structure and sequence in activated mammalian B cells, class switch recombination and somatic hypermutation. In class switch recombination, DNA deletion juxtaposes the rearranged and expressed heavy chain variable region to a new downstream constant (C) region, modifying the mode of antigen clearance without affecting antigen specificity. Switch recombination is region-specific, not sequence-specific, and junctions lie within 2–8 kb intronic G-rich sequences, called switch (S) regions. G-rich S regions occur upstream of all C regions that participate in class switch recombination, and S regions are transcribed from dedicated promoters to activate switch recombination (Chaudhuri and Alt, 2004). In somatic hypermutation, point mutations alter the sequences of the rearranged and transcribed heavy and light chain variable (V) regions (Maizels and Scharff, 2004). Hypermutation is coupled with clonal selection to increase antigen affinity while retaining specificity of antigen recognition.

A single factor, Activation-induced Cytidine Deaminase (AID), activates both class switch recombination and somatic hypermutation (Muramatsu *et al*., 2000; Revy *et al*., 2000). AID causes targeted deamination of transcribed immunoglobulin V and S regions, converting C to U, and thereby initiating mutagenic repair or recombination (Durandy, 2003; Neuberger *et al*., 2003).

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Class switch recombination and somatic hypermutation occur in B cells within germinal centers, highly organized microenvironments within lymph nodes, spleen, and other lymphoid tissues. AID expression is restricted to activated B cells, which populate germinal centers, and to tumors which derive from this cell type, including some DLBCL and Burkitt's lymphomas; and it is not expressed in pregerminal center normal B cells, or in tumors which derive from later stages of B-cell development (Muto *et al*., 2000; Greeve *et al*., 2003; Pasqualucci *et al*., 2004).

A requirement for AID in c-*Myc* translocation has been revealed in a murine model for Burkitt's lymphoma. The cytokine IL6 promotes development and proliferation of late stage B cells (Hirano *et al*., 1986). Mice expressing IL6 from a transgene develop plasmacytomas, characterized by reciprocal translocations between c-*Myc* and the immunoglobulin heavy chain locus (IgH), usually mapping to the Sμ or Sα switch region (Suematsu *et al*., 1992). IL6-induced translocations and tumorigenesis do not occur in *Aid*$-/-$ mice (Ramiro *et al*., 2004). Thus, AID is critical for translocation.

The prevalence of c-*MYC*/IgH translocations in B-cell tumors could simply reflect the proliferative advantage conferred by c-*MYC* deregulation. Alternatively, specific features of c-*MYC* regulation or genomic structure may render it a target for AID. The latter possibility is consistent with evidence that both translocated and nontranslocated c-*MYC* genes undergo hypermutation in tumors derived from germinal center B cells (Rabbitts *et al*., 1983; Raffeld *et al*., 1995; Pasqualucci *et al*., 2001), and with the requirement of AID for translocation (Ramiro *et al*., 2004).

Transcription of the heavy chain S regions is prerequisite to switch recombination (Chaudhuri and Alt, 2004). We have recently shown that S region transcription results in the formation of novel structures, called G-loops (Duquette *et al*., 2004). G-loops contain a stable, cotranscriptional RNA:DNA hybrid on the C-rich strand, and G4 DNA and single-stranded regions within the G-rich strand. AID can deaminate single-stranded but not double-stranded DNA (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Pham *et al*., 2003; Ramiro *et al*., 2003). G-loop formation will prolong denaturation of the DNA duplex, and thereby increase the probability of AID attack. Like the S regions, c-*MYC* is transcribed upon B-cell activation (Cole, 1986; Smeland *et al*., 1988); and it contains distinctly G-rich regions on the nontemplate DNA strand. This suggested that structures might form within the transcribed c-*MYC* gene and promote interactions with AID, thereby contributing to translocation or aberrant hypermutation of c-*MYC* in germinal center B cells.

