

Localization of DNA polymerases η and ι to the replication machinery is tightly co-ordinated in human cells

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Y-family DNA polymerases can replicate past a variety of damaged bases *in vitro* but, with the exception of DNA polymerase η (pol η), which is defective in xeroderma pigmentosum variants, there is little information on the functions of these polymerases *in vivo*. Here, we show that DNA polymerase ι (pol ι), like pol η , associates with the replication machinery and accumulates at stalled replication forks following DNA-damaging treatment. We show that pol η and pol ι foci form with identical kinetics and spatial distributions, suggesting that localization of these two polymerases is tightly co-ordinated within the nucleus. Furthermore, localization of pol ι in replication foci is largely dependent on the presence of pol η . Using several different approaches, we demonstrate that pol η and pol ι interact with each other physically and that the C-terminal 224 amino acids of pol ι are sufficient for both the interaction with pol η and accumulation in replication foci. Our results provide strong evidence that pol η targets pol ι to the replication machinery, where it may play a general role in maintaining genome integrity as well as participating in translesion DNA synthesis.

Keywords: DNA polymerase/replication foci/UV light/xeroderma pigmentosum variants

Introduction

DNA damage occurs ubiquitously in all cells. In order to maintain the stability of the genome, cells have evolved mechanisms not only to repair all types of DNA damage, but also to replicate DNA from which the damage has not been removed (post-replication repair). In the case of human cells, a major mechanism for carrying out post-replication repair involves translesion synthesis (TLS) past damaged sites. TLS is deficient in the variant form of the

sun-sensitive cancer-prone disorder xeroderma pigmentosum (XP). The gene defective in these XP variants (XP-V) encodes a DNA polymerase, pol η (Johnson *et al.*, 1999; Masutani *et al.*, 1999), which is able to replicate undamaged templates or those containing cyclobutane pyrimidine dimers (CPDs, the major UV photoproduct) with equal efficiencies (Masutani *et al.*, 1999). TLS by pol η is the principal mechanism for bypassing CPDs in human cells. Although the lack of pol η in XP-V cells does not confer substantial hypersensitivity to killing by UV light, UV hypermutability is increased to levels approaching those in classical XP cells, which are deficient in nucleotide excision repair (Maher *et al.*, 1976).

Pol η is a member of the recently discovered Y-family of DNA polymerases (Ohmori *et al.*, 2001), which have been best characterized for their lesion-bypassing properties (reviewed in Goodman, 2002). There are, however, few studies to date to indicate how these polymerases function inside cells. In previous work, we showed that in S-phase cells, pol η localizes in replication foci. On exposure to DNA-damaging treatments, we observed an accumulation of pol η -containing foci. These appear to represent replication factories in which replication forks are stalled at lesions (Kannouche *et al.*, 2001). The C-terminal 70 amino acids of pol η are required to localize it in the nucleus, and a further 50 are needed for the relocalization into replication foci (Kannouche *et al.*, 2001).

In addition to pol η , human cells possess three other Y-family polymerases (pol ι , pol κ and Rev1) (Woodgate, 1999; Friedberg *et al.*, 2002; Goodman, 2002). Pol ι , like pol η , is phylogenetically related to DNA polymerases in the *Saccharomyces cerevisiae* Rad30 branch of the Y-family (McDonald *et al.*, 1999; Ohmori *et al.*, 2001). It is extremely error prone on undamaged templates and, most strikingly, pol ι incorporates dGMP opposite T 3–10 times more frequently than the correct nucleotide dAMP (Johnson *et al.*, 2000b; Tissier *et al.*, 2000b; Zhang *et al.*, 2000c). Although pol ι can also insert bases opposite several DNA lesions, such as an abasic site or the 3' T of a 6–4 T–T photoproduct (Tissier *et al.*, 2000a; Zhang *et al.*, 2001), extension from the inserted base is often limited. It has been suggested that in such cases, the pol ι -dependent (mis)insertion might be extended by another polymerase, such as pol ζ (Johnson *et al.*, 2000b; Tissier *et al.*, 2000a). Using a model system containing human pol ι in conjunction with *S.cerevisiae* pol ζ , the combined action of the two polymerases leads to the complete bypass of abasic sites and 6–4 T–T photoproducts *in vitro* (Johnson *et al.*, 2000b; Guo *et al.*, 2001). Despite a wealth of data on the enzymatic properties of pol ι *in vitro* (McDonald *et al.*, 2001; Vaisman and Woodgate, 2001; Vaisman *et al.*, 2001; Zhang *et al.*, 2001), there are no data on the role of pol ι *in vivo* or on its functional relationship to other Y-family DNA polymerases.

Here, we show that the localization of pol ι following DNA damage is identical to that of pol η , and that recruitment of pol ι into replication foci is dependent on pol η . We also show by several criteria that pol η and pol ι physically interact. Taken together, our results suggest that an interaction between pols η and ι helps target pol ι to the replication machinery, where it can participate in genome maintenance and TLS.

Results

Pol ι does not correct the UV sensitivity of XP-variant cells

Since pol η and pol ι are paralogues and contain similar catalytic domains, we investigated whether overexpression of pol ι was able to correct the defect in XP-V cells. Although XP-V cells are only marginally hypersensitive to killing by UV, incubating them in caffeine following UV treatment confers a substantial sensitization to UV-induced cell killing, which is not seen in normal cells (Arlett *et al.*, 1975). pCDNA-pol ι was stably transfected into XP30RO cells and transfectant clones were analysed for resistance to UV + caffeine. As shown in Figure 1A, XP30RO cells overexpressing pol ι displayed the same UV + caffeine sensitivity as cells transfected with vector alone. In contrast, XP-V cells overexpressing pol η were resistant to UV + caffeine, with survival similar to that of the normal cell line MRC5. Thus, unlike pol η , pol ι is not able to correct the defect in XP-V cells.

Pol ι localizes into nuclear foci after UVC irradiation

In order to improve our understanding of the biological function of pol ι , we have analysed its cellular localization. The cDNA encoding enhanced green fluorescent protein (eGFP) was fused in-frame to the N-terminus of pol ι (eGFP-pol ι), and this construct was transfected into MRC5 normal fibroblasts. Despite the absence of any obvious nuclear localization signal (NLS) consensus motif in its amino acid sequence, eGFP-pol ι predominantly localized within the nucleus (Figure 1B, left) and appeared homogeneously distributed. In 10–15% of transfected cells, eGFP-pol ι was localized in many intranuclear foci (not shown). This localization pattern was strikingly similar to our previous observations with pol η (Kannouche *et al.*, 2001). Consequently, we asked whether the distribution of pol ι changes after DNA damage. We irradiated the transfected cells with 7 J/m² UVC and analysed the pattern of eGFP-pol ι 12 h later (Figure 1B, right). As with pol η , we found that in >60% of transfected cells, eGFP-pol ι was concentrated into foci throughout the nucleoplasm. We verified that the localization of eGFP-pol ι was not due to tagging artefacts by examining the distribution of untagged pol ι using a pCDNA-pol ι construct and anti-pol ι antibodies. Similar distributions were observed (Figure 1C). Furthermore, accumulation of nuclear foci was also observed after methyl methane-sulfonate treatment but not after γ -irradiation, indicating that pol ι focus formation is specific to certain classes of DNA lesions (data not shown). Using western blotting, we were able to show that there was no increase in the endogenous level of pol ι following UV irradiation of MRC5 cells (Figure 1D). This result suggests that the

