

S ϵ S μ and S ϵ S γ Switch Circles in Human Nasal Mucosa Following Ex Vivo Allergen Challenge: Evidence for Direct as Well as Sequential Class Switch Recombination¹

Lisa Cameron,^{2*†} Abdelilah Soussi Gounni,* Saul Frenkiel,* François Lavigne,* Donata Vercelli,[†] and Qutayba Hamid*

B cells switch to IgE under the influence of IL-4, IL-13, and CD40 costimulation through a multistep process involving ϵ germline transcription and class switch recombination. Classically, switching has been considered an event restricted to lymphoid tissues; however, ϵ germline transcripts (I(initiator) ϵ RNA) have been observed within lung, sinus, and nasal tissue of individuals with asthma, sinusitis, and rhinitis. Furthermore, nasal mucosal tissue from allergic rhinitis produces ϵ germline transcripts following ex vivo allergen challenge. Collectively, these studies raised the possibility that switching to IgE may occur locally, at sites of allergic inflammation. Although ϵ germline transcripts are considered necessary to target the IgE locus, it is class switch recombination that ultimately leads to de novo IgE production. In this study, we demonstrate that S ϵ S μ DNA switch circles (products of class switch recombination) as well as I ϵ and C ϵ RNA are produced within nasal tissue from allergic individuals following ex vivo allergen challenge. ϵ germline transcription was inhibited when tissue was cultured with a combination of allergen and neutralizing Abs against IL-4 and IL-13, indicating that de novo cytokine production mediated the isotype switch. We also show allergen-induced appearance of S ϵ S γ DNA switch circles and up-regulation of C γ 4 mRNA, illustrating that sequential switching to IgE also occurred. This work strongly suggests that B cells residing within the nasal mucosa undergo switching to IgE in the context of a local immune response to allergen. *The Journal of Immunology*, 2003, 171: 3816–3822.

Immunoglobulins are comprised of an Ag recognition domain (VDJ region) and an effector domain (C region). The five C region genes, C μ , C δ , C γ , C ϵ , and C α , are found downstream of the VDJ segments. Each C H , except C δ , is accompanied by its own promoter, I H exon, and switch region (S H).³ Isotype switching to the γ , ϵ , and α isotypes occurs through a multistep process that involves germline (GL) transcription and class switch recombination (CSR). B cells require two signals to undergo isotype switching. First, GL transcription is initiated when cytokines target the promoter of a specific isotype, resulting in the production of transcripts that have I H spliced directly to the acceptor site in the first exon of the C H gene (reviewed in Refs. 1 and 2). In the case of IgE, ϵ GL transcription is initiated by IL-4 and/or IL-13 (3, 4). Although GL transcripts are not translated, due to stop codons within I H (5), gene-targeted mutations of I H promoters have confirmed that they are necessary for CSR (6–8). It has been proposed that they may direct recombination by creating RNA/DNA hybrid structures recognized by processing enzymes (9, 10). However, synthesis of GL transcripts is not sufficient for DNA recombination to take place.

The second required signal for isotype switching is cross-linkage of CD40 on B cells, since defects in the CD40L gene are associated with X-linked hyper-IgM syndrome and deficiency in downstream Igs (11, 12). Activated T cells express CD40L (13), and engagement of CD40 on B cells in the presence of IL-4 or IL-13 induces CSR from IgM to IgE (4, 14). The recombinase activity induced by these pathways aligns the targeted S H region so that it is adjacent to S μ in the rearranged Ig locus. The DNA lying between S μ and S H is deleted, so that the targeted C H region is placed adjacent to VDJ, forming the template from which RNA coding for the new isotype is transcribed. The ends of the deleted DNA are spliced together at the S regions, giving rise to circular DNA products called switch circles (15, 16). In addition to direct isotype switching to IgE by IgM-expressing B cells, sequential switching to IgE through the γ locus also occurs. This was first observed in mice when switch circle fragments showed that in some cases the S ϵ and S μ were flanked by S γ 1 sequence (17) and was later confirmed in human B cells by demonstrating S μ S γ S ϵ junctions on the chromosome (18, 19).

