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MicroReview

Genomic analysis of protein–DNA interactions in bacteria: insights into transcription and chromosome organization

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Summary

Chromatin immunoprecipitation (ChIP) is a powerful method to measure protein–DNA interactions *in vivo*, and it can be applied on a genomic scale with microarray technology (ChIP-chip). ChIP-chip has been used extensively to map DNA–protein interactions across eukaryotic chromosomes. Here we review recent applications of ChIP-chip to the study of bacteria, which provide important and unexpected insights into transcription and chromosome organization.

Introduction

Protein–DNA interactions play a crucial role in transcription, replication, recombination, chromosome compaction and DNA repair. Historically, biochemical and genetic approaches have been used to study protein–DNA interactions. However, biochemical approaches do not study these interactions under physiological conditions, and genetic approaches are indirect and can be complicated by indirect effects of mutations.

Chromatin immunoprecipitation (ChIP) is a powerful method that directly measures both the position and strength of protein–DNA interactions *in vivo* (Fig. 1) (Aparicio *et al.*, 2004). Briefly, ChIP involves cross-linking of cells with formaldehyde, followed by cell lysis and sonication of the crude cell extracts, fragmenting the DNA to ~300–400 bp on average. The protein of interest is then immunoprecipitated, together with cross-linked DNA, the cross-links are reversed with heat, and the DNA is purified (Fig. 1). Hence the genomic regions that were bound by the protein of interest at the moment the formaldehyde was added to the cells will be specifically enriched. The levels of different genomic regions can then be measured using quantitative PCR and, typically, signals are reported as the enrichment of the region of interest relative to a control region.

ChIP has been combined with microarrays to create the ChIP-chip technique (sometimes referred to as ChIP-on-chip or ChIP^2) (Buck and Lieb, 2004). ChIP-enriched DNA is hybridized to a microarray and compared with a genomic DNA control or a mock immunoprecipitation control. This allows quantitative measurement of protein–DNA interactions across entire genomes. This powerful method has been used extensively to study protein–DNA interactions in eukaryotes. Until recently, ChIP-chip had been applied very little to bacteria, which, because of their small genome sizes, are ideally suited to methodologies involving microarrays. Advances in genome engineering permit rapid epitope tagging of proteins in many bacteria (Uzzau *et al.*, 2001; Court *et al.*, 2002; Cho *et al.*, 2006). Hence proteins for which no antibody is available can readily be studied using ChIP-chip.

Here we discuss the advantages of ChIP-chip over other techniques, we review advances in the study of bacterial protein–DNA interactions, and we suggest potential future applications.

Advantages of ChIP and ChIP-chip

In the past, bacterial DNA-binding proteins have been studied using a combination of genetic and biochemical approaches but ChIP has advantages over these methods. Unlike genetic analyses, ChIP does not require mutant cells, eliminating the possibility of indirect effects, and allowing the study of essential proteins. ChIP directly measures both the position and strength of protein–DNA interactions in living cells, in contrast to results from biochemical methods that may not accurately reflect the *in vivo* situation. Addition of formaldehyde to cells rapidly

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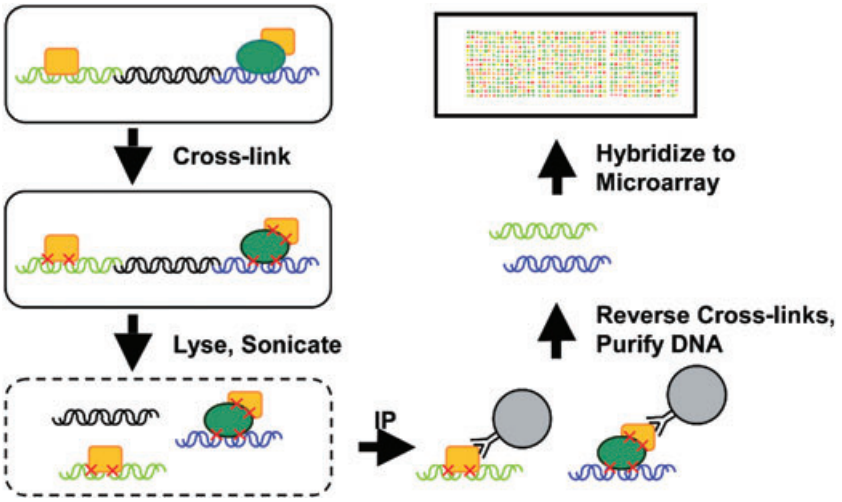

