CHAPTER 3

DNA Polymer as e activities in neuronal and Astroglial cell fractions in aging

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Introduction

DNA Polymerases are important components of replisome that are template directed machines catalyzing phosphoryl transfer reactions. They are also involved in recombination, repair. There is gradual increase in the discovery of number of polymerases, and today there are atleast 19 Polymerases. Since both DNA replication and repair are primary importance to cells, nature has created safety mechanisms by employing different Pols (Polymerases) for similar functional tasks. Many of the Pols contain other functional subunits and in addition to polymerizing subunit, often shows proof reading 3'5'-exonuclease. Substrates for DNA Polymerases vary from single nucleotide gaps to kilobase size gaps and from relatively simple gapped structures to complex replication forks in which two strands need to be replicated simultaneously.

Pol α was discovered in 1957 and it is localized in the nucleus. It lacks proof reading exonuclease activity and this fact created suspicion about its function in DNA replication. It is now thought to be involved in lagging strand synthesis (So and Downey, 1988; Waga and Stillman, 1994). Additional role for Pol a primase has been found in the checkpoint that couples S-phase to mitosis (D'Urso et al., 1995) Its role in DNA repair in yeast has not been identified although it may function in the repair of UV damage in Xenopus oocytes (Saxena et al., 1990; Oda et al., 1996).

Pol y and β were discovered in early 1970's. Pol β is small, highly conserved molecule and is essentially considered as a major DNA repair enzyme and has been extensively characterized enzymatically and by X-ray crystallography (Arnold et al., 1995;

Mullen and Wilson, 1997). Pol β has no proof reading exonuclease activity and is distributive in its catalytic function although short gaps with 5' phosphate are filled processively (Singal and Wilson, 1993). The Pol β has been proposed to function in DNA repair reactions involving filling in of very short gaps (Mullen and Wilson, 1997).

Pol y is involved in mitochondrial DNA replication and it has both 5'-3' and 3'-5' exonuclease activities (Graves, 1998).

Pol 8 was identified initially as a proof reading DNA Polymerase in mammalian cells (Lee et al., 1980; Hindges and Hubscher, 1997). It has tightly associated, intrinsic 3'-5' exonuclease activity and along with its auxiliary protein, proliferating cell nuclear antigen (PCNA) carries out replication of DNA processively (Tan et al., 1986; Prelich et al., 1987). Common characteristics of Pol δ are an active proofreading 3'-5' exonuclease activity, a low polymerase processivity in the absence of PCNA, sensitivity of the enzyme to aphidicolin, resistance to Pol a specific drug N2- (p -n-butylphenyl)-2'-deoxyguanosine 5-triphosphate (BuPhdGTP). These properties have been often applied to distinguish Pol δ from Pol a and Pol ε (Burgers et al., 1990).

Pol s which is previously known as DNA Polymerase II and the first report of it with proof reading activity was from yeast S.cerevisiae and was known as Polymerase B (Wintersberger and Wintersberger, 1970). It is distinguished from Pol δ because of its high processivity in the absence of PCNA (Burgers, 1991).

Novel Polymerases such as Pol ζ, Pol η, and Pol ι, Pol K and Rev 1 are involved in tranlesion DNA synthesis (Woodgate, 1999).

DNA Pols appear to possess a common catalytic active site. A two metal ion catalyses the phosporyl transfer mechanism which guarantees the incorporation of the

appropriate deoxynucleoside triphosphate base. Differences in the various aspects of structural architecture of the DNA Pols shows that the active site of the DNA Pol is conserved through evolution, where as the structure of the surface of the molecules might differ considerably (Foiani et al., 1997, Steitz, 1999).

Much of the information about the enzymology of DNA repair has originated from prokaryotic systems particularly from studies with E.Coli (Freidberg, 1985). Biochemical reconstitution studies often show specificity for certain repair enzymes in the recognition of the DNA damage and the incision steps of DNA repair, there is often little or no specificity for the enzymes required for the resynthesis steps. For example, gap filling during *in vitro* nucleotide excision repair can be carried out by Pol 8 or Pol ε holoenzyme (Aboussekhra et al., 1995) Pol δ or Pol ε also appear to be required for mismatch repair in eukaryotes. Some PCNA mutants of S.cerevisiae show defects in mismatch repair (Jhonson et al., 1996; Umar et al., 1996; Essenberg et al., 1997)

DNA Polymerase β, a smallest amongst the major DNA Polymerases was shown to be primarily involved in DNA repair. The mechanistic basis for the participation of Pol p in repair has been studied in more detail than that for Pol 8 or Pol e. In a post mitotic cell like neuron where replicative activity is absent the major DNA Polymerase activity found, is attributed to DNA Pol β (Waser et al., 1979). Evidence points out that Pol 8 as the primary DNA Polymerase for most DNA repair pathways, with Pol ε able to substitute for Pol 8 in the nucleotide excision repair pathway. Pol β is delimited to base excision repair.