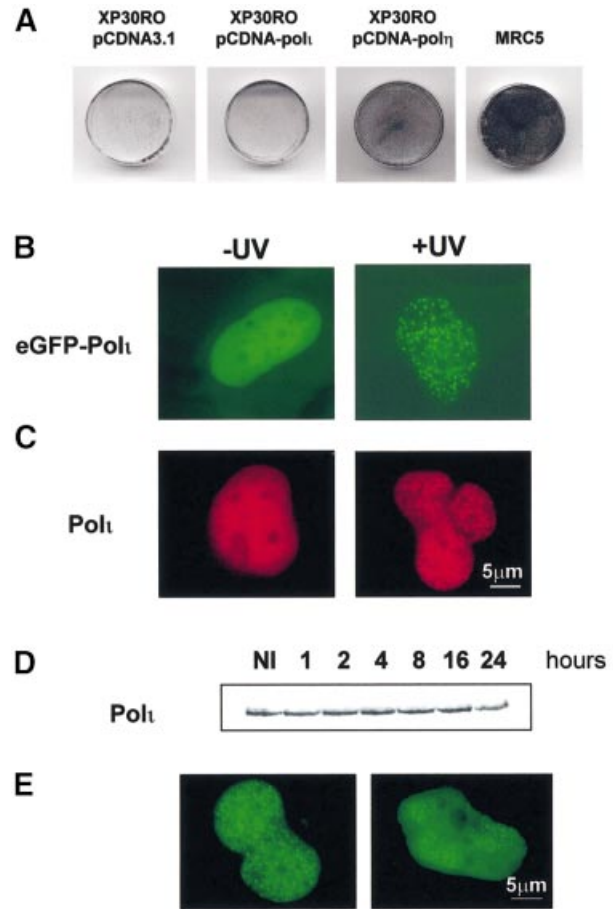


Fig. 1. Pol ι does not correct the UV + caffeine sensitivity of XP-V cells but localizes into nuclear foci after UV irradiation. (A) XP30RO cells were transfected with the plasmids pCDNA3.1 vector, pCDNA-pol ι or pCDNA-pol η as indicated. Stable clones were UV irradiated (7 J/m²) and then incubated in the presence of 75 μ g/ml caffeine. Right panel: MRC5 cells treated with the same doses of UV + caffeine. After 4 days, the cells were stained with methylene blue. (B and C) MRC5 cells were transfected with plasmids encoding either eGFP-pol ι (B) or pCDNA-pol ι (C). At 20 h post-transfection, cells were irradiated with 7 J/m² (right panels). After 12 h, the distribution of eGFP-pol ι was examined following paraformaldehyde fixation. For cells transfected with pCDNA-pol ι (C), pol ι distribution was revealed with anti-pol ι antibody and tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody. (D) Western blots of extracts of MRC5 cells irradiated with 7 J/m² and incubated for the indicated times. Blots were probed with anti-pol ι . In (E), MRC5 cells were transfected with eGFP-pol ι and irradiated either uniformly (left) or through a Millipore filter (right).

accumulation of pol ι in foci is not associated with induction of the protein.

Pol ι is recruited at sites of UV-induced DNA damage

By irradiating cells through a membrane filter containing holes of 8 μ m in diameter (Volker *et al.*, 2001), it is possible to induce local UV damage in the nucleus in cells transfected with peGFP-pol ι . After incubation for a further 12 h, cells were fixed and pol ι distribution was detected by autofluorescence of eGFP. In parallel, we irradiated the transfected cells without the membrane to compare the localization after total irradiation. As shown in Figure 1E (left), eGFP-pol ι is concentrated into foci throughout the nucleoplasm after total irradiation. In contrast, after local

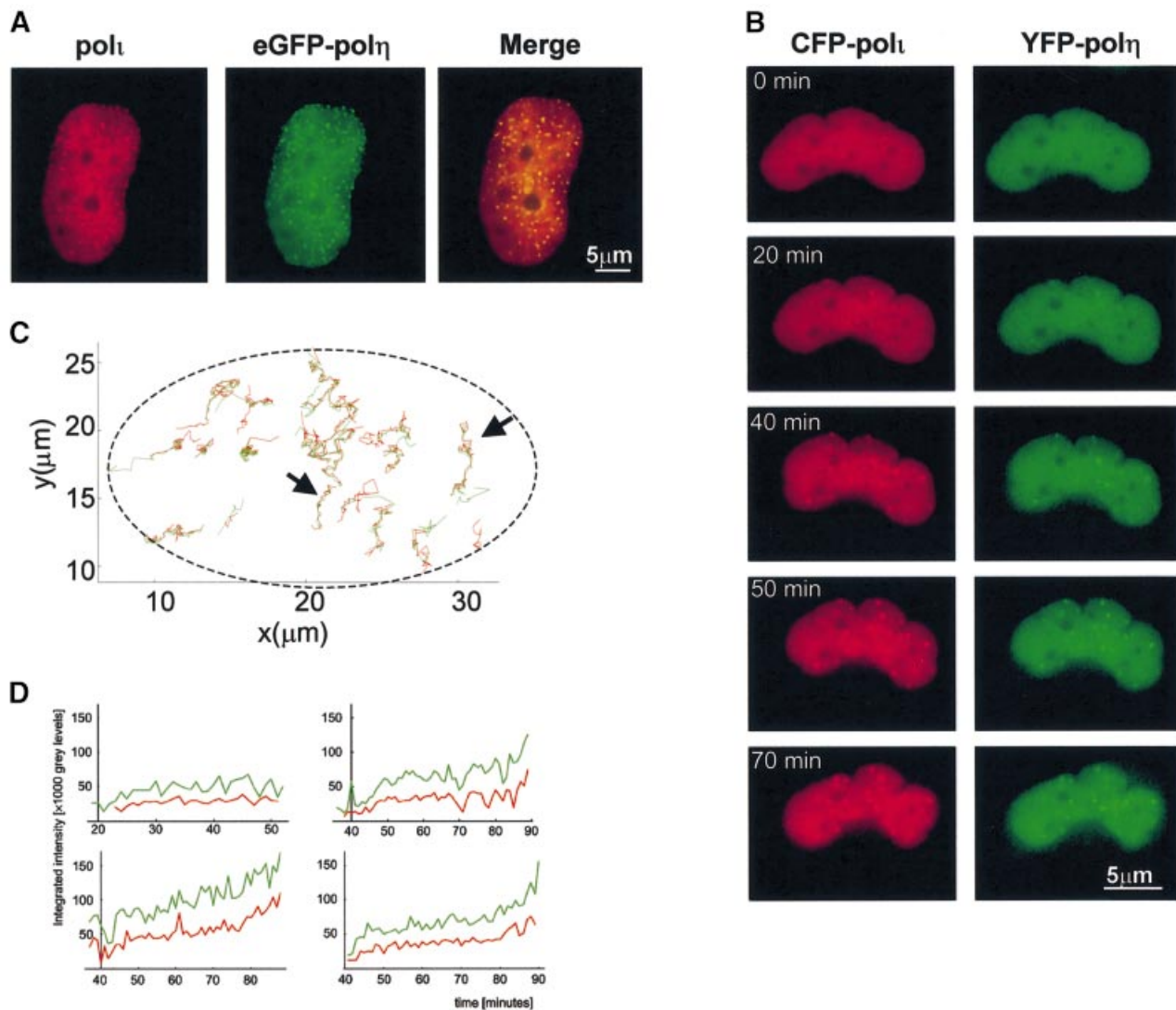


Fig. 2. Co-localization of $\text{pol}\eta$ and polt . (A) MRC5 cells co-transfected with eGFP- $\text{pol}\eta$ and pCDNA- polt were irradiated (7 J/m^2), fixed 12 h later and stained with anti- polt antibody and TRITC-conjugated secondary antibody. The staining pattern of polt (red) (left) and the autofluorescent signal of GFP- $\text{pol}\eta$ (green) (middle) in the same cell are shown. Co-localization of polt and eGFP- $\text{pol}\eta$ is indicated by a yellow pattern (right). (B–D) MRC5 cells were co-injected with peYFP- $\text{pol}\eta$ and peCFP- polt , and UV irradiated (10 J/m^2) 8 h later. The cells were examined by time-lapse microscopy using appropriate filters. (B) The fluorescent signals for the two tagged proteins at various times after irradiation. In (C), the co-ordinates of each focus in the cell were determined at different times. Each track represents the movement of a specific focus of $\text{pol}\eta$ (green) and polt (red). The dashed line represents the approximate position of the nuclear envelope. In (D), each of the four panels represents the intensity of the yellow and blue signals of an individual focus as a function of time. Green, $\text{pol}\eta$; red, polt .

irradiation, polt foci accumulated almost exclusively in the areas of the nucleus that had been exposed to UV (Figure 1E, right). This result supports the idea that polt foci accumulate at the sites of UV-induced DNA damage.