Allergen-specific IgE can be found within the nasal mucosa and lavage of allergic rhinitis following allergen exposure (20, 21). Although isotype switching has classically been considered an event restricted to germinal centers within lymph nodes and spleen, ϵ GL transcripts have been observed within nasal (22), ethmoidal (23), and bronchial (24) biopsies of individuals with rhinitis, sinusitis, and asthma following in vivo allergen exposure. Furthermore, the nasal mucosa from individuals with allergic rhinitis produces ϵ GL transcripts following ex vivo allergen challenge (25). These studies raise the possibility of local isotype switching to IgE at sites of allergic response. However, GL transcription is not followed by CSR unless additional signals are delivered to the B cells. Detection of molecular markers of DNA rearrangement is therefore needed to confirm that IgE switching occurs locally at sites of allergic inflammation. We reasoned that

*Meakins-Christie Laboratories, Department of Pathology and Medicine, McGill University, Montreal, Canada; and [†]Functional Genomics Laboratory, Arizona Respiratory Center, University of Arizona, Tucson, AZ 85724

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² Address correspondence and reprint requests to Dr. L. Cameron, Functional Genomics Laboratory, Arizona Respiratory Center, 1501 N. Campbell Avenue, Tucson, AZ 85724. E-mail address: lcameron@resp-sci.arizona.edu

³ Abbreviations used in this paper: S, switch region; CSR, class switch recombination; Dig, digoxigenin; GL, germline; I, initiator; PNU, protein nitrogen units.

ex vivo allergen stimulation would be ideally suited to prove that the ability to switch to IgE is a property intrinsic to the nasal mucosa. In this work, we show that, indeed, Se switch circles appear in the nasal mucosa in response to ex vivo allergen exposure. Allergen also induced an increase in ϵ GL transcription, the earliest molecular marker of switching, which was inhibited by blocking IL-4 and IL-13. Collectively, these findings strongly suggest that B cells undergo CSR to IgE within the nasal mucosa in the context of a local immune response. Furthermore, detection of allergen-induced $\text{SeS}\gamma$ as well as $\text{SeS}\mu$ DNA switch circles indicates that both direct and sequential switching occurred in this tissue.

Materials and Methods

Tissue culture

Tissue culture was performed, as described previously (25, 26). Briefly, nasal mucosal biopsies from the inferior turbinate were obtained outside the ragweed season from patients with allergic rhinitis and skin test positivity to ragweed ($n = 10$). Tissue was sectioned into multiple samples of $\sim 6 \text{ mm}^3$, placed in 2 ml of defined medium in six-well plates on filtered inserts of $0.4 \mu\text{m}$, and treated with ragweed or olive tree extract (500 protein nitrogen units (PNU)/ml; Hollister-Stier, Spokane, WA) in the presence or absence of neutralizing Abs for IL-4 and IL-13 ($10 \mu\text{g/ml}$; R&D Systems, Minneapolis, MN) or murine anti- β -galactosidase IgG1 Ab ($10 \mu\text{g/ml}$; Sigma-Aldrich, St. Louis, MO). After 24 h of culture at 37°C , tissue was fixed in 4% paraformaldehyde, washed in 15% sucrose/PBS, blocked in OCT embedding medium by immersion in isopentane (cooled in liquid nitrogen), and stored at -80°C until used. Tissue for PCR experiments was frozen at -80°C immediately following culture.

Probes

RNA probes complementary to I ϵ or C ϵ RNA (27) were labeled with ^{35}S by in vitro transcription. To this purpose, plasmids containing fragments of I ϵ or C ϵ cDNA were linearized and incubated with dNTP, ^{35}S -labeled UTP, and the appropriate RNA polymerase (T7 and Sp6, respectively). This gave rise to a 650-bp I ϵ probe and a 526-bp C ϵ probe with ^{35}S incorporated at every UTP site. A 30-bp consensus γ -chain oligonucleotide probe (27) and a $\gamma 4$ -specific oligonucleotide probe (TCCAAATATGTTC CCCATGCCCAT, position 902–926 of GenBank accession number K01316) were 3' end labeled with dd-11-UTP-digoxigenin (Dig) using terminal transferase (Roche Biochemicals, Indianapolis, IN), according to manufacturer's specifications.

