

Cell-Free Recombination of Immunoglobulin Switch-Region DNA with Nuclear Extracts

Ke Zhang¹ and Hai-Kit Cheah

The Hart and Louise Lyon Laboratory, Division of Clinical Immunology/Allergy, Department of Medicine, UCLA School of Medicine, Los Angeles, California 90095-1680

We have developed an *in vitro* recombination system employing cell-free nuclear extracts from human B lymphocytes capable of detecting the recombination between human μ switch ($S\mu$) and $S\epsilon$ sequences in a model plasmid. Nuclear extracts from CD40-stimulated B lymphocytes gave a higher frequency of recombination in the assay than the unstimulated B cells. Recombination between $S\mu$ and $S\epsilon$ was mediated by the nuclear extracts as the recombinational products could be amplified prior to bacterial transformation. Characterization of the recombination products demonstrated that the recombination process had the characteristics of immunoglobulin (Ig) isotype switching, as it was (i) switch-region-sequence specific, (ii) nonhomologous recombination, and (iii) enhanced by CD40 stimulation. Transcription through the S region DNA was not required for recombination in the system. These results demonstrate that Ig switch-region DNA recombination can be accomplished *in vitro* by cell-free nuclear extracts. This *in vitro* system for Ig switch-region DNA recombination using cell-free nuclear extracts will permit the dissection of the events involved in IgE class switch recombination, a critical event in the development of allergic diseases. © 2000

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INTRODUCTION

The diverse biological effector functions of humoral immunity depend on immunoglobulin (Ig) class switching by which B lymphocytes shift from production of IgM/IgD to other isotype(s), such as IgG, IgA, and IgE, while maintaining antigen specificity. Deletional recombination is the major mechanism accounting for isotype switching in primary B cells (1–3) although several other models have been proposed as mecha-

nisms for Ig isotype switching occurring under the certain circumstances (1–3). Ig class switch recombination (CSR) is a DNA recombinational process by which the μ switch ($S\mu$) region is rearranged to a downstream targeted S region, accompanied by looping out and excising the intervening DNA as switch circular DNA (i.e., a deleted switch circle) (1, 2). Ig S region DNA, featuring repetitive sequences and G-rich stretches, appears to provide the region-specific substrates for primary CSR. Although substantial homology exists among all the Ig S regions, CSR is clearly nonhomologous recombination (4).

At least two signals are necessary to achieve a given isotype switching (3, 5). Cytokines, acting as “switching factors,” direct the appropriately activated B lymphocytes toward certain isotype(s), thereby participating in the determination of the specificity of isotype switching. Cytokine-directed isotype switching is directly correlated with the ability to selectively induce germline transcription of the constant heavy-chain gene(s) that encode the Ig class subsequently switched to. Germline transcription of the corresponding CH genes are believed to render the switch-region DNA accessible to a putative switch recombinase that eventually accomplishes the deletional recombination process (1–7). While cytokines are necessary, they are not sufficient to cause isotype switching by themselves. A second signal in the form of an activation signal such as CD40 ligation is also required to accomplish the process of isotype switching (1–7).

Although the cellular processes required for Ig class switching have been intensively investigated, the nature of the putative switch recombinase that mediates Ig CSR is essentially unknown, though several molecules have been indirectly implicated in the Ig switch recombinase activity. These include DNA-dependent protein kinase (8), Ku protein (9–11), Rad51 (12), E2A protein (13), Endo-SR (14, 15), and SWAP complex (16). To analyze DNA sequences, regulatory elements, and transacting factors required for Ig switch recombination, plasmid- and retrovirus-based vectors containing Ig switch-region DNA have been developed and

¹ To whom correspondence and reprint requests should be addressed at 52-175, CHS, Division of Clinical Immunology/Allergy, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90095-1680. Fax: (310) 206-8107. E-mail: kzhang@mednet.ucla.edu.

transfected into B cells to detect the switch recombination activity (17–24). However, these cell-based systems are limited in their ability to dissect the exact basis of CSR, as Ig CSR is a complex process that likely requires the cooperation of host molecules. A cell-free system that mimics one or more of the steps in the complex switch recombinational processes would facilitate the dissection and understanding of CSR. We have constructed such a system employing model plasmids that undergo CSR *in vitro* upon incubation with the cell-free extracts from appropriately stimulated human cells. This report provides evidence that S μ and S ϵ region DNA recombination can be accomplished *in vitro* with cell-free nuclear extracts from human B lymphocytes.

MATERIALS AND METHODS

Construction of the Model Plasmid

The model plasmid for the *in vitro* recombination was constructed on the background of the pCR II vector (Invitrogen Corp., San Diego, CA). A 1.25-kb S ϵ fragment was generated by polymerase chain reaction (PCR) with a 5' primer (TGTCCCTTAGAGGACAGGTGCCAA) that corresponded to 1155–1180 of S ϵ (25) and a 3' primer (AAATCCCAGCAGAACTCAGTTTAAT) that corresponded to 2402–2378 of S ϵ (25). The amplified fragment was cloned into the multiple clone sites of the *lacZ* gene in PCR II vector by TA cloning method as described (26). The insertion of the 1.25-kb S ϵ fragment disrupted the internal *lacZ* gene expression. A 0.91-kb fragment containing S μ and its 5' flank sequences was amplified with a 5' primer (TCTAGACAAGGGGACCTGCTCATT) that corresponded to 1–24 of S μ (25) and a 3' primer (AGCACAGCACGCTCAACCCGTTTCAGCTTG) that corresponded to 914–885 of S μ (25) and cloned into the *EcoRV* sites of the pBluescripts by TA cloning (26). A *XbaI-XhoI* fragment containing S μ and its 5' flank sequences excised from pBluescripts was inserted into the pCR II vector containing a 1.25-kb S ϵ fragment. An intact *lacZ* gene (referred to as *lacZ'* to distinguish the internal disrupted *lacZ*) amplified from PUC 18 vector was cloned into the *ClaI-XhoI* sites of the pCR II vector between 0.91-kb S μ and 1.25-kb S ϵ fragments. A PCR-amplified 0.46-kb fragment containing human μ intronic enhancer sequences (E μ) was inserted into the *ApaI* sites (27) (Fig. 1). All the fragments in the construct were determined to be in the normal transcriptional orientation by restriction endonuclease mapping and partial DNA sequence analysis. The resulting plasmid, designated as p77D3.11, was used as a model for *in vitro* S-S recombination.

Cells and Cell Lines

Human primary B and T lymphocytes were prepared and purified from human tonsils as described (26). The B cells were harvested for nuclear extract preparation following culture for 3 days with or without 0.1 μ g/ml of CD40 monoclonal antibody G28.5 (26). Cell lines 2C4/F3 (kindly provided by Dr. F. Finkelman), JY, A11 (kindly provided by Drs. H. Yssel and J. de Vries), I.29 (kindly provided by Dr. J. Stavnezer), Ramos, AF-10, Jurkat, CHO, and Hela were maintained in complete RPMI 1640 (26). The 293 cell line (from ATCC) were maintained in complete DMEM medium.