***Pol* co-localizes with $\text{pol}\eta$ in replication foci**

The localization patterns of polt are strikingly similar to those of $\text{pol}\eta$ (Kannouche *et al.*, 2001). We therefore checked by immunofluorescence if these proteins co-localize after UV irradiation. MRC5 cells were co-transfected with pCDNA- polt and peGFP- $\text{pol}\eta$ plasmids and irradiated with 7 J/m^2 . At 12 h post-irradiation, polt was visualized using anti- polt antibodies (red staining, Figure 2A, left) and $\text{pol}\eta$ was detected by autofluorescence of eGFP (green staining, Figure 2A, middle). Interestingly, in the nucleus of cells displaying polt foci, we detected

co-localization with eGFP- $\text{pol}\eta$ dots. The overlapping of $\text{pol}\eta$ with polt is visualized as yellow foci (Figure 2A, right) and indicates that polt and $\text{pol}\eta$ are localized in the same regions after DNA damage. We obtained similarly striking co-localization using confocal microscopy (data not shown). These results strongly suggest that polt , like $\text{pol}\eta$, accumulates at replication forks stalled at sites of UV damage.

Co-ordination of $\text{pol}\eta$ and polt foci in living cells

In order to investigate the co-localization of $\text{pol}\eta$ and polt in more detail, we used dual labelling of $\text{pol}\eta$ and polt and time-lapse microscopy. The cDNAs encoding enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP) were fused in-frame to the N-termini of polt (peCFP- polt) and $\text{pol}\eta$ (peYFP- $\text{pol}\eta$),

respectively. Both plasmids (peCFP-pol ι and peYFP-pol η) were microinjected together into MRC5 cells and 8 h later the fibroblasts were UV irradiated (10 J/m²). Foci formation of pol η and pol ι was analysed in living cells using time-lapse microscopy. A time-lapse series following foci in a single nucleus is shown in Figure 2B. At each time after irradiation, the foci of pol η and pol ι appeared co-incidentally.

Co-ordination in time and in space. The positions of each focus of pol η and pol ι within a single nucleus are plotted as a function of time in Figure 2C. Red and green lines correspond to the tracks of eCFP-pol ι and eYFP-pol η , respectively, in the cell. There was a striking overlap of most of the red and green lines over the whole period of time, indicating that pol η and pol ι foci are tightly associated after UV irradiation and not just for a short time. The trajectories in Figure 2C are in absolute space. Visual examination of the relative positions of the individual foci shows that these trajectories reflect a combination of movement of the whole nucleus and that of the foci relative to the nuclear envelope.

Co-ordination in intensity and in time. In order to determine whether the two polymerases accumulate at the same time on unrepaired damage, we have plotted the appearance and intensity of the fluorescence due to pol η and pol ι in four individual foci as a function of time (Figure 2D). These plots show that both the time of appearance of the focus and the increase in intensity of the fluorescent signals were very similar for pol ι and pol η . This indicates that, inside the nucleus, these two polymerases are closely co-ordinated in both space and time after UV irradiation. We observed a similar co-localization with the fluorophores reversed, i.e. using eYFP-pol ι - and eCFP-pol η -expressing plasmids, thus ruling out the possibility of artefacts due to cross-talk (data not shown).

Pol ι relocation is dependent on pol η

In view of this co-ordinated response of pol η and pol ι after UV irradiation, we asked whether pol ι could relocate at sites of replication forks blocked by UV damage in the absence of pol η . We transfected XP-V (XP30RO) cells with peGFP-pol ι , irradiated the cells with 7 J/m² and analysed pol ι distribution 12 h after irradiation. Strikingly, the number of transfected cells containing pol ι foci was never more than 20%, in contrast to MRC5 cells in which >60% of cells contained pol ι foci after irradiation. In both cases, we also analysed the localization of proliferating cell nuclear antigen (PCNA; red staining). In MRC5 cells, eGFP-pol ι relocated in foci which co-localized with PCNA dots (Figure 3, left), whereas in XP30RO fibroblasts eGFP-pol ι displayed diffuse staining even in cells in which PCNA formed dots (Figure 3, right). The experiments were repeated several times and in each experiment we observed only 10–20% of irradiated XP30RO cells with eGFP-pol ι foci. We conclude that the accumulation of pol ι in UV-induced replication foci requires the presence of pol η . In contrast, we observed that pol ι does remain in the nucleus in XP30RO cells, demonstrating that it is only the foci formation and not nuclear localization that is dependent on pol η .

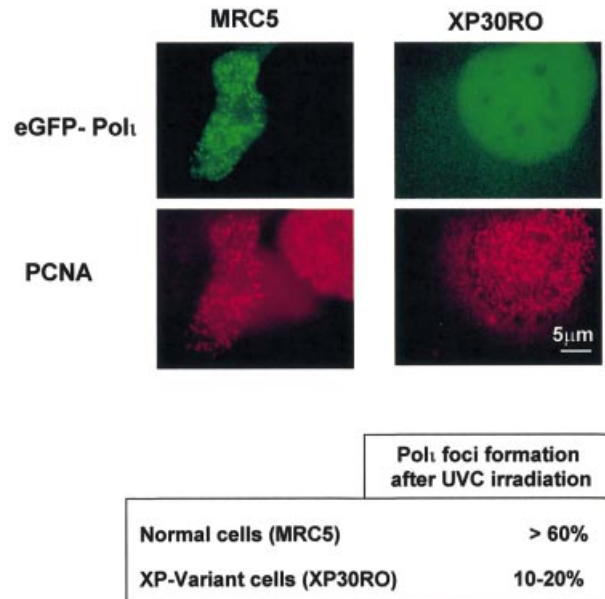


Fig. 3. Dependence of pol ι foci on pol η . MRC5 (left) or XP30RO cells (right) transfected with eGFP-pol ι were UV irradiated, fixed 12 h later and stained with anti-PCNA monoclonal and TRITC-conjugated secondary antibody. The staining patterns of the autofluorescent signal of GFP (green staining in upper panels) and PCNA (red staining in lower panels) in the same cell are shown.

Pol ι interacts with pol η

Our cellular localization data provide strong evidence that pol η and pol ι are closely linked in the nucleus. We therefore carried out several tests to determine if the two polymerases might interact directly. In the first series of experiments, we used the yeast two-hybrid system. pol η cDNA was cloned downstream of the GAL4 DNA-binding domain and pol ι cDNA downstream of the GAL4 activation domain. The plasmids were co-transfected into a yeast reporter strain, AH109. Interaction resulted in the ability of the yeast cells to grow in selective medium in the absence of tryptophan, leucine, histidine and adenosine. Figure 4A shows that only cells containing both pol η - and pol ι -expressing plasmids were able to grow in selective medium, whereas if either of the plasmids contained no insert, there was no growth. To determine which domain in pol ι was responsible for the interaction with pol η , we used three different pol ι deletion constructs. As indicated in Figure 4B, the C-terminal 224 amino acids of pol ι (residues 492–715) were sufficient to interact with pol η , whereas no interaction was observed with the N-terminal 492 amino acids containing the whole of the catalytic domain.

