

GENERATION OF SHORT PULSES OF HYDROXYL RADICALS BY SYNCHROTRON RADIATION FOR TIME-RESOLVED HYDROXYL RADICAL FOOTPRINTING

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Short pulses of hydroxyl radicals can be generated by synchrotron radiation and used to follow biological processes at time intervals on the millisecond scale.

A key process of the cell is the transcription of the genetic information encoded in the DNA. This process is facilitated by the enzyme DNA-dependent RNA polymerase (RNAP), which moves along the DNA “reading” the DNA sequence and synthesising a base-complementary RNA. We intend to use hydroxyl radicals (OH•) in order to follow the movement of RNAP on the DNA in the millisecond range. Hydroxyl radicals cleave the DNA at those regions which are not covered by the RNAP. Due to the ionising property of the X-ray beam, high-intensity X-ray pulses can be used to produce short hydroxyl radical pulses which can cleave the DNA in the same manner as chemically generated hydroxyl radicals (Figure 1a).

The DNA is cleaved without significant base specificity (Figure 1b) by interaction of the hydroxyl radicals with the sugar moiety. Those regions on the DNA that interact with the protein are precluded from hydroxyl radical attack and appear in the gel electrophoretic pattern as windows (footprints) in the otherwise regular cleavage pattern (Figure 2). The cleavage pattern is visualised by autoradiography of the radioactively-labelled fragments which are electrophoretically separated – a procedure similar to DNA sequencing.

Hydroxyl radicals are routinely generated chemically by the Fenton reaction and are used for probing protein-DNA contacts. The rate-limiting step in this footprint reaction is the production of hydroxyl radicals. In the Fenton

Fig. 1: a) Production of hydroxyl radicals (OH•) by ionising radiation, b) Cleavage reaction of the DNA-backbone after hydrogen abstraction at the C4'-atom by the electrophilic, highly reactive hydroxyl radical.

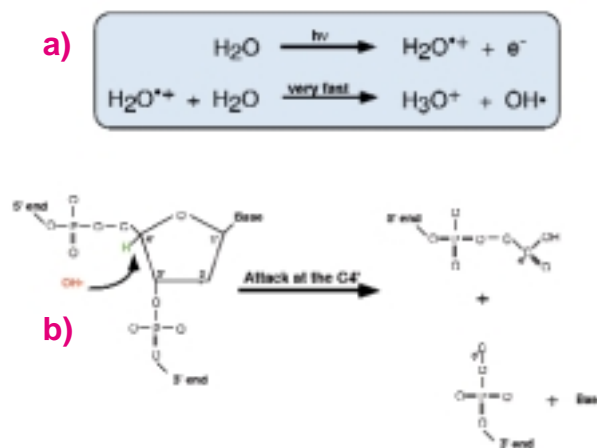
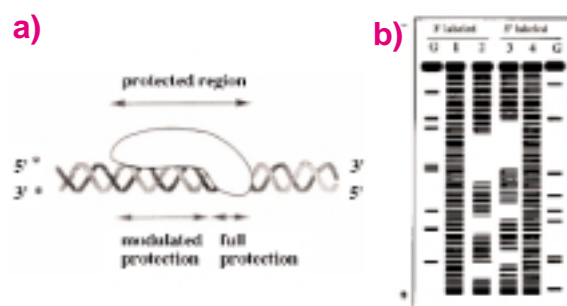


Fig. 2: Scheme of DNA-footprinting. a) The bound protein covers regions on the DNA and protects them from cleavage, b) This protected and therefore uncleaved region remains as missing bands in the cleavage pattern on the autoradiographic gel.



reaction the rate of chemically produced radicals is too low for fast footprinting. The integral amount of radicals for sufficient cleavage is reached after several seconds. Therefore, the time interval for subsequent footprints is limited to 20 seconds. Using the white beam of the X-ray synchrotron spectrum at ESRF's ID9

beamline, it is possible to generate short pulses of OH• which enables footprinting at 10 millisecond intervals by increasing the rate of Hydroxyl radical production by a factor of 10⁴.

