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13. ABSTRACT (Maximum 200) Defects in DNA polymerase delta and its accessory proteins could contribute to the molecular etiology of breast tumors. In addition to a central role in DNA replication, recent work has shown that polymerase delta and its accessory proteins are also involved in DNA repair and that there are linkages between polymerase delta and cell cycle regulation via protein-protein interaction of polymerase processivity factor PCNA with p21, a cyclin dependent kinase inhibitor. Besides using immunoaffinity chromatography techniques to show that the deregulation of the cell cycle machinery in breast cancer cells, our preliminary data also show that the regulation of polymerase delta gene expression is different between normal breast cells and abnormal breast cancer cells. Results from the proposed studies could provide an understanding of the linkage between the regulation of polymerase delta and breast carcinogenesis.				
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FOREWORD

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Marietta Lee 9/28/97
PI - Signature Date

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5. Introduction

DNA polymerase δ (pol δ) was discovered as a mammalian polymerase which possesses an intrinsic 3' to 5' exonuclease activity (Byrnes et al., 1976). This enzyme is critical for the maintenance of the fidelity of DNA replication. Studies using the SV40 system have established that pol δ plays a central role in chromosomal replication, and is part of a multiprotein complex involving pol δ and its accessory proteins (Waga and Stillman 1994). Using specific inhibitory monoclonal antibodies to pol δ and PCNA. Zeng et al., 1994 showed that both pol δ and PCNA are involved in DNA repair. In addition to nucleotide excision repair, recent reports also showed that PCNA, and pol δ are also involved in base excision repair and mismatch repair (Frosina et al., 1996, Longley et al., 1997). PCNA is found in quaternary complexes with cyclins (A, B and D), cyclin dependent kinases (CDK's 2, 4, 5) and p21. The interaction of PCNA with the cyclin dependent kinase inhibitor p21 shows a linkage between DNA replication machinery and the cell cycle machinery and provides a molecular basis for how DNA replication is halted until repair is completed when cells are subjected to DNA damage (Waga et al., 1994).

We have proposed in our grant application to examine the role of replication proteins in breast cancer cell lines and tissues in two major areas :

- 1) to seek for functional changes in DNA replication proteins and for changes in their protein-protein interactions and
- 2) by directly examining the genes for pol δ and PCNA to seek for mutational changes that could provide the basis for a molecular contribution to the cancer state.

6. Body

During the past year we have accomplished the following:

A) Studies of the multi-protein complexes of polymerase δ from MCF7 and MCF10 cell lines.

We have made use of our immunoaffinity column to study the pol δ complexes of MCF7 (breast cancer cell line) and MCF 10 A (a phenotypically normal, nontransformed cell line. The cells are a spontaneously immortalized line from a culture of human breast epithelial cells from a reduction mammoplasty). We have confirmed our initial observations (Table II of our grant application) that immunoblots of components of pol δ complexes isolated by immunoaffinity chromatography and HPLC gel permeation chromatography from MCF 7 and MCF10A cells are different. These complexes involve polymerase alpha, delta, epsilon, replication protein A (the human single stranded protein), replication factor C (RFC) and the cell cycle proteins (cyclins, cyclin dependent protein kinase). The latter are of importance for it is well known that the deregulation of the cell cycle machinery is a major facet of the cancer process.

We have also developed an affinity column using PCNA immobilized on Sepharose beads. This was done using recombinant PCNA which we have overexpressed in E. coli with yields of up to 10 mg of protein per liter of culture (Zhang et al., 1995). It was felt that the use of PCNA-Sepharose would be an important additional tool for our studies, as it is known to bind to cell cycle proteins, the cyclin/cdk's (Xiong et al., 1992, 1993) and also p21. Thus, this accessory protein which is critical for the ability of pol δ to act in a processive manner may be a key player in terms of its ability to interact with pol δ as well as p21 and the cell cycle proteins. We have tested the column using calf thymus extracts and established that cell cycle proteins are bound to this affinity column. This is consistent with published results that PCNA interacts with cell cycle proteins. The work on the calf thymus extracts has been submitted to Nucleic Acids Research (Loor et al., submitted). We have performed preliminary studies using MCF10A extracts and

demonstrated that DNA polymerases delta and epsilon, RFA, RFC and cell cycle proteins are also bound on this affinity column.

We have recruited Dr. Jinyao Mo, who is an expert in studying the fidelity of DNA replication. He will initiate in the next year all the fidelity studies involving MCF 7 and MCF10A and breast cancer tissue samples.

B) Expression of Pol δ Small Subunit in MCF7 and MCF10A cells after MMS (methyl methanesulfonate) treatment.

