Transcription, β -like DNA polymerases and hypermutation

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This paper discusses two aspects of immunoglobulin (Ig) gene hypermutation. In the first approach, a transcription termination signal is introduced in an Ig light chain transgene acting as a mutation substrate, and transgenic lines are generated with control and mutant transgenes integrated in tandem. Analysis of transcription levels and mutation frequencies between mutant and control transgenes clearly dissociates transcription elongation and mutation, and therefore argues against models whereby specific pausing of the RNA polymerase during V gene transcription would trigger an error-prone repair process. The second part reports the identification of two novel β -like DNA polymerases named Pol λ and Pol μ , one of which (Pol μ) represents a good candidate for the Ig mutase due to its higher lymphoid expression and its similarity with the lymphoid enzyme terminal deoxynucleotidyl transferase. Peculiar features of the expression of this gene, including an unusual splicing variability and a splicing inhibition in response to DNA-damaging agents, are discussed.

Keywords: Ig genes; transcription termination; Pol μ Pol λ

1. INTRODUCTION

It has been proposed that somatic mutation of immunoglobulin (Ig) genes and the SOS-adaptive mutation response of *Escherichia coli* represent analogous strategies. In one case, the attack of a foreign agent (the immunizing antigen) drives a mutagenic process whereby the B-cell receptor (the Ig gene) is able to change in order to recognize and eliminate the cause of the stress. In the other case, a block in the capacity of the cell to divide (by lesions in the DNA, or the lack of appropriate nutrient source) will trigger either a mutagenic bypass of the lesion or a (random) mutagenesis that allows the cell to adapt, e.g. to a previously inadequate metabolic supply (see a recent discussion in Radman (1999)). The (major) difference between both processes is the strict targeting of the mutation, in the case of the Ig gene, to the very locus involved in the response.

The recent discovery of mutagenic DNA polymerases involved in the SOS response, and the isolation of their mammalian equivalent, has reactivated this proposition (Radman 1999; Goodman & Tiffin 2000). In such a model however, it is necessary to assume that these polymerase activities, usually recruited at the site of a lesion, would be diverted to cope (with poor fidelity) with normal DNA synthesis at the Ig locus. A putative role of Pol ζ , a polymerase involved in mutagenic repair in yeast, has also been proposed for Ig gene hypermutation (Diaz *et al.* 1999).

We have pursued a different approach, hypothesizing that the Ig mutase is a polymerase specifically in charge of this process, and that, because of the specific pattern of Ig mutations, it could be related to another error-prone repair polymerase, Pol β (Bertocci *et al.* 1998). We report here the identification of two new β -like enzymes, one of which (Pol μ), isolated in parallel by the group of Luis Blanco (Dominguez *et al.* 2000), has a preferred lymphoid expression pattern and is closely related to terminal deoxynucleotidyl transferase (TdT), an enzyme whose unique function is the diversification of the T- and B-cell repertoire.

As far as targeting is concerned, we have also addressed the question of the role of transcription in hypermutation—either a direct role of the RNA polymerase II complex in the recruitment of the mutase or an indirect one, reflecting chromatin accessibility.

2. TRANSCRIPTION AND HYPERMUTATION: A DIRECT OR INDIRECT LINK?

Hypermutation and transcription have been shown to be tightly linked processes: (i) the mutation domain spans approximately one kilobase (kb) downstream from the promoter; (ii) transcriptional regulatory elements are necessary for proper mutation targeting; (iii) mutation frequencies correlate with transcriptional levels; and (iv) insertion of a promoter element upstream of an Ig constant region is able to generate a new mutation domain downstream of it, in a region otherwise spared from mutations (Lebecque & Gearhardt 1990; Betz *et al.*

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Figure 1. An immunoglobulin κ light chain transgene with a premature transcription termination signal. Constructs were derived from the *LκΔB* transgene (Yelamos *et al.* 1995; kindly given by M. Neuberger). A donor splice site mutation (CC instead of GT) and a Sal I restriction site were inserted in the leader intron, generating the control ‘MutSplice’ transgene. The ‘SPA-C2’ transgene was generated by insertion in the Sal I site of the MutSplice construct of a bipartite transcription termination signal, composed of a strong poly(A) addition site (SPA; Levitt *et al.* 1989) and a transcription stop element isolated from the C2 locus (Ashfield *et al.* 1994) (kindly given by N. Proudfoot). The transcription regulatory elements are figured in grey. L, V and C: leader, variable and constant region of the κ light chain Ig gene.

