

Project no. 69906
DOE grant number DE-FG07-99ER62875

Markers of the Low Dose Radiation Response
William S. Dynan, PI

An annual report was prepared in March, 2000, in connection with the EMSP National Workshop. Because the report was submitted early, it did not fully reflect progress in the first year. For this reason, this updated report has been prepared. New material is in italics.

The goal of this work is to develop a technology that will allow direct visualization of DNA double-strand break repair complexes in their original places in the nuclei of irradiated cells.

Ionizing radiation has a unique ability to induce damage simultaneously at multiple sites within a spatially restricted region of DNA. The resulting double-strand DNA breaks (DSBs) present a major threat to the integrity and stability of the genome. Our understanding of the origin and fate of DSBs is based primarily on studies at high radiation doses. Despite recent progress toward the elucidation of molecular mechanisms underlying DSB repair, technologies are still not available to visualize individual DSBs and DSB repair complexes in situ, in irradiated cells.

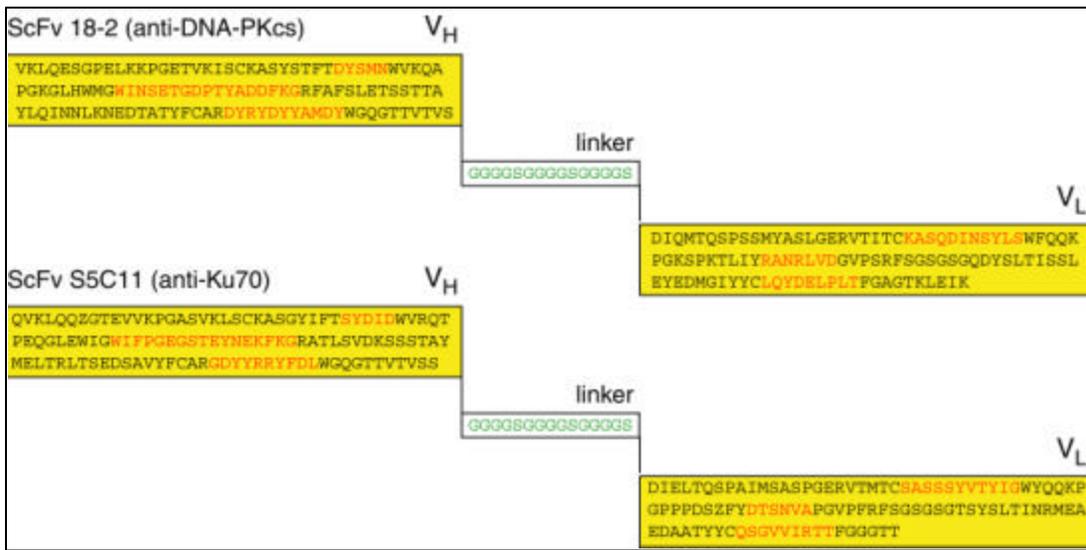
This project will involve development of a technology to allow direct in situ visualization of DSB repair complexes. These studies will help bridge the gap between biochemical studies of repair enzymes and an understanding of the process of repair as it actually occurs within the radiation-injured cell. Specific aims include:

Aim 1.

The development of tools for in situ visualization of DSB repair complexes. These tools will include fluorescently tagged repair proteins that can be used to visualize repair complex assembly in living cells. They will also include recombinant single-chain antibodies that will be used to detect changes in the conformation and phosphorylation state of DSB repair proteins that accompany the assembly of the repair complex.

Much of the first year has been spent in developing the recombinant single-chain antibody technology. In order to optimize our techniques and to prove, in concept, that single chain antibodies can be used to stain repair complexes in situ, we have cloned rearranged immunoglobulin genes from individual hybridoma lines that are already known to express antibodies directed against double-strand break repair proteins. Preliminary sequence of two single chain clones is shown in the figure below. We now have three recombinant single chain antibody clones that have been obtained and inserted in E. coli expression vectors. A preliminary analysis indicates that these clones are producing recombinant products that bind in vitro to purified Ku and DNA-PKcs proteins with the expected specificity. An additional two recombinant single chain antibodies are in intermediate stages of cloning. Once conditions have been optimized for cloning, expression, and detection of single chain antibodies, the next step will be to create a library of single chain antibodies to be used for phage display and in vitro selection as envisaged in the original proposal. This aspect of the project has benefited from the arrival of a trained postdoctoral fellow in May, 2000 (Dr. Shuyi Li). He will replace a temporary predoctoral fellow (Jessica Banko) who is initiated the project but is now returning to full-time graduate study elsewhere as of June, 2000.

