[23] Direct Visualization of Transcription Factor-Induced Chromatin Remodeling and Cofactor Recruitment *In Vivo*

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Introduction

The application of chromatin immunoprecipitation procedures, as described elsewhere in this volume, has greatly facilitated *in vivo* measurement of recruitment of various transcriptional activators and cofactors to specific *cis* sequences in a wide range of biological systems. The impact on the field of chromatin structure and gene regulation has been tremendous. However, this method does have two specific limitations. Not all proteins will cross-link efficiently to nearby DNA sequences. More importantly, perhaps, chromatin immunoprecipitation is a biochemical procedure involving averaging among cells in a population, precluding single-cell observations. This is a particular limitation when cell heterogeneity is present and/or when fine temporal ordering of events is important. Similarly, biochemical and molecular probes for chromatin remodeling have been limited to methods that measure averages over cell populations.

Our laboratory and others have begun to develop assays for chromatin remodeling and transcription factor and coactivator recruitment based on direct, microscopic observations within individual live cells. The experimental basis for this approach involves the visualization of chromosome regions containing multiple transgene copies. To date, two approaches have been used. The first uses direct repeats of bacterial operators to bind repressor fusion proteins as a means of tethering specific transcription factors to specific chromosomal sites. The second uses repeats of transgenes containing viral promoters with binding sites for known transcription factors. We anticipate a natural experimental progression will be to combine both of these approaches, using the bacterial operator repeats for tagging the chromosome regions, while using transgene repeats containing specific promoters with known transcription factor-binding sites and reporter constructs for monitoring gene expression.

These methods promise to allow real-time visualization of transcription factor dynamics and their relationship to changes in chromatin structure and gene expression. Here, we review previous work using these methods. We then discuss key methodologies used in these experiments together with ongoing technological developments in our laboratory. We conclude

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with several example protocols for specific experimental procedures used in these studies.

Review of Previous Work

The ability to tag chromosomes with green fluorescent protein (GFP), using direct repeats of bacterial operators and operator/repressor recognition, has provided a highly sensitive capability for localizing and measuring the dynamics of specific DNA sequences within living cells.[1] First described for the lac operator/repressor system,[2,3] this approach has now been extended to the tet operator as well,[4] and applied to a wide range of organisms, including bacteria, yeast, *Caenorhabditis elegans, Drosophila, Arabidopis,* and mammalian cells.[1] Although this approach readily allows detection of single-copy insertions of a 256-copy lac operator direct repeat in mammalian cells (and as low as 25 repeats under favorable conditions in *Drosophila;* A. Belmont, unpublished results), it is also possible to create cell lines containing labeled chromosome regions of varying size up to an entire chromosome arm in length, either by selection of cells that have integrated high copy numbers of the transfected plasmid[5] or by a secondary process of gene amplification.[2]

The first demonstration of large-scale chromatin unfolding induced by the tethering of a transcriptional activator used a lac repressor–VP16 AAD (acidic activation domain) fusion protein.[6] The CHO cell line A03_1 contains a gene-amplified chromatin array approximately 90 Mbp in size. This array is formed by repeating blocks of transgene repeats, several hundred kilobase pair blocks in size, spaced by large coamplified regions of genomic DNA, roughly 1 Mbp in size. Each transgene copy contains a 256-copy lac operator direct repeat and a dihydrofolate reductase (DHFR) cDNA construct. This array appears in metaphase as a homogeneously staining region (HSR) and has properties of heterochromatin, forming a condensed mass roughly 1 μm in diameter through most of interphase.[7]

Targeting the lac repressor–VP16 AAD fusion protein resulted in a dramatic uncoiling of the condensed chromatin array into extended, ~80-nm-diameter chromonema fibers up to 25–40 μm in length that accompanied gene activation.[6] In a related experiment, tethering of the

[1] A. S. Belmont, *Trends Cell Biol.* **11,** 250 (2001).
[2] C. C. Robinett, A. Straight, G. Li, C. Willhelm, G. Sudlow, A. Murray, and A. S. Belmont, *J. Cell Biol.* **135,** 1685 (1996).
[3] A. F. Straight, A. S. Belmont, C. C. Robinett, and A. W. Murray, *Curr. Biol.* **6,** 1599 (1996).
[4] C. Michaelis, R. Ciosk, and K. Nasmyth, *Cell* **91,** 35 (1997).
[5] Y. G. Strukov, Y. Wang, and A. S. Belmont, *J. Cell Biol.* **162,** 23 (2003).

