High-Resolution in Vivo Footprinting of a Protein−DNA Complex Using γ-Radiation

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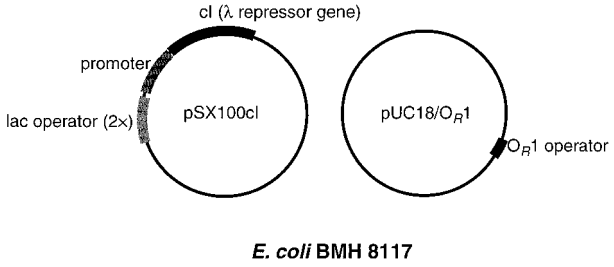
The imminent prospect of the sequencing of the human genome has focused attention on how the information encoded in DNA is expressed in living organisms. While much emphasis has been placed on enumerating the proteins encoded by the genome, how gene expression is regulated is at least of equal importance. Regulation of gene expression is accomplished by proteins which bind to specific DNA sequences nearby genes. Finding out which proteins occupy which DNA binding sites, and under what circumstances, is the essence of the problem. The footprinting experiment[1] gives a direct "image" of which DNA sites are occupied by protein and which are empty. Footprinting takes advantage of the ability of a bound protein to inhibit the action of a chemical or enzymatic reagent which degrades DNA. While footprinting is now routine for DNA−protein complexes prepared in vitro, it is of obvious interest to be able make footprints of proteins bound to DNA in a living cell. Methods for in vivo footprinting[2] have most often employed two classic footprinting reagents, deoxyribonuclease I and dimethyl sulfate. While these reagents can show which DNA binding sites are occupied by protein, they have the disadvantage of providing limited information on the structural details of a DNA−protein complex,[3] thus making it difficult to identify which protein is bound to a site.

Our laboratory introduced the use of the hydroxyl radical (generated by the Fenton reaction of iron(II) EDTA with hydrogen peroxide) as a high-resolution footprinting reagent,[4] capable of revealing structural details of protein−DNA complexes at single-nucleotide resolution.[5] The hydroxyl radical attacks the deoxyribose backbone of DNA by abstracting a hydrogen atom,[6] which leads ultimately to a single-strand break in the DNA chain. We have shown that the probability of attack of a particular deoxyribose hydrogen is directly proportional to the solvent-accessible surface area presented by that hydrogen atom.[6] Because of its high reactivity and low selectivity, the hydroxyl radical attacks every nucleotide within a naked double-stranded DNA molecule to a nearly equivalent extent, thus providing information on solvent exposure of the entire DNA backbone. This method yields what has been called the "footprint phenotype"[7] of a protein−DNA complex, a detailed pattern of DNA protection and exposure which can be used to classify the structural type of a DNA-binding protein and perhaps even lead to its identification. We now report the development of a new method that allows us to make such high-resolution footprints of a protein−DNA complex in a living cell, using not Fenton chemistry but ionizing radiation to produce the hydroxyl radical.

Our method involves irradiating cells in culture with γ-radiation, which is known to produce the hydroxyl radical. We earlier showed[8] that γ-radiation can be used to make a footprint of a protein−DNA complex in vitro. To develop the in vivo hydroxyl radical footprinting method we set up a test system to study the complex of the bacteriophage lambda repressor with one of its operator DNA binding sites, in a living *Escherichia coli* cell (Scheme 1). The experimental system involved co-transformation

