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**CHARACTERIZATION OF THE MAMMALIAN
DNA POLYMERASE GENE AND PROTEIN**

Annual Progress Report

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Progress Report

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

by

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

ABSTRACT

The long term goal of our research has been to understand the nature of the key enzymes involved in eukaryotic DNA transactions. We have purified and characterized at least three DNA polymerases from Chinese hamster ovary (CHO) cell line K1 in order to evaluate the roles of different polymerases in eukaryotic DNA replication. Pol α was the most abundant among different polymerase activities and it was neutralized by a monoclonal antibody raised against human pol α . Pol ϵ was separated from pol α and pol δ activities using DEAE Sephacell, phosphocellulose and hydroxylapatite columns. The enzyme proved to be sensitive to aphidicolin and carbonyldiphosphonate and was not stimulated by PCNA. Pol δ was the least abundant among the three enzymes. It was sensitive to aphidicolin and carbonyldiphosphonate and was stimulated by PCNA. It had a preference for template/primer poly (dA-dT).

Based on DNA sequence data of different eukaryotic polymerases PCR primers complementary to two neighboring conserved regions were synthesized. In PCR experiments - using CHO DNA as template - several PCR products were obtained which are assumed to be specific for the CHO polymerases.

We have also analyzed a large number of aphidicolin resistant mutants of CHO to identify mutants with altered DNA polymerases. Some of these mutants possessed different specific activity of the enzymes and possessed drug-resistance phenotype in *in vitro* assay. A particular aph mutant characterized in detail showed altered properties of the DNA polymerase δ and ϵ . These mutants also show restriction fragment length polymorphism (RFLP) for DNA polymerase gene. Also, a pulse chase analyses of DNA synthesis in the wildtype and aph mutant cell lines using aphidicolin and antibody against DNA pol δ showed the involvement of DNA pol α and DNA pol ϵ in the synthesis of the leading and lagging strands of DNA's during duplication in CHO cells.

Future studies will include the characterization that DNA polymerases particularly DNA polymerases δ and ϵ and cloning of these genes and their genetics. (DOE Contract #DE-FG09-90ER61040)

Introduction

We would like to use CHO cells as a biochemical genetic system in which it is possible to study the effects of drug resistance (for example aphidicolin resistance) mutations on the structure and function of different DNA polymerases. This way we would like to gain insight into the role of different polymerases in the replication process.

Pol α , δ and ϵ are the members of the α family of polymerases, whose catalytic subunits share six functional sequence motifs. Each shows enhanced activity in proliferating cells and each is present in mammalian cells. Pol β was assigned a DNA repair function, pol γ replicates mitochondrial DNA.

Since primase activity is associated uniquely with pol α , this enzyme is assumed to synthesize the primers for both leading and lagging strands. There is a certain controversy regarding function assignments of pol δ and pol ϵ . In viral systems pol δ appears to be required for leading strand extension. The synthesis of lagging strand Okazaki fragments is most probably not completed by the low processivity pol α . It may proceed following a switch to pol δ or pol ϵ . For yeast Morrison and Sugino suggest that it is pol ϵ that extends the leading strand while pol δ extends the lagging strand. The final steps in replication are removal of primer RNA and probably some primer DNA and then replacement with DNA. These steps are carried out by pol β and DNase V which it complexes. They carry out nick-translational repair in the same way as *E. coli* pol I.

Biochemical Characterizations of the Wild type CHO DNA polymerase of δ family (Feher and Mishra)

As a first step towards our goal we purified DNA polymerases from the wild type CHO cells. The cells were collected from roller bottles after 3-5 days of growth and were stored until use at -85 degrees. Cells harvested from 30 to 50 roller bottles were used in one experiment. The cells were disrupted by sonication, then a crude extract was prepared with ultracentrifugation which was dialyzed. The solutions contained PMSF, Na-bisulfate and pepstatin and EDTA as protease inhibitors throughout the purification. The purification steps are: ammoniumsulfate fractionation, DEAE-Sephacell, phosphocellulose and hydroxylapatite chromatographies.

On a DEAE-Sephacell column all three polymerases show up if we use two different template/primers to assay the fractions. There is one broad large peak with poly(dA)/oligo(dT) which is pol α and there are two peaks with poly(dA-dT). The first one is pol δ , the second one is pol ϵ . On the next two columns pol ϵ elutes at lower salt than pol α . After phosphocellulose the peak containing pol ϵ gives three major bands on the gel: Larger than 250 kD, 114 kD and 40 kD. The largest molecule is assumed to be the full size catalytic subunit, the 114 kD band is the pol ϵ^* enzyme. After hydroxylapatite only the pol ϵ^* band remained

detectable on the gel. So the 40 kD polypeptide does not seem to be required for activity, which is consistent with the data published on the human enzyme.

