CHAPTER 6

Genetic and Epigenetic Control of V Gene Rearrangement Frequency

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Abstract

The antibody repertoire is enormous and reflects the power of combinatorial and junctional diversity to generate a vast repertoire with a moderate number of V, D and J gene segments. However, although there are many V_H and $V\kappa$ gene segments, the usage of these genes is highly unequal. In this chapter, we summarize our studies elucidating many of the factors that contribute to this unequal rearrangement frequency of individual gene segments. Firstly, there is much natural variation in the sequence of the Recombination Signal Sequences (RSS) that flank each recombining gene. This genetic variation contributes greatly to unequal recombination frequencies. However, other factors also play a major role in recombination frequencies, as evidenced by the fact that some genes with identical RSS rearrange at very different frequencies in vivo. Analysis of these gene segments by chromatin immunoprecipitation (ChIP) suggests that differences in the structure of the chromatin associated with each gene is also a major factor in differential accessibility for rearrangement. Finally, transcription factors can direct accessibility for recombination, possibly by recruiting chromatin-modifying enzymes to the vicinity of the gene segment. Together, these factors dictate the composition of the newly formed antibody repertoire.

Introduction

The vast antibody repertoire is created by a combination of junctional diversity and combinatorial diversity. Each antibody heavy chain is encoded by a heavy chain and a light chain, the latter being encoded by either the kappa locus or the lambda locus. The heavy chain variable region is itself composed of three segments, V, D and J, while the light chain variable region has two segments, V and J. Combinatorial diversity is generated through the use of one each of the many V, D and J gene segments to encode the heavy and light chain exons and junctional diversity is generated by the deletion of a variable small number of nucleotides from the ends of each recombining gene segment and the random addition of a few nucleotides to the junction by TdT.[1] The BALB/c IgH locus contains ~50-100 functional V_H genes, 13 functional D_H genes and 4 functional J_H genes.[2-4] In the mouse, the random association of one V_H, one D_H and one J_H would theoretically create ~75 × 13 × 4 different H chains and the random association of light chain gene segments would similarly create ~50-100 $V\kappa$ × 4 $J\kappa$ kappa light chains and 4 different lambda chains. Further random association of heavy and light chains would thus create over a million different antibodies on the basis of combinatorial diversity alone. This theoretical diversity of combinations of gene segments has been thought to be a major factor in the size of the repertoire and given the large number of gene segments in the Ig loci, combinatorial diversity does contribute greatly to the antibody repertoire. However, we and others have shown that the rearrangement frequency

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of the different gene segments is very unequal and thus the contribution of some V gene segments to the repertoire is much smaller that that of other V genes.[5-8] Some of the genetic and epigenetic reasons for this difference in gene usage are summarized here.

Sequence Variation in RSS Can Greatly Affect Recombination

Each gene segment is flanked by a recombination signal sequence (RSS) which is composed of a conserved heptamer and nonamer, separated by a spacer of ~12 or ~23 bp.[9,10] The heptamer and nonamer have consensus sequences, but there is great natural variation in the sequences found in the Ig and TCR loci. The pioneering work of Gellert and colleagues using plasmid-based recombination substrates containing two RSS which can be varied in sequence clearly showed that the sequence of the heptamer and nonamer of the RSS were very important in determining the frequency of recombination.[9] By varying a nucleotide at each position of the heptamer and nonamer and varying the spacer length, general rules were established which showed that the first three bp of the heptamer were critical and changes in those positions away from the consensus almost abolished recombination. In contrast, variation in other positions showed a wide range of decreased recombination. These studies were complemented by the RSS database analysis by Ramsden and Wu of all published Ig and TCR RSS as of 1994.[11] They demonstrated that the first three base pairs of the RSS, CAC, were essentially invariant, whereas other positions of the heptamer and nonamer had more variability.

