

IgE isotype determination: ϵ -germline gene transcription, DNA recombination and B-cell differentiation

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Immunoglobulin class switching is the process which determines whether a B-cell secretes antibodies of the IgM, IgG, IgA or IgE class (or isotype). IgE is the antibody that mediates the allergic response by sensitising mast cells to allergens at the mucosal barrier. Class switching proceeds by three successive steps, culminating in the synthesis and secretion of antibody: these are germline gene transcription, DNA recombination and B-cell differentiation. We review here the present state of knowledge concerning the mechanisms involved in each of these steps, with particular reference to IgE. Intervention in the mechanisms that specify the selection of IgE may offer a means to combat allergy.

Immunoglobulin class switching

Immunoglobulin (Ig) molecules are made up of two identical heavy (H)- and two identical light (L)-chains, each with a variable (V) and constant (C) region. Igs have three functional domains, a pair of Fab arms, bearing the antigen combining sites at their tips, and the Fc domain, through which the molecule attaches to cell surface receptors. Human antibodies fall into nine different classes (IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE), differing in their heavy-chain 'constant' regions, which pair off in forming the Fc domain. Class-specific Fc receptors (FcR) are differentially distributed and differentially regulated across the broad range of cell types that mediate different effector functions. The effector function of an antibody, in other words, depends on its class and the location of its effector cells.

IgGs comprise the most abundant antibody classes in blood and lymph, while IgAs predominate in respiratory, digestive and urogenital secretions. IgE, which is the least abundant Ig in blood and lymph (1000 times less abundant than IgG), is sequestered in mucosal tissues by way of the high-affinity IgE receptor α -chain, Fc ϵ R1 α , on the resident mast

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cells and antigen-presenting cells. IgE sensitises mast cells to allergens in mucosal tissues to mediate the allergic response. (The only known beneficial effect is to provide protective immunity against parasites.) Crosslinking by allergen of the IgE-FcεRI complexes on mast cells results in the release of mediators of both the immediate and the late-phase responses.

The antigen specificity of an antibody is determined by intra-chromosomal recombination of variable (V, D and J, or collectively V) and constant (C) region sequences in the IgH- and IgL-chain genes of B-cell precursors in the bone marrow (Fig. 1A). The individual's antibody repertoire is continually renewed by the turnover of these cells. B-cells emerging from the bone marrow into the circulation express IgM and IgD on their plasma membranes (mIgM and mIgD). Cells migrate to secondary lymphoid organs, where the antigen specificity is fine-tuned by the process of somatic hypermutation of the V regions, and are then selected for high antigen affinity; unsuccessful B-cells undergo programmed cell death (apoptosis) and are replaced by the next cohort. Following antigen activation, the B-cells generally undergo class switching, with substitution of a new heavy-chain constant (CH) region. It is at this stage of B-cell differentiation that the antibody class is determined, and with it the commitment to IgE production.

Nine CH genes, Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2 defining, the nine antibody classes, lie in a tandem array on human chromosome 14 (Fig. 1A). Upstream of each CH gene are the associated intervening (I) and switch (S) regions, which play essential roles in class switching. Class switching involves DNA recombination between S regions, starting with Sμ, the furthest up-stream, as the donor, and proceeding directly or sequentially to the downstream S regions. The resulting hybrid S regions contain the 5' donor and the 3' acceptor sequences of the participating S regions. At each jump, the intervening DNA sequence is cut out to form a covalently closed circle. Then VDJ, linked to the new neighbouring CH, is transcribed into mRNA (Fig. 1B).

Class switching requires the stimulation of B-cell proliferation. In the T cell-dependent immune response *in vivo*, this is achieved by the interaction of B-cells and activated cognate T helper (Th) cells. Th cells can be activated by professional antigen-presenting cells or by B-cells. A B-cell can present a specific antigen through its antigen receptor or through a specific IgE antibody, bound to the low-affinity IgE receptor, CD23a. Th cell activation can be mimicked *in vitro* by exposure to anti-CD3 and anti-CD28, or to polyclonal T cell activating agents. The activity of Th cells on B-cells can be simulated *in vitro* by other B-cell mitogens, by membrane preparations from activated T cells, by a T cell membrane protein called CD40-ligand (CD40-L/CD154) or by anti-CD40.

Depending on local conditions, Th cells specialise to a greater or lesser extent in the secretion of one of two sets of cytokines^{1,2}. Highly polarised Th-1 cells produce interleukin-2 (IL-2) and interferon- γ (IFN- γ), while Th-2 cells produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Human Th-1 cells stimulate class switching to IgG1, IgG2 and IgG3, Th-2 cells to IgG4, IgE or, in the presence of TGF- β , to IgA1 and IgA2. The

