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PRINCIPAL INVESTIGATOR: Emily Anderson, Ph.D.
Dr. Deborah Wuttke

CONTRACTING ORGANIZATION: University of Colorado
Boulder, Colorado 80309

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13. ABSTRACT ***(Maximum 200 Words)***

Telomeres are the nucleoprotein structures that protect the ends of eukaryotic chromosomes. Telomere length regulation is controlled by the enzyme telomerase and a suite of telomere binding proteins. Anomalous telomeric replication and regulation are implicated in most forms of cancer, while telomeric shortening contributes to cellular aging. Cdc13p is an essential protein from *S. cerevisiae* that binds to the single-stranded ends of telomeres with high specificity and affinity. Genetically, Cdc13p has been shown to protect the end of the chromosome from degradation and to load telomerase. Biochemically, Cdc13p binds yeast single-stranded telomeric DNA (sstelo DNA) *in vitro* with high affinity (K_d=0.3 nM).

We are investigating the structural basis for high affinity binding and sequence specificity of the single-stranded DNA binding domain. We have expressed and purified the ssDNA binding domain in high yield. Its binding affinity and specificity have been examined with libraries of sstelo DNA randomized at each position. *In vitro* photocrosslinking experiments have been performed using 5-iodouracil substituted for thymine bases. Proteolytic digestion of the crosslinked products along with micro peptide sequencing have allowed us to identify sites critical for DNA binding. These experiments complement high resolution NMR studies of the protein/DNA complex in progress in our laboratory.

14. SUBJECT TERMS
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Annual Report for: Understanding Single-Stranded Telomere End Binding by an Essential Protein

Emily M. Anderson
Department of Chemistry and Biochemistry
University of Colorado at Boulder

TABLE OF CONTENTS

INTRODUCTION

Telomeres are the nucleoprotein complexes that protect the ends of linear eukaryotic chromosomes. Telomere replication and length regulation are controlled by the enzyme telomerase and a suite of telomere binding proteins. Anomalous telomeric replication is implicated in most forms of human cancer. Telomere metabolism is thus an active field in basic research for the eventual goal of developing inhibitors or modulators or telomere replication for cancer therapy. Cdc13p is an essential protein from the budding yeast *Saccharomyces cerevisiae* whose role is to protect the end of the chromosome from degradation and to load telomerase. Biochemically, Cdc13p binds to single-stranded yeast telomeric DNA with high affinity and specificity. We are investigating the structural basis for high affinity binding and sequence specificity of the DNA binding domain. One aspect of this research involves solving the high resolution solution structure of the domain complexed to DNA using heteronuclear, multidimensional NMR. Biochemical techniques are also being employed, including mapping regions of the domain in proximity to the DNA by photocrosslinking and investigating sequence specificity using libraries of DNA with varying sequences. The advantage of studying this protein using yeast as a model system is the power of combining structure, biochemistry, and genetics all in one system.

BODY

Significant progress toward accomplishment of the technical goals has been completed to date. Technical objective 1, outlined below, has been completed in full.

Technical Objective 1:

Express and purify DNA binding constructs	2 Months
Conduct binding assays with site-randomized DNA	4 Months
Conduct CD experiments of protein folding and DNA binding	1 Month

An optimized DNA-binding domain construct has been delineated using proteolysis and MALDI mass spectrometry. This construct has been subcloned, expressed and purified in high yield, suitable for high resolution structural characterization. The construct binds DNA with affinity comparable to that reported for the full-length protein as measured by both gel-shift binding assays and nitrocellulose filter-binding assays. Binding assays were conducted with site-randomized single-stranded DNA oligomers to determine bases in the DNA critical for binding affinity and specificity. These experiments are to be followed up by experiments involving chemical modification of the DNA with dimethylsulfate. Circular Dichroism experiments were performed to assess the secondary structural content of the domain, whether there are gross structural changes upon DNA binding, and to assess the thermostability of the domain in isolation. It was found that the domain in isolation forms a compact, stable, globular structure with both α helical and β sheet structure content. No major secondary structural changes occurred upon DNA binding.

Technical objective 2 is well on its way to completion.