Fig. 1. Schematic of ChIP-chip procedure. Cells are grown, cross-linked with formaldehyde and lysed. DNA is fragmented by sonication. The protein of interest is then immunoprecipitated, enriching for that protein and any DNA that is cross-linked either directly or indirectly to that protein. Cross-links are reversed with heat and the DNA is purified and hybridized to a DNA microarray.

cross-links cellular components, generating a 'snapshot' of the cell. Hence ChIP can be used over short time-courses. *In vivo* footprinting can also identify protein–DNA interactions *in vivo*, but this often requires mutant cells, is dependent on the identification of a clearly resolved signal, and cannot be easily applied to whole genomes. Other advantages of ChIP are that it can be used to detect protein–DNA interactions that are indirect (i.e. occurring via protein–protein interactions), and can be applied specifically to post-translationally modified forms of a protein. One disadvantage of ChIP is that it has a maximum resolution of ~50 bp which is lower than that of footprinting analyses.

ChIP is a powerful method for studying protein–DNA interactions. ChIP-chip, however, can address many questions that cannot be answered using ChIP alone, by measuring all protein–DNA interactions on a genomic scale. The majority of whole-genome studies of DNA-binding proteins have used transcript profiling, i.e. comparing the transcriptome in wild-type and mutant cells using microarrays. However, transcript profiling measures the consequences of binding of a protein rather than its actual binding and, thus, can be complicated by secondary effects on gene expression caused by mutating the gene of interest. ChIP-chip, in contrast, provides an absolute measure of protein–DNA interaction.

ChIP-chip of sequence-specific transcription factors

The genomic targets of almost all sequence-specific transcription factors in yeast have been determined using ChIP-chip (Lee *et al*., 2002; Harbison *et al*., 2004), as have the targets of several transcription factors in mammalian cells (Cawley *et al*., 2004; Boyer *et al*., 2005), providing many important insights into the functions of these proteins. The first use of ChIP-chip to study protein–DNA interactions in a bacterium was that of Laub *et al*. (2002) that identified 95 promoter targets of the transcription factor CtrA, a key cell-cycle regulator in *Caulobacter crescentus*. These included a surprising number of promoters for other transcriptional regulators. Equivalent studies have now been performed for several sequence-specific transcription factors in bacteria: Fur in *Helicobacter pylori* (Danielli *et al*., 2006), CodY (Molle *et al*., 2003a), Spo0A (Molle *et al*., 2003b) and SpoIIID (Eichenberger *et al*., 2004) in *Bacillus subtilis*, and MelR (Grainger *et al*., 2004), FNR (Grainger *et al*., 2007), CRP (Grainger *et al*., 2005), NsrR (S. Spiro, pers. comm.) and LexA (Wade *et al*., 2005) in *Escherichia coli*.

ChIP-chip can be used to study any DNA-binding protein, ranging from the *E. coli* transcription factor MelR that binds at single genomic locus (Grainger *et al*., 2004), to global regulators such as the *E. coli* transcription factors LexA and CRP that bind many genomic targets (Grainger *et al*., 2005; Wade *et al*., 2005). In addition to identifying novel genomic targets for DNA-binding proteins, these studies can reveal fundamental biological phenomena. For example, the ChIP-chip study of LexA revealed that essentially all matches to the LexA consensus sequence were bound by LexA *in vivo*, indicating that the entire *E. coli* genome is permissive to transcription factor binding, resolving a long-standing question (Wade *et al*., 2005).

The ChIP-chip approach also permits DNA binding profiles for a given factor to be compared under different conditions (Grainger *et al*., 2007). Such comparisons are not possible using indirect methods, such as transcriptional profiling and DNA sequence analysis. This work has demonstrated that some transcription factors bind under both activating and non-activating conditions, e.g. MelR ± melibiose (Grainger *et al*., 2004), whereas others bind only under activating conditions, e.g. FNR ± O_2 (Grainger *et al*., 2007).