Here we show that AID binds to G-loops, which form upon transcription of S regions and of c-*MYC*. In these experiments, we have used transmission electron microscopy (EM) to study interactions of AID with transcribed plasmid templates (Duquette *et al*., 2004; Larson *et al*., 2005), allowing us to visualize structure formation and protein binding within individual molecules. EM imaging shows that AID binds to the exposed G-rich nontemplate DNA strand, and not to the RNA/DNA hybrid within the C-rich strand, consistent with specificity of AID for deamination of single-stranded substrates *in vitro* (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Pham *et al*., 2003; Ramiro *et al*., 2003). We further show that G-loops formed upon c-*MYC* transcription map within the first exon and intron. This is the region which is most active in translocation to the immunoglobulin S regions and which is also the target for aberrant hypermutation. These results provide a mechanistic link between genetic instability of c-*MYC* in B-cell tumors and the AID-induced pathway of immunoglobulin gene diversification and class switch recombination, which is critical to normal B-cell development. They further suggest that the unusual G-rich sequence composition of c-*MYC* may underlie its genetic instability.

Results

AID binds to G-loops formed upon transcription of immunoglobulin S regions

G-loops form readily upon transcription of immunoglobulin S region sequences, both *in vitro* and intracellularly (Duquette *et al*., 2004). Following transcription of pPH600, which carries a 0.6 kb fragment from the murine Sγ3 switch region in a pBluescript vector, G-loops can be readily visualized in approximately 50% of transcribed molecules (e.g. Figure 1a). We asked if G-loops are targets for AID by incubating recombinant AID with transcribed pPH600 templates, crosslinking protein/DNA complexes, and visualizing AID by incubating with anti-AID antibodies, then decorating AID/antibody complexes with secondary antibodies conjugated to gold beads. EM imaging clearly identified the AID/DNA complex (e.g. Figure 1b). In a typical experiment, AID bound to 15% of G-loops (10 of 68 loops) in transcribed Sγ3 regions. In contrast, no G-loops were decorated if AID was omitted, but primary and secondary antibodies were included (0 of 130 loops). Two distinct filaments can typically be distinguished within each G-loop by EM imaging: a thicker filament, which corresponds to the RNA:DNA hybrid (e.g. Chow and Broker, 1989), and a thinner filament which corresponds to the G-rich strand and contains single-stranded regions and G4 DNA (Duquette *et al*., 2004). In all cases, AID bound to the thinner filament and not to the thicker filament. The thinner filament contains single-stranded regions, and this binding preference is consistent with the documented specificity of AID for deamination of single-stranded DNA (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Pham *et al*., 2003; Ramiro *et al*., 2003).

*G-loops form upon c-*Myc *transcription*

The c-*MYC* gene contains regions that are G-rich in the nontemplate strand. This biased sequence composition