Over the years the studies from this laboratory also revealed that Pol p is the most predominant DNA Polymerase in rat brain (Rao, 1997). Using whole brain extracts and specific inhibitors to the various DNA Polymerases like aphidicolin and 2'3'-

dideoxythymidine 5'-triphosphate to distinguish Pol a (aphidicolin sensitive) and Pol β (ddTTP sensitive), and monoclonal antibody to the Pol a, it was shown that the most predominant activity was that of Pol (3, while some activities can be contributed to the Pols a and 5/E (Prapurna and Rao, 1997).

Brain consists of heterogeneous populations of cells with two major types- neurons and astroglia with different characteristics.Therefore we have taken up the present study to examine DNA Polymerase activities in extracts of isolated neuronal and astroglial cell fractions from the rat cerebral cortex at three different ages. Rat brain neurons and astrocytes isolated from three age groups were designated as 'Young' (5 days postnatal), 'Adult' (6 months) and 'Old' (>2 years).

Methods

Preparation of neuronal and astroglial cell fractions from the rat cerebral cortex from 'Young', 'Adult' and 'Old' neuronal and astroglial cell enriched fractions were essentially prepared by the method of Usha Rani et al., (1983). DNA Polymerase assays and DNA Polymerase assay using inhibitors was carried out as described in Materials and Method, Chapter 2.

Results and Discussion

The relative proportions of DNA Polymerase a, (3 and δ/ε activities in isolated neuronal and astroglial cell fractions from developing, adult and aging rat brain cerebral cortex, was examined through a protocol that takes advantage of the reported differential sensitivities of different DNA Polymerases towards certain inhibitors like butylphenyl and butylanilino nucleotide analogs, 2', 3'-dideoxythymidine triphosphate (ddTTP), monoclonal antibody of human a Polymerase and the use of two template primers as substrates.

The concentrations of inhibitors used were arrived at after careful examination of the sensitivities exhibited to various inhibitors by Polymerases a, 5 and p and the I 05 (the amount of analog that gave 50% inhibition of the Polymerase activity) values reported by Lee et al., (1985) with respect to a given template primer. The reported sensitivity of β and 8 Polymerases towards ddTTP (were also taken into account (Dresler and Kimbro, 1987; Wahl et al, 1986). Accordingly, the Polymerase activity (with 'activated DNA' as substrate) inhibited by 1 μM BuPdGTP (N2- (p–n-butylphenyl)-2'deoxyguanosine-5'-triphosphate) or BuAdATP (N2- (p–n-butylanilino)-2'deoxyadenosine-5'-triphosphate) was considered as the activity of Polymerase a, whereas, at higher concentrations the additional reduction in activity is taken to be associated with 8 or e Polymerase. Similarly, the reduction in activity with 50 μM ddTTP was taken to be p Polymerase and further inhibition noticed at 1 mM level of ddTTP was attributed to 8 or E Polymerase. Polymerase a activity was considered to be the activity inhibited by the DNA Polymerase a specific monoclonal antibody, SJK 132-20. With Poly (dA). oligo (dT) $_{12\text{-}18}$ as substrate, the same procedure was followed, except that the two concentrations of BuPdGTP used were 10 and 100 μM and those for BuAdATP were 15 and 200 μM, respectively.

Table 2 shows the DNA Polymerase activities in isolated neuronal and astroglial cells from rat cerebral cortex of Young (5 days postnatal), Adult (6 months) and Old (>2 years). 'Activated DNA', Poly (dA). oligo (dT) $_{12\text{-}18}$ and Poly (dA.dT) were used as three different template primers to measure the polymerase activity. It can be seen from Table 2, the activity with 'activated DNA', both in neuronal and astroglial fractions, undergoes a significant decrease by the time the animal reaches adult stages of life. There is considerable variation in the activities from one animal to the other. However the pattern of changes with

