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Translesion DNA synthesis across non-DNA segments in cultured human cells

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ABSTRACT

DNA lesions that have escaped DNA repair are tolerated via translesion DNA synthesis (TLS), carried out by specialized error-prone DNA polymerases. To evaluate the robustness of the TLS system in human cells, we examined its ability to cope with foreign non-DNA stretches of 3 or 12 methylene residues, using a gap-lesion plasmid assay system. We found that both the trimethylene and dodecamethylene inserts were bypassed with significant efficiencies in human cells, using both misinsertion and misalignment mechanisms. TLS across these non-DNA segments was aphidicolin-sensitive, and did not require polη. In vitro primer extension assays showed that purified polη, polκ and polι were each capable of inserting each of the four nucleotides opposite the trimethylene chain, but only polη and polκ could fully bypass it. Polη and polι, but not polκ, could also insert each of the four nucleotides opposite the dodecamethylene chain, but all three polymerases were severely blocked by this lesion. The ability of TLS polymerases to insert nucleotides opposite a hydrocarbon chain, despite the lack of any similarity to DNA, suggests that they may act via a mode of transient and local template-independent polymerase activity, and highlights the robustness of the TLS system in human cells.

1. Introduction

DNA repair mechanisms have evolved to efficiently cope with a great variety of harmful agents constantly challenging the integrity of the genome. Most powerful are the error-free DNA repair mechanisms that eliminate the damaged nucleotides, and restore the original DNA sequence [1]. However, often replication commences prior to the completion of repair, and the unrepaired lesions form a barrier to replication fork progression. Such circumstances result in recruitment of the DNA damage tolerance mechanisms, either translesion DNA synthesis (TLS) [1–5] or homologous recombinational repair (HRR) [6–8]. The translesion mode of DNA synthesis is, for the most part, conducted by specialized DNA polymerases. It appears that the common denominator of these translesion DNA polymerases is a more spacious and flexible active site [9–14], which allows the bypass of even extremely bulky lesions, but resulting in lower fidelity of polymerization [5,15]. The multiplicity of TLS polymerases in mammalian cells serves, at least in part, to endow the TLS system with DNA damage specificity, as indicated by the ability of specific TLS polymerases to bypass particular lesions in vivo, with higher efficiency and higher accuracy than other polymerases, thus minimizing the otherwise mutagenic effect of the lesions [16–21].

One of the interesting questions concerning TLS is the range of the DNA lesions that it can overcome. It would have been anticipated that a robust TLS system would be able to replicate across a large variety of DNA lesions, and perhaps

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even novel lesions, which were not present during evolution. Such lesions can form by extreme changes in the environment, caused either by natural forces, or by man-made novel chemicals. Recently we have shown that the TLS system in *Escherichia coli* can bypass a non-DNA segment composed of a stretch of 3 or 12 methylene residues embedded in a ssDNA region, albeit at low efficiency [22]. This hydrocarbon segment represents an artificial and severe form of 'DNA damage', since it shares no similarity with either the base, sugar or phosphate components of DNA, and unlike DNA, it is non-polar and purely hydrophobic. Here we report that human cells are able to bypass these extreme types of DNA damage, and that this is done with an efficiency of about an order of magnitude higher than in *E. coli*. This is achieved by both misalignment and misincorporation mechanisms, illustrating the extraordinary ability of the mammalian TLS system to overcome severe types of DNA damage.

2. Materials and methods

2.1. Construction of DNA substrates

Oligonucleotides containing the methylene inserts M3 or M12 were synthesized by Genset (Evry, Cedex, France) by using hydrocarbon building blocks (phosphoramidite C3 and C12 CE phosphoramidite) from Glen Research (Sterling, VA). Oligonucleotides without a lesion were synthesized by Sigma. Gapped plasmids were prepared as described [19,22,23]. The DNA substrates for in vitro primer extension experiments were prepared by annealing a 5′-^{32}P-end labeled oligonucleotide primer to a hydrocarbon containing template oligonucleotide, followed by purification on a BioSpin 30 gel filtration column (Bio-Rad). Primer 5′-CTGGTTCAAGTAGCCCAGGGTTGAC-3′ was used for PT-51(-1) and primer 5′-CTGGTTCAAGTAGCCCAGGGTT-3′ was used for PT-51(-4). Analysis by electrophoresis on native gels revealed that >95% of the primers were annealed to the template oligonucleotides.

2.2. Cell cultures

The human large cell lung cancer cell line H1299 [24] was grown in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). The SV40-transformed fibroblast cells MRC5 (normal) and XP30RO (XPV, also designated GM3617) [25] were cultured in Eagle's minimum essential medium (Sigma) supplemented with 15% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in 5% CO_2 atmosphere. Aphidicolin treatment was conducted by adding aphidicolin (Sigma) prior to the transfection and throughout the incubation period. Aphidicolin was dissolved in 10% DMSO/40% ethanol to a final concentration of 25 μg/ml. Final concentrations of DMSO and ethanol were 0.25% and 1%, respectively.

2.3. In vivo TLS assay in cultured cells

The in vivo TLS assay system was previously described [19]. Briefly, it involved transient transfection of the cells with a DNA mixture containing the gap-lesion plasmid (bearing a kanamycin resistance marker; Kan^R), a normalizing gapped plasmid without a lesion (bearing a chloramphenicol resistance marker; Cm^R), and pUC18, a carrier plasmid. Depending on the efficiency of transfection 50–200 ng of gapped plasmid was used. Cells were usually harvested 24 h post-transfection, and plasmids were extracted from the cells by alkaline lysis, allowing for recovery only of completely filled-in plasmids. Plasmid survival was assayed by introduction of the recovered DNA mixture into an indicator *E. coli recA* strain, followed by plating on LB-Kan and LB-Cm to select for filled-in gap-lesion and normalizing plasmids, respectively. Lesion bypass was calculated by dividing the number of Kan^R colonies by the number of Cm^R colonies. Experiments with the GP21-M3 gapped plasmid (Tables 1 and 3) were performed using an earlier version of the assay system as described [26]. The actual number of transformant colonies obtained in the different experiments varied depending on the cell line and gap plasmid used, the gap filling efficiency and the transformation efficiencies. Therefore, different amounts of the extracted DNA mixture were taken to transform the indicator *E. coli* indicator strain, and different volumes of transformed bacteria were plated. In order to present the data in a simplified form, which allows comparison between experiment sets, the number of colonies was adjusted to a common volume of transformation mixture (100 μl). The numbers of transformants in experiments in which the earlier assay version was utilized were adjusted by dividing the actual number of Kan^R transformants obtained from filled gap-lesion plasmids by the Kan^R/Cm^R ratio obtained for the non-damaged plasmid in the same experiment. The assay is specific for TLS, since the lesion is on a ssDNA region, and there is no contribution from homologous recombination, as previously described [19,26].

