Isolation of differentially expressed genes upon immunoglobulin class switching by a subtractive hybridization method using uracil DNA glycosylase

Manabu Sugai, Shigeru Kondo+, Akira Shimizu1 and Tasuku Honjo*

Department of Medical Chemistry, Faculty of Medicine and 1Center for Molecular Biology and Genetics, Kyoto University Sakyo-ku, Kyoto 606, Japan

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ABSTRACT

Immunoglobulin class switch recombination enables B lymphocytes to sequentially express antibodies that have identical specificities but differ in class and effector function. Although several cis elements required for class switch recombination have been identified, few trans-acting factors which are directly related to class switching have been found. Previously we have developed an efficient in vitro class switching system using a cell line, CH12F3-2. To clarify the molecular mechanism of class switching, we intended to isolate genes induced with interleukin (IL)-4, transforming growth factor (TGF)-β and CD40L using the in vitro class switching system. For that purpose, an improved method for making subtracted cDNA libraries, using uracil DNA glycosylase, has been developed. This method can overcome a general problem of subtraction, that rare cDNAs are easily lost. This new subtraction method was applied to the CH12F3-2 switching system to isolate genes induced by stimulations with IL-4, TGF-β and CD40L, and cDNAs encoding deiodinase 1 and SS32, an alternatively spliced form of the muscle LIM protein, were obtained. Their expression patterns in response to various combinations of stimuli and the time courses of the induction supported the usefulness of this method.

INTRODUCTION

Immunoglobulin class switch recombination enables B lymphocytes to sequentially express antibodies that have identical antigen specificities but differ in classes carrying specific effector functions (1,2). Although several cis elements such as S regions (3), I region promoters (4–9) and the 3′ enhancer (10) have been shown to be required for class switch recombination, little is known about its molecular mechanism. Unlike VDJ recombination, class switch recombination does not take place at a specific site but occurs in a broad region called S located 5′ to each heavy-chain constant region (CH) gene which determines the class (11,12).

There are no trans-acting factors that have been shown to be directly involved in class switching except for the product of the scid gene (13). Although identification of such trans-acting factors is essential to clarify the molecular mechanism of class switch recombination, no functional assay system is available for the factors involved in class switch recombination.

Previously we have reported that a B lymphoma cell line, CH12F3-2, switches efficiently from IgM to IgA by stimulation with IL-4, TGF-β and CD40L (14). IgA+ CH12F3-2 cells are already detectable at 18 h after stimulation and reach 30 and 60% at 24 and 48 h, respectively, after stimulation. Interestingly, this cell line almost exclusively switches to the IgA class. As a first step toward understanding the molecular mechanism of class switch recombination, we intended to isolate genes induced in CH12F3-2 cells by stimulation with IL-4, TGF-β and CD40L. An obvious choice of strategy is to carry out subtractive hybridization between mRNAs (or cDNAs) from uninduced and induced CH12F3-2 cells. However, almost all the subtraction methods have a common problem, that differentially expressed clones in low abundance are easily lost, probably because of degradation during prolonged hybridization and inefficient separation steps. A good subtractive hybridization method, therefore, has to deal with the non-specific loss of target clones at all subtraction steps. This is particularly true when subtraction is done between cell populations with a subtle difference such as stimulated and unstimulated CH12F3-2 cells. For example, it is important to identify a few key genes that determine differentiation stages of the cell lineage. Stimulation-induced class switching of CH12 cell lines may depend on activation of a few genes that trigger series of the complex recombination reaction in collaboration with pre-existing molecules.