lac repressor–VP16 fusion protein to a peripherally located chromosome site containing the lac operator repeats resulted in this spot moving into the nuclear interior.[8] This initial work also demonstrated the ability to use this experimental system to detect *in vivo* recruitment of coactivators by the VP16 acidic activation domain. Histone H3 and H4 hyperacetylation was observed over the chromatin array, as well as recruitment of p300/CBP, PCAF, and GCN5, and splicing factors U2B and Sm100.[6] More recently, this work has been extended by using bead loading, rather than transient transfection, to load GFP–lac repressor–VP16 AAD directly into cell nuclei within several minutes.[9] This method was used to demonstrate asynchronous recruitment of different components of the SAGA histone acetyltransferase complex, and the mammalian SWI/SNF complexes. These results suggested that targeting of chromatin-modifying complexes to condensed chromatin may proceed through initial recruitment of "pioneer factors" consisting of individual components or partial subcomplexes that might act by themselves to produce initial chromatin unfolding.[9,10]

Similar approaches have now been taken using lac repressor fusions to other transcription factors to assay their effect on large-scale chromatin conformation. BRCA1, E2F1, and p53,[11] the estrogen receptor,[12] and several acidic activators including Gal4 and p65[13] have been demonstrated to have large-scale chromatin-unfolding activity. Interestingly, cancer-predisposing mutations in *BRCA1,* mapping to its BRCT 3′ repeats, were found to markedly enhance its chromatin-opening activity. When both the lac operator-tethered transcription factor and a recruited coactivator protein are labeled with color variants of GFP, recruitment can be visualized directly in living cells, as was done for the estrogen receptor and its coactivators SRC-1 and CBP.[12,14] This also allowed the kinetics and dynamics of recruitment to be observed and revealed weaker ligand independent interactions that had been overlooked in biochemical assays.

[6] T. Tumbar, G. Sudlow, and A. S. Belmont, *J. Cell Biol.* **145,** 1341 (1999).
[7] G. Li, G. Sudlow, and A. S. Belmont, *J. Cell Biol.* **140,** 975 (1998).
[8] T. Tumbar and A. S. Belmont, *Nat. Cell Biol.* **3,** 134 (2001).
[9] S. Memedula and A. S. Belmont, *Curr. Biol.* **13,** 241 (2003).
[10] C. L. Peterson, *Curr. Biol.* **13,** R195 (2003).
[11] Q. Ye, Y. F. Hu, H. Zhong, A. C. Nye, A. S. Belmont, and R. Li, *J. Cell Biol.* **155,** 911 (2001).
[12] A. C. Nye, R. R. Rajendran, D. L. Stenoien, M. A. Mancini, B. S. Katzenellenbogen, and A. S. Belmont, *Mol. Cell. Biol.* **22,** 3437 (2002).
[13] A. C. Nye, Ph.D thesis, Effects of transcription activators on a large-scale chromatin structure, Dept. of Cell and Structural Biology, University of Illinois, Urbana-Champaign, Urbana, 2003. *Work from ref. 13 is now in press:* A. E. Carpenter, A. Ashouri, and A. S. Belmont, *Cytometry,* in press.
[14] D. L. Stenoien, A. C. Nye, M. G. Mancini, K. Patel, M. Dutertre, B. W. O'Malley, C. L. Smith, A. S. Belmont, and M. A. Mancini, *Mol. Cell. Biol.* **21,** 4404 (2001).

An improved VP16 AAD targeting system was developed in the Spector laboratory by adding a protein reporter system to monitor gene activation simultaneous with chromatin conformation. This system also separated the lac operator/repressor chromosome visualization tag from the inducible transcription factor-targeting mechanism based on tet operator/repressor binding.[15] In this system, a significant lag between transcriptional initiation and detection of reporter protein exists, because of the time it takes for transcript buildup and protein accumulation. More recently, however, the Spector laboratory has added an RNA *in vivo* detection component to this system, incorporating repeats of a stem–loop structure in the RNA transcript to which bacteriophage MS2 protein binds.[16,17] Using this inducible system, it is now possible to simultaneously read out chromatin conformation and nascent RNA transcript accumulation within the 5–30 min following VP16 AAD targeting.[16] This represents a powerful system to investigate *in vivo* dynamics of the transcriptional machinery.

However, a major limitation of all the systems described above is that they rely on the tethering of a transcription factor to a 100- to 250-multicopy operator repeat. In a second experimental approach, a chromatin array containing multiple copies of a viral promoter was used to monitor changes in large-scale chromatin structure and coactivator recruitment after glucocorticoid receptor (GR) targeting. A GFP–GR fusion protein was expressed in a cell line[18] with ~200 copies of a plasmid containing the mouse mammary tumor virus (MMTV) promoter, with 4–6 GR-binding sites, upstream of the *ras* and bovine papilloma virus (BPV) genes.[19,20] Addition of ligand led to GR translocation to the nucleus and binding to the chromatin array. Recruitment to the array of GR coactivators SRC1 and CBP together with BRG1 and transcription factors NF1 and AP-2 accompanied targeting of GR.[18] Ligand-dependent GR targeting led to a cycle of large-scale chromatin decondensation and then recondensation, which roughly correlated with transcriptional activation and then downregulation.[18] A maximum fold induction of gene expression by GR of between 20 and 40 min correlated with maximum recruitment of GFP–pol2 over the array.[21]

