CHARACTERIZATION OF THE MAMMALIAN DNA POLYMERASE GENE AND PROTEIN

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Dr. Nawin C. Mishra
University of South Carolina
Columbia, SC 29208

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**Characterization of the mammalian
DNA polymerase gene(s) and protein(s)**

by

**N.C. Mishra
Principal Investigator**

Abstract

Methods were developed to purify the DNA polymerases of the α -family from Chinese hamster cells and their mutants selected as resistant to aphidicolin or specific inhibitor of DNA polymerases of the α -family. The wild type and mutant DNA polymerases were characterized with respect to their biochemical properties. A methodology was also developed to identify the replication intermediates and aphidicolin was found to inhibit a replication intermediate of the 24Kb size indicating the fact that aphidicolin inhibits the elongation process during DNA replication. This is the first demonstration of such role of aphidicolin in the eukaryotic DNA replication.

Characterization of the mammalian DNA polymerase gene(s) and protein(s)

by

N.C. Mishra Ph.D., Principal Investigator

In order to adopt a biochemical genetic approach to understand the role of DNA polymerases in mammalian DNA replication we chose to work with the Chinese hamster ovary cells because of the attributes of this mammalian cell line in genetic studies. These included that facts that this cell line is hypodiploid and grows indefinitely and can be easily used in somatic cell hybridization experiments to carry out genetic analysis such as dominance and recessiveness of allele and their linkage relationship. We chose to isolate DNA polymerase mutants of CHO cells and later that of human cells (W138) by their resistance to ara-C (an inhibitor of DNA replication) and/or to aphidicolin, a specific inhibitor of the DNA polymerase of α -family. (Figure 1)

Characterization of DNA pol α family from wild type cells (aph^S)

In order to determine the nature of changes in DNA polymerases of the aphidicolin resistant mutants we have isolated and characterized the DNA polymerases from the wild type (CHOK-1) cells. The DNA pol α and δ of the CHO cells have been characterized to some extent but when the simultaneous purification of these DNA polymerases of α family (α , δ , and ϵ) in human cells was described we adopted this methodology of isolation and purification of human DNA polymerases to purify and to characterize DNA pol of α family from the Chinese Hamster Ovary cellos. We have characterized these CHO enzymes with respect to catalytic and accessory subunits, primer template specificity, sensitivity toward different inhibitors including aphidicolin and associated proofreading activity identified as 3'→ 5' exonuclease (Feher and Mishra 1992).

Characterization of DNA polymerases from aph^R mutants

We have characterized these polymerases from at least three aphidicolin resistant mutants (JK-3-1-2A, BR-5 and BR-5-20) in greater detail. These mutants are characterized by changes in DNA polymerase α , δ , and ϵ as compared to the above properties of the wild type enzymes. The important feature shared by all of these three aph^R mutants is the fact that they possess an altered DNA pol δ and DNA pol ϵ in addition to DNA pol α . It seems that alteration in DNA pol δ is essential for acquisition of resistance to aphidicolin. In addition, all three mutants have changes in DNA pol α but these changes are different. In the aph^R mutant J-3-1-2A DNA pol α is resistance to aphidicolin in *in vitro* only on polydA-oligodT used as template but not on activated calf thymus DNA as template. In addition, this mutant J-3-1-2A possess altered 3'→ 5' exo nuclease activity of DNA pol δ and ϵ , whereas the aph^R mutants BR-5 and BR-5-20 possess DNA pol α which has become resistant to aphidicolin in *in vitro* but has no change in the exo nuclease activity of DNA pol δ . However, the proofreading activities of their DNA polymerases have not yet been examined. We have also found that the ratio of DNA polymerizing and exoactivity has been drastically changed for the DNA pol δ and DNA pol ϵ in JK-3-1-2A Cells but not in BR-5

or in BR-5-20a mutants. These properties of our *aph*^R are similar to those described for prokaryotic mutator and anti mutator DNA polymerase mutants. thus our *aph*^R mutants represent the first mammalian mutants with the mutator and anti-mutator properties described for the prokaryotic mutants.

In addition, the DNA pol α , from all these three mutants (as well as from AC6a which is resistant to ara-c and ara-A) showed decrease in their processivity as determined by their increased activity in truncated assay for enzyme activity in a reaction mixture which lacked one of the dNTPs. In such truncated experiments for example the one containing dATP, dCTP, dTTP (but missing dGTP) the incorporation of dNTP by the wild type enzyme in presence of aphidicolin was only about 40% as compared to control (i.e. absence of aphidicolin) whereas the level of dNTP incorporation by the mutant enzyme was consistently higher up to 135% in the presence of aphidicolin as compared to control (in absence of aphidicolin.)