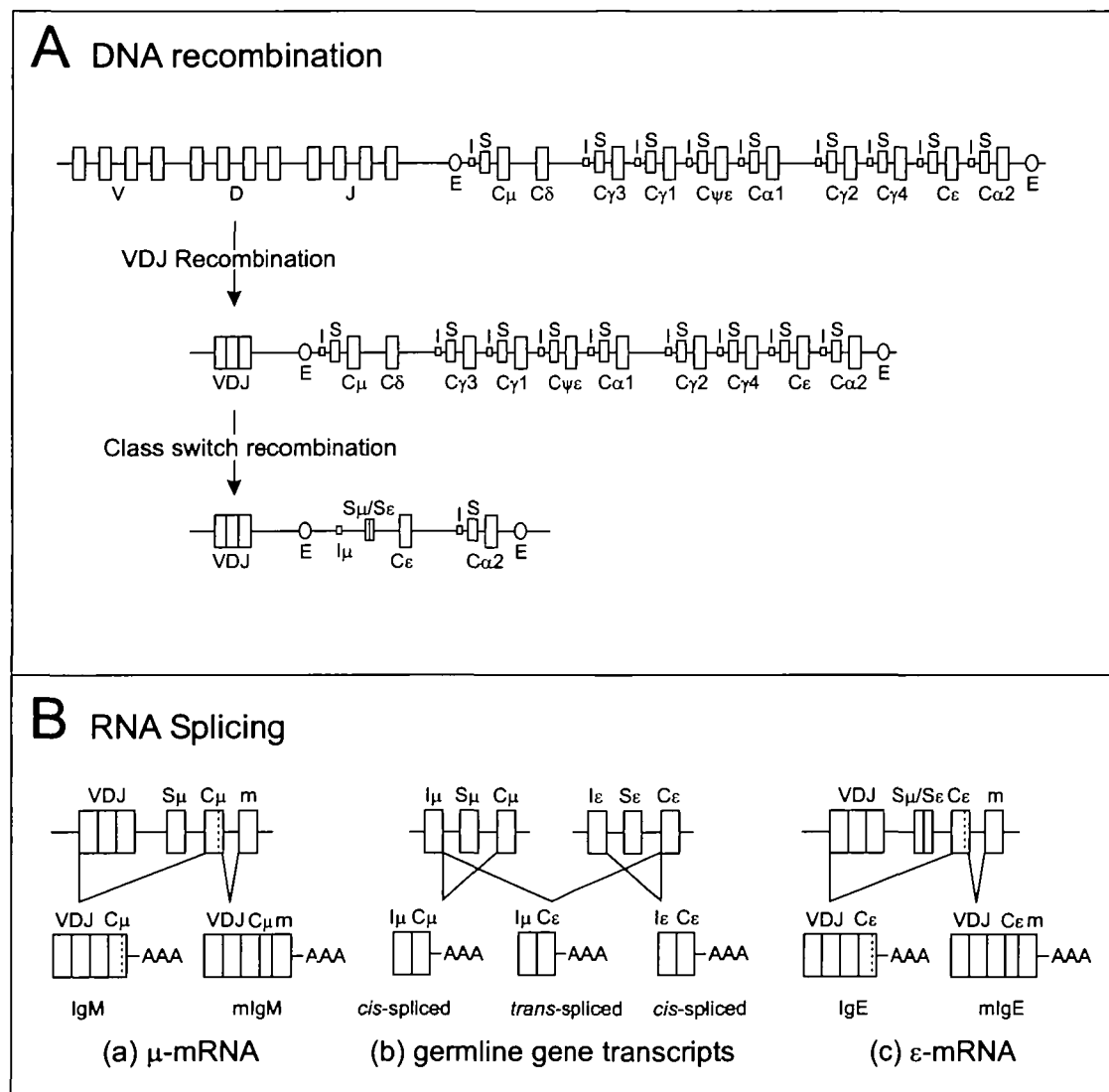


Fig. 1 Organisation of the human immunoglobulin heavy-chain gene locus and transcription products. (A) The heavy-chain gene locus on chromosome 14, showing the sequence of elements discussed in the text and the two somatic recombination events, VDJ recombination and class switch recombination (CSR), which occur during B-cell development. (B) RNA splicing to produce (a) mRNA for membrane-bound and secreted Ig μ -chains before CSR, (b) germline gene transcripts from C_μ and C_ϵ , and (c) mRNA encoding the membrane and secreted ϵ -chains after CSR.