In the second series of experiments, we constructed GST fusion proteins containing either full-length pol ι or the C-terminal 224 amino acids that interact with pol η in the two-hybrid assay. The proteins were electrophoresed in SDS-polyacrylamide gels, transferred to membranes and probed with ³⁵S-labelled *in vitro* translated pol η in far-western assays (Figure 4C). The results demonstrate that pol η interacts with both full-length GST-pol ι (lane 5) and GST-pol ι (492–715) (lane 6), whereas no interaction was seen with GST alone (lane 4). In order to determine which region of pol η interacted with pol ι , we repeated the far-western analysis using fragments of pol η containing

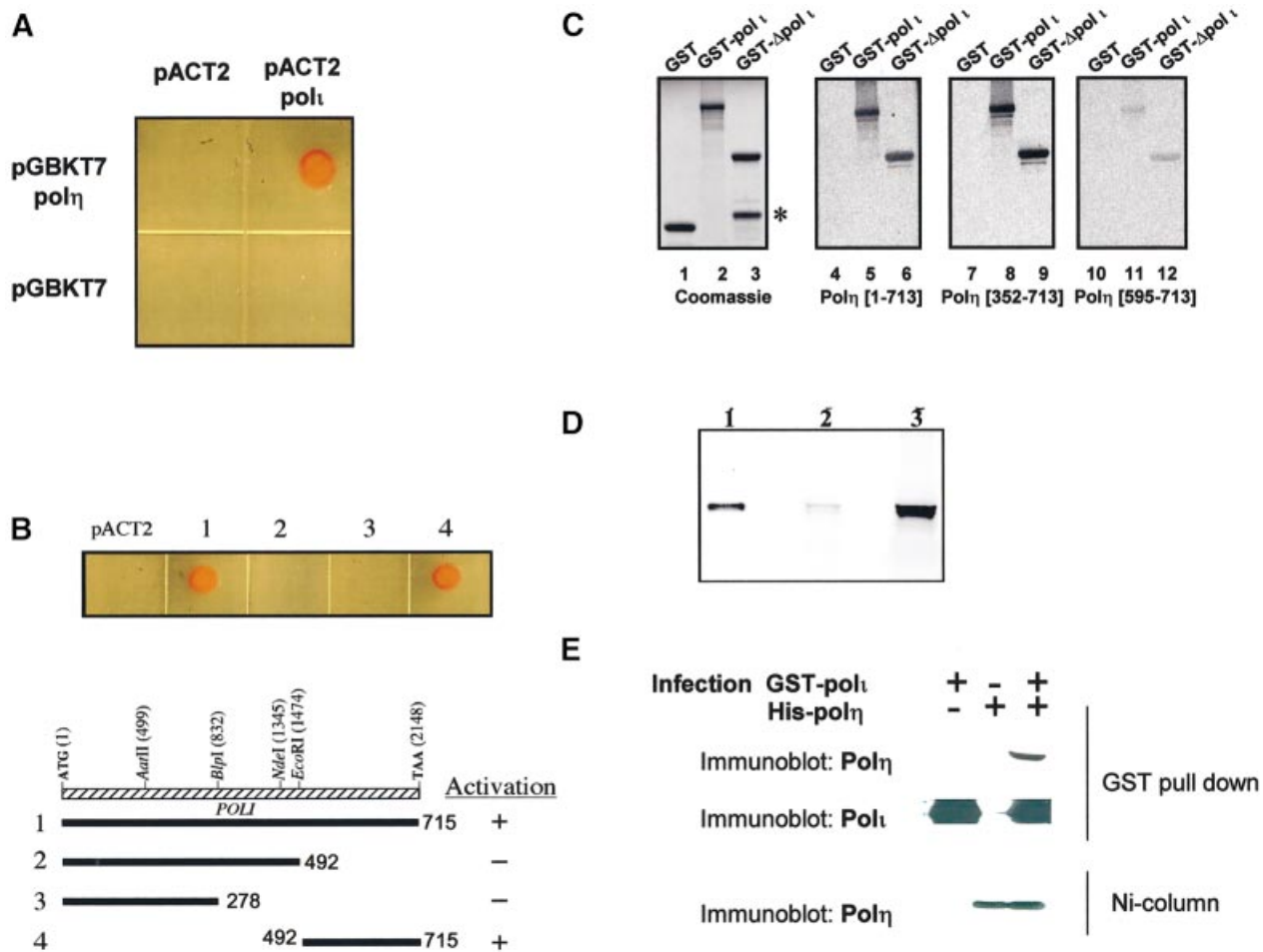


Fig. 4. Interaction between pol η and polt. (A and B) *In vivo* interaction of pol η and polt using the yeast two-hybrid system. (A) *Saccharomyces cerevisiae* strain AH109 was co-transformed with pGBKT7/pACT2, pGBKT7/pACT2-polt, pGBKT7-pol η /pACT2 and pGBKT7-pol η /pACT2-polt. A representative colony from each transformation was grown overnight at 27°C in selective medium and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 27°C for 3 days. (B) Determination of the minimal region of polt that interacts with pol η . *Saccharomyces cerevisiae* strain AH109 was co-transformed with pGBKT7-pol η /pACT2, or (1) pGBKT7-pol η /pACT2-polt, (2) pGBKT7-pol η /pAR218 (N-terminal 492 residues of polt), (3) pGBKT7-pol η /pAR216 (N-terminal 278 residues of polt) and (4) pGBKT7-pol η /pAR220 (C-terminal 224 residues of polt). (C) Far-western analysis of the interaction between polt and pol η . A Coomassie blue-stained SDS-polyacrylamide gel showing the expression and expected size of the GST-polt fusion proteins. A 1 μ g aliquot of GST (lane 1) was used as negative control; GST-polt (lane 2); GST- Δ pol η (492–715) (lane 3). The protein band indicated with an asterisk is a degradation product of the GST- Δ pol η (492–715) fusion protein. Far-western blots of equivalent samples after transfer to nitrocellulose membrane and incubation of the immobilized proteins with 35 S-labelled full-length pol η (lanes 4–6), pol η (352–713) (lanes 5–9) and pol η (595–713) (lanes 10–12). (D) GST pull-down assay demonstrating that pol η interacts with the C-terminal region of polt. *In vitro* translated 35 S-labelled pol η protein was incubated with glutathione-Sepharose beads and equal amounts of GST or GST- Δ pol η (492–715) as indicated in Materials and methods. Bound proteins were eluted, and resolved by 4–20% SDS-PAGE. A portion of the *in vitro* translated 35 S-labelled pol η corresponding to 10% of the labelled protein in the binding reaction was loaded as input (lane 1). The results show that pol η binds GST- Δ pol η (492–715) (lane 3) but not GST alone (lane 2). (E) Sf9 cells were infected with baculovirus supernatants containing GST-polt, His₆-pol η or both. Lysates were extracted with glutathione-Sepharose beads, and the extracted proteins analysed by SDS-PAGE and western blotting. Blots were probed with anti-pol η (top) or anti-polt (middle) To check the amounts of pol η in the lysates, parallel samples were extracted with nickel-agarose beads and analysed for the amount of pol η by western blotting.

either the C-terminal 362 amino acids (352–713) or the C-terminal 119 amino acids (595–713) as radiolabelled probes. Pol η (352–713) interacted with polt as well as the full-length protein (Figure 4C, lanes 7–9), whereas pol η (595–713) gave a much weaker signal (lanes 10–12). We conclude that the interaction takes place between the C-terminal 224 amino acids of polt and a region of pol η between amino acids 352 and 595, although we cannot exclude that residues 595–713 are also required.

