A new class of errant DNA polymerases provides candidates for somatic hypermutation

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The mechanism of somatic hypermutation of the immunoglobulin genes remains a mystery after nearly 30 years of intensive research in the field. While many clues to the process have been discovered in terms of the genetic elements required in the immunoglobulin genes, the key enzymatic players that mediate the introduction of mutations into the variable region are unknown. The recent wave of newly discovered eukaryotic DNA polymerases have given a fresh supply of potential candidates and a renewed vigour in the search for the elusive mutator factor governing affinity maturation. In this paper, we discuss the relevant genetic and biochemical evidence known to date regarding both somatic hypermutation and the new DNA polymerases and address how the two fields can be brought together to identify the strongest candidates for further study. In particular we discuss evidence for the *in vitro* biochemical misincorporation properties of human Rad30B/Pol ι and how it compares to the *in vivo* somatic hypermutation spectra.

Keywords: error-prone DNA polymerase; UmuC/DinB/Rad30/Rev1 superfamily; somatic hypermutation

1. INTRODUCTION

The creation of antibody diversity in mice and humans is a remarkable process whereby two different mechanisms are employed to generate an estimated 10^9 different variants of antibodies (Milstein 1987; Berek & Milstein 1988). Diversification of the immunoglobulin (Ig) genes occurs at discrete stages in the B-cell life cycle. The initial repertoire of antibodies results from a non-random recombination process between the V(D)J gene fragments. Following initial antigen recognition, B cells expressing the correct antibody become activated, leading to the second phase of diversity termed 'affinity maturation'. Somatic hypermutation is the process by which affinity maturation occurs and appears to take place in the dark zone of rapidly dividing cells in germinal centres (Zhang *et al.* 1988).

Mutations are targeted exclusively to the variable region of the immunoglobulin gene starting near the promoter and trailing off *ca.* 1–2 kb downstream (Kim *et al.* 1981; Gearhart & Bogenhagen 1983) at an estimated rate of $3\text{--}4 \times 10^{-4}$ per base pair per generation, nearly one millionfold higher than spontaneous background (Berek & Milstein 1988; Levy *et al.* 1989; Rada *et al.* 1991; Weiss *et al.* 1992). This dramatic increase in mutation rate is even more remarkable in that it does not seem to result from a global alteration in replication fidelity throughout the B cell, but is rather a specific targeted mutational mechanism which leaves other genes virtually untouched. There is one case, however, of somatic hypermutation reported in the *Bcl-6* gene in a Burkitt's lymphoma cell line (Migliazza 1995) but this gene does not appear to be

mutated in normal B cells isolated from germinal centres (Neuberger *et al.* 1998).

2. REQUIREMENTS FOR SOMATIC HYPERMUTATION

Although the exact mechanism of somatic hypermutation is unknown, some key components have been identified, mainly the *cis*-acting genetic enhancer elements located downstream of the variable region in light and heavy chain genes (Neuberger *et al.* 1998). For example, the intronic enhancer (E_i) and flanking matrix attachment region (MAR) of the κ light chain gene strongly stimulate the mutational process and completely eliminate somatic hypermutation when deleted (Betz *et al.* 1994). A second κ light chain 3' enhancer ($E_{3'}$) also affects the level of somatic hypermutation but to a lesser extent.

A promoter sequence upstream of the variable region of the immunoglobulin gene is also essential to the hypermutation process, although a non-immunoglobulin promoter can just as easily substitute (Betz *et al.* 1994). The fact that any promoter will do and that any DNA inserted into the variable region can be recruited to serve as a target for the mutations leaves only the enhancer regions as candidates for the targeting mechanism. The data seem to suggest the requirement for a particular DNA architecture, resembling that of transcription initiation, where enhancers and promoter regions are brought together by protein–DNA complexes (Winter *et al.* 1998).