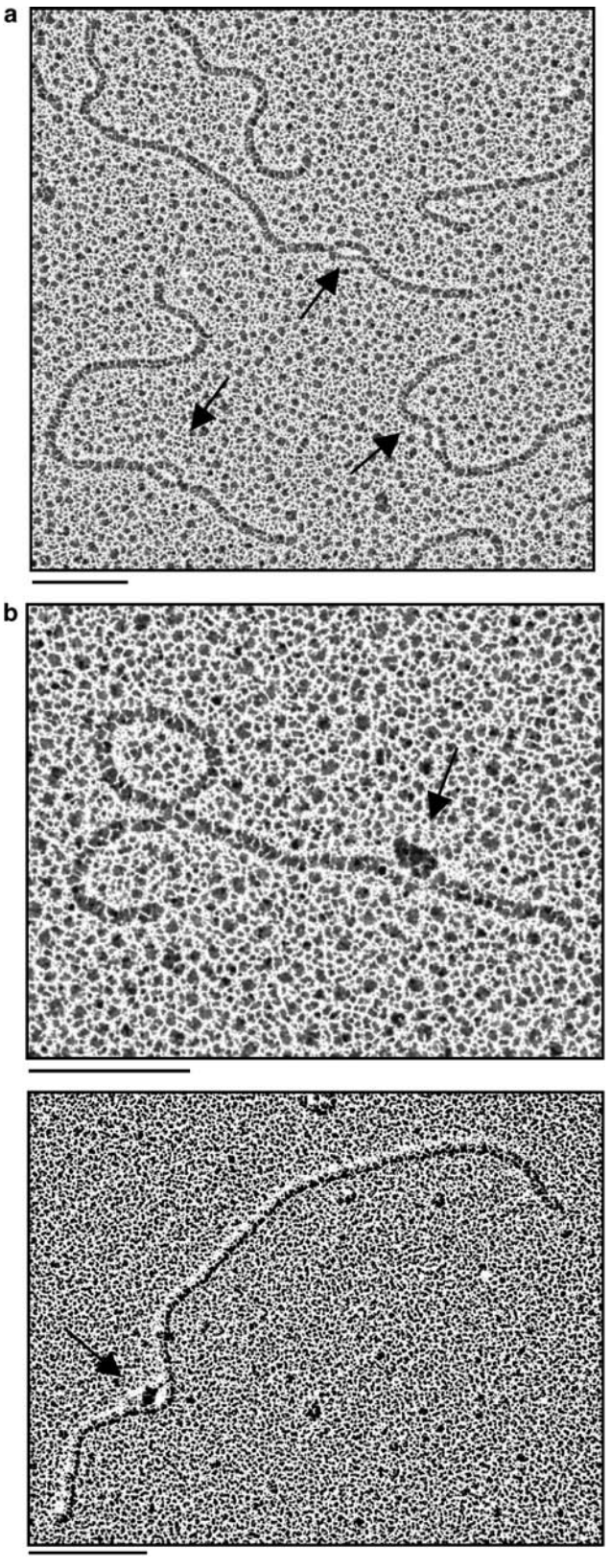

Figure 1 AID binds to G-loops within transcribed S regions. (**a**) Examples of G-loops in transcribed pPH600 (Sγ3). Arrows indicate G-loops. Bar, 200 nm. (**b**) Examples of AID bound to transcribed pPH600 (Sγ3). AID was decorated with anti-AID antibodies and anti-IgG conjugated to gold beads. Arrows indicate AID/DNA complexes. Bar, 200 nm

is similar to that of the S regions, which form G-loops upon transcription. To ask if transcription causes G-loops to form within c-*MYC*, the plasmid pMYC-8.3, which contains an 8.3 kb fragment spanning the human c-*MYC* locus (Krumm *et al*., 1992), was transcribed with T3 RNA polymerase, linearized, and imaged by EM. Large loops were clearly evident in the transcribed plasmids (e.g. Figure 2a). Loops were present in approximately 30% of transcribed substrates (97 of 327 molecules), and ranged from 120 to 1200 bp in size, with an average size of 430 bp in size. No loops were evident in plasmids which had not been transcribed (not shown).

Loops formed upon c-*MYC* transcription were shown to be G-loops using two specific reagents to probe nucleic acid structure. (1) RNase H cleaves the RNA strand of RNA:DNA hybrids. Following addition of RNase H to transcribed pMYC-8.3 templates, no loops could be visualized (0 loops in 75 molecules imaged). Loops formed within transcribed c-*MYC* thus contain an RNA:DNA hybrid. (2) Nucleolin-248 is a recombinant, truncated derivative of nucleolin which binds specifically and tightly ($k_D = 0.5$ nM) to G4 DNA (Hanakahi *et al*., 1999; Duquette *et al*., 2004). Nucleolin-248 was readily imaged bound to the loops in transcribed pMYC-8.3 (e.g. Figure 2b). In all cases, Nucleolin-248 bound to the thinner filament, corresponding to the G-rich DNA strand that forms the G4 DNA. Transcription of c-*MYC* thus promotes formation of G-loops, similar in structure to the G-loops formed upon transcription of S regions.

G-loops formed in transcribed c-Myc *are a target for AID*

We asked if AID binds to G-loops within c-*MYC* in experiments with transcribed pMYC-2.3 templates, which contain the region from exon 1 to the 5′ portion of exon 2. G-loops were abundant upon transcription of pMYC-2.3 templates: 26% of molecules (20 of 76 molecules) contained loops (e.g. Figure 3a). Moreover, AID was readily visualized bound to G-loops in transcribed pMYC-2.3 templates (e.g. Figure 3b). AID bound exclusively to the thinner filament, which corresponds to the G-rich DNA strand containing single-stranded regions. AID was not observed binding to the thicker filament, which contains the RNA:DNA hybrid.