A number of cDNA subtraction methods have been reported. In its prototype, a trace amount of cDNA from one population (tester) is hybridized with an excess amount of mRNA or cDNA from another population (driver), followed by separation of unhybridized cDNA (target) from hybridized sequences. Target cDNAs are usually separated by hydroxylapatite chromatography (15) or avidin–biotin binding (16,17). These methods are relatively inefficient for obtaining transcripts in low abundance and often require a large amount of mRNAs as a starting material. To

*To whom correspondence should be addressed. Tel: +81 75 753 4371; Fax: +81 75 753 4388; Email: honjo@mfour.med.kyoto-u.ac.jp

+Present address: Department of Integrated Art and Science, University of Tokushima, Minami-Josanjima 1-1, Tokushima 770, Japan

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overcome this problem, a new PCR-based technique was developed (16, 18–21). However, this method generated another problem, a high level of false positives due to biased PCR amplification, and therefore might be inappropriate for experiments in which only a few genes are differentially transcribed with a lower frequency. Zeng et al. (22) described a new subtractive hybridization method which incorporates PCR amplification, enhanced hybridization with phenol emulsion and exonuclease digestion of unhybridized cDNA. The authors reported that this method greatly improved the recovery of rare clones by reducing hybridization time using the phenol emulsion method (23). Separation of target cDNAs was also claimed to be improved by introducing exonuclease digestion coupled with protection of target clones by thiol nucleotide incorporation. However, this method still had limitations in the recovery of rare clones and degree of enrichment. In fact, there are few reports that new genes have been isolated by using this method.

Here we report an improved PCR-based cDNA subtraction method using uracil DNA glycosylase (UDG) (24, 25). Combination of UDG subtractive digestion and phenol emulsion hybridization has overcome the loss of differentially expressed transcripts in low abundance. This method enriches rare target clones which exist at least in the ratio of 1/10^6 in the tester cDNA. Application of the UDG subtraction method to our in vitro class switching system allowed us to isolate two inducible genes responding to the stimulation with IL-4, TGF-β, and CD40L. To obtain full-length cDNAs, ~2 × 10^5 PFUs were screened with subtracted cDNA fragments as probe, according to a standard method as described (30). Full-length cDNA fragments of SS32 and MMLP were used as probes to screen the commercially available 129SV mouse genomic library (Stratagene, USA). Approximately 1 × 10^6 PFUs were screened by a standard method (30). mRNA was resolved electrophoretically in 0.8% formaldehyde–agarose gels, and analyzed by a standard Northern blot protocol (30). S1 protection assay was performed to determine the transcription initiation site as described (30). To prepare the probe at the 5′ region of SS32, the genomic clone, which contains the 5′ fragment of SS32, was subjected to PCR amplification using a primer pair, S1F1 and S1R1. PCR-amplified fragments were digested with EcoRI and XhoI, and subcloned into BSKS(+). To prepare the S1 protection probe, an S ple and Apal-digested fragment was recovered. This fragment was dephosphorylated with CIP (Takara, Japan) and labeled at its 5′-end with [32P] by T4 polynucleotide kinase (Takara, Japan) (specific activity of 10^8 c.p.m./µg). The labeled probe was hybridized in excess (2 × 10^5) to 40 µg of total RNA at 50°C for 16 h in a 20 µl reaction mixture containing 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA and 400 mM NaCl, followed by digestion with 40 U of S1 nuclease (Takara, Japan). These products were analyzed on a 5% denaturing polyacrylamide gel. To quantify the RT–PCR product, a series of amplification cycle sets (27, 30, 33, 36, 39, 42 cycles) were performed and resolved electrophoretically in 2% agarose gels and visualized by ethidium bromide staining. These bands were quantitated using NIH imaging. Amplified product did not reach saturation in the range 27–36 cycles. In this range we quantitated the gene of interest.

