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Stable RNA/DNA Hybrids in the Mammalian Genome: Inducible Intermediates in Immunoglobulin Class Switch Recombination

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Although it is well established that mammalian class switch recombination is responsible for altering the class of immunoglobulins, the mechanistic details of the process have remained unclear. Here, we show that stable RNA/DNA hybrids form at class switch sequences in the mouse genome upon cytokine-specific stimulation of class switch in primary splenic B cells. The RNA hybridized to the switch DNA is transcribed in the physiological orientation. Mice that constitutively express an *Escherichia coli* ribonuclease H transgene show a marked reduction in RNA/DNA hybrid formation, an impaired ability to generate serum immunoglobulin G antibodies, and significant inhibition of class switch recombination in their splenic B cells. These data provide evidence that stable RNA/DNA hybrids exist in the mammalian nuclear genome, can serve as intermediates for physiologic processes, and are mechanistically important for efficient class switching in vivo.

Mammalian organisms require two types of DNA recombination to produce functional immunoglobulin (Ig) proteins. The first, called V(D)J recombination, mediates assembly of the variable domains of the Ig heavy and light chains in pre-B cells (*1*). Downstream of the V, D, and J segments is the region containing the Ig constant domains. In mice, this consists of eight distinct sets of constant domain exons (C_H), with the following organization: 5′-V(D)J-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′. In the second type of recombination, termed "class switch recombination" (CSR), the Cμ exons (and Cδ exons) are replaced by any one of the downstream C_H isotypes. This results in a deletion of the intervening genomic DNA as a circular product, which includes the Cμ exons (*2*). Replacement of Cμ ultimately causes a change from IgM to IgG, IgE, or IgA (*3–5*).

CSR from the IgM isotype to one or more of the downstream isotypes takes place anywhere within the several-kilobase G-rich (nontemplate strand) regions of repetitive DNA, termed "switch regions," which are located 5′ to each set of C_H exons (*6*). Immediately upstream of each mammalian switch region are intron promoters, which direct sterile transcripts into the switch and constant regions upon activation by particular cytokines (*4, 7*). Targeting of CSR to a given constant gene is considered to be tightly correlated with transcription from the corresponding upstream promoter (*3, 4, 8, 9*). Although the germ line transcripts appear to be required for CSR (*10, 11*) and in substrate studies (*12, 13*), their exact role in the targeting of class switch is unknown. Previous in vitro data showed stable RNA/DNA hybrid formation after transcription through switch sequences (*14, 15*). The RNA forms a hybrid with the DNA only when it is transcribed in the physiological direction (i.e., generation of G-rich RNA).

On the basis of this circumstantial evidence for RNA/DNA hybrid formation at switch sequences in vitro, we attempted to isolate RNA/DNA hybrids at several different murine switch sequences (Sμ, Sγ3, Sγ1, Sγ2b, Sε, and Sα) in the genome of B cells that are actively undergoing CSR [see supplementary Web material (*16*) for details]. The experimental design involved (i) enriching for small resting B cells from the spleens of wild-type C57Bl/6 mice, (ii) inducing the cells to undergo CSR by adding lipopolysaccharide (LPS) and appropriate cytokines, (iii) allowing the cells to proliferate for 2.5 days, (iv) isolating the genomic DNA, (v) treating the DNA with an excess of ribonuclease (RNase) A, and (vi) digesting all of the genomic DNA with deoxyribonuclease I. At this stage, the only nucleic acid remaining should be RNA that was stably hybridized to genomic DNA and, hence, protected from RNase A treatment.