[15] T. Tsukamoto, N. Hashiguchi, S. M. Janicki, T. Tumbar, A. S. Belmont, and D. L. Spector, *Nat. Cell Biol.* **2,** 871 (2000).
[16] D. L. Spector, T. Tsukamoto, E. Bertrand, D. Fusco, R. Singer, and S. M. Janicki, *Mol. Cell. Biol.* **13** (Suppl.), 2a (2002).
[17] E. Bertrand, P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer, and R. M. Long, *Mol. Cell* **2,** 437 (1998).
[18] W. G. Muller, D. Walker, G. L. Hager, and J. G. McNally, *J. Cell Biol.* **154,** 33 (2001).
[19] P. R. Kramer, G. Fragoso, W. Pennie, H. Htun, G. L. Hager, and R. R. Sinden, *J. Biol. Chem.* **274,** 28590 (1999).
[20] D. Walker, H. Htun, and G. L. Hager, *Methods* **19,** 386 (1999).

While providing a more physiological context by allowing analysis of chromatin remodeling and factor recruitment by real promoters, one technical difficulty of this second approach is in visualizing the chromosomal array in the absence of the fluorescently tagged *trans* factor. In the MMTV/GR experiments described above, the chromosomal array could not be examined before GR targeting, and the signal was sufficiently bright to allow detection of the chromosomal array in only a fraction of the cells after GR targeting. Similarly, many coactivators are present in various nuclear compartments and bodies at significant levels; therefore, differentiation between the chromosomal array and other nuclear concentrations of a given cofactor may be difficult. A natural progression for these *in vivo* methods will be to combine the two approaches, using natural promoters together with a direct operator repeat as a tag for the chromosomal array.

Methodology

Basic Approach

Our previous work used the lac operator/repressor system for transcription factor tethering. This required several experimental components. First, we needed to generate and subclone the lac operator repeat. Inverted repeats are highly unstable in bacteria. We therefore used a directional cloning method to force direct repeats of the lac operator, doubling the number of repeats with each cloning cycle.[2] Recombination between repeats was a significant problem in typical host cells. Reaching high copy numbers of lac operator repeats therefore required use of a special recA minus *Escherichia coli* strain designed for increased stability of direct repeats, switching to a moderate or low copy number plasmid at later stages of repeat amplification, and using nonstandard protocols for plasmid preparation. A detailed description of these methods for working with lac operator repeats has been published elsewhere.[22]

Second, we needed GFP–lac repressor fused with transcriptional activation domains. We have typically fused proteins at the 3′ end of the GFP–lac rep protein-coding region, mainly for cloning convenience. A unique *Asc*I restriction site was engineered downstream of the lac repressor and before the stop codon to facilitate convenient cloning of PCR products.[12] Caution should be exercised when designing a cloning scheme, as we have discovered that short (~10 amino acid) stretches rich in acidic and

[21] M. Becker, C. Baumann, S. John, D. A. Walker, M. Vigneron, J. G. McNally, and G. L. Hager, *EMBO Rep.* **3,** 1188 (2002).
[22] A. S. Belmont, G. Li, G. Sudlow, and C. Robinett, *Methods Cell Biol.* **58,** 203 (1999).

hydrophobic residues that resemble acidic activators are sufficient to direct dramatic large-scale chromatin unfolding.[13] This is consistent with previously published reports that random peptides with such properties can serve as potent transcriptional activators.[23,24] Therefore, linker regions should be avoided, or if used, a GFP–lac repressor fused to the linker alone should be used as control construct.

A significant number of lac repressor fusion proteins are inactive. Therefore, it is critical that functional assays be performed on lac repressor fusion proteins. In the case of transcriptional activators or repressors, the obvious functional assay is a direct measure of activation or repression, using a reporter system. We now typically use a transient transfection assay using a lac operator 8-mer adjacent to a minimal TATA box core promoter driving a luciferase reporter construct.[13] Additional functional criteria that can be used would include correct intranuclear localization, cofactor recruitment, and, where appropriate, normal ligand dose–response curves.[12] The ultimate functional test would be genetic complementation of the wild-type transcription factor. This has been used for a lac repressor–HP1 fusion protein.[25]

The third requirement is the generation of stable cell lines containing integrated plasmid arrays containing the lac operator repeats. In general, we created these cell lines in cells not expressing lac repressor constructs, giving us the flexibility of then expressing a variety of different lac repressor fusion proteins in a particular cell line. Two approaches can be used to generate large chromosomal arrays containing the transfected plasmids. The first is to select stable transformants with large copy numbers of the transfected plasmid. Alternatively, one can start with a given plasmid insertion and then use gene amplification to further increase the size of the chromosomal array. Detailed descriptions and protocols for both methods of cell transformation and cloning have been published elsewhere.[26]