Preparation of Nuclear Extracts

Nuclear extracts from primary human B cells and cell lines were prepared by the Nonidet P-40 (NP-40) lysis method (28) with important modifications. Briefly, 10⁸ (10⁷ for cell lines) purified fresh human tonsillar B lymphocytes (26) were harvested following culture for 3 days with or without 0.1 μ g/ml of CD40 monoclonal antibody G28.5 (26) and were lysed with 1 ml of sucrose buffer (0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.5 mM PMSF) containing 0.5% (vol/vol) NP-40 (BRL, Gaithersburg, MD). The cells were lysed immediately and the intact nuclei were pelleted by centrifugation at 2400 rpm/5 min at 4°C. The nuclei were washed twice (10⁸ B cells/ml) with ice-cold sucrose buffer lacking NP-40. The pelleted nuclei were suspended in 50 μ l/10⁸ B cells with low-salt buffer (20 mM Hepes, pH 7.6, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) followed by slowly adding 1 vol of high-salt buffer (20 mM Hepes, pH 7.6, 25% glycerol, 1.5 mM MgCl₂, 800 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) at 4°C for 30 min with agitation for nuclear protein extraction. The nuclear extracts were separated from genomic DNA and cell debris by centrifugation at 15,000 rpm/15 min at 4°C. The resulting nuclear extracts were dialyzed against 100 vol of buffer D (20 mM Hepes, pH 7.6, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) for 5 h at 4°C. The dialysates were microcentrifuged at 15,000 rpm for 10 min to remove any precipitates. The concentration of the nuclear protein was determined by Bradford assay. The resulting nuclear extracts were frozen as aliquots in liquid nitrogen and stored at –80°C.

In Vitro Recombination Assay

The p77D3.11 plasmid used for the *in vitro* recombination assay was prepared in large quantity and puri-

fied using QIAGEN plasmid kits (Qiagen Inc., Chatsworth, CA) as described by the manufacturer. The *in vitro* recombination reaction was carried out in a total volume of 20 μ l containing the following components: 1 μ g of p77D3.11 plasmid DNA, 1–5 μ g of nuclear extract protein (depending on the cell source), 0.125 mM γ NTP (Promega Corp., Madison, WI), 20 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 1 mM spermidine (Sigma, St. Louis, MO), 0.5 mM dNTP (Promega Corp.), 5 mM NaCl, 10 mM Hepes, 50 mM KCl, 0.1 mM EDTA, 0.75 mM DTT, and 10% glycerol. The reaction mixtures were incubated for 16 to 44 h at 25°C unless otherwise noted.

To test the effects of transcription through the S region DNA on *in vitro* recombination, varying amounts of T7 and Sp6 RNA polymerases (Promega Corp.) were included in the reaction mixture between 0 to 60 min at 37°C before the nuclear extracts were added.

Reactions were stopped by adding 80 μ l of H₂O and extracted with phenol to remove the nuclear extract protein followed by plasmid DNA precipitation. The precipitates were washed with 70% ETOH once and the resulting precipitated DNA was dissolved in 5 μ l of H₂O, of which 2 μ l of DNA was transformed for calculation of the white colonies vs the blue colonies. In the cases in which transformants were too many to count, DNA was diluted and the transformation was repeated. For calculation of the plasmid recovery rates, total numbers of transformants were scored based on DNA dilution factors.

Transformation

Transformation of plasmid DNA into *Escherichia coli* DH10 strain (BRL) was carried out by using Gene Pulser (Bio-Rad Laboratories, Hercules, CA) with 0.1 cm cuvettes at 1.5 kV, 200 ohm, and 25 μ F. Each transformation used 2 μ l of DNA and 50 μ l of electrocompetent cells prepared according to the BRL Cell-Porator Manual. The transformants were immediately complemented with 1 ml of SOC medium without antibiotics and incubated for 1 h at 37°C with shaking. The transformants were plated on LB agar plates containing 100 μ g/ml of ampicillin (for selection of ampicillin resistance), and 60 μ l of 0.1 M IPTG (isopropyl β -D-thiogalactoside) (Promega Corp.) and 20 μ l of 50 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) (Promega Corp.) for blue/white selection.

Polymerase Chain Reaction

Polymerase chain reaction was performed in a 50- μ l volume/reaction with 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.5 mM primer, and 2.5 U of *Taq* polymerase (Promega Corp.). For amplification of the S μ -S ϵ rearranged products, the precipitated plas-

mid DNAs from one recombination assay reaction were first digested with *Not*I (20 U) for 2 h. Such treatment disrupts unrearranged but not S μ -S ϵ -rearranged template plasmids and prevents their PCR amplification (see Fig. 1). PCR was carried out in 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 40 cycles with upstream primer S μ 1 (TCTAGACAAGGGGACCT-GCTCATT) and downstream primer S ϵ 4 (TTATC-CCAGCAGAACTCAGTTTAAATCAC). In order to achieve higher amplification specificity, second-round PCR was introduced to eliminate the possible nonspecific amplification from the first round PCR, with primer S μ 2 (GAGCCTAGACTAACAGGCTGAACT) and S ϵ 3 (GCCCAGTTCAGTTAACCTCAAC) (see Fig. 7C), although such second-round PCR is not necessary for the amplification of the recombinational products mediated by nuclear extracts.

Cloning of PCR Products

The pooled PCR products from a first- and second-round amplification were precipitated and directly cloned by TA cloning into PBK-CMV vectors (Stratagene, San Diego, CA) as described previously (29). The clones that contained inserts (white colonies) were enriched to master plates for screening of the positive clones to pS μ and pS ϵ by *in situ* hybridization. The clones that hybridized to both S μ and S ϵ probes were subjected to restriction endonuclease digestion and DNA sequencing analysis.

In Situ Hybridization

The transformed white colonies were transferred to nitrocellulose membranes (Nytran, Schleicher & Schuell Inc., Keene, NH). The membranes were then placed onto a Whatman 3-MM paper impregnated with 10% SDS for 5 min, denatured with 0.5 N NaOH, 1.5 M NaCl for 5 min, neutralized with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4) for 5 min, and finally rinsed in 2 $\times$ SSC for 5 min. The membranes were air-dried for 30 min and baked at 80°C for 2 h followed by Southern blot hybridization (see below).

