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The reverse transcriptase model of somatic hypermutation

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The evidence supporting the reverse transcriptase model of somatic hypermutation is critically reviewed. The model provides a coherent explanation for many apparently unrelated findings. We also show that the somatic hypermutation pattern in the human *BCL-6* gene can be interpreted in terms of the reverse transcriptase model and the notion of feedback of somatically mutated sequences to the germline over evolutionary time.

Keywords: immunoglobulin V(D)J genes; E_i/MAR-mediated reverse transcription; somatic hypermutation; error-prone copying; homologous recombination

1. INTRODUCTION

This paper briefly reviews the mechanics of the reverse transcriptase (RT) model of somatic hypermutation (Steele & Pollard 1987) and updates the wide array of data that now supports it (see also Steele *et al.* 1997; Blanden *et al.* 1998). We show how the RT model can provide a coherent explanation of apparently unrelated published data outside the ambit of DNA-based models (Umar *et al.* 1991; Umar & Gearhart 1995; Xu & Selsing 1994; Peters & Storb 1996) and why DNA repair-deficient mice still mutate their immunoglobulin V (IgV) genes (Wood 1998; Kelsoe 1998). We also show that recent data published on somatic hypermutation in the *BCL-6* gene expressed in human germinal centre B cells (Migliazza *et al.* 1995; Shen *et al.* 1998; Pasqualucci *et al.* 1998) and the pattern of differences between mouse and human germline *BCL-6* genes (Bernardin *et al.* 1997) are consistent with the RT model and with the notion that mutated somatic DNA has been fed back to the germline over evolutionary time (Blanden *et al.* 1998).

2. HISTORY

The reverse transcriptase model of somatic hypermutation was originally proposed to explain the locus-specific distribution of somatic point mutations in rearranged immunoglobulin variable genes, V(D)Js (Steele & Pollard 1987). At that time the molecular data were derived from hapten-specific B-cell hybridomas from immunized mice. The key issue was to explain how the mutations could be targeted to V(D)J regions of both heavy and light chains and their immediate 5′ and 3′ flanking regions. The occurrence of somatic point mutations at significant frequency ($\geqslant$1%) in the non-antigen selected J-C intron (Gearhart 1982; Gearhart & Bogenhagen 1983; Perlmutter *et al.* 1984) demanded a mechanism not restricted to the V(D)J coding region (Steele *et al.* 1991). These facts, plus the necessity to provide an explanation with a minimum number of key assumptions, led to the first iteration of the model (figure 1*a*). Rather than invoke a role for a special Ig locus-specific, error-prone DNA polymerase (e.g. DNA polymerase beta) we chose known evolutionarily ancient, enzymatic activities such as error-prone transcription and reverse transcription coupled to site-directed homologous recombination (gene conversion). Locus specificity was satisfied by predicting that error-prone reverse transcription would be initiated on the pre-mRNA in the J-C intron of the rearranged V(D)J gene downstream of the most distal J element and continue back through the V(D)J region to terminate naturally at the 5′-end of the mRNA (the cap site). Point mutations would be randomly scattered in the target region (apart from hot spots) at rates comparable with the known error rates of transcription and reverse transcription (10^{-3} to 10^{-4} per base pair per replication event). The homologous recombination step, inserting the mutated retrotranscript back into the same chromosomal site so as to replace the original V(D)J gene, would then ensure that both constant (C) regions and upstream promoter (TATA) elements were protected from the deleterious effects of point mutation. The location of RT priming in the J-C intron (providing the Ig locus specificity) was left unspecified but it must make priming possible 3′ of the most distal J element. The RT-priming process itself was not specified in detail. Alternatives suggested at the time were (i) premature termination of RNA synthesis; (ii) cleavage of pre-mRNA, coupled to hairpin loop RT priming; or (iii) specific primer binding sites by analogy with tRNA primers in retroviral reverse transcription (Steele & Pollard 1987). Later, multiple RT-priming sites in the J-C