Identifying DNA sequence motifs

ChIP-chip studies can lead to the identification of DNA sequence motifs that are bound by the protein of interest. This is particularly useful for determining the DNA-binding properties of uncharacterized transcription factors. Standard motif-searching algorithms such as AlignACE, MEME, BioProspector and MDScan (Liu *et al*., 2004) identify DNA sequences that are common to the most highly bound regions (Molle *et al*., 2003b; Eichenberger *et al*., 2004; Ben-Yehuda *et al*., 2005; Grainger *et al*., 2005; 2007; Wade *et al*., 2005; 2006). These methods determine a consensus binding site in the form of a matrix, rather than a single sequence, allowing for more sophisticated DNA sequence analyses. Thus, it is often possible to determine the precise genomic location of binding even when using microarrays with large PCR products. In cases where the protein of interest does not have an identifiable consensus sequence or binds to non-consensus sites, the position can be more accurately mapped using high-density oligonucleotide microarrays that are fully tiled (i.e. every base pair of the non-repetitive genome is represented at least once on the microarray) (Herring *et al*., 2005; Reppas *et al*., 2006).

Sequence-specific transcription factors often bind non-consensus sites

A recurring and unexpected feature of ChIP-chip analyses for sequence-specific transcription factors is that proteins often bind to non-consensus sites *in vivo*. For instance, many CtrA (*C. crescentus*), LexA (*E. coli*) and FNR (*E. coli*) targets identified by ChIP-chip do not contain a good match to the consensus sequence (Laub *et al*., 2002; Wade *et al*., 2005; Grainger *et al*., 2007), and 15% of *B. subtilis* Spo0A targets identified by ChIP-chip are not bound in an *in vitro* binding assay (Molle *et al*., 2003b). This strongly suggests that many bacterial sequence-specific transcription factors can bind to targets containing degenerate consensus sequences. Further analysis of one such non-consensus DNA target for LexA revealed an unconventional DNA site that is related to, but fundamentally different from the consensus sequence (Wade *et al*., 2005). A likely explanation for this phenomenon is that multiple transcription factors may bind cooperatively to adjacent DNA sites, in a manner akin to transcription factors binding to enhancers in mammalian cells. Cooperative interactions between transcription factors could reduce the requirement for consensus sequences, or perhaps alter the sequence preference of individual transcription factors. Alternatively, differences in local DNA topology might influence the sequence preference of transcription factors.

Transcription factors often bind DNA sites with no known function

Surprisingly, for many transcription factor targets identified by ChIP-chip there is no detectable effect on transcription of the neighbouring gene(s) when the transcription factor is deleted or depleted. Hence, ChIP-chip has identified many unexpected protein–DNA interactions that could not be identified using transcript profiling. For example, targets for CtrA and FNR were found upstream of genes whose transcription did not alter significantly in cells lacking these proteins (Laub *et al*., 2002; Grainger *et al*., 2007). Also, transcription of 36% of genes adjacent to LexA targets was unaltered following UV treatment that results in rapid LexA degradation (Wade *et al*., 2005). This may be due to a number of reasons:

(i) The promoter at which the transcription factor is functional has not been identified. Thus, deletion of the transcription factor may result in transcript changes that have not been detected simply because the gene has not been identified. Consistent with this, novel mRNAs and novel promoters have been identified using both ChIP-chip and transcriptomic approaches (Herring *et al*., 2005; Reppas *et al*., 2006; Wade *et al*., 2006).

(ii) The transcription factor may play roles other than in regulating transcription, e.g. as a nucleoid-associated protein (NAP) that controls chromosome organization (Dame, 2005). Hence some transcription factor binding sites may not influence the level of transcription of the neighbouring gene.

(iii) The transcription factor may function only in specific contexts. For example, many transcription factors bind to their DNA targets under both activating and non-activating conditions. If the transcription factor is deleted, under non-activating conditions there will be no effect on transcription of the corresponding genes. Similar situations may occur when multiple transcription factors cooperate in the regulation of an individual gene.