Technical objective 2:

Conduct photocrosslinking/identify contacts	3 Months
Design mutants/test *in vitro* and *in vivo*	6 Months

Photocrosslinking experiments with the chromophore iodouracil substituted for thymine have been performed. Upon proteolytic digestion and micro-Edman sequencing, peptides in the domain which crosslink to various substituted DNAs have been identified, along with the sites of crosslinking in the peptide. Although MALDI mass spectrometry was not successful in identifying the crosslinks, we are in the process of confirming their identity with electrospray ionization mass spectrometry, as this technique is less sensitive to peptide identity, sample preparation, etc. Mutations are currently being introduced at the sites of crosslinking to assess the effects of these mutations on DNA binding and crosslinking efficiency. The *in vivo* effect of these mutations will be assessed in the laboratory of our collaborator, Dr. Victoria Lundblad at the Baylor College of Medicine. A manuscript is currently in preparation to be published outlining the results of the photocrosslinking experiments.

Technical objective 3 involves primarily the high resolution NMR solution structure of the domain. Several of the tasks have been completed, and the rest are in progress. Two significant changes to the objective have been made. First, although the domain construct of the protein we have chosen exhibits adequate DNA binding and structural compactness by CD spectropolarimetry as mentioned earlier, we have not yet succeeded at keeping the domain alone in concentrated solution conditions long enough for any of the triple-resonance NMR experiments. However, the protein/DNA complex exhibits excellent solution behavior, and our structural efforts are focused in this direction. Second, the structural studies are now being completed in collaboration with another graduate student in the lab, Rachel Mitton-Fry. Technical objective 3 was stated originally as follows:

Technical Objective 3:

Optimize solution conditions of sample for NMR spectroscopy	1 Month
Protein alone –	
Collect heteronuclear NMR data for resonance assignment	6 Months
Assign resonances in the protein domain	6 Months
Collect heteronuclear NMR data for distance restraints	1 Month
Determine family of structures that satisfy restraints	6-12 Months
Protein/DNA complex –	
Titrate DNA into protein and conduct NMR experiments	6-18 Months

Solution conditions for the sample have been optimized through conducting a buffer screen of the protein/DNA complex. Samples of well over 1 mM concentration can be routinely prepared and NMR experiments can be conducted at 30 °C for several weeks to over a month. As mentioned earlier, work has focused on the protein in complex with DNA. The entire suite of triple resonance heteronuclear NMR experiments has been collected on a $^{15}N/^{13}C$ labeled protein sample with DNA, including HNCA, HNCO, HN(CO)CA, HNCACB, CBCA(CO)NH, etc. Rachel Mitton-Fry has completed the backbone amide assignments in the domain and has completed approximately half of the side-chain resonances. Carbon assignments are in progress as well. NOE experiments to determine distance restraints have been conducted and are in the process of being assigned and interpreted. This fall I will perform experiments to measure 3-bond scalar coupling which will add more restraints to allow for structure calculation. I will also examine the conformation of the unlabeled DNA in the complex using NMR by conducting isotope filtering experiments. Also this fall, a rotation student in the lab will continue efforts at optimizing solution conditions of the protein domain alone and conduct hydrogen exchange experiments by NMR to determine packed hydrophobic residues and surface expose residues.

KEY RESEARCH ACCOMPLISHMENTS

- An optimal construct of the Cdc13p single-stranded DNA binding domain has been expressed and purified in high yield
- DNA binding experiments have been conducted using site-randomized single-stranded telomeric DNA oligomers
- CD spectropolarimetry experiments of the domain have been performed to assess the folded state and DNA binding
- Photocrosslinking experiments have mapped key residues in the protein domain located at the protein/DNA interface
- Solution conditions have been optimized to perform high resolution NMR experiments of the protein/DNA complex
- Heteronuclear NMR experiments have been performed on the complex to assign residues in the protein domain
- Main-chain assignments of the domain have been completed, and approximately half of the side-chain resonances

REPORTABLE OUTCOMES

Manuscripts: The results of the photocrosslinking study (Technical Objective 2) are currently being prepared as a manuscript for publication.

Abstracts: The work in progress has been presented as a poster at several meetings: the 2000 Colorado Protein Stability Conference (Breckenridge, CO), the 14th Symposium of the Protein Society (Student Poster Award - San Diego, CA) and the 42nd Annual Rocky Mountain Conference on Analytical Chemistry (Broomfield, CO).

