

Specific Expression of Activation-induced Cytidine Deaminase (AID), a Novel Member of the RNA-editing Deaminase Family in Germinal Center B Cells*

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We have identified a novel gene referred to as activation-induced deaminase (AID) by subtraction of cDNAs derived from switch-induced and uninduced murine B lymphoma CH12F3-2 cells, more than 80% of which switch exclusively to IgA upon stimulation. The amino acid sequence encoded by AID cDNA is homologous to that of apolipoprotein B (apoB) mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC-1), a type of cytidine deaminase that constitutes a catalytic subunit for the apoB mRNA-editing complex. *In vitro* experiments using a glutathione S-transferase AID fusion protein revealed significant cytidine deaminase activity that is blocked by tetrahydrouridine and by zinc chelation. However, AID alone did neither demonstrate activity in C to U editing of apoB mRNA nor bind to AU-rich RNA targets. AID mRNA expression is induced in splenic B cells that were activated *in vitro* or by immunizations with sheep red blood cells. *In situ* hybridization of immunized spleen sections revealed the restricted expression of AID mRNA in developing germinal centers in which modulation of immunoglobulin gene information through somatic hypermutation and class switch recombination takes place. Taken together, these findings suggest that AID is a new member of the RNA-editing deaminase family and may play a role in genetic events in the germinal center B cell.

The germinal center (GC)¹ constitutes a highly specialized microenvironment required for the final maturation step of naive B cells toward antigen-specific memory cells or long-lived plasma cells (1, 2). Two prominent alterations of immunoglob-

ulin gene information are known to occur in this microenvironment (3–5). First, accumulation of massive point mutations in the variable region exon, a process referred to as somatic hypermutation (6), gives rise to affinity maturation of antibody in association with selection of B cells expressing high affinity immunoglobulins on their surface (7, 8). Second, class switch recombination (CSR) replaces the exons encoding the heavy chain constant region (9), which determines effector functions of the antibody including complement fixation. These two alterations of immunoglobulin gene information are critical for accounting for an effective humoral response to harmful microbes. The molecular mechanisms for these genetic events remain to be elucidated despite intensive study.

To dissect the molecular mechanism of class switching, we have isolated a murine B lymphoma clone CH12F3-2 in which CSR from IgM to IgA begins to occur within a few hours after stimulation with IL-4, TGF- β , and CD40L, giving rise to more than 80% IgA⁺ cells (10–12). Using CH12F3-2 cells, we have shown that CSR breakpoints are distributed not only within typical repetitive sequences (designated S region) but also in its flanking regions (11). However, the break points were rarely found in the I and C exons, which are separated by the S region, *i.e.* the intron of germ-line transcripts. Because accumulating evidence indicates that transcription from I to C exon and splicing of the transcripts are essential to CSR (13, 14), it is possible that the transcripts are involved directly or indirectly in CSR. We have thus proposed that a complex structure of DNA and RNA, but not S region sequence *per se*, is recognized for initiation of CSR (11). This idea gained further support with the finding that CSR can take place efficiently even when the S α region was replaced by the S ϵ or S γ 1 region in minichromosomal constructs introduced in CH12F3-2 cells upon cytokine stimulation (10).

Another type of genetic regulation, RNA-editing, is widely used as a means to create new functional genes from the restricted genome in plants and protozoa (15, 16). An increasing number of mammalian mRNAs are also known to be edited, including apolipoprotein B (apoB) mRNA, glutamate receptor mRNA, Wilms tumor-1 mRNA, α -galactosidase mRNA, neurofibromatosis type-1 mRNA, and tRNA^{Asp} (17). Although most of their molecular mechanisms are yet to be elucidated, that of apoB mRNA editing by APOBEC-1 (18, 19) is extensively documented. ApoB mRNA editing involves a site-specific C to U deamination of the first base of a CAA codon, encoding glutamine at residue 2153 in apoB100, and produces a UAA in-frame stop codon in apoB48 mRNA (20). ApoB100 and apoB48 are translation products of the unedited and edited apoB mRNAs, respectively, and these proteins have completely different physiological functions (21). APOBEC-1 requires an aux-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF132979.

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¹ The abbreviations used are: GC, germinal center; CSR, class switch recombination; apoB, apolipoprotein B; APOBEC-1, apoB mRNA-editing enzyme, catalytic polypeptide 1; CD40L, CD40 ligand; GST, glutathione S-transferase; THU, tetrahydrouridine; LPS, lipopolysaccharide; RBC, red blood cells; SRBC, sheep RBC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide; IL, interleukin; TGF, transforming growth factor; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s).

iliary factor(s) for site-specific RNA editing of apoB mRNA (18, 19). APOBEC-1 itself demonstrates cytidine deaminase activity on a monomeric nucleoside substrate and nonspecific, low affinity binding to AU-rich RNA (19, 20, 22, 23). Expression and activity of the auxiliary factors are found not only in organs that carry out apoB mRNA editing but also in those that have no detectable levels of APOBEC-1 or apoB mRNA (18, 19, 24). Such widespread expression of the auxiliary factors in the absence of APOBEC-1 implies that the auxiliary factors may be involved in either more general cellular functions or editing of other unknown RNAs. However, little is currently known concerning the identity or activity profile of these other components.