In situ hybridization

To prepare the tissue for hybridization, slides were incubated in Triton X-100 (3%) and proteinase K ($1 \mu\text{g/ml}$) to degrade membrane proteins and allow for probe entry. Sections were then hybridized with ^{35}S -labeled probe (0.75×10^6 cpm/slide) or Dig-labeled probe (0.55 ng/slide) and incubated overnight in a humid chamber at 40 – 42°C . Posthybridization washes were in decreasing concentrations of SSC (4 – $0.1\times$). Slides hybridized with RNA probes were treated with RNase A ($20 \mu\text{g/ml}$), to destroy excess single-stranded probe. Hybridization of radiolabeled probe was detected by dipping slides in liquid emulsion, with development following 15 days of exposure at 4°C . Slides hybridized with Dig-labeled probes were washed in 1% BSA/TBS to block nonspecific binding, and incubated for 4 h at room temperature with Ab against Dig. Signal was visualized following a 50-min development using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chromogens. With these methods, probe/mRNA hybrids were visualized as either a discrete accumulation of silver grains overlying the cells or as cell-associated purple staining, respectively.

Double in situ hybridization

To ascertain the coexpression of two RNA transcripts, double in situ hybridization was performed. ^{35}S -labeled I ϵ RNA and Dig-labeled C ϵ RNA probes were used, following a modified protocol in which slides were hybridized with both probes simultaneously and Dig staining was developed the next day. Slides were subsequently dipped in liquid emulsion for silver grain detection with an abridged exposure time of 7 days.

Quantification

In situ hybridization data were obtained using an Olympus Light Microscope (Carson Group, Montreal, Canada) at $\times 200$ magnification. Results

were quantified, as previously described (26, 28–30). Briefly, cells exhibiting hybridization signal for ^{35}S -labeled probes were readily identifiable as discrete, cell-associated clusters of silver granules overlying the cells. For radioactive in situ hybridization experiments, positive signal was first identified by the dark field filter, in which the silver granules are illuminated, and subsequently with phase contrast to be sure that the granules were indeed cell associated. Cells positive for Dig-labeled probe were observed under light field illumination. Due to the limitation of section size, because tissue was cut in cross section to have both epithelial and submucosal tissue represented, only three fields of vision, on average, were available for counting. Data in figures represent the mean number of positive cells counted for three fields of vision $\pm$ SEM. Statistical significance was determined using the Student's t test with WebStat v2.0 (www.stat.sc.edu/webstat).

Circular DNA fractions

Tissue from stimulated or control cultures was lysed with alkaline SDS (50 mM NaCl , 2 mM EDTA , $1\% \text{ SDS}$, $\text{pH } 12.4$). Tissue lysates were prepared by homogenizing with the piston of a 1-ml syringe, vortexing for 5 min, and centrifuging at 30°C for 1 h. The final lysates were neutralized with 1 M Tris-HCl (0.05 vol , $\text{pH } 7.1$) and 5 M NaCl (0.1 vol), followed by treatment with proteinase K ($100 \mu\text{g/ml}$) for 30 min at 37°C . After phenol extraction, the aqueous phase containing circular DNA was precipitated with absolute ethanol (2.5 vol). Precipitated DNA was digested with *EcoRI* and RNase A for 1 h at 37°C . Because the $\text{S}\mu$, Se , and $\text{S}\gamma$ regions do not contain *EcoRI* sites, digestion linearized the circles, but did not destroy the S region junctions. This DNA was used as PCR template for amplification of switch circle fragments.