As a technological alternative to the single chain antibodies, we are also preparing fusion proteins containing known repair factors joined to red or green fluorescent protein. At present, we have constructs for expression of red fluorescent Ku and green fluorescent RHA. These are currently being tested in transfection assays to determine levels of expression. They can potentially be used to study radiation-induced changes in localization of repair proteins in real time, in vivo, in living cells.



Aim 2.

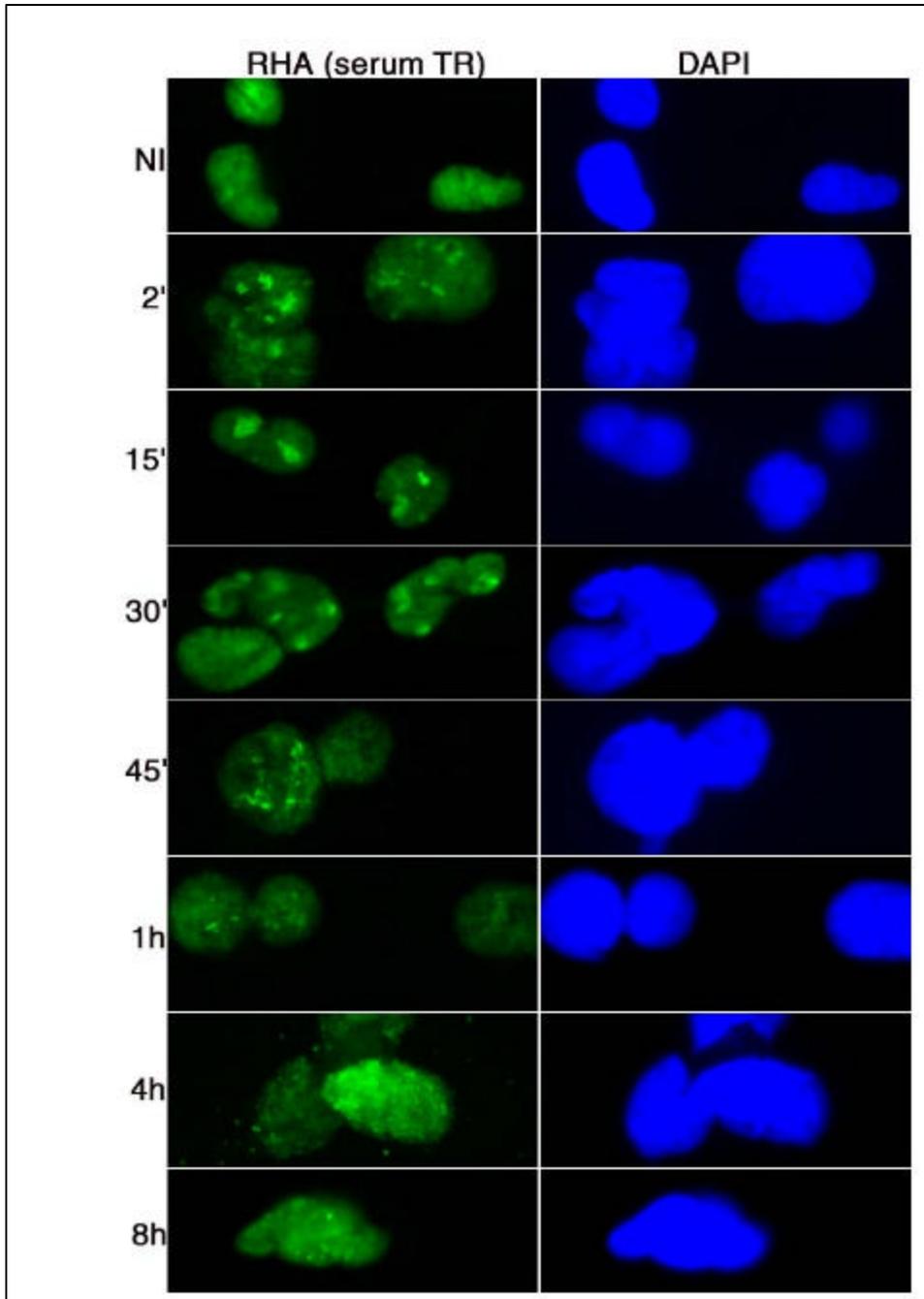
The use of these tools in combination with fluorescence microscopy to detect and characterize visible subnuclear structures associated with DSB repair. Control and reconstruction experiments will be performed to correlate visible structures with biochemically-defined repair complexes. Strategies will be developed to prolong the lifetime of repair complexes to make them more easily visible. Immunoprecipitation experiments will be performed to identify additional proteins that contribute to formation of repair structures.

