CHAPTER 5

Reduced DNA gap repair in aging rat neuronal extracts and its restoration *in vitro* by DNA Polymerase β

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Introduction

Mammalian genomic DNA suffers damage from a variety of physical and chemical agents leading to the base loss or alterations. In view of the generally protected situation of the brain, the damage to the genomic DNA of the brain is mainly due to small changes in the bases of the DNA. Apurinic or apyrimidinic (AP) sites arise in DNA as a result of spontaneous hydrolysis of the N-glycosidic bond or the removal of the altered bases by DNA glycolyases (Lindahl, 1993). Approximately 10,000 AP sites are formed in each mammalian cell per day under normal physiological conditions (Lindahl and Nyberg, 1972). This should be higher in brain due to its high metabolic activity. The AP site should be promptly removed since it is a noncoding lesion which can lead to the misincorporation during replication and transcription (Friedberg, 1995).

DNA synthesis catalyzed by DNA polymerases is a cyclic process consisting of recruitment and creation of a 3'-OH primer terminus (Kornberg, 1974). The polymerase regains its initial configuration with respect to the invariant structural elements of the primer template. This is achieved by two ways. 1) processive mechanism where after the addition of the given dNMP residue, the enzyme translocates on the primer template and consequently capable of adding another dNMP, thus it is able to replicate long stretch of the same template. 2) Non processive or distributive in which the enzyme regains its configuration through dissociation and reassociation process after each catalytic step.

The major repair pathway protecting the cells against single base damages is thought to be the base excision repair (BER). The other error free repair pathway such as nucleotide excision repair can also be involved. Repair of AP sites is initiated by AP endonuclease which binds AP site and hydrolyzes the phosphodiester bond 5' to the abasic site, generating 5'terminal sugar phosphate (Lindahl, 1990; Lindahl and Wood, 1999). The 5'terminal sugar phosphate (dRp) is removed by AP lyase activity associated with DNA polymerase β (Matsumoto and Kim, 1995; Sobol et al., 2000). Pol p removes the dRp and simultaneously adds one nucleotide to the 3' ends of the nick. The most preferred substrate for Pol p seems to be double stranded DNA with a single nucleotide gap in one of the strands which is the *in vivo* situation following the removal of a damaged base by appropriate glycosylase or by spontaneous depurination (Wang and Korn, 1980; Mosbaugh and Linn, 1983; Randahl et al.,1988; Singhal and Wilson, 1993).

BER in mammalian cells is mediated through atleast two sub pathways that are differentiated by repair patches and the enzymes involved. One sub pathway is Short patch BER or Single nucleotide repair and the other long patch repair pathway involving replacement of upto lOnucleotides (Matsumoto et al., 1994; Frosina et al., 1996; Wilson, 1998). The predominant repair pathway is short patch BER involving excision of a single damaged nucleotide and replacement catalyzed primarily by Pol β (Dianov et al., 1992; Klungland and Lindhal, 1997). In cases where the terminal sugar phosphate after the AP endonuclease incision develops a complex structure that cannot be acted upon by the dRpase activity of the Pol β (For example reduced or oxidized abasic site) the repair synthesis would nevertheless continue but in a strand displacement manner. This long patch synthesis is catalyzed by either pol p or pol 5/s with associated proof reading activity.

This pathway is stimulated by Proliferating Cell Nuclear Antigen (PCNA) and requires a "flap" structure specific enonuclease-1 (FEN1) (Harrinton and Lieber, 1994) activity to cut the flap like structure produced by the strand displacement type synthesis by Pol (3 (Wu et al., 1996; Klungland and Lindahl, 1997). The role of PCNA seems to be stimulation of FEN1 activity and the repair size is about seven nucleotides (Frosina et al., 1996)

A slightly different long patch BER pathway in which Pol 8 or Pol s is involved instead of Pol p. Pol p null embryonic fibroblast cells were proficient in repairing oxidative damage although they were defective in uracil initiated repair (Sobol et al., 1996) and that the neutralizing antibody to Pol P, which inhibited repair synthesis catalyzed by pure Pol p by approximately 90%, only suppressed repair in crude human cell extracts by a maximum of approximately 70%.