2.4. In vitro primer extension assay

Primer extension experiments were conducted in a solution containing 25 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin, 10% glycerol, 100 μM each of dATP, dCTP, dGTP and dTTP or of a single dNTP, and 25 nM of the appropriate primer/template. Polymerase concentrations in the reactions were 24, 15–25 and 30–100 nM for polη, polκ and polι, respectively (purchased from Enzymax, Lexington, KY). In reactions carried out with *E. coli* DNA polymerase I Klenow fragment (20 nM), 20 mM Tris–HCl (pH 7.5) was used instead of the phosphate buffer, the concentrations of bovine serum albumin and glycerol were 8 μg/ml and 4%, respectively, and 0.1 mM EDTA was added. Reactions were carried out at 37 °C for 5–20 min, after which they were stopped by addition of an equal volume of 99.5% formamide, 0.025% bromophenol blue and 0.025% xylene cyanol. Samples were fractionated by electrophoresis on 15% polyacrylamide gels containing 8 M urea, after which they were dried, visualized and quantified using a Fuji BAS 2500 phorphorimager. The extent of bypass was calculated by dividing the amount of bypass products by the amount of all extended primers. The extent of synthesis was calculated by dividing the total amount of extended products by the total amount of radiolabeled bands. TLS was calculated by dividing the amount of bypass products by the total amount of radiolabeled bands.

2.5. *Modeling DNA containing tri- and dodecamethylene chains*

Models of DNA duplexes in which the backbone in one of the strands is replaced by $(CH_2)_{12}$ were built, and energy minimized in a sphere of water. Several possibilities were tested: the $(CH_2)_{12}$ moiety was placed either opposite a single base, two bases, or three bases in the other strand. The unmodified DNA strand and the unmodified parts of the modified strand were fixed to their initial geometry in all the energy minimizations. This constraint maintained the double helix B-DNA geometry yet allowed the modified backbone fragment to adopt a low energy structure. The modeling and pictures were prepared using the InsightII package by Accelrys (San Diego, CA).

3. Results

3.1. *Mammalian cells bypass a trimethylene stretch embedded in ssDNA by both direct insertion and misalignment mechanisms*

In order to determine the capability of the mammalian TLS machinery to cope with a hydrocarbon chain embedded within the DNA we utilized the quantitative in vivo TLS model assay system previously described [19,26]. In these experiments, mammalian cells were transfected with a gapped plasmid, harboring a site-specific hydrocarbon chain 'lesion' and the kan^R gene. The DNA transfection mixture also contained a control gapped plasmid without the lesion (cm^R), as well as a carrier plasmid. Repaired plasmids were recovered from the cells and transformed into an *E. coli* indicator strain in order to determine survival (ratio of kan^R/cm^R colonies). Moreover, the results of single bypass events were determined by sequence analysis of isolated clones. This assay system was previously shown to respond to the DNA polymerase composition of cells [19].

TLS experiments conducted in the human large cell lung cancer cell line H1299 with the GP21-M3 plasmid, carrying the $-(CH_2)_3-$ chain, showed bypass levels of $47.3 \pm 13.5\%$ (Table 1). Control experiments, in which the DNA mixture was transformed directly into the *E. coli* indicator without prior passage in the mammalian cells, yielded a background bypass levels of $0.5 \pm 0.24\%$. Remarkably, sequence analysis of the gapped plasmid descendants showed that the vast majority of bypass events (91%; 39/43) were insertions of a single base across from the M3 chain, indicating 'well-behaved' TLS, despite the presence of the non-DNA segment. The majority of plas-

Table 1 – TLS across a trimethylene insert in human H1299 cells

Cell line	Gapped plasmid	*E. coli* transformants[a]		TLS (%)
		Kan^R	Cm^R	
H1299	GP21-M3	31	64	47.3 ± 13.5
No passage[b]	GP21-M3	100	21000	0.5 ± 0.24
H1299	GP51-M3	46	198	21.0 ± 2.6
No passage[b]	GP51-M3	18	6150	0.3 ± 0.22

Mutation type	Cell type/gapped plasmid			
	H1299		No passage[b]	
	GP21-M3	GP51-M3	GP21-M3	GP51-M3
Base insertions				
A	18 (42)	24 (55)	–	–
C	10 (23)	–	6 (35)	1 (7)
G	3 (7)	–	–	–
T	1 (2)	13 (30)	–	–
Complex events[c]	–	5 (11)	–	–
Double mutations (AA)	7 (16)	2 (4)	–	–
Total base insertions	39 (91)	44 (100)	6 (35)	1 (7)
Deletions				
(-1)	–	–	11 (65)	14 (93)
(-3) and higher	4 (9)	–	–	–
Total	43 (100)	44 (100)	17 (100)	15 (100)

A DNA mixture containing the indicated gap-lesion plasmid (kan^R) and the respective control gapped plasmid (cm^R) was introduced into H1299 human cells, after which plasmids were extracted and used to transform the indicator *E. coli* cells. The table shows the average of at least six transfections. Plasmids were then extracted from kan^R colonies and subjected to DNA sequence analysis. The table shows the DNA sequence opposite the trimethylene chain obtained from individual clones. Values in parentheses are in percent.

[a] The number of transformants obtained in a typical assay with 100 μl of transformation mixture.

[b] A DNA mixture containing the indicated gap-lesion plasmid was introduced into *E. coli* JM109*recA*, without prior passage through the human cells.

[c] Complex events in which flanking sequences were omitted yet nucleotides were inserted across from the hydrocarbon chain. For example, 5′-TTGACXXTTCTCGCG-3′, where XX represents the nucleotides inserted across the hydrocarbon chain, turned into 5′-TTGA·TTTTCTCGCG-3′ or 5′-TTGACATTT·TCGCG-3′.