In order to assess whether the natural variation in RSS could be responsible for the unequal rearrangement frequency, we first identified the frequency with which specific V genes rearranged in vivo before any biological selection could occur. We analyzed rearrangement of murine V_H genes in μMT mice, in which the mutation in the transmembrane exon of the heavy chain prevented differentiation past the pro-B-cell stage and we also analyzed rearrangement of Vκ genes in human cord blood cells.[7,8,12-15] In both cases we identified which genes rearranged more often than others in vivo. Then, using a modification of the recombination substrate approach, we determined if the RSS could be responsible for this nonrandom rearrangement. We designed "competition recombination substrates" in which, for example, two Vκ genes competed for rearrangement to a Jκ gene, as shown in Figure 1.[13] In this way, small differences in recombination could be assayed by determining the relative frequency with which the Jκ gene rearranged to each of the two Vκ genes. Each of the RSS in our plasmids were made by PCR so that they included ~50-100 bp of flanking DNA on either side of the RSS.

The analysis of two Vκ alleles provides a clear demonstration of the ability of a single base pair in the RSS to significantly affect recombination frequency. The VκA2 gene is used in the majority of anti-*Haemophilus influenzae* Type b (Hib) antibodies.[16] Navajos and genetically related Native

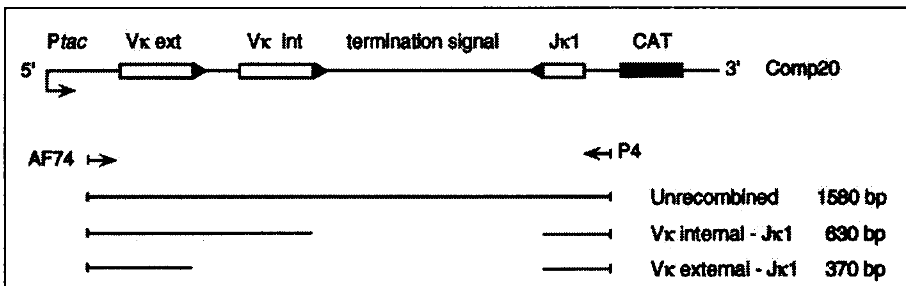

Figure 1. Competition recombination substrate. The top panel shows the basic design of the plasmid-based recombination substrate and the bottom panel shows the PCR assay used to determine the relative rearrangement of the Jκ gene to the internal or external Vκ. This basic design was used for all of our studies on the efficiency of various RSS. This figure is reproduced with permission from the Journal of Experimental Medicine, 1998, 187:1495-1503. Copyright 1998, The Rockefeller University Press.

Americans have a high incidence of Hib disease[17,18] and we discovered that they had a unique allele of the VκA2 gene, with one change from the predominant VκA2a allele at the 6th position in the heptamer.[15] Peripheral blood DNA from VκA2[a/b] heterozygotes showed that the VκA2a allele was rearranged ~5 times more often than this new Navajo VκA2b allele.[15] By placing the two VκA2 alleles in competition for a Jκ gene RSS in a recombination substrate, we were able to show that this single base pair change in the RSS was responsible for the difference in rearrangement frequency.[13] In this particular case, we hypothesized that this single nucleotide polymorphism was likely to play an important role in the increased incidence of Hib disease in Navajos, since impaired rearrangement of this Vκ gene would decrease the frequency of protective anti-Hib antibodies.[15] This would be one of the rare situations in which there was a genetic "hole" in the antibody repertoire with severe biological outcome: susceptibility to potentially fatal Hib disease.

RSS Is Not Always Responsible for Unequal Rearrangement

We found other examples where the rearrangement frequency in vivo was also recapitulated in the recombination substrate, demonstrating that the genetic basis for rearrangement differences was due to changes in the sequence of the RSS. For example, the small V_HS107 family has 3 functional V_H genes that rearrange at very different frequencies in vivo.[12] In pro-B-cells, the V1 gene rearranges 5 times more often than V11 and 40 times more often than V13. Using competition recombination substrates, we demonstrated that the V1 gene has an RSS that supports 3 times more rearrangement than the V11 RSS, thus accounting in large measure for the difference in rearrangement frequency in vivo.[14] However, V11 and V13 have very different rearrangement frequencies in vivo, yet their RSS are identical. Recombination substrate assays with ~100 bp fragments of V11 and V13 showed that the 5' and 3' DNA flanking the RSS also did not affect recombination frequency.[14] Hence, factors other than the RSS control the rearrangement frequency of these two V genes, as will be discussed later in this chapter.

