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]

Acknowledgments

This work was supported by grants from the National Institutes of Health to A. S. Belmont (R01-GM58460 and R01-GM42516). Anne E. Carpenter is a Howard Hughes Medical Institute Predoctoral Fellow.

[24] Measuring Histone and Polymerase Dynamics in Living Cells

By HIROSHI KIMURA, MIKI HIEDA, and PETER R. COOK

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,

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[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).
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0076-6879/04 $35.00

where, and how such histone exchange occurs in living cells and what role this exchange might play during transcription and replication.

Early studies on histone exchange and deposition in living cells utilized radiochemical labeling. In a seminal series of studies, cells were incubated in radioactive amino acids, and the assembly of the radiolabeled (newly made) histones into nucleosomes was monitored; H2A and H2B exchanged more rapidly than H3 and H4.[6,7] The stable association of H3 with H4 was also demonstrated using radiolabeled arginine.[8] HeLa cells were incubated in [^{3}H]arginine, which was then incorporated preferentially into the arginine-rich histones (i.e., H3 and H4); after fusion with mouse 3T3 cells, some of the nuclei in the resulting heterokaryons entered mitosis to give chimeric daughter nuclei. In these nuclei, autoradiography revealed that ^{3}H remained associated with the HeLa chromosomal territories, showing that the arginine-rich histones remained associated with HeLa DNA over several days.

Studies *in vitro* have also revealed a great deal about how RNA polymerase II transcribes naked DNA templates *in vitro;*[9,10] however, we still know little about how it transcribes natural templates *in vivo*. For example, the TATA-binding protein plays a critical role *in vitro,* but ''knockouts'' reveal it has little effect on activity *in vivo*.[11] Given this precedent, it seems studies *in vivo* will uncover other surprises.

Fortunately, the dynamics of proteins can be monitored in living cells after tagging them with the green fluorescent protein (GFP). A construct encoding GFP fused to the protein of interest is expressed in a cell, so the resulting fluorescent hybrid can be seen directly.[12] Mutant GFPs with altered fluorescence are available (e.g., enhanced cyan and yellow fluorescent proteins—ECFPs and EYFPs), and one—PAGFP—is photoactivated by irradiation with 413-nm light so its fluorescence (488-nm excitation) increases 100-fold.[13] Therefore, it is now possible to monitor histone and polymerase dynamics in real time, using these GFP tags and photobleaching techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP).[14–17] Here, we use

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[7] V. Jackson, *Biochemistry* **29,** 719 (1990).
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[15] A. B. Houtsmuller and W. Vermeulen, *Histochem. Cell Biol.* **115,** 13 (2001).

our studies on the dynamics of the core histones[18] and RNA polymerase II[19,20] as examples to illustrate these techniques.

Some Problems Associated with Use of GFP-Tagged Proteins

When using a GFP-tagged protein, it is essential to ensure that the tagged protein functions like the untagged protein. For example, when determining subcellular location, the requirement is simply that the protein localizes like the untagged protein. But even though a GFP-tagged protein might localize correctly, its dynamics will differ; it is inevitably 28 kDa larger—the size of the GFP tag—than the normal counterpart and it will diffuse more slowly. And when studying function, it becomes essential to demonstrate that the tag does not interfere with that function. Such a demonstration is best achieved by replacing all copies of the gene of interest with its counterpart encoding the GFP fusion under the control of the natural promoter; then, if the cell grows normally, the hybrid gene must be able to function normally.[21] However, this kind of demonstration proves to be practically difficult in higher eukaryotes. A second-best option is to use mutant cells that are defective in the gene of interest so that the GFP-tagged protein can be demonstrated to function normally, using genetic complementation. For example, the kinetics of the nucleotide excision repair factors have been analyzed with GFP-tagged proteins that complement the ultraviolet (UV)-sensitive phenotype in mutant cells defective in such repair.[22] In our case, the kinetics of transcription were analyzed with a GFP-tagged version of RNA polymerase II, which complemented a temperature-sensitive mutation in the polymerase;[19,20] as the mutant cells died at the restrictive temperature, it must have been the tagged version that kept the cells alive. If mutant eukaryotic cells are not available, it may be possible to demonstrate that the hybrid eukaryotic gene will complement the genetic defect in a homologous yeast mutant, if available. When mutants are unavailable, function may be demonstrable by enzymatic assay.[23] In the case of histones, no mutant eukaryotic cell lines are available, and it is difficult to prove decisively that the ectopically