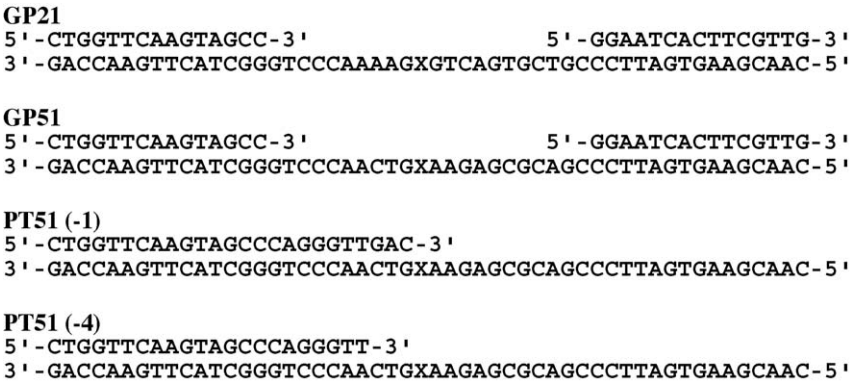

Fig. 1 – Structure of the DNA constructs used to assay TLS across hydrocarbon chains embedded in DNA. The DNA sequence in the vicinity of the hydrocarbon chain is shown for the two types of gap-lesion plasmids (GP21 and GP51) used in the in vivo experiments, and for the two primer-template constructs (PT51(-1) and PT51(-4)) used in the in vitro experiments. The hydrocarbon chains are represented by an X.

mids isolated from the control experiments, without passage through the mammalian cells, contained primarily (-1) deletions (11/17; 65%), consistent with previous results in *recA E. coli* cells.

In 42% of the plasmids (18/43) an A appeared at the position across from the lesion, while in 23% there was a C at that position (Table 1). The insertion of both C and A in the GP21 sequence context (5′-ACTGXGAAA-3′, X representing the hydrocarbon chain) could be explained either as a result of direct insertion of a nucleotide opposite the hydrocarbon chain, or as a result of a more elaborate misalignment and realignment mechanism (see Fig. 6; [27–29]). In order to distinguish between the two possibilities, we conducted the experiments utilizing an additional gapped plasmid, GP51-M3, in which the M3 chain was present in the 5′-AGAAXGTCA-3′ sequence context (Fig. 1). In this sequence context, there are no nearby downstream T's, and therefore the occurrence of an A at the position across from the hydrocarbon chain will indicate a direct insertion of a nucleotide by the polymerase. A misalignment–realignment mechanism involving the looping out of the M3 lesion will, in this sequence context, result in the insertion of a T at the same position. Bypass levels of the GP51 gapped plasmid were 21 ± 2.6%, about two-fold lower that GP21-M3 (Table 1). All of the recovered plasmids included base insertions opposite the M3 chain (44/44), with no deletions or other DNA rearrangements. The insertion of an A remained the most prevalent event (55%; 24/44), while a T appeared at the position across from the lesion in 30% (13/44) of the sequences obtained. In contrast to GP21, no C's were observed in the GP51 sequence context (<2.3%; 0/44; Table 1). The control plasmids that were not passed through the mammalian cells yielded primarily (-1) deletions (14/15; 93%; Table 1). Taken together these results suggest that TLS across the M3 insert occurs both via insertion and misalignment mechanisms (see Section 4 below).

3.2. *Mammalian cells can bypass a dodecamethylene segment within the DNA*

The length of the trimethylene hydrocarbon chain is similar to the length of a single nucleotide in the DNA backbone, and as described above, it did not form a significant barrier to the mammalian TLS machinery. We then proceeded to challenge the cells with the four-fold longer hydrocarbon chain containing 12 methylene residues (M12), spanning the length of approximately 2.5 nucleotides. Quantitative experiments conducted in H1299 cells with the GP51-M12 plasmid yielded plasmid survival levels of 8.9 ± 1.8%, 2.4-fold lower than TLS across the M3 chain (Table 2). The background level of the GP51-M12 that was not passed through the human cells was 0.14 ± 0.08% (Table 2). DNA sequence analysis of GP51-M12 descendants revealed that the majority of events in H1299 cells were insertions of one to three nucleotides across from the lesion site (32/50; 64%; Table 2). Most of these (28/50; 56%) were insertions of two nucleotides opposite the M12 chain, composed of TT (15/50; 30%) and AT (13/50; 26%). Insertions of one nucleotide (A, 2/50; 4%) and three nucleotides (2/50; 4%) were observed too (Table 2). In addition, more complex bypass events were observed (6/50; 12%), in which nucleotides were inserted but others deleted. For example, the 5′-C was omitted and an AT were added, changing the sequence from 5′-GAC__TT-3′ to GA-Δ-AT-TT-3′ (Table 2). Thus, remarkably, the TLS system can overcome this longer non-DNA insert, filling-in the gap primarily with two nucleotides, which roughly matches the length of the M12 chain.

3.3. *TLS across hydrocarbon chains is aphidicolin sensitive*

In an attempt to further analyze the bypass across the M3 and M12 inserts, we conducted the TLS assays in the presence of aphidicolin, a known inhibitor of the replicative DNA polymerases α, δ and ε [30–34]. TLS across the trimethylene insert in H1299 cells in the presence of aphidicolin was 14.1 ± 3.8%, 2.7-fold lower than bypass in the control experiment conducted with the same cells grown in the presence of ethanol–DMSO, the solvent used to dissolve aphidicolin (37.6 ± 15%; Table 3). Sequence analysis of plasmid descendants showed a spectrum generally similar to that obtained in the absence of aphidicolin (Table 3). Most events (28/31; 91%) involved single nucleotide insertions, and mostly A was inserted (21/31; 68%). These results suggest

Table 2 – TLS across a dodecamethylene chain in H1299 cells

Cell line	*E. coli* transformants[a]		TLS (%)
	Kan^R	Cm^R	
H1299	262	2814	8.9 ± 1.8
No passage[b]	24	21900	0.14 ± 0.08

Mutation type	Cell type	
	H1299	No passage[b]
Base insertions		
A	2 (4)	–
C	–	9 (82)
G	–	–
T	–	
AT	13 (26)	–
ATT	1 (2)	–
TT	15 (30)	–
TTT	1 (2)	–
Complex events[c]	6 (12)	–
Total base insertions	38 (76)	9 (82)
Large insertions	4 (8)	–
Deletions		
(-1)	1 (2)	2 (18)
(-3) and higher	7 (14)	–
Total	50 (100)	11 (100)

A DNA mixture containing the gap-lesion plasmid GP51-M12 (kan^R), the control gapped plasmid GP20-*cm* (cm^R), and the carrier plasmid pUC18 (amp^R) was introduced into H1299 human cells, after which the they were extracted and used to transform the indicator *E. coli* cells. The table shows the average of at least six transfections. Plasmids were then extracted from kan^R colonies and subjected to DNA sequence analysis. The table shows the DNA sequence opposite the M12 chain obtained from individual clones. Values in parentheses are in percent.