While most of the emphasis of the study of pol δ has been focused on the catalytic subunit, it possesses a small 50 kDa subunit. In yeast, the gene for the small subunit has been shown to be critical for cell survival, and mutations of the p50 gene lead to increased sensitivity to DNA damage. Thus, we felt it necessary to extend our investigation of pol δ to its p50 subunit. During this grant period we have isolated the cDNA for the small subunit of DNA polymerase δ and have defined its gene structure and cloned the promoter region of the small subunit (Perez et al., in preparation). Since we have obtained data on the effects of DNA damage on the expression of the pol δ catalytic subunit we have extended our studies to examination of the expression of the small subunit (pol δ p50). Northern blot analyses of the effects of MMS treatment on the expression of MCF7 and MCF10A cells have been performed. Preliminary data show that the message levels of both p125 and p50 are increased after 100 μ g/ml of MMS treatment of MCF10 A cells. There is no apparent change in message levels of p125 and p50 in the MCF7 cells (Fig 1, 2). We plan to confirm these findings by additional experiments, as they suggest that the control of the gene expression of these two pol δ subunits is altered in the cancer state.

C) Mutational analysis of the DNA polymerase δ promoter using a luciferase reporter gene and investigation of their response to DNA damage.

There is currently little experimental evidence on the important promoter elements of the pol δ gene. We are in the process of analyzing this promoter, with the goal of identifying the elements which are involved with response to DNA damaging agents, and also to artificially produce mutations which will enhance the response to DNA damaging agents. It is also planned to couple the study of these constructs with an examination of their behavior in normal and breast cancer cell lines. Four point mutants of the pol δ promoter in the luciferase reporter expression vector pGL2-basic (Promega Corp) were constructed (Fig 3). These were: (1) Introduction of a new CREB site by a point mutation in the -191/-182 region (GCGAGGCCAC to GTGACGTCAC). (2) Removal of an Sp1 binding site in the promoter region -162/-157 (GGCGG to GATGG). (3) Insertion of an Ap1 sequence (TGAGTCAG) at a Pst I site -149/-144. (4) Insertion of an Ap1 site by a one base change from GGAGTCAG to TGAGTCAG in the -53/-46 region. This creates a new regulatory element (Ap1) that follows the E2F site and overlaps with the ras-activated enhancer site. The construction of these mutants is shown diagrammatically in Fig. 3.

Six deletion mutants of the pol δ promoter which cover the promoter regions -110/-5, -214/-5, -324/-5, -435/-5, -534/-5 and -639/-5 respectively were constructed (Fig. 4). The transient expression analysis of pol δ and deletion constructs were determined. The promoter activity expressed from each deletion construct relative to the 1.8 kb promoter activity is shown in Fig 5 (right panel).

We tested the reporter activities of these mutants in MCF 7 breast cancer cells, as well as their response to different DNA damaging agents. The different agents used were UVB (50 J/m²), H₂O₂/FeSO₄ (200 μ M/200 μ M), cisplatin (45 μ g/ml), N-methyl-N'-nitro'-N-nitrosoguanidine (MNNG, 10 μ M) methyl methanesulfonate (MMS, 30 μ g/ml). We found that MNNG or MMS were the most effective (Fig. 6) and 10 μ M was the optimum concentration for

MNNG (Fig. 7). Therefore MNNG was the reagent of choice for further studies. The reporter gene activity was determined by luciferase assay; the pSV β -gal reporter gene was used as a control and its activity was determined by β gal assay. Our preliminary results show that deletion mutants had a much higher response to MNNG than the full length 1.8 kb promoter (Fig. 8). These results suggest that deletions in the pol δ promoter can result in an enhanced response to DNA damaging agents. These findings would be consistent with a situation in which modifications of the promoter lead to up regulation of pol δ expression; this may be a required response for enhanced proliferation rates.

D) Electrophoretic mobility shift assays (EMSAs) showing DNA-nuclear protein interaction in breast cells.

We have initiated studies using electrophoretic mobility shift assays (EMSAs) to investigate the transcriptional factor elements in normal and breast cancer cells that are associated with the pol δ promoter. The promoter deletion mutant fragments were labeled with ^{32}P and used as probes for the EMSA analysis. The results show that the patterns of the DNA-protein complexes between MCF-10A and MCF-7 are different. Fig. 9 shows an example in which the DNA-protein complex bands in MCF-7 cells are obviously more pronounced than those in MCF-10A cells when the region of -110 to -5 was used as a probe. These preliminary studies indicate that different DNA-nuclear protein interactions involving the pol δ promoter may be present in normal and abnormal breast cells. It is possible that the changes in patterns of DNA-nuclear protein interactions reflect a functional difference between normal breast cells, MCF-10A, and abnormal cancer cells, MCF 7.

E) Immunolocalization of polymerase δ in breast cancer cell lines.