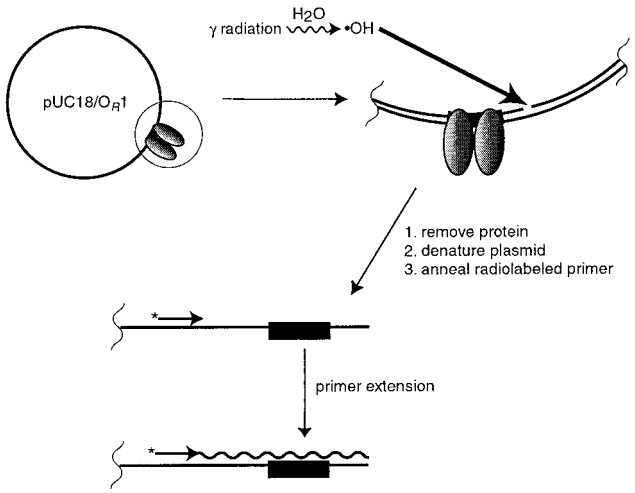
Scheme 1



of *E. coli* with a plasmid expression vector for lambda repressor,[9] along with another plasmid harboring one of the natural binding sites for lambda repressor, the O_R1 operator site.[10] We chose the lambda repressor−DNA complex for these studies because this system is very well-characterized structurally[11] and genetically,[12] and because we have used it extensively in our laboratory as a test system, for example, to develop the hydroxyl radical footprinting[4] and missing nucleoside[13] experiments.

A major challenge in the development of this method is how the footprint will be detected, since conventional end-labeled linear DNA obviously cannot be used in vivo. We found that the method of primer extension footprinting[14] could be applied to hydroxyl radical footprinting performed on supercoiled plasmid DNA (Scheme 2).[15] In this method the footprinting reaction is

Scheme 2



performed on the complex of a protein with unlabeled supercoiled DNA. A radiolabeled primer is then hybridized to the plasmid

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(10) Plasmid pSX100cI, an expression vector for the lambda repressor, was the generous gift of B. Müller-Hill.[9] An oligonucleotide containing the sequence of the O_R1 operator was synthesized and cloned into the *Pst*I site of plasmid pUC18, to give pUC18/O_R1. For in vitro iron(II) EDTA plasmid hydroxyl radical footprinting experiments we also used plasmid p*K*B280,[8] which we prepared by inserting the O_R1 operator sequence into a different expression vector for lambda repressor.

10.1021/ja000285f CCC: $19.00
Published on Web 06/03/2000

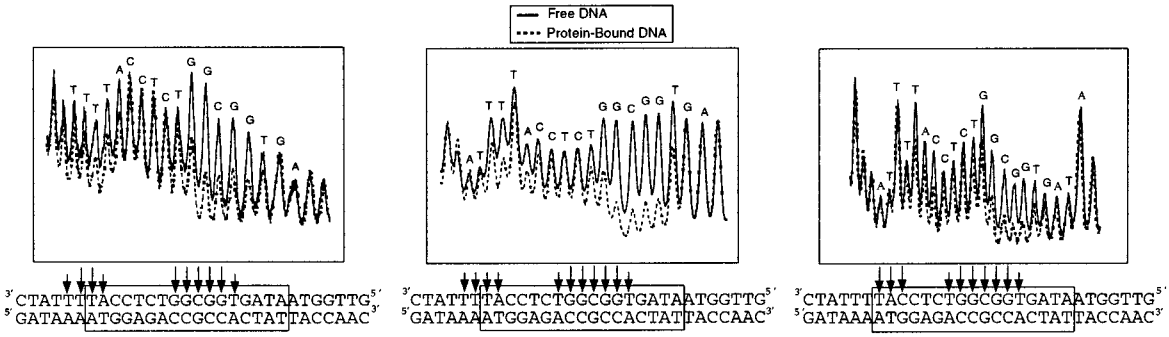

Figure 1. Hydroxyl radical footprints of the lambda repressor-O_R1 complex, in vitro and in vivo. Left, conventional iron(II) EDTA hydroxyl radical footprint of lambda repressor bound to linear end-labeled DNA; center, in vitro iron(II) EDTA hydroxyl radical footprint of repressor bound to pKB280[8] plasmid DNA, analyzed by primer extension; right, in vivo primer extension γ-ray footprint of lambda repressor bound to its operator in plasmid pUC18/O_R1 in living *E. coli*. Solid line, no repressor present. Broken line, repressor present. Below each panel is shown the 17 base pair O_R1 operator (boxed) and surrounding sequence. Vertical arrows denote nucleotides protected from cleavage by bound repressor.

and extended by use of DNA polymerase and nucleoside triphosphates. When the polymerase encounters a gap left by hydroxyl radical cleavage, it stops extension. Where the protein was covering the DNA backbone no cleavages occurred, and therefore no polymerase stops at these nucleotides are found in the primer extension reaction mixture. The set of radiolabeled, extended primers thus constitutes the footprint.