Initially, we attempted to detect RNA/DNA hybrids at Sμ and Sγ3 by reverse transcription–polymerase chain reaction (RT-PCR) on RNA purified from B cells stimulated with LPS and interleukin-10 (IL-10) (*17*). When we examined the 5′ end of Sμ and Sγ3, we found that an RT-PCR product of the correct size is generated from an RNase A–resistant RNA species at both loci (Fig. 1B, lanes 1 and 4, respectively). The RNA species is also present at the 3′ end of Sγ3, as evidenced by the generation of the correct-sized RT-PCR product (Fig. 1B, lane 7). Lanes 3 and 6 show that these bands are undetectable in the absence of RT, eliminating the possibility that the observed PCR product is a consequence of genomic DNA contamination (Fig. 1B). To confirm that the RNA is involved in hybrid formation, we treated the genomic DNA with RNase H (which only hydrolyzes RNA involved in hybrid formation) and RNase A simultaneously. Upon treatment with RNase H, the RT-PCR products disappear (Fig. 1B, lanes 2, 5, and 8).

To confirm that RNA/DNA hybrid formation is a general property of mouse switch sequences, we examined hybrid formation at Sγ1 and Sε following stimulation of the B cells with IL-4 rather than IL-10. RT-PCR products of the expected sizes were produced in the absence (lanes 9 and 11) but not in the presence of RNase H (lanes 10 and 12) (Fig. 1B), thus confirming the presence of a hybrid at these sequences. For Sμ, Sγ3, and Sγ1, we verified that the RNA in the hybrid was the G-rich RNA being generated in the physiologic direction by performing RT-PCR with strand-specific primers during RT (RBT49, RBT08, and RBT14 in Fig. 1A) [see Web fig. 1 (*16*)].

To confirm the RT-PCR data, we attempted to detect hybrid formation at the Sμ and Sγ3 genomic loci by Northern blot analysis (Fig. 2) (*18*). As a positive control, we tried to detect germ line transcripts by Northern blot

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using the respective I regions or C regions as probes (*19*). Use of these probes demonstrated that germ line and mature heavy chain transcripts are being produced at both the μ and γ3 loci (Fig. 2B, lanes 11 and 13, respectively) (*20*).

Upon stimulation of class switch with LPS and IL-10, a distribution of RNase A–resistant RNA species (appearing as a smear) is