age are quite consistent. In case of the synthetic oligos the activity in either type of cells is far less when compared to activated DNA. Also no age dependent changes were observed in both neuronal and astroglial cells when synthetic substrates Poly (dA). oligo $(dT)_{12-18}$ and Poly(dA.dT) were used. It is possible that the low activities with these synthetic substrates shows that these substrates are not the preferred ones for Pol p. It was shown by Syvaoja et al.,(1990) that Pol 5 and E are active towards these substrates and Pol ε prefers a template primer such as Poly (dA). Oligo$(dT)_{12-18}$ that contains long stretches of single stranded Poly(dA). Conversely, Polymerase 5 exhibit equal preference towards 'activated DNA and Poly (dA).Oligo$(dT)_{12-18}$ (Crute et al.,1986; Wahl et al.,1986). However, the activity of Pol 5 on Poly(dA).Oligo$(dT)_{12-18}$ is highly influenced by the inter primer nucleotide distance in the substrate and the presence and the presence of PCNA (Crute, 1986; Syvoaja et al., 1990). It is therefore taken that this low but steady activity is due to Polymerase 5/s present in the cell extracts.

Table 3 and 4 shows the extent of inhibition exerted by the various inhibitors used with 'activated DNA' as substrate. The activity without any inhibitor (control) is taken as 100 and other values are relative to the control. The Polymerase activity in young neurons inhibited by 1 uM BuPdGTP is 35%. This activity was therefore attributed to Polymerase a. This assumption appeared to be reasonable since the monoclonal antibody to human a Polymerase also exerted inhibition to a similar extent. However, the activity was inhibited by 71% when the concentration of BuPdGTP was raised to 200 uM. The rise in inhibition by 36% was thought to be due to the inhibition of Polymerase 8 and/or Polymerase e. A similar rationale applies to our interpretation of data with the other nucleotide analog inhibitor, BuAdATP. When ddTTP was used at a concentration of 50 uM level, Polymerase

activity was inhibited by 50% and the inhibition went up 78% when the concentration of ddTTP was raised to 1 mM. The percentage of activity inhibited by 50 uM ddTTP was attributed to Polymerase β activity and the additional inhibition at 1 mM level was taken as that of 5 and /or e Polymerase activity.

Table 4 shows the range of inhibitions exerted by the same inhibitors with astroglial extracts. The results indicate that a relatively higher levels of Pol a and lower levels of Pol δ/ε in astroglia than in neurons which is a notable difference between these types of cells.

Table 5 shows the calculated relative percentages of different DNA Polymerases, out of the total Polymerase activity. It is shown in the table the value obtained for each of the inhibitor used with respect to calculating the percentage of a given polymerase and the average is taken to minimize the error. It can be seen that in both types of cells and at all the three ages studied, Pol β appears to be the predominant polymerase, which confirms earlier reported observations. Polymerase activity is attributable other than Pol β is due to Pol 5/e activity which is closely behind the Pol β activity in neurons whereas Pol a is the second predominant polymerase in astroglia. It can be seen from the table 5 that the relative percentages of Pol β in young, adult and old neurons are 50, 43 and 42 respectively. Where as the values for Pol a in young, adult and old neuronal cells the values are 33, 20 and 21. For Pol 5/e, the values read 34, 32 and 42. In astroglia, the relative abundance of Pol β at young, adult and old ages is 67,50, and 53 respectively; for Pol a in the same order 47, 24, and 35; for δ/ε, the figures are 25, 19 and 16.

Tables 6, 7 the results of similar experiments with Poly (dA). oligo $(dT)_{12-18}$. as template primer are shown. Firstly, the basal activity itself is very low with this substrate (see Table 2). Compared to activated DNA as substrate the pattern of results with various inhibitors, in both neurons and astroglia, is similar. The results from the tables 6,7 showed striking difference between the two cell types in that, the neurons showed higher percentage of Polymerases δ/ε activity even in aging brain and astroglia showed a more sustained Pol a activity. When ddTTPS was used as the inhibitor, the inhibition is less potent in astroglia with the synthetic substrate leading to lower percentages of Pol β and Pol 5/s (compare the data in Table 5 and 8). Hence it appeared that this synthetic primer might be more useful to distinguish various DNA Polymerase activities with pure proteins (Syvaoja et al., 1990) rather than with crude extracts. Nonetheless, the data do indicate the presence of activity that is attributable to Polymerases δ/ε throughout the lifespan.

Table 8 shows the relative percentages of Pol β in young, adult and old neurons. Relative pe..entages for Pol p in young adult and old neuronal cells are 41, 41 and 27 respectively. Where as the values for Pol a in young, adult and old neuronal cells the values are 39, 20and 21. For Pol δ/ε, the values are 20, 11 and 30. In astroglia, the relative abundance of Pol β at young, adult and old ages is 37,36, and 39 respectively; for Pol a in the same order 31 24, and 24; for 5/e, the figures are 16, 15 and 9.