Southern Blot Hybridization

DNA samples of either restriction endonuclease-digested plasmid DNAs or PCR products were electrophoresed on 1% agarose gel (NuSieve and Seakem agarose 1:1 mixture, FME Bioproducts, Rockland, ME) in 0.5 $\times$ TBE buffer. DNA was then transferred to nylon membranes (Nytran, Schleicher & Schuell Inc.) in 0.4 M NaOH and blots were analyzed by hybridizing with random-labeled DNA probes. Blots were prehybridized for 2 h at 68°C in 5 $\times$ SSPE Denhardt's solution, 0.5%

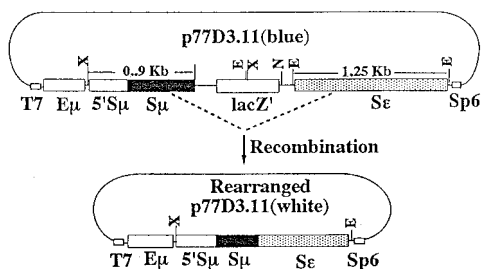


FIG. 1. Diagram of plasmid p77D3.11 and *in vitro* switch recombination in this switch substrate. The $E\mu$, $S\mu$, $lacZ'$ gene, and $S\epsilon$ fragments are constructed in the natural transcriptional orientation. The $lacZ'$ gene is flanked by the 5' $S\mu$ and 3' $S\epsilon$ fragments, as shown. The tandemly repeated sequence regions in $S\mu$ and $S\epsilon$ are indicated by heavy shading and light shading, respectively. Restriction endonuclease sites that are utilized in the manipulation and analysis of this plasmid are shown. Recombination between $S\mu$ and $S\epsilon$ deletes the $lacZ'$ gene, resulting, after transformation, in white colonies in the presence of IPTG and X-gal. X, *Xba*I; E, *Eco*RI; N, *Not*I.

SDS, and 250 ng/ml of salmon sperm DNA. Hybridization was carried out overnight at 72°C for the $S\mu$ and $S\epsilon$ probes and at 68°C for the $lacZ'$ gene probe. The blots were then washed for 20 min at room temperature with $2\times$ SSC plus 0.1% SDS and then twice more at 70°C with $0.2\times$ SSC plus 0.1% SDS for 20 min.

DNA Sequencing

Nucleotide sequences of the inserts and recombined regions were determined by the standard dideoxy chain termination method using a kit purchased from USB (USB, Cleveland, OH). Sp6 and T7 primers as well as PCR primers and synthesized oligonucleotides were used as sequencing primers as described (22).

RESULTS

Rationale and Outcomes of the *in Vitro* Ig Switch-Region DNA Recombination Assay

A model plasmid p77D3.11 that contained a portion of human $S\mu$ (0.91 kb) and $S\epsilon$ (1.25 kb) sequences with an intact $lacZ'$ gene (referred to as $lacZ'$) interposed between them in the vector pCR II backbone (Fig. 1) was constructed as the basis for our *in vitro* system to detect Ig switch-region DNA recombination to be mediated by cell-free nuclear extracts. This construct makes it possible to monitor DNA recombination between $S\mu$ and $S\epsilon$ by blue/white selection. Recombination deletes the $lacZ'$ gene, resulting in the formation of white colonies, whereas the nonrecombined plasmid gives blue colonies as the interposed $lacZ'$ gene remains intact (Fig. 1). The frequency of the $S\mu$ - $S\epsilon$ DNA recombination could be calculated by scoring the white vs blue colonies for different conditions from the same

transformation. As the DNA fragments in the construct were in their normal transcriptional orientation and under the control of bacteriophage T7 RNA polymerase, the construct could also be used to study the effects of transcription through switch-region DNA sequences on *in vitro* recombination. This approach is not able to detect recombination events within the $S\mu$ (or $S\epsilon$) alone, as such recombinants would not delete the $lacZ'$ gene.

Restriction endonuclease analysis of the resulting white colonies using *Xba*I plus *Eco*RI can reveal recombination between various elements (e.g., $S\mu$ - $S\epsilon$, $S\mu$ - $lacZ'$, $lacZ'$ - $S\epsilon$) as schematically diagrammed in Fig. 2. Recombination between $S\mu$ and $S\epsilon$ in the model plasmid generates a single digested product when mapped with *Xba*I + *Eco*RI, whereas the nonrecombined plasmids give three visible products of 0.3, 1.1, and 1.25 kb (also see Fig. 5). Recombination between $S\mu$ and the $lacZ'$ gene would result in two or three digested products as indicated. Recombination between the $lacZ'$ gene and $S\epsilon$ would generate a single or two digested products depending on where the recombination site occurs. If the recombination occurs upstream of the *Eco*RI and/or *Xba*I sites of the $lacZ'$ gene, a single digested product would be detected while recombination occurring downstream of the *Eco*RI and/or *Xba*I sites of the $lacZ'$ gene would generate two digested products. Recombination between $S\mu$ (or $S\epsilon$) and the plasmid DNA sequence would result in loss of the *Eco*RI site in the 3' end of $S\epsilon$ (or *Xba*I site in the 5' end of $S\mu$). Digestion with *Eco*RI + *Xba*I would only linearize the circular plasmid due to the single remaining

Recombination models	Mapping fragments by X+E	Colony analyzed	Ratio (%)
	3		
	1	269	87.6%
	1	ND	ND
	2	3	1.0%
	2	3	1.0%
	3	3	1.0%
	0 (linearized)	8	2.6%
	0 (linearized)		
	0 (supercoiled)		
	21		6.8%
	Total	307	100%

FIG. 2. The patterns of the recombination in p77D3.11. The possible recombination patterns are diagrammed on the left, whereas the recombination frequencies of the each pattern detected are listed on the right. Among all the clones analyzed, 87.6% of the recombination events occurred between the two S DNA. The recombination frequency that occurred between two S regions was significantly higher than other types of recombination ($P < 0.0001$). For details, see the text. ND, not determined.

restriction site. Plasmids remaining supercoiled after digestion with *EcoRI* + *XbaI* would indicate that recombination has taken place between the vector sequences with the loss of the *EcoRI* and the *XbaI* sites. Recombination between the *lacZ'* gene and vector sequences (resulting in deletion of the entire *S μ* or *S ϵ* fragment) would also generate a single digest product that is distinguished from that derived from *S μ* -*S ϵ* recombination by Southern blot analysis with a *lacZ'* gene sequence probe.