[a] The number of transformants obtained in a typical assay with 100 μl of transformation mixture. Using the control gapped plasmids GP20 and GP50 (without the methylene chains) yielded similar results.

[b] A DNA mixture containing the indicated gap-lesion plasmid was introduced into *E. coli* JM109*recA*, without prior passage through the human cells.

[c] Complex events in which flanking sequences were omitted yet nucleotides were inserted across the hydrocarbon chain (see examples in Table 1).

that an aphidicolin-sensitive DNA polymerase is involved in the bypass across nearly two-thirds of the trimethylene inserts. When inhibited, other polymerase(s) can carry out the reaction, with reduced efficiency, but with a similar specificity.

We then examined the effect of aphidicolin on TLS across the dodecamethylene insert. Interestingly, the efficiency of plasmid recovery was similar with or without aphidicolin (4.4 ± 1.4% and 5.9 ± 1.5%, respectively) (Table 3). However, DNA sequence analysis of recovered plasmids revealed a major difference. Whereas without aphidicolin 54% (18/33) of the plasmids contained base insertions of one to three nucleotides, in the presence of aphidicolin, only 18% (6/33) contained nucleotide insertions (Table 3). Most other events (24/33; 73%) included deletions, which have arisen from mechanisms other than TLS (see Section 4). Taking this into account, TLS across the M12 chain is reduced by 3.3-fold in the presence of aphidicolin, similar to the effect on M3 bypass.

3.4. *TLS across hydrocarbon chains does not depend on polη*

In order to examine whether polη is required for TLS across the M3 chain we conducted the TLS assay in the XPV cell line XP30RO, which lacks polη, and the control cell line MRC5. Plasmid survival was only slightly reduced in the XPV cell line (6.0 ± 1.7%) compared to the control cell line (10.4 ± 2%; Table 4). Sequence analysis of plasmids isolated from these two cells lines showed similar spectra, and those were similar to the sequences obtained in cell line H1299. The majority of events involved insertion of a single A across from the M3 insert (52% and 47% in MRC5 and XP30RO, respectively), while the second most frequent event involved the insertion of a T, the expected product of a misalignment–realignment mechanism, at that position (26% in both cell lines) (Table 4).

Similar experiments were performed with a gapped plasmid containing the M12 chain. As can be seen in Table 4, similar plasmid survival levels were obtained in MRC5 and XP30RO cell lines (14.3 ± 1.1% and 14.9 ± 1.1%, respectively; Table 4). DNA sequence analysis of specific plasmid clones showed similar results from both cell lines. However, in contrast to the M3 chain, in this case only 23–24% of the events involved nucleotide insertions representing TLS, while most other events involved large deletions (Table 4). Base insertions were of two nucleotides exclusively (Table 4).

3.5. *Purified DNA polymerases η and κ can bypass a trimethylene stretch embedded in DNA*

We conducted in vitro primer extension experiments with purified human DNA polymerases η, κ and ι. Both polη and polκ conducted efficient bypass, reaching around 40% at 20 min (Fig. 2). Polι was capable of inserting a nucleotide across from the lesion, but not extending past it (Fig. 2). In order to examine the specificity of nucleotide insertion opposite the M3 insert we used a primer-template in which the primer terminus was located opposite the template base preceding the lesion (standing-start assays; Fig. 1), and conducted primer extension experiments with single dNTPs. As can be seen in Fig. 3, polη inserted each of the four dNTPs at comparable efficiency. Polκ and polι each inserted A, G and T with comparable efficiency at the M3 chain, whereas C insertion was significantly weaker.

Similar in vitro primer extension experiments were conducted with a substrate carrying the M12 chain. Unlike the M3 insert, the M12 insert severely blocked all three DNA polymerases (Fig. 4). Polκ was completely blocked by the M12 lesion, whereas both polη and polι were each capable of efficiently inserting a nucleotide across from it (Figs. 4 and 5). However, only polη exhibited very low levels of full bypass (Fig. 4). Primer extension standing-start assays conducted with single dNTPs showed that both polη and polι inserted each of the four dNTPs at the M12 insert.

Table 3 – TLS across hydrocarbon chains in H1299 cells under aphidicolin treatment

Gapped plasmid	Treatment	*E. coli* transformants[a]		TLS (%)
		Kan^R	Cm^R	
GP21-M3	Solvent	67	188	37.6 ± 15
	Aphidicolin[b]	19	100	14.1 ± 3.8
GP51-M12	Solvent	34	580	5.9 ± 1.5
	Aphidicolin[b]	19	490	4.4 ± 1.4

Mutation type	Gapped plasmid/treatment		
	GP21-M3 aphidicolin[b]	GP51-M12 aphidicolin[b]	GP51-M12 solvent
Base insertions			
A	21 (68)	–	1 (3)
C	4 (13)	–	–
G	1 (3)	–	–
T	–	–	1 (3)
AT	–	2 (6)	6 (18)
ATT	–	–	4 (12)
TT	–	3 (9)	2 (6)
TTT	–	1 (3)	1 (3)
Complex events[c]	–	–	3 (9)
Double mutations	2 (7)	–	–
Total base insertions	28 (91)	6 (18)	18 (54)
Large insertions	1 (3)	1 (3)	3 (9)
GP20	–	1 (3)	–
Deletions			
(-1)	1 (3)	1 (3)	1 (3)
(-2)	1 (3)	–	1 (3)
(-3) and higher	–	24 (73)	10 (31)
Total	31 (100)	33 (100)	33 (100)

The experiment was conducted as described in the legends to Tables 1 and 2, except that cells were treated with either aphidicolin or the control solvent solution (DMSO/ethanol) prior to transfection. Values in parentheses are in percent.

[a] The number of transformants obtained in a typical assay with 100 μl of transformation mixture.

[b] Aphidicolin was at a concentration of 25 μg/ml, known to inhibit replicative DNA polymerases [30–34].

[c] Complex events in which flanking sequences were omitted yet nucleotides were inserted across the hydrocarbon chain (see examples in Table 1).

4. Discussion

The results presented in this study show that hydrocarbon chains of 3 or 12 methylene residues, inserted into the DNA backbone, can be bypassed in mammalian cells. These non-DNA inserts share no common features with DNA, representing an extreme model of artificial DNA damage.