We have initiated studies of the localization of the p125 and p50 subunits of pol δ by immunofluorescence using the confocal microscopy core facility at the Univ. Miami run by Dr. L. Bourguignon. My graduate student, Mr Bao Qing Li has performed the initial experiments with MCF 10 A cells using pol δ p125 subunit antibody (78F5) and p50 small subunit antibody (13D5). He found that both proteins are localized at the perinuclear region (Fig. 10). These very preliminary experiments are quite surprising as we have immunolocalized both p125 and p50 subunits of pol δ to the nucleus (Zhang et al., in preparation). The perinuclear localization and the failure to find significant localization in the nucleus of MCF 10 A cells could be a matter of technique, i.e differences in permeabilizing the cells. We are now trying to systematically examine the conditions for preparation of the cells as well the effects of growth conditions. After the protocol is established, we will repeat the experiments using MCF 10 A and MCF 7 cells in different stages of the cell cycle. It may be that nuclear localization occurs in a specific stage of the cell cycle. Nuclear localization before and after treatment with damaging agents will also be studied.

7. Conclusions

We initiated studies of pol δ in a normal and a breast cancer cell line at several levels: isolation and characterization of the promoter of the gene of pol δ ; effects of DNA damaging agents on gene expression of the two subunits of the core enzyme; characterization of the replication complexes isolated by immunoaffinity chromatography; EMSA analysis using probes based on the pol δ promoter. These studies point to significant differences between the normal and breast cancer cell lines. We have shown that there are significant differences in the responses of MCF10A (phenotypically normal breast cell line) and MCF7 (malignant breast tumor cell line) to damaging agents. Our work provides new evidence that pol δ and its accessory proteins are a likely loci that could underlie, or contribute to the molecular etiology of breast cancer. Further studies are clearly warranted.

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- Zhang, P., Zeng, X-R., Zhang, S.J., Li, B.C. and Lee, M.Y.W.T. Overexpression and Characterization of the Human DNA Polymerase delta Catalytic Subunit p125, its deletion mutants and with its small subunit p50. (in preparation).
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9. Appendices.

See Figures 1-10

Publications.

- Loor, G., Zhang, S.J., Zhang, P., Toomey, N.L., and Lee, M.Y.W.T. Identification of DNA replication and Cell Cycle Proteins that Interact with PCNA (submitted)
- Perez, A., Hsu H., and Lee, M. (1997) Promoter Analysis and Gene Structure of the Polymerase delta small subunit Gene (POLD 2) Promoter. *Eukaryotic DNA Replication*. Cold Spring Harbor Laboratory p. 184.
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Zhang, P., Sun, Y., Hsu, H., Zhang, L., Zhang, Y., and Lee, M.Y.W.T. (1997) The Interdomain Connector Loop of Human PCNA is Involved in Direct Interaction with Human Polymerase Delta. Cold Spring Harbor Laboratory P. 273.

List of Personnel receiving pay from this grant:

- 1) Marietta Lee Ph.D., (25%)
- 2) Peng Zhang, Ph.D., Post doctoral research associate (100 %)
- 3) Lan Toomey, Technician (50%)
- 4) Jinyao Mo, Ph.D., Post doctoral research associate (100%) started in July.
- 5) Li Liu , graduate assistant (50%)