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expressed GFP-tagged protein is fully functional and behaves exactly like the endogenous protein. In this case, a wide range of different biochemical and cytological analyses were used to demonstrate that the endogenous and GFP-tagged proteins behaved similarly, including copurification/sedimentation with nucleosomes, coextraction with the endogenous counterparts by different salt concentrations or nuclease treatments, and immunoprecipitation of nucleosome-sized DNA, using anti-GFP antibody from micrococcal nuclease-treated nucleosomes.[18,24,25] Even so, there is always the suspicion that the tagged proteins might not be incorporated into nucleosomes exactly like their untagged counterparts.

It is also important to demonstrate that the GFP-tagged protein is expressed at the appropriate level, as both dynamics and function are likely to be affected by the expression level. For example, if the tagged protein is expressed in addition to the untagged protein, the combined concentration is likely to be higher than that normally found, and this will affect dynamics through mass action. Expression levels are particularly difficult to control when the tagged protein is introduced by transiently transfecting cells with the hybrid gene; then, different cells receive different numbers of plasmids and expression levels can vary widely from cell to cell in the population. Therefore, it is generally better to use stable and clonal cell lines so that each cell studied will contain the same copy number. But even then, it is still unlikely that the hybrid gene will be expressed at the same level as the endogenous gene. Just as in proving functional equivalence, proof that expression levels are equivalent is best achieved by replacing all copies of the gene of interest with its counterpart encoding the GFP fusion under the control of the natural promoter,[21] but—as before—this is practically difficult in higher eukaryotes. In the case of the histones, which are present at $>10^7$ molecules in a human cell, an expression of H2B–GFP at 10% of total H2B gives bright fluorescence.[18,24] The amount of the GFP-tagged protein relative to its endogenous counterpart is often determined by ''western blotting,'' as the GFP-tagged protein is 28 kDa bigger than the untagged protein. Although this method can be applied to relatively large proteins, the blotting efficiency of which is less affected by the extra 28 kDa, it cannot be used for GFP–histones because the blotting efficiency of GFP-tagged histone is quite different from that of nontagged histone. Therefore, it is better to purify nucleosomes, run sodium dodecylsulfate (SDS)-polyacrylamide gels, and estimate the relative amount of GFP-tagged histone by Coomassie staining.[18,24]

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Constructing Expression Plasmids and Establishing Stable Cell Lines

Choice of End for Attaching GFP-Fusion

Experience shows that the GFP tag can be attached to either the amino- or C-terminal end of many proteins without affecting their function,[12] and this seems to be true of the histones. Fusion at the N terminus might appear attractive as the N termini of all core histones extend from the nucleosome and do not form a defined structure detectable by X-ray crystallography.[1] One might worry that such a tag would affect the posttranslational modifications occurring at this end and that are known to play important roles,[26] but such N-terminal tags have been used with H2B, H2A, and H3 without any obvious problems.[24,27,28] The C termini of H2A and H2B are also attractive candidates because they are also located at the surface of the nucleosome. Indeed, H2B fused with GFP at its C terminus has become the most commonly used GFP-tagged histone, and an appropriate plasmid vector is commercially available from BD Biosciences Pharmingen (San Diego, CA), in which the C terminus of H2B is connected with GFP through six amino acids. In contrast to H2A and H2B, the C termini of H3 and H4 are located at the center of the nucleosome, and we failed to obtain stable HeLa cell clones showing bright fluorescence when using GFP attached through the same six amino acids.[18] However, many stable clones expressing high levels of H3– or H4–GFP were obtained by using a longer linker with 23 amino acids; this suggests the longer linker enabled the GFP moiety to be placed outside the particle. Note, however, that a different six-amino acid linker has been used successfully in *Drosophila* cells.[29,30] In the case of the linker histone H1, the C-terminal GFP fusion is the only one reported[21,25,31] and the linker connecting H1 and GFP can be as short as one alanine residue.[25] In conclusion, both ends of histones can be used for attaching the tag, but the length (and perhaps flexibility) of the linker must be considered for C-terminal GFP fusions with H3 and H4.