Polymerase chain reaction

PCR was performed, as described by Zhang et al. (31), in a $50 \mu\text{l}$ vol reaction with $10\% \text{ DMSO}$, 50 mM KCl , 20 mM Tris-HCl ($\text{pH } 8.4$), 2.5 mM MgCl_2 , $80 \text{ pM primer/reaction}$, and $2.5 \text{ U Taq polymerase}$ (Platinum; Invitrogen, Life Technologies, Carlsbad, CA). From the circular DNA fraction, $2.5 \mu\text{l}$ was used as template DNA for each reaction. The PCR mix was incubated at 94°C for 10 min (without *Taq* polymerase), 65°C for 10 min (*Taq* polymerase added), 72°C for 10 min, and then 40 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 5 min, with a 15-min extension at 72°C in the final cycle. In some cases, template amplified using primers 1 and 2 or 1 and 5 was further amplified using nested primers 3 and 4 ($\text{SeS}\mu$) or 3 and 6 ($\text{SeS}\gamma 4$; Fig. 1 and Table I). Second round PCR was done at 94°C for 7 min, 40 cycles of 94°C for 1 min, 68°C for 1 min, 72°C for 1 min, and an extension of 15 min at 72°C in the final cycle.

DNA sequencing

PCR products were purified using GeneClean II Kit (Bio 101, Vista, CA), subcloned by TA cloning (Promega, Madison, WI), and sequenced.

Results

Ex vivo allergen exposure regulates the expression of ϵ GL transcripts in the nasal mucosa

GL transcription and CSR are both necessary for isotype switching. To determine whether allergen-dependent CSR occurs within the nasal mucosa, we analyzed nasal tissue for the presence of molecular markers signaling the occurrence of these critical events. GL transcripts have I ϵ spliced to C ϵ (I ϵ -C ϵ), while mature ϵ mRNA transcripts contain the VDJ segment spliced directly to C ϵ (VDJ-C ϵ). Therefore, the I ϵ probe we used detects exclusively ϵ GL transcripts, while the C ϵ probe identifies both GL as well as mature ϵ RNA. Our probes were highly specific because I ϵ transcripts were detected in tissue cultured with ragweed (Fig. 2B), but not medium alone (Fig. 2A). Fig. 3 demonstrates that ragweed extract induced a dose-dependent increase in the numbers of cells expressing I ϵ and C ϵ RNA in sections of nasal mucosal tissue from ragweed-sensitive patients ($n = 3$) after 24 h of culture. Tissue cultured in medium alone exhibited baseline numbers of both I ϵ (1.3 ± 0.29) and C ϵ (1.3 ± 0.63) RNA $^+$ cells; however, there were more cells expressing both these transcripts in tissue cultured with ragweed allergen. This response was steadily elevated with increasing concentrations of allergen and was maximal at 500 PNU/ml (I ϵ , 7.3 ± 0.88 ; C ϵ , 9.7 ± 0.29 ; $p < 0.01$), accounting for a 5.6- and 7.5-fold induction, respectively. As such, additional

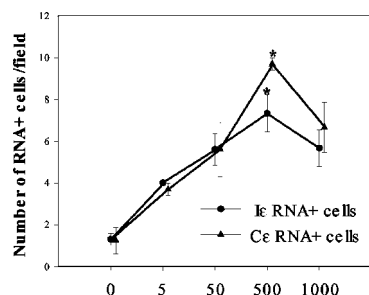


FIGURE 3. Ex vivo allergen exposure regulates the expression of ϵ GL transcripts in the nasal mucosa. Nasal mucosal tissue obtained from asymptomatic allergic individuals was cultured in the presence of increasing concentrations of ragweed extract (5–1000 PNU/ml) or medium for 24 h. Sections of tissue underwent in situ hybridization with ^{35} S-labeled RNA probes. Data represent the number of cells expressing I ϵ (●) or C ϵ (▲) RNA calculated from three fields of vision/tissue section from three individuals (a total of nine fields) (*, $p < 0.01$).