In this paper we report isolation of cDNA encoding a novel activation-induced cytidine deaminase (AID) that is structurally related to the apoB RNA-editing enzyme, APOBEC-1. The restricted expression and inducibility of AID within B cells in GC suggests that AID may play a role in genetic events in GC.

MATERIALS AND METHODS

Construction and Screening of Subtracted cDNA Library—PCR-select cDNA subtraction kit (CLONTECH) was used to generate a library enriched for inducible genes upon CSR according to the manufacturer's instruction. CH12F3-2 cells were cultured and stimulated as described before (12, 25). Poly(A)⁺ RNAs for a tester and a driver cDNA were isolated from stimulated and unstimulated CH12F3-2 cells, respectively. *Hae*III-digested ϕ X174 phage DNA was added to the tester DNA to monitor the efficiency of subtraction. The subtracted cDNA was cloned into T-vector (Promega), and the plasmid library was generated. Two thousand colonies from the subtracted library were screened by differential hybridization as described previously (25). The tester cDNA probe used in this study were derived from CH12F3-2 cells stimulated for 5 and 12 h. To discriminate clones containing *Hae*III-digested ϕ X174 phage DNA, replica filters were hybridized with *Hae*III-digested ϕ X174 DNA. After subtraction, the final cDNA pool was ~100-fold enriched. Plasmids from 115 positive colonies that showed stronger signals with the cDNA probe derived from stimulated cells were sequenced, and their inserts were subjected to Northern blot hybridization to demonstrate up-regulated expression upon CSR in CH12F3-2 cells. Twenty-three cDNA fragments were found to be up-regulated by stimulation with IL-4, TGF- β , and CD40L, including 8 ABCD-1/MDC, 2 interferon- γ receptor, 1 I-a (major histocompatibility complex class II), 3 AID, 7 15B11, 1 8B9, and 1 16A9. Full-length cDNA clones encoding AID gene was obtained by cDNA library screening (25).

Production of GST Fusion Protein and Cytidine Deaminase Activity—AID cDNA was subcloned into pGEX4T1 vector (Amersham Pharmacia Biotech) after PCR amplification with AID-138 and AID-161 primers and sequenced on both strands. AID cDNA was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* cells. The fusion protein was purified essentially as described previously (22) with the exception that the GST fusion protein was purified by two rounds of glutathione-agarose chromatography. The purified GST fusion protein was confirmed by Western blot analyses as described previously (26) using anti-AID antisera, which were prepared by immunizing rabbits with a multiple-antigen peptide (27) containing residues 185–198 (EVDDL RDAFRMLGF). Cytidine deaminase activity was determined as described previously (22).

Mouse Immunization and Fractionation of Splenic Cells—BALB/c mice (6–12 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan). Purified splenic B cells were obtained and stimulated as described previously (25). In this study, dead cells were removed by Ficol gradient after T-cell depletion and 50 μ g/ml LPS from *Salmonella typhosa* (Sigma) were used to stimulate cells. Mice were intraperitoneally immunized with 1×10^8 sheep red blood cells (SRBC; Cosmo Bio). Nylon fiber (Wako Chemicals) was used to separate T cells from RBC-depleted splenic cells (28). The final T cell fraction contained more than 90% CD3⁺ cells and less than 3% B220⁺ cells. To enrich or deplete B cells, magnetic beads-conjugated anti-CD19 antibody (Miltenyi Biotec) was applied, and the mixture was passed over a separation column. The CD19⁺ cell depleted fraction contained less than 5% B220⁺ B cells, whereas the CD19⁺ cell-enriched fraction contained more than 60% B220⁺ B cells.

In Situ Hybridization—An *Eco*RI/*Xho*I fragment of AID cDNA was subcloned into pBleuscriptSK(+) (Stratagene). The plasmid was linearized with *Eco*RI or *Xho*I and used for RNA transcription with T3 or T7

RNA polymerase to generate antisense and sense probes, respectively, in the presence of digoxigenin-labeled rUTP (Roche Molecular Biochemicals). *In situ* hybridization procedures and detection of riboprobes were conducted as described previously (29). The slides were examined under a standard light transmission microscope.

Nucleic Acid Analyses—PCR amplification of looped-out circular DNA-containing S-regions were performed as described previously (11) using α F1 and μ R3(first) primers for primary PCR and α F1 and μ R3(second) primers for secondary PCR. Total RNA and poly(A)⁺ RNA were purified as described previously (25). Northern and Southern blot hybridizations were performed according to standard procedures. Amounts of AID mRNA were radio-quantitated from Northern blot data after normalization with intensities of GAPDH bands and represented as relative folds of nonstimulated samples. RT-PCR was carried out as described previously (10). GAPDH cDNA was amplified using GF and GR primers (10). AID cDNA was amplified using AID-118 and AID-119 primers. Because an intron exists between AID-118 and AID-119 primers,² the PCR product derived from contaminating genomic DNA was easily distinguished. Sequence comparison was performed with GENE-TYX MAC 9.0, and a data base search was done with BLAST (30) using GenBank[®] as DNA data bases and SwissProt as protein data base. Motif search analysis was performed on line at Prosite (31).