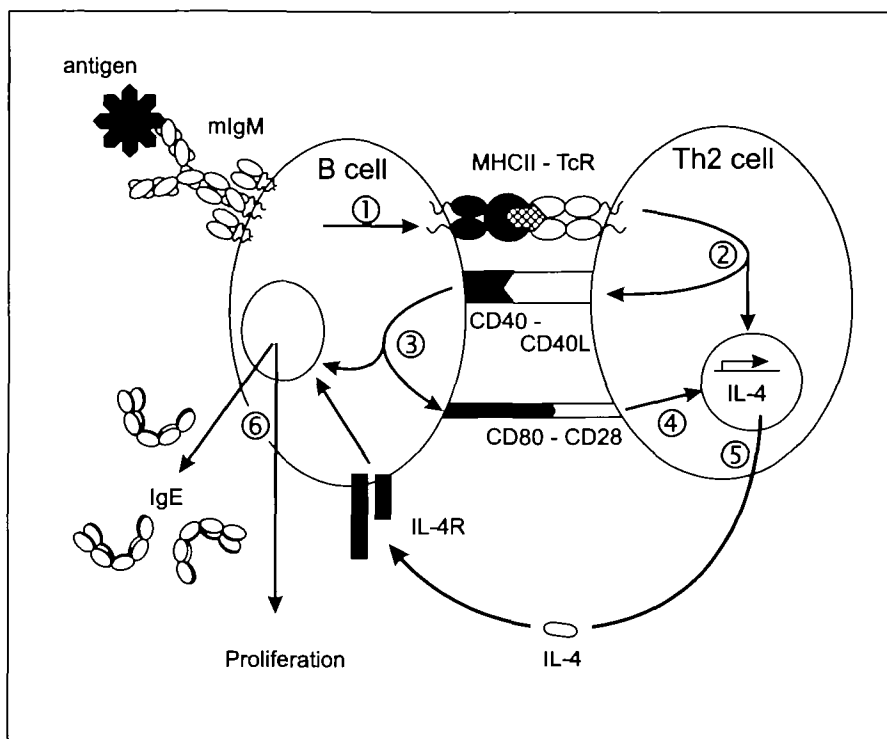


Fig. 2 Dynamics of Th cell-B-cell collaboration in heavy-chain switching to IgE. Numbers denote the sequence of events: (1) Presentation of antigen (hatched) to the Th cells. (2) Up-regulation of CD40-L on the Th cell and interaction of CD40-L with CD40 on the B-cell. (3) Up-regulation of CD80 on the B-cell and interaction of CD80 with CD28 on the Th cell, together with stimulation of B-cell proliferation. (4) Up-regulation of IL-4 synthesis in the Th cell. (5) Secretion of IL-4 and binding to the IL-4R on the B-cell. (6) Stimulation of class switching to IgE in the B-cell.

dynamics of the Th-2 cell-B-cell interaction, leading to class switching to IgE, are illustrated in Figure 2. The antigen (or allergen) is internalised and processed and the resulting peptide is presented to Th cells in the context of the major histocompatibility complex (MHC) class II antigen. The peptide-MHC class II antigen complex is recognised by the Th cell receptor (TcR) on cognate Th cells (step 1), resulting in transient expression of CD40-L on the Th cell (step 2). Interaction of CD40-L with constitutively expressed CD40 on the B-cell up-regulates CD80 on the B-cell and stimulates B-cell proliferation (step 3). The binding of CD80 to CD28 on the Th cell stimulates IL-4 synthesis (step 4) and secretion (step 5). Binding of IL-4 to the IL-4 receptor on the B-cell directs class switching to IgE and cell proliferation (step 6).

The initial action of the cytokines is to stimulate germline gene transcription from promoters in the 5' flanking sequence of the I region,

corresponding to the target CH gene. Transcription proceeds from the initiation sites through I, S and CH and terminates in the 3' flanking region of CH; finally, the transcripts are polyadenylated. This germline gene transcript (GLT) is spliced from the 3' end of the I region to the 5' end of the CH gene, and the mature transcript thus contains an I exon unique for each antibody class. The I exon in the germline gene transcript occupies the position of the VDJ exon in mRNA, synthesised after class switch recombination (CSR; Fig. 1B).

After CSR the B-cell expresses the new mIg, which allows further affinity maturation and antigen presentation to Th cells, leading to cell death or clonal expansion. mIg may also be a signal for isotype selection, as described below (B-cell differentiation). B-cells must undergo a substantial phenotypic change into the terminally differentiated plasma cell if they are to synthesise and secrete Igs. The choice between membrane and secreted Ig is made at the level of RNA splicing (Fig. 1B). To form the message for mIg the cell uses a splice site within the RNA sequence encoding the terminal exon (Cε4) which defines the secreted form; this is spliced to a down-stream membrane exon (m). The splice donor site at which this occurs is inactive in plasma cells. The plasma cell has a highly developed endoplasmic reticulum and devotes up to 50% of its protein synthesis activity to the secreted Ig.

Molecular mechanism of class switching to IgE

We now consider in more depth the sequence of steps involved in class switching, focusing, as far as the evidence allows, on human IgE. Information in this area is derived from studies on transgenic mice or mice with engineered gene defects (knock-out mice), as well as from human and murine cell systems and recombinant proteins and DNA sequences *in vitro*. The evident similarity of the known aspects in mouse and man justifies extrapolation to the human system. For example, CD40-L is required for heavy-chain switching to any of the antibody classes; hence, patients with defects in this gene present with the X-linked hyper-IgM syndrome and CD40-L knock-out mice exhibit a similar condition. The IL-4 receptor (IL-4R) and CD40 signalling cascades require highly homologous proteins in the two species. We discuss in turn germline gene transcription, DNA recombination and B-cell differentiation.