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Only one of the subunits of RNA polymerase II has been tagged with GFP—the largest catalytic subunit.[19,32] This subunit was chosen because a cell line with a temperature-sensitive mutation in this subunit was available, and this enabled genetic complementation to be used to demonstrate that the hybrid protein was functional. Here, the N terminus was chosen for tagging, as the C terminus plays such an important role in regulation and message production.[33] Various subunits (i.e., RPA194, RPA43, RPA40, and RPA16) of RNA polymerase I have also been tagged at either the N or C terminus.[34]

Choice of Expression System

GFP expression vectors for mammalian cells are available commercially (e.g., from BD Biosciences Clontech, Palo Alto, CA). We found the use of one of these, in which expression of the fusion protein was under the control of the constitutive elongation factor 1α promoter, led to uneven distribution of H3– and H4–GFP; this was traced to a preferential assembly of nucleosomes during DNA replication.[18] Therefore, the use of a promoter that enables more natural[21] or inducible expression[29] has advantages. For analysis of the largest subunit of RNA polymerase II tagged with GFP, we used the cytomegalovirus (CMV) promoter.[19]

Cell Line

The use of established clonal cell lines rather than transient transfections has many advantages (see above). Their use is essential in the particular case of the core histones, which exchange so slowly that it takes several days before the GFP-tagged histone has fully equilibrated.

FRAP and FLIP

Overview

With the introduction of confocal microscopes and laser illumination, FRAP and FLIP have become popular techniques for analyzing the kinetics of molecules in living cells, and many good reviews are now available.[14–17,35] For FRAP, a small part of a cell expressing the GFP-tagged

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[33] T. Maniatis and R. Reed, *Nature* **416,** 499 (2002).
[34] M. Dundr, U. Hoffmann-Rohrer, Q. Hu, I. Grummt, L. I. Rothblum, R. D. Phair, and T. Misteli, *Science* **298,** 1623 (2002).
[35] J. Lippincott-Schwartz, N. Altan-Bonnet, and G. H. Patterson, *Nat. Cell Biol.* Suppl., S7–14 (2003).

