EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

http://193.49.43.2:8080/smis/servlet/UserUtils?start

Reports supporting requests for additional beam time

Reports can now be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

<table>
<tr><td> </td><td>Experiment title: Kinetics of E.coli RNA Polymerase binding and escape by time-resolved x-ray footprinting</td><td>Experiment number:
LS-1852</td></tr>
<tr><td>Beamline:
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31-08-2001
Received at ESRF:</td></tr>
<tr><td>Shifts:
6</td><td>Local contact(s):
Michael Wulff</td></tr>
<tr><td colspan="3">Names and affiliations of applicants (* indicates experimentalists):
Bianca Sclavi*
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</table>

Report:

Time-resolved X-ray footprinting is a relatively new methodology which was developed at NSLS[1]. It is based on the production of hydroxyl radicals by the radiolysis of water caused by the X-rays. These radicals are used to cleave a polynucleotide (either DNA or RNA) at its solvent-accessible sites. If a protein covers the DNA for example it will protect the DNA from cleavage only at those sites that are in contact with the protein. From an hydroxyl-radical footprinting experiment one can determine, at a single base resolution, where are the specific contacts between the protein and the DNA. The advantage of using a synchrotron X-ray beam to produce the hydroxyl radicals is that they can be produced fast enough (a few milliseconds) to carry out time-resolved experiments to study the structural kinetics of formation of protein-nucleic acid complexes.

During our first run (July 2000) we established that this kind of experiments could be carried out at ESRF and we obtained some very encouraging preliminary results indicating that the sample exposure time at ESRF is shorter (2 ms) than that used at NSLS (20ms) and that the pattern of DNA protection was the expected one. These experiments were carried out using ^{32}P labeled DNA. For our second run we decided to use fluorescently labeled DNA to avoid using radioactive samples. In addition, the use of fluorescently labeled DNA increases the data resolution and could potentially decrease the time required to acquire and analyze the data.

Fluorescently labeled DNA is currently used to sequence DNA or to qualitatively determine the location of a binding site of a specific protein, however, it still has not been used for a quantitative study of binding affinities or kinetics. One of the aims for our second ESRF run therefore was to determine whether the latter would be possible. In order to do this we developed a new data analysis protocol that would allow

[1] Sclavi, B., Woodson, S. A., Sullivan, M., Chance, M. R. and Brenowitz, M. (1998). Following the Folding of RNA with Time-resolved Synchrotron X-Ray Footprinting: *Methods in Enzymology*, **295**, 379-402.

us to follow the cleavage changes at each base on the DNA starting from the output of a DNA sequencing apparatus.[2]

In figure 1 the different steps of the data analysis are described. The analysis shown is of an experiment we carried out during our last run at ID9 where *E. coli* RNA polymerase was mixed with a DNA fragment containing a promoter sequence. The aim of this kind of experiment is to follow the location and the kinetics of the contacts made by the polymerase on the promoter. There are at least two known intermediates in the pathway: an initial recognition complex, an isomerized complex and finally a complex where the two DNA strands are separated at the transcription start site. Figure 1D shows the rate at which three bases in the –10 consensus region become protected from cleavage. Usually the data from three separate experiments is needed in order to have higher confidence in the results.

We were worried that the fluorescent probe might be damaged by oxygen radicals and that we would lose the intensity of the signal, even at these short exposure times. Our preliminary results show that although there is a decrease in signal after exposure it can be overcome by increasing the concentration of labeled DNA in the sample.

During this last run we also tested a new kind of quartz capillary as a sample holder which however were not as resistant to long exposures to the white beam and had to replaced frequently, losing a large amount of precious beamtime and sample. The setup of the stopped-flow apparatus also will need to be changed in order to automate several of the steps that have to take place before each time point can be collected.

