CHAPTER 6

GENERAL DISCUSSION

SUMMARY

REFERENCES

General discussion

The process of aging is one of the most intriguing problems of today's world. Aging, although a basic and general phenomenon is a complex interplay of genetic and epigenetic factors resulting in the functional deterioration. Aging changes are manifest at all levels of organization -molecular to organismic level. It is generally accepted that changes occurring after attaining reproductive maturity comprise the phenomenon of aging or senescence. However, fundamental molecular mechanisms involved in aging remains controversial and largely unproven and the major reason for this is the obvious complexity of the problem. The nature of biochemical and molecular mechanisms underlying the aging process had been the subject of considerable speculation. Several theories have been proposed based on genetic and stochastic events as the triggering events that eventually lead to senescence. DNA damage and repair theory of Hart and Setlow (1974) has evoked tremendous attention among the scientists all over the world.

According to this concept, repair of the damaged DNA is a primordial molecular process, fundamental to the maintenance of life and genetic diversity on earth. There is extensive correlative evidence that DNA damage and mutations increase with age. In addition, there are studies that have demonstrated a corresponding decrease of DNA repair. This decrease in DNA repair may in part account for the increased DNA damage levels and mutation frequencies observed with age. The contribution of DNA damage and repair in processes impinging on Man's mortality, such as aging and carcinogenesis, have become of particular interest.

The importance of DNA repair to the nervous system is most graphically illustrated by the neurological abnormalities observed in patients with hereditary DNA repair disorders. As discussed in the Chapter 1, most of the alterations in the DNA are the result of continuous exposure of living organisms to DNA damaging agents like radiations, certain environmental components and products of cellular metabolism. In mammals, cell types that do not divide like long-lived neurons, differentiated muscle cells, and the cell types that divide only slowly, accumulate DNA damage with age (Bernstein and Bernstein, 1991; Rao 1997; Rao, 2002; Nouspikel and Hanawalt, 2002). It is likely that these cells may govern the rate of overall mammalian aging. Brain is composed of cells with a variety of developmental histories, functions and fates, and the capacity to repair DNA may be profoundly affected by developmental status of individual cells. The level of DNA repair is low in brain, endogenous damages accumulate, mRNA synthesis declines, and protein synthesis is reduced with age (Price, 1971; Chetsanga et al., 1977; Mori and Goto, 1982; ZS-Nagy and Semsei, 1984; Sajdel-Sulkowska and Marolta, 1985). Thus, for the brain, there appears to be a direct relationship between the accumulation of DNA damage and the important feature of aging. In contrast to non-dividing or slowly dividing cells cell populations, at least some types of rapidly dividing cell populations appear to cope with DNA damage by replacing lethally damaged cells through replication of undamaged ones. Examples include duodenum and colon epithelial cells and hemopoitic cells of bone marrow (Bernstein and Bernstein, 1991).

The last 50 years have seen great strides of advancement in the understanding of the various pathways of DNA repair both in prokaryotes and higher organisms including humans. These aspects have already been discussed in Chapter 1 of this thesis.

Among the four different pathways involved in repairing the DNA damage, Base excision repair pathway (BER) is the most important and relevant DNA repair pathway as far as the brain is concerned.

Five major DNA polymerases have been known for some time, the a, p, *y,* 5 and 8. In addition, a number of DNA polymerases are being discovered in recent past and the total number of DNA polymerases has now reached to 17 (Hubshcer et al., 2002). A characteristic feature of these newly identified polymerases seems to be their ability to affect traslesional DNA synthesis. It appears that cell can utilize these polymerases under the conditions of desperation both for synthesis and repair of DNA. As far as the five major DNA polymerases are concerned, DNA polymerase a is classically regarded as a major enzyme associated with cell replication. Polymerase p is essentially a repair enzyme at least for some forms of damage and polymerase y is involved in mitochondrial replication. Polymerase 8, associated with its intrinsic 3'-5' exonuclease activity and high processivity in the presence of PCNA has been assigned a role in DNA replication, more precisely in leading strand DNA synthesis at the replication fork. DNA polymerase s which is PCNA independent also associated with 3'-5' exonuclease is considered to be involved in DNA repair. (Budd, 1997; Burgers, 1998; Hubshcer et al., 2002). In a post mitotic cell like neuron, replication is absent and it has been shown over the years that whatever the DNA-polymerase activity observed in this organ was found to be exclusively DNA polymerasep. However, the possibility still exists that brain cells may possess other DNA Polymerases