Ex vivo allergen challenge induces IgG4 RNA expression in the nasal mucosa

Because sequential switching from IgM to IgE through IgG4 has been reported (18, 19, 31), we examined the presence of IgG4-expressing B cells within nasal mucosal biopsies. Due to exceptionally high sequence similarity between the IgG subclasses, it was impossible to design a probe specific for $\gamma 4$ GL transcripts. However, using a probe complementary to the hinge region of IgG4 (sequence unique to $\gamma 4$) we were able to investigate the expression of C $\gamma 4$ RNA. Fig. 5 demonstrates that unstimulated tissue exhibited only a few cells expressing C $\gamma 4$ RNA transcripts (1.83 ± 0.17), while ragweed stimulation resulted in a 3.5-fold induction of C $\gamma 4$ RNA-expressing cells (6.3 ± 1.9 , $p < 0.05$, $n = 3$). Additional experiments with a consensus C γ probe demonstrated that the increase in total C γ RNA (2.9-fold) was highly similar to the increase in C $\gamma 4$ RNA, suggesting that the majority, if not all, of IgG RNA within the nasal mucosal biopsies was likely to be IgG4 (data not shown). The increase in C $\gamma 4$ RNA was not observed within tissue cultured with a combination of ragweed extract and Abs against IL-4 and IL-13 (1.67 ± 0.33). Furthermore, like the I ϵ and C ϵ response, the number of C $\gamma 4$ RNA $^{+}$ cells was not affected by culture with olive tree extract (1.83 ± 0.17). Thus, induction of $\gamma 4$ RNA was both allergen dependent and al-

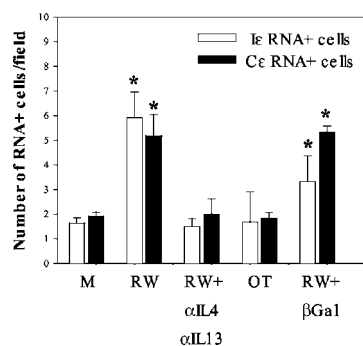


FIGURE 4. Allergen-induced expression of I ϵ and C ϵ RNA is inhibited with neutralizing Abs to IL-4 and IL-13. Nasal mucosal tissue was cultured for 24 h in medium (M), ragweed extract (RW; 500 PNU/ml), ragweed with neutralizing Ab to IL-4 and IL-13 (10 μ g/ml each), olive tree extract (OT; 500 PNU/ml), or ragweed with murine anti- β -galactosidase IgG1 Ab (10 μ g/ml) for 24 h. Data represent the number of cells expressing I ϵ or C ϵ RNA calculated from three fields of vision/tissue section from six individual independent patients (a total of 18 fields) (*, $p < 0.01$).

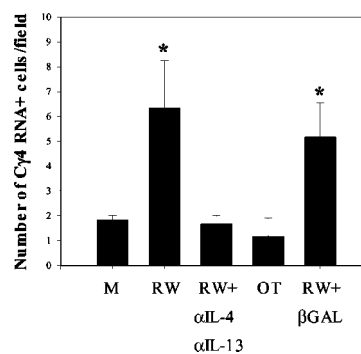


FIGURE 5. Expression of C $\gamma 4$ RNA following ex vivo allergen exposure. Nasal mucosal tissue was cultured for 24 h in medium (M), ragweed extract (RW; 500 PNU/ml), ragweed with neutralizing Ab to IL-4 and IL-13 (10 μ g/ml each), olive tree extract (OT; 500 PNU/ml), or ragweed with murine anti- β -galactosidase IgG1 Ab (10 μ g/ml) for 24 h. Data represent the number of cells expressing I ϵ or C ϵ RNA calculated from three fields of vision/tissue section from three independent patients (a total of nine fields) (*, $p < 0.01$).

lergen specific and also required local production of the Th2 cytokines IL-4 and IL-13.