Oligonucleotides and Probes—Oligonucleotides used in this study are as follows; AID-118, 5'-ggctgaggttaggttccatctcag-3'; AID-119, 5'-gagg-gagtaagaagaatcacgctgg-3'; AID-138, 5'-ggaattcgccatggacagccttctgat-gaa-3'; AID-161, 5'-gcccgtcagtgcaaaatcccaacatagaa-3' (restriction sites underlined). DNA fragments used as probes are as follows; 1020-base pair AID probe, nucleotides 847–1866 of AID cDNA; GAPDH probe, nucleotides 566 to 1016 (GenBank[®] U32599) generated by RT-PCR with GF and GR primers (10); α A probe, the 1155-base pair *Hind*III-*Eae*I fragment isolated from the 10-kb *Eco*RI fragment (IgH703) (GenBank[®] accession number D11468, nucleotide numbers 1993–3148) (10).

RESULTS

Construction and Screening of CH12F3-2 Cell cDNA Libraries Enriched for Gene Sequences Induced upon CSR—Within hours of stimulation of CH12F3-2 cells with IL-4, TGF- β and CD40L, it is possible to detect looped-out circular DNA containing S regions, which represent the earliest signs of CSR (11). Because looping-out of the circular DNA was inhibited with cycloheximide, we reasoned that up-regulation of proteins is required in a very early phase after stimulation for CSR to take place (Fig. 1, A and B). To isolate such early response genes with CSR induction, we performed PCR-based subtraction using cDNAs obtained from CH12F3-2 cells, shortly after and before stimulation. 115 clones that showed significantly stronger signals for the stimulated probe compared with the non-stimulated probe were sequenced. Seven independent sequences were obtained from the 115 clones, all of which were up-regulated upon stimulation in CH12F3-2 cells, as confirmed by Northern blot hybridization. The seven sequences contained four novel genes and three known genes, which include I-a (major histocompatibility complex class II), ABCD-1/MDC, and interferon γ receptor. I-a and ABCD-1/MDC are known to be up-regulated by IL-4 and CD40L in splenic B cells (32), indicating that the subtraction was effective. Among four novel genes, a clone encoding AID was selected for further analysis because its basal expression in nonstimulated CH12F3-2 cells was low, whereas drastic up-regulation (10 folds at 6 h) was observed upon stimulation (Fig. 1C).

Isolation and Structure of a Novel Gene, AID—To obtain a full-length AID cDNA clone, a 1020-base pair AID fragment was used as a probe for screening a cDNA library of stimulated CH12F3-2 cells. One clone (1.2 kb) revealed a single open reading frame and one polyadenylation site. Three clones, each 2.4 kb, contained two polyadenylation sites and a 5' 1.2-kb portion identical to the shorter one. Two major transcripts (2.4 and 1.2 kb), observed in Northern blot (Fig. 1C), appear to

² M. Muramatsu and T. Honjo, unpublished data.

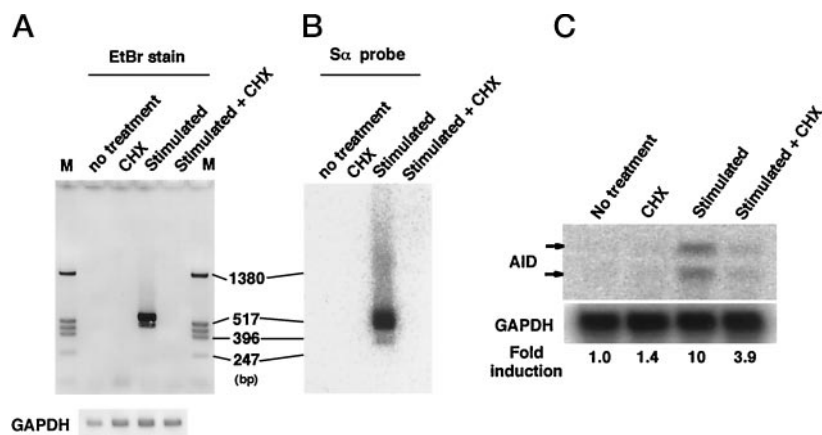


FIG. 1. Inhibition of CSR and AID induction by cycloheximide (CHX). CH12F3-2 cells were treated with or without stimulants (IL-4, TGF- β , and CD40L) in the presence or absence of 200 ng/ml CHX for 6 h. Genomic DNAs were extracted and subjected to PCR to amplify circular DNA containing S μ and S α sequences or GAPDH genomic DNA. *A*, ethidium bromide (*EtBr*) staining of amplified DNA. Formation of switch circular DNA is inhibited by CHX treatment. *M*, molecular mass standards. *B*, Southern blot hybridization of the same gel in *A* with S α probe. *C*, CHX abrogates the up-regulation of AID mRNA. CH12F3-2 cells were treated for 6 h with either medium, CHX (200 ng/ml), stimulants, or CHX plus stimulants. Northern blot hybridization of 10 μ g of total RNA was carried out with AID and GAPDH cDNA probes. Induction fold of AID mRNA expression is indicated below.