Table 1. Mutation frequency and transcription level of *MutSplice* and *SPA-C2* transgenes in double transgenic mice

transgenic line	29.12		29.7	
	MutSplice	SPA-C2	MutSplice	SPA-C2
copy number	2	1	3–4	2–3
% mutation	0.34	0.29	1.0	0.33
(% per mutated sequence)	(0.63)	(0.55)	(1.1)	(0.55)
relative transcription	100	1.2	98	2.3

1994; Peters & Storb 1996; Goyenechea *et al.* 1997; Fukita *et al.* 1998). Accordingly, the favoured models of hypermutation propose an active role of the RNA polymerase II complex (by pausing or colliding with a replication fork) in recruiting an error-prone DNA synthesis process (Peters & Storb 1996; Goyenechea *et al.* 1997; Storb *et al.* 1998; Diaz *et al.* 1999). Such error-prone repair remains to be defined, since mice or men with invalidating mutations of most known repair pathways (nucleotide-excision repair, transcription-coupled repair, etc.) failed to show any incidence of the mutation frequency of their Ig genes (Wagner *et al.* 1996; Kim *et al.* 1997; Shen *et al.* 1997; Jacobs *et al.* 1998).

On the other hand, the correlation observed between transcription and hypermutation could simply reflect the simultaneous accessibility of an Ig locus to both processes. Allowing transcription to initiate on an Ig mutation substrate and inducing a premature termination of transcription should help to distinguish between these possibilities.

In eukaryotes, the 3'-end of an mRNA is specified by cleavage of the nascent transcript at a poly(A) addition site. Termination of transcription does not however immediately ensue. Dissociation of the Pol II complex takes place progressively, while further elongating, as a consequence of both destabilization of the nascent transcript

with a now exposed, uncapped 5'-end, and the possible loss of an elongation factor from the RNA polymerase complex when passing the poly(A) addition site (see review by Proudfoot (2000)). In some cases, however, strong transcription terminators exist: when genes are closely spaced, non-dissociation of the Pol II complex before encounter of the next downstream promoter would lead to transcriptional interference by promoter occlusion. Such a termination signal has been characterized between two genes of the major histocompatibility complex class III locus (the C2 complement and B1 gene, located 0.492 kb apart), which contains a binding site for a specific protein, acting as transcription terminator, and is also capable *per se* to behave as a pause site for the RNA polymerase in the context of an upstream poly(A) addition signal (Ashfield *et al.* 1994; Yonaha & Proudfoot 2000).

We therefore constructed an Ig mutation substrate derived from the *LκΔB* transgene (Yelamos *et al.* 1995), in which a strong poly(A) addition signal and the transcription stop sequence of the C2 locus were introduced in the leader intron (named ‘SPA-C2’) (figure 1; Frey *et al.* 2001). A donor splice site mutation was also made, to prevent any competition between splicing of the leader intron (which would excise the transcription stop signal from the RNA) and transcription termination. A control transgene harbouring only the splice site mutation was also

type of modification	(G) ₆ repeat												number of cases /27 sequences analysed
	G	C	C	T	G	G	G	G	G	C	T	C	
transient misalignments	-	-	-	G	-	-	-	-	-	-	-	-	4
	-	-	G	G	-	-	-	-	-	-	-	-	1
	-	-	G	G	-	G	-	-	-	-	-	-	1
insertions	-	-	-	-	GGG	-	A	-	-	-	-	T	1
deletions	-	-	-	-	-	()	-	-	-	-	-	-	1
point mutations	-	T	T	-	-	-	-	-	-	-	-	-	1
	-	-	-	-	-	-	-	-	C	-	-	-	1
	-	-	-	-	-	-	C	-	-	-	-	-	1
	-	-	-	-	-	-	-	-	-	-	-	T	1
	-	-	-	-	-	-	-	-	-	-	-	-	1

Figure 2. The mutation spectrum of a natural mononucleotide repeat in the human heavy chain J_{H4}-J_{H5} intron reveals typical DNA polymerase slippage events. Mutation events are compiled from a sample of 27 intronic sequences from peripheral memory B cells (Levy *et al.* 1998) and the number of cases indicated. DNA modifications typical of an error-prone DNA polymerase are in bold: insertions, deletions and transient misalignments (Kunkel & Soni 1988). The latter, the most frequently observed on this short sequence, corresponds to a DNA copying process of the coding strand (from right to left in the figure).

constructed ('MutSplice'). To avoid the variability introduced by the integration site in the comparison of transcription and mutation between both transgenes, double transgenic mice were generated, harbouring the two constructs inserted in tandem.