In Figure 1 we show a comparison of three hydroxyl radical footprints of lambda repressor: *left*, performed in the conventional way, using a linear, end-labeled DNA molecule and iron(II) EDTA as the source of hydroxyl radical;[4,5] *center*, using the primer extension method described above[15] on repressor bound to the O_R1 site in plasmid p*K*B280[8] in vitro, with iron(II) EDTA as the source of hydroxyl radical; and *right*, the primer extension footprint of repressor bound to the O_R1 operator site in plasmid pUC18/O_R1 in vivo in *E. coli*, using γ-radiation to produce hydroxyl radical for footprinting.[16] All three footprints are very similar and reveal essentially the same contacts of repressor with the DNA backbone. As we have pointed out before,[4] this footprint is consistent with the three-dimensional structure of the lambda repressor−DNA complex as determined by X-ray crystallography.[11]

We have analyzed the in vivo footprint using the GelExplorer software developed in our laboratory for quantitation of footprints.[17] GelExplorer provides a straightforward way to compare the footprint pattern to the control cleavage pattern, so that the complex cleavage pattern of DNA in vivo can be accounted for. A very clear footprint emerges from this analysis (Figure 2).

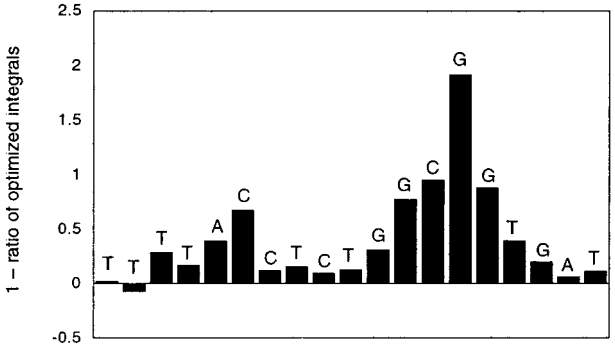

Figure 2. GelExplorer analysis of the in vivo γ-ray primer extension footprint of lambda repressor bound to its operator in *E. coli*. The experimental data shown in Figure 1 (right panel) were analyzed by calculating the ratio of the integral of a band for free DNA to the integral of the same band for the footprint, and then subtracting this ratio from 1. Positive features represent nucleotides protected by bound repressor.

Hydroxyl radical footprinting has been used to study a large number of protein−DNA complexes in vitro.[18] Of particular interest for the study of eukaryotic gene regulation is the ability of hydroxyl radical footprinting to reveal the presence of nucleosomes,[19] which the methylation protection experiment cannot.[3] Our demonstration here that hydroxyl radical footprinting can be used to obtain a high-resolution "image" of a protein−DNA complex in vivo opens the way for the study of a large number of systems. We look forward to the further application of this new method.

Acknowledgment. We thank B. Müller-Hill for providing the expression vector for lambda repressor, and for his help in developing this experiment. This work was supported by NIH Grant GM 41930.

JA000285F

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(15) We used the −40 forward primer for pUC18 from New England Biolabs (Beverly MA) for primer extension analysis of the hydroxyl radical footprint of lambda repressor bound to pUC18/O_R1. The primer was radiolabeled at the 5′ end using [γ-^{32}P]dATP and polynucleotide kinase. After treatment with hydroxyl radical, plasmid DNA was isolated by ethanol precipitation. The primer extension protocol involved denaturation of the plasmid at 90 °C for 5 min, annealing of the primer, and then one round of primer extension at 72 °C for 10 min, using 1 unit of $Vent_R$ polymerase (New England Biolabs).
(16) Plasmids pSX100cI[9] and pUC18/O_R1 were transformed into *Escherichia coli* strain BMH 8117 (Scheme 1). Expression of the cI gene that codes for lambda repressor is constitutive in these cells. A control culture was prepared by transforming *E. coli* with only plasmid pUC18/O_R1. After overnight growth, cells were suspended in 20 mM potassium phosphate buffer (pH 7.5) in a 1.5 mL Eppendorf tube, placed on ice, and irradiated for 15 min in a Shepherd ^{137}Cs γ-ray source (Oncology Department, Johns Hopkins School of Medicine).The exposure rate was approximately 50 Gy/min. After irradiation cells were immediately lysed, and the DNA was isolated by standard methods.
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