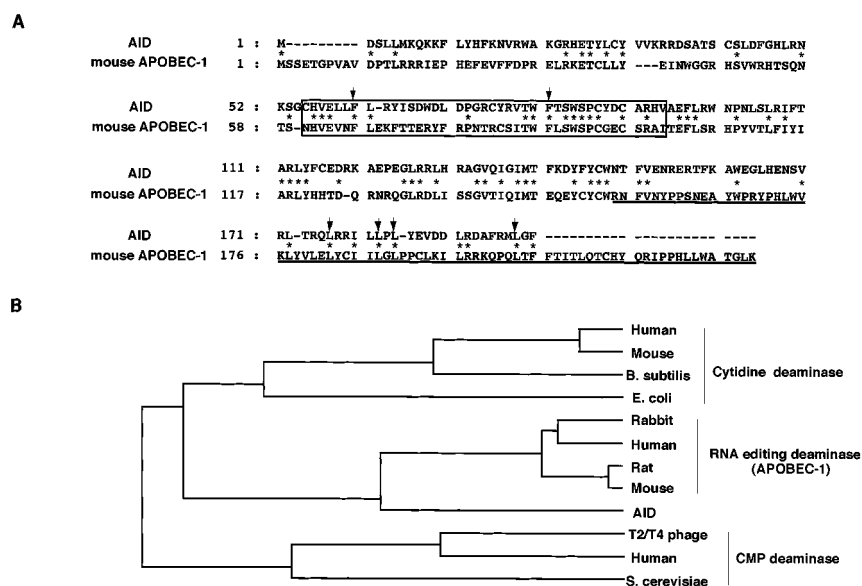


FIG. 2. Alignment of amino acid sequences of AID and mouse APOBEC-1 and the phylogenetic tree of the cytosine deaminase family. *A*, identical amino acids are marked by asterisks. Hyphens represent gaps in the sequence generated by the program to allow better alignment. The conserved active site of cytidine deaminase is indicated by an open box. Arrowheads represent phenylalanines and leucines, which are conserved among rat, mouse, rabbit, and human APOBEC-1. Underlines indicate the pseudoactive domain. *B*, The phylogenetic tree was derived by alignment of the active site of cytidine deaminases, using the UPGMA method. Accession numbers of amino acid sequences used here are as follows; human cytidine deaminase, L27943; mouse cytidine deaminase, AA388666; *Bacillus subtilis* cytidine deaminase, U18532; *E. coli* cytidine deaminase, X63144; rabbit APOBEC-1, U10695; human APOBEC-1, L25877; rat APOBEC-1, U10695; mouse APOBEC-1, U21951; T2/T4 phage CMP deaminase, J05172; human CMP deaminase, L12136; *Saccharomyces cerevisiae* CMP deaminase, U10397.

correspond to the two species of cDNA utilizing the 3' and 5' polyadenylation sites, respectively.

The open reading frame of AID cDNA encodes a 198-residue protein with a predicted molecular mass of 24 kDa. Homology search against data bases of known protein sequences revealed significant homology (34% amino acid identity) with mouse APOBEC-1 (Fig. 2). A data base search revealed the presence of an active site for cytidine deaminases, which is conserved in the large cytosine deaminase family. The family can be classified into RNA-editing deaminases, cytidine deaminases, and CMP deaminases, based on substrate specificity and homology of the active-site sequence (20). As shown in Fig. 2*B*, the phylogenetic tree shows that the active site of AID is closer to the subgroup of RNA-editing deaminases rather than those of cytidine and CMP deaminases. The leucine-rich region located in

the C terminus of APOBEC-1 has been proposed to be important for protein-protein interaction (24, 33). The AID amino acid sequence also contains the C-terminal leucine-rich region, in which four leucines of AID are conserved among rabbit, rat, mouse, and human APOBEC-1 (Fig. 2*A*).

Cytidine Deaminase Activity of the AID Protein—Recombinant APOBEC-1 is known to catalyze *in vitro* deamination of apoB mRNA (multimeric substrate) as well as cytidine (monomeric substrate) (20). Because the cytidine deaminase motif in AID is completely conserved, we investigated the cytidine deaminase activity of AID using a purified GST-AID fusion protein. As shown in Fig. 3*A*, the purified GST-AID fusion protein migrated to the expected position on SDS-polyacrylamide gel (49 kDa). The purified protein was confirmed by an anti-AID peptide antibody recognizing the C terminus of AID