Figure 2b shows a schematic footprint of RNA polymerase bound specifically to

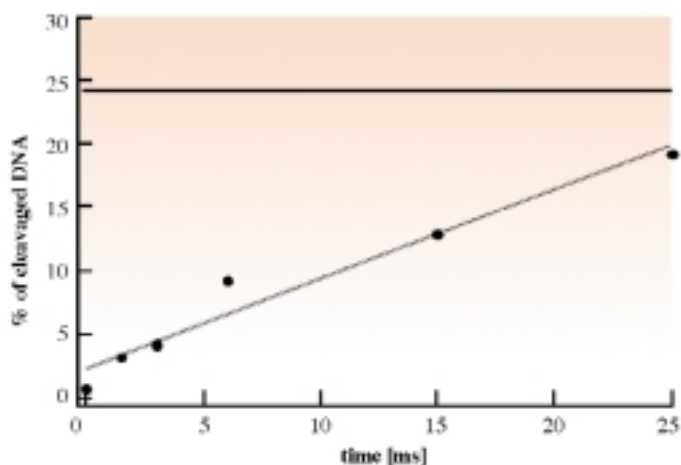


Fig. 3: Linear increase in cleavage with exposure time; the solid line indicates the percentage of cleavage using chemically generated hydroxyl radicals after a reaction time of 1 minute.

its cognate DNA fragment. The following information can be extracted from the hydroxyl radical footprinting patterns:

- The size of the DNA region interacting with the protein, which is obtained from the positions of the windows.
- The mode of interaction, which can be derived from the variation of the intensity of the bands within the interacting region. Protection of both strands indicates that the protein wraps around the DNA, whereas a window at both strands shifted by 10 bases indicates protein binding towards one side of the DNA.

In order to obtain quantitative data about the interaction of DNA and protein, the exposure time has to be adjusted in such a way that not more than 10 - 20% of the DNA molecules are cut. A simple criterion for the quality of the cleavage procedure is a linear increase in the band intensity with the exposure time. This linearity is demonstrated for our cleavage studies in Figure 3.

Cleavage by hydroxyl radicals occurs not only in the DNA but also in the protein. However, this does not interfere with the interpretation of the footprinting data, since the DNA is cleaved much more efficiently than the protein due to its higher sensitivity towards the hydroxyl radical. Only the DNA fragments which are cleaved by hydroxyl radicals produce the characteristic band pattern on the electrophoresis gel. Direct strain breaks of the DNA-helix due to radiation damage would produce DNA fragments of every size and lead to a strong background on the gel. Therefore the exposure time has to be adjusted to obtain good cleavage with minimal radiation damage.

In order to follow the movement of the RNA polymerase along the DNA, the footprints have to be determined in the millisecond time range. One prerequisite is rapid mixing of the components, which is achieved by a fast mixing device having a dead-time below 20 ms. The machine consists of a system of syringes and produces a continuous flow of mixed sample through the irradiated capillary. A stepping motor controls the flow-rate, permitting a minimal exposure time of 0.5 ms/ μ l. This device was installed at the ID9 beamline using the wiggler (gap at 30 mm) without any beamline optics. The

sample was irradiated with a 2.5 x 1 mm² beam using a quartz capillary (OD 1 mm; walls 0.01 mm). Damage to the capillary was avoided by using glassy carbon and aluminium attenuators.

Figure 4 shows the first footprint of RNA polymerase obtained by X-ray synchrotron radiation in 10 ms. On the gel, the protected regions are visible as areas with less pronounced or missing bands (Figure 4a). The larger region indicates the full protection of the DNA by the protein and the smaller regions above, separated by windows of ~10 base-pairs, the half protection of the DNA. Further analysis of the pattern is done by integrating the profile of the pattern (Figure 4b). In the profile the protected regions, compared to unbound DNA are shown. The pattern is compatible with that obtained by chemically generated OH-radicals after 20 s.