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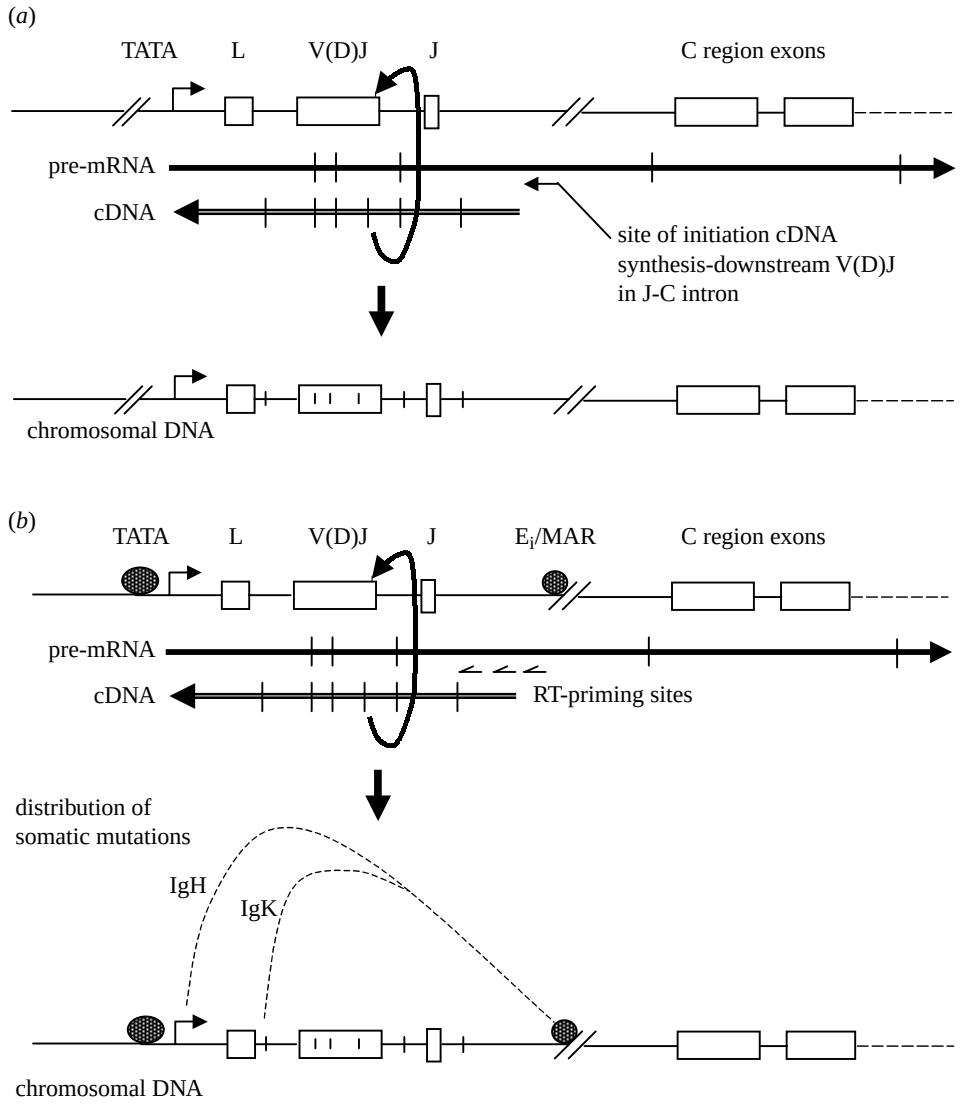

Figure 1. The reverse transcriptase mutatorsome. (*a*) Original RT model (Steele & Pollard 1987). (*b*) RT-mutatorsome concept updated to handle the original predictions and highlight the new data published over the ensuing ten years (Steele *et al*. 1997; Blanden *et al*. 1998). The relative positions of the promoter (TATA, larger shaded oval) the transcription start or mRNA cap site (right-pointing arrow), leader (L), rearranged variable region (indicated by the generic symbol V(D)J), an unrearranged J element (J), the J-C intronic enhancer/matrix attachment region (E_i/MAR, smaller shaded oval) and the constant (C) region exons. Point mutations introduced by error-prone transcription and reverse transcription indicated as vertical lines in the pre-mRNA or cDNA (vertical lines), and shorter vertical lines in the chromosomal DNA. The looping thick arrow indicates the homologous recombination step (one-way gene conversion) of the mutated cDNA retrotranscript back into the site of the V(D)J so as to replace the original unmutated sequence. The mutation frequency in RNA and cDNA is exaggerated to emphasize the error-prone nature of the DNA→RNA→DNA copying loop. Thin lines and rectangles are the 3′ to 5′ strand of the DNA with coding regions, thick filled lines with arrows indicate the direction of synthesis of RNA, and double lines with arrows indicate the direction of the back synthesis of cDNA. For all other explanations see § 2. Note that recently it has been shown unequivocally that deletion of promoter regions drastically reduces the rate of hypermutation, that an RNA polymerase-I-dependent promoter can replace the normal RNA polymerase-II promoter and that the level of transgene-specific pre-mRNA correlates with mutation frequency (Fukita *et al*. 1998). Furthermore, as with the interpretation of the somatic mutation distribution pattern for the human *BCL-6* gene (see figure 3 and § 2; Peters & Storb 1996, fig. 2*b*), the location of the E_i/MAR control region for mouse Vλ (Motoyama *et al*. 1991) and mouse TCR-Vα (Marshall *et al*. 1999) would have to be downstream of the 3′-end of the C region.