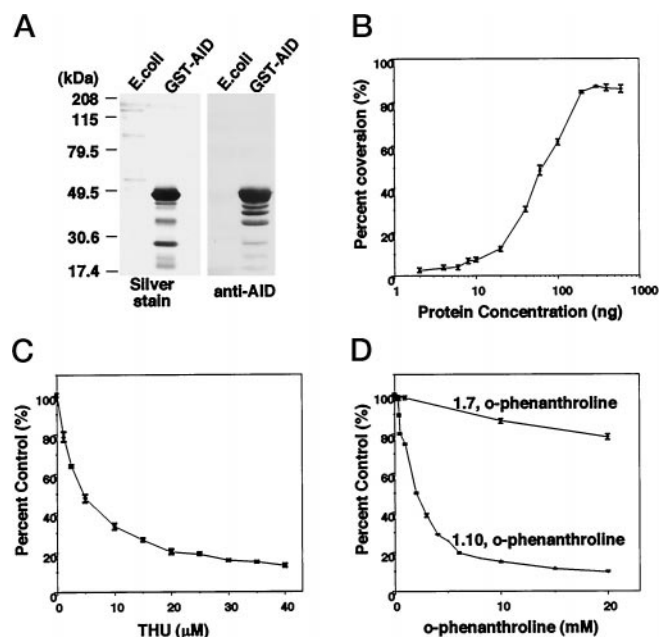


FIG. 3. Cytidine deaminase activity of AID. *A*, purification of GST-AID and Western blot analysis. Lysate derived from nontransformed or transformed cells by GST-AID expression vector were subjected to purification of glutathione-agarose affinity chromatography. The purified GST-AID fusion protein and material from nontransformed *E. coli* extract were electrophoresed in 10% SDS-polyacrylamide gels and silver-stained (*left*) or electrotransferred to a membrane and probed with anti-AID antisera (*right*). *B*, cytidine deaminase activity was determined by *in vitro* incubation of the indicated amounts of the purified GST-AID fusion protein, and the product was quantitated by thin layer chromatography. *C*, inhibition of cytidine deaminase activity by THU. Incubation was performed with 300 ng of GST-AID, with increasing concentrations of THU (0–40 μ M). *D*, inhibition of cytidine deaminase activity of 300 ng of GST-AID by zinc chelation using increasing concentrations of 1,10-*o*-phenanthroline (0–20 mM) or its inactive isomer 1,7-*o*-phenanthroline.

protein. Minor bands seen below the major band for GST-AID in silver staining may be degradation products, some of which were detected by Western blot with the anti-AID antibody. Fig. 3*B* demonstrates that the cytidine deaminase activity of the AID fusion protein increases in a dose-dependent manner. K_m and V_{max} values were calculated to be 1.6×10^{-5} M and 1.33 unit/mg, respectively. The deaminase activity of AID was inhibited by a cytidine deaminase-specific inhibitor, tetrahydrouridine (THU) (Fig. 3*C*). The activity is also sensitive to a zinc chelator, 1,10-*o*-phenanthroline, with 91% inhibition at 20 mM but only 13% inhibition by its inactive isomer, 1,7-*o*-phenanthroline, indicating that, like APOBEC-1, AID is a zinc-dependent cytidine deaminase (Fig. 3*D*). Recombinant APOBEC-1 is known to bind AU-rich RNA (17, 20, 22, 23) and to mediate apoB RNA-editing in the presence of chicken extracts containing auxiliary factors. An RNA substrate of APOBEC-1 was challenged by AID to test its RNA-editing activity. Although AID is a functional cytidine deaminase with structural similarity to APOBEC-1, a gel retardation assay showed no binding capacity of AID to the AU-rich RNA, and the *in vitro* apoB RNA-editing assay (22) showed no C to U conversion (data not shown).

Specific Induction of AID mRNA Expression in Activated B Cells in Lymphoid Organs—Northern blot analysis of different mouse tissues revealed that AID mRNAs were abundant in mesenteric lymph nodes, scarce in spleen, and undetectable in other nonlymphoid tissues (Fig. 4*A*). Expression of AID mRNAs in various lymphoid organs was further investigated by RT-PCR (Fig. 4*B*). Transcripts for AID were observed in peripheral lymphoid organs such as Peyer's patches, and lymph nodes,

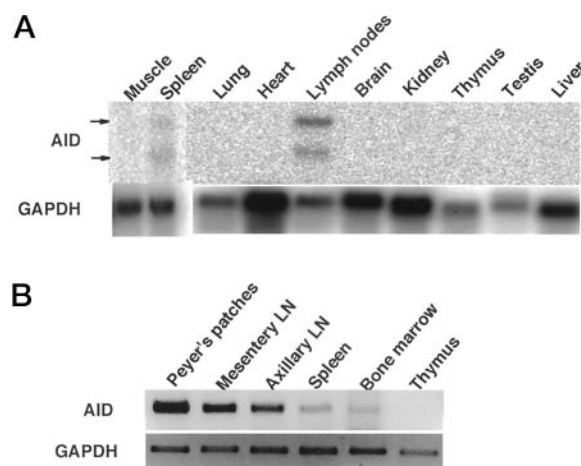


FIG. 4. AID mRNA expression in mouse tissues. *A*, Northern blot analyses of AID mRNA expression. Each lane contains 2 μ g of poly(A)⁺ RNA from indicated cell sources. The filter was hybridized with AID and GAPDH cDNA probes. *B*, RT-PCR analysis of AID mRNA expression. Total RNA from the indicated cell sources were reverse-transcribed, and first-strand cDNAs were used as PCR templates to amplify AID and GAPDH cDNAs.

with lower levels in bone marrow. AID mRNA was not detected in thymus.