In another example of genes with identical RSS rearranging at different frequencies, we analyzed the 20-member V_H7183 gene family. This is the most proximal V_H family, along with the V_HQ52 family that is interspersed with it in the 250 kb at the 3' end of the V_H locus. The most 3' functional V_H gene in this family, 81X, has been shown by several groups to rearrange at an extremely high frequency,[19,20] but the frequency of rearrangement of the other members of the family had not been determined. We analyzed the rearrangement frequency of the entire V_H7183 family in pro-B-cells and showed that the genes rearranged with a wide range of frequencies.[8] We cloned and sequenced each of the genes in the family and the RSS fell into two major groups. One group, which we termed Group I, had an RSS that was closer to the consensus than Group II and in competition recombination substrates, we showed that the Group I RSS supported a higher frequency of rearrangement than the Group II RSS, as would be predicted.[8] However, the rearrangement frequency of V_H genes with identical RSS was quite different in vivo in many cases and the Group I genes did not rearrange at a higher frequency than the Group II genes. Thus, factors other than the RSS were more important than the differences in the efficiency of the RSS in controlling gene rearrangement frequency for this V_H gene family. We mapped all of the V_H genes in the family and found a much higher correlation between chromosomal location and V gene rearrangement frequency.[8] The genes closest to 81X at the 3' portion of the locus rearranged more than the V_H genes in the middle of the locus and the genes in the 5' third of the locus rearranged very poorly, with the exception of the last V_H gene in the family, 61-1P. We propose that the chromatin structure may be different at these different portions of the V_H7183 part of the V_H locus, resulting in the observed different rearrangement frequencies for genes with identical RSS scattered throughout this 250 kb region.

Chromatin as the Gatekeeper of Accessibility

The process of gene rearrangement is lineage-specific, in that TCR genes do not rearrange in B-cells and Ig genes do not rearrange in T-cells, other than some D_H-J_H rearrangements.[21] Furthermore, this process of V(D)J rearrangement is highly ordered: D_H to J_H, followed by V_H to

DJ_H, followed by kappa rearrangement and lastly lambda rearrangement. The same order is observed in T-cells, with TCRβ rearrangement occurring before TCRα. Over 2 decades ago, Alt and colleagues proposed the "accessibility hypothesis" to explain these observations.[22] This hypothesis stated that accessibility to recombination would be limited to only certain small portions of the Ig or TCR loci in any given lymphocyte precursor population, e.g., the D_H and J_H subloci in early pro-B-cells. This hypothesis was supported by the observation that germline transcription of unrearranged genes precedes gene rearrangement, thus suggesting that this transcription reflected the induced accessibility for RAG binding and rearrangement.[23] The mechanism by which regions were maintained in inaccessible status until the proper time for their rearrangement was not clear at that time, but it is now generally agreed that chromatin structure is likely to be the key factor.[24]

The tails of histone proteins protrude from the core nucleosome and they can be posttranslationally modified by acetylation, methylation, phosphorylation and ubiquitinylation.[25,26] In general, lysines on tails of histones H3 and H4 are acetylated on active genes. Methylations are more complex and methylation of specific lysines, such as lysine 9 (K9me) or lysine 27 (K27me) on H3, are associated with repressed genes in general, while methylation of lysine 4 (K4me) on H3 is associated with active genes. It has been shown that V, D and J genes that are rearranging are more highly associated with acetylated H3 and H4 and less frequently associated with repressive modifications such as H3K9me, than genes that are not rearranging at that particular stage in lymphocyte development.[27-30] Thus, the status of histone posttranslational modifications (PTM) may control the accessibility of V, D and J genes.

Since histone acetylation appears to affect the accessibility of V, D and J genes, we hypothesized that perhaps the V genes that did not rearrange as well were associated with histones that did not have as high an extent of this positive PTM and were higher in negative PTM such as H3K9me. We therefore analyzed the V_HS107 gene family by chromatin immunoprecipitation (ChIP) with antibodies against acetylated H3 and H4. We were particularly interested in determining if there were any differences between V11 and V13, since they had identical RSS yet rearranged at such different frequencies. Indeed, there was an excellent correlation between the relative rearrangement frequency of the three V_HS107 genes and their enrichment in acetylated H3 and H4 (Fig. 2).[31] Furthermore, there was an inverse relationship between the level of the repressive modification

