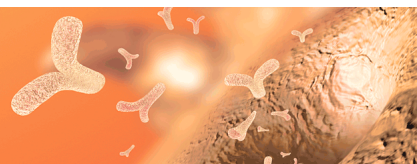




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## Human MSH6 Deficiency Is Associated with Impaired Antibody Maturation

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# Human MSH6 Deficiency Is Associated with Impaired Antibody Maturation

Pauline Gardès,<sup>\*,†</sup> Monique Forveille,<sup>‡</sup> Marie-Alexandra Alyanakian,<sup>§</sup> Pierre Aucouturier,<sup>¶,||</sup> Denisa Ilencikova,<sup>#</sup> Dominique Leroux,<sup>\*\*</sup> Nils Rahner,<sup>††</sup> Fabienne Mazerolles,<sup>\*,†</sup> Alain Fischer,<sup>\*,†,‡,‡</sup> Sven Kracker,<sup>\*,†,1</sup> and Anne Durandy<sup>\*,†,‡,‡,1</sup>

**Ig class-switch recombination (Ig-CSR) deficiencies are rare primary immunodeficiencies characterized by defective switched isotype (IgG/IgA/IgE) production. Depending on the molecular defect, defective Ig-CSR may also be associated with impaired somatic hypermutation (SHM) of the Ig V regions. Although the mechanisms underlying Ig-CSR and SHM in humans have been revealed (at least in part) by studying natural mutants, the role of mismatch repair in this process has not been fully elucidated. We studied in vivo and in vitro Ab maturation in eight MSH6-deficient patients. The skewed SHM pattern strongly suggests that MSH6 is involved in the human SHM process. Ig-CSR was found to be partially defective in vivo and markedly impaired in vitro. The resolution of  $\gamma$ H2AX foci following irradiation of MSH6-deficient B cell lines was also found to be impaired. These data suggest that in human CSR, MSH6 is involved in both the induction and repair of DNA double-strand breaks in switch regions. *The Journal of Immunology*, 2012, 188: 000–000.**

**I**mmunoglobulin class-switch recombination (Ig-CSR) deficiencies are rare primary immunodeficiencies with an estimated frequency of  $\sim 1$  in 100,000 births. These deficiencies are characterized by normal or elevated serum IgM levels and a decrease in (or an absence of) IgG, IgA, and IgE (1). As a result of this molecular defect, defective Ig-CSR may be associated with faulty generation of somatic hypermutations (SHMs) in the Ig V region. The molecular identification and analysis of several Ig-CSR immunodeficiencies has provided new insights into the mechanisms underlying Ig-CSR and SHM, both of which are key elements in the maturation of Ab responses (2).

Investigation of CD40L/CD40 deficiencies has provided evidence for an essential role of this activation pathway in both CSR and SHM (3). Of the Ig-CSR deficiencies caused by an intrinsic B cell defect, the autosomal recessive form due to mutations in

the gene coding for activation-induced cytidine deaminase (AID) is characterized by impairment of both CSR and SHM (4); this emphasizes AID's master role in Ab maturation. The enzyme selectively changes cytosine residues into uracils within the ssDNA of transcribed switch (S) and V regions (5, 6). Uracil N-glycosylase [which is also mutated in another reported Ig-CSR defect (7)] removes the uracil residues introduced into DNA by AID (8) and thus produces an abasic site. In the mouse, it has been shown that abasic sites are eventually cleaved by apurinic-apyrimidinic endonucleases (9). The cleavage ultimately leads to the formation of single-strand DNA breaks which, if present on both strands of the DNA, lead to the double-strand breaks (DSBs) required for CSR. Indeed, it has been shown that AID can deaminate both the nontemplate and template strands in transcription bubbles (10). Recently, the RNA exosome has been found to be involved in AID's targeting of both DNA strands (11). DNA lesions in the S and V regions are repaired by different mechanisms. In S regions, Ig-CSR-induced DSBs are mostly repaired through the classical nonhomologous end-joining (c-NHEJ) pathway; however, a recently described alternative end-joining pathway can also perform repair based on microhomology (12). The error-prone repair of SHM-induced DNA lesions involves the MSH2/MSH6 complex (a component of the mismatch repair [MMR] machinery) and error-prone DNA polymerases (13).

According to in vitro studies, the MSH2/MSH6 (MutS $\alpha$ ) complex recognizes small insertions and single-bp mismatches (14) [including U:G mismatches (15)] and then recruits PMS2/MLH1 (MutL $\alpha$ ) and exonuclease-1 (EXO1) (16). The latter has 5'  $\rightarrow$  3' exonuclease activity and initiates strand degradation from a nick (either pre-existing or induced by PMS2 endonuclease activity) located on the 5' side of the mismatch (17). Once the mismatch is removed, EXO1 activity is inhibited, and the gap is filled by polymerases and DNA ligases. Another complex (MSH2/MSH3, also known as MutS $\beta$ ) is involved in recognizing insertion/deletion loops of 2–16 bp (14, 18) but can also bind some single-bp mismatches, at least in yeast (19).

The MMR proteins' roles in Ig-CSR and/or SHM have been described thanks to appropriate knockout mouse models (20–22).

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Abbreviations used in this article: AID, activation-induced cytidine deaminase; c-NHEJ, classical nonhomologous end-joining; DSB, double-strand break; EXO1, exonuclease-1; Ig-CSR, Ig class-switch recombination; MMR, mismatch repair; S, switch; sCD40L, soluble CD40L; SHM, somatic hypermutation.

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In humans, it has been reported that: 1) MSH5 variants can be associated with common variable immunodeficiency and IgA deficiency phenotypes (23); and 2) four patients with MSH2 or MSH6 deficiency reportedly exhibited an IgA deficiency (24–26). More recently, we showed that a PMS2 deficiency is associated with defective Ig-CSR, whereas SHM generation is unaffected (27). The Ig-CSR defect was found to occur upstream of the Ig-CSR-induced DSBs in S regions, suggesting that PMS2's endonuclease activity has a role in this process. The latter hypothesis was recently confirmed in PMS2 endonuclease-deficient mice (28). Thus, MMR has been shown to play a role in DSB generation during Ig-CSR in both mice (29) and humans (27). The MutS $\alpha$  complex (but not the MutL $\alpha$  complex) is involved in SHM in mice (30) and recruits the EXO1 and polymerase  $\eta$  (10, 31, 32) that ultimately introduce mutations at AT nucleotides. The fact that polymerase  $\eta$  deficiency in humans results in a significant decrease in the number of AT mutations (33) provides evidence for a similar role in SHM in humans.