Germline gene transcription

More comprehensive reviews and extensive references are cited elsewhere³⁻⁸. Activation of ε-germline gene transcription is an essential

gateway to IgE expression (see below). Both IL-4 and IL-13 induce ϵ -germline gene transcription in human B-cells, especially in synergy with a second signal through CD40. The second signal is also required for DNA recombination to S ϵ , in common with other switch variants. Human ϵ -GLT synthesis is initiated at multiple start sites within 90 bp of the furthest 3' start site. Transcription proceeds through I ϵ , S ϵ and C ϵ , terminates in the 3' flanking sequence of C ϵ , and is followed by polyadenylation of the RNA precursor. The I ϵ exon of ~0.5 kb is spliced to C ϵ in the RNA, with deletion of the 3.5 kb intervening sequence, to yield the 1.7 kb ϵ -germline gene transcript; the ϵ -chain mRNA by contrast is 2.2 kb. I ϵ , like other mouse and human I exons, contains stop codons in all three reading frames and ϵ -GLT cannot, therefore, be translated into a protein fragment containing C ϵ .

The role of IL-4 in the activation of ϵ -germline gene transcription is now understood in terms of the signalling pathway between IL-4R on the cell membrane and the ϵ -germline gene in the cell nucleus (Fig. 3). The mouse and human ϵ -GLT promoters are highly homologous and conserve many of the same regulatory sequences. One such is the IL-4 response element, which is also common to the ϵ - and other genes, including those for the MHC class II antigen, the Fc ϵ R1 α -chain, CD23b and the IL-4 gene itself. (Co-ordinate control of these five and possibly even more genes may be essential for orchestrating the effector functions IgE, once it is present.) IL-4R is a heterodimer of an α - and a γ -chain; the γ -chain is common to several cytokine receptors (IL-2, IL-7, IL-9 and IL-15). The IL-13 receptor (IL-13R) contains the same α -chain and a ligand-specific chain in place of the γ -chain. Dimerisation of IL-4R, contingent on ligand binding, leads to activation by cross-phosphorylation of Jak1, attached to the α -chain, and Jak3, attached to the γ -chain. The ligand-specific chain of the IL-13R binds Tyk2 instead of Jak3. JAKS (Janus kinases) are a family (comprising Jak1, 2 and 3 and Tyk2)

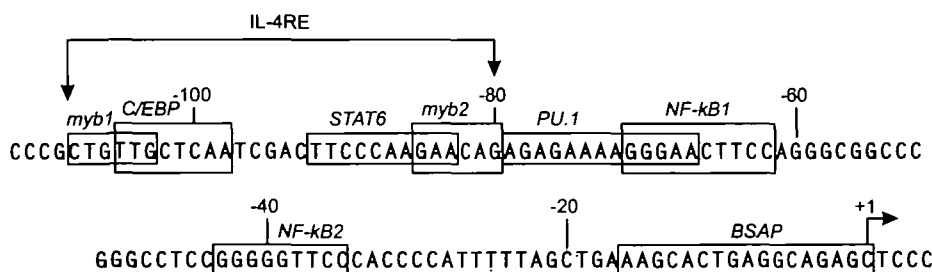


Fig. 3 The human ϵ -germline gene promoter. The 5' flanking sequence of the template strand of the ϵ -germline gene, the I ϵ promoter sequence, with its various regulatory protein binding sites or response elements (REs) boxed. The response elements are identified in the text.

of tyrosine kinases involved in signal transduction through various cytokine receptors. The α -chain is then phosphorylated to generate docking sites for Stat6.

STATs (signal transducers and activators of transcription) are a family (comprising Stat1–6) of transcription factors. Tyrosine phosphorylation of the Stats allows them to form homodimeric or heterodimeric transcription factors, which migrate to the nucleus and there activate transcription from the IL-4 response elements. The sequence motif recognised by Stat6 is 5'-TTC(N_{3 or 4})GAA-3', where N can be any of the four bases. The different Stats recognise very similar sequences with subtle differences in affinity. Stat1 has a preference for N₃ and Stat6 for N₄. It may be significant that Stat6 binding sites, which will not bind other STAT proteins, are present in IL-4-inducible genes. The Stat6 site in the murine ϵ -germline gene does however bind the B-cell lymphoma (Bcl)-6 gene product, which may act to repress transcription in the absence of Stat6⁷.

Full activation of the human I ϵ promoter in response to IL-4 and CD40 engagement requires a constellation of nuclear factors, which are either constitutively expressed or recruited to the promoter by specific activating signals (Fig. 3). The ϵ -germline promoter contains binding sites for endogenous myb proteins and a Stat6 element, partially overlapping the 3' myb site. Because myb proteins are known to act as cell cycle sensors, the presence of myb binding sites may reflect a mechanism for the synchronisation of germline transcription, switch recombination and cell division in the cell cycle, as described below. Both Stat6 and myb interact with two proteins, p300 and CBP, which possess acetyltransferase activity and may, therefore, play a part in chromatin remodelling, as well as in the interaction with the basal transcription machinery, required for RNA chain initiation.