The time course of AID mRNA induction in CH12F3-2 cells was further determined by Northern blot hybridization (Fig. 5*A*). Induction of AID mRNAs was detectable by 3 h after stimulation with IL-4, TGF- β , and CD40L, reached a maximal level (13–14-fold induction) at 12 h, and declined gradually from 48 h. To determine which stimulant was responsible for induction of AID mRNAs, different combinations of reagents were tested for expression of AID mRNAs by Northern blot hybridization (Fig. 5*B*). Although a single stimulation with either IL-4, TGF- β , or CD40L slightly induced AID expression, simultaneous addition of the three was most effective as previously shown for CSR induction from IgM to IgA in CH12F3 cells (10, 12). Up-regulation of AID mRNA appears to require *de novo* synthesis of proteins, because its induction was profoundly reduced by cycloheximide treatment (Fig. 1*C*).

We next investigated whether AID mRNAs are induced in splenic B cells by reagents shown to activate B cells and induce CSR. Fig. 6 demonstrates that AID mRNAs were strongly up-regulated in splenic B cells cultured in the presence of either LPS, LPS + IL-4, or LPS + TGF- β . Stimulation of splenic B cells with CD40L resulted in AID mRNA induction, albeit to a lower level. We further investigated whether expression of AID mRNAs is up-regulated by antigen stimulation *in vivo*. An immunization with SRBC is known to initiate immunological responses, followed by clonal expansion and germinal center formation, in which CSR and affinity maturation take place. Poly(A)⁺ RNA was extracted from spleens of mice immunized with SRBC and subjected to Northern blot hybridization (Fig. 7*A*). AID mRNA expression was barely detectable in spleens without immunization, and significant up-regulation (3–5-fold induction) was demonstrated 5 and 13 days after immunization. To determine which cells express AID mRNAs in immunized spleen, RT-PCR was performed using fractionated splenic cells. Fig. 7*B* shows that AID mRNAs were observed in the non-T and CD19⁺ cell fractions, indicating that SRBC immunization induced AID mRNA expression in CD19⁺ splenic B cells.

Specific Expression of AID mRNA in Germinal Centers—Because the timing of AID mRNA up-regulation in the spleen was roughly synchronous with the onset of GC formation after immunization (Fig. 7*A*), we next investigated the precise local-

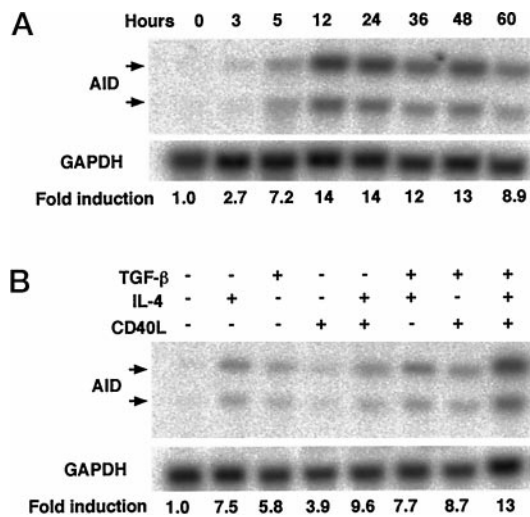


FIG. 5. **Induction of AID mRNAs in CH12F3-2 cells.** Each lane contains 10 μ g of total RNA from nonstimulated or stimulated CH12F3-2 cells. Northern blot filters were hybridized with the AID and GAPDH cDNA probes. Induction fold of AID mRNA expression was indicated below. *A*, CH12F3-2 cells were treated by IL-4, TGF- β , and CD40L for indicated periods. *B*, specificity of stimulants were tested by stimulation of CH12F3-2 cells with indicated stimulants for 12 h.

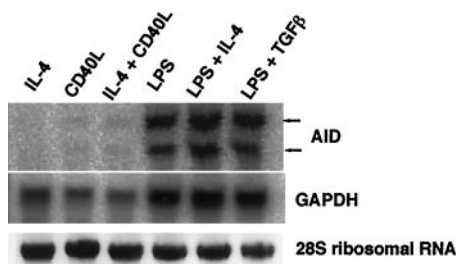


FIG. 6. **Induction of AID mRNAs in splenic B cells stimulated *in vitro*.** Purified murine splenic B cells were cultured with the indicated stimulants for 4 days. Total RNA (15 μ g) from stimulated cells were electrophoresed, stained with ethidium bromide, and analyzed by Northern blot hybridization with an AID cDNA probe. The same filter was hybridized with a GAPDH cDNA probe.

ization of AID mRNAs in lymphoid organs using *in situ* hybridization. When the antisense AID cRNA was used, distinct focal signals were seen in spleen sections from mice immunized with SRBC for 5 days (Fig. 8, *E* and *H*). By contrast, no signals were detected in unimmunized spleen sections with the same probe, in agreement with the data obtained by Northern blot hybridization (Fig. 7A). Peanut agglutinin staining of serial sections (Fig. 8, *C*, *F*, and *I*) showed that both Peyer's patches and immunized spleens contained GC, and that signals derived from the antisense probe colocalized with GC (Fig. 8, *E* and *H*). On the other hand, regardless of immunization, the sense probe produced almost no signal in spleens or Peyer's patches (Fig. 8, *A*, *D*, and *G*). GCs were rarely observed in spleens from unimmunized mice (Fig. 8C). Taken together, these data demonstrate that AID mRNAs are specifically induced in GCs, which contain mostly activated B cells after encounter with antigens.