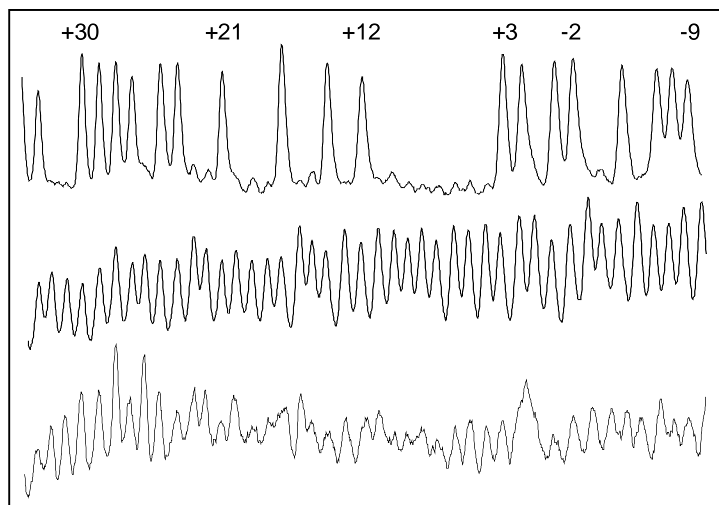
Even though some specific technical changes are still needed to our experimental set-up we are confident that in the future they will result in a greater amount of time dedicated to time-resolved data collection.

In conclusion we have determined that fluorescently labeled DNA can be used for time-resolved X-ray footprinting experiments. We are currently developing a data analysis protocol that results in a single base analysis of the changes in protein-DNA contacts during recognition of the protein binding site. The initial time-resolved results are very encouraging.

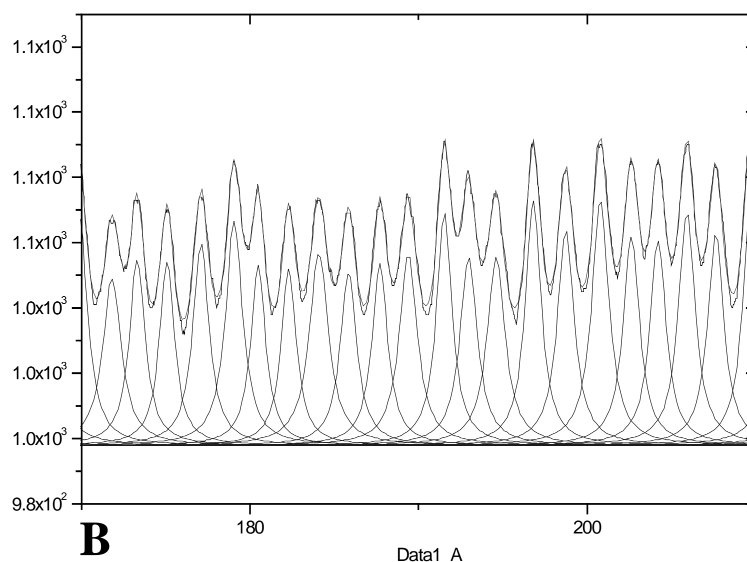
This technique, generally applicable to a time-resolved analysis of any given nucleoprotein complex, will be applied in our laboratory to obtain a detailed picture of the contacts made by the *E. coli* RNA polymerase as it recognizes the promoter and then as it escapes into transcription. This kind of study will reveal the structural changes taking place during transcription initiation and the role of the regulators of trancsription activity at each step in this process.

This work was carried out in collaboration with the members of Hermann Heumann's laboratory from the Max-Planck Institut fuer Biochimie, Marinsried.