The final step is to express the GFP–lac repressor fusion protein in the cell, detect the chromosomal array, and monitor cofactor recruitment. We typically have used transient transfection to examine steady state levels of large-scale chromatin decondensation and cofactor recruitment.[6,9] For time courses of opening and factor recruitment, we have used ligand-dependent inducible systems[12] or bead loading as a method for rapidly

[23] D. M. Ruden, J. Ma, Y. Li, K. Wood, and M. Ptashne, *Nature* **350,** 250 (1991).
[24] K. Melcher, *J. Mol. Biol.* **301,** 1097 (2000).
[25] Y. Li, J. R. Danzer, P. Alvarez, A. S. Belmont, and L. L. Wallrath, *Development* **130,** 1817 (2003).
[26] Y. G. Strukov and A. S. Belmont, *in* "Live Cell Imaging: A Laboratory Manual" (D. L. Spector and R. D. Goldman, eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2003.

introducing a recombinant protein into the cell.[9] Factor recruitment can be done either with antibodies to detect endogenous proteins or with GFP- or epitope-tagged transfected proteins. The use of GFP-tagged coactivator proteins provides the unique capability of monitoring coactivator recruitment in live cells. However, there are always concerns when expressing exogenous proteins, particularly by transient transfection methods, with regard to mislocalization and alterations in binding kinetics due to protein overexpression. We have preferred to conduct initial work with antibodies to monitor the distribution of endogenous proteins, reserving the use of GFP-tagged coactivators for more specific follow-up experiments.

Ongoing Methodology Development

pSP Plasmids. Our laboratory is moving to combine the two approaches used to date for *in vivo* analysis of transcription factor-mediated changes in large-scale chromatin structure and cofactor recruitment. The idea is to separate the chromatin tag, using lac operator or other repeats, from the targeting of transcription factors, allowing the use of natural promoters. At the same time, where appropriate, protein and/or RNA reporter constructs can be included for direct visualization of gene expression,[15,16] as described earlier.

Toward this end, the pSP plasmids, which contain a synthetic 258-bp polylinker, were designed with several intended goals.[13] First, they contain a large number of unique restriction sites, many of them rare, 8-bp recognition sequences, to facilitate cloning of multiple components including upstream regulatory elements, promoter, reporter gene, repetitive DNA-binding sequences (such as lac operators), and a selectable marker. Because of the large number of unique rare restriction sites, the design of the polylinker allows cloning of four components, up to ~20 kb, by simple cloning methods. Although we have designed the polylinker for generating plasmids to make stable cell lines, it is also useful for any cloning project where several large inserts must be combined.

Second, the plasmid contains a combination of restriction sites allowing directional cloning of repeats. In the first step, a DNA sequence of interest is cloned into the blunt *Pme*I site. The *Spe*I and *Nhe*I sites surrounding *Pme*I have complementary cohesive ends that do not recreate either restriction site when ligated together. Multiple copies can be cloned by cutting out the DNA sequence of interest, using *Nhe*I and *Asc*I, and ligating it into the *Spe*I and *Asc*I sites of a separate aliquot of the same plasmid. In this way, the number of DNA sequences of interest can be doubled in each round of cloning, as was done previously to generate 256 tandem repeats of the lac operator sequences.[2] In addition, *Sal*I, *Xho*I, and any nearby unique

site can be used for directionally cloning a second set of repeats of a DNA sequence. Alternatively, existing lac operator repeats flanked by *Xho*I and *Sal*I sites can be cloned into these sites.

Third, because the plasmid must stably carry repetitive DNA sequences, it must be present at low copy within bacteria in order to prevent recombination and therefore loss of some repeats. We chose the pMB1 origin of replication, which is used in the plasmid pBR322. This origin of replication includes the *rop* gene, which is responsible for maintaining the plasmid at about 20 copies per cell.[27] Last, because plasmids become more difficult to clone as they approach 20 kb in size, we made the plasmid as small as possible. At under 3 kb, the pSP2 plasmid is able to contain the average 13–16 kbp of inserts required for the typical project.

We have used this pSP plasmid for a number of different constructs containing different combinations of regulatory elements, promoters, and reporter genes, together with lac operator repeats.[13] The mammalian selectable marker chosen for these plasmids was puromycin.[28] Because this is an uncommon marker, it allows other plasmids with different markers to be stably cotransfected into mammalian cells. Puromycin also allows cells to survive even with low-level expression of the puromycin resistance gene, which might prevent bias of clones toward integration sites with high-level expression.

For the repetitive DNA-binding sites, we inserted previously constructed 64- and 256-copy lac repressor-binding sites.[2] Several promoters were inserted: the simian virus 40 (SV40) promoter and enhancer, which has high transcriptional activity; the cytomegalovirus (CMV) core promoter, which has high transcriptional activity, although not as high as the entire CMV promoter; the F9 polyoma promoter, which has moderate activity; and the vitellogenin B1 TATA[29] and the E1b TATA, both of which have low basal activity but are capable of induction when strong activators are tethered nearby. Although we did not directly compare these two TATA-based reporters, preliminary tests indicated that the E1b TATA is capable of about 2-fold higher induction than the vitellogenin B1 TATA.