Ex vivo allergen challenge induces direct (μ to ϵ) as well as sequential (γ to ϵ) switching within the nasal mucosa

Although GL transcription is required for isotype switching (8), the presence of these transcripts does not prove that switching has actually taken place. To determine whether isotype switching to IgE occurred de novo within the nasal mucosa upon allergen challenge, the circular DNA fraction was extracted from tissue cultured for 24 h with or without ragweed extract and used as a PCR template. In all individuals examined ($n = 3$), products were amplified only from allergen-stimulated tissue. Primer sets (Fig. 1, Table I) designed to amplify S ϵ S μ junctions within the switch circle generated amplicons from ragweed-stimulated tissue isolated from subjects 1 and 2 (Fig. 6A). No S ϵ S μ amplification product was observed in tissue derived from the third subject. Since allergen exposure occurred ex vivo, and S ϵ S μ fragments were not observed within tissue cultured in medium alone, these results strongly suggest that B cells residing within the nasal mucosa underwent local CSR. Sequencing confirmed that the switch fragment of subject 1 was derived from S ϵ and S μ sequence (Fig. 6C), indicating that indeed direct switching from IgM to IgE had taken place.

To determine whether sequential switching to ϵ through the γ locus occurred within the nasal mucosa following ex vivo allergen challenge, PCR was performed with primers for S ϵ S γ switch circles. Product was amplified and cloned from tissue cultured with allergen (subjects 2 and 3, Fig. 6B), but not medium alone (data not shown), and sequencing confirmed that the fragments contained S ϵ S γ junctions (Fig. 6C). This finding suggests that at least a proportion of B cells within the nasal mucosa underwent allergen-dependent IgG $\rightarrow$ IgE class switching. Although the primers were designed from S $\gamma 4$ sequence, there is extremely high sequence similarity between the S γ regions (32). Although the S γ portion of all the switch fragments generated with the S ϵ S $\gamma 4$ primer set aligned to S $\gamma 4$ genomic sequence (GenBank accession number 56796), they also aligned to the other S γ regions (S $\gamma 1$, S $\gamma 2$, and S $\gamma 3$). In one case (subject 2), however, the S γ fragment included diagnostic nucleotides, indicating that it was derived from either the $\gamma 4$ or $\gamma 2$ S region (Fig. 6D).

sole population giving rise to C ϵ RNA-expressing cells. It is also possible that we underestimated the number of C γ 4-expressing cells. Due to the high sequence similarity between the γ subclasses, we were forced to use a short (25-bp) Dig-labeled DNA probe corresponding to the γ 4 hinge, the only region unique to γ 4 (39). The RNA probe used for C ϵ RNA detection was longer (526 bp) and more intensely labeled, because we used *in vitro* transcription with ³⁵S-labeled UTP. These differences in probe labeling, if anything, may result in the detection of lower numbers of C γ 4-expressing cells. We therefore conclude that the levels of allergen-induced C γ 4 transcripts may be at least equal to, if not higher than, C ϵ . Mills et al. (32) have demonstrated chromosomal switch junctions between S ϵ and all four of the γ subclasses, indicating that sequential switching to IgE can occur through any of the S γ regions. Based on sequence, we could rule out S γ 1 and S γ 3, but could not definitively identify the fragment as S γ 4. However, because the induction of IgG4 RNA in response to allergen challenge very nearly accounted for the total IgG RNA induction, it seems likely that the S γ 4 region gave rise to the observed S ϵ S γ switch fragments.

The S regions are composed of tandemly repetitive palindromic sequences thought to be involved in the formation of stem-loop secondary structure (40, 41). This three-dimensional structure, rather than the primary sequence of S regions, has been suggested to be the target for class switch recombinase, because S region replacement with a palindrome-rich multicloning site was still

functional (42). Computer-predicted structures for S regions indicate that their denaturation during GL transcription may result in transition from double-stranded stem regions to single-stranded loop regions (40). Chen et al. (43) recently demonstrated that S μ /S α junction sites on predicted secondary structure mapped close to transitions from single-stranded to double-stranded regions. Similarly, we observed that the breakpoints in the S ϵ S μ and S ϵ S γ fragments amplified from allergen-stimulated nasal tissue mapped to stem-loop structures on genomic sequence (Fig. 7). Furthermore, we noted the association of these breakpoints with the AGCT motif, which is also found on the palindromic sequences and stem-loop structures of hotspots of somatic hypermutation (41, 44, 45). To our knowledge, these observations may represent the first evidence from human primary B cells to support this structural recognition pattern for CSR.