3. MODELS FOR THE MECHANISM OF SOMATIC HYPERMUTATION

Several models have been proposed to explain the polarity and localization of somatic hypermutation. One

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model tries to explain the link between enhancers for transcription and the mutational process as transcription-coupled repair (TCR) gone awry (Storb 1996). This model suggests transcription forks may become stalled in the variable region where the paused fork serves as a signal to recruit TCR proteins. TCR machinery could then repeatedly replicate the same region thereby increasing the chance of mutations in that area. The main problem with this model is the requirement for repeated rounds of TCR on the same stretch of DNA, a very wasteful and inefficient process. TCR is known to exhibit a significant strand bias to repair the actively transcribed strand. The large mutational data set for somatic hypermutation has provided questionable evidence for strand bias (Milstein *et al.* 1998; Foster *et al.* 1999; Cowell & Kepler 2000) and argues against TCR as the mechanism.

Another model put forward suggests the involvement of an error-prone reverse transcriptase, which could synthesize a cDNA copy from an elongating mRNA that replaces the gene by homologous recombination (Steele *et al.* 1997). However, replacing an entire segment of the immunoglobulin gene with a cDNA copy seems a rather indirect way to get mutations on the chromosomal copy of the gene.

Yet another model has been proposed involving a stalled transcription fork colliding with a moving DNA replication fork, which causes the replicative DNA polymerase(s) in the fork to misincorporate bases (Winter *et al.* 1998). Such an extreme alteration in the intrinsic polymerase fidelity seems highly unlikely. The replicative eukaryotic DNA polymerases Pol δ and Pol ϵ have estimated error frequencies in the order of 10^{-6} to 10^{-8} . In order to achieve the observed mutation frequency in somatic hypermutation, the errors need to be in the order of 10^{-2} to 10^{-3} . Another version of this model involves the first step of a stalled transcription fork colliding with a DNA replication fork, but rather than altering the fidelity of the replicative polymerase, the stalled replication fork would serve as a signal to recruit an error-prone polymerase to replace Pol δ or Pol ϵ (Diaz & Flajnik 1998).

We believe a more likely somatic hypermutation model would involve a member of the newly discovered family of errant DNA polymerases spanning prokaryotic and eukaryotic organisms (Goodman 2000; Woodgate 1999). The existence of these remarkably low fidelity DNA polymerases could easily account for observed hypermutation rates after a single round of replication through the variable region (Lederberg 1959; Brenner & Milstein 1966; Lebecque & Gearhart 1990; Manser 1990). The activating signal may or may not be the collision between replication and transcription forks. We can only guess as to whether the error-prone replication mechanism acts via a substitution event in the replicative fork moving through the target Ig region or if it occurs by the independent assembly of a mutagenic replication bubble. The various possibilities are complicated by the fact that human chromosomes are littered with different replication origins in addition to long and short patch post-replication DNA repair systems, all of which, to some degree, can act independently of one another. One thing does seem certain—some sort of open complex or repair patch must serve as an intermediate onto which an error-prone polymerase can be loaded and is probably localized by the unique

Figure 1. A schematic model of enhancer-mediated targeting of hRad30B/Pol ι . Transcription factor(s) (light grey) bind to the (E_i) intronic enhancer elements and with the help of co-activator proteins (hatched lines) interact with the Ig promoter (P). Once an open DNA complex is present in this region (whether by a fork moving through or direct opening of the DNA), enhancer associated targeting factors recruit hRad30B/Pol ι (dark grey) to replicate the gene in this localized region.

physical interaction between the Ig promoter and E_i enhancer.

Evidence that the enhancer–promoter interaction targets the mutation factor by a specific DNA architecture is found in the fact that the hypermutation process can be redirected to act downstream in the constant region by simply duplicating the Ig promoter just upstream of that area (Peters & Storb 1996). Initially this evidence was interpreted to reflect a requirement for active transcription in order to achieve somatic hypermutation, but rather we believe that it simply reflects a requirement for the enhancer–promoter interaction to enable loading of the error-prone polymerase.

4. CANDIDATE ERROR-PRONE POLYMERASES FOR SOMATIC HYPERMUTATION

The error-prone polymerase model has been strengthened by the recent discovery of a new family of low fidelity DNA polymerases, the UmuC/DinB/Rad30/Rev1 superfamily (Nelson *et al.* 1996; Tang *et al.* 1998, 1999; Johnson *et al.* 1999, 2000; Reuven *et al.* 1999). These remarkable DNA polymerases have error rates as high as 10^{-2} to 10^{-3} . Three other polymerases have also been identified in humans that do not fall into the UmuC/DinB/Rad30/Rev1 superfamily: the terminal deoxynucleotidyl transferase-like Pol μ (Dominguez *et al.* 2000), the biochemically uncharacterized POLQ gene product (Sharief *et al.* 1999), a member of the type A family of DNA polymerases including *Escherichia coli* Pol I, and a Pol β -like gene *Pol L* (GenBank accession number AJ131890). Pol μ is an interesting candidate as it is expressed at elevated levels in lymphoid tissues, although not only in activated B cells (Dominguez *et al.* 2000).