Presentations: This work has been presented as a talk in two formats: the RNA Club Meeting of the University of Colorado in March, 2000 and the Biotechnology Program Summer Student Seminar in August, 2000.

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Biochemical Investigation of a Sequence-Specific, Single-Stranded DNA Binding Protein at the Telomere

E.M. Anderson[1], R.M. Mitton-Fry[1],T.R. Hughes[2], V. Lundblad[2], D.S. Wuttke[1]

[1]Dept. of Chemistry and Biochemistry, U. of Colorado, Boulder, CO 80309
[2]Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, 77030

Telomeres are the nucleoprotein structures that protect the ends of eukaryotic chromosomes. Telomere length regulation is controlled by the replicative enzyme, telomerase, and a suite of telomere binding proteins. Anomalous telomeric replication and regulation are implicated in many forms of cancer, while telomeric shortening contributes to cellular aging. Cdc13p is an essential protein from *S. cerevisiae* that binds to the single-stranded ends of telomeres with high specificity and affinity. Genetically, Cdc13p has been shown to protect the end of the chromosome from degradation and to load telomerase. Biochemically, Cdc13p binds yeast single-stranded telomeric DNA (sstelo DNA) *in vitro* with high affinity (K_d=0.3 nM).

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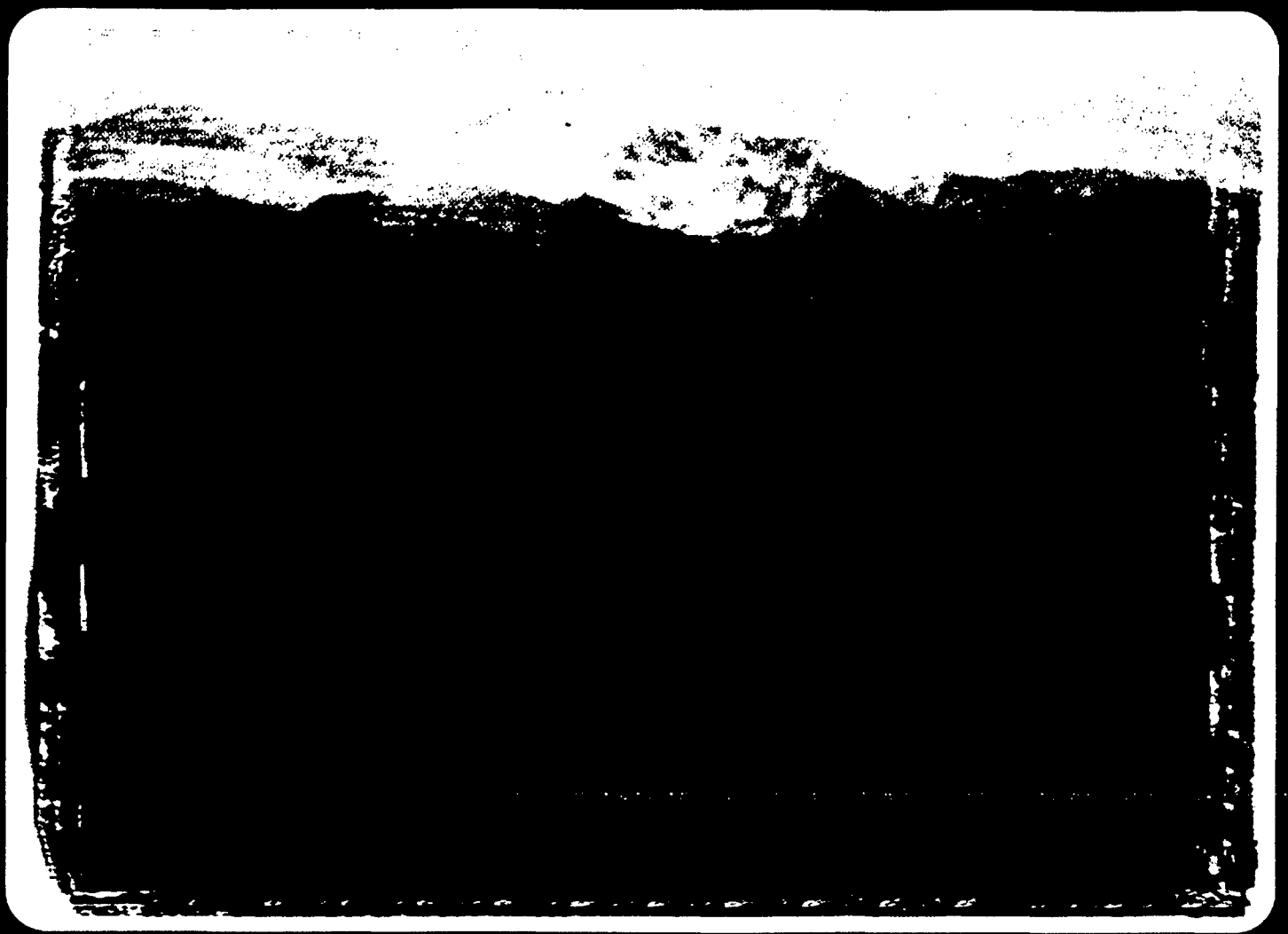