A critical property of the hydrocarbon chain that influences TLS is its length. Indeed, the trimethylene insert, whose length corresponds to approximately one nucleotide, was bypassed more efficiently by the TLS system than the dodecamethylene chain, corresponding to approximately 2.5 nucleotides in length. Strikingly, a common outcome of bypass was the net addition of one to three nucleotides at the site of the hydrocarbon chain, as if the polymerase continued DNA synthesis opposite that segment, despite the absence of any similarity to DNA. In H1299 cells the majority of bypass events involved an insertion of a single A (42%) or C (23%) at the position across from the M3 lesion in GP21-M3. The sequence context of the lesion in this plasmid (5′-GACTGXGAAAAC-3′; Fig. 1), does not allow a distinction between mechanisms of insertion or misaligment–realignment (Fig. 6). Although the most straightforward explanation would be a direct insertion of a nucleotide across from the trimethylene chain by a DNA polymerase (Fig. 6A), it may as well be a result of a misalignment–realignment mechanism. In a misalignment event, in which the hydrocarbon chain is looped out, an insertion of a C would occur across from the next template G. Subsequent realignment and continued synthesis would result in the appearance of a C at the position across from the lesion (Fig. 6C). Such a mechanism for the bypass of an abasic site has been previously shown for DNA polβ [28] and polμ [29]. On the other hand, if both the hydrocarbon chain and the adjacent 3′ template G were to be looped out, the 3′ C of the misaligned primer will be base paired with the 5′ template G, and the next insertion would be of an A. Subsequent realignment and continued synthesis would result in the appearance of an A at the same position (Fig. 6D). These possibilities were distinguished using gapped plasmid GP51, harboring the sequence context 5′-GAGAAXGTAAC-3′ (Fig. 1). In this sequence context, there are no nearby template T's downstream to the lesion, and the next two template nucleotides are A's. The fact that once more, in this sequence context, most repaired plasmids contained

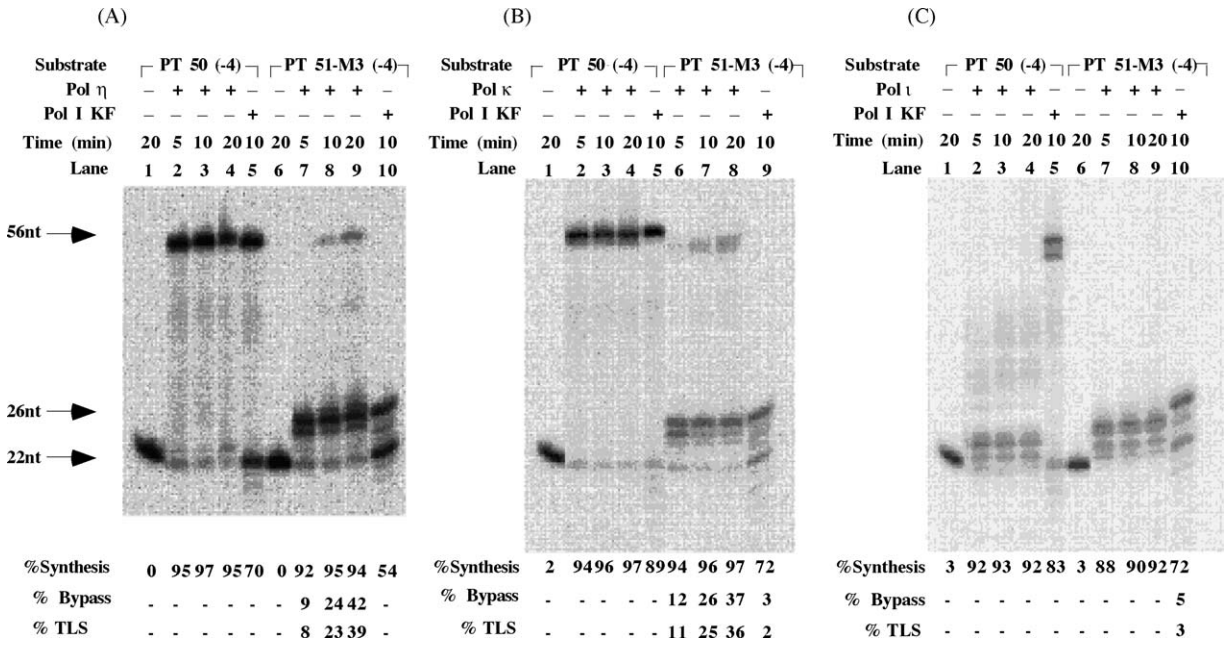

Fig. 2 – In vitro bypass across a trimethylene insert by purified Y family human DNA polymerases. Primer extension assays were performed with polη (A), polκ (B) or polι (C), using either a non-damaged oligonucleotide [PT50(-4)], or an oligonucleotide harboring a trimethylene insert (M3) in the template strand [PT51-M3(-4)]. A 22-mer is the unextended primer, a 26-mer represents replication up to the hydrocarbon chain, and products of 27–56 nt represent bypass products. The bands of oligonucleotides shorter then 22 nt (lanes 5 and 10 in panels A and C, and lane 9 in panel B) represent excision products of the 3′–5′ proofreading activity of polI KF. See Section 2 for definitions of calculating synthesis, bypass and TLS.