NF- κ B binds to two distinct sites in the ϵ -germline gene promoter and co-operates with Stat6 in synergistic activation of transcription. In accordance with the well-known ability of CD40 signalling to induce NF- κ B activation, the participation of these proteins may explain the CD40-dependent enhancement of IL-4-induced ϵ -germline gene transcription. NF- κ B activity is at the same time essential for IL-4 responsiveness. Both effects most probably reflect the physical association between NF- κ B/Rel proteins and Stat6^{9,10}. Interaction with NF- κ B appears to induce profound changes in the activity of Stat6: DNA binding affinity is substantially enhanced and most importantly *trans*-activating activity appears¹⁰. Activation of Stat6 or NF- κ B requires no *de novo* protein synthesis, and both pass rapidly to the nucleus. More recently, a PU.1 binding site, overlapping with an NF- κ B site further up-stream in the sequence of the promoter, has been found essential for activity of promoter constructs; This introduces another level of cell cycle regulation of ϵ -germline gene transcription¹¹.

Of special note is the binding site for the B-cell-specific activator protein (BSAP), which is expressed in a stage-dependent manner in the B-cell lineage from the pro-B to the mature B-cell, but not in plasma cells. BSAP binds to a highly conserved region immediately upstream of the major I ϵ transcription initiation site in murine and human B-cells. Overexpression of BSAP stimulates IgE and inhibits IgA expression in a mouse B-cell line¹². Antisense-mediated down-regulation of BSAP inhibits cell proliferation, perhaps because BSAP represses transcription of the tumour suppressor gene, p53, and eliminates a block between the G1 and S phases of the cell cycle. TGF- β also inhibits transcription of the ϵ -germline gene and enhances transcription of the α -germline gene. IFN- γ secreted by Th-1 cells acts on bystander cells to inhibit ϵ -germline gene transcription by an unknown mechanism. The numerous regulatory elements in the ϵ -germline gene promoter may be needed to integrate the various signals reaching a B-cell at successive stages of its development and migration.

A different pathway is apparently required for the stimulation of B-cell proliferation by both IL-4 and IL-13. After phosphorylation of the α -chains by Jak 1 and Jak3, the insulin receptor substrates-1 and 2 (IRS-1 and IRS-2) are recruited to IL-4R and IL-13R, where they are phosphorylated by Fes and thus enabled to activate the phospho-inositol-3 (PI-3) kinase and the small ribosomal subunit protein, S6, kinase (p70^{S6K}) pathways, leading to cell proliferation. Increase in the B-cell clonal population is also promoted by the inhibition of apoptosis. Binding of SHIP, an inositol phosphatase, to IL-4R results in the phosphorylation of SHIP, and accompanying partial dephosphorylation of the products of PI-3 kinase; the latter activate the anti-apoptotic kinase, Akt. The negative regulation of these pathways has also been elucidated in great detail⁸.

DNA recombination

More comprehensive reviews and extensive references are cited elsewhere^{7,13-17}. The targets for class switch recombination (CSR) are the 1–10 kb S regions up-stream of all but one CH; the exception is C δ , which is expressed by differential splicing of an mRNA precursor containing C μ and C δ . The S regions contain short (pentanucleotide) and long (20–80 bp) repeats, which show varying degrees of similarity to each other and to those in other S regions. Switch junctions can occur almost anywhere in the S regions, and to a lesser extent in the flanking sequences. They appear to be excluded from the I regions. No common sequences have been found at any fixed distance from or in the switch junctions, in contrast to the signal sequences in VDJ recombination sites¹⁸⁻²⁰.

Moreover, class switching occurs normally in mice deficient in the products of the recombination activating genes (Rag-1 and Rag-2), the nucleases responsible for site-specific DNA recognition and cleavage during VDJ recombination.

One peculiarity of S sequences is the presence of G-rich and C-rich runs, the latter concentrated in the template DNA strand. A possible function for these sequences is implied by the discovery of RNA loops (R-loops), formed by nascent germline gene transcripts following transcription of S region DNA *in vitro*²¹. An R-loop is an RNA–DNA hybrid, in which one strand of the DNA duplex is replaced by RNA complementary to the other strand of DNA (Fig. 4A). Such hybrids are a general feature of DNA transcription, persisting up to a length of 10–12 bp behind the last ribonucleotide to be added to the RNA chain. Beyond this length, as it reaches the trailing edge of the polymerase–protein complex the heteroduplex is actively dissociated to regenerate the double-stranded DNA template, with liberation of single-stranded RNA.

In switch regions, the nascent RNA–DNA hybrids may reach several hundred base pairs in length²¹. It is known that rG–dC polymers are more thermostable than the corresponding dG–dC polymers. As mentioned above, the template strand of S is rich in oligo-dC runs and this may well account for the unusual resistance of the RNA–DNA hybrid as RNA polymerase works through the S region. The R-loops formed by transcription of S regions *in vitro* can be cleaved by the nucleotide excision repair nucleases, XPF-ERCC1 and XPG²¹, which function in general DNA repair processes. These may not be the nucleases that act during CSR *in vivo*.

For CSR to occur, two S regions, up to several megabases apart, must be cleaved on both strands with release of three DNA fragments, and the two outermost fragments must then be joined. How can chromosome 14 be cut into three fragments without falling apart? In VDJ recombination, this problem was in principle solved with the discovery of the ‘synaptic complex’ by Rag-1 and Rag-2 proteins, apposed at the two sites of recombination before and after DNA cleavage^{22–25}. This mechanism is not available for CSR.