G-loops map to the region of c-Myc *active in translocation and aberrant hypermutation*

G-loops were mapped using NIH Image software to define loop positions within transcribed and linearized DNA templates. G-loops mapped to the first exon and first intron of c-*MYC* (Figure 4a). No G-loops were evident in the downstream region of the c-*MYC* gene, which includes the 3′ region of exon 2 to exon 3. To rule out the possibility that formation of G-loops within upstream regions might impair transcription and thus formation of structures within downstream regions, we compared G-loop formation in full-length pMYC-8.3;

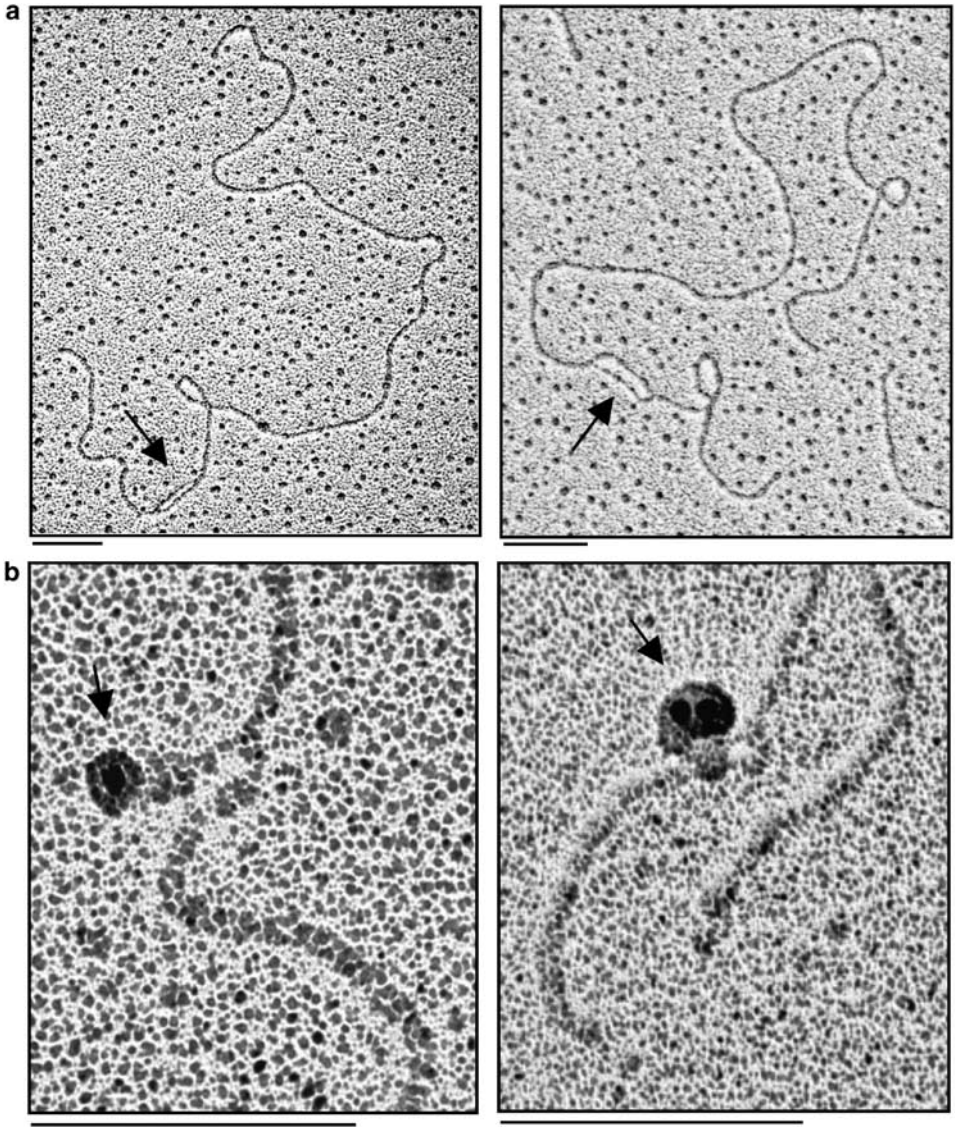

Figure 2 G-loops form within transcribed c-*MYC*. **(a)** Examples of loops formed within transcribed pMYC-8.3. Arrows indicate G-loops. Bar, 200 nm. **(b)** Examples of transcribed pMYC-8.3, probed with Nucleolin-248 to identify G4 DNA, and decorated with secondary antibody conjugated to gold beads (15 nm). Arrows indicate protein/DNA complexes, which are clearly evident on one arm of the loop. Bar, 200 nm

in pMYC-2.3, which contains the region from the P1 promoter to the 5′ portion of exon 2 and in pMYC-3.4, which contains the region from the 5′ portion of exon 2 to the 3′ end of exon 3 (Figure 4b). Loops formed readily in transcribed pMYC-8.3 and pMYC-2.3 templates (e.g. Figures 2 and 3). In contrast, no loops were evident in transcribed pMYC-3.4 templates (0 of 75 molecules; e.g. Figure 4c). Thus, formation of transcription-induced G-loops is limited to the region of the transcribed c-*MYC* gene just downstream of the P1 promoter, including the first exon and first intron. This corresponds to the region of c-*MYC* that undergoes translocation in sporadic Burkitt's lymphoma (Neri *et al*., 1988; Apel *et al*., 1992; Muller *et al*., 1995) and aberrant hypermutation in B-cell malignancies (Pasqualucci *et al*., 2001).