(iv) The protein may be bound upstream of a gene where it has little impact on levels of transcription, perhaps because of the overriding influence of another regulatory protein or because protein's role is to 'tweak' levels of promoter activity.

(v) The transcription factor may bind to target sites with no functional relevance. Such sites are likely to exist because bacterial genomes are constantly evolving. As the *E. coli* genome, and presumably other bacterial genomes, are permissive to transcription factor binding, close matches to transcription factor consensus sequences that are created in functionally irrelevant locations by chance will be bound in all cases

(Wade *et al*., 2005). Hence the presence of such sites will be difficult to avoid without a specific mechanism to prevent or reverse this phenomenon.

ChIP-chip of nucleoid folding proteins

Bacterial chromosomes are packaged into nucleoid structures by NAPs, a group of histone-like proteins including Fis, H-NS, HU, IHF and StpA, that are conserved across most bacterial species (Dame, 2005). ChIP-chip is well suited to the study of NAPs as they generally bind DNA with low sequence specificity and it is difficult to predict genomic sites of association using sequence information alone. Recently, four groups have used ChIP-chip to study the genome-wide association of H-NS in both *E. coli* and *Salmonella*, with fascinating results (Grainger *et al*., 2006; Lucchini *et al*., 2006; Navarre *et al*., 2006; Oshima *et al*., 2006). They found that H-NS has a preference for binding A/T-rich DNA which results in preferential binding to foreign genetic elements (Lucchini *et al*., 2006; Navarre *et al*., 2006; Oshima *et al*., 2006). This results in specific silencing by H-NS of DNA acquired by lateral gene transfer, a process referred to a 'xenogeneic silencing'. Additionally, H-NS in *Salmonella* does not colocalize with RNA polymerase (RNAP), indicating that H-NS silences transcription by occluding the binding of RNAP (Lucchini *et al*., 2006). In contrast, H-NS binding in *E. coli* correlates somewhat with that of RNAP, both at promoters and within coding sequences (Grainger *et al*., 2006; Oshima *et al*., 2006). This supports the notion that H-NS can repress transcription by trapping RNAP at promoters (Dame *et al*., 2002) and that H-NS is deposited along genes as a result of active transcription. Grainger *et al*. (2006) showed that a second NAP, Fis, also associates with actively transcribed genomic regions, suggesting a similar role for Fis and H-NS in the structural organization of transcriptionally active DNA. IHF, on the other hand, does not associate with actively transcribed regions, and is predominantly bound at intergenic regions (Grainger *et al*., 2006).

ChIP-chip of RNAP identifies promoters and the organization of transcribed sequences

Several groups have used ChIP-chip to study the genomic distribution of RNAP and associated factors (Grainger *et al*., 2005; Herring *et al*., 2005; Lucchini *et al*., 2006; Oshima *et al*., 2006; Reppas *et al*., 2006; Wade *et al*., 2006; Rodrigue *et al*., 2007). Using ChIP-chip, Grainger *et al*. (2005) demonstrated that, in rapidly growing *E. coli*, the majority of RNAP associates with ~90 transcribed regions, indicating that most transcription occurs at a small fraction of genes. By adding rifampicin, a chemical that inhibits transcription elongation by blocking the RNA exit channel of RNAP, Grainger *et al*. (2005) and Herring *et al*. (2005) identified all potential promoters. This demonstrated that there are at least 1100 potential promoters in *E. coli*.

Similar studies by Reppas *et al*. (2006) determined the genomic association of both RNAP (β-subunit) and the predominant σ-factor, σ^{70}, in rapidly growing *E. coli*, using very high-density microarrays. This permitted identification of 1286 σ^{70}-dependent promoters and showed that, on average, RNAP spends ~50 times longer at a promoter than at a given position within the coding sequence. This report also compared binding profiles of RNAP and RNA levels directly for the first time. Unexpectedly, at almost a quarter of σ^{70}-bound promoters, the corresponding gene is not detectably transcribed, consistent with the results of Herring *et al*. (2005) who detected RNAP association with promoters that were predicted to be transcriptionally inactive.