Until recently, DNA pol β , was considered a candidate based on its average mutation rate of 10^{-3} to 10^{-4} per base pair per generation (Kunkel & Alexander 1986). But Pol β is no longer a tenable candidate because mice with reconstituted immune systems from foetal liver cells of embryonic *Pol β ^{-/-}* knockouts are able to somatically

Figure 2. Gapped DNA construct for *in vitro* assay. The variable (VJ) region of *V_κOx1* is replaced with the *lacZ* gene from *E. coli* and an f1 phage origin. A 450-nucleotide gap is generated in the *lacZ* target gene, keeping the remainder of the immunoglobulin transgene intact, including the leader (L), promoter (P), intronic enhancer (E_i), constant region (C_κ), and the 3' enhancer (E_{3'}).

hypermutate their antibody genes and mount a relatively normal immune response (Exposito *et al.* 2000).

One member of the UmuC/DinB/Rad30/Rev1 superfamily, XPV (xeroderma pigmentosum variant) was eliminated as a somatic hypermutation candidate before the gene was known to code for human Pol η , when patients with inherited XPV disease were shown to have normal levels of somatic hypermutation in their B cells (Kim *et al.* 1997). The polymerase encoded by human DINB1 (denoted Pol θ by the Prakash group, and Pol κ by the Ohmori group) (Johnson *et al.* 2000; Ohashi *et al.* 2000), was recently shown to have an error rate in the order of 10^{-3} to 10^{-4} , but it is widely expressed throughout human tissues (Gerlach *et al.* 1999) that show no evidence of somatic hypermutation.

5. RAD30B/POL ι AS THE TRANS-ACTING MUTATION FACTOR

We believe that the human (and mouse) Rad30B/ pol ι polymerase could be the key *trans*-acting factor responsible for somatic hypermutation. Our focus on Rad30B as opposed to other members of the superfamily stems from our own work in SOS targeted and untargeted mutagenesis in *E. coli* by UmuC, a member of the errant polymerase superfamily. UmuD'₂C (*E. coli* Pol V), the polymerase responsible for SOS mutagenesis (Tang *et al.* 2000), is part of a tightly regulated mutagenic complex, the Pol V mutasome (Echols & Goodman 1990; Tang *et al.* 1999).

Several key features about the mutagenic mechanism of Pol V ring similar to the requirements of somatic hypermutation. First and foremost is the targeting mechanism, which for Pol V is mediated by the single-stranded DNA-binding protein SSB (P. Pham and M. F. Goodman, unpublished data) and RecA (Sweasy *et al.* 1990; Bailone *et al.* 1991; Frank *et al.* 1993). Without RecA, Pol V has a very low probability of using a DNA template, a feature that limits Pol V activity only to areas of severe replication fork blockage (Tang *et al.* 2000). Remarkably RecA protein also triggers Pol V polymerase activity by increasing it *ca.* 15 000-fold (Tang *et al.* 2000).

In parallel, the mutation factor involved in somatic hypermutation would need to be relatively inactive on other DNA templates, which lack the appropriate targeting and activation factors. In fact hRad30B has a very weak intrinsic polymerase activity on primer-template DNA *in vitro* (Tissier *et al.* 2000), possibly making it an unlikely competitor for DNA substrate without help from an auxiliary targeting factor in a cell with possibly ten other polymerases.

It is believed that Pol V interacts with RecA and SSB via the unique C-terminal region of the UmuC protein.

Our suggestion that Rad30B may be required in somatic hypermutation is partially based on its similarity to UmuC in its domain structure. Like UmuC, Rad30B has a unique protein C-terminus, which could mediate the necessary interactions to stimulate its activity and, most importantly, to target the polymerase to the immunoglobulin gene by an analogous rather than homologous process (figure 1). The other members of the UmuC/DinB/Rad30/Rev1 superfamily have more well-characterized domain structures and *in vitro* have the ability to replicate DNA with substantial activity in the absence of accessory proteins.