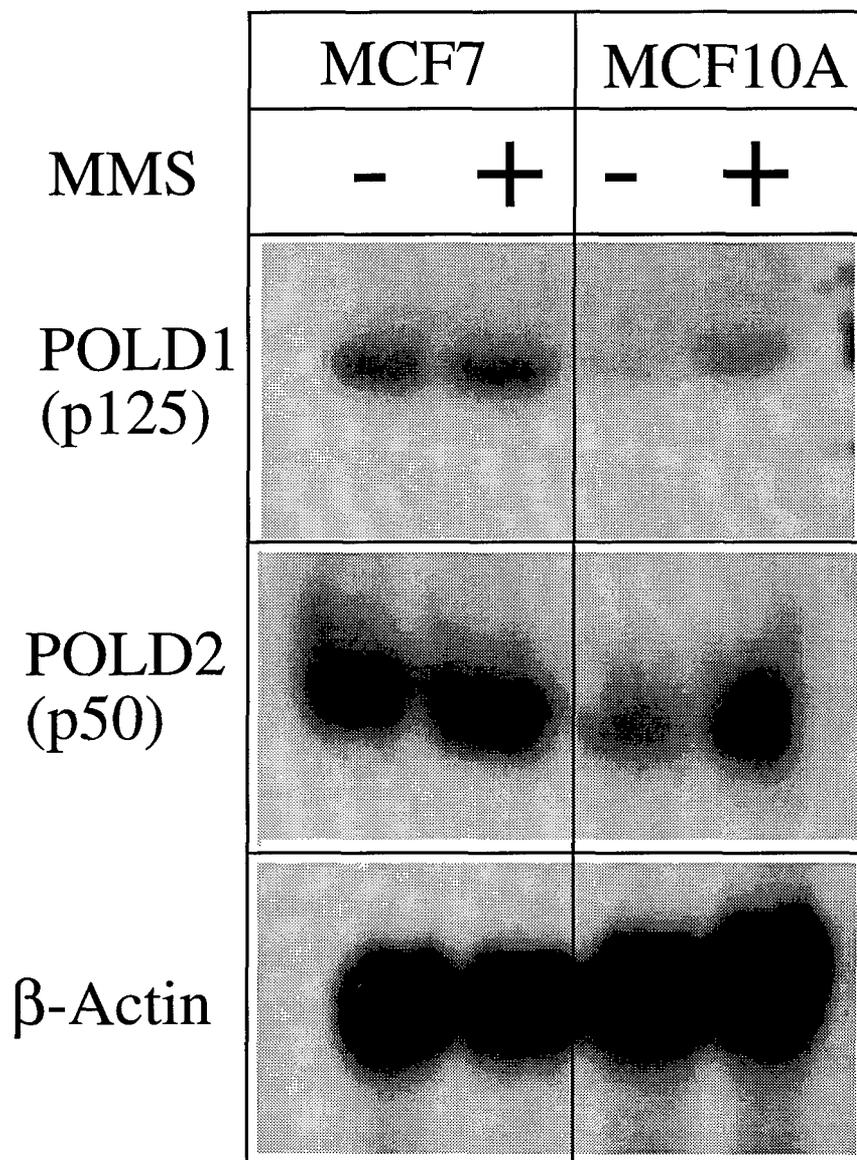


Fig. 1. Northern Blot Analysis of the Expression of the mRNAs for the p125 and p50 subunits of pol δ .

MCF7 and MCF10A cells were grown to 90% confluence and treated with 100 $\mu\text{g}/\text{ml}$ MMS. After two hours the cells were harvested. RNA was then extracted from the control (-) and treated (+) cells and Northern blotted using antisense RNA probes for the p125 and p50 subunits of pol δ , and for actin.