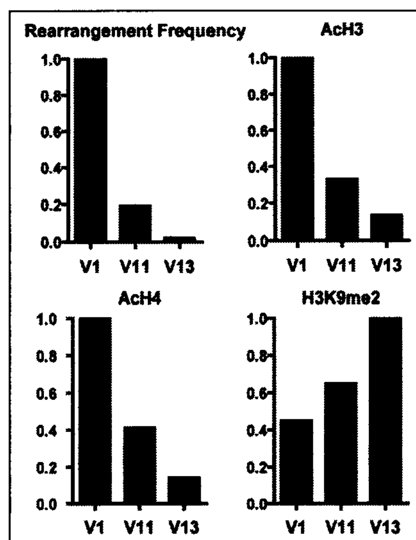

Figure 2. Relative rearrangement frequency in vivo of the three functional V_HS107 genes correlates positively with the extent of histone acetylation and negatively with the extent of histone K9 methylation.

H3K9me2 and the rearrangement frequency (Fig. 2).[32] Thus, the histone PTM status accurately reflects the relative accessibility for rearrangement of these genes.

We further investigated the histone PTM status of the 81X gene. This gene rearranges at a very high frequency in fetal life and also rearranges at a high frequency in adult bone marrow, although not as frequently as in fetal liver. We compared the histone acetylation status of this gene as compared to the rest of the V_H7183 family, using an 81X-specific primer and a primer that amplifies all V_H7183 genes except 81X. 81X was more highly enriched in acetylated histones than the remainder of the V_H7183 family and the extent of enrichment was greater in fetal life than in adult life, correlating with the relatively higher rearrangement of 81X in fetal life (Fig. 3).[31] As with the V_HS107 genes, there was a reciprocal relationship of these genes with the repressive H3K9me2 PTM.[32]

Further evidence that histone PTM may influence rearrangement frequency is demonstrated by analysis of mice deficient in the histone methyltransferase Ezh2, which adds the repressive K27 methylation PTM. The pro-B-cells from these mice do not rearrange the V_H genes in the distal half of the locus, although the proximal half rearranges at near normal frequency.[33] We have shown that the H3K27me PTM is found on the proximal V_H genes in pro-B-cells (C.-R. Xu and AJF, unpublished data) and thus we propose that the presence of this repressive PTM on the proximal V_H genes is necessary for the distal V_H genes to rearrange at normal frequency.

Role of Transcription Factors in Controlling Rearrangement

Although there clearly seems to be a good correlation between histone PTM patterns and accessibility for recombination, it is not clear what determines the histone modification status of genes. Histone acetylases, deacetylases and methylases are often recruited into large multi-protein complexes and it is likely that the specificity of these complexes derives from DNA-binding transcription factors. We have investigated the role of transcription factors in inducing accessibility for recombination. Mice that are deficient in EBF, Pax5, or E2A, are all devoid in B-cells, demonstrating the essential role of these transcription factors in B-cell differentiation.[34-37] The first two factors are B-cell specific and are essential for B-cell development. E2A is a widely expressed transcription factor, but it is only in B-cells that it is present as a homodimer and this probably explains the specific loss of B-cells in the E2A-deficient mice.[38]

Using a novel system, devised by our collaborator Dr Cornelis Murre in which expression vectors for E2A or EBF were transiently transfected, along with expression vectors for RAG1 and RAG2, into a nonlymphoid cell line, the ability of transcription factors to induce accessibility of genes for recombination was revealed.[39] Transient transfection with EBF resulted in the induction of rearrangement of Vλ3 genes, but not of any kappa genes. Conversely, the ectopic expression of E2A resulted in recombination of many VκI genes in this cell line. Importantly, although the three major Vκ

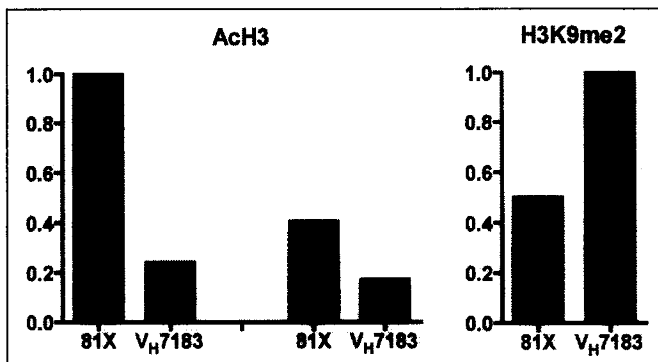

Figure 3. The frequently rearranging 81X gene is more highly enriched for histone acetylation and less enriched for H3K9 methylation than the remainder of the V_H7183 family.