Potential applications of the footprinting technique include the analysis of the structural rearrangements of RNA polymerase and DNA during formation of the transcription competent initiation complex. During formation of this complex at least three different intermediate complexes are transiently formed, namely the closed complex,

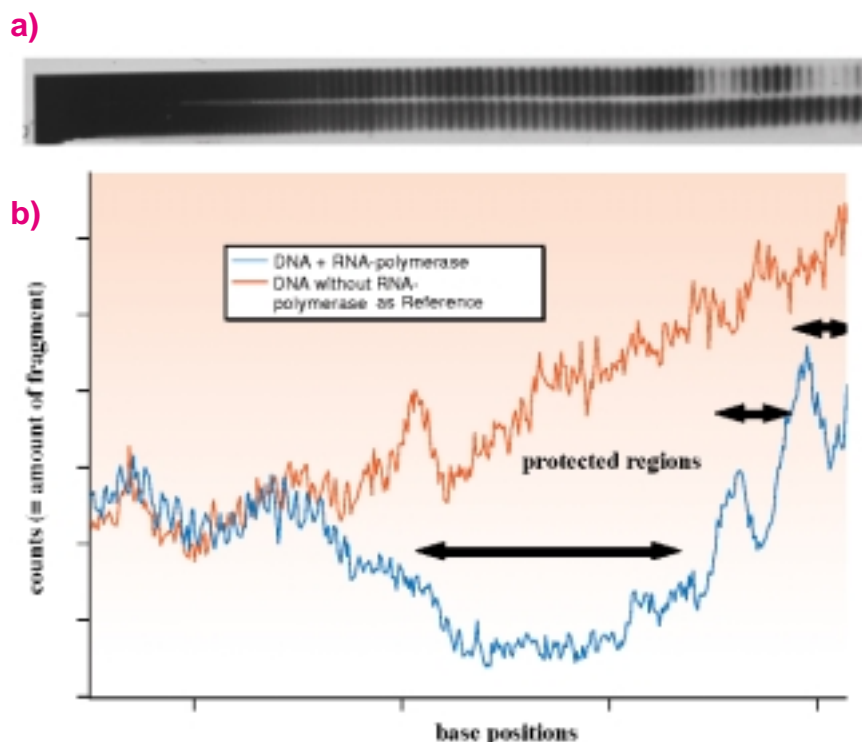


Fig. 4: Footprint of the RNA-polymerase on the DNA. a) Experimental data: Upper pattern RNA-polymerase + DNA; lower pattern DNA alone, as reference, b) Profile analysis of the cleavage pattern from a).



the intermediate complex and the transcription competent open complex. These intermediates are characterised by kinetic studies and by chemical footprinting. The later required lowering of the temperature in order to “freeze” the intermediate complexes. Fast Footprinting by synchrotron-generated hydroxyl radical pulses would allow direct structural characterisation of the intermediates.

Another fascinating application of this technique would be the analysis of the formation of initiation complexes which are controlled by helper proteins

such as the Fis-protein. These helper proteins play an important role for the control of gene expression not only in bacteria but also in eucaryotic cells.

A related project which could also be tackled by Fast Footprinting is the analysis of the translocation of RNA polymerase along DNA during the transcription process in real time – a “cinematographic” analysis. This process is probably not monotonous and does not occur in parallel with incorporation of each nucleotide into the RNA. The latter process occurs approximately every 50 - 500

milliseconds. In order to follow this process in real time, Fast Footprinting using synchrotron-generated OH-radicals is presently the only direct approach to follow the movement of the enzyme on the DNA. This translocation process recently gained special attention, since RNA polymerase can be considered as a highly processive linear molecular motor.

ACKNOWLEDGEMENTS

We thank the DFG for support.

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