**Analysis of nucleic acids**

A cDNA library was constructed using the lambda-zap cDNA synthesis kit (Stratagene, USA) with CH12F3-2 mRNA isolated 24 h after stimulation with IL-4, TGF-β and CD40L. To obtain full-length cDNAs, ~2 × 10^5 PFUs were screened with subtracted cDNA fragments as probe, according to a standard method as described (30). Full-length cDNA fragments of SS32 and MMLP were used as probes to screen the commercially available 129SV mouse genomic library (Stratagene, USA). Approximately 1 × 10^6 PFUs were screened by a standard method (30). mRNA was resolved electrophoretically in 0.8% formaldehyde–agarose gels, and analyzed by a standard Northern blot protocol (30). S1 protection assay was performed to determine the transcription initiation site as described (30). To prepare the probe at the 5′ region of SS32, the genomic clone, which contains the 5′ fragment of SS32, was subjected to PCR amplification using a primer pair, S1F1 and S1R1. PCR-amplified fragments were digested with EcoRI and XhoI, and subcloned into BSKS(+). To prepare the S1 protection probe, an S ple and Apal-digested fragment was recovered. This fragment was dephosphorylated with CIP (Takara, Japan) and labeled at its 5′-end with [32P] by T4 polynucleotide kinase (Takara, Japan) (specific activity of 10^8 c.p.m./µg). The labeled probe was hybridized in excess (2 × 10^5) to 40 µg of total RNA at 50°C for 16 h in a 20 µl reaction mixture containing 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA and 400 mM NaCl, followed by digestion with 40 U of S1 nuclease (Takara, Japan). These products were analyzed on a 5% denaturing polyacrylamide gel. To quantify the RT–PCR product, a series of amplification cycle sets (27, 30, 33, 36, 39, 42 cycles) were performed and resolved electrophoretically in 2% agarose gels and visualized by ethidium bromide staining. These bands were quantitated using NIH imaging. Amplified product did not reach saturation in the range 27–36 cycles. In this range we quantitated the gene of interest.

**UDG subtractive hybridization**

One round of UDG subtractive hybridization consists of three cycles of hybridization and UDG/S1 digestion, followed by additional exonuclease digestion and PCR amplification. A schematic representation of the UDG subtractive hybridization is shown in Figure 1. Two rounds of subtractive hybridization were carried out to construct subtracted libraries.
cDNA synthesis and amplification. To reduce the contamination of trace amounts of genomic DNA, polyA RNA was subjected to DNase I treatment. Oligo(dT) was used to prime cDNA synthesis from 5 μg of polyA+ RNA. Double-stranded cDNA was synthesized using a Time Saver cDNA synthesis kit (Pharmacia, USA), and divided into four aliquots. To prepare cDNA fragments suitable for PCR amplification, four aliquots of double-stranded cDNA were digested separately with AluI, with AluI plus RsaI, with HaeIII or with HaeIII plus AccII. The aliquots digested with AluI and with AluI plus RsaI were mixed. Similarly, those digested with HaeIII and with HaeIII plus AccII were combined (Fig. 1). The two mixtures were ligated with 10 μg of double-stranded phosphorylated oligodeoxynucleotide linkers. Different sequences were chosen for the linkers of the tester and driver. To monitor rare clones the AluI fragments of Drosophila genomic clone DR3 (450 bp + 600 bp) were added to tester and driver cDNAs before the linker ligation step at the weight ratio 1/10⁶. After synthesis of the second strand cDNA, the double-stranded cDNAs were fractionated with Sephacryl S400 (Sigma, USA). These products were subjected to digestion with the indicated restriction enzymes, and then quantitated with A₂₆₀. The control DR3 fragments were also quantitated with A₂₆₀. DR3’s lengths were longer than the average length of digested cDNA as estimated by the agarose gel fractionation. Therefore, the DR3 molar ratio was probably less than at 1/10⁶. After phenol–chloroform extraction, the unligated linkers were removed by centrifugation through a Sephacryl S400 column. Then the ligated cDNAs were dissolved in 3.1 ml of water. An aliquot of 10 μl of cDNA solution was amplified in a 100 μl PCR reaction mixture by Taq polymerase (Takara, Japan) through 10–15 cycles, using the following parameters: tester, 94 °C for 30 s, 57 °C for 1 min, 72 °C for 2 min; driver, 94 °C for 30 s, 53 °C for 3 min, 72 °C for 8 min. Driver cDNA amplification by PCR was carried out by substitution of dTTP with dUTP. To protect PCR-amplified tester cDNA fragments from exonuclease III digestion, incorporation of dNTP-α-S into 3′ ends was done by Klenow fragments as described previously (22).