families are interspersed, only the VκI genes are induced to rearrange, but not the neighboring VκII or VκIII genes (Fig. 4).[40] Thus, this means that the Vκ locus is not made accessible as a whole unit by the action of E2A, but that individual V genes, or related V genes such as members of a Vκ family, are induced on a localized level to become accessible. Since members of a Vκ or V_H family arose by gene duplication, their coding and flanking sequences are very similar. Therefore, we proposed that there are transcription factor binding sites in the vicinity of all functional V genes and that the binding of the appropriate transcription factor could then recruit chromatin modifying enzymes such as histone acetyltransferases or deacetylases, histone methyltransferases or demethylases, or ATP-dependent chromatin remodeling complexes, which would then change the chromatin structure of the V gene, making it accessible, or inaccessible, for recombination.[40]

We hypothesized that the expression of E2A would increase the histone acetylation of the VκI genes, but not of the VκII and VκIII genes which were not induced to undergo recombination after ectopic E2A expression. Similarly, we hypothesized that expression of EBF would increase the histone acetylation of Vλ3 genes specifically. We assessed this by ChIP, using primers that flanked the RSS and we found that this was indeed the case (P Goebel and AJF, unpublished data). Surprisingly, however, we found that the extent of acetylation of the appropriate genes was very modest. We

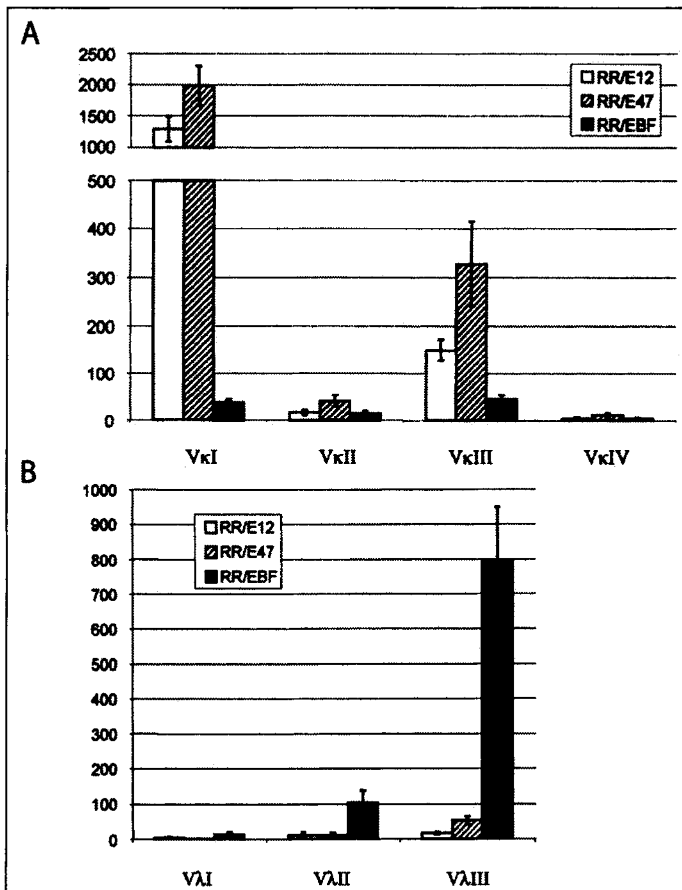

Figure 4. Ectopic expression of E2A in a nonlymphoid cell line induces preferential rearrangement of VκI genes (A), while ectopic expression of EBF induces preferential rearrangement of Vλ3I (B). This figure is reproduced with permission from the *Journal of Experimental Medicine*, 2001, 194:645-656. Copyright 2001, The Rockefeller University Press.

therefore proposed that only a small fraction of the Vκ genes are induced to become acetylated by E2A and that these genes are preferentially used for recombination. Similarly, our data showing that within the V_HS107 family, the V1 gene is most highly enriched in acetylated histones and V13 the least, most likely reflects the fact that more V1 genes within the population of pro-B-cells that we investigated were associated with acetylated histones.[31] This may suggest that the limiting factor for recombination is the number of V genes that are acetylated at any given time.