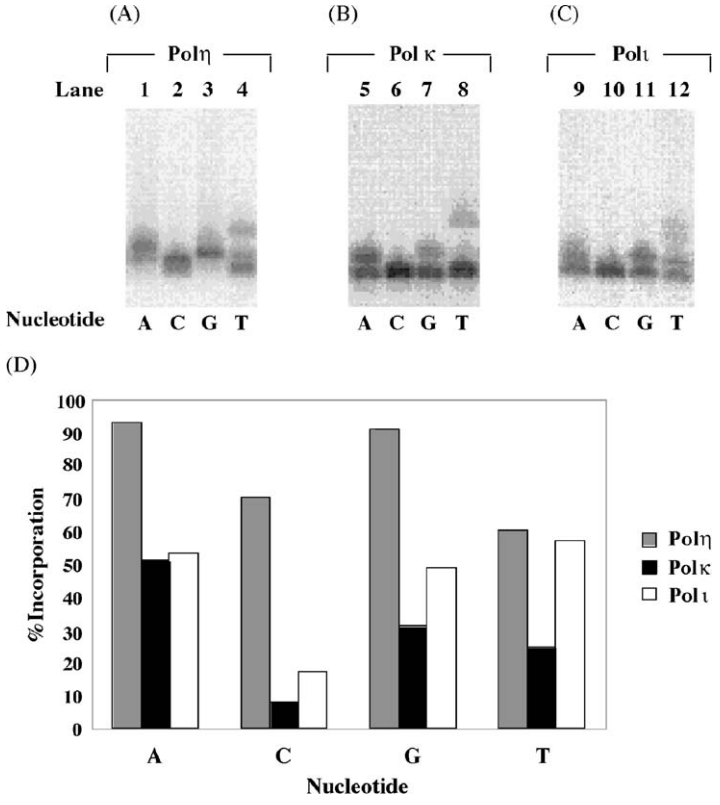

Fig. 3 – Specificity of nucleotide incorporation across the trimethylene chain by Y family human DNA polymerases. Standing-start primer extension experiments were conducted with polη (A), polκ (B) or polι (C), using the PT51-M3(-1) primer-template, and providing a single dNTP (100 μM) in each reaction. Panel D shows a comparison of nucleotide insertion efficiencies of the three polymerases based on quantification the gels presented in panels A–C.

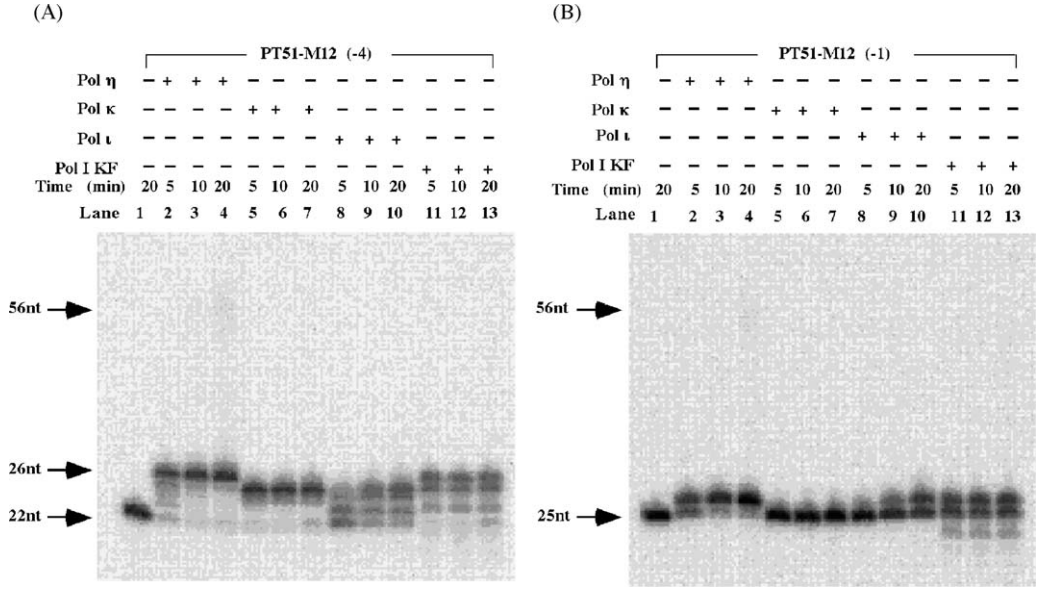

Fig. 4 – In vitro bypass across a dodecamethylene insert by purified Y family human DNA polymerases. Primer extension assays were performed with the indicated DNA polymerase using an oligonucleotide template bearing the dodecamethylene insert (M12) and primed either four bases upstream to the lesion (PT51-M12(-4), panel A) or up to the base preceding the lesion (PT51-M12(-1), panel B). Lane 1 in each panel shows a control incubation of the reaction mixture without any DNA polymerase. Other lanes containing the product of reactions performed with the following DNA polymerases: polη (lanes 2–4), polκ (lanes 5–7), polι (lanes 8–10), and *E. coli* polI (Klenow fragment) (lanes 11–13). For each DNA polymerase three time points of the reaction are shown.

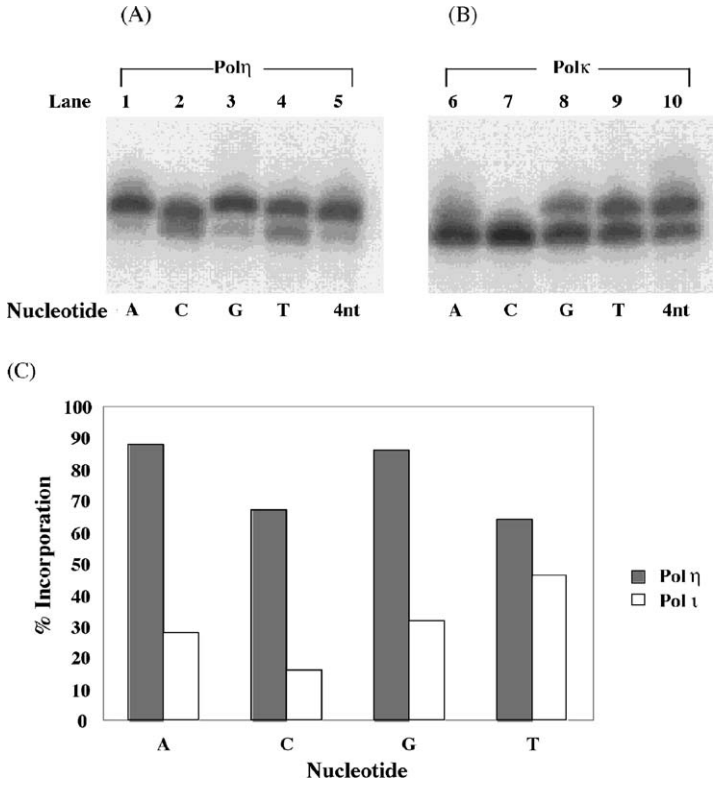

Fig. 5 – Specificity of nucleotide incorporation across the dodecamethylene insert by human polη and polι. Standing-start primer extension experiments were conducted with polη (A) or polι (B) using the PT51-M12(-1) construct. Lanes 1–4 in each panel show reactions with the indicated single dNTP (100 μM), and lane 5 is a reaction with all four nucleotides. Reactions times were 20 min. Panel C shows a comparison of nucleotide insertion efficiencies of the two polymerases based on quantification the gels presented in panels A and B.