in very minor proportions that may escape detection due to the type of template primers and the sensitivity of the methodology used. Moreover, each cell type of brain may harbor different proportions of the polymerases. Indeed the results presented in Chapter 3 do show that both neurons and astrocytes possess small proportions of other polymerases, the pol a and pol 5/E. This is the first report to suggest the presence of pol 5/E activity in isolated cell fractions of developing as well as aging brain. It is seen that one notable difference between neurons and atrocities is the relatively higher levels of pol a and lower levels of pol 6/s in astroglia than in neurons. In both the cell types and at all the three ages studied, pol β appeared to be the predominant polymerase which is in line with many earlier reports. Once again, the results demonstrate that pol β activity decreases with age. Next predominant activity to pol β is that of pol 6/E in neurons whereas pol a is the next predominant polymerase in astroglial cells. This could be due to the completely non replicative nature of neurons and some residual replicative activity in astrocytes. Some amount of pol 5/e may become handy for a neuron when it has to perform long patch BER in a contingent situation that demands long patch repair mode of BER.

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) $_{12\text{-}18}$. It may be argued that this is an oversimplification of a complex situation and the values for each polymerase presented in Chapter 3 may not represent absolute values but only an approximation. Indeed that is all that is being claimed from that data. The percentages of different polymerases arrived at are, of course, to an approximation. The value of that data does not lie in denoting the exact level of a given DNA polymerase but in suggesting that some level of the

given polymerase does exist in the brain cells. Complete characterization and assessment of these polymerases and perhaps of those that are being discovered now, may constitute the heart of a future study from some laboratory.

It is easy to predict that base excision repair pathway would be the main guardian to ensure genomic stability in a highly active organ like brain. When compared to the different polymerases found in the nuclei of mammalian cells the predominant enzyme that takes part in the BER is DNA polymerase β (Wood RD, 1997; Wilson III DM et al., 1997; Fortini et al., 1998). It thus appears that genomic maintenance in brain cells is largely taken care by Pol β dependent BER pathway. In some situations where long patch BER is required, the neurons may still able to affect it since it is noticed that neurons do possess some levels of pol δ/ε.

The present study constitutes part of the overall efforts that have been going on in this lab to examine the link between DNA-repair potential and aging phenomenon with special reference to Brain. BER pathway enjoys special relevance in brain. Pol β has a crucial role in BER. It is already established that pol β activity decreases in brain with age. Can we put these facts together and conclude that pol p plays a crucial role in the process of aging? May be a more precise assay procedure for measuring the functionally relevant activity of pol β is needed before conclusions are drawn in this aspect. This study is an attempt towards that end.

An overview of the results presented in Chapter 4 and 5 reveal a few interesting facts. Firstly, pol β is able to extend a primer in a linear synthetic oligo template primer. In fact this is perhaps nothing new in that 'activated DNA' is routinely used as a substrate for

measuring the DNA synthetic activity of pol p. Many years ago, Wang and Korn (1982) have shown that pol p is capable of adding a long stretch of nucleotides to a primer although slowly. Even in recent years pol β is observed to add nucleotides to a primer to fill up the gap to join the down stream primer or some times even without a down stream primer although with varying speeds and under different experimental conditions like time of incubation and pol β concentration (Singhal and Wilson, 1993; and Chagovetz et al, 1997) It therefore appears that attributing a narrow function of filling up a single nucleotide gap only to pol β may be far from appropriate. From the present studies it does appear that pol P can extend primers with or without downstream primer, with or without a phosphate group on 5' side, with varying efficiencies and in a distributive and strand displacement manner. The results of Chapter 4 also show that while extending a primer helical distortion (mismatched bases) at the extending end is not tolerated and results in decreased efficiency. It appears that an independent 3'-5' exonuclease activity helps circumventing this problem.

Perhaps the most important outcome of the results presented in this thesis is the demonstration that pol β dependent primer extension activity is drastically decreased in the neurons of aging rat brain and that this lost activity can be restored *in vitro* by simply adding pure recombinant rat liver pol p to the reaction mixture under appropriate conditions.