Ig S-S-Region DNA Recombination Can Be Mediated by Cell-Free Nuclear Extracts

To test whether Ig switch-region DNA recombination could be achieved *in vitro* with cell-free nuclear extracts, plasmid p77D3.11 was incubated with the nuclear extracts from human tonsillar B cells that presumably contained the putative Ig class switch recombinase because germinal center B cells in the human tonsil have been demonstrated to contain active Ig class switch recombination activity (30). The data presented in Fig. 3A show that the recombination ratio was always less than 0.1% (0.017–0.101%, $n = 10$) in the absence of nuclear extracts. Thus, the spontaneous recombination between the *S μ* and the *S ϵ* was very rare when the vector propagated in the host bacterial strain DH10B, a strain that has been frequently employed for assaying the *in vitro* switch recombination (17, 18, 22). In contrast, the recombination frequency occurring in the presence of fresh tonsillar B cell nuclear extracts was 0.895% (0.594–1.40%, $n = 5$), a rate about 20-fold higher than that in the controls. The recombination rate was further increased when CD40-stimulated B cell nuclear extracts were used, reaching an average of 3.63% (1.12–10.56%, $n = 18$). The recombination effect was abolished by heating the nuclear extracts at 80°C for 30 min (Fig. 3A), suggesting that the protein components in the nuclear extracts were required for the recombination activity. Addition of EDTA (10 mM) to the recombination reaction almost completely blocked the activity, indicating that Mg^{2+} was required for the recombination (Fig. 3A). ATP was necessary for the assay as its depletion resulted in the recombination rates close to the background (0.24%, $n = 7$) (Fig. 3A). Kinetic determination of the switch recombination activity shows that the activity was dose-dependent at nuclear protein concentrations from 1 to 5 μ g/reaction (Fig. 4A). The reactions were incubation temperature-dependent, with optimized temperature at 25°C (Fig. 4B), and incubation time-dependent, with best results achieved between 16 and 44 h (Fig. 4C). Overall, these results indicate that recombination between *S μ* and *S ϵ* could be achieved by nuclear extracts from B cells *in vitro*.

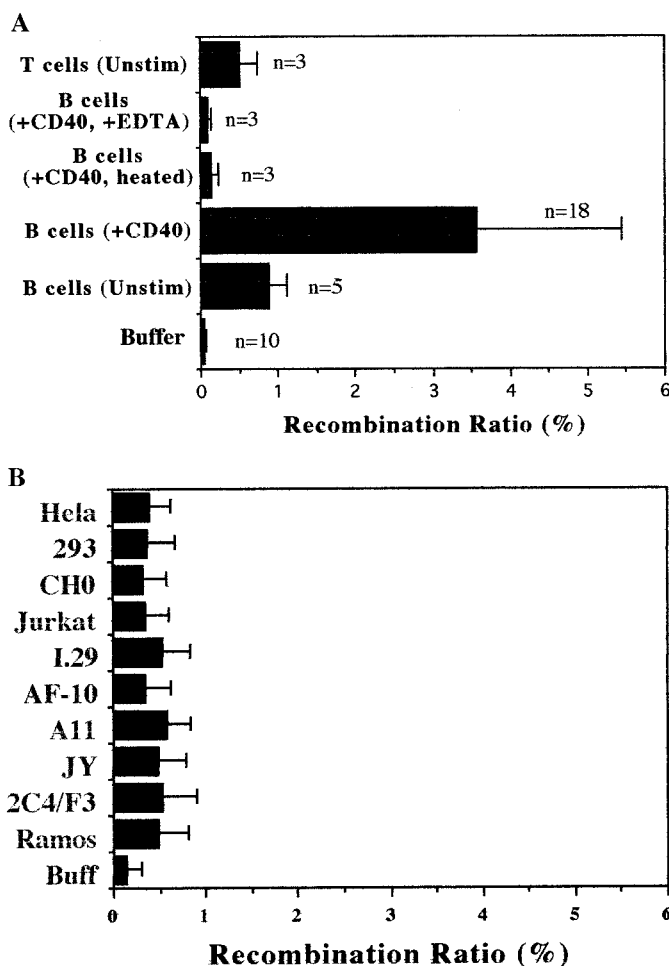


FIG. 3. Frequency of the *in vitro* switch recombination with nuclear extracts. The recombination ratio was calculated by scoring the recombined colonies (white) vs the nonrecombined colonies (blue) from the same transformation dish. The bars represent the average value from the number of experiments (n), as indicated above the error bars. The error bars represent one standard deviation. (A) Recombination frequencies from primary B and T cells with 5- μ g nuclear extracts incubated for 16 h under the conditions indicated. (B) Recombination frequencies from the cell lines with 1- μ g nuclear extracts incubated for 16 h.

The recombination activity from other types of primary cells and cell lines was also investigated. Nuclear extracts from primary human tonsil T cells showed lower (0.52%) but consistent recombination activity under conditions comparable to B cells (e.g., the protein concentration from T cells is the same as that from B cells) (Fig. 3A). Such lower recombination activity also can be detected, although varied, in all the cell lines tested so far, including lymphoid cells (2C4/F3, JY, Ramos, A11, AF-10, Jurkat, I-29) and nonlymphoid cells (293, CHO, Hela) (Fig. 3B). The recombination frequencies generated from these various cell lines, however, cannot be directly compared to each other or to primary B and T cells. The nuclease activity in these

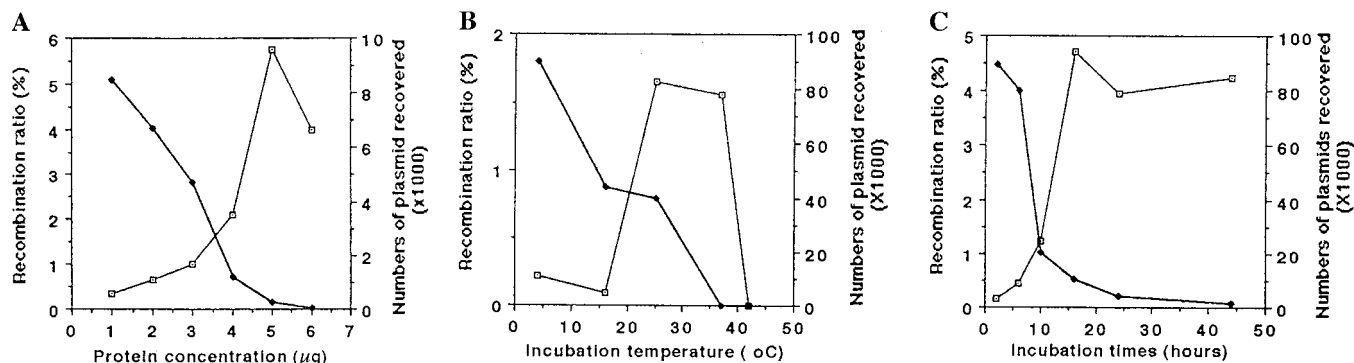


FIG. 4. Optimization of the switch recombination assay *in vitro*. The open squares represent the recombination ratio, whereas the filled squares represent the plasmid recovery rates. The nuclear extracts for the experiments were from CD40 mAb-stimulated tonsillar B cells. The plasmid recovery rates were calculated by scoring the transformed plasmids from a given dish. The final numbers were multiplied by the dilution factors for each reactions. The data represent the average value from three experiments. (A) The protein concentration (from 1 to 6 μ g) vs recombination ratio and rates of plasmid recovery. (B) The incubation temperature vs recombination ratio and rate of plasmid recovery. (C) The incubation time vs recombination ratio and rate of plasmid recovery.

cell lines was much higher than in primary B cells and greatly different from each other since the same concentration of the nuclear protein from cell lines and from primary B cells (5 μ g/reaction) almost completely degraded the input plasmids (data not shown). Overall, the results suggested that the recombination activity was not restricted but significantly higher in primary B cells stimulated with CD40.