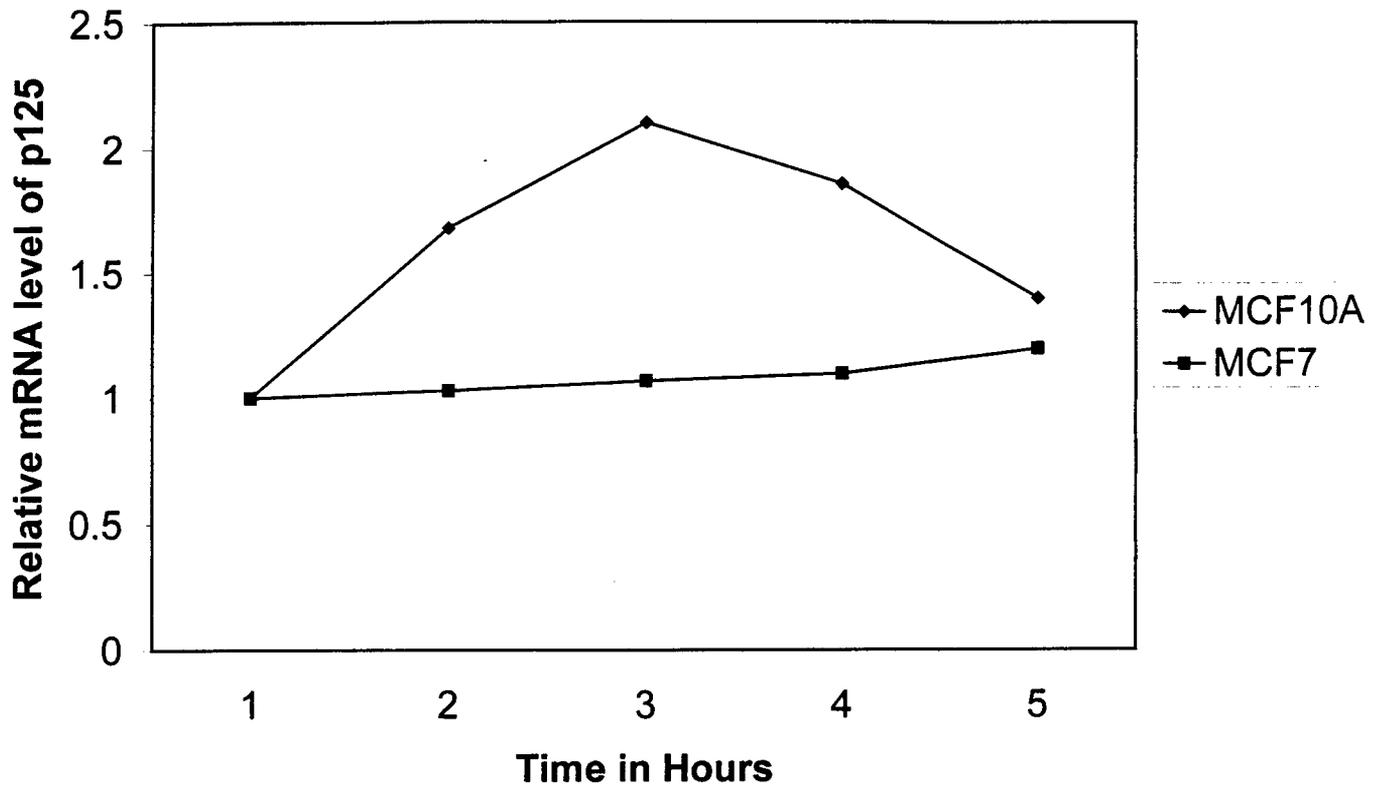


Fig. 2. Effect of MMS treatment on the expression of the message for p125 subunit as a function of time.

MCF10A and MCF7 cells were treated with MMS (100 μ g/ml); cells were harvested at 1,2,3,4 and 5 hrs. Northern blot analyses were performed as in Fig. 1., and levels of message were estimated by phosphorimaging.

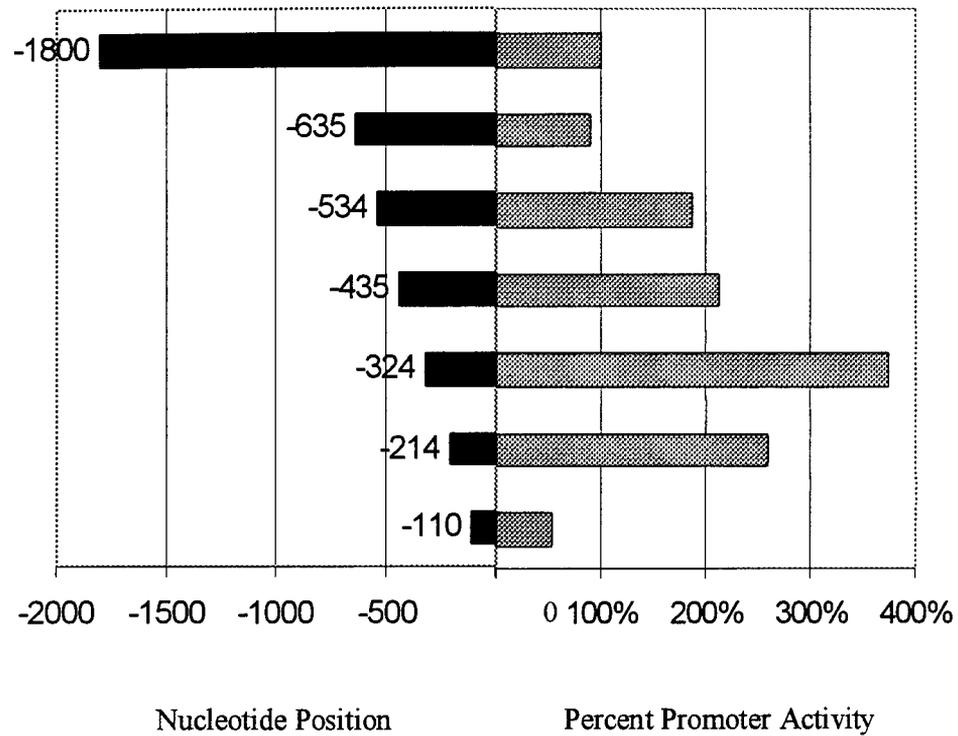


Fig. 5. Transient expression analysis of the POLD 1 promoter and its deletion constructs. The POLD 1 promoter and its deletion constructs were inserted into luciferase reporter gene plasmids and transfected into actively growing MCF7 cells. The promoter activity is expressed as a percentage of that for the full length 1.8 kb promoter. For convenience a map of the constructs is shown on the left.