intron were invoked (Steele *et al*. 1992) to explain the asymmetrical distribution of somatic point mutations (Both *et al*. 1990; Lebecque & Gearhart 1990; Weber *et al*. 1991; Steele *et al*. 1992; Rothenfluh *et al*. 1993) (figure 1*b*). More recently, multiple priming sites and a number of alternative RT-priming mechanisms have also been advanced to attempt to explain both the asymmetry of the distribution and focusing to the particular V(D)J rearrangement undergoing hypermutation (discussed in Steele *et al*. 1997; cf. critical discussions on this point in Peters & Storb (1996) and Tumas-Brundage *et al*. (1996)).

3. KEY DEVELOPMENTS SINCE 1987

In our view, apart from the general analysis of data provided elsewhere (Steele *et al*. 1997; Blanden *et al*. 1998),

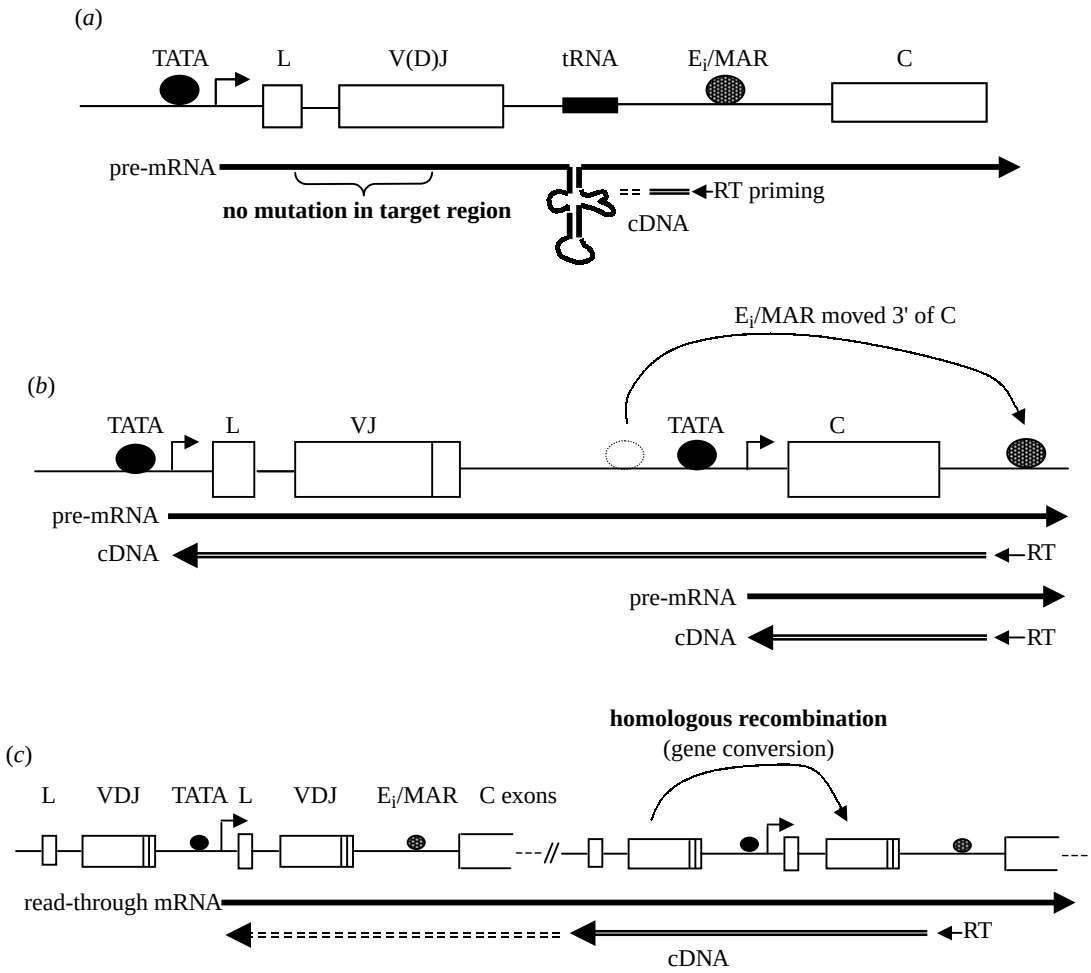

Figure 2. Apparently unrelated findings from the laboratories of Gearhart, Storb and Selsing that can be interpreted in terms of the RT model. See legend to figure 1 for definition of general terms within the gene diagrams. (*a*) A summary of the findings in Umar *et al*. (1991) and Umar & Gearhart (1995). Somatic hypermutation is inhibited in the V(D)J target region because reverse transcription, initiated in the J-C intron and controlled by E_i/MAR, is inhibited by the tRNA secondary structure. (*b*) A summary of the construct used by Peters & Storb (1996). The key modification of the standard VJκ transgene was the relocation of E_i/MAR from the J-C intron to a position downstream of C. An additional Ig promoter was positioned in front of the C region. These manipulations will ensure cDNA synthesis in two target regions: VJ and C. See § 4 for further discussion. (*c*) A summary of the construct used by Xu & Selsing (1994). The two closely linked and highly homologous VDJs are positioned upstream of an E_i/MAR and C region exons. The construct is integrated as multiple copies, ensuring read-through transcription from the multiple functional promoters in front of the downstream VDJ partner.

there have been three key developments since 1987 that are important in evaluating the validity of the RT model. The first has been the identification by Betz, Neuberger, Milstein and their colleagues (Betz *et al*. 1994) of the intronic enhancer/matrix attachment region (E_i/MAR) in the J-C intron as essential for somatic hypermutation. We interpret E_i/MAR as the 'locus-specific device' locating the initiation site(s) of RT priming on the pre-mRNA in the J-C intron (Betz *et al*. 1994). The importance of E_i/MAR for IgH loci has been confirmed *in vitro* for the hypermutating 18-81 B-cell line (Bachl *et al*. 1998).