The fact that germline gene transcription always precedes CSR, and the conserved nature of the transcripts, both imply that germline gene transcripts are an essential feature of the CSR mechanism. It is now clear that transcription of the germline gene does not merely unfold the chromatin so that recombinases can gain access to the DNA²⁶. Such a simple mechanism is at odds with several pieces of evidence: (i) when the I exon and its promoter are deleted from the mouse genome, no CSR occurs; (ii) when the I exon and its promoter are replaced by another promoter-gene construct, transcription ensues, but not necessarily CSR; (iii) if CSR is to occur, the replacement gene must contain an intron; and

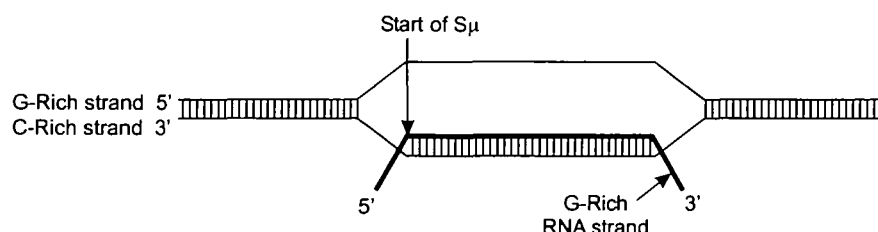
(iv) removal of a 114 bp sequence containing the splice site from the replacement gene abrogates switching. It thus appears that transcription upstream of S and splicing of the RNA precursor are both required for CSR. The I exon of the germline gene transcript therefore plays more than a passive role in CSR.

A novel mechanism for tethering the fragments of chromosome 14 during CSR is based on the existence of hybrid germline gene transcripts (Fig. 1B)²⁷. These contain an up-stream I spliced to a down-stream CH, but not the CH directly linked to I that can be merely one of several products of the splicing machinery at the site of germline gene transcription. Kinoshita and Honjo have suggested that the stalling of RNA polymerases in switch regions may allow random assembly of the donor and acceptor spliceosome components, leading to the generation of hybrid germline gene transcripts²⁸. The volume within which this search proceeds would of course be limited by the length of DNA between the S regions. Ligation of the chromosome 14 fragments to breaks in other chromosomes, which could lead to oncogenic transformation of the B-cell (see Hiom *et al.*²⁹), may thus be strictly contained.

The early stages of VDJ recombination are now well understood, thanks to the development of *in vitro* reconstitution systems. This approach is not yet available for the study of CSR. The late stages of VDJ recombination are less clear and may be similar in CSR. Both must occur by the process known as non-homologous recombination/end joining (NHEJ) or illegitimate recombination. Mammalian enzymes implicated in the NHEJ pathway fall into four complementation groups, XRCC4, XRCC5, XRCC6 and XRCC7³⁰⁻³³. XRCC4 associates with mammalian DNA ligase IV and may enhance its activity. The other three gene products are components of the DNA-activated protein kinase (DNA-PK)-Ku complex. DNA-PK is a member of the PI-3 kinase superfamily. Ku is a heterodimeric DNA end-binding protein that is the DNA-binding factor of the DNA-PK complex. XRCC5 and XRCC6 encode the 86 kDa and 70 kDa subunits of Ku, while XRCC7 encodes the kinase. Gene defects in any of the three loci block class switching, as well as VDJ recombination, and implicate these proteins in the repair mechanisms.

Even supposing that the 5'- and 3'-ends of the DNA are in random motion after scission of the two S regions involved in recombination, there may yet be a way for the two strands to unite without complementary base-pairing. Experiments with purified components *in vitro* showed that Ku was required for efficient ligation by DNA ligase IV under the stringent conditions (short or zero overlap, physiological salt and temperature) required for end-joining in cells³⁴. Thus, while the formation of RNA-DNA hybrids and *trans*-splicing may tether the broken ends of the DNA, Ku may be required to bring them together (Figs 1B & 4B).

A R-loop



B Class switch recombination

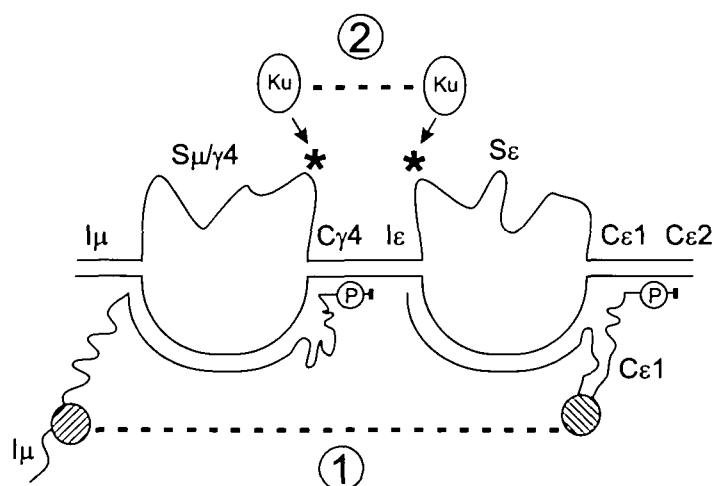


Fig. 4 Scheme for maintaining the continuity of chromosome 14 during CSR from IgG4 to IgE. (A) The RNA-loop (R-loop) generated by germline gene transcription through an S region²¹. (B) After formation of R-loops at S μ and S ϵ , spliceosome subunits (hatched) bring the nascent RNAs into contact and catalyse splicing of I μ to C ϵ (1). This is followed by cleavage of the DNA (*) and then non-homologous end-joining and repair facilitated by Ku (2). Stalled polymerases (P) may provide time for these events to occur.