After filling the single nucleotide gap by DNA polymerase p the nick is sealed by DNA ligase. In case of the short patch repair pathway DNA ligase III along with its partner XRCC1 seals the nick (Cappelli et al., 1997). Where as DNA ligase I/III joins the nick in case of long patch repair pathway (Kim et al., 1998). Eukaryotes, in contrast to prokaryotes contain more than one DNA ligase, and these enzymes have distinct roles in DNA metabolism.

In view of the reported precise role of pol β in short gap repair, the present study has been extended to examine Pol β aided BER with a model oligoduplex substrate containing a gap of 1 or 4 nucleotides. The gapped DNA substrates are formed as described in materials and methods (chapter 2). Using these gapped oligoduplexes the ability of the neuronal extracts to fill the gap is tested . The gap filling ability is also tested with respect to the age of the animals.

Methods

The sequences of the oligonucleotides used for generating the gapped oligoduplex are described in Chapter 2 and also shown in figures. Briefly, gapped oligoduplexes are formed by annealing a 14 mer (5'-kinased with ^{32}P-γ-ATP and T4 polynucleotide kinase) and unlabeled 14 or 17mer to a 32 mer. When the two 14 mers are annealed to the 32 mer, a 4 nucleotide gapped substrate is formed and when a 5' end labeled 14 mer and 17 mer were annealed to the 32 mer, a single nucleotide gap is formed. The annealing of these oligoduplexes were done as described in materials and methods (Chapter 2).

Gap repair assays

Gap repair assays with neuronal extracts was carried as follows. The final reaction volume of 30μl contained 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, 0.1mg/ml Bovine serum albumin, 2% glycerol and 20 μM of all the four dNTPS. 400 femtomole of the gapped oligo duplex containing 1 gap and 4 gap were incubated with 5μg of neuronal extract protein at 37^{0}C for 20minutes. The reaction was stopped by heating at 70°C for 10minutes. When the experiment is carried out with supplementation of pol β, 2.5 units of pure rat liver recombinant enzyme was added in the reaction along with neuronal extracts.

2 units of the T4 DNA ligase and/or 1 mM ATP were added to the reaction mixture as and when mentioned. Purification of the repaired products was done on sephadex G-50 spin column and the products were separated by sequencing gel electrophoresis.

Results and discussion

Figure 23 shows the gap filling activity in the presence of the 'young', 'adult' and 'old' neuronal extracts. Gap filling activity is very low in 'adult' and 'old' neuronal extracts (lanes 3-6). On the other hand, excision of labeled 14-mer to shorter lengths is seen. However, 'young' neuronal extracts showed the gap filling activity both with 1 gap and 4 gap substrates (lanes 1 and 2). But the extended 14-mer is not apparently joined to the down stream 17-mer and hence no labeled 32 –mer product is seen. Also, the number of nucleotides added to 14-mer appear to be more than the gap revealing a tendency of continued strand displacement synthesis. When the neuronal extracts are supplemented with pure recombinant rat liver DNA pol p (2.5units), a major spot corresponding to the length of 32-mer is seen. However, while the predominant spot is 32-mer, several spots/bands below 32-mer are also noticed . This ladder like bands are taken to indicate that pol β is no doubt filling up the gap but also adding nucleotides beyond the gap in a strand displacement manner reading the lower strand as template. This general pattern is the same at all the ages(lanes 7-12). In those tubes where only pol p was present (lanes 13 and 14) the pattern of extension is not very much different essentially suggesting that this is the property of the exogenously added pol β .