We chose several reporter genes: CFP-PTS and YFP-PTS are derivatives of the green fluorescent protein that are cyan and yellow, respectively. Fluorescent proteins are also used to label the lac operator array in the nucleus, so as to prevent overlap of the lac operator spots in the nucleus with the reporter genes, CFP-PTS and YFP-PTS are tagged with a

[27] M. Lusky and M. Botchan, *Nature* **293,** 79 (1981).
[28] S. de la Luna, I. Soria, D. Pulido, J. Ortin, and A. Jimenez, *Gene* **62,** 121 (1988).
[29] T. C. Chang, A. M. Nardulli, D. Lew, and D. J. Shapiro, *Mol. Endocrinol.* **6,** 346 (1992).

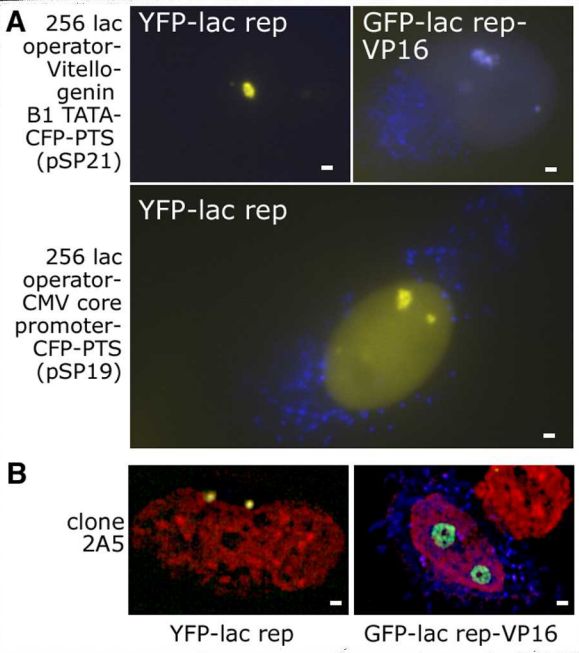

FIG. 1. Testing pSP plasmids in transient transfections. (A) CFP-PTS observed by microscopy. pSP plasmids were transfected into wild-type CHO-K1 cells along with YFP–lac rep or GFP–lac rep–VP16. GFP bleeds through in the CFP channel, so the GFP–lac rep–VP16 protein is not distinctly visible. Single optical sections are shown. Scale bar: 1 μm. (B) Stable cell lines containing pSP plasmids. Chromatin unfolding and reporter activation in response to VP16 activator in stable clone 2A5 with the 256 lac op–vitellogenin B1 TATA–CFP-PTS plasmid (pSP21) integrated into the genome. DNA stained with DAPI is shown in red, the YFP channel is shown in green, and the CFP channel is shown in blue. A single optical section from a deconvolved image series is shown. Scale bar: 1 μm. (See color insert.)

peroxisome-targeting signal.[15,30] This tag directs the fluorescent proteins to the peroxisomes, which appear as a pattern of spots in the cytoplasm. These reporters are compatible with live, individual cell observations by microscopy or flow cytometry. For more routine analysis of cell populations, the luciferase reporter is more convenient.

During plasmid construction, we tested pSP plasmids by transient transfection; this represents a significant time saving over generating stable cell lines. For plasmids with a fluorescent CFP-PTS or YFP-PTS reporter gene, transiently transfected CHO-K1 cells were observed by microscopy (Fig. 1A). For example, the 256 lac op–vitellogenin B1 TATA–CFP-PTS plasmid (pSP21) was found to show low CFP-PTS reporter expression

[30] S. J. Gould, G. A. Keller, and S. Subramani, *J. Cell Biol.* **107,** 897 (1988).

when cotransfected with YFP–lac rep. In contrast, the plasmid showed strong CFP-PTS expression in most transfected cells when cotransfected with GFP–lac rep–VP16 acidic activation domain, a strong transcriptional activator. When the constitutively active 256 lac op–CMV core–CFP-PTS plasmid (pSP19) was transiently cotransfected with YFP–lac rep, nearly 100% of transfected cells expressed CFP-PTS (Fig. 1A).

Interestingly, despite the fact that the plasmid was not integrated into the genome, it was possible to see some structure of the plasmids even in the transiently transfected state. Aggregates of the 256 lac op–vitellogenin B1 TATA–CFP-PTS plasmid (pSP21) detected by YFP–lac rep were typically condensed whereas plasmid aggregates detected by GFP–lac rep–VP16 were usually less condensed. Although not as dramatic, these results mimic the results previously seen with stably integrated plasmids.[6] It is known that transiently transfected DNA takes on some, but not all, properties of plasmids integrated into chromatin.[31]

We also examined the behavior of transiently transfected plasmids by flow cytometry and by luciferase assays, where appropriate.