DISCUSSION

To dissect the molecular mechanism of CSR, we screened subtracted cDNA libraries from nonstimulated and stimulated CH12F3-2 cells on the assumption that induction of trans-acting factors, such as switch recombinase, are required for CSR. In support of this, it was demonstrated that cycloheximide treatment inhibited the formation of looped-out circular DNA in stimulated CH12F3-2 cells (Fig. 1, *A* and *B*), indicating the requirement for *de novo* protein synthesis for CSR to take place.

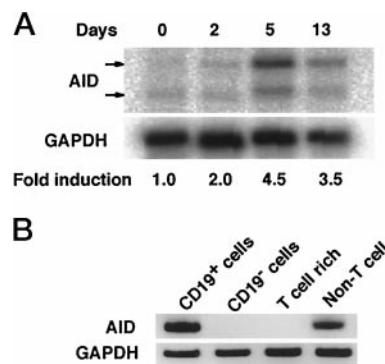


FIG. 7. **Induction of AID mRNAs *in vivo*.** *A*, induction of AID mRNAs in murine splenic B cells following immunization with SRBC. Mice were intraperitoneally immunized with SRBC. At the indicated time, five spleens were resected and pooled to extract RNA, and 2 μ g of poly(A)⁺ RNA was isolated and subjected to Northern blot hybridization. Induction fold of AID mRNA expression was indicated below. *B*, expression of AID mRNAs in fractionated splenic B cells obtained from mice immunized for 5 days. Spleens were resected 5 days after immunization with SRBC, and the splenic cells were fractionated by either MACS using anti-CD19 antibody (1st and 2nd lanes) or nylon-fiber separation (3rd and 4th lanes). RNA from each fraction was subjected to RT-PCR.

Among four novel genes thus isolated, we have characterized AID, which is specifically induced in GC B cells upon immunization. AID contains the active site for cytidine deaminase and catalyzes deamination of cytidine *in vitro* (Fig. 3). The inhibitory effect of THU and of zinc chelation on enzyme activity suggests that the deamination process may be similar to that of other cytosine deaminases, including APOBEC-1(19). Phylogenetic analysis revealed that AID is located closer to the RNA-editing deaminase rather than other cytosine deaminases (Fig. 2B), despite the fact that AID lacks RNA editing activity on an apoB RNA template. Mutagenesis studies indicate that Phe-66, Phe-87, His-61, Glu-63, and Cys-93 in mouse APOBEC-1 are essential for RNA binding (17, 20, 22, 23). These residues were noted to be conserved in the AID primary structure (Fig. 2A). However, as noted above, AID was not found to exhibit apoB RNA binding activity. X-ray crystallographic studies on the *E. coli* cytidine deaminase has revealed its three-dimensional structure, which has similarities with the predicted structure of APOBEC-1(34). The cytosine deaminase family can be divided into two groups in the view of the quaternary organization (35). *E. coli* cytidine deaminase and APOBEC-1 have a pseudoactive site domain in the C terminus, which is required to form a homodimer, whereas the other group, *e.g.* CMP deaminase, lacks such domain and forms homotetramers. AID contains a leucine-rich C terminus, which is shorter than but similar to the pseudoactive site domain of APOBEC-1 (Fig. 2A), indicating that AID belongs to the APOBEC-1/*E. coli* cytidine deaminase group. The predicted structural similarity of AID to APOBEC-1 implies that AID may be a novel RNA-editing deaminase induced in GC B cells, although it remains to be determined whether AID assembles into either a homodimer or associates into a heteromeric with the auxiliary factors used by APOBEC-1. Further resolution of this issue will require formal identification of the auxiliary factors themselves.

AID mRNA was induced in CH12F3-2 cells within 3 h after cytokine stimulation (Fig. 5). The appearance of AID mRNAs and the onset of CSR in CH12F3 cells thus coincide temporally. AID mRNA expression was induced in splenic B cells after *in vitro* treatment with LPS and cytokine, which activates naive B cells to initiate CSR (Fig. 6). AID mRNAs were also up-regulated in splenic B cell *in vivo* when mice were immunized with the T cell-dependent antigen, SRBC. Furthermore, *ex vivo* experiments (Fig. 7) and *in situ* hybridization (Fig. 8) studies