The pattern of results seen in Fig 23 brought up many questions. Firstly why even a short gap of 1 or 4 nucleotides is not filled and ligated by neuronal extracts even when supplemented with pure pol p. This is borne out by the fact that several bands/spots are seen below the 32-mer suggesting that pol p added nucleotides all the way to extend the 14-mer to 32-mer. It is also possible that both processive and distributive addition of nucleotides was taking place since considerable radioactivity is found in 32-mer spot apart from the

several bands below. It is pertinent to mention that Singhal and Wilson (1993) and Prasad et al., (1994) have shown that pol β can fill up a gap of upto 6 nucleotides in a processive manner and for that to happen the down stream primer must have a phosphate group on the 5' end. It is also reported that the most favored substrate for pol (3 is 1 nucleotide gap. In view of this existing information, the results seen in Fig 23 are not altogether unexpected in that the down stream primer in the model oligo substrate used did not have a phosphate group on 5' side. As a result of this, ligation after the addition of nucleotide(s) might not have taken place and the enzyme continued to add nucleotides, albeit slowly, up to the length of the template. It is not known whether the neuronal extracts used in this study possess the necessary activities to 5' phosphorylate the downstream primer and ligate the strands.

T4 DNA ligase is a bacteriophage ligases and is ATP-dependent (Armstrong et al., 1983, Nilsson and Magnusson 1982, Rossi, 1997). The ATP dependent DNA ligases catalyze the joining of single strand breaks (nicks) in the phosphodiester back bone of the double stranded DNA in a three step mechanism (Timson et al., 2000). The first step is the formation of a covalent enzyme-AMP complex and the ATP is cleaved to pyrophosphate and AMP, with the AMP being covalently joined to highly conserved lysine residue in the active site of the ligase. The activated AMP residue is then transferred to the 5'Phosphate of the nick, before nick is sealed by phosphodiester bond formation and AMP is eliminated.

In order to check these possibilities, the reaction was carried out in the presence of 1 mM ATP either alone or together with T4 DNA ligase. The results are shown in Fig 24. It may be seen that there is some improvement with ATP but still there are faint spots seen below the 32-mer spot. Addition of T4 DNA ligase did not show any benefit over and

above that already conferred by ATP. In any case it can be taken that ATP supplementation may be causing the phosphorylation of the down stream primer thus helping ligation of the added nucleotides to the down stream primer at least to some extent.

Therefore, the above experiments were repeated where in the oligo duplex with 1 and 4 gaps has a down stream primer which has been already phosphorylated on the 5' side. The results of these experiments are shown in Figs 25 and 26.

To begin with, neuronal extracts themselves, without any supplementation, have shown some improvement in repairing the gaps. In particular, 'young' neuronal extracts (lane 1 and 2, Fig 25) have shown detectable extension of the 14-mer to 32-mer. Very careful observation also reveals the even in the case of 'adult and 'old' neuronal extracts, very faint spots corresponding to 32-mer could be discerned. This means, there is significant gap repair activity present in 'young' neurons while in 'adult' and 'old' neurons, this activity is drastically reduced (lanes, 3-6). It is also apparent that for this gap repair activity to take place , presence of 5' phosphate on the down stream primer is very helpful. However, it is also clear that the gap repair is not solely processive and addition of nucleotides in a distributive and strand displacement manner is also taking place. Once the neuronal extracts were supplemented with pol p, there is a considerable increase in the 32-mer product at all the ages. This result is taken once again to pointing out that aging neurons are deficient of pol p. It is also seen that while pol β is improving the gap repair and formation of 32-mer, it is essentially through a slow strand displacement addition of nucleotides. As a positive control, cell free extracts from testis are also used in the experiment. In testis, as can be seen the gap repair is affected largely by a processive insertion of nucleotide to fill the gap followed by ligation.

When the neuronal extracts were supplemented with either ATP alone or together with T4 DNA ligase apart from pol (3, no marked difference was seen in the pattern of results (Fig 26). Thus the results are essentially similar to those seen when the extracts were supplemented with pol p alone (compare the lanes,7-12 of Fig 25 with all the lanes of Fig 26).