Generation of Stable Cell Lines. Multiple copy repeats are subject to transgene silencing, a phenomenon still not well understood. Work is in progress to find transformation conditions that minimize the effects of transgene silencing and increase the fraction of chromosome arrays that show inducible expression. In a previous study that used 96 copies of the tet operator to target the VP16 AAD to a chromosome array, only 3 of 71 stable transformant clones showed both good growth and inducible expression of the reporter gene.[15] Using the pSP21 plasmid, containing the 256 lac op–vitellogenin B1 TATA–CFP-PTS plasmid, only 1 of 200 clones showed under basal conditions a large, condensed chromosome array that also visibly unfolded and showed significant inducible expression when transfected with a GFP–lac repressor–VP16 AAD expression vector. This was the 2AS cell line containing two large chromosome arrays per nucleus (Fig. 1B).

The pSP plasmid contains a low-copy origin of replication. The *rop* gene sequences that make the plasmid low-copy are called "poison" sequences because they have been found to reduce expression of transfected proteins when transfected into mammalian cells.[27,32] We tested the effects of removal of this sequence, using the 256 lac op–vitellogenin B1 TATA–CFP-PTS plasmid (pSP21). Transfection of linear DNA increased the proportion of stable transformants showing both a large, visible chromosome array and inducible expression after expression of GFP–lac

[31] C. L. Smith and G. L. Hager, *J. Biol. Chem.* **272,** 27493 (1997).
[32] D. O. Peterson, K. K. Beifuss, and K. L. Morley, *Mol. Cell. Biol.* **7,** 1563 (1987).

repressor–VP16 AAD, with 4 of 18 positive colonies from 0.6 μg of linear DNA with the poison sequences removed versus 3 of 50 from 5 μg of intact, supercoiled plasmid.[13]

Therefore, removal of these prokaryotic poison sequences appears to improve efficiency of inducible chromosome array isolation. A second improvement we have found is to cotransfect plasmid DNA with genomic DNA. Using the 256 lac op–CMV core–CFP-PTS plasmid (pSP19), we found that cotransfection of genomic DNA increased CFP-PTS expression. Greater than 80% of stably transfected cells cotransfected with genomic DNA expressed the CFP reporter versus ~50% without genomic DNA in cells expressing GFP–lac repressor. Of 18 stable colonies with visible chromosomal arrays, 15 showed CFP expression in cells expressing YFP–lac repressor.

Automated Microscopy of Chromatin Structure and Coactivator Recruitment. Our initial work used qualitative or semiquantitative measures of array opening and/or coactivator recruitment. For instance, percentages of cells with small or large array opening or weak or strong coactivator recruitment were typical measures.[6,9,11] Even for these rough classification schemes, it became important to collect statistics on large numbers of cells, which was time consuming and tedious when working with transient transfection efficiencies of 0.5–5%. Later work used quantitative measures of array size to better analyze experimental results.[9,12] To analyze a larger number of proteins and their mutant versions for effects on large-scale chromatin structure, it became clear that manual microscopy was not sufficiently high-throughput.

We therefore have adapted a commercially available motorized microscope and software package to automate data collection and analysis. The program we developed runs unattended, scanning a slide in search of the 0.5–5% of cells expressing the transiently transfected, fluorescently labeled lac repressor.[13] It finds the optimal focus and exposure for the chromosome array, collects images, and measures the array size. We further adapted this automated routine to analyze not only the size of the lac repressor-labeled chromatin array but also the recruitment of a second protein to that region of the nucleus, using two different colors of GFP.[13] An algorithm determines the ratio of fluorescence intensity at the spot versus fluorescence intensity in the remainder of the nucleus to determine quantitatively the extent of colocalization. This automation made possible the analysis of chromatin unfolding and recruitment of 25 different fusion proteins within a matter of weeks, with each data point, duplicated in two independent experiments, consisting of measurements of 100–200 transiently transfected nuclei.[13]

Using such an automated data collection approach should greatly facilitate the adaptation of these cell systems to provide more general assays of chromatin remodeling and coactivator recruitment.

Selected Protocols

Directional Cloning in pSP Plasmids

Note: Tris = 10 m*M* Tris-HCl, pH 8.0 or 8.5.