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duction. In this study the aqueous fraction of bio-oil, generated from fast pyrolysis, was catalytically steam reformed at 825 and 875°C, high space velocity (up to 126,000 h-1) and low residence time (26 ms). Using a fixed-bed micro-reactor interfaced with a molecular-beam mass-spectrometer (MBMS). This provided a relatively short test in which changes to the full slate of products could be monitored. A variety of research and commercial nickel-based catalysts were tested. The cobalt-promoted nickel and chromium-promoted nickel supported on MgO-La_2O_3-alpha-alumina catalysts showed the best results of the research catalysts. At the reaction conditions used progressive catalyst deactivation was observed leading to a decrease in the yields of hydrogen and carbon dioxide and an increase in carbon monoxide. The loss of activity also resulted in the formation of higher amounts of methane, benzene and other aromatic compounds. Commercial steam-reforming catalysts proved to be more efficient for hydrogen production from bio-oil than most of the research catalysts mainly due to the higher water-gas shift activity. Supported by the U.S. DOE Hydrogen Program and the Secretaría de Estado de Universidades, Investigación y Desarrollo (Spain).

MS, GC/MS, LC/MS Oral Session—Shane Needham, Alturas Analytics, Inc., 1282 Alturas Drive, Moscow, ID 83843, Tel: 208-883-3400, Fax :208-882-9246, E-mail: sneedham@alturasanalytics.com

180. SURFACE ENHANCED RAMAN IMMUNOASSAY (SERIA): MEASUREMENT OF PHARMACEUTICALS AND DISEASES. Jason Guicheteau, Roberta Sulk, Keith Carron, Robert Corcoran, University of Wyoming, Department of Chemistry, Laramie WY 82071-3838

Surface Enhanced Raman cattering (SERS) spectroscopy offers a unique approach to immunoassays through its highly localized enhancement of materials at the surface of the metal substrate. The localized enhancement allows us to perform sandwich or competitive assays in the presence of normally interfering species. In particular we are able to perform assays in the presence of excess reporters since only those bound directly to the antibody/antigen complex will be observed in the SERS spectrum. The aspect of our approach is very important as it eliminates the washing steps that introduce error and spread biohazardous waste in conventional immunoassays. We will discuss the instrumentation involved in the SERIA measurements and the methodologies. Appropriate dyes for tagging antibodies will be presented. Partial Least Squares (PLS) techniques were used for data quantization. Particular systems that will be discussed are human growth hormone, thyroid stimulating hormone, pesticides, illicit drugs, and prion diseases.

MS, GC/MS, LC/MS Oral Session—Keith Carron, University of Wyoming, Department of Chemistry, Laramie, WY 82071-3838. Tel: 307 766-2811, Fax: 307 766-2807, E-mail: carron@uwyo.edu

MS, GC/MS, LC/MS Poster Sessions

181. *BIOCHEMICAL INVESTIGATION OF A SEQUENCE-SPECIFIC, SINGLE-STRANDED DNA BINDING PROTEIN AT THE TELOMERE.* E.M. Anderson[1], R.M. Mitton-Fry[1], T.R. Hughes[2], V. Lundblad[2], D.S. Wuttke[1], [1]Dept. of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309 and [2]Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, 77030

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MS, GC/MS, LC/MS Poster Session—Emily Anderson, Dept. of Chemistry and Biochemistry, University of Colorado at Boulder, Campus Box 215, Boulder, CO, 80309-0215. Tel: (303) 492-2369, Fax: (303) 492-5894, E-mail: Emily.Anderson@colorado.edu