To better characterize the role of the MSH2/MSH6 complex in Ab maturation in humans, we studied the Ig-CSR and SHM processes in B cells from eight MSH6-deficient patients. In this study, we report that MSH6 deficiency leads to a partial Ig-CSR defect and an abnormal SHM pattern. Our data suggest that MSH6 is not only able to convert staggered DSBs to blunt DSBs but is also involved in S junction DSB repair.

## Materials and Methods

### Patients

Blood samples were collected from eight MSH6-deficient patients from five families [one of which has already been reported (34)]. The samples were obtained after provision of informed consent by the patients themselves or (for children) their parents. This study was approved on May 20, 2005, by the local independent ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale no. 05632, Paris Saint Antoine) and was conducted in accordance with the Declaration of Helsinki.

Patient P1 presented systemic lupus erythematosus at 16 y of age and colorectal cancer at 17. P3 (the oldest patient, 52 y of age) also developed colorectal tumors at the age of 45 and 49 y, together with multiple colon adenomas and polyps. P2, P5, and P6 also showed colon polyps (at the ages of 11, 11, and 10 y, respectively). Moreover, P5 developed T cell lymphoma when she was 6. P4 developed glioblastoma in early childhood and died at the age of 14 as a result of tumor progression. Patient P8 is P4's cousin and carries the same mutations. No health problems have been reported in the 10-y-old P7 and the 2-y-old P8 (the two youngest patients), who are regularly examined. None of the patients were reported as being peculiarly susceptible to bacterial infections.

Concentrations of IgM, IgA, IgG, and IgG subclasses were determined in the serum of each patient. However, because IgG4 can go undetected in some healthy female controls (35), only results from male patients were taken into consideration for this isotype.

### Expression of MMR gene transcripts

Total RNA was prepared from EBV-B cell lines or soluble CD40L (sCD40L) and IL-4-stimulated PBLs. Single-strand cDNA was transcribed as described previously (4). RT-PCR was performed with primers for AICDA, PMS2, MLH1, MSH2, and MSH6 (available upon request).

### Abs used for Western blotting and immunoprecipitation assays

The following Abs were used: anti-MSH6 (44/MSH6; BD Pharmingen), anti-MSH2 (G219-1129; BD Pharmingen; and N-19; Santa Cruz Biotechnology), anti-MSH3 (52/MSH3; BD Pharmingen; and S-16; Santa Cruz Biotechnology), anti-MLH1 (G168-728; BD Pharmingen; and N-20; Santa Cruz Biotechnology), anti-C-terminal part of PMS2 (A16-4; BD Pharmingen); anti-N-terminal part of PMS2 (E-19; Santa Cruz Biotechnology); and anti-PI3K (Millipore).

### MMR protein expression and immunoprecipitation

After washing, EBV-B cell lines were pelleted and lysed for 30 min on ice in lysis buffer (50 mM Tris/HCl [pH 8], 150 mM NaCl, 5 mM EDTA, 50 mM

NaF, 1% Nonidet P-40, 0.5 mM PMSF, and 0.8  $\mu$ g/ml leupeptin). Lysates were clarified by centrifugation at  $14,000 \times g$  for 15 min. The protein concentration of supernatants was determined according to the Bradford method (Bio-Rad), with BSA as a standard. The same amount of each supernatant was resolved on 4–12% bis Tris gel (Invitrogen). For immunoprecipitation experiments, 1 mg each supernatant was incubated for 1 h at 4°C with 4  $\mu$ g appropriate goat or mouse Ab and recovered by incubation with 50  $\mu$ l protein G-Sepharose beads for 45 min at 4°C. Next, the supernatants were incubated overnight at 4°C with 2  $\mu$ g corresponding specific Ab. Immunoprecipitates were recovered by incubation with protein G-Sepharose beads for 45 min at 4°C and washed in lysis buffer prior to dissociation in reduced Laemmli sample buffer and resolution on a 4–12% bis Tris gel. Following migration, protein bands were electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore) for 2 h at 25 V. Nonspecific binding was blocked with reconstituted powdered milk and the blots were incubated with the different Abs. Proteins were visualized with an HRP-coupled antimouse Ab or protein A (Amersham Biosciences), using a chemoluminescence detection system (the ECL+ from Amersham Biosciences).