Discussion

We have shown that AID binds to G-loops formed upon transcription of S regions and c-*MYC*. G-loops map to the region that is targeted for translocation and aberrant hypermutation within exon 1 and intron 1 (Figure 5). The ability of AID to bind G-loops formed within the transcribed c-*MYC* gene suggests that AID is a key source of genetic instability of c-*MYC* in activated B cells. AID attack may initiate destabilization of genomic integrity at c-MYC just as it does at the immunoglobulin loci, causing translocation to the S regions and aberrant hypermutation.

We propose that the sequence composition and regulation of c-*MYC* combine to make this gene a target for AID in activated B cells. The first exon and

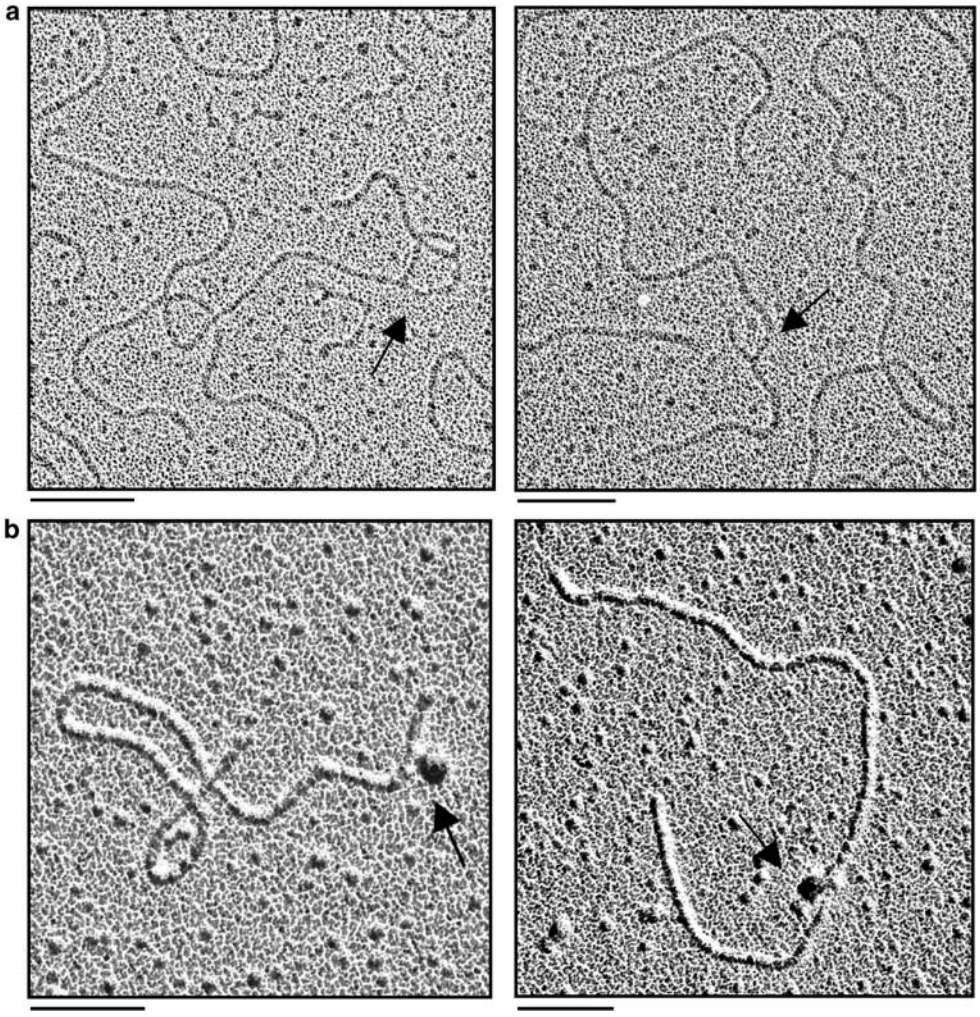

Figure 3 AID binds G-loops in the transcribed c-*MYC* gene. (**a**) Examples of G-loops within transcribed pMYC-2.3 templates. Arrows indicate G-loops. Bar, 200 nm. (**b**) Representative examples of AID bound to G-loops formed on transcribed pMYC-2.3. Arrows indicate AID/DNA complexes. Bar, 200 nm