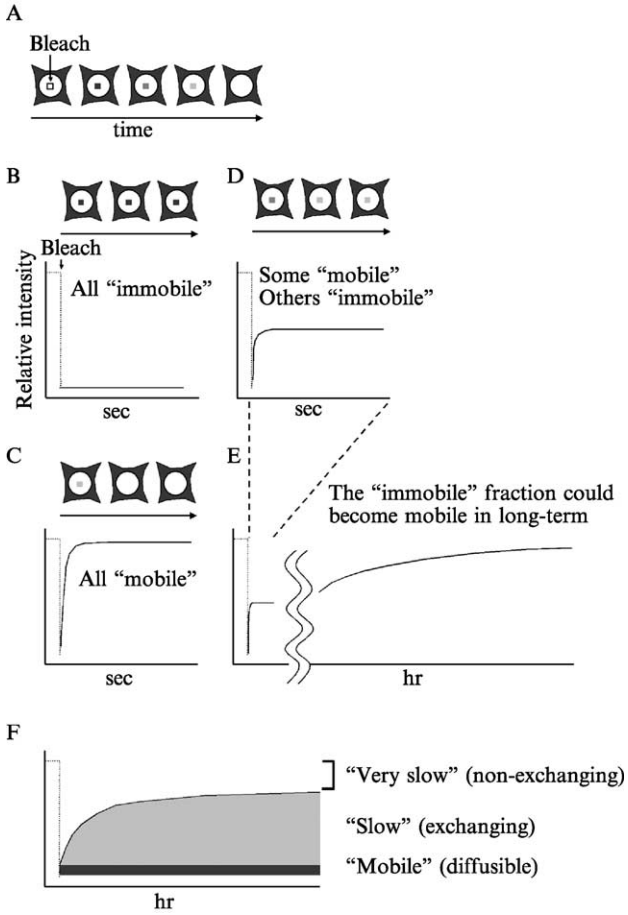

FIG. 1. Fluorescence recovery after photobleaching (FRAP) and molecular kinetics. (A) A schematic illustration of FRAP. Part of a nucleus expressing a GFP-tagged protein (e.g., a histone) is bleached with an intense laser pulse. Bleaching irreversibly damages the GFP fluorophor, and recovery of fluorescence in the bleached area depends on new fluorescent molecules diffusing from the unbleached area into the bleached area. Analysis of the rate of recovery allows the rates of diffusion and association/dissociation to be uncovered. (B) If all molecules are fixed, the intensity of the bleached area never recovers. (C) If all molecules are free to diffuse, the fluorescence in the bleached area quickly recovers almost to the original level; the diffusion rate can be determined by analyzing the recovery curve. (D) If both mobile and immobile fractions exist, the fluorescence recovers with diffusion kinetics but reaches a plateau at a level that reflects the size of the two fractions. (E) Sometimes, an ''immobile'' fraction defined by short-term analysis is seen to be mobile over the longer term; here, the recovery curve contains two components—rapid diffusion and the slower exchange. (F) The recovery of histone–GFP, where there are three fractions with differing mobilities.

protein is irradiated with an intense laser pulse to bleach the fluorophor (Fig. 1). Different microscopes have different mechanisms for defining the region of interest (ROI) to be bleached; one is an acoustical optical tunable filter (AOTF), which allows almost any area with any shape to be bleached. [Even if the microscope does not feature special ROI tools, a small area can still be bleached, using a high zoom (e.g., ×100); in this case, the middle of the field is usually bleached.] Because the GFP fluorophor is bleached irreversibly, the recovery of fluorescence in the bleached area depends on the influx of unbleached molecules. If all molecules are fixed, the bleached area remains bleached (Fig. 1B); in contrast, if all molecules are free to diffuse, the fluorescence in the bleached area recovers quickly almost to the original level as unbleached molecules equilibrate throughout both bleached and unbleached areas (Fig. 1C). Then, the diffusion rate can be determined by analyzing the recovery curve. When both immobile and mobile fractions coexist, the recovery curve reaches a plateau well below the original level, depending on the relative size of the two fractions (Fig. 1D).

FLIP is complementary to FRAP, and the two should usually be used in conjunction so that the results obtained by one method can be confirmed by the other. In a typical FLIP experiment, a field containing two cells—or in our case, two nuclei—is selected, and raster scanned repeatedly with the laser. (One nucleus is used for reference.) For most of each scan, a low laser power sufficient for imaging is used; then, power is increased for bleaching whenever the laser scans through the ROI (in our case, a rectangle) containing the bottom half of the lower nucleus. This process is then repeated until most fluorescence disappears from the top half of the bleached nucleus. Now the intensity in the unbleached (top) half of the bleached nucleus is expressed relative to its original (unbleached) intensity, and values are further corrected for the slight effects of bleaching during imaging (using the reduction in fluorescence seen in the other unbleached nucleus). Whereas bleaching precedes image collection in FRAP, here the two processes are interspersed. If all the GFP-tagged molecules are freely diffusible, bleaching the bottom half should progressively reduce the (relative) intensity in the top half to zero because unbleached molecules have plenty of time to diffuse into the target area and be bleached; this is the result obtained in control cells expressing GFP. If all are immobile (as in fixed cells), the relative intensity remains at unity because immobile molecules in the top half can never enter the bleaching zone. The results obtained with GFP–Pol lay between these two extremes, and were consistent with the existence of a large ''mobile'' (diffusing) population, and a smaller ''immobile'' (engaged) population that could be eliminated by incubation with the transcriptional inhibitor

5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). As many of the same techniques are used for both FRAP and FLIP, only those used for FRAP are described.