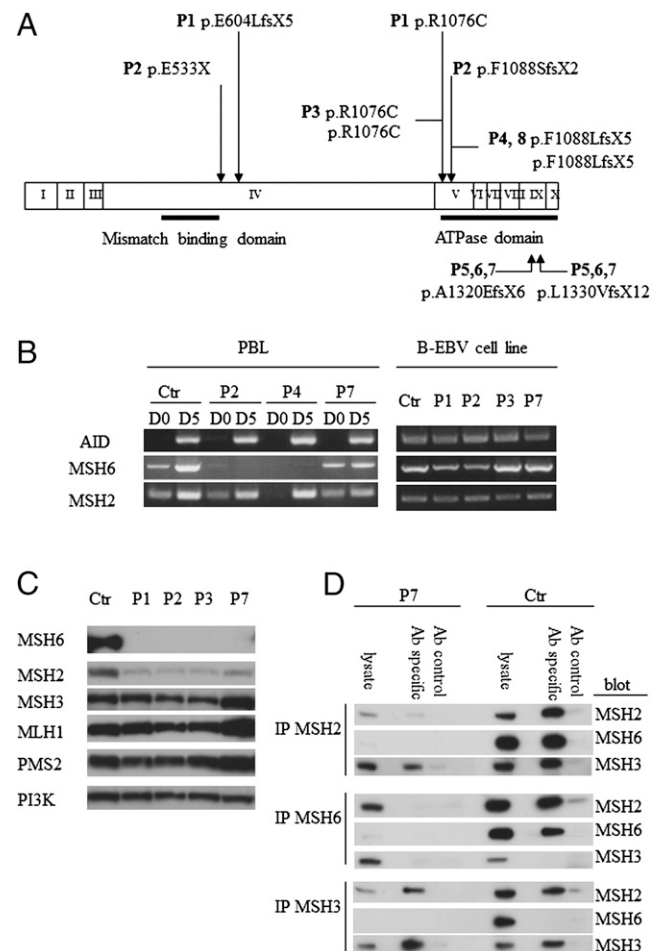


**FIGURE 1.** MSH6 deficiency. **A**, Description of the patients' mutations in the MSH6 gene. **B**, Induction of AICDA, MSH2, and MSH6 RNA transcripts during CSR. RT-PCR was performed on: 1) PBLs from control, P2, P4, and P7 before (D0) and after (D5) 5 d of activation with sCD40L and IL-4; and 2) EBV-B cell lines from control, P1, P2, P3, and P7 (number of experiments;  $n = 1$ ). **C**, MMR protein expression in EBV-B cell line from MSH6-deficient patients. The Western blot assays were performed at least three times for each patient. **D**, MSH2 interaction with MSH3 and MSH6. Immunoprecipitation (IP) using Abs against MSH2, MSH6, or MSH3 (specific Ab) was assessed in total cell lysates from P7 and control EBV-B cell lines ( $n = 2$ ). Mouse or goat Abs (control Ab) were used as negative controls for immunoprecipitation. For each IP, the presence of MSH2, MSH6, and MSH3 was assessed (blot).

### Flow cytometry analysis of B cells

Cells were stained with fluorochrome-conjugated Abs against CD19, CD27, IgM, and IgD (as described previously in Ref. 27) and analyzed with an FACSCanto II system (BD Biosciences).

### Lymphocyte proliferation and CSR

PBLs were isolated from patient samples on a Ficoll-hypaque gradient and then suspended at a concentration of  $1 \times 10^6$  cells/ml in: 1) RPMI 1640 medium (Life Technologies) supplemented with antibiotics and 10% human AB serum (for proliferation); or 2) IMDM medium (Life Technologies) supplemented with antibiotics, 10% heat-inactivated FCS (Life Technologies), insulin transferrin (Sigma-Aldrich), and linoleic (Sigma-Aldrich) and oleic acids (Sigma-Aldrich) (for IgE production); or 3) RPMI 1640 medium supplemented with 10% heat-inactivated FCS (for IgA production). The PBLs were activated with 500 ng/ml sCD40L (Alexis) and 100 U/ml IL-4 (R&D Systems) for IgE production or 100 U/ml IL-10 (R&D Systems) for IgA production. Levels of IgE and IgA in the culture supernatants were assayed in duplicate (ELISA) after 12 d or 7 d of culture, respectively. Proliferation was assessed in 5-d sCD40L and IL-4-activated cultures by [ $^3$ H]thymidine uptake. Data are reported as the mean cpm of duplicate samples.

### Amplification and analysis of $S\mu$ - $S\alpha$ junctions

Genomic DNA was purified from patient or control PBLs. The  $S\mu$ - $S\alpha$  junctions were amplified as described previously (36) with GoTaq polymerase (Promega) processing, gel purification (Roche), cloning into a TOPO-TA vector (Invitrogen), and sequencing on an automated fluorescent sequencer (MilleGen).

The alignment among  $S\mu$ ,  $S\alpha 1$ , or  $S\alpha 2$  region sequences and the junction sequence enabled us to determine the microhomology usage at the junction and the mutations occurring in the  $S\mu$  region during Ig-CSR. Microhomology was defined as successive nucleotides shared by  $S\mu$  and  $S\alpha$  at the S junction, with no mismatches. A nucleotide at the breakpoints that was not identical to either of the S regions was defined as an insertion. Polymorphisms in the S region were excluded from the mutation analysis.

### Analysis of SHM in variable regions of Ig genes

Peripheral blood CD19<sup>+</sup>CD27<sup>+</sup> cells were sorted and processed to obtain cDNA, and PCR was performed using VH3-23 (GenBank U96286.1) and C $\mu$  primers and Pfu Taq polymerase. The DNAs were then cloned and sequenced: each studied clone was unique according to its CDR3 sequence. Mutations in CDR3 were not taken into consideration in the study of frequency and pattern of SHM (4).

### $\gamma$ H2AX repair foci detection by immunofluorescence

EBV-B cell lines were submitted to 5 Gy irradiation and then incubated in fresh RPMI 1640 medium supplemented with 10% FCS for 30 min or 24 h. Then cells were harvested, allowed to adhere to polylysine-coated coverslips, and then fixed with 4% paraformaldehyde. After permeabilization in 0.5% Triton X-100 in PBS, cells were labeled with mouse anti- $\gamma$ H2AX Ab (Upstate), washed, and then incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). Slides were mounted in Vectashield with

DAPI (Vector Laboratories) and analyzed by fluorescence microscopy (Axioplan; Carl Zeiss Microimaging). Images were taken with a Cool Snap HQ camera (Photometrics) and saved with PMCapture Pro 6 software (Photometrics). Grayscale images were processed using Photoshop (Adobe Systems). Cells with more than five foci were counted as  $\gamma$ H2AX positive.

## Results

### Mutations and protein expression

Eight patients in five families were diagnosed with MSH6 deficiency because of the occurrence of cancers in five patients (P1 to P5) and a family history of cancer in three patients (P6, P7, and P8). Patients with cancer were studied prior to chemotherapy or at least 6 mo afterward. All patients presented MSH6 biallelic mutations: two patients from the same family (P4 and P8) had a homozygous premature stop codon, and four patients from two different families (P2, P5, P6, and P7) had compound premature stop codons. Patient P3 carried a homozygous missense mutation (p.R1076C, located in the MSH6's ATPase domain) that was also found to be heterozygous and associated with a stop codon in P1 (Fig. 1A). MSH6 RNA transcripts in sCD40L and IL-4-activated PBLs were readily detectable in P7 but barely so in patients P2 and P4, suggesting that the latter's nonsense mutations lead to RNA instability. However, MSH6 RNA transcripts were detectable in EBV-B cell lines from P1, P2, P3, and P7 (Fig. 1B). The expression of MSH6 RNA transcripts was not studied in patients P5 and P6 because they had the same mutations as P7. In contrast, Western blot analysis did not evidence any MSH6 protein in EBV-B cell line extracts from the four tested patients (P1, P2, P3, and P7). Interestingly, MSH2 protein expression was found to be significantly lower (although detectable) in these four patients than in controls (Fig. 1C). Coimmunoprecipitation studies revealed that MSH2 was able to interact with MSH3 in EBV-B cells from P7 and healthy controls. An MSH2-MSH6 interaction was detected in controls but not in the tested patient (P7) (Fig. 1D).