Conditions for hybridization. Basically we used the PERT technique as described previously (22,23) with following modifications: (i) we used twice as much quantity of starting tester cDNA as used in Zeng et al. (22); (ii) the volume of the second and the third hybridization solution was reduced to 1/10 of that described in Zeng et al. (22); and (iii) the hybridization period of the second and third hybridization was prolonged to 48 h. For the first hybridization, 0.5 μg of tester cDNA fragments was combined with 5 μg of driver cDNA in a 122 μl buffer containing 40 mM Tris–HCl, pH 8.0 and 4 mM EDTA. After denaturation at 95 °C for 5 min, hybridization was carried out by the phenol–emulsion reassociation technique (PERT) in a 500 μl solution containing 2 M NaSCN and 8% phenol at room temperature for 24 h with continuous agitation, as described previously (22,23). For the second and third hybridization, 5 μg of driver cDNA was added to once-subtracted cDNA fragments and hybridization was performed in 50 μl of the buffer by PERT for 48 h. After hybridization, the solution was extracted with chloroform and cDNA was precipitated with ethanol.

Digestion with UDG and S1 nuclease. The cDNA fragments were incubated with 8 U of UDG (Perkin-Elmer, USA) in a 20 μl reaction mixture containing the buffer recommended by the manufacturer for 30 min at 37 °C, followed by digestion with 60 U S1 nuclease (Takara, Japan) in 200 μl of the S1 buffer for 30 min at 37 °C.

Additional nuclease digestion. After the third hybridization and UDG/S1 digestion cycle, the subtracted cDNA fragments were further digested with exonuclease III (300 U) and exonuclease VII (20 U) to reduce amplification of cDNA with partially digested fragments as primer. The exonuclease digestion conditions were as described (22), except for the amounts of the enzymes used.

Construction of subtractive library and differential hybridization screening. After two rounds of subtraction, differentially enriched subtracted cDNAs were ligated into T vector (Promega, USA). These libraries were transformed into Escherichia coli DH5α with a modified Hanahan’s procedure. 20 000 colonies were differentially screened with tester and driver cDNA probes. The differential screening procedure was basically done as described previously (30). Two sets of colony-replicated nitrocellulose filters were subjected to differential hybridization screening. The first transferred filters were for the tester cDNA probe hybridization and the second for driver. To prepare the tester and driver cDNA probes, 10 μg of polyA RNA were primed with oligo dT and reverse transcribed using Superscript (Gibco, USA) with [α-32P]dCTP. Then the RNAs were hydrolyzed with 0.3 N NaOH. A set of 20 filters (130 × 90 mm) was hybridized with
2 × 10^8 c.p.m. of each probe in a 60 ml hybridization buffer for 60 h. Radiolabeled cDNA probes that had hybridized to the colonies were detected by autoradiography.

RESULTS

UDG subtractive hybridization method

Initially, we tried to use the method by Zeng et al. (22) for isolation of cDNA induced in stimulated CH12 F3-2 cells, but this method did not allow us to obtain reproducible results even for model experiments using Drosophila DNA described below. We often experienced incomplete and uneven elimination of driver cDNAs after hybridization. We therefore extensively modified this method by readjusting conditions and adding new steps using UDG. The differentially expressed cDNAs (targets) are assumed to be present in the tester cDNA but absent (or present at much lower levels) in the driver cDNA. The tester and driver double-stranded cDNAs were digested with four-base restriction enzymes that yield blunt ends (Fig. 1). The tester and driver cDNA fragments were ligated with different linker primers, and subjected to PCR reactions. To remove driver cDNAs by digestion with UDG, dUTP was incorporated into the driver cDNAs during the PCR amplification reaction, in which dUTP was substituted for dTTP. Additional exonuclease III and exonuclease VII digestion of the subtracted cDNA fragments was necessary to reduce amplification of artifacts produced by priming with partially digested fragments. Digestion of the tester cDNAs were specifically blocked by incorporation of thionucleotides at their 3′ ends, as described previously (22).