Most studies involving FRAP and FLIP monitor the kinetics over several seconds to a few minutes. Then, most of the population of core histones tagged with GFP appear as immobile because the pool of free protein is so small and the others are so tightly bound to DNA. However, if the kinetics are monitored for longer, the intensity of fluorescence in the bleached area gradually becomes more intense as bound molecules in the unbleached area dissociate, diffuse into the bleached area, and rebind to the DNA (Fig. 1E). Because diffusion is so fast compared with the rate at which a molecule dissociates and rebinds, the recovery curve over the long term can be modeled by first-order dissociation/association kinetics. Unlike the tagged histones, there proves to be a large pool of free GFP–Pol that rapidly enters the bleached area, plus a second fraction that enters more slowly; the latter is probably the engaged polymerase as it is sensitive to the transcriptional inhibitor DRB.

Microscopy

The details of live cell microscopy have been described in this series.[36,37] For our studies, cells expressing GFP-tagged histone are plated a few days before analysis in a 35-mm glass-bottomed dish [e.g., from MatTek (Ashland, MA) or Matsunami (Osaka, Japan)]. It is important to transfer cells quickly from the incubator used for growth to the heated stage, as otherwise the temperature falls and it can take a significant time (usually $\sim$0.5 h) for reequilibration, which is seen as a stabilization of the focal plane.

It is important to keep cells alive during image collection, and the issue of phototoxicity has been discussed elsewhere.[36] It is also difficult to prove formally that a cell does remain alive and physiologically intact during imaging—it often depends on how one defines ''alive.'' However, passage through mitosis provides one good practical indication that the cell is alive enough to pass through the necessary checkpoints. Morphological changes such as the subsequent appearance of membrane blebs can also be used as another practical and sensitive indicator of the health of the cell.[38] Therefore, it is essential to image the cells (usually using differential interference, or phase-contrast optics, which are less damaging than the light used

[36] P. M. Conn, ed., *Methods Enzymol.* **302** (1999).
[37] P. M. Conn, ed., *Methods Enzymol.* **309,** (1999).
[38] D. Zink, *Cytometry* **45,** 214 (2001).

for GFP imaging) after the FRAP (or FLIP) data have been collected to check that the cells remain more alive than dead. In our experiments using HeLa cells expressing H2B–GFP,[18] most cells go through mitosis after having been scanned >60 times over 8 h [0.3% power of 25-mW argon laser; pinhole aperture, 4; fast scan mode; ×7 zoom; ×63 PlanApo objective, NA 1.4; Bio-Rad (Hercules, CA) μRadiance]. Locating a cell over such long periods is aided by using a glass-bottomed dish with a grid (MatTek or Matsunami). If cells suffer after imaging, survival can be improved by reducing the laser power, scan number, zoom factor, resolution, or by increasing the rate of scanning.

Often only one optical plane is scanned to reduce photodamage. The problem then arises of ensuring that the same optical section is imaged over time, especially over long periods during which the focal plane may shift as the temperature changes or the cell moves. Therefore, the focus must be readjusted manually every time an image is collected, and—for ease of refocusing—an equatorial plane or some specific cellular feature can be selected.