The second development has been the universal failure of any of the DNA repair knockout mice to show significant reductions in the rate of somatic hypermutation (reviewed in Wood 1998; Kelsoe 1998; and also see Frey *et al*. 1998; Phung *et al*. 1999; Ehrenstein & Neuberger 1999). The core process of hypermutation remains intact in such mice despite a deficiency in a range of DNA mismatch repair and DNA excision repair genes. In addition, Park *et al*. (1998) have shown that isolated human centroblasts (from germinal centres) have an intact DNA mismatch system making it unlikely that downregulation of mismatch repair occurs *in vivo* during somatic hypermutation in germinal centres. More recently, Rajewsky's group have shown that mice reconstituted with foetal liver cells deficient in the error-prone DNA polymerase beta mutate their V(D)J genes normally (Esposito *et al*. 2000). All these data render DNA-based models unlikely (see Steele *et al*. (1991) for a more formal description of 'DNA-based models').

A third development has been the finding that spontaneous hypermutation of the non-productively rearranged T-cell receptor (TCR)-Vα in a T-cell hybridoma *in vitro* was correlated with the presence of mutated cDNA reverse transcripts of the TCR α-chain 'suggesting a role for reverse transcriptase in the generation of the mutations' (Marshall *et al*. 1999). This work also confirmed two earlier results showing IgV-like somatic

hypermutation in rearranged TCR-V loci, *in vivo* studies with hapten-immunized mice reported by Zheng *et al.* (1994) and in HIV patients by Cheynier *et al.* (1998).

4. DYNAMICS OF THE RT MODEL EXEMPLIFIED BY DATA OUTSIDE THE AMBIT OF DNA-BASED MODELS

We have found the RT model useful in the critical evaluation of the molecular data derived from transgenic mice carrying either productive or passenger V(D)J and non-Ig transgenes (Steele *et al.* 1997; Blanden *et al.* 1998). Taken together, all extant molecular evidence strongly favours a hypermutation model based on error-prone reverse transcription coupled to homologous recombination, as outlined in figure 1.

Key features that distinguish the RT model from all the alternative DNA-based models include its ability to account for Ig locus specificity and targeting to the V(D)J and immediate 5′ and 3′ flanks, its dependence on transcription and synthesis of a pre-mRNA template for reverse transcription, and the obligatory site-directed homologous recombination step. All these features are compatible with apparently unrelated findings from the laboratories of Gearhart, Storb and Selsing (figure 2).