Mutator and antimutator phenotype of the *arac*^R and *Aph*^R mutants:

Furthermore, among the mutants isolated and characterized in our laboratory there are two groups of them based on their mutability phenotype; one group characterized by the mutator phenotype was represented by the mutants designated as AC6a. The second group characterized by the lack of mutability is represented by mutant designated on JK3-1-2A and BR-5. Thus AC6a showed 50-100 fold increase in mutation of HPRT Locus as compared to the wild type CHOK-1 cells in which the mutation frequency at the HPRT Locus is $1/10^7$ cells. In contrast, the mutants JK-3-1-2A and BR-5 showed 0.1-0.5X decrease in mutability frequency at the HPRT as compared to the wild type.

Development of new methodology to analyze the role of different DNA polymerases in DNA replication: Visualization of DNA Replication intermediates by autoradiography

In order to examine the effect of aphidicolin on DNA synthesis, Lonn and Lonn analyzed the ³H labelled human DNA (synthesized in the absence or presence of aphidicolin rendered into single stranded DNA by treatment with mild (0.03M) NaOH followed by size fractionation upon gel electrophoresis; each gel was sliced and slices were then examined for radioactivity. Following this strategy Lonn and Lonn (1983) showed that control human cells (i.e. Synthesizing DNA in absence of aphidicolin) made okazaki fragments, and the 10Kb DNA intermediates whereas in aphidicolin treated cells the replication fork is topped and there is no formation of replication intermediates. They also showed that 10Kb DNAs intermediates formed before aphidicolin blockage were however ligated into high mol weight DNA where as already formed okazaki fragments accumulated in the cells. Moreover, in cells released from aphidicolin blockage the 10KbDNA intermediates were formed. Thus these workers showed that okazaki fragments and the 10KbDNA respond differently to aphidicolin.

Since we have *aph*^R cell lines, therefore, we wanted to check this important finding of Lonn and Lonn (1983). However, we decided to visualize the different products of DNA replication by autoradiography after gel electrophoresis; instead of slicing the gel and then

examining each slice for measurement of radioactivity as done by Lonn and Lonn (1983). During the present study, the nature of DNA synthesized during DNA replication was examined after incorporation of ^{32}P labelled dNTP in synchronized and permeabilized mammalian cells. In such experiments DNA from cells exposed to ^{32}P dNTP was denatured, electrophoresed and then examined by autoradiography. Such autoradiograph showed several bands of DNA (as seen in Figure 2) ranging in size representative of Okazaki fragments to the size of leading and lagging DNA strands. It was further seen that the synthesis of leading DNA strand was inhibited in the wild type cells when grown in the presence of aphidicolin whereas the synthesis of this DNA strand was carried out even in the presence of aphidicolin by the *aph^R* mutant cells such as JK-3-1-2A, BR-5, BR-5-20a and others. (See figure 2). From these experiments it became obvious that the synthesis of leading DNA strand in the wild type mammalian cells (CHOK-1, Mouse, Human) is inhibited by the presence of aphidicolin and also by ara-C. The fact that in the *aph^R* mutant the synthesis of this leading DNA strand remains unaffected by the presence of these drugs is consistent with the altered properties of DNA polymerase α and δ in the mutant cells. In the mutant cells the leading DNA strand is synthesized by the DNA pol δ which has altered properties as a result of mutation. It is also possible that the synthesis of leading DNA strand in mutant may also be facilitated by the mutant DNA pol δ in conjunction with mutant DNA pol α .

Development of this system offers great possibilities in analyzing the role of different DNA polymerases and accessory proteins in the DNA replication as well as in evaluating the role of different drugs on a specific aspect of DNA replication and repair.

Robert Singleton, a graduate student working on this project defended his Ph.D. dissertation successfully in Jan 1993 and left the University of South Carolina to join the Genetics division of the Dow Chemical Company as a junior staff scientist.

APHIDICOLIN

ara-C

Fig. 1: **Structure of DNA replication inhibitor**

Fig - 2

Effect of aphidicolin on the synthesis of a particular DNA band of 24 Kb in the wild type and *aph^R* mutant cells. (Lane 3-8).