### Defective *in vivo* and *in vitro* Ig-CSR in cases of MSH6 deficiency

A serum Ig level assay revealed mild abnormalities in all MSH6-deficient patients other than P1 (Table I). Serum IgM levels were elevated (relative to the normal reference range) in P4, P5, P6, P7, and P8, who are the youngest patients. IgG levels were slightly depressed in three patients (P2, P3, and P5) and more strongly depressed (in combination with an IgG1 deficiency) in P2. Patient P8 also presented an IgG1 deficiency. IgG2 levels were below

Table I. Ig characteristics of seven patients with MSH6 deficiency

| Gender  | Age (y) | Serum Ig Levels (g/l) |             |             |            |              |                       |              | CD19 <sup>+</sup> CD27 <sup>+</sup><br>(/ $\mu$ l) | IgM <sup>+</sup> IgD <sup>+</sup> CD19 <sup>+</sup><br>CD27 <sup>+</sup> (/ $\mu$ l) |
|---------|---------|-----------------------|-------------|-------------|------------|--------------|-----------------------|--------------|----------------------------------------------------|--------------------------------------------------------------------------------------|
|         |         | IgM                   | IgG         | IgG1        | IgG2       | IgG3         | IgG4                  | IgA          |                                                    |                                                                                      |
| P1      | F       | 22                    | 1.76        | 9.66        | 11.4       | 1.08         | 0.87                  | 0.016        | 3.24                                               | <b>13</b>                                                                            |
| P2      | F       | 16                    | 1.37        | <b>4.27</b> | <b>3.6</b> | <b>0.23</b>  | 0.48                  | 0.05         | 0.75                                               | <b>34</b>                                                                            |
| P3      | M       | 50                    | 1.71        | <b>6.35</b> | 4.14       | 1.93         | 0.27                  | <b>0.001</b> | 1.95                                               | <b>46</b>                                                                            |
| P4      | M       | 13                    | <b>1.71</b> | 10.7        | 6.15       | 0.5          | 1                     | <b>0.008</b> | 1.55                                               | 50                                                                                   |
| P5      | F       | 12                    | <b>1.83</b> | <b>5.17</b> | 4.05       | <b>0.009</b> | 1.15                  | 0.002        | 0.48                                               | 58                                                                                   |
| P6      | M       | 11                    | <b>2.33</b> | 7.81        | 6.7        | <b>0.06</b>  | 1.02                  | <b>0.003</b> | 0.9                                                | 52                                                                                   |
| P7      | M       | 8                     | <b>2.01</b> | 7.57        | 6.2        | <b>0.12</b>  | 1.02                  | <b>0.001</b> | 0.51                                               | 25                                                                                   |
| P8      | M       | 2                     | <b>2.47</b> | 6.8         | <b>3.8</b> | <b>0.4</b>   | 0.56                  | <b>0.02</b>  | 0.66                                               | <b>2</b>                                                                             |
| Control | 1–2     | 0.54–1.06             | 5.3–10.1    | >3          | >0.3       | >0.12        | 0.04–1.95             | 0.34–0.78    |                                                    |                                                                                      |
| Control | 9–16    | 0.53–1.62             | 5.82–12.29  | >4          | >0.5       | >0.17        | 0.04–1.95             | 0.46–2.03    | 17–100                                             | 5.5–26                                                                               |
| Control | >16     | 0.50–2.09             | 6.65–12.3   | >4          | >0.6       | >0.17        | F 0–2.10;<br>M > 0.03 | 0.7–3.44     | 19–76                                              | 10–27                                                                                |

The serum Ig levels from the patients, as well as their numbers of peripheral memory B cells (CD19<sup>+</sup>CD27<sup>+</sup>) and switched memory B cells (IgM<sup>+</sup>IgD<sup>+</sup> among CD19<sup>+</sup>CD27<sup>+</sup>), are presented. Abnormal values are shown in boldface.

F, Female; M, male.

normal in five patients (P2, P5, P6, P7, and P8), whereas a low IgG4 level was found in P3, P4, P6, P7, and P8 (Table I).

Total memory CD19<sup>+</sup>CD27<sup>+</sup> B cell counts were normal in all tested patients but P1, whereas the number of in vivo-switched IgM<sup>+</sup>IgD<sup>+</sup>CD19<sup>+</sup>CD27<sup>+</sup> B cells was lower than normal in all patients but P4 (Table I). In vitro CSR toward IgE was found to be defective in the six tested patients, despite robust sCD40L and IL-4-induced cell proliferation (Fig. 2). The same defect was observed for CSR toward IgA for the three tested patients (Fig. 2B). However, the CSR defect was significantly less drastic than the one observed in AID-deficient B cells (Fig. 2). It is noteworthy that under the same culture conditions, CD40L-deficient PBLs containing very few switched B cells (0.3 and 0.005%) were able to switch to IgE (Fig. 2A). Moreover, activation of purified control naive B cells with sCD40L and IL-4 leads to an important pro-

duction of IgE (44 and 120 ng/ml). Hence, our in vivo findings and in vitro assay results provide evidence of a role for MSH6 in Ig-CSR in humans.