Similarly the results with what is generally considered to be the most physiologically relevant substrate for pol p, a duplex with a short gap in one of the strands (Chapter 5), are of some consequence. Whether these observations can be extended to the overall BER pathway is essentially a matter of speculation at this time. However, it may be logical to assume that once pol β dependent activity is low in aging brain, the same may be

applicable to over all BER pathway since pol p is a main player in BER pathway (Cabelof 2002, Intano et al., 2003). Nevertheless it is possible that any of the other factors involved in BER pathway may be more affected during aging than pol β. This possibility , however, appears to be less likely since , as is shown in this study, addition of pol p alone is able to bring back the activity once the mismatch removal is affected. Moreover, Intano et al., (2003) have very recently reported an 85% decrease in BER activity in aging mouse brain nuclear extracts and there was a decreased abundance of pol p, but not of other BER proteins, in old mice. On the other hand they could not restore the BER activity by the addition of pure pol p to the reaction mixture. The reasons for this variation from the present results are not known at this time. A close comparison of the conditions used in the two labs may yield some clues.

The importance of pol β in the general maintenance of genomic stability in brain is becoming pointedly apparent in recent years. For example, (Sugo et al., 2000) have shown that mice deficient in pol β suffer from neonatal lethality and abnormal neurogenesis. Pol p also appears to be repairing the DNA-damage following cerebral injury due to a variety of reasons like hypoxia, ischemia etc., (Liu et al., 1996; Englander et al., 1999, Lin et al., 2000) and the fidelity with which such repair is carried out may determine the chances of recovery. Pol β is also found to confer protection against the cytotoxicity of oxidative DNA damage (Horton et al., 2002) Moreover, there are a number of genetic disorders that show elevated genomic damage and neurodegeneration with coupled symptoms of premature aging in some cases (reviewed in Brooks, 2002 and Rao, 1997). The involvement of pol β in the maintenance of genomic stability is also becoming apparent from the results being

reported from a different direction. For example, induction of oxidative stress was found to increase the levels of pol p in mouse monocytes and fibroblasts. It was also found that it is the pol p dependent BER which was conferring protection against DNA damage (Chen et al.,1998) In other words, there is an up regulation of pol β dependent BER when cells are subjected to DNA damage.

Interestingly, the up-regulation of pol β is also connected to the genomic instability. Thus Canitrot et al.,(1998; 1999) have proposed that over expression of pol β could be a genomic instability enhancer process and indeed it has been found that pol β is up-regulated in some types of adenocarcinomas and cell lines (Srivastava et al, 1999). A role for deregulated pol β in inducing chromosome instability and tumorigenesis has also been envisaged by Bergoglio and co workers (2002). Further, regulated over expression of pol β is found to mediate early onset of cataract in mice (Sobol et al., 2003) these diverse observations surface the possibility that pol β may have slightly different roles in different tissues and a balanced level of this enzyme may be required for its proper function.

Be as it may, the present studies revealing that pol β may be one of the, if not the sole, most limiting factors for carrying out BER in aging neurons and that this deficiency can be rectified, *in vitro,* by the addition of pure pol p, should be of considerable importance. A thorough study of BER in normal and pathological/experimental brain and examination of ways and means to bring back the lost DNA repair activity may yield results with far reaching consequences.

Summary and Conclusions

1. The hypothesis that "decreased DNA repair capacity in the brain is atleast one of the major biochemical markers that is associated with advancing age and deterioration of brain function" has been tested in this investigation.
2. DNA Polymerase activities were studied in extracts of isolated neuronal and astroglial cells fractions from the rat cerebral cortex at three different ages of 'young' (4 days postnatal),'Adult' (6months), 'Old' (> 2 years).
3. DNA polymerase activity undergoes a significant decrease with the advancement of age with 'activated DNA' as the substrate in spite of the fact that considerable variation in this activity was noticed from one animal to the other.
4. DNA polymerase activity towards synthetic oligos as substrate in either type of cells is far less when compared to 'activated DNA'. No age dependent changes were observed when synthetic substrates Poly (dA). oligo $(dT)_{12-18}$ and Poly(dA.dT) were used.
5. Relative abundance of DNA polymerases using differential sensitivities of these enzymes towards inhibitors like BuPdGTP, BuAdATP, ddTTP, monoclonal antibody of human a polymerase, was assessed. The results pointed that Pol β is the predominant polymerase at all the post-natal ages, where as some amounts of Pols a,5,e are also present . Pol 5/e activity was closely behind the Pol β activity in neurons whereas Pol a is the second predominant polymerase in astroglia.
6. Base excision repair (BER) being the main DNA-repair mechanism in brain cells and Pol β being a main player of that pathway, a more *in vivo* relevant functional assay for Pol p activity was standardized.