Results from mapping of the recombinant colonies are summarized in Fig. 2. Of a total 307 white colonies obtained from 12 independent experiments, 269 colonies (87.6%) gave a single band with *Xba*I plus *Eco*RI digestion, indicating that the recombination in these colonies occurred between $S\mu$ and $S\epsilon$. Other types of recombination were observed much less commonly, with frequencies ranging from 1 to 6.8% (Fig. 2). These results show that the majority of the detected recombination events occurred between $S\mu$ and $S\epsilon$ regions, as occurs with CSR. Figure 5 shows one mapping experiment in which 29 randomly picked recombined clones were analyzed by *Xba*I + *Eco*RI digestion. In this experiment all the clones gave a single digested band (Fig. 5A) and all but one hybridized to either $S\epsilon$ (Fig. 5B) or $S\mu$ probes (Fig. 5C) but not the probe containing *lacZ'* gene sequences (Fig. 5D). Those clones had undergone the $S\mu$ - $S\epsilon$ recombination in this *in vitro* assay.

Ig S-S DNA Recombination Catalyzed by Nuclear Extracts Occurs Prior to Transformation

To definitively show that the S-S recombination is mediated by the nuclear extracts *in vitro* rather than during or following bacterial transformation, we performed PCR analysis on the $S\mu$ - $S\epsilon$ recombination products following nuclear extract reaction but prior to bacterial transformation. The precipitated plasmid

DNA resulting from incubation with or without nuclear extracts was used as DNA templates for PCR amplification. To optimize the opportunity to amplify $S\mu$ - $S\epsilon$ -recombined products, we digested the template DNA with the restriction endonuclease *Not*I prior to PCR amplification as the majority of the input plasmids were not rearranged. Such treatment disrupts unrearranged but not $S\mu$ - $S\epsilon$ -rearranged template plasmids (see Fig. 1). As the amplified products appeared as a smear rather than a set clear band when resolved on agarose gels (data not shown), we directly cloned the PCR products by TA cloning methods from the whole PCR mixture (26). The resulting clones were screened by differential *in situ* hybridization with $S\mu$ and $S\epsilon$ probes (Fig. 6). Seventeen out of 357 clones screened hybridized to both $S\mu$ and $S\epsilon$ probes (Fig. 1), showing that they represented $S\mu$ - $S\epsilon$ -rearranged products that were further confirmed by sequence analysis (Fig. 7B). By using the amplification strategy for detection of the looped-out circular DNA products and the DNA inversion events in the constructs, we also identified the products representing deleted circular DNA and DNA inversion (33, 34) through cloning and sequencing analysis (data not shown). These results demonstrate that recombination of human $S\mu$ and $S\epsilon$ DNA is catalyzed by the cell-free nuclear extracts from human B cells when added to the *in vitro* assay.

Ig S-S Recombination Mediated by Nuclear Extracts in Vitro Is Nonhomologous Recombination

One characteristic feature of Ig class switch recombination is that the deletional recombination between two involved S regions is region specific but site non-specific; i.e., it falls in the category of "illegitimate" recombination (4). To show that the recombination

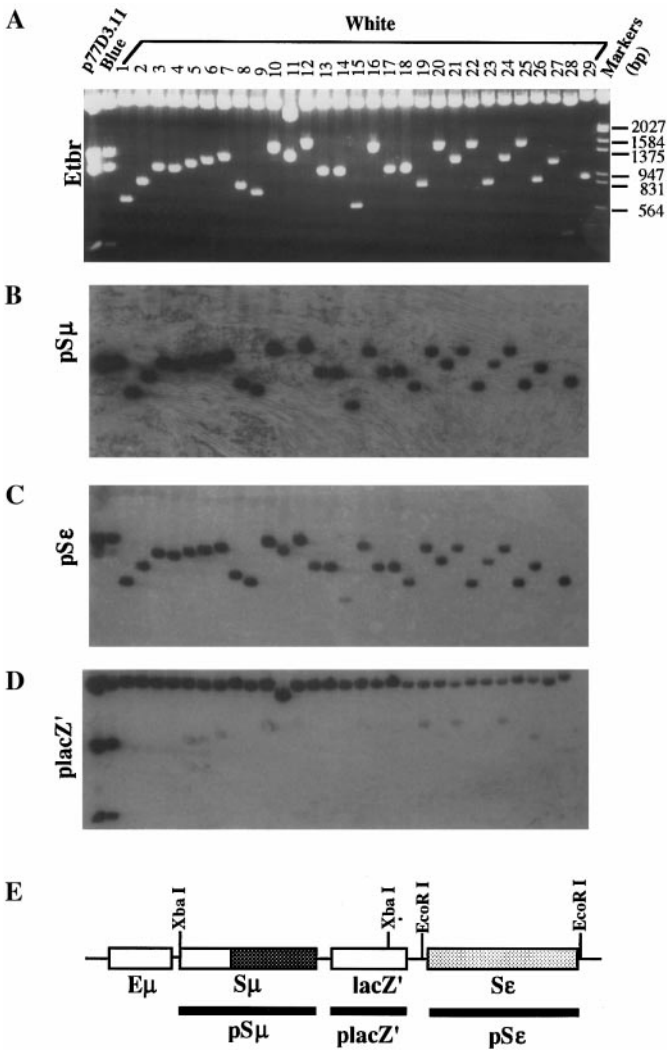


FIG. 5. Analysis of the recombinational clones by restriction mapping and Southern blot hybridization. (A) Restriction mapping of clones by *Xba*I + *Eco*RI. Twenty-nine randomly picked recombined clones (white colonies), as well as one unrearranged clone (blue colony), were digested with *Xba*I + *Eco*RI. The digested plasmids and products were resolved in 1% agarose gel and stained by EtBr. (B) The restriction-mapped products shown in A were hybridized with probe pSμ. (C) The restriction-mapped products shown in A were hybridized with probe pSε. (D) The restriction-mapped products shown in A were hybridized with probe lacZ'. The weak signals on the blot were due to the incomplete strip of the previous hybridization to pSμ and pSε as sequence analysis conferred that there were no lacZ' gene sequences in those weak positive clones. (E) Diagram of the probes used in the hybridization shown in B–D.