Pax5 also has an important role in V(D)J recombination, in addition to its role in controlling the expression of hundreds of genes critical for B-cell function.[41] Mice deficient in Pax5 are blocked at the late pro-B-cell stage of differentiation.[42] Although the proximal V_H7183 family rearranges at almost normal frequency, the distal V_HJ558 genes seldom rearrange and the V_H families in between these two families rearrange at intermediate levels.[43] An explanation for this could be that the V_H locus in Pax5-deficient pro-B-cells fails to undergo the compaction that appears to be critical to bring the distal V_H genes closer to the D-J_H locus.[44] In this extended configuration, the distal V_HJ558 genes, which are located 1-2.5 Mb from the D-J region, would be too far away from the DJ_H genes to undergo rearrangement. In addition, it has been shown that V_H genes in Pax5-deficient B-cells are enriched in the repressive modification H3K9me2 and it has been suggested that Pax5 is required for the histone exchange necessary to make the V_H genes associate with acetylated histone H3 and not K9 methylated H3.[45]

We have described another function for Pax5 which is important for V(D)J recombination. Although transcription factor binding sites are traditionally found in promoters and enhancers, we searched for Pax5 binding sites within V_H coding regions since we hypothesized that the RAG complex may bind Pax5. The reason for this hypothesis was the fact that the core RAG2 knock-in mice had a defect in V to DJ rearrangement, although DJ and kappa rearrangement was not impaired.[46] Since Pax5-deficient mice were originally reported to have the same generalized defect in V_H rearrangement, but not DJ rearrangement,[42] we hypothesized that perhaps the non-core region of RAG2 might bind to Pax5 which would stabilize its interaction with the RSS. A search of V_H gene sequences with the sequences of the Pax5 binding sites in KI, KII, RAG, CD19, showed several potential matches (AFJ, unpublished data) and EMSA analysis showed Pax5 did bind to several of these sites, with varying affinities.[32,47] The V_HS107 gene V1 had the highest affinity site and the V_H7183 genes had strong Pax5 binding sites also. The V_HJ558 genes also had Pax5 binding sites, although their affinity estimated by cold target competition was lower. ChIP analysis showed that Pax5 was bound to V_H genes in pro-B-cells.[47] Our collaborators, Zhixin Zhang and Max Cooper, showed that Pax5 interacted with RAG complex, although they showed that Pax5 also bound to complexes made with the core RAG1/2,[47] thus rendering our initial hypothesis that Pax5 may bind to the non-core portion of RAG2 unlikely. Using an in vitro assay, our collaborators found that Pax5 increased recombination, suggesting that the interaction of Pax5 with the RAG complex did stabilize the interaction.[47] In addition to this role of Pax5 in interacting with the RAG complex, we propose that these Pax5 sites located throughout the *IgHV* locus may be the reason that Pax5-deficient mice cannot undergo locus contraction and thus the function of these Pax5 sites in V_H genes may be to initiate *IgHV* locus contraction. Mice deficient in the transcription factor YY1 also have a defect in rearranging distal V_H genes and also do not undergo locus compaction[48] and thus a complex containing Pax5 and YY1 complex may be involved in the contraction of the locus.

Conclusion

The antibody repertoire derives part of its size from the combinatorial diversity generated when different V, D and J genes are used to encode the two chains of the receptor heterodimers. However, all V, D and J genes are used at very different frequencies. We have summarized work showing that part of this unequal representation is due to the natural variation in the sequences of the RSS flanking each gene. Since the RSS is the DNA binding site for the RAG recombinase, the mechanism for the influence of these genetic variations is clear. However, the differences in the chromatin structure of nucleosomes associated with individual V genes can override the simple

direct effect of the genetic variation in RSS efficiency in recruiting and stabilizing RAG binding. We found that the extent of positive or negative histone PTM can affect the ability of individual V genes to undergo rearrangement. One of the important unanswered questions is to determine what directs the histone modifications to occur on specific genes within the V loci. We hypothesize that specific transcription factors bind to sites near the V genes, in the promoter or even in coding regions as we have shown for Pax5. These proteins may then recruit histone-modifying enzymes, chromatin remodeling complexes and DNA methyltransferases. These epigenetic modifications would then render a gene more or less accessible or inaccessible to undergo rearrangement.

Acknowledgements

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