Mutation and transcription were analysed in Peyer's patch PNA^{high} B cells from two double transgenic lines. Transcription elongation was estimated by a PCR assay, quantifying the amount of RNA polymerase bypass up to 0.33 kb downstream of the transcription stop signal, i.e. up to FR2. Additional controls, i.e. RNase protection and actinomycin D treatment to quantify transgenic RNA half-lives, were performed on lipopolysaccharide (LPS)-stimulated blasts to confirm the adequacy of our PCR assay for estimating the relative transcription of both transgenes.

Transcription of transgenes harbouring the transcription stop signal was reduced 50-fold or 100-fold in the two transgenic lines compared with control transgenes. In contrast, the mutation frequency of their V gene was either similar (in one line) or slightly reduced (threefold in the other line) compared with MutSplice transgenes (table 1; Frey *et al.* 2001).

Taken as such, these results indicate that transcription elongation and mutation can be dissociated, and therefore are not in favour of models in which the RNA polymerase complex would specifically recruit the mutator activity. One nevertheless cannot exclude at this stage that a minimal transcription activity might still be required for mutations to occur at the Ig locus. If a high level transcription *per se* is not necessary, what then delimits the mutation domain downstream of the promoter site? Whether assembly of transcription factors at the promoter is sufficient to delimit such a domain in the context of a downstream enhancer, or whether transcription initiation is required, inducing for example a negative supercoiling of the downstream region, are obvious questions that will have to be addressed.

One should also consider that this experimental set-up might have created a unique configuration that would not be easily transposable, e.g. by knock-in, to a natural locus. According to this view a gene, for which only transcription initiation but not elongation would occur, might not experience sufficient transcriptional activity to be

Figure 3. Amino-acid similarities within human DNA polymerases—nucleotidyl transferases of the Pol X family. A two-by-two comparison was performed by the BLAST program (Altschul *et al.* 1997) between the four members of the human Pol X family. The two figures are percentages of amino-acid identities and similarities, respectively. The highest identity-similarity values are marked in a grey box (β-λ and TdT-μ).

maintained in an active chromatin configuration (e.g. due to acetylase activities conveyed by the RNA polymerase complex). Strikingly enough, most of the single transgenic lines studied, i.e. harbouring only SPA-C2 constructs, were totally transcriptionally silent, at both initiation and elongation levels, rendering them non-informative for our initial question. One could thus envision that, in the double transgenic configuration, the transcriptionally active MutSplice transgenes might maintain the adjacent SPA-C2 copies in a chromatin configuration accessible to mutations, either by inducing long-range chromatin alterations, or by allowing its passive co-localization to specific nuclear subdomains. In this case, the link between transcription and mutation would merely reflect chromatin accessibility and the definition of the mutation domain would remain a totally open question.

3. THE MUTASE: AN ERROR-PRONE POLYMERASE?

The proposition that Ig gene diversification could be created by somatic mutations introduced by an error-prone

Figure 4. Pol λ , Pol μ and TdT share a similar protein domain organization. The different functional domains are schematically represented along the proteins: the DNA polymerase domain (Pol X); two helix–hairpin–helix motifs, involved in non-specific DNA binding (Doherty *et al.* 1996) (grey motifs contain residues differing from consensus at the Gly-(hydrophobe)-Gly core positions); and a BRCT domain; (BRCA-1 C-terminal domain; Bork *et al.* 1997) that probably mediates protein–protein interactions.