1. Check that the promoter of interest lacks *Nhe*I, *Spe*I, and *Asc*I sites. Clone the promoter into the blunt *Pme*I site of pSP12 and check the orientation, noting that the reporter gene runs counterclockwise (3′ to 5′). Clone enhancers of interest into the *Pme*I site of pSP21 or pSP32, which already contain TATA core promoters. Check that the promoter works in this context with a transient transfection.
2. Prepare the vector for cloning, using the plasmid resulting from step 1, which contains one copy of the promoter.
   a. Digest ~2 μg of the plasmid with *Spe*I (in buffer H from Roche Molecular Biochemicals, Indianapolis, IN) for 2 h at 37°. Check an aliquot on a gel to make sure the digest is complete. *Spe*I is allowed to digest alone before the *Asc*I digest (step 2c) because *Spe*I may not cut well near the end of DNA. That is, if *Asc*I cuts first, the *Spe*I site will be close to the end of the fragment and may not be cut.
   b. Purify DNA on a cleanup column (e.g., QIAquick PCR purification column; Qiagen, Hilden, Germany). Elute in ~40 μl of Tris.
   c. Digest with *Asc*I (in NEB4 buffer; New England BioLabs, Beverly, MA) overnight at 37°.
   d. Heat kill the *Asc*I at 65° for 20 min.
   e. Reserve 2 μl of phosphorylated vector to ligate as a control. If it is truly cut by both *Spe*I and *Asc*I, it should not yield any colonies. Dephosphorylate the remainder with shrimp alkaline phosphatase (SAP; Roche Molecular Biochemicals) for ~4 h. Dephosphorylation is theoretically not necessary because the vector should have incompatible ends, but there appears to be always some vector that is cut by only one enzyme.
   f. Purify the DNA on a cleanup column or run on an agarose gel, excise without exposing to UV light (see Generating Stable Cell

Lines, below), and extract with a QIAquick gel extraction column. Elute in 30 μl of Tris.

3. Prepare the insert for cloning, using the plasmid resulting from step 1, which contains one copy of the promoter.
   a. Digest ~4 μg of the plasmid with *Nhe*I and *Asc*I (in NEB4 buffer) for 2 h at 37°.
   b. Run the DNA on an agarose gel, excise the fragment without exposing to UV light, and extract with a QIAquick gel extraction column. Elute in 30 μl of Tris.
4. Run 2 μl of vector and 2 μl of insert on a miniature gel to compare their concentrations.
5. Ligate. As controls, ligate vector plus ligase, and phosphorylated vector plus ligase.
6. Check the colonies for the appropriate insert. Because pSP is a low-copy plasmid, prepare 1.5 ml of bacterial culture and use about half of the 50-μl product for each diagnostic digest.
7. After obtaining a plasmid with two copies of the promoter, repeat the cloning to obtain 4 copies, 8, 16, 32, and so on. To obtain enough DNA for cloning, prepare 5 ml of culture, using one QIAquick spin miniprep column, or use the chloramphenicol amplification procedure.[33] Recombination and loss of repeats are problematic for large numbers of repeats (roughly eight or more), so the chloramphenicol amplification procedure should not be used and STBL2 cells from Invitrogen (Carlsbad, CA) should be used and grown at 30°.

Generating Stable Cell Lines

1. *Test pSP plasmid in a transient transfection.* Transfect the pSP plasmid with transcription factors of interest to make sure that the reporter responds appropriately and strongly, given that most plasmids will have much lower expression when stably integrated than when transiently transfected. pSP19 (CFP-PTS reporter) or pSP32 (luciferase reporter) can be used as a positive control: these plasmids should express the reporter in nearly all transfected cells. Cotransfect a plasmid that allows detection of which cells are transfected, for example, YFP–lac rep or CMV–β-galactosidase.
2. *Determine transfection conditions.* Optimize transfection conditions for the cell line of choice, using a convenient constitutively active reporter (e.g., CMV–β-galactosidase or GFP). Choose the transfection reagent, the amount of reagent and DNA to use, and the size of the dish. We use 60-mm dishes, 2 μg of DNA, 40 μl of FuGENE6 (Roche Molecular Biochemicals), and 300 μl of serum-free medium for the easily transfectable CHO-K1 cell line. Other cell lines would probably require a 75- or 150-cm^2 flask.
3. *Test sensitivity of the cell line of interest to puromycin.* Transfect cells with pPUR (BD Biosciences Clontech, Palo Alto, CA; contains puromycin selectable marker) as a positive control and some other DNA (e.g., pUC19) as a negative control. The day after selection begins, cells can be passaged to a larger flask if necessary. Passaging 1 day after selection seems to get rid of nonexpressing cells quickly, because dying cells do not reattach. The minimal concentration of puromycin should be used because the selection reagent slows the growth even of resistant cells. The concentration selected for CHO-K1 cells was 7.5 μg/ml. Puromycin (Sigma, St. Louis, MO) is dissolved in H_2O. Store aliquots at −20°.
4. *Prepare pSP DNA for transfection.*
   Make DNA. Preparing DNA of pSP plasmids is not an easy task, because the 256 lac operators are unstable. For this reason, the plasmid is low-copy (to minimize recombination) and is maintained in STBL2 cells (GIBCO/Life Technologies, Grand Island, NY), which exhibit repeat-stabilizing properties when grown at 30°. To grow a culture for DNA preparation, streak from a frozen glycerol stock or a stab culture onto an LB-Amp plate and grow overnight at 30°. The next day around noon, pick 10–20 colonies into 2–3 ml of liquid LB-Amp cultures and grow at 30° overnight. The next day, prepare 1.5 ml of culture, digest 40 of the 50 μl of plasmid DNA in a 50-μl restriction digest that should cut on either side of the lac operator repeats, and run the digests on a gel (with fairly large wells to hold the large volume). Choose the cultures that have full-length repeats and use the leftover "miniprep" culture to inoculate a large culture. Grow at 30° overnight. It is best to choose two different positive clones and do one 60-ml culture for one Bio-Rad (Hercules, CA) "midiprep" for each in case one of the cultures loses its repeats in culture overnight, although this rarely occurs. Elute the midiprep DNA, using 400 μl of hot 10 m*M* Tris-HCl, pH 8.5. Each midiprep usually yields 100–300 μg of DNA, using the instructions for a low-copy plasmid. Do not use larger culture volumes than suggested.
   Remove poison sequences. If it is desirable to eliminate the poison sequences, digest the pSP plasmid with enzymes cutting at sites on either side of the DNA of interest (e.g., *Bgl*II and *Fse*I, or an *Xmn*I