To promote the efficiency of reassociation, we changed conditions of the phenol emulsion-enhanced hybridization technique from those described by Zeng et al. (22). This technique allows such rapid reassociation kinetics that even rare sequences can be annealed to completion. Thiol nucleotide-incorporated tester cDNA was hybridized to an excess of dUMP-incorporated driver cDNA. During hybridization, the cDNA fragments form three kinds of hybrid molecules as follows: tester–tester homo-hybrids without dUMP, driver–driver homo-hybrids with dUMP on both strands and tester–driver hetero-hybrids with dUMP on one strand (Fig. 1). Tester-specific cDNA should always form homo-hybrids without dUMP.

Subsequently, the tester–driver hetero-hybrid and the driver–driver homo-hybrid molecules containing dUMP were removed by digestion with UDG. UDG excises uracil bases from DNA and generates abasic sites. Multiple abasic sites provide substrates for S1 nuclease, which can digest both strands of cDNA fragments if one strand has abasic sites. Thus S1 nuclease completely degrades the tester–driver heterohybrid and driver–driver homohybrid molecules while leaving the tester–tester homohybrid molecules intact. The recovered tester–tester homohybrid molecules were subjected to two additional cycles of the subtraction step. After three cycles of the subtraction process, additional digestion was performed by treating the reaction mixture with exonuclease III, followed by exonuclease VII. The tester cDNAs were specifically protected from exonuclease digestion by incorporation of thionucleotides at the 3′ ends. This process reduces artifacts by non-specific amplification with partially digested fragments as primers during the subsequent PCR step (22). Finally, the remaining cDNA fragments were amplified by PCR, and another round of subtraction was carried out prior to cloning.

Model experiments using the UDG subtractive hybridization method

To monitor the efficiency of subtraction, two AluI fragments of a control clone DR3 (DR), a genomic fragment of Drosophila, was added to tester cDNA in a weight ratio of 1 × 10^-6. As shown in Figure 2A, the mixture (TDR), starting tester (T), driver (D) and subtraction PCR products were analyzed by Southern blotting. To determine the efficiency of this technique, (TDR)–(TDR) subtraction was performed. Only faint bands hybridized with DR3 were detected in TDR, but after one cycle of subtraction, the DR3 band was augmented at least 100 times compared with TDR. To confirm that the amplification of DR3 is specific, (TDR)–(TDR) subtraction was performed. After subtraction, the DR3 bands disappeared completely. These results suggest that specific sequences in tester cDNA were enriched by the UDG subtraction. Subtracted fragments were cloned into the T-vector and this library was screened with DR3 probe. The DR3 actually exists at the ratio of 12/10^5. One of the positive clones was sequenced and subtraction PCR products were analyzed by Southern blotting. To determine the efficiency of this technique, (TDR)–(TDR) subtraction was performed. Only faint bands hybridized with DR3 were detected in TDR, but after one cycle of subtraction, the DR3 band was augmented at least 100 times compared with TDR. To confirm that the amplification of DR3 is specific, (TDR)–(TDR) subtraction was performed. After subtraction, the DR3 bands disappeared completely. These results suggest that specific sequences in tester cDNA were enriched by the UDG subtraction. Subtracted fragments were cloned into the T-vector and this library was screened with DR3 probe. The DR3 actually exists at the ratio of 12/10^5. One of the positive clones was sequenced and confirmed to be DR3. To further confirm specificity of subtraction, Southern blot hybridization was performed with probes for CD72 (31), β-actin and RBP-J (32), which are equally expressed in the tester and driver. A single subtraction cycle eliminated CD72 (Fig. 2A) and β-actin (data not shown) sequences to undetectable
levels. In the case of RBP-J, subtraction was not complete (data not shown) and two rounds of the subtraction process were required. These findings indicate that the UDG subtraction method can efficiently eliminate common sequences and enrich specific sequences present at a low frequency as low as $10^{-6}$.