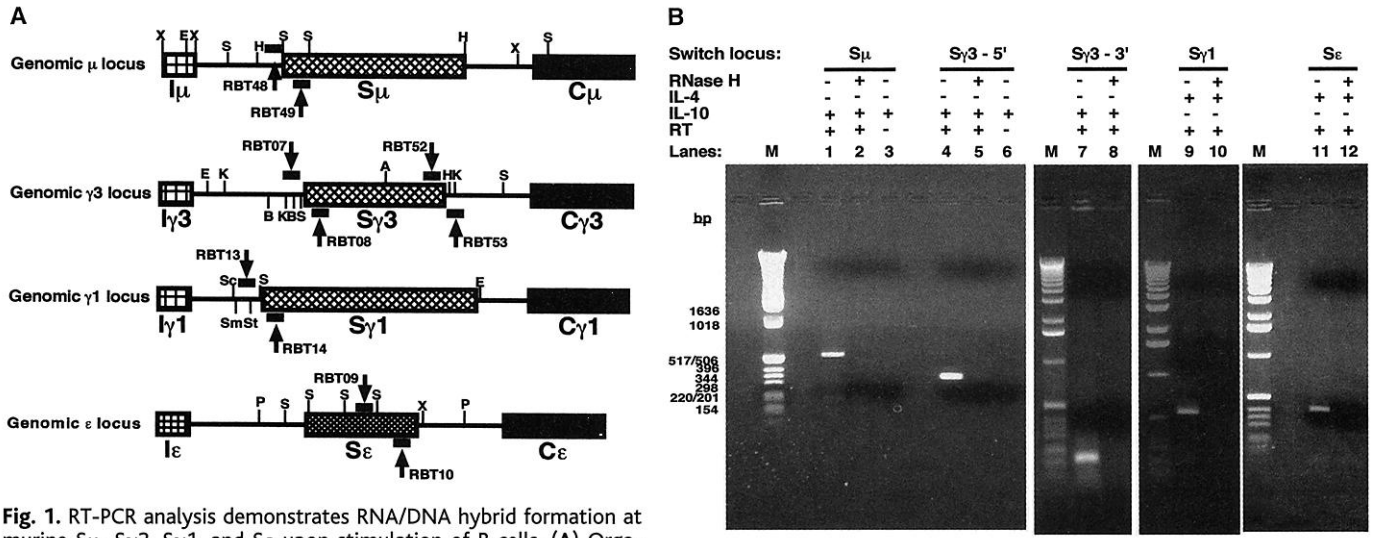
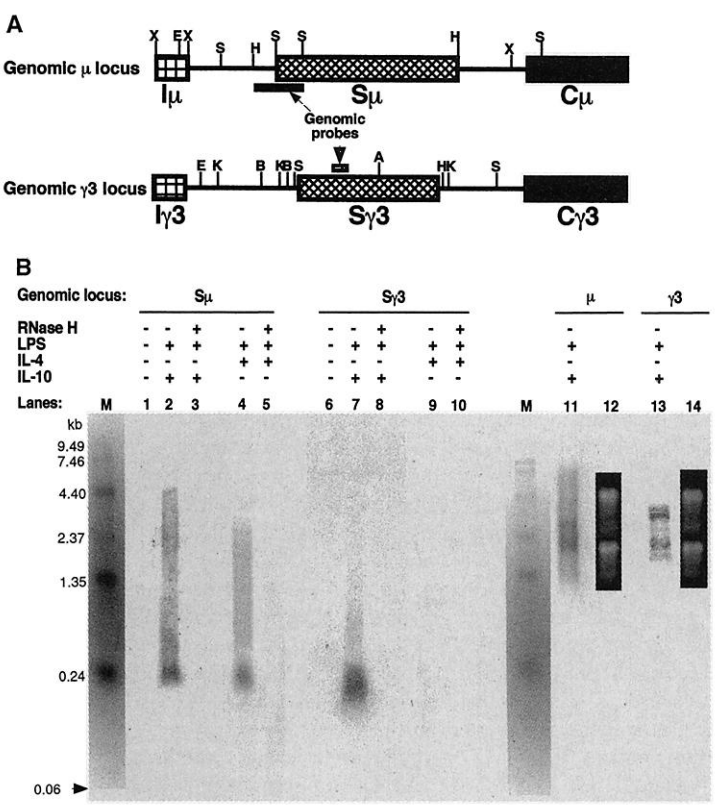


Fig. 1. RT-PCR analysis demonstrates RNA/DNA hybrid formation at murine Sμ, Sγ3, Sγ1, and Sε upon stimulation of B cells. (**A**) Organization of the murine μ, γ3, γ1, and ε loci (from top to bottom) includes the upstream promoter region (I), the switch region (S), and the constant domain (C). The location of selected restriction enzyme sites (A, Ava I; B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; S, Sac I; Sc, Sca I; Sm, Sma I; St, Stu I; X, Xba I) and locations of the primers used for PCR are shown for each genomic locus. (**B**) RT-PCR analysis of RNA/DNA hybrid formation at Sμ, Sγ3, Sγ1, and Sε. cDNA transcribed from purified RNA was amplified with primers RBT48 and RBT49 to detect hybrid formation at Sμ (lanes 1 through 3), primers RBT07 and RBT08 for the 5′ end of Sγ3 (lanes 4 through 6), primers RBT52 and RBT53 for the 3′ end of Sγ3 (lanes 7 and 8), primers RBT13 and RBT14 for Sγ1 (lanes 9 and 10), and primers RBT09 and RBT10 for Sε (lanes 11 and 12). A 1-kb DNA ladder was used as a molecular weight marker (M). bp, base pairs. "+" denotes present and "−" signifies absent.