DNA polymerase was formulated by Brenner & Milstein (1966). Since then, the recruitment of a specific error-prone activity has not always been implicit in many of the current models of hypermutation, and, as long as somatic mutations were considered as single base substitutions, many other DNA modification processes could be considered. However, the very large mutation database that was recently accumulated in particular from unselected sequences, as well as from human sequences where the mutation load is strikingly high, has revealed a much more complex panel of DNA alterations, including duplications, insertions (from one to several hundred base pairs) and slippage events on short repeated sequences (Wilson *et al.* 1998; Goossens *et al.* 1998; Levy *et al.* 1998; Bertocci *et al.* 1998). Such slippage events, typical of a DNA copying devoid of further correction processes, can be observed, for example, on a six nucleotide repeat in the human J_{H4} – J_{H5} intronic sequence of human memory B cells (figure 2). On the other hand, when longer microsatellites (e.g. 11(GT) or 18(T)) were introduced in the V region of an Ig mutation substrate, slippage events were much less frequent, suggesting that such repeat lengths exceed the average patch of synthesis of the polymerase involved (Bertocci *et al.* 1998). We therefore proposed that Ig gene hypermutation is indeed generated by a short patch error-prone DNA synthesis. Moreover, because the mutation pattern observed resembles the one generated by Pol β *in vitro*, we proposed that this putative DNA polymerase could be a ‘ β -like’ enzyme (Bertocci *et al.* 1998), the involvement of Pol β itself being excluded by the normal incidence of Ig gene mutations observed on Pol β -deficient mice (Esposito *et al.* 2000).

We searched for new members of this family as possible candidates. The Pol X family contains only two members in the genome of higher eukaryotes: Pol β and TdT, the terminal deoxynucleotidyl transferase. Using the G(S,G)(Y,F)(N,R)(L,R)G(X)5,6D(L,I,V,M)DXL motif as a consensus including both the Pol X family and the poly(A) polymerase (Holm & Sander 1995), one sequence was retrieved from expressed sequence tag (EST) databases and shown to correspond to a new Pol β homologue, which was named Pol λ . Surprisingly, a direct search using the protein sequence of TdT retrieved a second sequence, in addition to Pol λ ; this sequence was

closer to TdT and was named Pol μ (an ideal name for a putative mutase). In a parallel investigation, the same two enzymes were characterized by the group of Luis Blanco (Dominguez *et al.* 2000; Garcia-Diaz *et al.* 2000). A more recent search on the draft of the human genome failed to sort additional members for the Pol X family. The overall similarities in the human Pol X family are shown in figure 3. The close relatedness of TdT and Pol μ is further attested by the identical exon–intron structure of their genes, whereas Pol λ gene organization differs completely from Pol β (Aoufouchi *et al.* 2000).

The structure of TdT, Pol μ and Pol λ is very similar: it contains a polymerase domain, two HhH motifs (helix–hairpin–helix, involved in non-specific binding of DNA; Doherty *et al.* 1996) and a BRCT domain (figure 4). This domain, named from its initial characterization in the breast cancer type 1 susceptible protein (BRCA-1) C-terminal region, is found in a variety of proteins, collectively described as ‘DNA damage-responsive cell cycle checkpoint proteins’ (even though TdT is, among this group, the one for which this definition would seem the least adequate) (Bork *et al.* 1997). The BRCT domain has been suggested to mediate not only interaction with DNA but also protein–protein interaction, and it might represent an ideal tool for isolating specific partners of these polymerases.

The tissue distribution of expression of Pol μ and Pol λ differs completely. Both enzymes show a low level of expression with a wide variety of tissues. Pol λ expression is highest in the testis (as is expression of many of the translesion polymerases, e.g. λ , κ , ι), but also in foetal liver. In contrast, expression of Pol μ is more lymphoid, its highest expression being observed in thymus, tonsillar B cells (CD19+, CD38+, i.e. B cells from germinal centres) and Burkitt’s lymphomas (i.e. the transformed counterpart of germinal centre centroblasts) (Aoufouchi *et al.* 2000). Among all other DNA polymerases described to date, Pol μ thus appears the most lymphoid of all, even though its expression is clearly not restricted to the sole B-cell population undergoing mutation of their Ig genes. For Burkitt’s lymphomas in particular, its expression appears similar, whether the cells are undergoing hypermutation constitutively or not (e.g. the Ramos cell line; Sale & Neuberger 1998), and in the case of the BL2

Figure 5. An unusual variety of mRNA species for the Pol μ gene in Burkitt's lymphomas. Representative example of splice variants, obtained by RT-PCR amplification of poly-adenylated mRNA from the Ramos or the BL2 cell line, are depicted. The exon structure of the coding region is represented, as well as the domain organization of the protein (BRCT, HhH and Pol X domains). Multiple events of alternative splicing are frequently observed within a sequence (see last three examples). A similar frequency of splice variants is observed in normal lymphoid tissues (thymus, lymph nodes).

Burkitt's lymphoma, which can be induced for hypermutation upon proper stimulation (IgM cross-linking and helper T-cell co-stimulation; Dénépoux *et al.* 1997), its expression is not modified upon induction.