**Isolation of cDNA clones induced in CH12F3-2 upon class switching**

The UDG subtraction method was applied to isolate cDNAs that are induced in CH12F3-2 cells upon class switching. Tester cDNAs were prepared from CH12F3-2 at 18 h and 24 h after stimulation with IL-4, TGF-β and CD40L because IgA+ CH12F3-2 cells can be detected at 18 h after stimulation. Driver cDNAs were prepared from the same cell line without stimulation. The procedure was monitored quantitatively by agarose gel electrophoresis of the PCR-amplified cDNA fragments from each cycle. The subtraction efficiency of each cycle was estimated quantitatively by comparing the PCR products with the serially diluted standard fragments (data not shown). Following two rounds of subtraction (i.e. six hybridization and UDG digestion steps), a plasmid library of subtracted cDNA was prepared. 20 000 clones of the subtracted library were screened by differential hybridization using tester and driver cDNA probes. Unexpectedly, most of the clones were hybridized with both tester and driver cDNA probes, indicating that they are derived from mRNA commonly present in abundance. We picked up nine clones which had no signal to either probes, indicating that they are rare clones. These nine clones were analyzed further by Northern blotting or RT-PCR. These nine clones were specifically found in the tester cDNA but not in the driver cDNA. Of these nine clones, seven clones were derived from genomic DNA fragments contaminating the starting tester cDNA preparation. The two genuine differentially expressed cDNAs were obtained. One cDNA encodes deiodinase 1, involved in thyroid hormone metabolism (33,34) and the other, termed SS32, contains a nucleotide sequence similar to the gene for the mouse muscle LIM protein (MMLP) (35). To confirm that these two genes are really enriched in this subtraction procedure, the PCR products of each step were analyzed by Southern blotting (Fig. 2B). Indeed, the deiodinase 1 and SS32 bands were undetectable in the tester cDNA but strongly visible after one round of subtraction. As a control these bands completely disappeared in the tester–tester subtraction. To confirm further that the amplification of deiodinase 1 and SS32 were specific, Southern blot hybridization was performed with probes for CD72, β-actin and RBP-J. A single subtraction cycle eliminated CD72, RBP-J and β-actin (data not shown) sequences to undetectable levels.

**SS32 is an alternatively spliced form of MMLP**

The 3′-403 bp portion of SS32 is 97% identical to the published sequence of MMLP, while the remaining 254 bp sequence of SS32 is completely different from MMLP. To determine whether SS32 is an alternatively spliced form of MMLP, we isolated its genomic clones from a mouse genomic library and analyzed its structure. The MMLP gene is composed of six exons, as shown in Figure 3A, and exons 5 and 6 are shared with SS32.

![Figure 3](https://academic.oup.com/nar/article-abstract/26/4/911/2902350/10.1093/nar/26.4.911)
transcription initiation site of SS32 is located within the intron between exons 4 and 5 of the MMLP gene. Comparison of sequences of the MMLP and those of SS32 cDNA derived from five cDNA clones from the CH12F3-2 cDNA library and 10 cDNA clones directly isolated by PCR revealed that SS32 is spliced correctly according to the GT/AG rule and that SS32 cDNA potentially encodes a putative 53-amino-acid polypeptide in a frame different from that of MMLP (Fig. 3B). We therefore concluded that SS32 is an alternatively spliced form of MMLP.

To clarify the transcription initiation site, we performed an S1 protection assay using murine heart RNA and the probe S1P (Figs 3B and C). A heart-RNA-specific protected band appeared at around 164 bases with shorter smear bands (Fig. 3C), indicating that the major transcription initiation site is located at around position 0 in Figure 3B, with multiple initiation sites downstream.

**Deiodinase 1 and SS32 are induced by stimulation with IL-4, TGF-β and CD40L in CH12F3-2**

Sequence analysis of the 5′ flanking region of SS32 revealed that the SS32 promoter region contains a TATA box, IL-4-responsive elements (IL-4RE), a TGF-βRE (36–38) and an SP-1 site (Fig. 3B). Although AP-1 and SP-1 are universal transcription factors, in some cases TGF-β can stimulate gene expression through the AP-1 or SP-1 binding site (39–42). The previously published sequence of the 5′ flanking region of the human deiodinase 1 gene also contains a TATA box, an IL-4RE, a TGF-βRE and several AP-1/SP-1 sites. To confirm that SS32 and deiodinase 1 are inducible in CH12F3-2, mRNA was isolated after stimulation with IL-4, TGF-β and CD40L, and studied by Northern blot analysis (Fig. 4). Deiodinase 1 mRNA expression was detectable 6 h after stimulation and reached a plateau at ~24 h (Fig. 5B). These expression profiles were compared with the time course of class switching. Both deiodinase 1 and SS32 mRNAs were undetectable before stimulation and their expression proceeds after surface IgA expression.