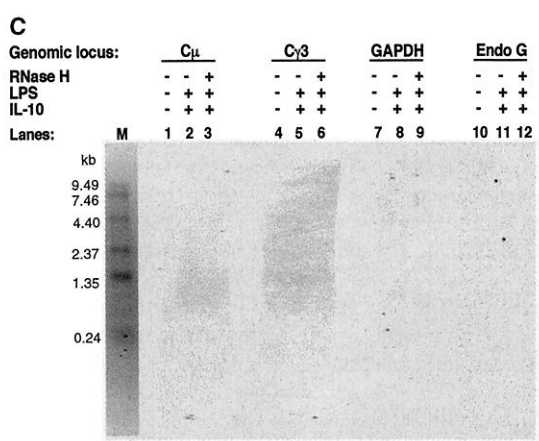


Fig. 2. Northern blot analysis demonstrates the formation of a stable RNA/DNA hybrid at murine Sμ and Sγ3 upon cytokine-specific stimulation of B cells. (**A**) Organization of the murine μ (top) and γ3 (bottom) loci includes the upstream promoter region (I), the switch region (S), and the constant domain (C). The location of selected restriction enzyme sites and the positions of probes used to detect the RNA hybridized to the Sμ and Sγ3 DNA are shown. The loci are not drawn to scale. (**B**) Northern blot analysis of RNA/DNA hybrid formation at Sμ and Sγ3. Purified RNA was probed either with a fragment of Sμ (lanes 1 through 5) or a fragment of Sγ3 (lanes 6 through 10). Lanes 11 and 13 are controls from the same experiment showing germ line and mature transcript production at the μ and γ3 loci, respectively, using the C-region probes. As a control for sample loading, lanes 12 and 14 show a photograph of the ethidium bromide–stained gels for the μ and γ3 transcripts, respectively (levels of 28*S* and 18*S* ribosomal RNA are shown). "+" denotes present and "−" signifies absent. (**C**) Northern blot analysis of RNA/DNA hybrid formation at nonswitch sequences. Purified RNA was probed with either a fragment of Cμ (lanes 1 through 3), Cγ3 (lanes 4 through 6), glyceraldehyde phosphate dehydrogenase (lanes 7 through 9), or Endonuclease G (lanes 10 through 12). An RNA ladder was used as a molecular weight marker (M). The results are representative of multiple independent experiments.

apparent at both the Sμ and Sγ3 loci (Fig. 2B, lanes 2 and 7, respectively). The size of the RNA at Sμ ranges from ~0.20 to 4.4 kb, and the size of the RNA at Sγ3 ranges from ~0.18 to 1.6 kb. The maximum length of the range of RNA species for Sμ and Sγ3 is similar to the known sizes of the Sμ and Sγ3 DNA sequences (*21*). The lack of a distinct band at both sequences could be a consequence of the fact that RNase A can cleave the RNA species in RNA/DNA hybrids at a very low, but still significant, efficiency (*22*) [see (*16*) for additional discussion]. To verify hybrid formation, we added RNase H during treatment of the genomic DNA with RNase A and found that the RNA signal disappears (Fig. 2B, lanes 3 and 8 for Sμ and Sγ3, respectively).

Because a particular cytokine(s) controls switching to a certain Ig isotype, we evaluated whether RNA/DNA hybrid formation only occurs when B cells are stimulated with the appropriate cytokine. As expected, when B cells were stimulated with IL-4 instead of IL-10, RNase A–resistant, RNase H–sensitive RNA is present at Sμ (lanes 4 and 5) but is absent at Sγ3 (lanes 9 and 10) (Fig. 2B). Switching to IgG3 is increased by the addition of IL-10 and suppressed by IL-4 (*4*). In addition, hybrid formation requires stimulation (Fig. 2B, lanes 1 and 6).

When we examined other switch sequences by Northern blot analysis, we found that an RNase A–resistant, RNase H–sensitive RNA species also exists at Sγ2b and Sα [see Web fig. 2 (*16*)]. Similar to Sγ3, hybrid formation at these switch sequences is cytokine dependent. In addition, we found that RNA/DNA hybrid formation is specific to switch sequences, because when we tested for hybrids at several different actively expressed genes from splenic B cells, the RNase A–resistant RNA species was undetectable (Fig. 2C, lanes 2, 5, 8, and 11).