In a typical FRAP experiment, several images are collected at low laser power before bleaching, and the intensity of the zone to be bleached is averaged to provide the initial intensity. Then, scanning the bleaching zone one to four times with full laser power is usually enough to reduce GFP fluorescence significantly. In general, the size of the bleached area should remain constant throughout a series of experiments that are going to be averaged, because the bleaching period and efficiency are directly affected by the number of scan lines and the zoom factor. Note that the frequency of imaging depends on the kinetics to be analyzed. If monitoring the kinetics of a diffusible fraction, many images are usually collected over the next few seconds; if monitoring the transcription cycle of GFP–Pol or the reassociation of histone–GFPs, they are monitored for minutes or even hours.

Measurements of Relative Intensity

For FRAP, the intensity of the bleached area, the whole nucleus, and the background should be measured with an image analysis tool. We use imageJ (provided by W. Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) for analysis; however, others may find it easier to use the built-in software associated with the microscope or a commercial package like MetaMorph (Universal Imaging, Downingtown, PA; http://www.image1.com). For each time point, the net intensity is obtained by subtracting the background intensity from the intensity of the bleached area or the whole nucleus, and normalizing it relative to the intensity found

before bleaching. Then, the relative intensity is determined by the following equation: Relative intensity $[I_{\text{relative}}\ (n)] = [I_{\text{bleach}}\ (n)/I_{\text{nucleus}}\ (n)]/\ [I_{\text{bleach}}(\text{O})/I_{\text{nucleus}}\ (\text{O})]$, where $I_{\text{bleach}}\ (n)$ is the net intensity of the bleached area at time n, $I_{\text{nucleus}}\ (n)$ is the net intensity of the whole nucleus at time n, $I_{\text{bleach}}\ (\text{O})$ is the average net intensity of the bleached area before bleaching, and $I_{\text{nucleus}}\ (\text{O})$ is the average net intensity of the whole nucleus before bleaching.

Curve Fitting

An example involving the slow recovery during FRAP of histone–GFP is given here (Fig. 1F); the bleached molecules dissociate from DNA to be replaced by unbleached molecules, and first-order kinetics apply. If only one exchanging fraction is present, the recovery should occur exponentially and be governed by $I_{\text{relative}} = C + P(1 - \exp^{-kt})$, where C is a constant value at time 0, P is the plateau value, k is an association constant, and t is time. Curves can be analyzed by nonlinear regression using software such as Prism (GraphPad, San Diego, CA; http://www.graphpad.com), Origin (OriginLab, Northampton, MA; http://www.originlab.com), or SAAM II (SAAM Institute, Seattle, WA; http://www.saam.com). The constant value (C) represents the sum of unbleached and diffusible fractions. The unbleached fraction, or bleaching efficiency, can be determined by bleaching cells that have been fixed with paraformaldehyde. The plateau value essentially represents the exchanging fraction; considering the unbleached and diffusible fractions, the exchanging fraction $= (P - C)/(1 - C)$. The association half-time of this exchanging fraction is calculated from the association constant k $[t_{1/2} = -\ln(1/2)/k]$. Then, the immobile fraction (which has a $t_{1/2}$ of more than the observation period) $= 1 -$ (exchanging fraction + unbleached fraction). If there are two exchanging fractions with distinct kinetics, recovery is governed by $I_{\text{relative}} = C + P_1(1 - \exp^{-k_1 t}) + P_2(1 - \exp^{-k_2 t})$, where P_1 and k_1 refer to population 1 and P_2 and k_2 refer to population 2. When the curve is fitted to a model including more than two different fractions, the different properties of these fractions should be demonstrated (e.g., in the case of H2B–GFP by a differential sensitivity to an inhibitor such as DRB, or by different kinetics in different compartments[18]).