#### Study of Ig-CSR-induced S junction repair

Sequencing of ex vivo-rearranged S $\mu$ -S $\alpha$  junctions during Ig-CSR revealed the increased usage of microhomology by MSH6-deficient B cells because the frequency of junctions using  $\geq 10$  bp microhomology was significantly higher than in control cells (26 versus 4%, respectively;  $p < 0.001$ ) (Fig. 3A, Supplemental Fig. 1). In addition, the frequency of mutations close to the junctions (i.e., within 15 bp) was lower in MSH6-deficient cells than in control cells ( $p < 0.01$ , data not shown). The limited number of mutations prevented us from comparing mutation patterns. Furthermore, S junctions were preferentially located in the part of the S $\mu$  region (275–760 bp) that presented the strongest homology with S $\alpha$  ( $p < 0.001$ ) (Fig. 3B). This finding indicates that in the

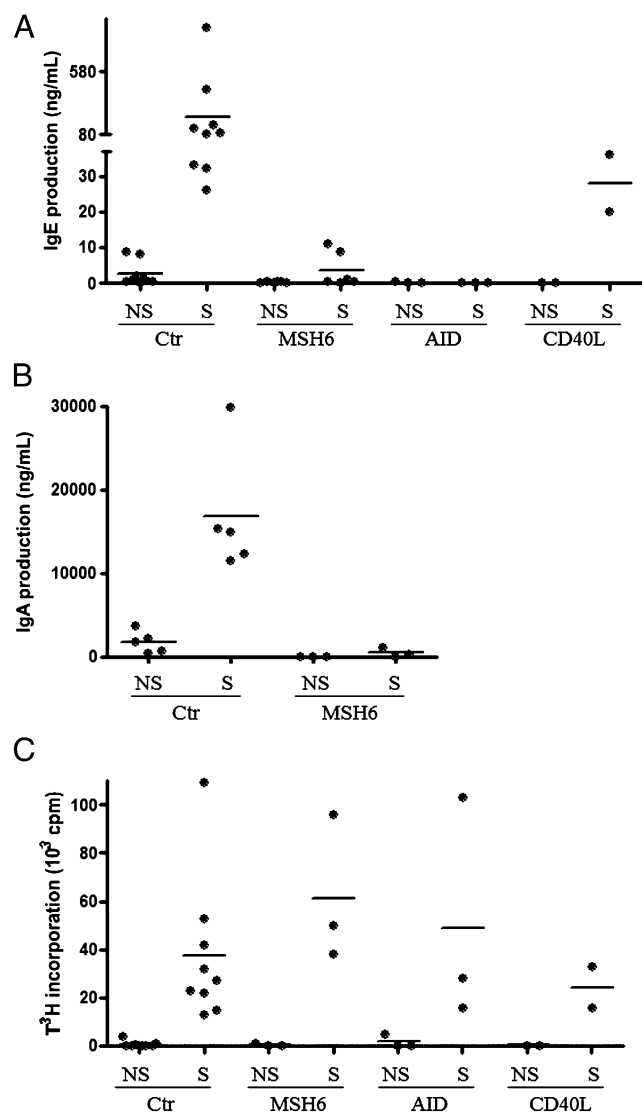


**FIGURE 2.** In vitro Ig-CSR defects. *A*, In vitro IgE production. PBLs from MSH6-deficient patients (P1, P2, P4, P6, P7, and P8), two CD40L-deficient patients, and three AID-deficient patients were cultured for 12 d in the absence (NS) or presence (S) of sCD40L and IL-4. IgE in the supernatants was quantified by ELISA ( $n = 1$ ). *B*, In vitro IgA production. After PBLs from patients 2, 4, and 8 were cultured for 7 d in the absence (NS) or presence (S) of sCD40L and IL-10, IgA in the supernatants was quantified by ELISA ( $n = 1$ ). *C*, In vitro cell proliferation was assessed by [<sup>3</sup>H]thymidine uptake after 5 d of culture, under the same conditions as described in *A* ( $n = 1$ ).

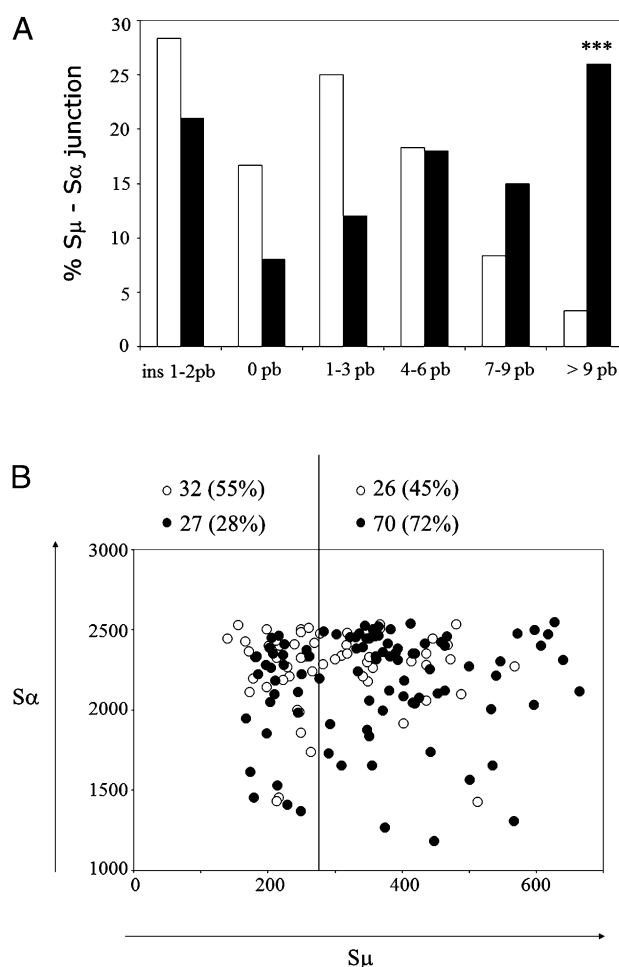


**FIGURE 3.** S junction recombination. *A*, Pattern of the S $\mu$ -S $\alpha$  junctions. Sixty junction sequences were obtained from two control genomic DNAs (white bars), and 100 sequences were obtained from P1, P2, P3, P4, and P6 (black bars). The graph shows the proportion of junctions as a function of the perfectly matched microhomology length. Data on some of the control junctions have already been published (36). Statistically significant differences in a  $\chi^2$  test are indicated by \*\*\* ( $p < 0.001$ ). *B*, A scatter plot analysis of S $\mu$ -S $\alpha$  breakpoints. The positions of each breakpoint in the S $\mu$  (x-axis) and the S $\alpha$  (y-axis) regions are shown. The breakpoints of the junctions from MSH6-deficient patients (black circles) were significantly ( $p < 0.001$ ) more frequently located in the S $\mu$  region (275–760 bp), which showed the highest degree of homology with S $\alpha$  (beyond the vertical line) relative to controls (white circles).

context of MSH6 deficiency, S junction repair preferentially involves a c-NHEJ-independent pathway.