intron of c-*MYC* are G-rich, and this sequence composition will predispose the c-*MYC* gene to become structured upon transcription, and form a cotranscriptional RNA:DNA hybrid, which derives its stability from the unusual stability of the rG : dC base pairs between the template DNA and newly synthesized transcript (Duquette *et al*., 2004). B-cell activation causes a rapid induction of c-*MYC* transcription, which is necessary to promote structure formation. Within G-loops, AID binds specifically to the filament containing single-stranded regions (Figure 3), consistent with demonstrated substrate-specificity of the AID deaminase activity (Bransteitter *et al*., 2003; Chaudhuri *et al*., 2003; Pham *et al*., 2003; Ramiro *et al*., 2003). The presence of a stable RNA:DNA hybrid within a G-loop may enhance exposure of single-stranded regions to AID by prolonging the DNA denaturation that accompanies transcription.

The results reported here identify a mechanistic link between AID and translocation and aberrant hypermutation targeted to c-*MYC*. The immunoglobulin loci and c-*MYC* are in proximity within the nuclei of normal B cells (Roix *et al*., 2003), and proximity may contribute to the probability of their reciprocal translocation. We have recently shown that MutSα can bind to G4 DNA and G-loops to promote synapsis of transcriptionally activated S regions (Larson *et al*., 2005). MutSα might similarly promote synapsis of transcriptionally activated S regions and c-*MYC*.

Genetic analysis has shown that AID is required for reciprocal IgH/c-*Myc* translocation in murine IL-6-induced plasmacytomas, in which c-*Myc* typically translocates to the Sμ or Sα switch region (Ramiro *et al*., 2004). Reciprocal c-*Myc*/IgH translocations similarly result from treatment of BALB/c mice with pristane, which promotes tumor formation by inducing an inflammatory response. This response is mediated by macrophages and neutrophils, accompanied by oxidative damage and release of cytokines including IL6 (Potter and Wiener, 1992). Pristane has been reported to induce c-*Myc*/IgH translocations in *Aid*-deficient mice (Unniraman *et al*., 2004), although the assay used to measure translocation in those experiments has come into question (Ramiro *et al*., 2005). Moreover, chronic

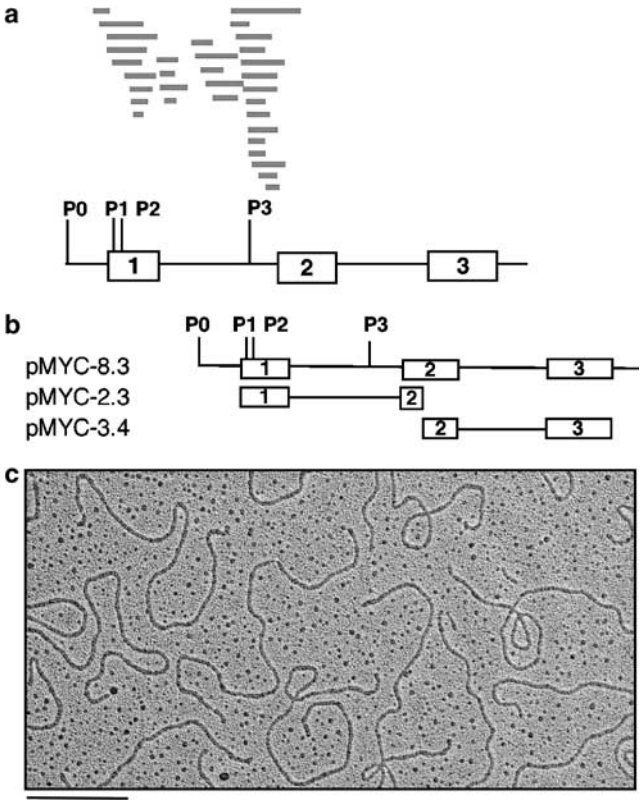

Figure 4 G-loops map to exon 1 and intron 1 of c-*MYC*. (**a**) Loops formed within transcribed pMYC-8.3 were measured and mapped to the c-*MYC* locus (grey bars). P0, P1, P2, and P3 are c-*MYC* promoters. Exons 1, 2, and 3 of c-*MYC* are boxed. (**b**) Diagrams of the regions of the c-*MYC* gene carried in the plasmid templates analysed for G-loop formation. (**c**) Examples of transcribed pMYC-3.4 templates, visualized by electron microscopy. Bar, 200 nm