**Induced expression of SS32 mRNA in stimulated splenic B cells**

Tissue distributions of MMLP and SS32 mRNAs were examined with RT–PCR using specific primers. MMLP mRNA was expressed predominantly in heart, skeletal muscle and lung (Fig. 6). Weak expression was detected in thymus, but not in spleen. In contrast, SS32 showed a more ubiquitous expression pattern and the strongest signal was seen in heart. SS32 mRNA was weakly expressed in spleens of BALB/c and C57B6. Mouse embryos (d17) expressed both MMLP and SS32 mRNAs. Deiodinase 1 mRNA expression was not detected in spleen (data not shown). We confirmed that in vitro cultured splenic B cells
are impossible to eliminate, and continue to expand. However, no other methods gave rise to complete elimination of even selected species of abundant common cDNAs. Complete elimination of many common sequences was achieved by (i) enzymic digestion with UDG and S1 nuclease; and (ii) repeating the hybridization and digestion cycle three times.

Using the UDG subtraction method we have shown that a rare clone present at the frequency of $10^{-6}$ in tester can be enriched at least 100 times after one round of subtraction. Two rare clones (deiodinase 1 and SS32) differentially expressed in stimulated CH12F3-2 cells were isolated by the UDG subtraction method. The frequencies of deiodinase 1 and SS32 mRNAs in the induced CH12F3-2 mRNA (24 h) were estimated to be $2.5 \times 10^{-8}$ by the screening of total cDNA libraries with respective probes. One problem of the UDG subtraction method is biased amplification of unexpected sequences by PCR. Such non-specific amplification appears to be inevitable as long as PCR amplification is involved. This is the reason why we introduced the differential hybridization step to pick up final candidates from the subtracted library. Abundantly expressed common sequences can be easily eliminated, and abundant differential clones were easily picked up at this step. Rare clones may not hybridize to either tester or driver probe regardless of whether or not they are differentially expressed. In fact, all the rare clones isolated were differentially present in the tester cDNA. Taken together, the UDG subtraction method is useful and effective for isolation of rare clones in the tester.

**Are deiodinase 1 and SS32 involved in class switching?**

Expression of deiodinase 1 and SS32 mRNAs is induced in CH12F3-2 cells upon stimulation to class switching. Both class switching and expression of these mRNAs are most strongly augmented by stimulation with the combination of CD40L, TGF-β and IL-4, followed by that of IL-4 and TGF-β. Although deiodinase 1 mRNA is induced as early as 6 h after stimulation, its induced expression was not seen in stimulated spleen B cells. The results cast doubts on the possibility that deiodinase 1 may play an important role in class switching. Induction of SS32 mRNA is relatively slow and detectable only a few hours before appearance of IgA+ cells. However, SS32 mRNA is also induced in stimulated spleen B cells. MMLP was first identified as a novel regulator of myogenesis (35). A disruption of cardiac cytoarchitectural organization was reported in MMLP-deficient mice, in which SS32 expression was likely to be intact because the MMLP-deficient mice were created by replacing the second exon of the MMLP gene (44). Unfortunately, there is no description about B lymphocytes in the MMLP deficient mice.

Transcription of SS32 mRNA from the MMLP gene reminds germline transcription of immunoglobulin heavy chain constant region genes because SS32 transcription is initiated within the intron of the MMLP gene. Specific induction of germline transcripts from promoters adjacent to targeted S regions has led to the proposal that accessibility to a particular S region determines a switch recombination target (34–47). A number of experiments demonstrate that the specificity of germline transcript expression correlates well with that of the switch recombination target (3,47,48), suggesting that the germline transcription and its splicing are required for class switch recombination (3,47,49,50). However, the functional relation...
between the two events is still unclear. It remains to be seen whether SS32 mRNA or its translation product plays a role in class switching.

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