Pol p is considered as a repair polymerase. Several lines of evidence suggest a major role for this enzyme in gap filling repair and that too in the repair of short gaps . Even in the case of long patch BER, Podlutsky et al., (2001) have shown that in human cell extracts pol β is the major DNA polymerase incorporating the first nucleotide during the repair of reduced AP sites, thus initiating the long patch BER synthesis. With all these observations regarding the unique role of pol β in single or short gap DNA repair, the possible role for this small DNA polymerase in filling up long patches of gaps in a double strand DNA is largely ignored. The ability of pol β to extend a primer with long stretches of nucleotides, albeit slowly, using the other strand as template is also ignored.

For example, almost 30 years ago Wang et al., (1974) showed that Pol β is intrinsically capable of synthesizing 10-20 nucleotides at each nick by limited strand displacement manner. Using duplex primer template that contained limited numbers of nicks or gaps of defined average sizes, the same laboratory (Wang and Korn, 1980) again showed that KB and human liver DNA polymerase β performs limited synthetic reaction and incorporation of about 15 nucleotides at each nick where as polymerase a is unreactive towards gapped substrates. It was shown that Pol β prefers small gaps approximately 10 nucleotides long. Recently Wilson and Singhal (1993), using different levels of pol β on different gapped

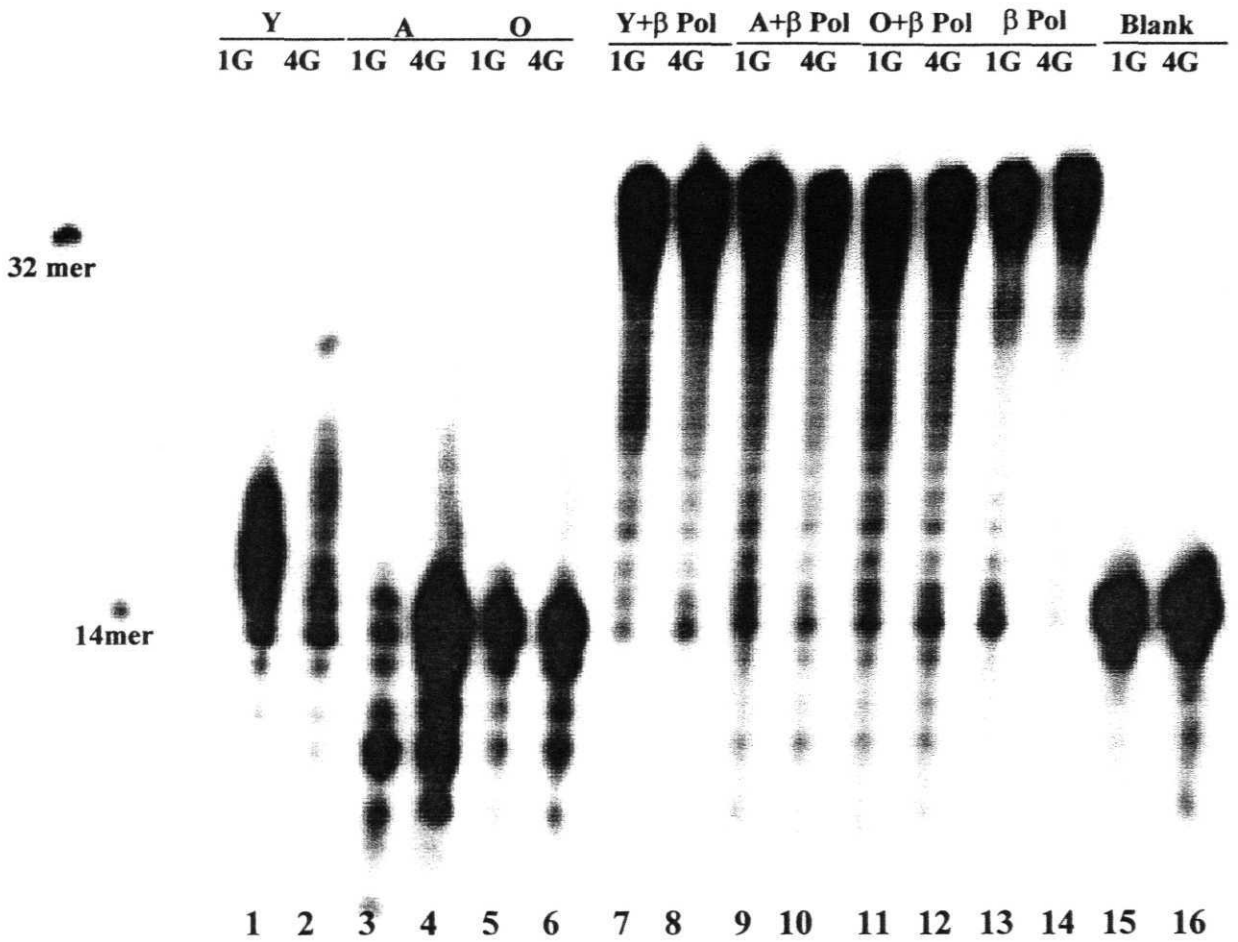
substrates showed that reactions with large amount of the enzyme, showed limited strand displacement synthesis. They have also shown that the extent of strand displacement by pol β is dependent on the time of incubation and the enzyme concentration. Limited strand displacement was observed even at low enzyme levels with longer incubation periods where as higher enzyme levels resulted in more extensive strand displacement. The results presented in this as well as in Chapter 4 indeed substantiate these earlier observations and demonstrate that pol p can incorporate a considerable number of nucleotides both to an open ended primer as well as to that in a gap. The addition, of course, seem to be a slow and distributive one displacing the down stream primer. It is possible that higher amounts of enzyme have been used in the present studies.