Interference with the RNA template and/or the ability of the RT mutatorsome (Steele *et al.* 1997) to synthesize cDNA is predicted to inhibit somatic hypermutation. Therefore V(D)J transgenes with a reporter tRNA in the J-C intron upstream of E_i/MAR should not mutate in the V(D)J target region because cDNA synthesis will be prevented from proceeding through the tRNA sequence (figure 2*a*). The data published by Umar *et al.* (1991) and Umar & Gearhart (1995) support this prediction (also see Steele *et al.* (1992, 1997) for further explanation and analyses of these data).

Since the E_i/MAR determines where, and in what orientation, cDNA synthesis is initiated, its location in a construct will dictate which DNA region is mutated. This explains the hypermutation of the C region in the Igκ transgene construct of Peters & Storb (1996) because the E_i/MAR was removed from its normal location in the J-C intron and repositioned downstream of C (figure 2*b*). Explanations of this type (coupled to the additional concept of read-through transcription in tandem transgene arrays, see figure 2*c*) can be applied to the negative results reported in Hengstschlager *et al.* (1994) and the partial rescue of mutation recorded in Klix *et al.* (1998). In the latter case the E_i/MAR was positioned upstream of the transcription start site and the transgene was integrated as two copies per genome (presumably in tandem; Klix *et al.* 1998). Also of relevance here is the transfection data from the 18-81 B-cell line where repositioning of the IgH E_i/MAR in the reverse orientation reduced the mutation frequency by an order of magnitude compared with the correctly orientated control VDJ (Bachl *et al.* 1998).

The obligatory requirement for homologous recombination separates the RT model from all publicly articulated DNA-based models as this step is intrinsic to the hypermutation process. It is predicted that hypermutation will be inhibited or facilitated if this step is disrupted or enhanced, respectively. Thus Xu & Selsing (1994) prepared a double IgH transgene construct with two highly homologous VDJ genes closely linked 1.5 kb apart

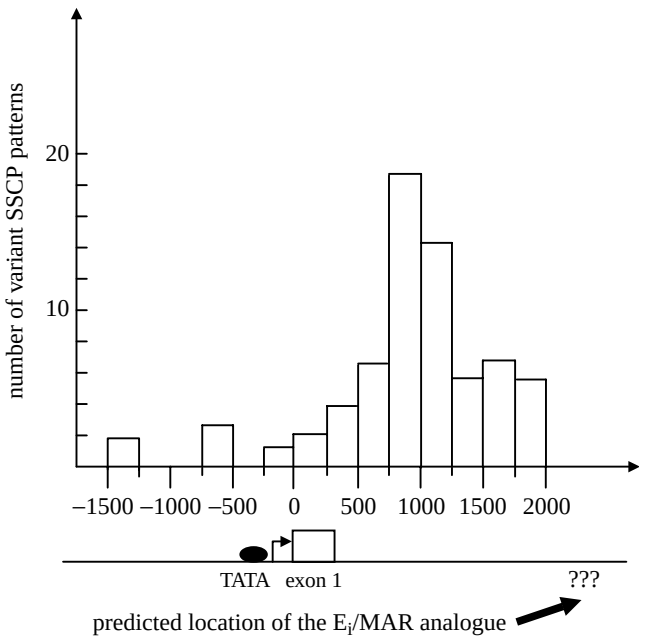

Figure 3. Distribution of single-strand conformational polymorphism variants around the 5′ non-coding region of the human *BCL-6* gene. This frequency distribution is plotted from the data presented in fig. 3 of Migliazza *et al.* (1995). The 250 bp intervals correspond approximately to the 15 tandemly overlapping PCR fragments amplified from 30 diffuse large-cell lymphomas (DLCL) and 15 follicular lymphomas (FL). Most *BCL-6* loci were normal (unrearranged) apart from ten DLCL lines with rearranged loci. The relative positions of the promoter (TATA), the transcription start or mRNA cap site (right arrow) and exon 1 are shown. The putative 'locus-specific device' (E_i/MAR analogue) is indicated to lie beyond 2 kb downstream of exon 1 in intron 1. See § 5 for further discussion.