The purification of pol α involved essentially the same steps as described for pol ϵ . The hydroxylapatite-purified pol α gives five major bands on an SDS-polyacrilamide gel. The three upper bands of this five are 180, 135 and 120 kD, respectively. The 120 kD subunit is the catalytically active one. The two lower bands may correspond to the 58 and 48 kD subunits described by Linn in HeLa cells. Since most of the described pol α 's contain four subunits, one of the bands possibly the 141 kD, could be a proteolytic product. We attempted to purify pol α further on an antibody column. The obtained product was pure, but most of the activity was lost in this step. We estimate the overall degree of purification to be at least 500-fold for pol α , and at least 2-300-fold for pol ϵ . These values are affected by the loss of activity during purification and storage. We also worked out an alternative purification scheme using Q-Sepharose and S-Sepharose. On Q-Sepharose pol α and pol ϵ form two very close peaks, but they are well separated from pol δ . On S-Sepharose pol α and pol ϵ are sufficiently well separable.

The preparation of pol δ proved to be more difficult than the other two polymerases. It is the least abundant, easily inactivates and needs a cofactor-PCNA - for significant activity. Using the scheme on the left side we achieved at least 100-fold purification, but could not get a gel picture of this polymerase because we had too little amount of it. Q-Sepharose provides a clear cut separation from pol α and pol ϵ but further steps are required to obtain a more pure product. After examining the partially purified product with SDS-gel electrophoresis, more than 10 bands were visible, but among the major bands there was a 129-130 kD and a 51-52 kD protein which very much resemble with the subunits of pol δ .

The prepared polymerases were tested for sensitivity to different polymerase-specific inhibitors. All three were sensitive to aphidicolin, pol δ was very sensitive to carbonyldiphosphonate (COMDP) and was stimulated by PCNA. Pol α was not stimulated by PCNA and was inhibited by an anti-pol α monoclonal antibody. Pol ϵ proved to be moderately sensitive to COMDP, it was inhibited by PCNA and DMSO and was not affected by the anti-pol α antibody.

The prepared polymerases were also tested for their primer/template preference. Pol α showed preference to activated (nicked) calf thymus DNA template, but it was also very active on poly(dA)/oligo(dT) single primed template. Pol ϵ was most active on poly(dA)/oligo(dT) and on alternating poly(dA-dT). Poly(dA-dT) was the best template/primer for pol δ . The primer/template preference and the inhibitor sensitivity data are in most cases consistent with those of the published polymerases and served as a basis for their identification. Other aspects of identification were: the behavior of proteins on different columns and their subunit composition.

PCR- Analysis (Feher and Mishra)

The members of the polymerase α family have six regions which show significant sequence similarity. These six regions were conserved throughout the evolution from bacteria to humans. Regions I, II and III appear to be the basic core regions of the pol α -like polymerase family. We selected region I and II and designed two PCR primers homologous to them. We were able to amplify polymerase specific DNA fragments from CHO and HeLa cells. We detected more amplified fragments with HeLa, since the matching was more perfect. We know that the 412 bp long PCR product is a fragment of the pol α gene. The amplified fragments can be used as hybridization probes for library screening and cloning polymerase genes from the pol α family.

Characterization of Mutant DNA Polymerases (Feher, Moore and Mishra)

Since one of our goals was the characterization of aphidicolin resistant mutants, we attempted a partial purification of polymerases from an aphidicolin resistant mutant BR5-20a. After an ammonium sulfate fractionation we used DEAE-Sephacell chromatography. The elution profile was practically the same as for the wild type. Using fraction 10 for pol δ , fraction 15 for pol α and fraction 20 for pol ϵ we determined specific activity and compared it with specific activity of corresponding fractions from the wild type. The specific activity of the mutant pol α is about 2/3 of that of the wild type. Pol ϵ and pol δ isolated for the mutant has a specific activity of more than 2 times higher than those from the wild type.

We determined the aphidicolin resistance of the same fractions. The columns represent the enzymatic activity in the presence of 100 μ g/ml aphidicolin. The residual activity of pol α in the presence of aphidicolin is 1.4-1.7 times higher in the aph^r mutant. For pol ϵ it is 2.2-3 times higher in the aph^r mutant. No significant difference was observed in the case of pol δ .

These results may indicate that the mutation or mutations causing the aphidicolin resistant phenotype may have occurred in pol ϵ or pol α genes or in both. This question could be answered by RFLP studies and by cloning the wild type and the mutant gene. We are using cloned DNA polymerase gene or PCR generated probe for such study. This is our future goal. We also want to purify pol δ to near homogeneity and to make comparisons between aphidicolin resistance mutant and wild type DNA polymerases. We are also planning to characterize other aph^r mutant to reveal the nature of changes in the different DNA polymerases among the mutants. The genetic analysis of different DNA polymerase mutants via somatic cell hybridization.

Nature of DNA Synthesis and Okazaki Fragments in aph' Mutants
(Singleton and Mishra)

Study of the effect of aphidicolin and DNA pol δ antibody on the formation and joining of okazaki fragments during DNA replication in the CHO K-1 (wild type) and BR-5 (aph' mutant) cells indicated that aphidicolin affects the synthesis of the lagging strand of DNA and thus acts on DNA pol ϵ where as antibody affected the synthesis of leading strand mediated by DNA pol α . These results are consistent with the properties of α -family of DNA polymerases characterized in detail in another aph' mutant BR-5-20a as described by Feher and Mishra (see above).

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