182. *HIGH THROUGHPUT ANALYSIS OF COMBINATORIAL LIBRARIES BY FIA AND LC\MS ANALYSIS.* Meg Hermann, Kathy Halm, Kevin Ash, Adam Cook, Mark Munson, Alan Florjancic, Gary Hingorani; Conrad Hummel; Greg Miknis; John Josey, Array BioPharma Inc., 1885 33rd Street, Boulder, CO 80301

Traditional sequential medicinal chemistry methods have been augmented by combinatorial synthesis methods yielding a higher number of compounds for high throughput screening against a disease targets and consequently a greater number of compounds that need to be analyzed. Initially, the analysis of compounds was accomplished by flow injection mass spectrometry of all wells in a 96-well plate. A well was passed or failed based on the presence of the molecular ion with at least 25% base peak intensity. In trying to coordinate library information with the RP-HPLC/UV purity data it became obvious that having LC/UV/MS data on libraries was crucial. Therefore, LC/UV/MS analysis was performed on the same wells that were being analyzed in parallel by RP-HPLC/UV for purity. The sampling protocol for purity determination at Array BioPharma is three columns per plate. This work was performed using a Gilson 215 autosampler with a TSP 4000 pump and TSP 2000

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15th Annual Symposium, July 28–August 1, 2001, Philadelphia, PA

16th Annual Symposium, August 17–21, 2002, San Diego, CA

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486-T

Dynamic studies of Single Molecule *E. Coli* RNA Polymerase D. Izhaky, [1] N. Forde, [1] G. J.L. Wuite, [2] R. Landick, [1] C. Bustamante[1,3]. [1]Howard Hughes Medical Institute and Department of Molecular and Cell Biology and [2]Department of Physics, University of California, Berkeley, CA 94720-3206, USA. [3]Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA.

Transcription is the central event in the regulation of gene expression. In prokaryotes, this process is carried out by DNA-dependent RNA polymerases (RNAP) which are typically large, multi-subunit enzymes. It has been shown that *E. Coli* RNAP translocates along the DNA discontinuously during the elongation phase of transcription, spending proportionally more time at some template positions, known as pause sites, that at others.

Recently, it has been shown in our laboratory using optical trapping/flow control that RNA polymerase molecules possess different intrinsic transcription rates and different propensities to pause and stop[1]. It was also shown that reversible pausing is a kinetic intermediate between normal elongation and the arrested state.

To further characterize the dynamics of RNAP, we are carrying out additional single molecule experiments, in order to more closely address the roles of mechanical force and substrate chemistry on the enyzmatic reaction pathway. To this end, we are varying the concentration of NTPs and PPi, and are applying force both hindering and aiding transcription.

1. Davenport, RJ; Wuite, GJL; Landick, R; Bustamante, C. Single-molecule study of transcriptional pausing and arrest by E. Coli RNA polymerase SCIENCE , 2000 V287:2497-2500.

487-S

BIOCHEMICAL INVESTIGATION OF A SEQUENCE-SPECIFIC, SINGLE-STRANDED DNA BINDING PROTEIN AT THE TELOMERE E.M. Anderson[1], R.M. Mitton-Fry[1], T.R. Hughes[2], V. Lundblad[2], D.S. Wuttke[1]. [1]Dept. of Chemistry and Biochemistry, U. of Colorado, Boulder, CO. 80309 and [2]Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, 77030

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488-M

Characterization of CRS2: A Group II Intron Splicing Factor.
G.J. Ostheimer, B.D. Jenkins, A. Barkan, and B.W. Matthews. Institute of Molecular Biology, University of Oregon. Howard Hughes Medical Institute

CRS2 is a nuclear encoded protein that is required for the efficient splicing of a subset of chloroplast group II introns. Although some group II introns self-splice *in vitro*, no chloroplast group II introns are known to do so. Presumably, chloroplast introns require additional cellular factors to facilitate the folding of these large RNAs into their active conformations. We believe CRS2 to be such a factor. Density gradient and gel filtration experiments with chloroplast extract demonstrate that *in vivo* CRS2 is found in very large complexes with molecular weight greater than 600 kD. Northern blot analysis indicates that CRS2 co-migrates with RNAs containing the introns that require CRS2 for splicing. In addition, nuclease treatment reduces that apparent mass of CRS2 to approximately 100 kD. The combination of these results suggests that CRS2 is found in complex with the introns that require CRS2 for efficient splicing. Interestingly, CRS2 is predicted to have a molecular weight of 24 kD, but *in vivo* it is found in a 100 kD nuclease resistant complex. Currently, we are working towards identifying the other constituents of this nuclease resistant complex with the long term goal of characterizing the roles that CRS2 and its co-factors play in group