Problems Associated with Analysis over Long Periods

Many FRAP experiments can be completed in a few seconds or minutes, but analysis of histone–GFPs required analysis over many hours, and then nuclear movements made it difficult to identify the bleached

area precisely. It turns out that HeLa cells do not move as much as some other cells (e.g., human fibroblasts, CHO cells, and BHK cells). When using rapidly moving cells, a larger area (e.g., half of the nucleus) can be bleached to facilitate identification of the bleached region.[39] Another problem is caused by the rotation of the nucleus in the *xy* axis, which also makes identification of the bleached area difficult; in our case, this kind of rotation did not occur frequently in HeLa cells.[18] Another problem is caused by the synthesis of histone–GFP during the long period required for imaging, as the newly made protein may contribute to the recovery of fluorescence seen. Although a protein synthesis inhibitor such as cycloheximide can be added,[18] the accompanying side effects (e.g., inhibition of DNA replication, inhibition of cell cycle progression, etc.) can complicate analysis. In such cases, the use of an inducible expression vector should be considered.

Present Results and Future Directions

FRAP and FLIP have now been used by different groups to analyze the exchange of various histone–GFP constructs. The linker histone H1 exchanges rapidly even when it is in heterochromatin or a mitotic chromosome,[21,25,31,40] whereas the core histones exchange much more slowly.[18] For example, H2B exchanges more rapidly than H3 and H4, even though all form part of the same structure, the nucleosome. About 3% of H2B (probably the transcriptionally active fraction) exchanges within minutes ($t_{1/2}$, ~6 min), ~40% (probably the euchromatic fraction) more slowly ($t_{1/2}$, ~130 min), and another ~50% (probably in heterochromatin) remains bound permanently ($t_{1/2}$, >8.5 h). More than 80% of H3 and H4 is also bound permanently. These results are consistent with the mobile components—H1, H2B (and perhaps H2A)—facilitating immediate access of transcription factors and polymerases to the DNA, whereas the immobile components—H3 and H4—act as stable epigenetic markers. Although this global view of histone exchange in living cells has emerged, the underlying control mechanisms that govern it remain largely unknown. We are also only beginning to analyze the kinetics of the different histone variants; for example, the *Drosophila* H3.3 variant—which differs from the major from of H3 in only a few amino acids—is incorporated into transcriptionally active chromatin independently of DNA replication whereas H3 is not.[30] And we have yet to analyze how the histone code modifies those

[39] N. Daigle, J. Beaudouin, L. Hartnell, G. Imreh, E. Hallberg, J. Lippincott-Schwartz, and J. Ellenberg, *J. Cell Biol.* **154,** 71 (2001).
[40] F. Catez, D. T. Brown, T. Misteli, and M. Bustin, *EMBO Rep.* **3,** 760 (2002).

kinetics. Similar studies have also revealed two kinetic fractions of RNA polymerase II in living nuclei: most diffuses freely, but a small but significant fraction becomes transiently immobile (association $t_{1/2}$, ~20 min) during transcription.[20,32] One challenge now is to analyze what happens as a single tagged polymerase transcribes a specific nucleosomal template in a living cell.

Acknowledgments

The authors' work was supported by the Wellcome Trust. Miki Hieda is a Research Fellow of the Japanese Society for the Promotion of Science.

[25] Measurement of Dynamic Protein Binding to Chromatin *In Vivo,* Using Photobleaching Microscopy

By ROBERT D. PHAIR, STANISLAW A. GORSKI, and TOM MISTELI

Introduction

Chromatin-binding proteins play a crucial part in every aspect of chromatin structure and gene expression.[1] Direct binding of proteins to chromatin maintains and regulates higher order chromatin structure, and leads to histone modifications and transcriptional activation. Once a gene is activated, components of the RNA polymerase machinery directly contact DNA and mediate transcription. Despite the crucial importance of chromatin proteins, most of what we know about the interaction of these proteins with DNA comes from *in vitro* experiments. Regardless of whether the DNA used in *in vitro* assays consists of naked DNA or reconstituted chromatin, it is unlikely that these templates reflect the physiological binding substrates that are found in a cell nucleus or that the buffer conditions accurately reproduce the ionic environment in a cell. Methods are required to probe the binding of proteins to native, unperturbed chromatin in intact cells.

An experimental approach to studying the binding of protein to chromatin in living cells is the use of photobleaching methods.[2–9] In these

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