The ability of the nasal mucosa to support CSR to IgE has profound implications for the pathogenesis of allergic disease. Indeed, the nasal mucosa has multiple features that make it an ideal site for immediate hypersensitivity reactions. It has been estimated that as a result of local switching, IgE-committed B cells are enriched by 100- to 1000-fold in the nasal mucosa compared with peripheral blood B cells (46). IgE synthesis persists in this tissue even in the absence of allergen exposure (47), and the rate of local IgE synthesis generously exceeds the minimum requirement for saturation of IgE receptors on mast cells (46). All of this suggests that the IgE network in the nasal mucosa of allergic individuals is poised to react whenever allergen exposure occurs. The IgE self-sufficiency of the nasal mucosa may also explain how many individuals diagnosed as nonatopic, based on skin prick tests and low serum IgE levels, nevertheless manifest allergy-like symptoms. Indeed, elevated levels of ϵ GL transcripts and mature ϵ mRNA have been observed within the ethmoid sinus and the lung even in individuals diagnosed with nonatopic sinusitis and asthma (23, 24). Interestingly, these data parallel observations of IgA isotype switching within the gut lamina propria (38), suggesting that local synthesis of Igs may be a property of peripheral tissues strategically located at the site of Ag entry.

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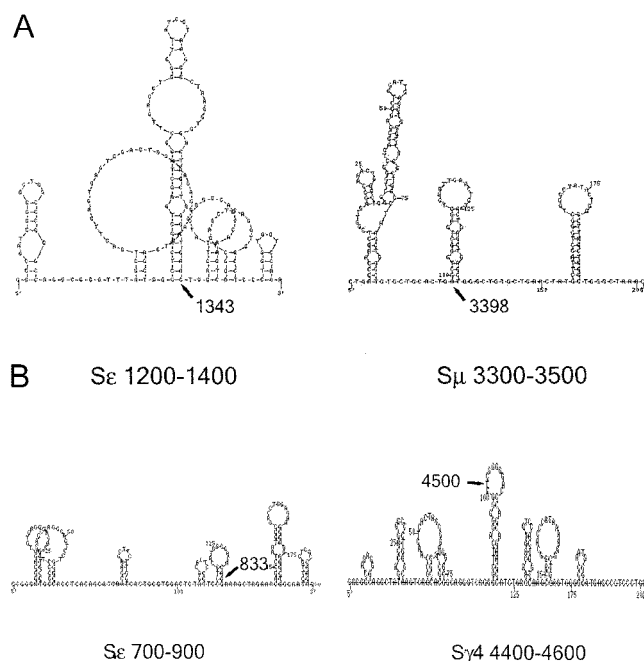


FIGURE 7. Association of S region breakpoints with secondary structure. Predicted secondary structure of 200 bases surrounding the S region breakpoints identified in switch circle fragments (GenBank accession number, S ϵ , X56797; S γ 4, X56796; and S μ , X56795) was generated using MFOLD software (bioinfo.math.rpi.edu/~mfold/dna/). The S ϵ /S μ and S ϵ /S γ junction sites all mapped to stem-loop structures. A, The S ϵ breakpoint of subject 1 (position 1343) was mapped to a stem 3 bases from the nearest loop, while the S μ breakpoint (position 3398) was on a stem within 4 bases of a loop. B, The S ϵ break of subject 2 (833) was on a stem 2 bases 3' of a loop, while the S γ 4 breakpoint (4500) was on a loop. Note the frequency of the AGCT motif surrounding these breakpoints, which is consistent with previous reports that these sequences may play a role in forming the stem-loop structure (43). The temperature parameter was set at 37°C, and salt concentration was 150 mM Na⁺.

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