In the third set of experiments, the same GST-polt (492–715) construct was used in a ‘pull-down’ assay. *In vitro* translated 35 S-labelled pol η was incubated with glutathione-Sepharose beads coupled with either GST or

GST-polt (492–715). The bound proteins were resolved by SDS-PAGE. Figure 4D shows that *in vitro* translated pol η was indeed bound to the beads coupled with GST-polt (492–715), but not with GST alone. In these assays, the bound protein represents ~10% of the total input protein.

In the final series of experiments, we looked for direct interaction of pol η and polt inside cells using co-immunoprecipitation. We were unable to detect co-immunoprecipitation inside human cells, so we over-expressed both proteins in insect cells using baculovirus constructs, in which pol η was tagged with His₆ and polt was tagged with GST. After infection of Sf9 cells with

both constructs, extracts were either incubated with glutathione–Sepharose beads to extract polt, or passed down a nickel column to extract pol η , and interacting proteins were analysed by western blotting using appropriate antibodies. The results are shown in Figure 4E. Pol η was found associated with GST–polt on the beads (lane 3), but not in controls without GST–polt (lane 2). The bottom panel shows that similar amounts of pol η were present in both extracts. The amount of pol η associated with polt represents a small proportion of the total pol η in the insect cell extract, suggesting that under these assay conditions, the pol η –polt interaction is not particularly robust.

Taken together, these experiments provide convincing evidence that pol η and polt interact physically, and this correlates with their tight co-localization within nuclear foci.

Deletion analysis of polt

In contrast to pol η , the primary sequence of polt contains no clear NLS. To identify the regions of polt required for nuclear localization and UV relocalization, a series of GFP-tagged deletion mutants was constructed, and their localization is shown in Figure 5.

Nuclear localization. As shown in Figure 5, pBC28, which contains a deletion of the C-terminus from amino acid 451 to 715, was located in the cell nucleus but did not relocalize into foci after UVC irradiation. Thus, the last 265 amino acids of polt are not required for transport of the protein into the nucleus but are required for foci formation. However, using pBC34, in which the C-terminal deletion was increased to 317 amino acids (400–715), the protein was no longer nuclear. On removal of the N-terminal 218 amino acids (pBC41), the protein was still nuclear (and was able to relocalize into foci), and the construct containing amino acids 219–451 (pBC52) was sufficient to confer nuclear localization. These results indicate that the sequences necessary for nuclear localization are between amino acids 219 and 451 and are likely to be between amino acids 400 and 451.

Foci formation. Foci formation was not affected by the removal of the first 218 amino acids (pBC41) but was abolished by removal of the last 80 amino acids (pBC26). To investigate the role of C-terminal sequences in foci formation, we added an artificial NLS [corresponding to the SV40 large T antigen NLS (PKKKRKV)] to the last 175 amino acids of polt (pBC30). This construct, though clearly nuclear, did not form foci after UV irradiation. When we added the same artificial NLS to the last 224 amino acids of polt (pBC36), the result was different. In this case, the protein was able to relocalize into foci. These results indicate that the domain required for foci formation is distinct from the NLS. The foci formation domain requires the last 80 amino acids as well as sequences located between amino acids 490 and 539.

Discussion

Recent studies indicate that human cells possess four Y-family DNA polymerases (pol η , polt, polk and Rev1) along with a B-family polymerase, pol ζ (Nelson *et al.*, 1996), which, based on *in vitro* replication assays (for a

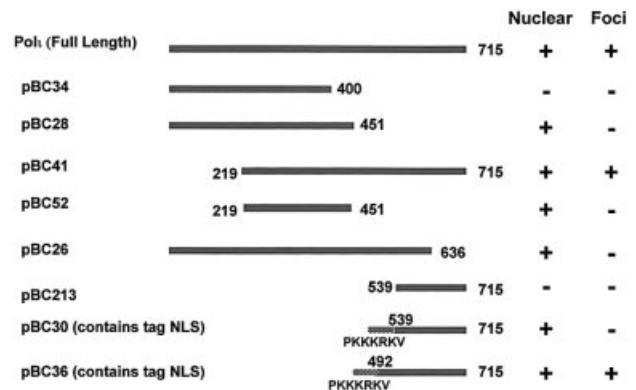


Fig. 5. Deletion analysis of polt localization. Polt deletion constructs were made as described in Materials and methods. The fragments were cloned downstream of the eGFP tag and transfected into MRC5 cells. The cells were UV irradiated (7 J/m²) 24 h later and, after a further 8 h, the cells were fixed and analysed for nuclear location and focus formation as indicated.

review, see Goodman, 2002), all appear capable of participating (with varying efficiencies) in lesion bypass. An important question that needs to be addressed is how each of these polymerases is physically recruited to any particular DNA lesion *in vivo* and, once there, how the cell determines which polymerase should be employed to bypass the lesion.

Tight co-ordination of pol η and polt in the nucleus

In our previous work, we showed that in human cells, pol η , which is required for translesion synthesis past UV photoproducts in DNA, is localized in replication factories (Kannouche *et al.*, 2001). Following DNA damage, the pol η -containing foci accumulate as replicating forks stall at the sites of DNA damage. In the present work, we have shown that the localization pattern of human polt is very similar to that of pol η . In both undamaged cells and cells damaged either uniformly or at specific sites in the nucleus, the localization patterns are the same for both polymerases (Figure 2). We have shown, using time-lapse microscopy, that pol η and polt accumulate at unrepaired lesions during DNA replication at the same time and with the same changes in intensity. This strongly suggests that the assembly of the two paralogues into replication foci is tightly co-ordinated. This important result indicates that the recruitment of these different polymerases is not sequential in the nucleus. A complex containing both polymerases is likely to be pre-formed in the nucleoplasm before it relocalizes in replication factories. We have been obliged to use overexpression in all our experiments, as none of our antibodies is able to detect endogenous levels of polymerases. However, we have previously presented arguments which strongly suggest that our results reflect the localization patterns of endogenous proteins (Kannouche *et al.*, 2001).

How does polt relocalize into intranuclear foci?

Figure 6A shows the different domains that we have identified in pol η and polt. We previously showed that the C-terminal 119 amino acids of pol η (595–713) were sufficient for localization into replication foci (Kannouche *et al.*, 2001). This domain contains a putative C2H2 zinc finger, a bipartite NLS and a potential PCNA-binding site.