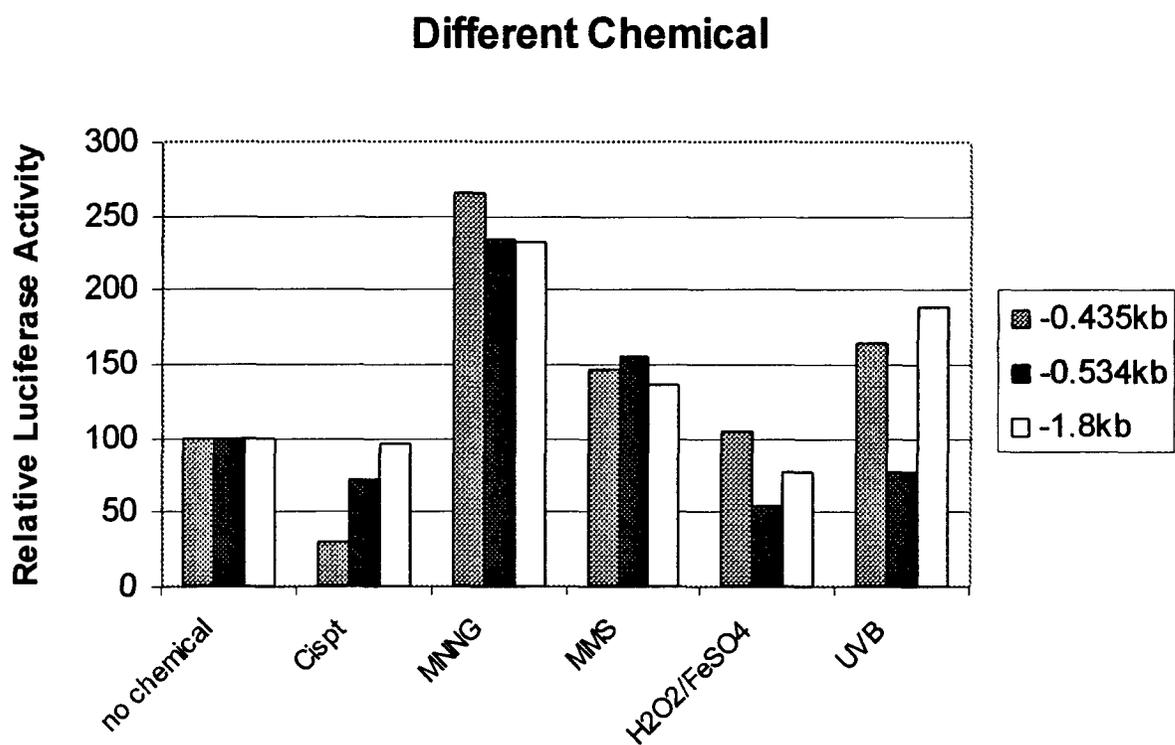


Fig. 6. Effects of treatment of MCF7 cells with various DNA damaging agents on the activities of the POLD 1 promoter.

The -435, -534 and full length (1.8 kb) promoter plasmids were transfected into MCF7 cells treated with the agents as shown. The pSV β -gal reporter gene was co-transfected. Promoter activity was determined by luciferase assay, normalized to the β -gal assays.

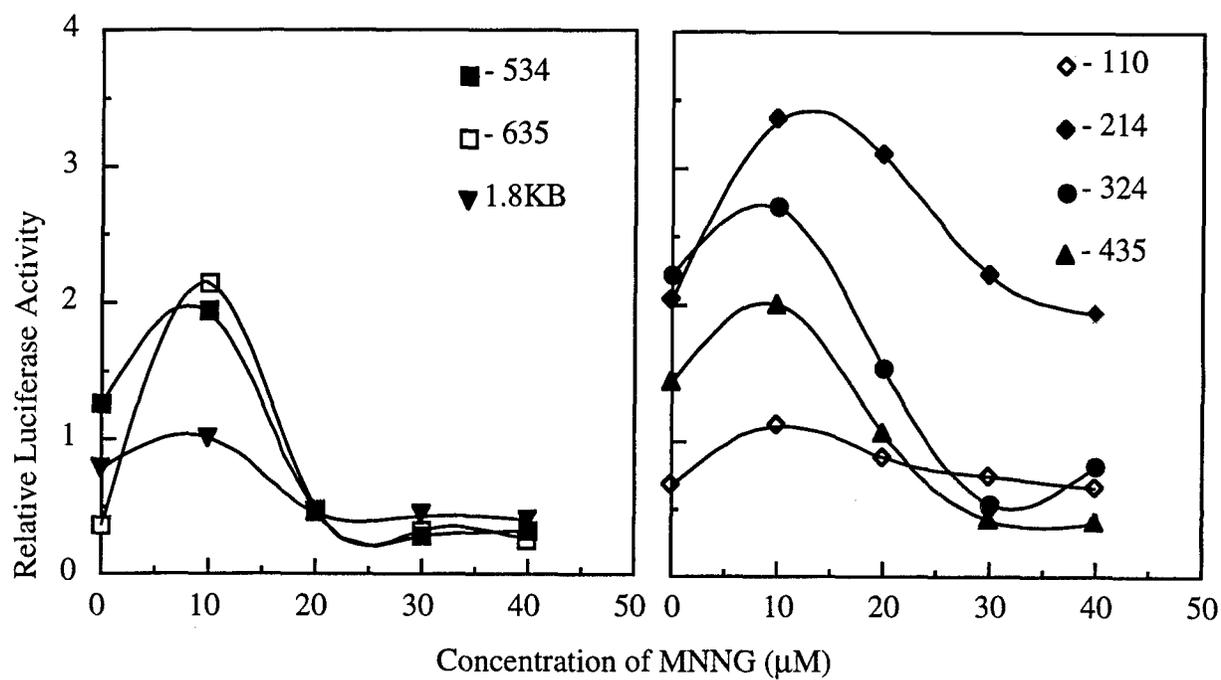


Fig. 7. Effects of treatment of MCF7 cells with different concentrations of MNNG on the activity of the POLD1 promoter.