Ig-CSR-induced mutations occurring in S $\mu$  regions outside the switched junctions (i.e., located at least 15 bp upstream of the junction) had a normal frequency, and the GC targeting did not appear to differ from that seen in controls. In contrast, the frequency of transitions on GC residues was much higher than in controls. Transitions on AT residues were not modified (Table II). These results suggest that MSH6 has a role in the introduction of mutations in S $\mu$  regions during the Ig-CSR process in humans.

#### Study of irradiation-induced foci in EBV-B cell lines

In view of recent studies suggesting that MSH6 can interact with the c-NHEJ pathway (37), we used  $\gamma$ H2AX staining to investigate the DNA repair capacity of B cells following irradiation-induced DSB formation. Thirty minutes after 5 Gy irradiation, a  $\gamma$ H2AX signal was found to a similar extent in EBV-B cell lines from P1, P2, P3, P7, and controls. Twenty-four hours after irradiation, the  $\gamma$ H2AX signal decreased in the control cells but persisted in the cells from all tested MSH6-deficient patients (Fig. 4). The persistence of irradiation-induced foci was observed in the context of a major NHEJ defect (i.e., Cernunnos deficiency) but was not found in two patients with a PMS2 deficiency.

This result indicates that in MSH6-deficient B cell lines, DNA damage sensing is potentially active, but DNA repair is defective.

#### Somatic hypermutation

The introduction of mutations into V regions during the SHM process was analyzed in CD19<sup>+</sup>CD27<sup>+</sup> B cells from four MSH6-deficient patients. The SHM frequency in the VH3-23 region of IgM was found to be within the control range (2.3–6.5%) in P1, P3, and P4 (2.3, 3.9, and 2.9%, respectively) but slightly lower than normal in P2 (1.9%). A skewed nucleotide substitution pattern was observed in the four tested patients, with a significantly higher proportion of mutations on GC residues and transitions on these residues (Supplemental Fig. 2, Table II). These findings suggest that MSH6 is involved in the SHM process in humans.

### Discussion

The present results (including low serum IgG2 and IgG4 levels, low numbers of circulating switched B cells, inability to undergo CSR in vitro, and preferential usage of microhomology at S junctions by patients' B cells) indicate that MSH6 deficiency impairs Ig-CSR. Child patients present also with increased levels of IgM, which is an indirect sign of CSR deficiency. IgM levels were, however, found normal in older patients, likely because IgG- and IgA-

producing long-lived plasma cells compensate the CSR defect as observed in some CSR-deficient patients (38). The CSR deficiency observed in MSH6-deficient patients is reminiscent of the findings for MSH6-deficient mice (21, 22), although the murine S junctions exhibited a normal pattern (21). This discrepancy may simply be due to the fact that S $\mu$ -S $\alpha$  junctions were analyzed in human peripheral blood cells, whereas S $\mu$ -S $\gamma$ 3 junctions were studied in murine splenocytes after in vitro activation.

As previously reported, another MMR deficiency (PMS2 deficiency) also leads to a partial Ig-CSR defect in humans. The lack of PMS2 endonuclease activity (27) results in a lower frequency of DNA breaks in S regions during the CSR process. The MSH2/MSH6 complex is known to recruit the PMS2/MLH1 complex at DNA mismatches. One can thus hypothesize that the Ig-CSR defect associated with MSH6 deficiency results from the impaired triggering of PMS2 activity. However, PMS2 deficiency leads to a much more profound Ig-CSR defect, leading to clinical susceptibility to infection; three out of nine PMS2-deficient patients were reported as suffering from a clinical Ig deficiency (27 and S. Kracker, A. Fischer, and A. Durandy, unpublished observations). This difference may result from a compensatory effect exerted by other MMR proteins when MSH6 is absent. A direct interaction between MSH2 and MSH3 was evidenced in EBV-B cell lines from MSH6-deficient patients. Hence, MSH3 may exert a partially compensatory role in the absence of MSH6. However, it has been shown that MSH2/MSH6 and MSH2/MSH3 complexes do not recognize the same mismatches on DNA (14) and that *Msh3*<sup>-/-</sup> mice exhibit normal Ig-CSR (21, 22). Whereas *Msh6*<sup>-/-</sup> and *Ung*<sup>-/-</sup> mice exhibit a partial Ig-CSR defect, *Msh6*<sup>-/-</sup>*Ung*<sup>-/-</sup> mice exhibit a complete in vivo CSR defect (39), showing that (at least in the mouse) MSH3 does not play a role in CSR, even in the absence of MSH6. Nevertheless, other MMR molecules could also be involved; for example, MSH5 has been shown to play a role in Ig production in humans (23) but not in the mouse (40).

As also observed in PMS2 deficiency (27), the Ig-CSR defect in MSH6-deficient B cells was associated with preferential usage of microhomology in DNA repair in S regions. Preferential microhomology usage has been found to be associated with defective DNA lesion induction (36) and defective DNA repair (41). Ig-CSR-induced DSBs generation might be impaired in MSH6-deficient B cells, as suggested by: 1) their preferential location in the downstream part of S $\mu$  region; and 2) the biased mutation pattern observed in proximity to the junctions. Hence, in addition to contributing to the induction of DNA DSBs in S regions, MSH6 might also be involved in DSB resolution. Recently, it has been

Table II. Characteristics of mutations in S $\mu$  core and V region

|                       | Percentage of Mutations |       | Percentage of Mutations at GC |    | Percentage of Mutations at AT |    | No. of Mutations Analyzed | Frequency per 100 bp |
|-----------------------|-------------------------|-------|-------------------------------|----|-------------------------------|----|---------------------------|----------------------|
|                       | On GC                   | On AT | Ts                            | Tv | Ts                            | Tv |                           |                      |
| Mutations in Sμ core  |                         |       |                               |    |                               |    |                           |                      |
| Controls              | 71                      | 29    | 48                            | 52 | 70                            | 30 | 68                        | 0.6–0.9              |
| Patients              | 76                      | 24    | 88                            | 12 | 77                            | 23 | 128                       | 0.65                 |
| <i>p</i> Value        | NS                      |       | < 0.001                       |    | NS                            |    |                           |                      |
| Mutations in V region |                         |       |                               |    |                               |    |                           |                      |
| Controls              | 65                      | 35    | 56                            | 44 | 53                            | 47 | 875                       | 2.3–6.5              |
| Patients              | 87                      | 13    | 67                            | 33 | 48                            | 52 | 412                       | 2.8                  |
| <i>p</i> Value        | < 0.001                 |       | < 0.001                       |    | NS                            |    |                           |                      |

S $\mu$  core mutations were studied by amplification and sequencing of S $\mu$ -S $\alpha$  junctions from the patients' genomic DNA. SHMs were analyzed by amplification and sequencing of the VH3-23 region of IgM from the patients' peripheral memory B cell RNA. Statistically significant differences obtained by  $\chi^2$  test by comparing the data from the controls and the patients are shown.