Be that as it may, the above results show that with gapped DNA as the substrate, the gap filling activity in neurons decreases with age of the animal and the supplementation of the reaction mixture with pure recombinant pol β restores this activity predominantly by a slow distributive strand displacement type of addition of nucleotides to the full length of that of template (in the present case, 32-mer). Save the exact mechanism of gap filling activity , the ability of pol p, the most predominant DNA polymerase present in brain, to restore the lost activity in aging neurons should be of considerable importance. This may raise possibilities for a potential with therapeutic consequences.

Figure 23

Gap filling activity in the **presence of the 'Young', 'adult' and 'old' neuronai extracts and supplemented with recombinant pure rat liver DNA polymerase β or β alone with no 5'- phosphate on the downstream primer.**

A typical autoradiogram is shown .Lanes 1-6 neuronai extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O,> 2 years) with out added pol β. Lanes 7-12(Y+P pol, A+P pol, O+ β pol) with added P-pol. Lanes 13 and 14 are with **p** pol alone. Lanes 15 and 16 are without any neuronai extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Lanes 1,3,5,7,9,11,13,15 are with 1gap(lG). Lanes 2,4,6,8,10,14,16 are with 4 gap (4G) oligo duplex.



1 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c -3' (17 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

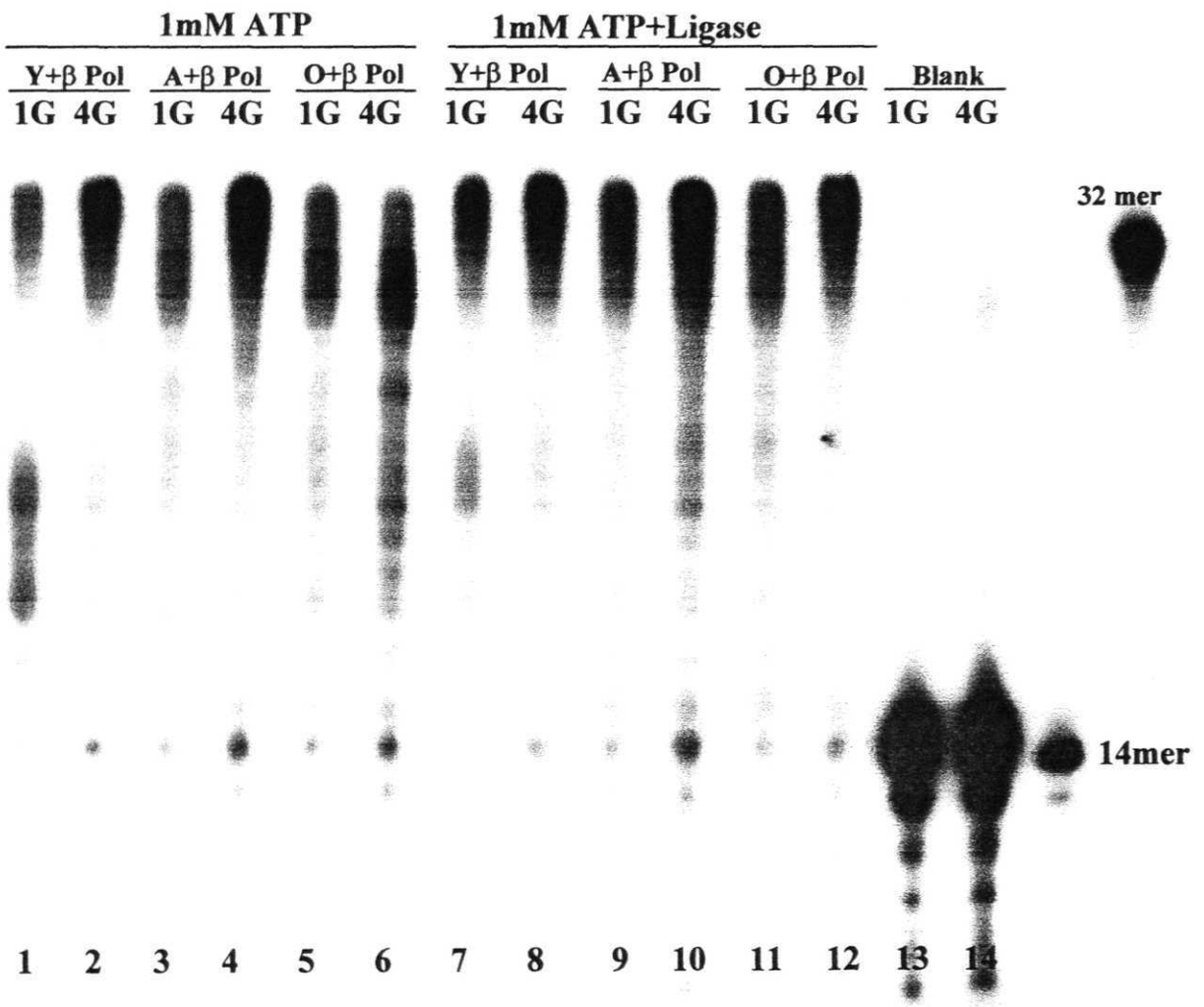
4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3' (14 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

Figure 24

Gap filling activity in the presence of 1mM ATP alone or together with T4 DNA ligase with 'Young', 'adult' and 'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase (3 with no 5'-phosphate on the downstream primer.

A typical autoradiogram is shown. Lanes 1-6 neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol β and lmM ATP. Lanes 7-12(Y+β pol, A+p pol, O+ p pol) with T4 DNA ligase and lmM ATP. Lanes 13 and 14 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Other notations are similar to the Figure 23



1 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c -3' (17 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