[33] J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

digest, as long as the enzymes do not cut within the promoter of interest). Run the DNA on a low-percent (0.75%) SeaPlaque GTG gel (Cambrex Bioscience, Rockland, Rockland, ME), which should be poured and allowed to set at 4° because it will be flimsy. The gel can be run at room temperature. Fresh agarose and Tris–acetate–EDTA (TAE) should be used in a clean gel box, to avoid contamination of the sample. It is helpful to cut out a minimal gel slice to aid purification later. Avoid exposing the DNA to UV light or ethidium bromide when cutting out the large band containing the pSP fragment of interest. Once the band has been excised, there are two options.

a. Heat the agarose at 65° for 10 min or until melted and transfect the unpurified DNA. In this case, there is no need to check the concentration if the amount of DNA loaded onto the gel is known and if the majority of the band has been cut.
b. Use a QIAEX II gel extraction kit. Use 30 μl of reagent. Do not vortex the pellet—gently flick to avoid shearing. Use hot elution buffer to elute the large DNA fragment. Elute twice. Because yields of 5–20% are expected for a fragment of this size, this method requires that a great deal of DNA be run on the gel. In addition, the concentration of the DNA fragment should be checked before transfection.

   Option (a) is recommended because it is much easier and provides much higher yields of DNA. We obtained similar size arrays with both protocols. There is, of course, the possibility of contamination when transfecting the unpurified melted agarose gel, whereas the QIAEX II protocol includes an ethanol wash. In practice, the nonsterile melted gel does not produce contamination as long as antibiotics are present in the cells' medium.

5. *Transfect cells with the linear fragment or supercoiled plasmid to make a stable cell line.* Use the conditions determined in steps 2 and 3, including positive and negative control transfections.
6. *Screen clones for properties of interest.* Cells can be analyzed as a mixed population (which may rapidly change in properties over time, because some clones grow faster than others), or they can be subcloned to obtain a pure population. We note, however, that subcloned populations do tend to display variety in the number and appearance of chromatin arrays, presumably because of genomic instability, which is particularly evident in CHO-K1 cells. An aliquot of the stably transfected mixed population should be frozen as a backup in case subcloning fails. To subclone, the cells are passaged and serially diluted, flow sorted, or plated for filter paper cloning in order to obtain individual clones in a 96-well plate. For filter paper cloning, dilute cells are plated in large 150-mm dishes and grown until colonies contain ~20–50 cells. Trypsin-treated bits of sterile filter paper are then placed on well-isolated colonies for several minutes until they detach, and the paper with some attached cells is transferred to a 96-well plate. Further details on cell cloning are provided elsewhere.[26]

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[24] Measuring Histone and Polymerase Dynamics in Living Cells

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Introduction

In eukaryotic cells, DNA is packaged into nucleosomes by wrapping it around histone octamers; each octamer contains two copies of H2A, H2B, H3, and H4.[1] In dividing mammalian cells, where DNA is made during the S phase, DNA is first wrapped around the $(H3–H4)_2$ tetramer before the addition of two H2A–H2B dimers.[2] Once assembled, these core histones are so tightly bound to DNA that they resist extraction with salt concentrations below 0.63 *M*.[3] Therefore, it is assumed that histone–DNA interactions must be loosened or remodeled to allow access of proteins such as polymerases to DNA.[4,5] Various factors mediating chromatin assembly, disassembly, and remodeling have been identified; some slide nucleosomes along the DNA without dissociating the octamer, others displace some or all of the histones. However, important questions remain as to when,

[1] K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargant, and T. J. Richmond, *Nature* **389,** 251 (1997).
[2] A. Verreault, *Genes Dev.* **14,** 1430 (2000).
[3] R. H. Simon and G. Felsenfeld, *Nucleic Acids Res.* **6,** 689 (1979).
[4] J. L. Workman and R. E. Kingston, *Annu. Rev. Biochem.* **67,** 545 (1998).
[5] A. P. Wolffe and J. J. Hayes, *Nucleic Acids Res.* **27,** 711 (1999).