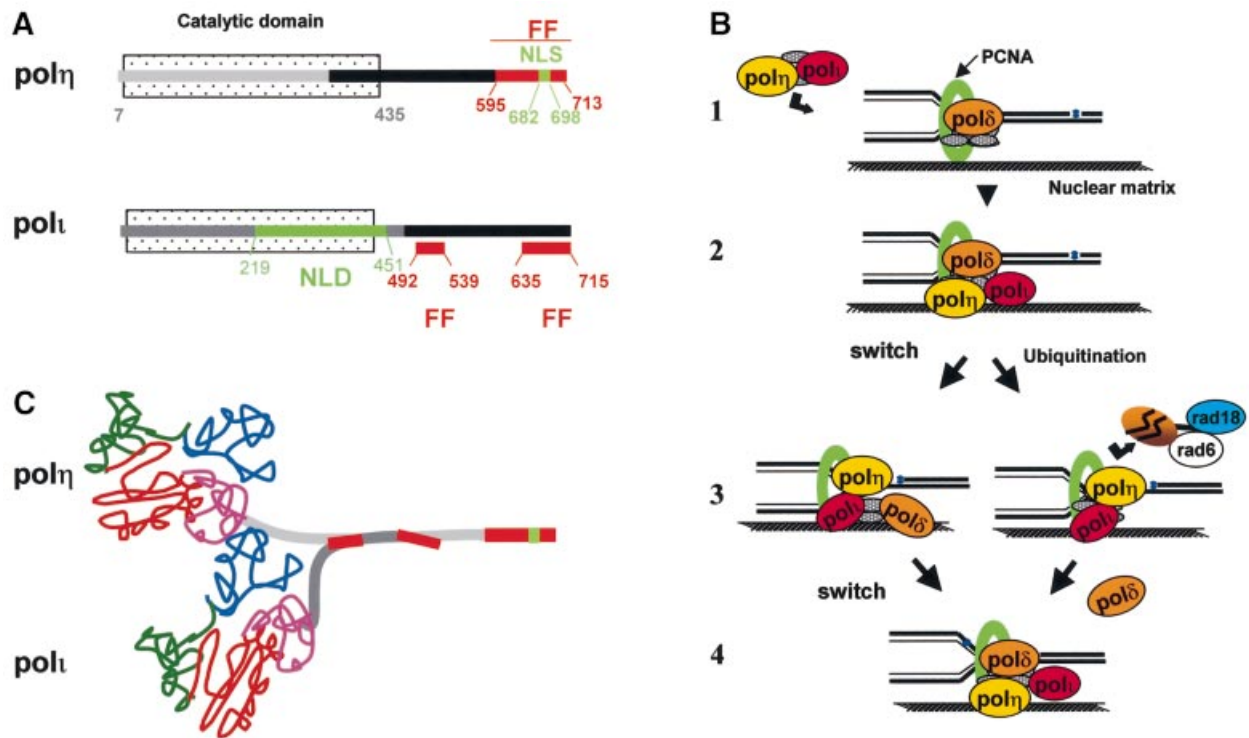


Fig. 6. Interactions between pol η and pol ι , and involvement in TLS. **(A)** Domain structures of pol η and pol ι . FF, domains required for foci formation; NLS, classical nuclear localization signal; NLD, nuclear localization domain. The black bar indicates regions of interaction between the two polymerases. **(B)** Involvement of polymerases in bypass of damage. (1) The structure shows a replication fork attached to a nuclear matrix structure in a replication factory, with the DNA being pulled through the fork. Pol η and pol ι are either co-localized in the factory and get recruited to the replisome or, in (2), they are an intrinsic part of the replisome, possibly by interacting with PCNA. (3) When pol δ is blocked by damage, it either disengages from the primer terminus or is removed following modification, allowing pol η or pol ι to bypass the damage. In (4), pol δ is restored to the replication fork after the damage has been bypassed. **(C)** Scheme for interaction of polymerases. The depicted N-terminal catalytic domain is drawn in schematic form from the crystal structure of a Y-family DNA polymerase from *Sulfolobus solfataricus* (Ling *et al.*, 2001), with the finger, palm, thumb and little finger domains indicated in blue, red, green and purple, respectively.

This site recently has been shown to bind PCNA and, in doing so, stimulates the catalytic activity of pol η *in vitro* (Haracska *et al.*, 2001a). We have shown that the zinc finger, NLS and PCNA-binding motifs are all required for efficient localization into nuclear foci (results to be published elsewhere). Pol ι has neither a zinc finger nor an NLS, but has a potential PCNA-binding site. Like pol η , the *in vitro* catalytic activity of pol ι is stimulated in the presence of PCNA (together with RFC and RPA) (Haracska *et al.*, 2001b). Such findings prompted Haracska *et al.* to suggest that the interaction between pol ι and PCNA targets pol ι to the replication machinery (Haracska *et al.*, 2001b). We find, however, that in XP-V cells, even though PCNA assembles into replication foci, which accumulate after UV irradiation, pol ι does not. Although our data do not exclude a role for PCNA in the localization of pol ι into replication foci, they strongly suggest that it is pol η which targets pol ι into these foci, since (i) pol ι directly interacts with pol η (Figure 4); (ii) the C-terminal 224 amino acids (492–715) of pol ι are sufficient both for interaction with pol η and for localization into replication foci (Figure 6A); and (iii) in pol η -defective XP-V cells, the relocalization of pol ι into foci is drastically diminished (Figure 3).

Figure 6B shows several possible mechanisms for polymerase–polymerase interactions taking place at the replication fork. Pol η and pol ι are localized together in the replication factories, but within the factory they could

either be within the body of the factory and recruited to the replisome when required, as indicated in Figure 6B,1, or they may actually be part of the replisome complex (Figure 6B,2). When the replisome encounters a lesion, there may be a switch from replicative to TLS polymerase (Figure 6B,3, left) or, alternatively, the replicative pol δ may be modified by ubiquitylation (Figure 6B,3, right) and possibly degraded, and then replaced by a TLS polymerase. Since the Rad6/Rad18 E2 ubiquitin-conjugating enzyme is known to be absolutely required for TLS, at least in *S.cerevisiae* (Broomfield *et al.*, 2001), it could perform the proposed ubiquitylation reaction. The TLS polymerases are inherently distributive so that, shortly after lesion bypass occurs, they would dissociate from the primer template and be replaced by pol δ , either by re-engaging the ‘switched out’ polymerase or by incorporation of a new pol δ molecule into the replisome (Figure 6B,4). The model of removing pol δ by ubiquitylation is reminiscent of that proposed to remove RNA polymerase II blocked at lesions to enable transcription-coupled repair to take place (Ratner *et al.*, 1998). A related model whereby the polymerase switching is mediated by ubiquitylation of PCNA has been proposed by Hoege *et al.* (2002).

Pol ι interacts with the central region of pol η

A model showing how pol η and pol ι might interact is shown in Figure 6C. Pol η contains three distinct functional

domains. Based on the three-dimensional structure of yeast pol η , the N-terminal 435 amino acids comprise the polymerase catalytic site (Trincao *et al.*, 2001). The C-terminal 119 amino acids (595–713) are involved in nuclear and foci localization (Kannouche *et al.*, 2001), but no function has as yet been attributed to the intervening sequences (435–595). We have now shown (Figure 4C) that the region of pol η that interacts with polt is likely to be contained within this domain. Interestingly, this sequence is not conserved in *S.cerevisiae* pol η (Rad30), indicating that this domain is not necessary in yeast. This suggests that it could be involved in interaction with other proteins, which, like polt, are only present in higher eukaryotes.

Domain structure of polt

Despite the absence of any obvious NLS consensus motif in the amino acid sequence of polt, we observed that its nuclear localization (as opposed to relocalization into foci) is not dependent on pol η . The mechanism of nuclear accumulation remains to be determined. Although pol η and polt are paralogues, contain a similar N-terminal domain that comprises the polymerase catalytic site and show an identical nuclear localization into foci, the organization of their C-terminal domains is completely different. Whereas in pol η the C-terminal 119 amino acids were sufficient for nuclear localization and foci formation (Kannouche *et al.*, 2001), localization of polt in the nucleus requires sequences within amino acids 219–451, and the domain involved in foci formation may be bipartite: sequences within amino acids 492–539 and 636–715 (Figure 6A).