Table 4 – TLS across hydrocarbon chains in human XPV and control cell lines

Gapped plasmid	Cell line	*E. coli* transformants[a]		TLS (%)
		Kan^R	Cm^R	
GP51-M3	MRC5	42	425	10.4 ± 2
	XP30RO	42	914	6 ± 1.7
GP51-M12	MRC5	53	414	14.3 ± 1.1
	XP30RO	77	540	14.9 ± 1.1

Mutation type	Gapped plasmid/cell line			
	GP51-M3		GP51-M12	
	MRC5	XP30RO	MRC5	XP30RO
Base insertions				
A	14 (52)	14 (47)	–	–
C	–	–	–	–
G	1 (4)	–	–	–
T	7 (26)	8 (26)	–	–
AT	–	–	–	3 (14)
TT	–	–	3 (18)	1 (4.5)
Complex events[b]	1 (4)	1 (3)	1 (6)	1 (4.5)
Double mutations	3 (11)	3 (10)	–	–
Total base insertions	26 (96)	26 (86)	4 (24)	5 (23)
Deletions				
(−1)	–	2 (7)	–	–
Multiple bp	1 (4)	2 (7)	13 (76)	17 (77)
Total	27	30	17	22

The experiment was conducted as described in the legends to Tables 1 and 2, except that the XP-V derived (XP30RO) or normal (MRC5) human cell lines were used. Values in parentheses are in percent.

[a] The number of transformants obtained in a typical assay with 100 μl of transformation mixture.

[b] Complex events in which flanking sequences were omitted yet nucleotides were inserted across the hydrocarbon chain (see examples in Table 1).

an A across from the M3 insert (55%), despite the absence of downstream template T, suggests a mechanism of insertion by the polymerase opposite the M3 chain, without the involvement of misalignment. However, it seems that bypass also occurs via a misalignment–realignment mechanism, dictated by the template base 5′ to the M3 chain. While in GP21 we observed 23% C across from the M3 chain, in GP51 no C's were observed, but a similar proportion of the plasmids (30%) bore a T, which is complementary to the template nucleotide 5′ to the M3 chain. Similar mutation spectra were observed in both the MRC5 and XP30RO cell lines with the GP51-M3 plasmid. Interestingly, in all three cell lines insertion seems to be twice as frequent as misalignment.

Strikingly, TLS across the longer M12 chain in H1299 cells also involved primarily nucleotide additions (76%). This is in sharp contrast to *E. coli*, where the vast majority of TLS events (83% [22]) involved precise elimination of the M12 chain, without net addition or loss of nucleotides (Fig. 6B). Such an event was uncommon in mammalian cells (1/50; 2%). Most insertion events (28/38; 74%) involved the net insertion of two nucleotides, consistent with the lengthier insert, although some insertions of one or three nucleotides were observed as well. It appears that the majority of bypass events of the M12 chains involved some form of misalignment. All but two of the insertion events (36/38; 95%) involved an insertion of at least one T consistent with a misalignment mechanism, as the sequence 5′ to the M12 chain contains AA. However, it is important to note that an A was also inserted, indicating that some form of direct insertion by a polymerase did occur. Interestingly, very little occurrences of dCMP addition opposite the M3 and M12 chains were observed, indicating that incorporation by REVI is not a mechanism for the bypass of these methylene chains [2–5].

We modeled the M12 chain into dsDNA (Fig. 7). The hydrocarbon chain does not impose an extreme deformation in the DNA, and it can adopt either partial or fully looped-out structures. These structures may be responsible for the one-nucleotide insertions and the small deletions, as well as the misalignment during TLS. In the bypass of the M12 chain we also observed complex events in which there was a combination of nucleotide insertion, and deletion of adjacent nucleotides. All of these events involved an insertion of at least one T across from the lesion. These events could result from a non-precise misalignment event involving looping out of the adjacent non-damaged template nucleotides, or perhaps reflect repeated bypass attempts, accompanied by dynamic changes in the conformation of the M12 hydrocarbon chain.

The severity of the M12 lesion is clearly seen when assayed in the SV40-transformed human cell MRC5. Whereas bypass across the M3 lesion in this cell line was similar to bypass in cell line H1299, and consisted mainly of single base additions (26/27; 96%), bypass across the M12 lesion was fundamentally different. Whereas in H1299 cells the majority of recovered plasmids contained mainly one to three nucleotide additions (38/50; 76%), in MRC5 cells this class of events accounted for only 24% of recovered plasmids (4/17). The majority of the plasmids contained deletions of multiple nucleotides in the region corresponding to the M12 lesion (13/17; 76%). Such events are likely to be the result of breakage of the plasmid at the ssDNA region, followed by non-homologous end joining, the most common mechanism for the repair of double strand breaks in mammalian cells [35–37]. This result highlights the differences in the capacity of different cell lines to bypass lesions, perhaps due to differences in the composition of TLS polymerases, and the importance of sequence information of recovered plasmids in this assay system, as rescued plasmids can be obtained by either TLS or NHEJ of broken plasmids.

The aphidicolin sensitivity of TLS across both the M3 and M12 lesions is interesting, since this drug is known to inhibit replicative DNA polymerases. It is consistent with the in vitro primer extension results showing that polκ and polι cannot bypass the M12 lesion, and that polη only very weakly bypasses it. Interestingly, both polη and polκ can bypass the M3 lesion, however, at least polη is not essential for this bypass in human cells. The identity of the aphidicolin-sensitive polymerase responsible for the bypass is unknown, and it may be a TLS rather than a replicative DNA polymerase, since the aphidicolin-sensitivity of some TLS polymerases is unknown.