Could there be other levels of regulation that might restrict the expression of a functional Pol μ enzyme to a specific cell subset? A very peculiar feature of this gene is the unusually large number of splice variants observed, most of which affect the polymerase domain (figure 5). In a sample of 35 cDNAs amplified from the BL2 cell line (induced or uninduced), only three encode the Pol μ enzyme. Eighteen different mRNA species were characterized in this sample and the combinatorial assortment of splice alterations at several exon junctions led us to expect a larger number of possible species. Half of the cDNAs contain a 53 bp insertion at the last exon junction (between exon 10 and 11), that leads to a shift in the reading frame of exon 11 and the translation of the first 288 bp of the 3' untranslated region. In most other cases of splice variants, a premature stop codon is created that, surprisingly with regard to these variants accounting for the majority of the species present, suggests that no efficient nonsense-mediated decay is occurring for these transcripts (Li & Wilkinson 1998). A specific search aimed at identifying a particular variant that would be unique to the BL2 induced for hypermutation failed to reveal any candidate species. Whether such variants exist, or whether the complex array of these different species—most of which are truncated polymerases with a complete BRCT domain—could act as dominant negative, obviously remain to be studied. In this last hypothesis,

only a specific balance between these different forms would allow the biological function of the Pol μ polymerase.

Whereas we have found so far no biological function for this unusual splicing regulation, regulation of Pol μ expression by splicing inhibition was surprisingly observed in response to various DNA-damaging treatments induced in the Ramos Burkitt's lymphoma cell line. Downregulation of Pol μ mRNA expression and accumulation of an unspliced RNA precursor occurred in response to UV light, γ -rays and H_2O_2 treatments, in a dose- and time-dependent fashion (Aoufouchi *et al.* 2000). Such a regulation might ensure that Pol μ is not recruited in the repair of these lesions.

Could overexpression or inhibition of Pol μ alter the capacity of Burkitt's lymphomas to mutate their Ig genes? Overexpression in the Ramos cell line (constitutive for the mutation process) or BL2 (inducible) cell line failed to generate any increase (or even decrease) in hypermutation. Induction of mutation in the BL2 cell line can be observed at the V gene level four days after induction (Dénépoux *et al.* 1997): transfection of anti-sense oligonucleotides that resulted in strong Pol μ mRNA downregulation over the next 24 h, did not induce any phenotypic alteration either. Finally, the overexpression of a Pol μ mutant, in which the two aspartate residues that coordinate the metal ions in the active site are replaced by alanines, did not alter the induction of mutation in the BL2 cell line either. That the Pol μ protein was indeed expressed from the transfected cDNA was indirectly assessed by visualizing it upon tagging with green fluorescent protein.

All of these assays thus failed to suggest a possible implication of the Pol μ enzyme in the process of Ig gene hypermutation, and one has probably to await gene inactivation experiments to obtain definitive conclusions. In spite of these negative data, what are the arguments in favour of this enzyme that would make it a better candidate than the Π enzymes that precede it in the Greek alphabet? At first and as mentioned before, is its strong lymphoid expression, even though we are far from the RAG-1, RAG-2 and TdT paradigm, where gene expression is tightly restricted to the precise cells undergoing Ig gene rearrangement. Strikingly enough in this context, all subtractive approaches attempted by many different groups between mutating and non-mutating cells, failed to identify a specifically induced polymerase activity. In contrast, the group of Honjo identified an activity acting probably upstream in the centroblast differentiation pathway (see Muramatsu *et al.* 2000). A second argument is the remarkable infidelity of Pol μ reported by the group of Blanco (Dominguez *et al.* 2000), even though several translesion DNA polymerases (η and ι , in particular) appear competitive in this respect (Matsuda *et al.* 2000; Tissier *et al.* 2000). Finally, mainly its relatedness with TdT is the most appealing feature for the immunologist—two enzymes would have evolved in higher eukaryotes: TdT, to generate diversity of CDR3 by random nucleotide insertion at the V–D–J junction; and a TdT-like polymerase with a poor fidelity to diversify the whole V gene at later differentiation steps.

Considering the number of groups involved in knock-outs from ζ to μ , we will probably know very soon whether such analogy applies, or whether somatic mutation of Ig genes evolved as a gene-targeted adaptation of the bacterial SOS response, or even whether new polymerase activities remain to be discovered.

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