7. With a synthetic staggered oligoduplex DNA substrate the primer extension activity was low in ‘ Young' neuronal extracts and almost undetectable in adult and old brain neuronal extracts.

8. Restoration of the lost activity by supplementing the neuronal extracts with pure DNA polymerases(calf thymus a polymerase, E.coli DNA polymerase I and rat liver DNA polymerase P) was examined *in vitro.* Only polymerase β gave consistent and encouraging results. Extension of the primer was slow and distributive in nature and primers with a mismatched base at 3’-end were extended much less efficiently than the correctly paired primer.

9. Processivity and efficiency of pol β aided primer extension (including those with a mismatch) can be enhanced by preincubating the synthetic oligoduplexes with only neuronal extracts and supplementing pol β along with dNTPS and Mn^{++} in the second step. These results pointed that pol β can restore the DNA-repair / synthetic activity of the neuronal cells irrespective of the age of the animal.

10. Pol β aided extension of a primer with a 3' mismatch will occur optimally only after the removal of the mismatch. This was established by a 3 step reaction where the products of the second step were restrict digested with HinP 1 and looked for the predicted labeled 12-mer. These experiments also suggested the existence of 3’-5’exonuclease activity in neuronal extracts.

11. Since pol β doesn't possess the proof reading ability, studies were extended to establish the actual existence of an independent 3’-5’exonuclease activity in neuronal extracts. The results indicated that indeed neuronal extracts do possess such activity. Duplex DNA Model duplex oligo is excised from the 3’-end in a sequential and time

dependent manner with no particular specificity towards a mismatched base. However, at the initial stages of the reaction, a mismatched base is removed faster

12. With age there is a decrease in 3'-5' exonuclease activity but still maintained at significant levels even in old neuronal extracts. It is concluded that this 3'-5' exonuclease activity is facilitating the extension by pol β of a primer with a mismatch at the 3'-end.

13. It was observed that neuronal extracts were also able to excise bases from 3'-end of single stranded oligos. Excision activity towards single strand oligos (14-mer and 21-mer) showed that extracts of all the ages show increasing activity with time and 'young' extracts show higher activity where as 'adult' and 'old' extracts still retain considerable activity towards primer (14 mer). No striking changes in the activity could be seen with respect to age towards 21-mer. The physiological relevance of this activity is not clear at this time.

14. Since the most preferred substrate for pol (3 is reported to be a duplex DNA with a one nucleotide gap, the ability of neuronal extracts to fill up 1 and 4 nucleotide gaps in oligo duplex was studied.

15. The results reveal that the gap filling activity decreases with age. 'Adult' and 'old' neurons could hardly exhibit any gap filling activity. Supplementation of the extracts with pure pol p restored the gap filling activity largely through a slow distributive strand displacement addition of nucleotides to the up stream primer all the way to the length of the template (32 nucleotides). This conclusion was reached because of the several radioactive spots representing various lengths between 14 and 32 nucleotides, seen on

the autoradiogram. Additional supplementation of the extracts with ATP alone or together with T4 DNA ligase did not change the situation significantly.

16. When the above experiments were repeated with the down stream primer having a phosphate group on 5'-side, still the improvement seen due to the addition of pol β was no different from what was seen in the previous set of experiments (when there was no phosphate group on the down stream primer). It was concluded that that gap filling is achieved by added pol (3 both by a processive mechanism where the filled gap is immediately ligated as well , perhaps more predominantly, by extending the up stream primer in a slow distributive manner until the template length is achieved.
17. Be as it may, the present studies show that pol β is the most predominant DNA polymerase present in brain and plays a major role in repair and maintenance of DNA in brain. It restores the lost primer extension as well as gap filling activities in aging neurons. These results surface the exciting possibility of restoring the BER activity in aging neurons to normal level, which could have far-reaching consequences.