Ts, Transitions; Tv, transversions.

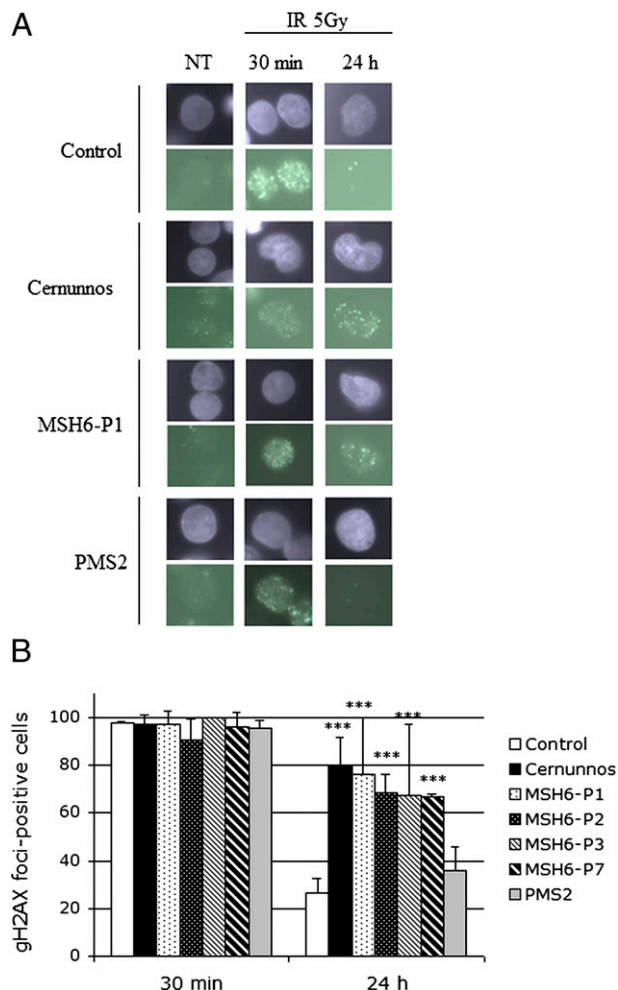


**FIGURE 4.** Irradiation-induced focus formation. **A**, Representative images for the detection of  $\gamma$ H2AX foci in EBV-B cell lines. Lines from two healthy controls, a Cernunnos-deficient patient, P1, P2, P3, and P7, and two PMS2-deficient patients (PMS2) were irradiated or not (NT). Thirty minutes or 24 h after irradiation, cells were labeled with mouse anti- $\gamma$ H2AX Ab and Alexa Fluor 488 anti-mouse Ab. Nuclei were stained with DAPI. Original magnification  $\times 1000$  for all panels. **B**, Quantitative analysis. Cells with  $>5$   $\gamma$ H2AX foci were counted as positive. The percentage was calculated by dividing the number of  $\gamma$ H2AX foci-positive cells by the number of DAPI-stained cells. Error bars represent the SD in two or three independent experiments for each individual. At least 30 nuclei were counted in each experiment. Statistically significant differences in a  $\chi^2$  test (relative to healthy controls) are indicated by \*\*\* ( $p < 0.001$ ).

reported that AID binds cooperatively with uracil N-glycosylase and MMR proteins to S regions (42). Because this association requires the C-terminal part of AID [which is important for CSR but not for SHM (43)], one can legitimately hypothesize that the latter domain guides AID into a molecular complex (that includes MSH2/MSH6) required for DSB recombination. This hypothesis is supported by the following observations: 1) overexpressed AID binds the DNA-dependent protein kinase catalytic subunit through its C-terminal part (44); 2) MSH2–MSH6 binds to Ku70–Ku80 key molecules in the NHEJ repair (37); and 3)  $\gamma$ H2AX repair foci persist 24 h after irradiation of four different MSH6-deficient EBV-B cell lines (Fig. 4). Defects in DSB DNA repair lead to both defective CSR with abnormal S junctions and susceptibility to cancers, as observed in ataxia-telangiectasia and NHEJ defects (45–47).

Analysis of the mutation pattern in the  $S_{\mu}$  region from rearranged S junctions showed that GC targeting was similar to that

observed in control cells; this finding is consistent with the results obtained in *Msh2*<sup>−/−</sup> murine B cells (48). In contrast, SHM in the V<sub>H</sub> region was affected by the MSH6 deficiency, a feature reminiscent of what has been observed in MSH2- or MSH6-deficient B cells in the mouse (21, 22, 30) and provides evidence of a role for MSH2/MSH6 in SHM generation. MSH3 appears to be unable to compensate for the lack of MSH6 in the SHM process, an observation already suggested in mice (48) and possibly related to different DNA sequence specificities for MSH2/MSH6 and MSH2/MSH3 complexes.

However, as MSH2 expression was found diminished in the four tested MSH6-deficient patients, one cannot exclude that reduced MSH2 expression could contribute to the phenotype. Study of MSH2-deficient patients could potentially help to address this question.

Although the Ig-CSR and SHM defects observed in MSH6-deficient B cells do not appear to have a significant impact on overall B cell function in vivo, our observations (together with those concerning the Ig-CSR defect caused by PMS2 deficiency) highlight a significant role for the MMR pathway in Ab maturation in humans. Biallelic mutations of one of the MMR genes should be considered in patients with subtle CSR deficiency, especially when familial history is evocative of predisposition to cancers. Moreover, this humoral defect has to be taken into account for an accurate follow-up of the patients, especially when upon chemotherapy for cancer treatment.