We have recently learned (Tissier *et al.* 2000), that hRad30B (Pol ι) has a remarkable behaviour unlike any other known polymerases to date. hRad30B actually prefers to incorporate G opposite a template T to make the G:T wobble base pair 3:1 over the Watson-Crick A:T base pair (Tissier *et al.* 2000). Surprisingly, the wobble base pair is only favoured by hRad30B when the T is in the template, because when G is the template, the polymerase prefers to put in the correct base C 2:1 over the wobble T. In stark contrast, the exact opposite effect is seen when the A is found in the template, with the correct T nucleotide incorporated 10:1 over G incorporation. This odd behaviour is accompanied by an extremely low overall fidelity in the order of 10^{-2} (Tissier *et al.* 2000).

The Pol ι misincorporation pattern is very much in line with the signature observation of Ig mutational spectra where A mutates more often than T and transitions are favoured 2:1 over transversions. For Pol ι , the most common errors are to mutate A to G and C to T, i.e. both transitions, although other errors are also observed at elevated levels (Tissier *et al.* 2000). The asymmetrical mutation frequency between A and T (Golding *et al.* 1987; Foster *et al.* 1999) observed in somatic hypermutation may be a signature of Pol ι activity rather than a reflection of strand bias in the mechanism.

6. A POSSIBLE ASSAY TO STUDY THE BIOCHEMICAL MECHANISM OF SOMATIC HYPERMUTATION

We are testing a model of Pol ι targeting to the immunoglobulin gene by taking an *in vitro* biochemical approach analogous to what we have used to study the targeting of *E. coli* Pol V to damaged DNA template sites (Rajagopalan *et al.* 1992; Tang *et al.* 1998, 1999, 2000). It is important, however, to emphasize that a key distinction between the two systems is that while Pol V is targeted to DNA template lesions, the putative polymerase responsible for somatic hypermutation is probably acting on an undamaged DNA template. In an attempt to construct an *in vitro* model to explore somatic hypermutation, we have generated a gapped plasmid construct derived from the

V_kOx1 gene (Sharpe *et al.* 1991; Betz *et al.* 1994) (figure 2). The variable region of V_kOx1 has been replaced with *lacZ* to allow rapid detection of forward mutations by blue–white screening in *E. coli* following *in vitro* replication reactions (Bebenek & Kunkel 1995). Using purified Pol ι protein and extracts from the Ramos Burkitt's lymphoma cell line, we will monitor if Pol ι is stimulated and localized to the gapped template by factors in the extract that bind to the enhancers in the V_kOx1 construct. The essential targeting factors for somatic hypermutation are likely to be present at induced levels in the Ramos cells because this cell line undergoes constitutive somatic hypermutation in culture (Sale & Neuberger 1998). Detection of targeting events by this approach should enable us to purify factors that stimulate Pol ι activity by fractionation and subsequent purification of cell extract components.

7. CONCLUSION

This *in vitro* approach is designed to link together genetic evidence for *cis*-acting promoter and enhancer elements with a direct biochemical assay to elucidate potential *trans*-acting mutagenic factors. A major advantage of using such an *in vitro* system is that it eliminates the wait time associated with current methodologies of passaging cells or generating transgenic animals before changes in mutation frequencies can be detected.

If a biochemical role can be established for Pol ι in somatic hypermutation by our approach, the obvious next step would be to validate it genetically *in vivo*. But a role for Pol ι would only be the beginning of the story. Many laboratories are actively pursuing the identification of key transcription factors that occupy the E_i and E_{3'} enhancers during the antigen stimulated phase in B cells (Reya & Grosschedl 1998). The transcription factors Oct-2 (Tang & Sharp 1999), TFE3 (Merrell *et al.* 1997), C/EBP β (Hatada *et al.* 2000) and the co-activator OBF-1/OCA-B (Schubart *et al.* 2000) are thought to act specifically during this stage, but many others remain unidentified. Defining a line between transcription activation and hypermutation targeting will be a crucial step to elucidate the components of the targeting mechanism for somatic hypermutation.

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