To investigate whether stable RNA/DNA hybrids play a substantial role in the mechanism of CSR, we generated transgenic mice that constitutively express the *Escherichia coli* RNase H gene from a cytomegalovirus promoter. We generated these mice to determine whether disruption of the hybrid in the animals interferes with their ability to perform CSR. This tests whether RNA/DNA hybrid structures can act as physiological intermediates during CSR. All of the transgenic mice have two to three copies of the RNase H gene, and they express it in their splenic B cells. In addition, they are anatomically, histologically, and immunologically normal in comparison to their wild-type counterparts [see (*16*) for other details].

Initially, we determined what effect the constitutively expressed RNase H transgene had on the level of the RNA/DNA hybrid formation at the switch sequences in stimulated splenic B cells. For these mice, only a trace amount of RNase A–resistant, RNase H–sensitive RNA was present at Sμ (lanes 3 and 4), Sγ3 (lanes 7 and 8), and Sα (lanes 11 and 12) (Fig. 3). In contrast, wild-type mice displayed significant amounts of the RNA species at all three regions (Fig. 3, lanes 1 and 2, 5 and 6, and 9 and 10, respectively). In the RNase H mice, the amount of signal was reduced 75-fold at Sμ, 70-fold at Sγ3, and 62-fold at Sα. C-region probes established that germ line and mature transcripts are being produced and are of the correct size at both the μ and γ3 loci in the transgenic mice (Fig. 3, lanes 13 and 15, respectively).

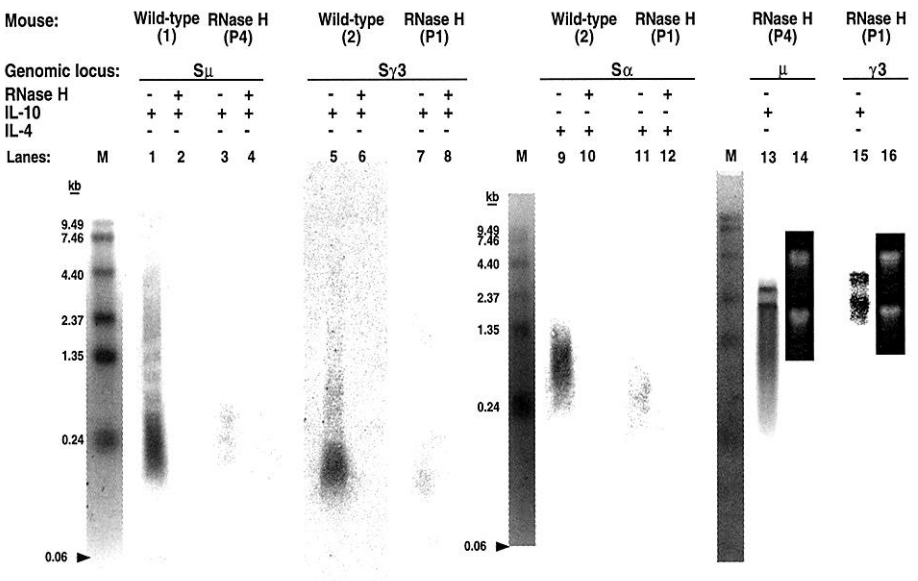


Fig. 3. Stable RNA/DNA hybrid formation at murine Sμ, Sγ3, and Sα is reduced in RNase H transgenic mice. RNA purified from wild-type mice or RNase H transgenic mice was probed with a fragment of Sμ (lanes 1 through 4), a fragment of Sγ3 (lanes 5 through 8), or a fragment of Sα (lanes 9 through 12). Lanes 1, 3, 5, and 7 contain RNA that was isolated from B cells that were stimulated with LPS and IL-10 for 2.5 days. Lanes 9 and 11 contain RNA from B cells that were stimulated with LPS and IL-4. Lanes 2, 4, 6, 8, 10, and 12 contain RNA isolated from B cells that were stimulated for 2.5 days; the RNA was subsequently treated with RNase H. Lanes 13 and 15 are controls from the same experiment showing germ line and mature transcript production at the μ and γ3 loci, respectively, in RNase H transgenic mice P4 and P1. The results are representative of multiple independent experiments.