FIG. 7. Nucleotide sequences surrounding the retained recombination breakpoints. The recombinational breakpoints are indicated by arrows with the referenced nucleotide position according to the published sequences (25). The sequences homologous between Sμ and Sε are bold. (A) Nucleotide sequences surrounding the recombination breakpoints derived from recombination assay-derived clones. (B) Nucleotide sequences surrounding the recombination breakpoints derived from direct PCR-generated clones without bacterial transformation. (C) Summary of location of all the recombination breakpoints defined from recombination assay and PCR amplification assigned to Sμ and Sε regions in p77D3.11. The arrows in the top row represent the recombination breakpoints defined from PCR amplification, whereas those in the lower row represent the recombination breakpoints defined from recombination assay. The primer position for amplification of the Sμ–Sε recombinational products also is indicated.

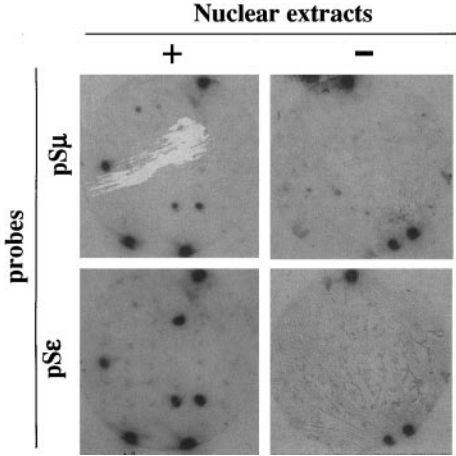
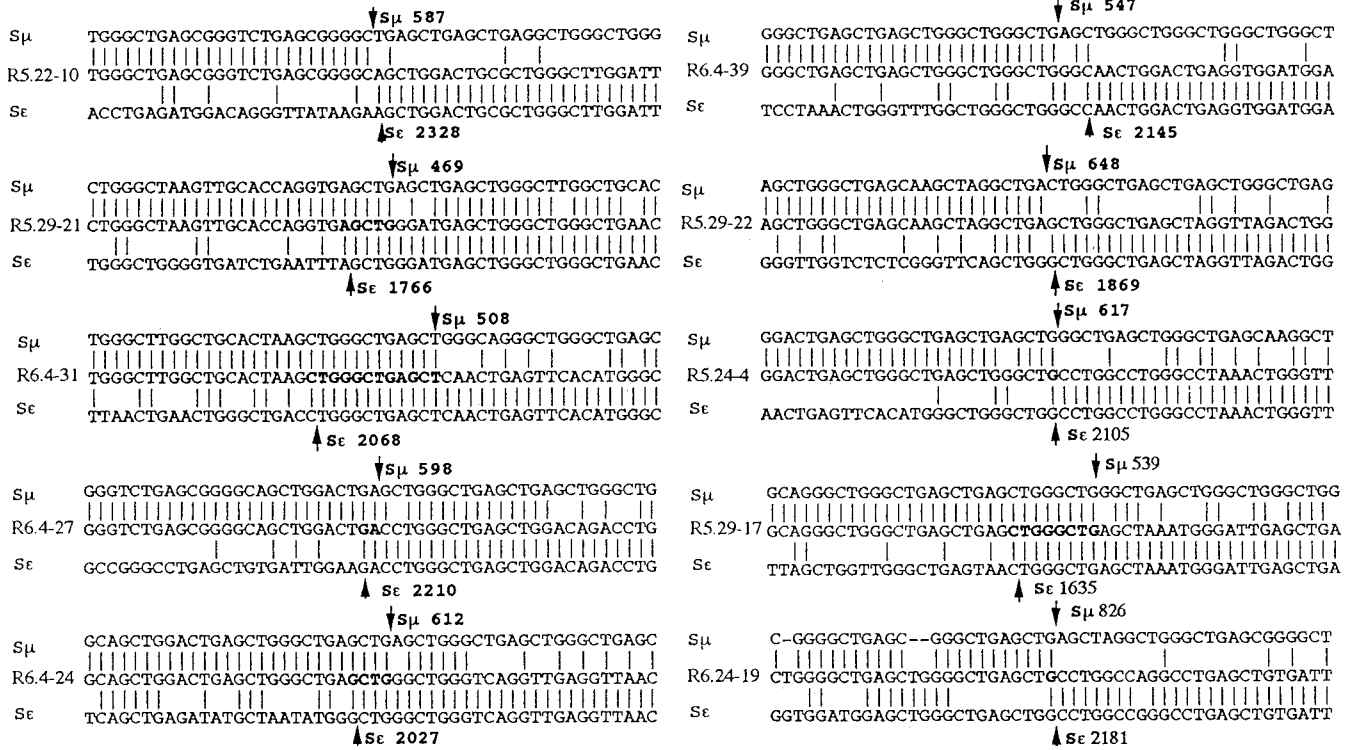


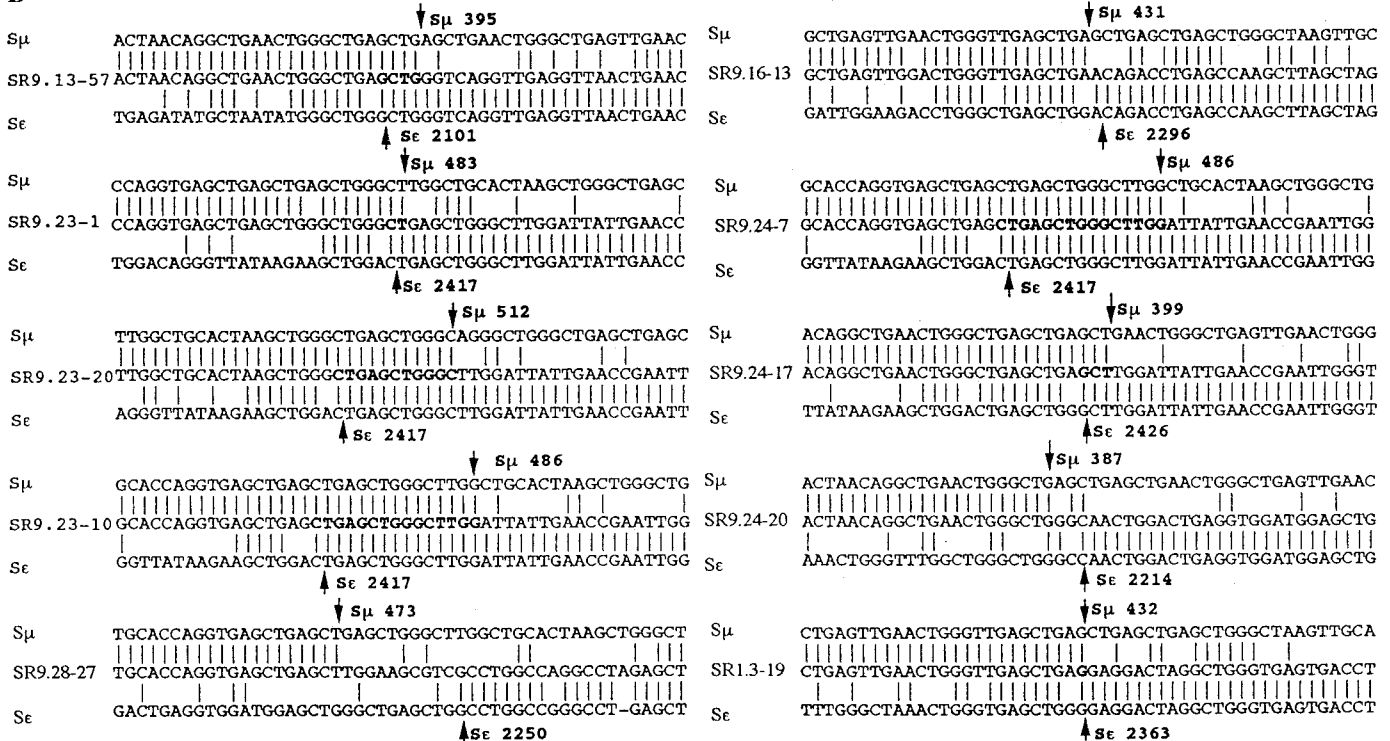
FIG. 6. *In situ* hybridization for detection of the positive colonies for Sμ and Sε in products derived from PCR amplification. The blots were individually hybridized to pSμ and pSε, as diagramed in the figure legend for Fig. 5. The three strong positive dots in the periphery of each blot are hybridization-positive controls and serve for blot orientation. They contain the unrearranged plasmid p77D3.11. The blots derived from the extracts (+) can be seen to have at least five positive colonies to both pSμ and pSε, while only the hybridization controls are seen in the absence of the extracts.