The full length (1.8 kb) and deletion promoter plasmids were transfected into MCF7 cells treated with different concentrations of MNNG. The pSV β -gal reporter gene was co-transfected. Promoter activity was determined by luciferase assay, normalized to the β -gal assays.

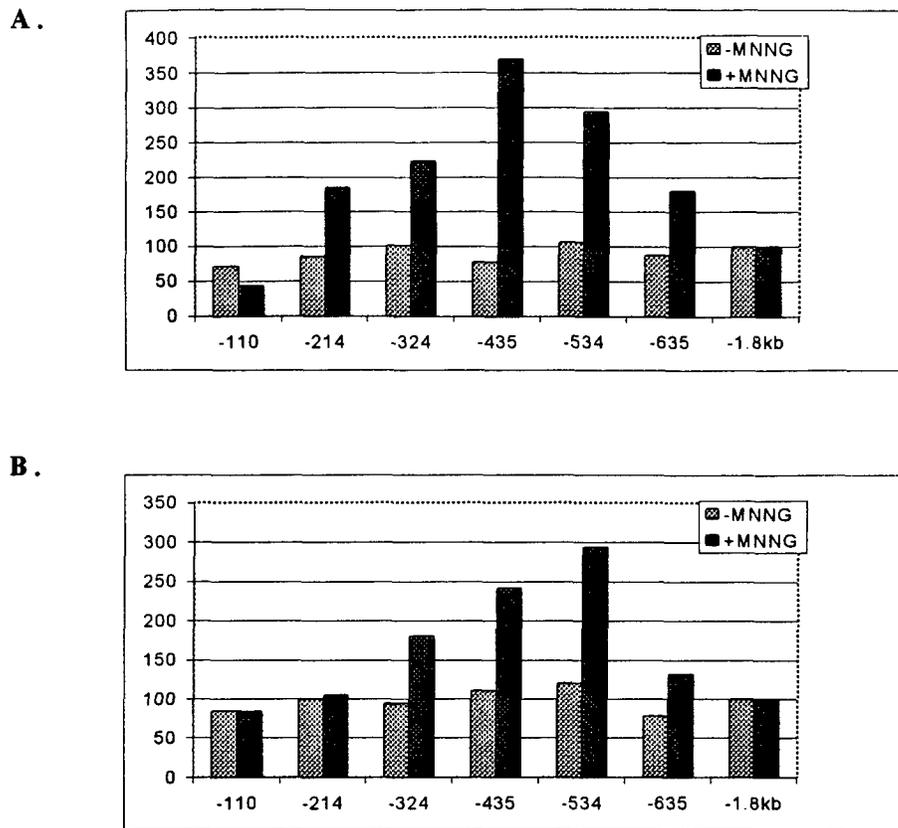


Fig. 8. Activity of the POLD1 promoter in MCF7 cells at 4 and 8 hours of MNNG treatment.

The full length (1.8 kb) and deletion promoter plasmids were transfected into MCF7 cells treated with 10 μ M MNNG. The pSV β -gal reporter gene was co-transfected. Promoter activity was determined by luciferase assay, normalized to the β -gal assays. Panel A and Panel B refer to experiments where the cells were treated for 4 and 8 hrs, respectively.