The role of polt in human cells

Although several studies have been carried out on the activities of polt *in vitro* (Johnson *et al.*, 2000b; Tissier *et al.*, 2000a; Vaisman and Woodgate, 2001; Vaisman *et al.*, 2001; Zhang *et al.*, 2001), there has been much speculation, but as yet no convincing evidence, as to its role *in vivo*. Our findings that the localization of polt is tightly co-ordinated with that of pol η suggest that polt, like pol η , is involved in TLS past DNA damage. Like other members of the Y-family, it appears able to accommodate a variety of DNA lesions in its active site but, as yet, no lesion has been identified that polt can bypass efficiently without the assistance of another polymerase. Its role in the bypass of UV photoproducts is particularly unclear due to conflicting reports on the ability of the enzyme to incorporate bases opposite T–T CPDs or T–T 6–4 photoproducts *in vitro* (Johnson *et al.*, 2000b; Tissier *et al.*, 2000a; Haracska *et al.*, 2001b; Zhang *et al.*, 2001). As pol η is able to bypass T–T CPDs efficiently (Masutani *et al.*, 2000; Matsuda *et al.*, 2000), it is unlikely that polt plays a major role in TLS past this photoproduct in normal cells. However, pol η and polt appear to possess roughly similar abilities to incorporate a base opposite the 3' T of a T–T 6–4 photoproduct (Masutani *et al.*, 2000; Tissier *et al.*, 2000a; Zhang *et al.*, 2000a). Neither polymerase can bypass the lesion unassisted. If, however, the (mis)incorporated bases can, as shown *in vitro*, be extended by another polymerase such as pol ζ , it is possible that polt may play a role in the bypass of T–T 6–4 photoproducts. Last, but not least,

all the *in vitro* studies assaying the ability of Y-family polymerases to bypass UV photoproducts have been performed using only T–T photoproducts, since C-containing photoproducts are inherently unstable. It is possible that polt might be involved in the bypass of C-containing photoproducts. It is also likely that together with pol ζ , polt may similarly be involved in TLS past other adducts that normally block DNA replication.

In XP-V cells lacking pol η , a back-up process is able to synthesize intact DNA molecules, albeit at reduced efficiency (Lehmann *et al.*, 1975). This process is highly mutagenic as XP-V cells are hypermutable to UV (Maher *et al.*, 1976). Presumably, in XP-V cells, another polymerase substitutes for pol η and bypasses the UV photoproducts with low efficiency and low fidelity. polt is a candidate for the alternative polymerase, as neither polk nor Rev1 appear able to incorporate bases opposite UV photoproducts (Johnson *et al.*, 2000a; Ohashi *et al.*, 2000; Zhang *et al.*, 2000b, 2002). However, our observation that polt focus formation is dramatically reduced in XP-V cells would appear inconsistent with such a hypothesis. The possibility remains, however, that the reduced number of polt-containing foci might be sufficient to carry out some TLS past UV photoproducts, albeit with reduced efficiency and fidelity, thus conferring the hypermutable phenotype observed in XP-V cells.

Finally, our observation that polt is located in replication foci in undamaged cells suggests that it may also play a general role in the maintenance of genome integrity during DNA replication. This might occur though the incorporation of guanine opposite uracil resulting from deamination of cytosine (Vaisman and Woodgate, 2001), or opposite the AP site generated during the subsequent base excision repair of the uracil.

Materials and methods

Cell lines and culture conditions

The SV40-transformed human fibroblasts MRC5V1 (normal) and XP30RO(sv) (XP-V) (also designated GM3617) (Cleaver *et al.*, 1999) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The designations of the cell lines are abbreviated to MRC5 and XP30RO. Transfection using Fugene, isolation of stable clones and UV irradiation were carried out as described previously (Kannouche *et al.*, 2001).

Construction of expression vectors

pCDNA-pol η was constructed as previously described (Kannouche *et al.*, 2001). Full-length cDNA encoding polt was obtained by PCR using pJM296 (McDonald *et al.*, 1999) as template, with *Pfu* DNA polymerase and primers I-A and I-B (Table I).

The PCR product was digested with *EcoRV* and *BamHI* and inserted into the *EcoRV*–*BamHI* sites of pCDNA3.1zeo downstream of the CMV promoter (Invitrogen), producing the plasmid pCDNA-polt.

peGFP-pol η was produced as previously described and was able to correct the UV sensitivity of pol η -defective XP-V cells (Kannouche *et al.*, 2001). To generate peYFP-pol η , pol η cDNA lacking the ATG initiator codon was amplified by PCR using pCDNA-pol η as template, *Pfu* DNA polymerase and the primers I-C and I-D (Table I). The product was digested with *XhoI* and *BamHI* and inserted into the *XhoI*–*BamHI* sites of the peYFP-C1 vector. PeCFP-polt was produced in a similar way, using pCDNA-polt as template and the primers I-E and I-F.

A series of eGFP-tagged deletion mutants of polt was generated to study the cellular organization of polt. All polt deletion mutants were cloned in-frame downstream of the eGFP cDNA in peGFP-C3.1. Polt cDNA fragments were generated using PCR with primers (see Table I) containing an *XhoI* site at the 5' end and a *BamHI* site at the 3' end. The PCR products were digested with *XhoI* and *BamHI* and cloned into

Table I. Primers used in deletion constructs

Primer name	Sequence
I-A	5'-GATATCATGGAAGTGGCGGACGTGGGGGCG-3'
I-B	5'-GGATCCTTATTTATGTCCAATGTGGAAATC-3'
I-C	5'-CTCGAGTGGCTACTGGACAGGATCGA-3'
I-D	5'-GGATCCCTAATGTGTAAATGGCTTAAAAAATGA-3'
I-E	5'-CTCGAGTGGAACTGGCGGACGTGGGGGCG-3'
I-F	5'-GGATCCTTATTTATGTCCAATGTGGAAATC-3'
I1	5'-CTCGAGCTCGAACTGGCGGACGTGGGGGCG-3'
I2	5'-GGATCCTTATTTATGTCCAATGTGGAAATC-3'
I3	5'-CTCGAGCTCGAACTGGCGGACGTGGGGGCG-3'
I4	5'-GGATCCTGGCATCTCACATTCACCATATTTTCGAAAAAG-3'
I5	5'-CTCGAGCCAAAAAGAAGCGCAAGGTCGCCTCTAGAGCAGTATTATCT-3'
I6	5'-GGATCCTTATTTATGTCCAATGTGGAAATC-3'
I7	5'-CTCGAGCCAAAAAGAAGCGCAAGGTCATGAATGCCACTCTGTTCACACTCC-3'
I8	5'-GGATCCTTATTTATGTCCAATGTGGAAATC-3'
I9	5'-CATTCATTTCCAAACCTGCAGAGTGAGCAAC-3'
I10	5'-GTTGCTCACTCTGCAGGTTTGGAAATGAATG-3'

peGFP-C3 digested with the same enzymes. For full-length polt (pBC21), we used primers I1 and I2. Plasmid pBC28 was obtained by digesting pBC21 with *XmnI* and *BamHI* to remove a 1 kb fragment of polt cDNA from the 3' end of the open reading frame (ORF). The ends were made blunt with the Klenow enzyme and vector was religated. Plasmids pBC34, pBC30 and pBC36 were constructed using pBC21 as template and PCR with primers I3 and I4 (5' 1200 bp), I5 and I6 (3' 531 bp) and I7 and I8 (3' 672 bp), respectively. To construct plasmid pBC26, a *PstI* site at nucleotide 1913 of the cDNA was introduced by site-directed mutagenesis. First, plasmid pBC21 was used as template, and primers I9 and I10. The resulting plasmid pBC25 contained a silent change with a *PstI* site in the cDNA sequence at nucleotide 1913. pBC26 was obtained by digesting pBC25 with *PstI* and *BamHI* to remove a 0.2 kb fragment of polt cDNA located in the 3' end of the ORF. The ends were made blunt with the Klenow enzyme and the vector was religated. All PCR products were checked by DNA sequencing.