To begin to evaluate whether disruption of the hybrid interferes with the ability to perform CSR, we compared the level of production of two classes of immunoglobulins (IgM and IgG) in both wild-type and RNase H transgenic mice in response to the antigen, dinitrophenyl conjugated to keyhole limpet hemocyanin (DNP-KLH). We tested for antibodies early in a secondary immune response in transgenic mice (H1, H2, I2, J2, and L2) and in their wild-type littermates (*23*). The average levels of DNP-KLH–specific IgM, after 4 days, were essentially identical between the transgenic and wild-type mice at all serum dilutions (Fig. 4A). However, when the average levels of DNP-KLH–specific total IgG were examined, the transgenic mice had a significantly reduced level of total IgG at all serum dilutions (~4.5-fold) (Fig. 4B).

Because the serum levels of IgG were substantially reduced, we attempted to directly test whether disruption of the hybrid inhibits its CSR in the splenic B cells of the transgenic mice. By enzyme-linked immunosorbent assay (ELISA) (*23*), we found that the RNase H mice secreted a significantly diminished level of IgG3, IgG1, IgG2b, IgE, and IgA from their B cells [see Web fig. 3 (*16*)]. Analysis of surface expression of the same Ig's by flow cytometry (*24*) showed that they were also significantly reduced [see Web table 1 (*16*)]. Using the digestion-circularization–PCR assay (*25*), we found that Sμ-Sγ1 and Sμ-Sα DNA recombination was significantly decreased in the RNase H mice [see Web fig. 4 (*16*)].

Our data show that stable RNA/DNA hybrids are formed on chromosomes inside mammalian cells, where they play an important physiological role during the CSR mechanism. On the basis of this data, we propose a model for CSR in which the RNA/DNA hybrids act as intermediates during the cutting phase of the mechanism (Fig. 5). The process is initiated by activation of the intron promoters upstream of the switch sequences (in this case, Sμ and Sγ3) by a B cell activator(s) and specific cytokine signals (Fig. 5A). The transcript is processed such that the exon located 5′ of the switch sequences (either Iμ or Iγ3) is spliced to the C_H exon (either Cμ or Cγ3) to generate a mature germ line transcript (*11, 26*). We propose that the

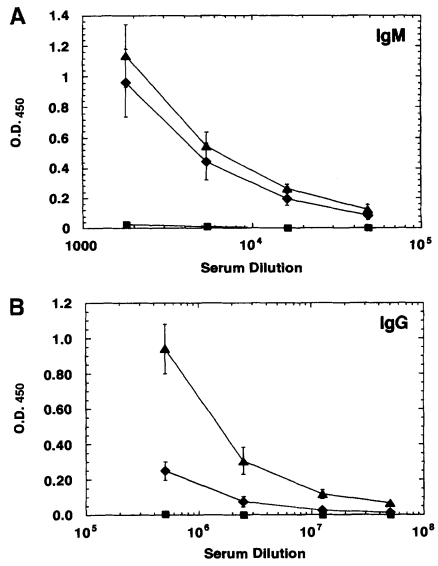


Fig. 4. RNase H transgenic mice display significantly reduced levels of serum IgG to the antigen DNP-KLH. Initially, the mice were injected subcutaneously with 100 μg of DNP-KLH in Freund's incomplete adjuvant. After 25 days, the mice were immunized a second time with 100 μg of DNP-KLH in phosphate-buffered saline. Secondary immune response of (**A**) IgM or (**B**) total IgG to DNP-KLH in both wild-type (triangles) and RNase H transgenic mice (diamonds) or in wild-type mice that were not immunized with DNP-KLH (squares) after 4 days. Serum dilutions were 1:1800, 1:5400, 1:1.6 × 10^4, and 1:4.9 × 10^4 for IgM and 1:5 × 10^5, 1:2.5 × 10^6, 1:1.25 × 10^7, and 1:5.0 × 10^7 for IgG. The reported values are the average from seven DNP-KLH–treated wild-type mice, five DNP-KLH–treated transgenic mice, or seven untreated wild-type mice. Results are expressed as optical density at 450 nm ($O.D._{450}$) of IgM- or IgG-specific ELISA as a function of serial serum dilutions. Error bars indicate the standard deviation.