[2] The cleavage pattern of the DNA is usually detected by separating the different DNA fragments on a denaturing polyacrylamide gel. If the DNA is end-labeled with ^{32}P the gel is stopped when the smaller fragments have reached the bottom, it is then fixed, dried and exposed to a phosphorimager screen. The screen is later scanned and a digital image of the gel is obtained. The intensity of each band on this image corresponds to the amount of DNA of a specific size on the gel. These images are analyzed with ImageQuant software. If the DNA is end-labeled with a fluorescent probe it is run on the same kind of denaturing gel. In this case however the gel is part of a machine that has a laser at the bottom of the gel that excites the fluorescent probe and several small detectors, each one aligned with a different DNA sample. In this way as the DNA fragments migrate to the bottom of the gel their fluorescent signal is detected and recorded in real time in digital form. Since all of the DNA has to reach the bottom of the gel to be measured the resolution, band separation, is increased. Therefore the latter method has several advantages: the DNA is not radioactive, the gel does not need to be dried and exposed to a screen, the resolution of the bands is increased and the acquisition of data is in real time, as the gel is running.



A



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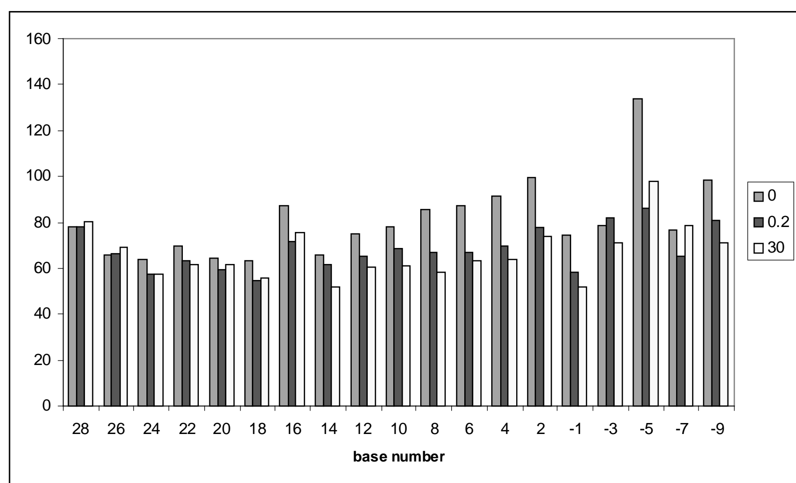

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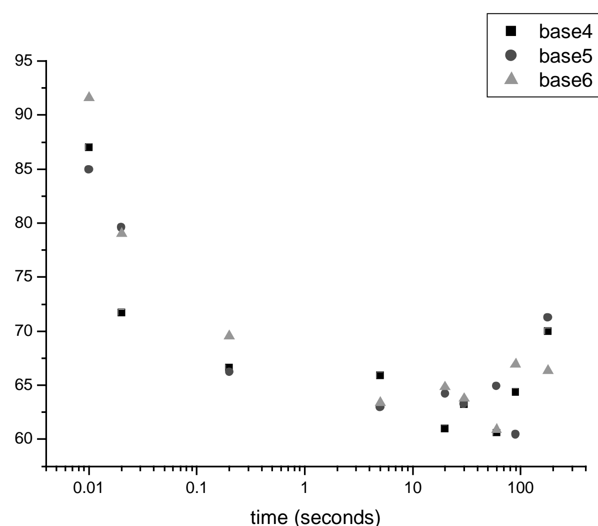

D

Figure 1. A. Output from the sequencing machine. Each peak is the intensity of a fragment of DNA one base longer than the one on its left. The first lane corresponds to a chemically induced cleavage of the DNA at all G and A nucleotides allowing identification of the positions od the bases on the DNA fragment. The second lane corresponds to the DNA alone, while the third one is the "footprint" of the RNA polymerase on the DNA. **B.** Result of the fitting session. Each peak is fitted to a lorenzian curve. **C.** The area of each peak determined from the fitting is plotted as a function of its position. Three different time points are shown, the DNA alone, after 0.2 and 30 seconds of mixing with the RNA polymerase. The protection can be clearly seen in the region from +12 to –9. The total protection of the RNA polymerase in the final complex extends up to the base at –50. **D.** Kinetics of appearance of the protection at bases at + 4, 5 and 6.