Live cell microscopy and image analysis

peCFP-polt (0.1 µg/ml) and peYFP-polη (0.1 µg/ml) were microinjected into MRC5 cells. After 8 h, cells were irradiated with 10 J/m². For monitoring living cell cultures, MRC5 cells were incubated in a phenol red-free medium including 10% FCS. Fluorescence images of cell nuclei were acquired on an Axiovert TM 135 microscope (Carl Zeiss) equipped with a 100× NA 1.4 objective lens and an Orca ER CCD camera (Hamamatsu) using Acquisition Manager (Kinetic Imaging). Real time excitation measurements were made by alternating excitation at 440 ± 12.5 and 500 ± 10 nm, and detection of CFP and YFP emission at 465 ± 12 and 530 ± 15 nm, respectively. A CFP/YFP filter set (Omega XF 135) was used with excitation and emission filter wheels. The separate pairs of excitation and emission filters suppress the cross-talk, and a stationary shared dichroic mirror ensures that there is no lateral shift between the two fluorescent channels. Integrated intensity of either channel in cell nuclei did not show systematic decline. Therefore, we concluded that bleaching was either insignificant or was compensated by continuing production of the proteins. Foci in cell nuclei were detected by Sobel image enhancement followed by thresholding in every individual frame of a movie, and their positions and integrated intensities were calculated using intensity values from the original images. These data were used to track an individual focus automatically through space and time. Processing of images and data was performed in Mathematica (Wolfram Research), and the images and plots were finalized using Illustrator (Adobe).

Immunofluorescence microscopy and western blotting

Fixation of the cells and immunofluorescence microscopy and western blotting were carried out as described previously (Kannouche *et al.*, 2001). Polt protein was detected using a rabbit polyclonal antibody directed against a keyhole limpet haemocyanin (KLH)-conjugated peptide corresponding to the extreme C-terminal 15 amino acid residues (AEWKRTGSDFFHIGHK) of polt. The antibody was diluted 1:500 for immunofluorescence and 1:4000 for western blotting. Polη protein was

detected using a rabbit polyclonal antibody (Kannouche *et al.*, 2001) diluted 1:2500. PC10 monoclonal anti-PCNA protein was from Santa Cruz Biotechnology. For immunofluorescence, all antibodies were diluted in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). No foci were observed in cells transfected with empty vectors, and no immunofluorescent signals were observed without primary antibody.

Two-hybrid interactions

An interaction between human polη and polt was demonstrated *in vivo* using the *S.cerevisiae* two-hybrid Matchmaker III system (Clontech, Palo Alto, CA). The human polη gene was cloned as an ~2.5 kb *NdeI-XhoI* fragment from pET21-polη into the *NdeI-SalI*-digested GAL4 DNA-binding domain plasmid, pGBKT7, to generate pAR204. The full-length human polt gene was cloned into the pACT2 activation domain vector by ligating an ~2.5 kb *NcoI-SalI* fragment from pJM299 (Tissier *et al.*, 2000b) into the *NcoI-XhoI*-digested vector to generate pAR116. Both pAR116 and pAR204 were then transformed into the *S.cerevisiae* strain AH109. Transformants were selected on DOBA-Trp-Leu (Bio 101) plates. Colonies were subsequently replica plated on DOBA-Trp-Leu-His-Ade + X-α-Gal (Bio 101, Vista, CA) plates, to confirm the activation of the reporter genes.

Deletion derivatives of the pACT2-polt plasmid, pAR116, were constructed as follows. Plasmid pAR216 containing the N-terminal 278 residues from polt was generated by ligating a 0.9 kb *NcoI-XhoI* fragment from pAR124 (A.R.Fernández de Henestrosa and R.Woodgate, in preparation) into the similarly digested pACT2 vector. pAR218, containing the N-terminal 492 amino acids of polt, was constructed by cloning a 1.5 kb *NcoI-EcoRI* fragment from pAR110 (A.R.Fernández de Henestrosa and R.Woodgate, in preparation) into the similarly digested pACT2. Finally, a plasmid, pAR220, expressing the C-terminal 224 amino acids of polt, was constructed by cloning a 1 kb *NcoI-DraI* fragment from pAR130 (A.R.Fernández de Henestrosa and R.Woodgate, in preparation) into pACT2. Analysis was as described above.

In vitro transcription/translation of polη

In vitro transcription/translation of full-length polη (1–713) and two N-terminal truncated proteins, polη (352–713) and polη (595–713), was performed using a TNT-coupled lysate system (Promega) according to the manufacturer's instructions. The expression vectors encoding the full-length polη (pGBKT7-polη) and its truncated versions (cDNAs cloned in pET29a) were added separately to the reaction mixtures and then incubated for 90 min at 30°C in the presence of [³⁵S]methionine. Reaction products were analysed directly by SDS-PAGE and used in both far-western and GST pull-down assays.

GST fusion proteins

Full-length GST-polt was purified from Sf9 insect cells infected with pJM299 baculovirus, as previously reported (Tissier *et al.*, 2000b). A plasmid, pAR208, expressing GST fused to the C-terminal 224 residues of polt was constructed by cloning a 0.7 kb *EcoRI-SalI* fragment

encoding the C-terminus of pol θ from pAR110 (a pGBKT7 derivative expressing full-length pol θ ; A.R.Fernández de Henestrosa and R.Woodgate, in preparation), into the similarly digested vector, pGEX4T1 (Amersham Pharmacia Biotech). Extracts were made from 500 ml of exponentially growing DH5 α /pGEX4T1 or DH5 α /pAR208 cells that had been induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h. Both the wild-type GST protein and the GST-pol θ (492–715) protein were subsequently purified by glutathione–Sepharose affinity chromatography following the manufacturer's instructions (Amersham Pharmacia Biotech).

Far-western blot analysis

Purified GST fusion proteins were separated by 4–20% SDS–PAGE (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Invitrogen). Membranes were incubated overnight with ³⁵S-labelled Pol η protein at 4°C and then washed three times for a total time of 14 min at 4°C, dried briefly and scanned with a FujiFilm FLA-3000 phosphorimager.

GST pull-down assay

Equal amounts of GST or GST-pol θ (492–715) (~100 μ g) coupled to glutathione–agarose beads (15 μ l) were mixed with ³⁵S-labelled pol η in 200 μ l of binding buffer (PBS, 0.5% BSA and 0.2% Triton X-100), containing 'Complete' protease inhibitors (Roche Molecular Biologicals, Indianapolis, IN) for 15 min at 37°C. The beads were subsequently washed four times with binding buffer and the bound proteins separated on a 4–20% SDS–polyacrylamide gel.

Co-infection in Sf9 insect cells and pull-downs of pol θ and pol η

Recombinant virus expressing pol θ tagged with GST at its N-terminus was made as previously described (Tissier *et al.*, 2000b). Virus expressing pol η tagged with His₆ at its C-terminus was kindly provided by Fumio Hanaoka (Masutani *et al.*, 2000). Sf9 cells were infected or co-infected with baculovirus expressing GST-pol θ , His₆-pol η or both. In brief, the cells were harvested 72 h after infection, washed with ice-cold PBS, resuspended in 8 vols of ice-cold buffer A (20 mM sodium phosphate pH 7.3, 10% glycerol, 10 mM β -mercaptoethanol, 300 mM NaCl, 1% NP-40, 1 \times protease inhibitor cocktail) and incubated on ice for 30 min with occasional agitation. The suspension was centrifuged at 12 000 g for 50 min.

GST pull-down experiments. The supernatants were incubated with glutathione–agarose beads (Amersham Pharmacia Biotech) equilibrated with buffer A for 2 h at 4°C. Beads were washed three times with 10 vols of buffer A, and the bound proteins were detected by western blotting.

Ni²⁺ pull-down experiments. The supernatants were adjusted to 20 mM imidazole and incubated with Ni-NTA–agarose (Qiagen) for 2 h at 4°C. Beads were washed three times with 10 vols of buffer A plus 20 mM imidazole, resuspended in the last wash, and subsequently packed into a disposable column. The bound proteins were eluted with buffer A containing 250 mM imidazole. Fractions were then analysed by western blotting.

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