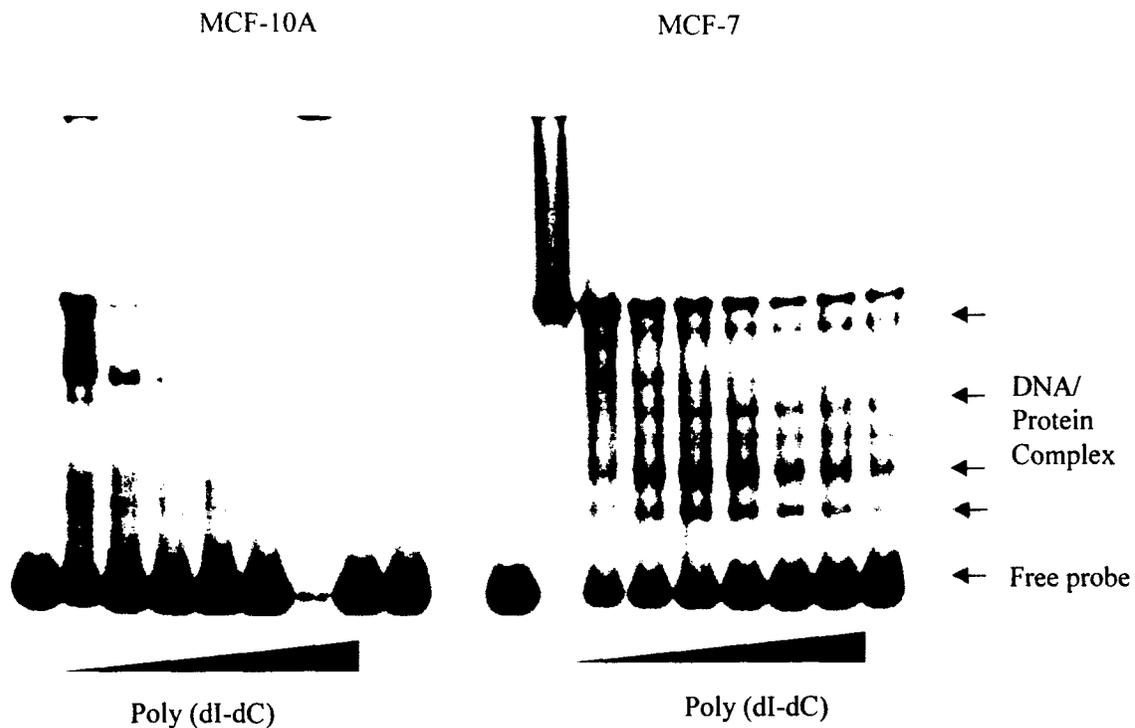


Fig. 9. Electrophoretic mobility shift assays for the -5 to -110 sequence of the POLD1 promoter.

Nuclear extracts of MCF10A and MCF7 cells were prepared. The extracts (ca. 15 μ g protein) were incubated with end labeled oligonucleotide corresponding to the -5 to -100 region of the POLD1 promoter for 30 min at room temperature. The extracts were then subjected to polyacrylamide gel electrophoresis in the presence of increasing concentrations of poly(dI-dC). The amounts of poly(dI-dC) 100, 200, 400, 800, 1600, 3200 and 6400 ng respectively.

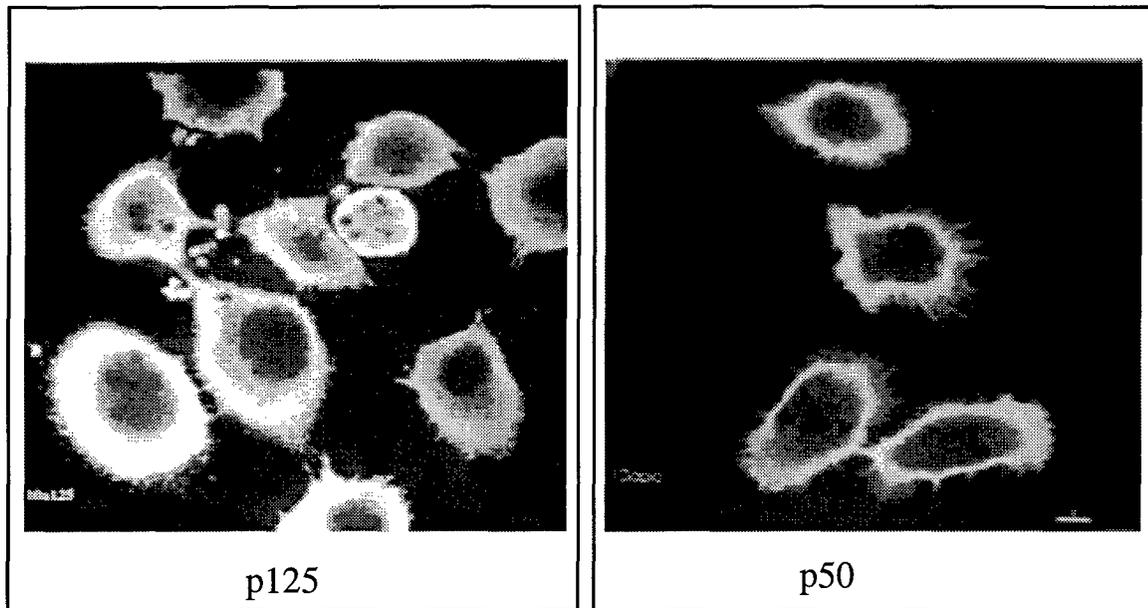


Fig. 10 . Preliminary experiments on the immunolocalization of p125 and p50 in MCF10A cells.

MCF10A cells were washed in PBS, fixed with 0.5 % glutaraldehyde for 30 min at room temperature and quenched with 0.1 M glycine. The cells were then permeabilized with 90% ethanol for 30 min on ice. After an additional wash with 0.1 M glycine in PBS, the cells were incubated for 30 min with 1:100 diluted 78F5 primary monoclonal antibody against pol δ catalytic subunit (Jiang et al., 1995) or against p50 pol δ small subunit antibody (Sun et al., 1997). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma Co.) diluted 1:100 was applied for 30 min. Cell on glass coverslips were examined on a MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Inc.) and photomicrographs were taken on Kodak Tmax 100 film.



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PHYLLIS M. RINEHART
Deputy Chief of Staff for
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