489-T

Structure of the RNA polymerase domain of E. coli primase J.L.Keck, D.D. Roche, A.S.Lynch, J.M. Berger. Dept. MCB, Univ. California, Berkeley, CA 94720 (JLK, JMB) Tularik, South San Francisco, CA 94080 (DDR, ASL)

All cellular organisms use specialized RNA polymerases called "primases" to synthesize RNA primers for the initation of DNA replication. In this presentation, we describe the first high-resolution crystal structure of an active primase domain, comprising the catalytic core of the E. coli DnaG protein. The core contains an active-site architecture that is unrelated to other DNA or RNA polymerase palm folds, but is instead related to the toprim fold. Based on the structure, it is likely that DnaG binds nucleic acid in a groove clustered with invariant residues and that DnaG is positioned within the replisome to accept DNA directly from the replicative helicase. Supported by The Jane Coffin Childs Memorial Fund for Medical Research (JLK) and the G. Harold and Leila Y. Mathers Charitable Foundation (JMB).

490-S

Zinc finger is the nuclear localization signal of transcription factor Sp1
J. Kuwahara, M. Azumano, T. Takeda, Y. Watanabe, K. Itoh. Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770-8505, Japan

The bidirectional traffic between the nucleus and cytoplasm of a growing mammalian cell is routed through nuclear pore complexes (NPCs). Globular proteins of greater than 60 kDa can not cross the NPC by simple diffusion at a significant rate and therefore, transport of large proteins into the nucleus is an active process that requires the presence of a suitable nuclear localization signal (NLS). Sp1 is an ubiquitous transcription factor isolated from HeLa cell. Although the protein is supposed to be actively transported into the nucleus because of its relatively large molecular mass (95 kDa/105 kDa), none of NLS in Sp1 has been reported. The goal of our research is to identify the NLS of Sp1 and to understand its nuclear transport mechanism. We show here subcellular localization of Sp1. Full length or truncated fragments of human Sp1 cDNA were ligated to green fluorescent protein (GFP) gene from jerry fish, that is expressed under the control of CMV promoter. These plasmid constructs were transfected into HeLa cell by lipofection. Localization of transiently expressed GFP-Sp1 fusion proteins was detected using confocal laser scanning microscopy. Fusion of native Sp1 accumulated GFP in the nucleus of HeLa cell, whereas GFP alone was localized throughout the cell. We found that the three contiguous repeats of Cys2His2-type zinc finger were sufficient to localize GFP in the nucleus. These results strongly suggest that the two functional domains for DNA binding and nuclear localization would be spatially close or would overlap each other.

491-M

Stereoselectivity Study of WT DNA Polymerase Beta and Mutant D276R J. Liu, X. Zhong, and M-D. Tsai. Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210

DNA polymerase beta (Pol β) is a DNA repair enzyme that requires magnesium ions to catalyze the nucleotidyl transfer reaction. In the current study, based on the crystal structures and rational prediction, pre-steady-state kinetic analysis, site-directed mutagenesis and stereo phosphorothioate isomers (dATPαS) were used to study the stereoselectivity of Pol β. Several important findings have been obtained: (1) Sp isomer was highly preferred for WT pol β in the presence of Mg2+ ion, while this stereoselectivity towards Sp was decreased dramatically when Mg2+ was replaced by Mn2+. This result suggests that metal ions are one of the important factors that control the stereoselectivity of WT Pol β. (2) D276R mutant was designed to introduce an extra hydrogen bonding interation between the Arg side chain and the pro-Sp oxygen of α-phosphate. The kinetic data showed that the stereoselectivity of D276R is "relaxed" compared with WT pol β, supporting that the newly introduced protein side chain interacts with α-phosphate and influences the stereoselectivity of the enzyme. Acknowledgments: This work was supported by the NIH

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