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## Disclosures

The authors have no financial conflicts of interest.

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### **Supplemental figure 1: S $\mu$ -S $\alpha$ junctions' sequences**

Each S $\mu$ -S $\alpha$  junction sequences from patients (P1-P5) are represented. Sequences from controls are not shown since a part of them has been already published (36). Bold characters represent insertions and sequence homology between junctions and S $\mu$  and S $\alpha$  .

### **Supplemental figure 2: somatic hypermutation in Vh3-23**

Sequence of human VH3-23 RNA (Gen bank U96286.1) and mutations observed in patients.

Bold characters represent CDR1 and CDR2 regions. Deletions are shown as .....

Each clone was unique according to its CDR3 sequence (not shown since mutations were not taken into consideration for frequency and pattern analysis) as published in (4).

Downloaded from [www.jimmunol.org](http://www.jimmunol.org) on January 17, 2012

|       |                                                                                                                                                   |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| clone | GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTC |
| 1     | T                                                                                                                                                 |
| 2     |                                                                                                                                                   |
| 3     | A A C G                                                                                                                                           |
| 4     | T A A A                                                                                                                                           |
| 5     | T A A A                                                                                                                                           |
| 6     |                                                                                                                                                   |
| 7     | T                                                                                                                                                 |
| 8     | A AT                                                                                                                                              |
| 9     | G A C A T                                                                                                                                         |
| clone | AGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCG |
| 1     | AG AA A G                                                                                                                                         |
| 2     |                                                                                                                                                   |
| 3     | G A C AT A                                                                                                                                        |
| 4     | G A T T                                                                                                                                           |
| 5     | T A C T A A                                                                                                                                       |
| 6     |                                                                                                                                                   |
| 7     | T                                                                                                                                                 |
| 8     | G C A T T A                                                                                                                                       |
| 9     | AG ----- T T A                                                                                                                                    |

P1

|       |                                                                                                                                                   |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| clone | GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTC |
| 1     |                                                                                                                                                   |
| 2     | T C T A G T A                                                                                                                                     |
| 3     | T G A                                                                                                                                             |
| 4     |                                                                                                                                                   |
| 5     | C                                                                                                                                                 |
| 6     | T C A C A                                                                                                                                         |
| 7     | A A                                                                                                                                               |
| 8     | G                                                                                                                                                 |
| 9     | A                                                                                                                                                 |
| 10    | AT T G A A T                                                                                                                                      |
| 11    | A A G                                                                                                                                             |
| 12    | G A                                                                                                                                               |
| 13    | G T                                                                                                                                               |
| clone | AGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCG |
| 1     | G G GT                                                                                                                                            |
| 2     | TGC ----- C G                                                                                                                                     |
| 3     | C AA T                                                                                                                                            |
| 4     |                                                                                                                                                   |
| 5     | A                                                                                                                                                 |
| 6     | T A T                                                                                                                                             |
| 7     |                                                                                                                                                   |
| 8     |                                                                                                                                                   |
| 9     |                                                                                                                                                   |
| 10    | G T                                                                                                                                               |
| 11    |                                                                                                                                                   |
| 12    | G G                                                                                                                                               |
| 13    | G A A A                                                                                                                                           |

P2

|       |                                                                                                                                                   |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| clone | GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTC |
| 1     | T T C T A                                                                                                                                         |
| 2     |                                                                                                                                                   |
| 3     | A A G A A TG A A C C T                                                                                                                            |
| 4     |                                                                                                                                                   |
| 5     | G A C A TT T C CG                                                                                                                                 |
| 6     | A A C T A T                                                                                                                                       |
| 7     | G A AA AA C T A T                                                                                                                                 |
| 8     | A T A A T                                                                                                                                         |
| 9     | C T T G A GA T A T A                                                                                                                              |
| 10    | T T G G T A C                                                                                                                                     |
| 11    | A C T A C                                                                                                                                         |
| 12    | G A G AGG A T T G A A T                                                                                                                           |
| 13    | A T T G A CT G                                                                                                                                    |
| 14    |                                                                                                                                                   |
| 15    | T                                                                                                                                                 |
| clone | AGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCG |
| 1     | A A A A CT T                                                                                                                                      |
| 2     | T A G T A T C                                                                                                                                     |
| 3     |                                                                                                                                                   |
| 4     | G A A A                                                                                                                                           |
| 5     | G G G C A A                                                                                                                                       |
| 6     | G G G C C C T A                                                                                                                                   |
| 7     | A C AA C C C A                                                                                                                                    |
| 8     | A G C C T A A                                                                                                                                     |
| 9     | G AC A G C                                                                                                                                        |
| 10    | A T A G G                                                                                                                                         |
| 11    | A ----- A AT AT                                                                                                                                   |
| 12    | A --- C T C                                                                                                                                       |
| 13    | T AT C GA G T                                                                                                                                     |
| 14    | A G A A A                                                                                                                                         |
| 15    |                                                                                                                                                   |

P3

|       |                                                                                                                                                   |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| clone | GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTC |
| 1     | G T C A A A                                                                                                                                       |
| 2     |                                                                                                                                                   |
| 3     | CA A T A                                                                                                                                          |
| 4     | T A C                                                                                                                                             |
| 5     | T A A G TT T CA A A                                                                                                                               |
| 6     | T T G T C A                                                                                                                                       |
| 7     | T A                                                                                                                                               |
| 8     |                                                                                                                                                   |
| 9     | G A A C C                                                                                                                                         |
| 10    | C T                                                                                                                                               |
| 11    |                                                                                                                                                   |
| clone | AGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCG |
| 1     | ----- T TG TAG A A A A T                                                                                                                          |
| 2     | G A AA T T                                                                                                                                        |
| 3     | T A A GT T C                                                                                                                                      |
| 4     | AG G A T C                                                                                                                                        |
| 5     | A A A A                                                                                                                                           |
| 6     | A A A A T T                                                                                                                                       |
| 7     |                                                                                                                                                   |
| 8     | G                                                                                                                                                 |
| 9     | G A AC C C                                                                                                                                        |
| 10    | A A                                                                                                                                               |
| 11    |                                                                                                                                                   |

P4