sites employed following nuclear extract-driven recombination demonstrate this feature, we sequenced the recombination junctures of recombined clones. Thirty-two randomly selected colonies that hybridized to both Sμ and Sε probes from the *in vitro* recombination assay and 17 colonies derived from direct PCR amplification were sequenced. The breakpoints were defined by alignment of the clone sequences with the Sμ and Sε sequences in plasmid p77D3.11. The sequences around the recombination junctions from 10 clones derived from recombination assay and 10 clones from PCR amplification are shown in Figs. 7A and 7B, respectively. The homology between Sμ and Sε in these sequence-identified clones from both recombination assay and PCR assay varies from a homology of 15 (clone SR9.23-10) nucleotides to 0 (e.g., clones R5.22-10, R5.29-22, SR9.16-13). Clones that contained unmatched sequences in the breakpoint to both Sμ and Sε are also presented (clones R6.4-39, SR9.24-20, and SR9.28-27). Thus, although clones derived from the *in vitro* recombination assay often share a short stretch of

A



B



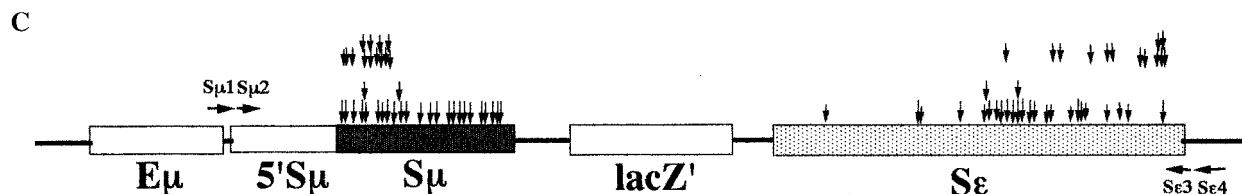


FIG. 7—Continued

homology for $S\mu$ and $S\epsilon$ in the junctional sites as seen in *in vivo* CSR, homology between $S\mu$ and $S\epsilon$ was not required for switch-region DNA recombination in our system. The frequently observed short homologous stretches between $S\mu$ and $S\epsilon$ at the recombination junctions likely reflect the internal features of the $S\mu$ and $S\epsilon$ constructs used to make p77D3.11, with its tandemly repetitive sequences GGGCT, GGGGT, and GAGCT densely distributed throughout the $S\mu$ and $S\epsilon$ segments.

Ig S-S DNA Recombination in Vitro Preferentially Targets Tandemly Repetitive Sequences

The sequenced recombination breakpoints assigned to $S\mu$ and $S\epsilon$ from both the recombination assay and the PCR assay are summarized in Fig. 7C. Strikingly, all 41 recombination breakpoints are located in the tandemly repeated regions in $S\mu$ and $S\epsilon$. None of them occurred in the $lacZ'$ gene or 5' to $S\mu$ (Fig. 7C). Although the recombination breakpoints in the PCR-generated clones may represent selection biased by the PCR assay itself and/or cloning procedures employed, the targeting of breakpoints to the tandemly repetitive $S\mu$ and $S\epsilon$ regions in the recombination assay-generated clones demonstrates that the recombination activity detected in the *in vitro* recombination assay preferentially targets the tandemly repetitive S region sequences. Otherwise some of the recombination sites would be expected to scatter in the $lacZ'$ gene and/or regions 5' to $S\mu$.

To further examine the sequence requirements for recombination activity in our assay, we replaced the entire $S\epsilon$ sequences in p77D3.11 with an 0.9-kb fragment derived from a sequence immediately 5' to $S\epsilon$, a sequence that does not contain typical G-rich, tandemly repetitive sequences. The modified construct, p77D3.11a, when compared with p77D3.11, generated a >10-fold lower recombination frequency (0.20% with p77D3.11a vs 3.63% with p77D3.11 in average). The recombination rate with p77D3.11a is barely above the highest background level (0.1%). These results further support the observation that recombination activity in this system preferentially targets G-rich, tandemly repetitive S sequences as recombination substrates.

Transcription through the S Region Is Not Required for Ig S DNA Recombination in the in Vitro Recombination System

It has been suggested that transcription through the targeted S region is a prerequisite for *in vivo* switch recombination. All the results shown were obtained under the conditions without transcription activity through the S region (i.e., without addition of bacterial T7 RNA polymerase) in the recombination reaction. Thus, the *in vitro* recombination system does not require transcription through the S region for $S\mu$ - $S\epsilon$ recombination. While p77D3.11 contained the $I\mu$ promoter and μ enhancer sequences ($E\mu$ fragment) that initiated $I\mu$ transcription *in vivo* (35, 36), this was not required for $S\mu$ - $S\epsilon$ recombination in the system as the deletion of the $E\mu$ fragment from p77D3.11 did not significantly alter the recombination frequency nor the nature of the recombination events observed (data not shown). The effects of transcription through S regions on recombination in our *in vitro* recombination system have been tested with bacterial T7 or Sp6 RNA polymerases and relationships between transcription activity and switch recombination frequencies have not been firmly established in this system (data not shown).