VDJ and CSR recombination may occur following the recruitment of the DNA repair complex, termed MRN (comprising Mre11, Rad50 or Rad51 and Nbs1), to the broken DNA segments. Pieces of this puzzle have been assembled in the case of the more general double-strand break repair processes, *e.g.* X-irradiation-induced double-stranded breaks^{35,36}. Histone 2AX (H2AX), a sub-type of histone 2A (H2A), associated with DNA in the region of double-stranded breaks, is found to be

phosphorylated at a specific residue (ser139) in the C-terminal tail. (H2A is one of the four histones that make up the nucleosome structure of chromatin. The C-terminal tail is located near the linker region of DNA between nucleosomes, where it may affect the accessibility of the DNA.) Within several hours, H2AX recruits successively the breast cancer-related tumour suppressor gene, Brac1, product and the MRN complex. Mre11 acts as a 3' to 5' exonuclease and an endonuclease on single-stranded DNA and hairpin structures. Mre11 promotes the joining of non-complementary ends *in vitro* by pausing at sequences of short homology within the DNA sequences. Switch regions may thus be an ideal substrate for such activity. The Nijmegen breakage syndrome gene product (Nbs1) stimulates the unwinding of DNA duplexes and hairpin cleavage, while Rad50 is responsible for ATP binding by the MRN complex³⁷. The participation of the MRN complex, and perhaps an error prone DNA polymerase, may account for the abundance of mutations, insertions and deletions observed in switch junctions.

A recent discovery by Honjo and colleagues promises to implicate yet another protein in CSR^{38,39}. In their studies of class switching to IgA, induced by IL-4, TGF- β and anti-CD40 in the murine B lymphoma line, CH12F3-2B, they observed that the protein synthesis inhibitor, cycloheximide, inhibits CSR. They accordingly created a subtracted cDNA library, using mRNA from induced *versus* uninduced cells. This threw up a cDNA clone encoding a protein with homology to a family of cytidine deaminases. The closest homologue is APOBEC-1, which changes a C to a U in the mRNA for apolipoprotein, thus generating a termination codon. The resulting apolipoprotein mRNA codes for a truncated protein. The new protein from CH12F3-2B-cells is called activation-induced cytidine deaminase or AID.

Induction of AID begins in the CH12F3-2B-cells within 3 h, reaching a maximum at 48 h. Cycloheximide prevents the synthesis of AID, although germline gene transcription proceeds normally. Germline gene transcription depends on protein phosphorylation but not protein synthesis, as already mentioned. AID is expressed only in lymphoid tissue. It is up-regulated in mouse spleen by immunisation and appears during the period of class switching. AID knock-out mice exhibit a hyper-IgM syndrome, while overexpression of the protein leads to lymphoid hyperplasia, resembling that seen in human patients with the X-linked hyper-IgM syndrome (deficient in CD40-L; see above). A different group of patients with a hyper-IgM syndrome are now known to have lesions in the AID rather than the CD40-L gene⁴⁰. The human AID gene has been mapped to chromosome 12⁴¹. The AID defect inhibits somatic hypermutation as well as CSR, but not VDJ recombination. The substrate of AID must be an RNA (ribozyme or mRNA) that participates in the first two processes only.

B-cell differentiation culminating in immunoglobulin synthesis and secretion

More comprehensive reviews and extensive references are cited elsewhere^{42,43}. Class switching occurs in antigen (or surrogate antigen)-activated cells that are stimulated into cell cycle and proliferation. After a number of cell divisions, the cells may undergo one of three possible fates: they may die, or they may differentiate into memory B-cells or into plasma cells. Somewhere along the survival pathway they may switch to another antibody class. In the germinal centres of secondary lymphoid organs, on which most studies of somatic mutation, antibody affinity maturation and class switching have focused, cells mature along a defined pathway as they pass from one compartment to the next. Naïve B-cells are activated by specific antigen and cognate interaction with T cells outside of the germinal centres in the T cell-rich zone. Depending on the level of CD40 signalling, such B-cells differentiate into primary plasma cells secreting IgM or into founder B-cells, which enter the germinal centre reaction. After migrating into the dark zone of the germinal centre, the founder B-cells undergo rapid proliferation (6 h cycling time) and become centroblasts. Somatic hypermutation occurs at this stage. Migrating into the light zone as smaller centrocytes, the B-cells then compete for antigen, presented by follicular dendritic cells, and either die or progress into the extrafollicular T cell-rich zone. There they differentiate into memory B-cells or plasma cells. The different cell types are characterised by cell markers, on the basis of which separations can be achieved. Germline gene transcripts (GLTs) and switch circles are found only in centroblasts and centrocytes⁴⁴: this fixes the time of class switching during B-cell development.