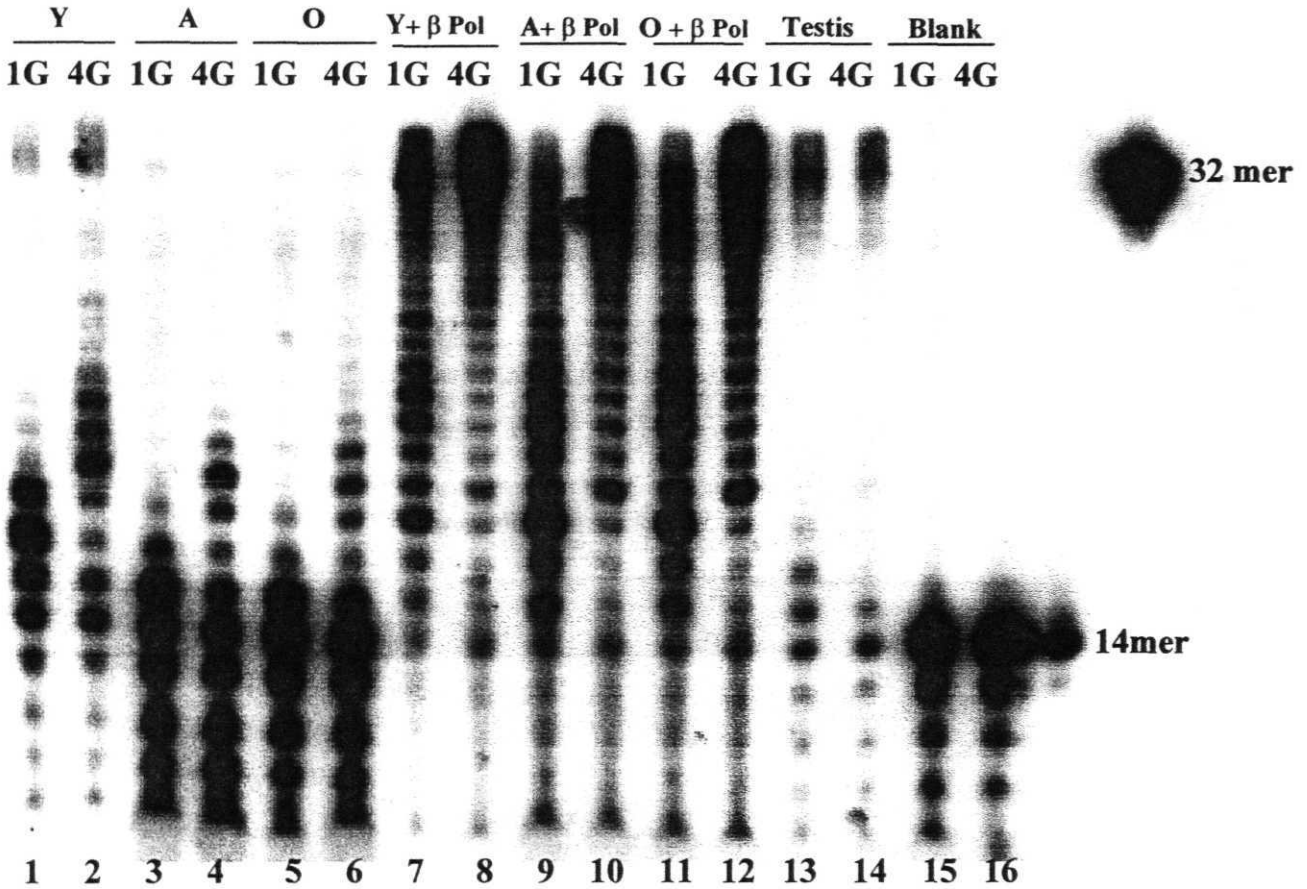
4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3' (14 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

Figure 25

Gap filling activity in the presence of the 'Young', 'adult' and 'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase β or β alone with 5'-PO_4 on the downstream primer.

A typical autoradiogram is shown. Lanes 1-6 neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol β. Lanes 7-12(Y+p pol, A+P pol, 0+ β pol). Lanes 13 and 14 are with testis extracts alone. Lanes 15 and 16 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown.