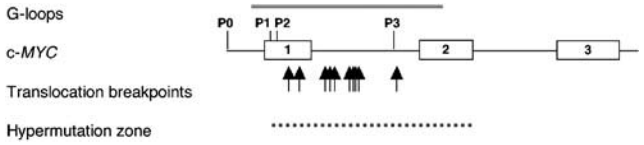

Figure 5 G-loops form with the region of c-*MYC* that undergoes translocation and hypermutation in B-cell lymphomas. The c-*MYC* gene, annotated as in Figure 4. Above, region to which G-loops mapped, grey line (and see Figure 4). Below, arrows indicate sites of balanced translocation breakpoints between c-*MYC* and the S regions, identified in sporadic Burkitt's lymphoma (Neri *et al*., 1988; Apel *et al*., 1992; Muller *et al*., 1995) and multiple myeloma (Bergsagel *et al*., 1996; Shou *et al*., 2000; Avet-Loiseau *et al*., 2001; Bergsagel and Kuehl, 2001); dotted line indicates zone targeted by aberrant hypermutation in DLBCL (Pasqualucci *et al*., 2001)

inflammation induced by pristane can lead to oxidative DNA damage (Potter, 2003), which in turn could contribute to genetic instability in pristane-treated mice. Guanine has the lowest oxidation potential of all four bases, and G's within runs are especially prone to oxidation (Senthilkumar *et al*., 2003). G-rich genes and genomic regions may therefore be particularly susceptible to oxidative damage, and this may provide a complementary or alternative pathway to AID-dependent translocation (Figure 5).

What enables AID to attack some genes, while leaving others untouched? Transcription is necessary but not sufficient for either switch recombination or somatic hypermutation, and only a small fraction of transcribed genes are targets of genomic instability in tumors of germinal center-derived B cells. Our results suggest that formation of DNA structures within a transcribed region may contribute to AID targeting to a specific subgroup of transcribed genes. In addition to c-*MYC*, genes shown to be targeted for hypermutation and translocation in B-cell malignancies include the B29 and MB1 B-cell receptor genes (Gordon *et al*., 2000; Gordon *et al*., 2003), and the BCL-6 (Migliazza *et al*., 1995; Pasqualucci *et al*., 1998; Shen *et al*., 1998; Akasaka *et al*., 2000), CD95/FAS (Muschen *et al*., 2000a, b), RHO/TTF (Preudhomme *et al*., 2000), PAX-5, and PIM1 proto-oncogenes (Pasqualucci *et al*., 2001). Whether any of these genes form transcription-induced structures similar to those formed by c-*MYC* can be readily tested using the EM assay we have developed.

Activation of c-*MYC* provides a powerful driving force toward malignancy (Levens, 2003), and c-*MYC* expression is deregulated in many tumor types by a variety of mechanisms, including not only translocation and mutation but also amplification. AID is expressed exclusively in activated B cells and will therefore not contribute to genetic instability of c-*MYC* in other cell types (Muto *et al*., 2000; Greeve *et al*., 2003). However, related cytidine deaminases with a wider expression profile, such as Apobec3G, Apobec3F, or Apobec1, could promote c-*MYC* genetic instability in other tissues, functioning by a mechanism analogous to that of AID (Beale *et al*., 2004; Conticello *et al*., 2005). Formation of DNA structures may also contribute to genetic instability induced by mechanisms other than deamination. For example, DNA structures may cause replication fork stalling and produce DNA ends which can initiate aberrant recombination or amplification (Mills *et al*., 2003; Michel *et al*., 2004; Pasqualucci *et al*., 2004). If formation of transcription-induced structures correlates with genetic instability, then it should be possible to develop algorithms that explain or predict instability at other loci and in other cell types.