B-cell development has also been followed after stimulating the human B lymphoma line, CL-01, with IL-4, IL-10, IL-6 and CD40-L or anti-CD40 *in vitro*⁴⁵. As before, GLTs and switch circles appear in the centroblast and centrocyte fractions. IL-6 is required for differentiation of the cells to plasmacytes, a process that is blocked by anti-IL-6. Anti-TGF- β inhibits the synthesis and secretion of IgA, while anti-IL-10 inhibits the synthesis of IgG (but not of Cy CSR). Clearly then, despite the critical stages of germline gene transcription and DNA recombination, the fate of a B-cell is further determined, and IgE synthesis and secretion regulated, by post-CSR events accompanying cell differentiation.

Informative results have also emerged from studies on class switching in cytokine-stimulated B-cells as a function of cell cycle stage and the number of cell cycles completed. Hodgkin and co-workers separated murine B-cells, stimulated with IL-4 and anti-CD40, into populations which had divided from 1 to 10 times. Analysis of the antibodies produced by these cells showed that IgG appeared after about three rounds of replication and IgE after five⁴⁶. These authors concluded that

CSR was a stochastic process with a probability determined by the number of cell divisions. *Trans*-splicing (Fig. 4B) could operate as a stochastic mechanism.

Other workers separated the B-cells at different stages of the cell cycle⁴⁷. Germline gene transcripts were found only in cells that had progressed into cycle and had reached the G1/S boundary. Low numbers were found at the G2/M stage, when the DNA is replicated and the cells divide. If class switching involves DNA breakage and repair, this would make excellent sense, because genome integrity is required for viability during mitosis. This cell cycle connection may also shed light on the results of Hasbold and co-workers, mentioned above⁴⁶, as germline gene transcription is required for CSR and may need to be repeated in successive cycles for the chance event(s) to occur with sufficient frequency (Fig. 4B).

IL-4 does not uniquely specify IgE, for it also stimulates class switching to IgG and IgA. The probability of switching to IgE increases with the number of cell divisions, perhaps because the cell can switch to IgG or IgA1 before settling on IgE. Alternatively, the longer distance between the donor S and acceptor S ϵ may affect the probability of switching. Less obvious characteristics of the S region sequences, such as the length and conformation of single-stranded segments of the DNA, the nature and number of protein binding sites, the distribution of polymerase pausing sites and the existence of 'hot spots' for DNA cleavage, may also play a part.

Isotype selection at the post-CSR is a neglected subject. Some light is now beginning, however, to fall on this dark corner. We have dwelt on the synergism between IL-4R and CD40 in promoting ϵ -germline gene transcription. IL-4 also stimulates, by a less well-characterised pathway, changes in the cytoskeleton of B-cells, which engender the extrusion of microvilli and onset of motility⁴⁸. IL-4 promotes homotypic association of B-cells and heterotypic association of B with T cells. These interactions may be important because the strength or duration of the B-cell-T cell contacts may govern the number of times the B-cell proliferates before differentiation into a plasma cell; this may determine whether CSR continues from IgG or IgA1 to IgE. Another Th-2 cell cytokine, IL-10, has different activities, and instead promotes the differentiation of cells that have undergone CSR at the C γ 1 or C γ 3 loci into IgG1- and IgG3-secreting plasma cells⁴⁹. Premature differentiation may deprive the immature B-cells of the option to undergo a further switch to IgE. As B-cells proliferate and detach from the cell clusters, they must be exposed to autocrine and paracrine IL-6, which promotes cell differentiation.

IL-4 stimulates transcription of the gene for the low-affinity IgE receptor, CD23b, which first appears on the membranes of B-cells and is then proteolytically cleaved with release of a soluble fragment (sCD23). sCD23 induces B-cell differentiation into plasma cells and up-regulates

Bcl-2, thereby rescuing B-cells from apoptosis⁵⁰. Bcl-2 may enable B-cells to complete a developmental programme without abortion. sCD23 has also been shown to stimulate class switching to IgE⁵¹. CD23 co-ligates sIgE and CD21 on the surface of B-cells (see Fig. 3 in Aubry *et al.*⁵²). This provides a synergistic signal for the preferential growth of cells committed to IgE synthesis⁵³ and is thus a model for isotype-, as distinct from antigen affinity-based B-cell selection.

Conclusions

At the conclusion of Ig class switching, a B-cell secretes an antibody characterised by one of nine antibody classes and any one of an effectively infinite number of possible antigen specificities. The antigen specificity is fixed (except for somatic hypermutation), but class switching specifies the antibody class. Each of the three stages of this process – germline gene transcription, DNA recombination and B-cell differentiation – is biochemically complex, and the range of antibody classes that the B-cell may ultimately express is reduced at each stage. For IgE the activation of ϵ -germline gene transcription is essential, and the requirement for multiple rounds of cell division is critical for class switch recombination. At the final stage of B-cell differentiation, additional factors are involved in isotype determination, and IL-4 is once again required for IgE expression.

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