The percentage calculations for each type of nuclear DNA Polymerase is based on the extent of inhibition exerted by various known inhibitors for these Polymerases and with two different substrates, the activated DNA and Poly (dA). oligo $(dT)_{12-18}$. It may be argued that this is an oversimplification of a complex situation since the inhibitors used in this study are not absolutely specific and the calculated percent distribution of the polymerases

is only approximate. In spite of this limitation, these data do give sufficient hint regarding the relative abundance of the various DNA Polymerase activities in two major cell types of rodent brain as sufficient care was taken in choosing the two different concentrations of the inhibitors.

Table 2: DNA Polymerase activity in rat neuronal and astroglial cells of different ages with three different Template-Primers.

	Substrate and cell Fraction used	AGE YOUNG	ADULT	OLD
I.	'Activated **DNA'**			
	Neurons	2023±1076	719±541*	568±412*
	Astroglia	1471±550	822±512*	694±652*
II.	**Poly(dA).Oligo(dT)$_{12-18}$**			
	Neurons	18.4±3.9	17.7±3.2	17.7±3.6
	Astroglia	37.6±18.6	33.0+14.9	31.2±11.8
III.	Poly dA-dT			
	Neurons	16.7±3.0	18.0±4.8	19.1±3.6
	Astroglia	35.9±16.5	35.7±14.9	34.5±12.0

Values are averages ± S.D. and expressed as picomoles of the radioactive deoxynucleotide Incorporated into the acid insoluble fraction in 1hr/mg protein.

*These values are significantly different ($p<0.001$ for neurons and 0.02 for astroglia respectively) from the corresponding value at 'Young'.

Table 3: Effect of various inhibitors on the activity of DNA Polymerases in extracts of neuronal cells isolated from rat brain of different ages using *Calf Thymus 'Activated DNA'* as Template-Primer.

Inhibitor concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuPdGTP, 1µM	65.0±4.8	77.3±12.2	82.2±3.2
+ BuPdGTP, 200µM	28.7±7.4	42.1±0.6	33.5±15.0
+BuAdATP, 1µM	68.6±2.7	92.3±2.2	78.5±12.4
+ BuAdATP, 200µM	31.1*±4.7	49.5±7.5	27.3±9.4
+ddTTP 50µM	50.0±14.6	57.4±1.6	57.6±13.0
+ddTTP, 1mM	22.3±5.5	37.3±6.0	31.3±8.4
+SKJ132-20 ab, 1 µg	68.4±1.3	72.1 ±6.2	77.5*±3.7

Activity expressed as in Table 2The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments except in two cases marked with an asterisk.

Table 4: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of astroglial cells isolated from rat brain of different ages using *Calf Thymus "Activated" DNA* as Template-Primer

Inhibitor concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuPdGTP, 1µM	57.7±7.4	80.8±7.0	58.6±11.3
+ BuPdGTP, 200µM	24.5±2.0	60.2±4.2	44.7±4.2
+BuAdATP, 1 µM	49.8±1.0	70.8±7.66	79.4±2.9
+ BuAdATP 200µM	19.7±2.2	46.3±10.5	79.4±2.9
+ddTTP, 50µM	33.6±6.2	49.7±6.2	47.2±7.6
+ddTTP, 1mM	21.8±1.4	39.4±5.8	33.5±5.2
+SKJ132-20 ab, 1µg	50.9±13.1	76.1±8.5	57.6±4.5

Activity expressed as in Table 2.The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 5: Percentage of different DNA Polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different Ages using *calf thymus `activated' DNA* as Template-Primer

	NEURONS									***ASTROGUA***								
	a			**δ / ε**			P			a			5 / E			P		
	Young	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**
BuPdATP	35	23	18	**36**	35	49	**NS**	**NS**	**NS**	42	19	41	33	21	14	**NS**	**NS**	**NS**
BuAdGTP	31	8	22	38	42	51	**NS**	**NS**	**NS**	50	29	21	30	24	20	**NS**	**NS**	**NS**
ddTTP	**NS**	**NS**	**NS**	28	20	27	50	43	42	**NS**	**NS**	**NS**	12	10	14	66	50	53
SJK132-20	32	28	22	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	49	24	42	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
Average	**33**	**20**	**21**	**34**	**33**	**42**	**50**	**43**	**42**	**47**	**24**	35	**25**	**19**	**16**	**66**	**50**	**53**

These data are recalculated, as explained in text, from the values in tables 3 and 4. NS = not sensitive

Table 6: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of neuronal cells isolated from rat brain of different ages using *Poly(dA).Oligo(dT)$_{12-18}$* as Template-Primer