Materials and methods

Plasmids

The plasmid pPH600 contains a 604 bp fragment of the murine Sγ3 switch region downstream of a T7 promoter (Duquette *et al*., 2004). A plasmid containing an 8.3 kb *Hind*III–*Eco*RI fragment spanning the human c-*MYC* locus (Krumm *et al*., 1992) was the generous gift of Dr A Krumm (University of Washington Medical School), and is referred to herein as pMYC-8.3. A 2.3 kb *Xho*I–*Eco*RV fragment from this plasmid (spanning the region from just downstream of P1 to the 5′ portion of exon 2) was subcloned into pBluescript KS+ (Stratagene, La Jolla, CA, USA) to generate pMYC-2.3; and a 3.4 kb *Eco*RV–*Eco*RI fragment (spanning the 5′ end of exon 2 to the 3′ end of the gene) was subcloned to generate pMYC-

3.4. Plasmids were prepared for *in vitro* transcription using the Qiagen maxiprep procedure (Qiagen, Valencia, CA, USA).

Transcription and EM

Transcription was carried out for 15 min at 37°C in reactions containing 100 μg/ml supercoiled plasmid DNA, 1 mM each NTP, and 50 U/ml T3 RNA polymerase (Roche Applied Science, Indianapolis, IN, USA) or T7 RNA polymerase (New England Biolabs, Beverly, MA, USA) in manufacturer's buffer supplemented with 40 mM KCl. Free RNA was digested by incubation with 20 μg/ml RNase A for 15 min at 37°C. DNAs were linearized at unique *Afl*III sites by incubation with 200 U/ml *Afl*III (New England Biolabs) in 1 × NEB3 buffer at 37°C for 1 h. *RNase*H digestion was carried out at 37°C for 30 min with 17 U/ml RNaseH (Roche). Samples were spread for EM as previously described (Duquette *et al*., 2004), imaged using a JEOL 1010 transmission electron microscope at 60 kV. Images were captured using a Gatan ultrascan camera (Gatan, Pleasanton, CA, USA) and acquired using Gatan Digital Micrograph software.

EM imaging of protein : DNA complexes

Purification and buffer exchanges were carried out using filter centrifugation columns (Microcon®100 kDa cutoff; Millipore, Bedford, MA, USA), referred to henceforth as FCC-purification. Glutaraldehyde crosslinking was carried out in 0.2% glutaraldehyde (EM grade; Sigma-Aldrich, St Louis, MO, USA). To image AID : DNA complexes, transcribed DNA was FCC-purified; incubated at 37°C for 10 min with 200 nM AID-GST prepared according to Bransteitter *et al*. (2003), in Buffer A (40 mM HEPES, pH 7.5, 40 mM KCl, 5 mM EDTA); glutaraldehyde-crosslinked at 37°C for 20 min; FCC-purified; incubated with 50 μg/ml rabbit polyclonal anti-AID antibody (ab5197, Abcam, Cambridge, MA, USA) in Buffer A at 4°C for 4 h; glutaraldehyde-crosslinked at 4°C for 16 h and FCC-purified; incubated at 4°C for 4 h in Buffer A with anti-rabbit IgG conjugated to 15 nm gold beads (Electron Microscopy Sciences, Hatfield, PA, USA), glutaraldehyde-crosslinked at 4°C for 16 h and FCC-purified into 10 mM Tris, pH 7.0, 20 mM KCl. Plasmids were linearized as described above, spread and imaged by EM. Nucleolin-248 was expressed as an MBP fusion (Duquette *et al*., 2004). To image Nucleolin-248 : DNA complexes, transcribed DNA was FCC-purified; incubated at 37°C for 30 min with 20 nM Nucleolin-248 in 20 mM HEPES, pH 7.4, 100 mM KCl, 0.2 mg/ml BSA, 1 mM DTT; glutaraldehyde-crosslinked at 37°C for 20 min; FCC-purified; and incubated in Buffer A with 40 μg/ml biotinylated anti-MBP antibody (Vector Laboratories, Burlingame, CA, USA) at 4°C for 1 h. Following glutaraldehyde-crosslinking and FCC-purification with buffer exchange into 1 × NEB3 (New England Biolabs), plasmid DNA was linearized as described above; EDTA was added to 2 mM; protein : DNA complexes decorated by incubation at 37°C for 20 min with 1 μg/ml streptavidin gold beads (Electron Microscopy Sciences, Hatfield, PA, USA); and DNA was then spread and visualized by EM.

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