1 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c -3' (17 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

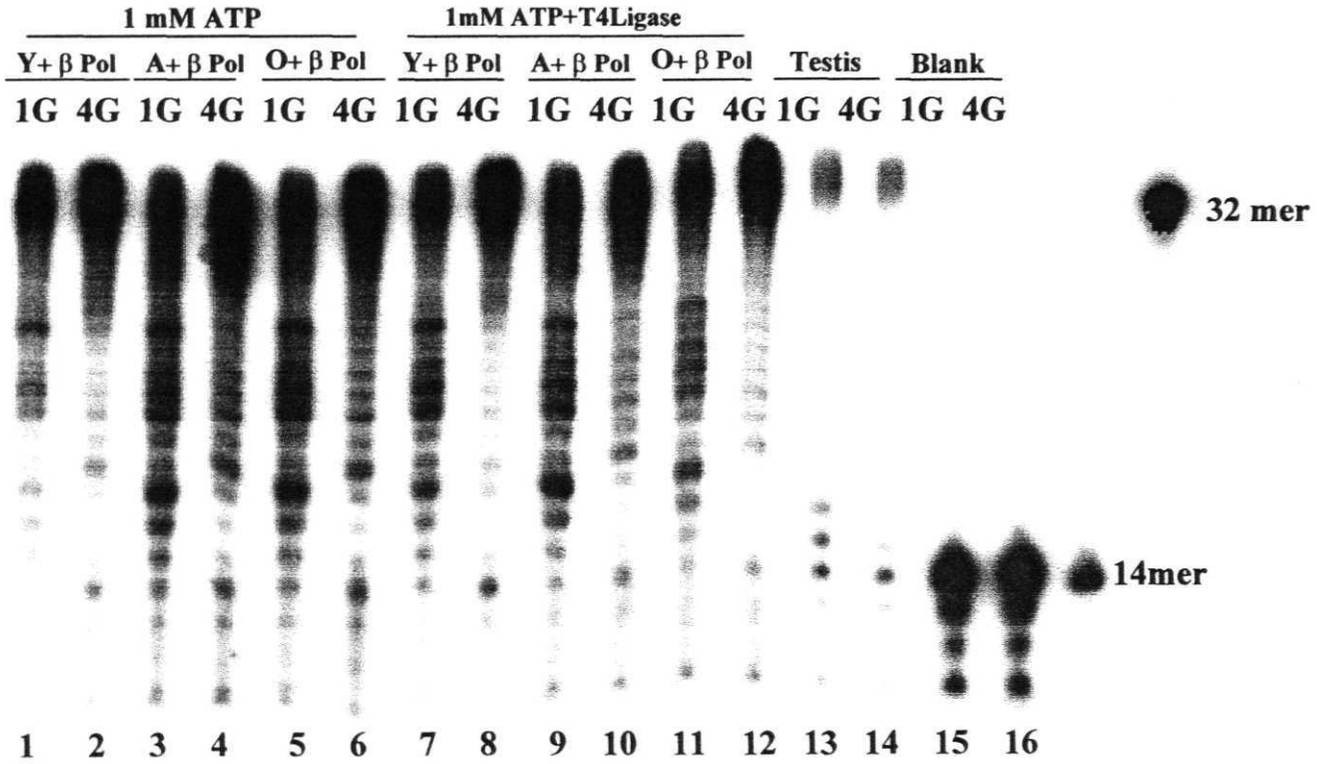
4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3' (14 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

Figure 26

Gap filling activity in the presence of lmM ATP alone or together with T4 DNA ligase with 'Young', 'adult' and **'old' neuronal extracts and supplemented with recombinant pure rat liver DNA polymerase β with 5'-phosphate on the downstream primer.**

A typical autoradiogram is shown. Lanes 1-6 (Y+p pol, A+p pol, 0+ β pol) neuronal extracts from young brain (Y, 5 days postnatal), adult brain (A, 6 months) old brain (O, > 2 years) with out added pol p and lmM ATP. Lanes 7-12(Y+p pol, A+p pol, O+ β pol) with T4 DNA ligase and lmM ATP. Lanes 13 and 14 are with testis extracts alone. Lanes 15 and 16 are without any neuronal extracts (Enzyme blanks). The mobility of labeled standard 14-mer and 32-mer are also shown. Other notations are similar to the Figure 23



1 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c -3' (17 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - - - - t t t t t t g c g g t g c c -3' (14 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)