DISCUSSION

As IgE plays a central role in human allergic disorder, the molecular and cellular mechanisms for controlling human IgE production and regulation has been intensively investigated for many years. In this report we describe a cell-free system modeling $S\mu$ and $S\epsilon$ DNA recombination driven by nuclear extracts from human B lymphocytes. The resulting $S\mu$ - $S\epsilon$ DNA recombination possessed several key features of Ig S-S recombination *in vivo*. First, the sequences around the recombination breakpoints revealed that the $S\mu$ - $S\epsilon$ recombination was nonhomologous even though tandemly repetitive sequences are densely distributed in both $S\mu$ and $S\epsilon$ fragments in the recombination substrate. Second, there was restricted distribution of the recombination breakpoints to G-rich S region sequences with the requirement for switch rather than

nonswitch-region G-rich DNA, indicating that this system has preferential targeting of G-rich switch-region DNA sequences. Third, enhancement of S-S recombination by the nuclear extracts from CD40-driven B lymphocytes agrees with the requirement of the CD40 stimulation on B cells for Ig class switching. Taken together, these results strongly suggest that the recombination activity assayed in the *in vitro* recombination system reflects the normal processes involved in the Ig CSR *in vivo*. The biochemical nature (makeup) of the Ig switch recombinase activity which is believed to take part in the process of switching to all Igs including IgE is largely unknown. Our *in vitro* recombination system that can specifically detect and quantify Ig CSR will provide a tool for characterizing the nature of the Ig switch recombination processes, determining the components involved, and eventually isolating the putative switch recombinase and/or the components participating in this process.

There are notable differences between the *in vitro* S region DNA recombination and the *in vivo* Ig CSR. Switch recombination *in vivo* requires orchestration of at least two independent intracellular processes, e.g., Ig germline transcription that is thought to render the S region DNA accessible for the targeting of the putative switch recombinase and deletional switch region DNA recombination. The *in vitro* S DNA recombination system employs a small model construct and nuclear extracts in the absence of the germline transcripts or the need for transcriptional activity through S region DNA. It was not surprising that transcription through the S region would be not required, as the plasmid S region DNA is likely already in an accessible configuration for switch recombinase activity in such artificial constructs. These results were consistent with the observation that transcription activity was not required for the switch recombination in a switch construct that was stably transfected into a cell line (24). The observed inhibition of recombination by T7-driven transcription through the S region DNA likely represents a nonphysiological phenomenon *in vitro* due to overaccumulation of RNA, a situation which would not occur *in vivo* where regulatory balances control the rates of RNA production. Alternatively, transcription through S region(s) may play a role in changing the configuration of the S region (37, 38) for "recombinase" targeting rather than participating in the catalytic recombinational process itself. The recent finding that transcriptional silencing factors participate in DNA repair and illegitimate recombination indicates that the DNA end-joining processes involved in illegitimate recombination require transcriptional suppressive environments in the sites where DNA end-joining processes are carried out (39).

As G-rich switch-region DNA undergoes rearrangement in bacteria (4), the possibility of S μ -S ϵ recombi-

nation during the bacterial transformation process and the fidelity of the *in vitro* recombination mediated by nuclear extracts required special scrutiny. Plasmid p77D3.11, which contains several inserted DNA fragments including S μ and S ϵ , is stably propagated in the bacteria competent cells with a recombination background below 0.1% (Fig. 1). This property was required to ensure that the measured recombination rates were not caused by bacterial recombination during the transformation process. To conclusively demonstrate that the cell-free nuclear extracts were driving the recombination processes *in vitro*, recombination products were amplified and cloned through a PCR-based strategy without having been transformed into bacteria. The products so analyzed showed that S region recombination events resulted from the actual *in vitro* recombination system.

The nature of the S region DNA dependency and nonhomologous recombination in the *in vitro* recombination system distinguishes it from previously described nuclear extracts-driven homologous and illegitimate recombination systems (40–44). The preferential targeting of G-rich, tandemly repetitive sequences in S μ region by the nuclear extract recombination activity in the assay resembles primary switch recombination events as defined by the analysis of switch circular DNA that preferentially targets the S region sequences (4, 26, 27, 31, 32). This is significantly different from the recombination sites defined from Ig class switched B cell lines (4, 31) and some of the switch recombination assays reported (18, 19, 22, 38), in which the switch recombination breakpoints were frequently located in non-G-rich, tandemly repetitive sequences outside of the S region.

Characterization of the types of recombined products from our recombination substrate further defines the events observed as representative of the outcomes occurring *in vivo*. Definition of both excised circular DNA and DNA inversion products (data not shown) in this system provides strong evidence that the S-S DNA recombination is mediated through the predicted processes (33, 34). These findings also demonstrate that the looping-out, cleavage, and religation processes occurring in the *in vitro* recombination are tightly coupled processes that are likely being mediated by the switch recombinase complexes rather than occurring by random cleavage and ligation reaction mediated by nonspecific endonuclease and DNA ligase that might exist in the nuclear extracts. The appearance of nucleotides unmatched to the template switch region DNA (S μ and S ϵ) at the recombination breakpoints in some clones (clones R6.4-39, SR9.24-20, and SR9.28-27) is a phenomenon that is frequently observed at switch recombination breakpoints generated *in vivo*. This suggests that the proposed *in vivo* "error-prone" mecha-

nism also operates appropriately in our *in vitro* recombination assay (4).

The recombination activity detected in our assay from primary B cells as well as non-B cells indicates that, although it is not specifically restricted in B cells, the recombination activity is higher in primary B cells; more significantly, it is up-regulated by CD40 stimulation. The nonrestricted expression of the recombination activity suggests that the actual Ig CSR *in vivo* may be controlled at a transcriptional level other than in the S DNA recombination level, as the "accessibility" model suggested (1–7), since germline transcription correlated with the specificity of a given isotype switch. Alternatively, switch-region DNA-specific recombination machinery (presumably the switch recombinase complex) may share components with the recombinase activity that participates in non-switch-region DNA recombination that is expected to exist in all types of cells; for example, Ku protein and DNA-dependent protein kinase have been shown to be involved in VDJ recombination and CSR (8–11). Under these circumstances, the recombinase activities are expected to be non-B cell restricted in the *in vitro* recombination assay.

Overall, our results demonstrate that Ig switch-region DNA recombination can be accomplished *in vitro* using a model switch vector and cell-free nuclear extracts. Characterization of the recombination products demonstrated that the recombination process had the characteristics of Ig isotype switching, as it was (i) switch region sequence specific, (ii) nonhomologous recombination, and (iii) enhanced by CD40 stimulation. Transcription through the S region DNA was not required for recombination in this system. This *in vitro* system for Ig switch-region DNA recombination using cell-free nuclear extracts will permit further dissection of the events involved in IgE class switch recombination.

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