Inhibitors concentration	Activity (%) at different ages		
	YOUNG	ADULT	OLD
Control	100	100	100
+BuP dGTP, 10μM	61.9±3.9	78.9±1.6	77.5±1.5
+BuP dGTP, 100μM	43.4±2.5	68.3±3.6	43.5±0.9
+BuAdATP, 15 μM	64.1±2.4	82.5±1.8	81.7±4.6
+BuAdATP, 200μM	48.6±3.5	70.7±3.3	48.9±2.7
+ddTTP, 50μM	59.5±5.2	59.3±3.7	73.1±1.3
+ddTTP, 1mM	35.0±4.4	50.5±1.4	50.1±3.8
+SKJ132-20 ab, 1μg	56.6±11.5	78.2±4.6	76.4±7.8

Activity expressed as in Table 2.The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 7: Effect of various Inhibitors on the activity of DNA Polymerases in extracts of astroglial cells isolated from rat brain of different ages using *Poly(dA).Oligo(dT)*$_{12-18}$ as Template-Primer

	Activity (%) at different ages		
Inhibitors concentration	**YOUNG**	**ADULT**	**OLD**
Control	100	100	100
+BuPdGTP, 10μM	73.0±0.4	80.2±0.6	73.3±1.2
+ BuP dGTP, 100μM	59.8±1.3	61.3±1.3	61.2±5.1
+BuAdATP, 15 μM	65.1±1.4	71.1±2.6	78.3±1.2
+BuAdATP, 200μM	49.9±0.6	58.4±2.5	70.4±0.4
+ddTTP, 50μM	63.4±0.8	63.9±1.1	61.2±2.3
+ddTTP, 1mM	44.5±1.2	50.8±2.4	53.3±1.0
+SKJ132-20 ab, 1μg	68.8±8.4	76.6±1.3	75.7±0.2

Activity expressed as in Table 2The control value is taken as 100 and the other values in the presence of various inhibitors are expressed average ±SD. The values are from three different experiments.

Table 8: Percentage of different DNA Polymerases present in neuronal and astroglial cell fractions isolated from rat brain of different Ages using Poly(dA).Oligo$(dT)_{12-18}$ template- primer

	NEURONS									***ASTROGLIA***								
	a			6 / E			β			*a*			δ / ε			P		
	Young	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**	**Young**	**Adult**	**Old**
BuPdATP	38	21	22	19	11	34	**NS**	**NS**	**NS**	27	20	27	13	19	12	**NS**	**NS**	**NS**
BuAdGTP	36	18	18	16	12	33	**NS**	**NS**	**NS**	35	29	22	15	13	8	**NS**	**NS**	**NS**
DdTTP	**NS**	**NS**	**NS**	24	9	23	41	41	27	**NS**	**NS**	**NS**	19	13	8	37	36	39
SJK132-20	43	22	24	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	31	23	24	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
Average	**39**	**20**	**21**	**20**	**11**	**30**	**40**	**41**	**27**	**31**	**24**	**24**	16	**15**	**9**	**37**	**36**	**39**

These data are recalculated, as explained in text, from the values in tables 6 and 7. NS = not sensitive

DNA Pol β is the predominant polymerase in the adult brain. Traces of other polymerase activity was suspected to be the a type (Waser et al, 1979; Shrivastaw et al, 1983; Subrahmanyam and Rao, 1988; Rao, 1997).These observations appeared logical since only DNA repair but not replication is required in the post mitotic tissue like brain and the Pol p is considered to be predominant repair enzyme (Wood and Shivji, 1997; Hubscher, 2000). Neuronal cells, once differentiated, do not replicate, but they show high metabolic activity and the gene expression in these cells is 2 to 3 times more as compared to the cells in rest of the body (Chaudhari and Hahn, 1983; Tobin, 1994). Also the genomic DNA in brain cells is shown to suffer various types of damage due to endogenous sources (Rao, 1997). Abasic sites and many types of base modification alterations in DNA can be repaired in brain cells through Base Excision Repair pathway (BER) and it is well established now that Pol p is the enzyme that participates in BER. There is now considerable evidence that even in adult brain, some progenitor stem cells are present capable of differentiating into mature neurons (Van Praag et al, 2002). It is possible that a small portion of the different polymerase activities noticed in this study could have come from these maturing neurons. It is not however, clear what are the *in vivo* stimuli that would induce this differentiation and that too in the specific area of brain used in this study viz., the cerebral cortex. The present results may largely reflect the situation in terminally differentiated neurons which are essentially incapable of dividing.