Although we do not yet have the single-chain antibody tools available, we have had an exciting serendipitous result, which has led to the identification of a protein that is potentially a new marker of the radiation response. This protein is RNA helicase A (RHA), previously described as a protein involved in RNA biogenesis. We discovered that RHA physically associates with the double-strand break repair protein, Ku, in vitro and in vivo. This prompted us to examine the physical localization of RHA in irradiated and nonirradiated cells. Like many repair proteins, RHA is a target of autoantibodies present in patients with certain autoimmune diseases. Using patient sera provided by Dr. Yoshihiko Takeda, we have performed immunofluorescence staining to examine localization of RHA before and after irradiation. We find that RHA dramatically reorganizes into subnuclear foci at the earliest timepoints that can be measured after irradiation (i.e. 15 min. of irradiation, followed by 2 min. recovery and staining). A representative series of images is presented below. These are SV40 transformed human fibroblasts stained with patient antisera as primary antibody and FITC-conjugated anti-human IgG as secondary antibody (green). They have been counterstained for DNA with DAPI dye (blue). Similar results have been obtained with monospecific rabbit anti-RHA.

The exciting aspect of this result is that the foci appear and disappear on the same time scale as double-strand DNA breaks. That is, they form immediately and largely disappear within 1 h. In this respect, they differ from other known types of "ionizing radiation induced foci," which tend to form only after a period of hours, when most breaks have already been repaired. Although the initial discovery of the RHA rearrangement was made at high doses (15 Gy), the immediacy of the effect suggests that RHA may be useful as a marker of the low dose radiation response. Experiments to examine the dose-response of RHA focus formation

are planned. In addition, we suspect that the rearrangements probably reflect post-translational modification of RHA. In principle, antibodies sensitive to the modification could be developed (e.g. anti-phosphopeptide antibodies) which could serve as more sensitive markers of RHA modification for use at very low doses.

The RHA system has been developed principally by Dr. Yoshihiko Takeda. Imaging was performed by Mr. Serguei Zavgorodni. Mr. Zavgorodni left the project in April, 2000 to take a position with a web start-up venture and has been replaced by Dr. Mohamed Labazi, a postdoctoral fellow who is continuing the work and who collected the images shown below. Dr. Steven Vogel, co-investigator, has provided advice on imaging technology throughout.



Aim 3.

A demonstration of the practical utility of the tools and assays developed in Aims 1 and 2 by quantitative studies of DSB repair in different cell types. The formation of repair complexes at high and low radiation doses will be compared. The effect of a low radiation dose on the response to subsequent, higher doses will be determined. The relative prevalence of the Ku-dependent end joining and Rad51-dependent recombinational pathways will be investigated.

The ability to visualize single DSB repair complexes formed at the sites of DNA damage will provide an improvement of two orders of magnitude in sensitivity over existing physical methods of measuring double-strand breaks. It will open up exciting prospects for direct investigation of the low-dose radiation response.

Experiments in Aim 3 have not yet been initiated.

Personnel:

As of March, 2000.

William S. Dynan	Principal Investigator	Medical College of Georgia	IMMAG/Medicine
Steven Vogel	Co-investigator	Medical College of Georgia	IMMAG/Medicine
John Barrett	Co-investigator	Medical College of Georgia	Radiology
Yoshihiko Takeda	Assistant Professor	Medical College of Georgia	IMMAG/Medicine
Serguei Zavgorodni*	Research Assistant	Medical College of Georgia	IMMAG
Farlyn Hudson	Research Assistant	Medical College of Georgia	IMMAG
Jessica Banko*	Predoctoral Fellow	Medical College of Georgia	IMMAG

**departed April, 2000*

***departing June, 2000*

New personnel:

<i>Shuyi Li</i>	<i>Postdoctoral Fellow</i>	<i>Medical College of Georgia</i>	<i>IMMAG</i>
<i>Mohamed Labazi</i>	<i>Postdoctoral Fellow</i>	<i>Medical College of Georgia</i>	<i>IMMAG</i>

Budgetary update:

It is not anticipated that there will be significant unspent project funds remaining at the end of the project year.

Contact: William S. Dynan

Telephone: (706) 721-8753; email: wdynan@mail.mcg.edu

Note that this is a new email address for the Principal Investigator.