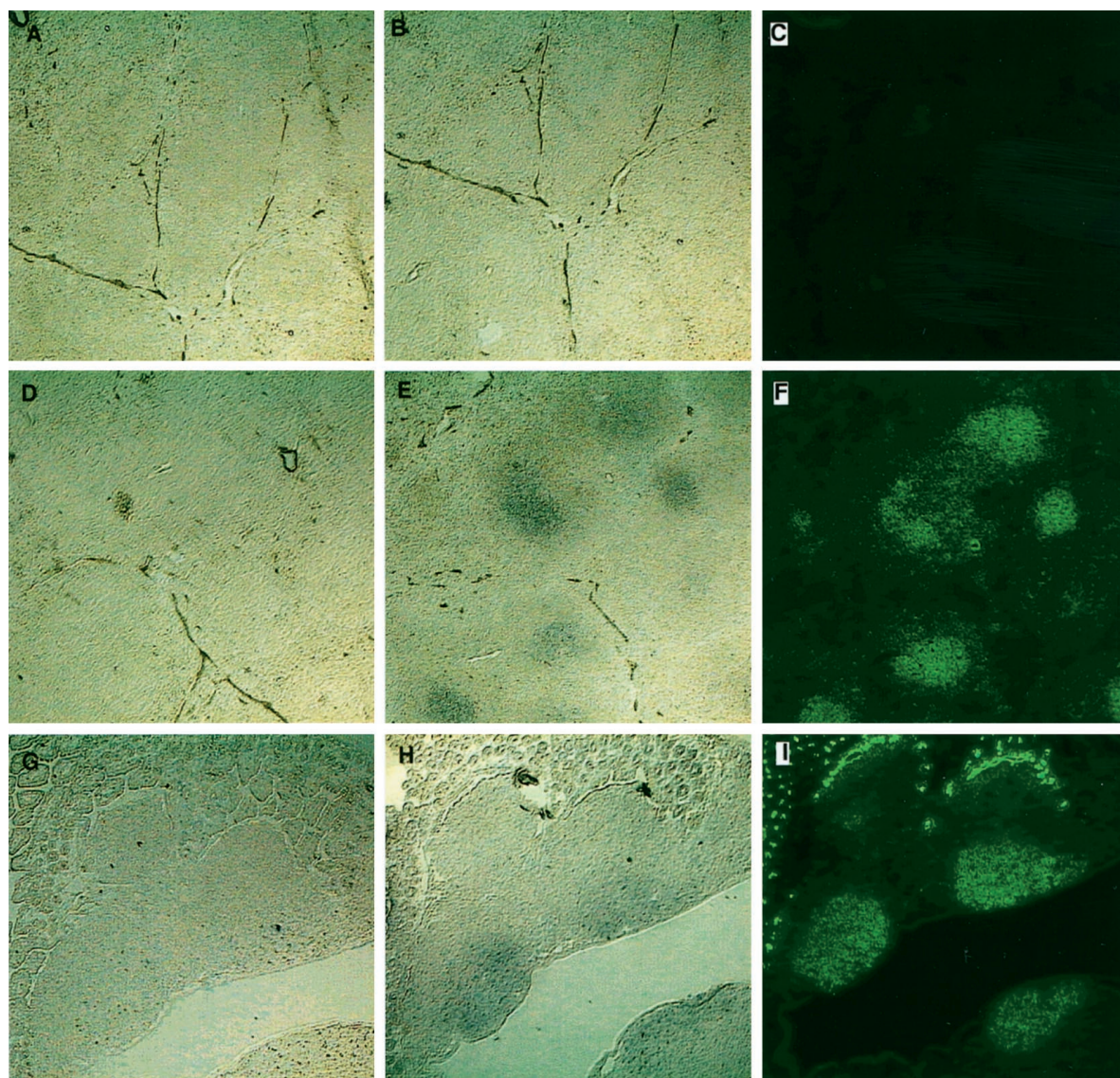


FIG. 8. Localization of AID mRNA in lymphoid organs. Paraformaldehyde-fixed frozen sections were generated from normal spleen (A–C), normal Peyer's patches (G–I), or from the spleen of mice immunized for 5 days with SRBC (D–F). The sections were hybridized with antisense (B, E, and H) or sense (A, D, and G) AID cRNA probe labeled with digoxigenin. The hybridized probes were detected with the anti-digoxigenin antibody conjugated to alkaline phosphatase, and localization of bound antibodies were identified as dark purple deposits. Serial sections indicate foci of germinal centers by staining with fluorescein isothiocyanate-conjugated peanut agglutinin (Vector Laboratories, Inc.) (C, F, and I). Original magnification: $\times 50$.

revealed that AID mRNA was specifically detected in GC B cells, which are competent to perform somatic hypermutation and CSR. In addition, none of the cell lines examined that do not support CSR, including LyD9, BA/F3, 70Z/3, WEHI231, X63, WEHI-3, EL-4, 2B4, F2, P815, L929, NIH3T3, and ST2, were found to express AID mRNA (data not shown). Taken together, these data indicate that AID mRNA is a GC B cell-specific gene induced upon antigen stimulation. Highly restricted expression of the AID gene in GC B cells together with the concordant onset of AID induction and CSR suggest its role in GC function. Furthermore, the possibility of RNA-editing activity on other templates inspires us to speculate that AID may participate in regulatory steps unique to GC function such as somatic hypermutation and CSR. It is of note that the efficiency of CSR in CH12F3-2 cells is increased up to double when cells were stimulated in the presence of THU (data not shown). The physiological function of AID is currently being investigated by gene targeting and transgenic techniques.

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Specific Expression of Activation-induced Cytidine Deaminase (AID), a Novel Member of the RNA-editing Deaminase Family in Germinal Center B Cells

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