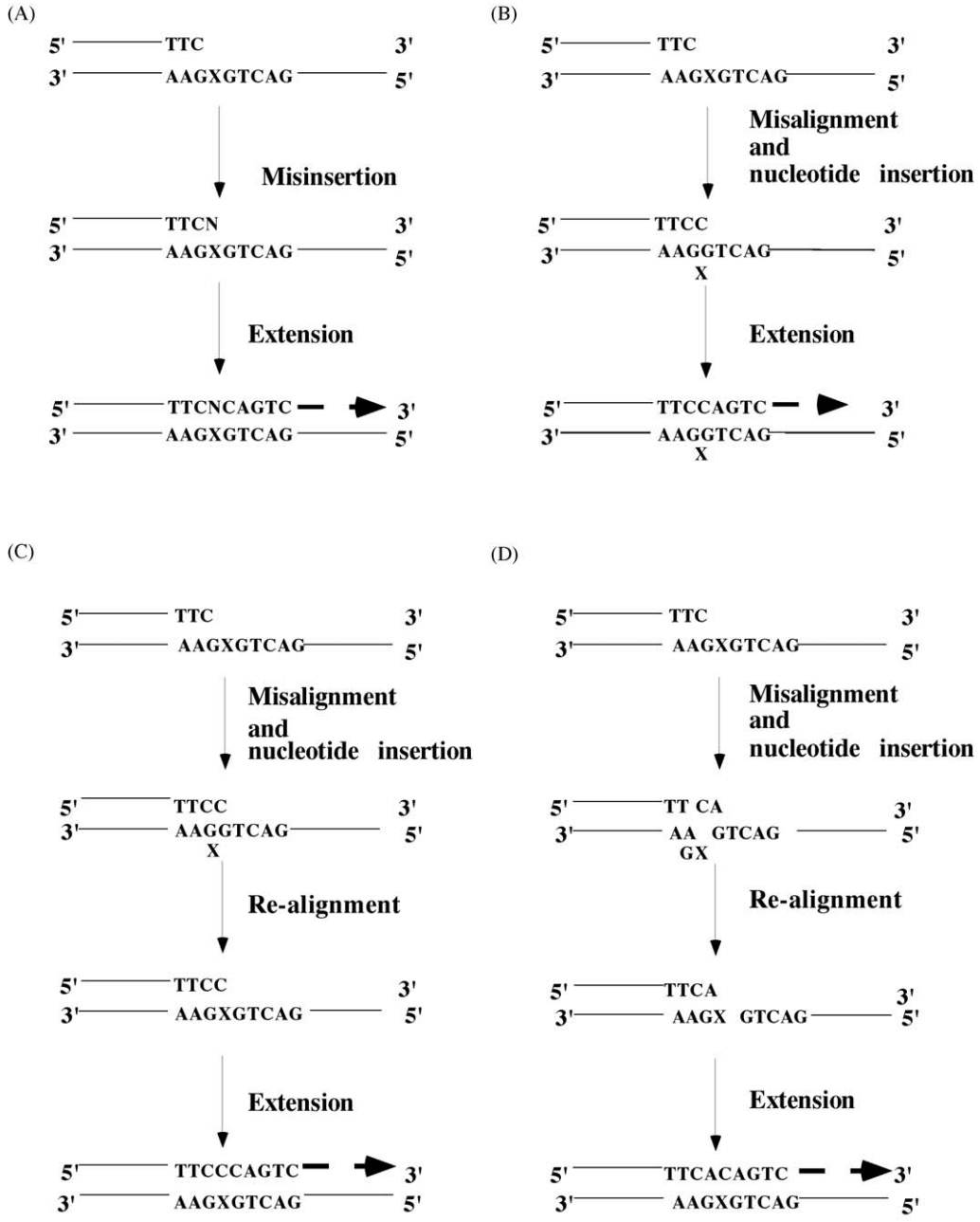

Fig. 6 – Possible scenarios for TLS across the trimethylene chain in the GP21 sequence context by DNA polymerases. (A) Insertion of a nucleotide across from the M3 chain. (B) Misalignment, pairing of the 3′ terminal primer nucleotide with a nucleotide proximate and 5′ to the lesion, resulting in a (-1) deletion. (C) Misalignment, insertion of a nucleotide across from the proximate 5′ template G, followed by a subsequent realignment step and insertion using the 5′ template G once more, resulting in a C at the location across the lesion without a frameshift. (D) Misalignment of the primer C, to pair with the template G 5′ to the M3 chain, followed by addition of one nucleotide, the next 3′ A, and subsequent realignment. This would result in the insertion of an A opposite the lesion, without a frameshift. See text for details.

Bypass across the M3 insert, when not occurring via misalignment, included almost exclusively the insertion of A's. This does not correlate with the ability of purified polη, polκ and polι to insert each of the four nucleotides opposite the M3 chain. However, it does resembles the A rule, whereby DNA polymerases insert an A opposite an abasic site [38–41]. The common features of the M3 lesion and the abasic site are lack of coding information, and their small size. Therefore, a similar mechanism may govern the bypass across an abasic site and the M3 chain. This is supported by the aphidicolin-sensitivity of bypass across the two lesions [26].

When TLS in mammalian cells is compared to *E. coli* using the gapped plasmid assay system, mammalian TLS is at least an order of magnitude more efficient than in *E. coli*. This is based on previous experiment with an abasic site and a benzo[*a*]pyrene-G adduct [19,26,42], and on the results pre-

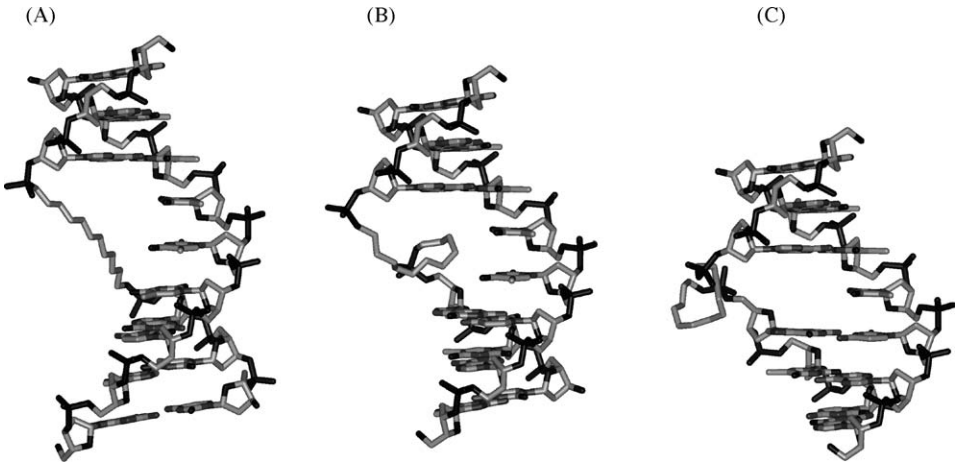

Fig. 7 – Models of DNA duplexes in which the backbone in one of the strands was replaced by $(CH_2)_{12}$. The hydrocarbon can form an extended chain allowing for three unpaired bases (A). Looping out is possible allowing for two or one unpaired bases (panels B and C). Carbon atoms are colored in light gray, nitrogen atoms in dark gray and the phosphates and oxygens are in black.

sented here with hydrocarbon chains. This may be attributed to a more sophisticated TLS system, including the presence of multiple TLS polymerases, and illustrates the broad range and robustness of the TLS system in mammalian cells.

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