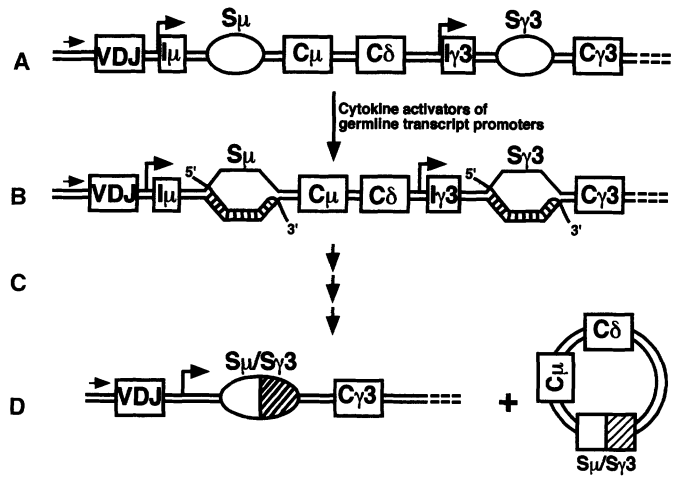


Fig. 5. Model for CSR. Only a portion of the Ig heavy-chain locus is shown. Details of the model are discussed in the text. In (**A**), the right-angle arrows denote germ line transcript promoters; the horizontal arrow represents the promoter used to generate the complete Ig; the ellipses represent the repetitive G-rich switch sequences (S); the constant domains are symbolized as a single rectangle (C); and downstream, there are five additional sets of I (untranslated sterile transcript exon), S, and C regions in mice that mediate the switch to IgG1, IgG2b, IgG2a, IgE, and IgA. See text for explanation of (**B**), (**C**), and (**D**).

intronic switch region, which was spliced out, actually remains hybridized to the duplex switch DNA at both Sμ and Sγ3 (Fig. 5B) (*11, 26*), thereby serving as an intermediate for a nuclease (Fig. 5C). The resulting double-strand breaks (*27*) are joined by nonhomologous DNA end joining (Fig. 5D) (*2, 28*).

A distinctive feature of the switch regions is that they permit stable RNA/DNA hybrid formation upon transcription. We would argue that at least a part of this stability is due to the fact that a purine RNA strand is substantially more stabilizing to RNA/DNA hybrid formation than is a pyrimidine RNA strand (*29*). In agreement, we only find stable hybrids in the chromosome (this study) and on minichromosomes (*15*) when the G-rich RNA strand is transcribed through the switch regions. This feature and the repetitive nature of the switch regions may permit the RNA from one repeat to thread back into the DNA of the previous repeat to form an R loop (see Fig. 5B for an example of R = loop structure) (*30*). Further arguing for the potential importance of the G-rich RNA to the mechanism are studies that have shown that efficient class switching requires switch sequences to be in the physiological orientation (*13, 31*).

The causal link between generation of a particular germ line transcript and targeting of switching to the corresponding isotype strongly argues for the necessity of the sterile transcript in CSR. In fact, the existence of the RNA/DNA hybrid structure now provides a rational explanation for the seven intron promoters located upstream of the seven murine switch regions. This would also explain why the human and mouse switch regions are conserved not necessarily in their primary sequences, but rather in their being repetitive, G-rich on the nontemplate DNA strand, C-rich on the template strand, and downstream of intron promoters (*32*).

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18. Northern blot analysis was performed as described (*33*). See (*16*) for a detailed summary of probes used.
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