(figure 2*c*). The upstream VDJ did not have a conventional promoter region and was assumed to be transcriptionally silent. However the resultant transgenic mice carried tandem arrays of 10–50 copies of the construct, a situation conducive to read-through transcription of the entire tandem array initiated by the multiple functional promoters of the downstream partner (figure 2*c*). When such mice were immunized, hybridomas displayed sequence transfers between the two genes. Point mutations were also common, indicative of somatic hypermutation. The most striking feature was the obligatory association between VDJ-region-targeted homologous recombination and the occurrence in the same hybridoma sequence of somatic point mutations. This apparent association between somatic hypermutation and homologous recombination was articulated by the authors: 'all the hybridomas that exhibited transgene conversion also showed somatic hypermutation even though half of the IgG-producing anti-Ars hybrids showed no evidence of hypermutation' and 'the marked absence among these hybridoma panels of cells that exhibit transgene conversion without accompanying hypermutation' (Xu & Selsing 1994). Although many of the molecular steps are unclear, this conclusion fulfils a key prediction of the RT model. Indeed, in a later review paper Selsing and colleagues tentatively entertained a possible role for reverse transcription in somatic hypermutation (Selsing *et al.* 1996).

An obligatory requirement for homologous recombination in the V(D)J and immediate vicinity may explain why rearranged Ig loci are hypersusceptible to homologous recombination during antigen-driven somatic hypermutation (Gerstein *et al.* 1990; Giusti & Manser 1993; Umar & Gearhart 1995; Tumas-Brundage *et al.* 1996).

Finally we should point out that the RT interpretation of somatic hypermutation can also be extended to explaining the gene conversion process, which generates diversity in rearranged V(D)J genes in B lymphocytes of chickens, unifying molecular processes that have hitherto been thought of as quite different (Blanden & Steele 1998).

5. E_i/MAR AND LOCUS SPECIFICITY OF SOMATIC HYPERMUTATION

We have outlined the central role of E_i/MAR in somatic hypermutation of Ig loci. It is conceivable that an analogue of this region could have been transposed during evolution to other loci in the genome, thus rendering the DNA upstream of it hypermutable if the locus is expressed in lymphocytes undergoing hypermutation. We would argue however that such hypermutation would have no consequence unless it impaired the function of a gene or its product. This argument is consistent with recent findings that the human *BCL-6* gene hypermutates only in its non-coding region (near the 5′ non-coding exon; see figure 3) and only in germinal centre B cells (Migliazza *et al.* 1995; Shen *et al.* 1998; Pasqualucci *et al.* 1998). Storb and colleagues have established that a range of other genes expressed in germinal centre B cells are not mutated (Shen *et al.* 1998; Storb *et al.* 1998). The features of the hypermutation phenomenon in human *BCL-6* are identical in every respect to V(D)J genes except for the fact that it is operating in a non-Ig locus. Thus the RT model (figure 1) predicts that an E_i/MAR analogue will be located downstream in the intron 1 region, beyond 2000 bp 3′ of exon 1. Since the RNA substrate for error-prone cDNA synthesis will be generated from the promoter in front of exon 1, the transcription start site should be the extreme 5′ boundary of mutation. However the intervention of RNA splicing at the exon 1–intron 1 border (coupled to 5′-end trimmings of the RNA) could mean that this region is the 5′ boundary for *BCL-6* somatic mutations. This prediction is consistent with the mutation distribution data (figure 3).

Another interesting feature of *BCL-6* genes concerns DNA sequence homology between the human and mouse genes, as illustrated in fig. 1 of Bernardin *et al.* (1997). When this figure is scrutinized in conjunction with fig. 3 of Migliazza *et al.* (1995) it can be seen that the most pronounced lack of homology between the human and mouse sequences corresponds to the site of most pronounced somatic hypermutation in the human gene. These data are consistent with the notion that over evolutionary time hypermutated somatic *BCL-6* gene sequences generated in lymphocytes have been fed back to germline DNA (Blanden *et al.* 1998).

6. CONCLUSIONS

We have shown that many of the current experimental findings on somatic hypermutation can be interpreted under the RT model. The basic principles can also be applied to gene conversion processes, for example in chicken Ig loci, hitherto thought of as being mechanistically unrelated to somatic hypermutation (Blanden & Steele 1998). Furthermore, the paradoxical appearance of the somatic hypermutation signature in the germline unrearranged IgV segment repertoire (Blanden *et al.* 1998) is consistent with soma-to-germline feedback of mutated sequences from B cells over evolutionary time. This concept has been strengthened by reports of uptake and integration by mammalian sperm of DNA and RNA, and RT activity in sperm which provide a mechanism for movement of somatic gene sequences to the germline (Giordano *et al.* 2000; Zoraqi & Spadafora 1997; Perry *et al.* 1999), e.g. from apoptotic memory lymphocytes that have migrated to germ tissues (Rothenfluh 1995).

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