Grainger *et al*. (2005; 2007) showed that ChIP-chip of RNAP can be used to study transcriptional changes induced by environmental or genetic perturbation. The authors demonstrated that treating cells with salicylic acid results in RNAP levels changing within genes identified as being regulated by salicylic acid in transcript profiling experiments (Grainger *et al*., 2005). Importantly, such experiments also allow the RNAP binding profile across transcribed regions to be measured. Hence, Reppas *et al*. (2006) showed that σ^{70} rarely associates with elongating RNAP *in vivo*. In stationary-phase *E. coli*, it was shown that not only is RNAP dramatically redistributed across different transcribed regions but also becomes more skewed towards promoters (Grainger *et al*., 2007). Thus, ChIP-chip of RNAP provides more information than transcriptional profiling, as it determines both the change in the absolute level of transcription and the distribution of RNAP across a transcribed region.

Substantial overlap of σ-factors revealed by ChIP-chip

In addition to studying σ^{70}, ChIP-chip has also been used to determine the genome-wide distribution of alternative σ-factors. Wade *et al*. (2006) determined the genome-wide association of an alternative σ-factor, σ^{32}, using ChIP-chip. Alternative σ-factors have historically been thought of as regulating largely distinct subsets of genes. By comparing the ChIP-chip data for σ^{32} with those for σ^{70}, Wade *et al*. (2006) showed that, surprisingly, the majority of σ^{32}-dependent promoters can also be transcribed by σ^{70}. This is also true for a second alternative σ-factor, σ^{E}. Thus, alternative σ-factors often share promoter targets with the 'housekeeping' σ-factor. Most ChIP-chip studies

of σ-factors have focused on *E. coli*, but a recent study identified novel targets for several different σ-factors in *Mycobacterium tuberculosis* (Rodrigue *et al*., 2007).

ChIP-chip of proteins not involved in transcription

All the ChIP-chip experiments described thus far involve proteins involved in transcription. However, ChIP-chip can be used to study any protein that associates with DNA. To date, only three bacterial proteins not involved in transcription have been studied using ChIP-chip (Jeong *et al*., 2004; Ben-Yehuda *et al*., 2005; Breier and Grossman, 2007). Two of these, RacA and Spo0J, are *B. subtilis* proteins involved in chromosome segregation following replication (Ben-Yehuda *et al*., 2005; Breier and Grossman, 2007). Ben-Yehuda *et al*. (2005) showed that RacA binds predominantly to origin-proximal sequences and is likely to play a crucial role in chromosome segregation by attaching the chromosome to the cell pole. Breier and Grossman (2007) showed that Spo0J also binds largely to origin-proximal sequences, although there are at least two binding sites for Spo0J located distal to the origin, and Spo0J can spread from sites of initial binding. Hence ChIP-chip has provided important insights into processes in bacteria other than transcription.

Future applications of ChIP-chip

The many remaining potential applications for ChIP-chip include the study of transcription, replication, DNA repair, recombination, chromosome segregation and chromosome organization. Some proteins are ideal candidates for ChIP-chip analysis. For example, it would be interesting to determine the genome-wide binding profiles of all seven *E. coli* σ-factors to subdivide the genome into different functional categories based on σ-factor preference, and to determine the degree of overlap between alternative σ-factors and the housekeeping σ-factor, σ^{70}. Similar studies could be performed in other bacteria, e.g. *B. subtilis*, which possesses 17 σ-factors, several of which are believed to be highly specific for particular developmental processes. Further studies of NAPs will be particularly informative in regard to how bacterial chromosomes are organized. This could be extended to other proteins that are involved in chromosome organization such as condensins.

We anticipate many more ChIP-chip studies of individual sequence-specific transcription factors. As these data accumulate it will be possible to compare the data sets for different transcription factors and identify groups of proteins that colocalize. An equivalent study in human cells revealed the unexpected colocalization of the Oct4, Sox2 and Nanog proteins (Boyer *et al*., 2005). In addition, it will be possible to compare these ChIP-chip data sets with transcript profiling data sets. These analyses have already been performed for individual transcription factors. However, a more powerful approach is to analyse these experiments on a large scale, incorporating data sets from many different studies. This approach can define complete transcriptional networks and has been used successfully for yeast transcription factors (Bar-Joseph *et al*., 2003).

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