Chromatin Dynamics in Interphase Cells Revealed by Tracking in a Two-Photon Excitation Microscope

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ABSTRACT Increasing evidence points to a dynamical compartmentalization of the cell nucleus, yet the mechanisms by which interphase chromatin moves and is positioned within nuclei remain unclear. Here, we study the dynamics of chromatin in vivo applying a novel particle-tracking method in a two-photon microscope that provides ~10-fold higher spatial and temporal resolutions than previous measurements. We followed the motion of a chromatin sequence containing a lac-operator repeat in cells stably expressing lac repressor fused with enhanced green fluorescent protein, observing long periods of apparent constrained diffusion interrupted by relatively abrupt jumps of ~150 nm lasting 0.3–2 s. During these jumps, the particle moved an average of four times faster than in the periods between jumps and in paths more rectilinear than predicted for random diffusion motion. Additionally, the jumps were sensitive to the temperature and absent after ATP depletion. These experimental results point to an energy-dependent mechanism driving fast motion of chromatin in interphase cells.

INTRODUCTION

Significant advances in the fields of microscopy and molecular biology have in recent years allowed direct visualization of chromatin structure and dynamics in live cells, changing the initial vision of an interphase nucleus containing randomly arranged and static DNA (1).

A significant breakthrough in the field was the development of a novel approach to label specific DNA sequences in live cells, which consist of the insertion of a lac-operator repeat in the DNA and the expression of the lac repressor protein fused to the enhanced green fluorescence protein (EGFP) (2,3). This new approach made it possible for the first time to study the dynamics of chromatin in vivo.

Using this methodology, several studies in *Saccharomyces cerevisiae* (4), mammalian cells (5), and *Drosophila* (6) have described the motion of specific chromatin regions as undergoing apparent Brownian motion limited to a nuclear subregion.

However, the general dynamic organization of DNA seems to be more complex. Heun et al. (7) reported that early and late origins of replication in *S. cerevisiae* are more mobile in the G1 phase than in the S phase. Also, the movement in the G1 phase was highly sensitive to ATP depletion and to changes in metabolic status.

The interior versus peripheral intranuclear location of early- versus late-replicating DNA regions is established early in G1 (8); similarly, changes in the transcriptional activity of certain genes have been shown to be correlated with changes in their intranuclear location (9). In a simplified experimental system, the targeting of the VP16 transcriptional activator to a specific DNA region in Chinese hamster ovary (CHO) cells leads to a repositioning of this region from the nuclear periphery to the interior (10).

These results suggest that important nuclear functions such as DNA transcription are accompanied by defined changes in the position of the sequence. However, as concluded by Gasser (11), there is little experimental support to date for an active mechanism driving these chromatin movements.

This divergence could be explained by the fact that the methods used for the observations do not have the temporal and/or spatial resolutions required for detecting such a motion. Also, it is important that the phototoxicity generated under imaging conditions that produce no other obvious phenotypes can significantly change chromatin dynamics (A. S. Belmont and C. H. Chuang, unpublished data).

In this work, we reexamine interphase chromatin dynamics using a new, two-photon microscopy fluorescent particle-tracking method (12). This method has spatial and temporal resolutions of 20 nm and 30 ms, respectively, i.e., ~10-fold higher than the resolution of methods previously used to study chromatin dynamics in vivo. The method is especially well suited for biological applications since it provides significantly lower out-of-focus photodamage and photobleaching (13).

Our results reveal that chromatin undergoes an apparently confined random motion alternating with moments of fast curvilinear motion. These jumps are ATP-dependent and appear to be the manifestation of active, versus passive diffusive, mechanisms for interphase chromosome movements.

MATERIALS AND METHODS

Establishment of DHFR-BAC cell line

A 256-copy lac-operator direct repeat (14) was inserted into the dihydrofolate reductase (DHFR) bacterial artificial chromosome (BAC)

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(clone 057L22 from the CITB mouse library). This BAC clone contains the 31-kb DHFR locus together with 132 kb of upstream and 5.5 kb of downstream sequence inserted into the pBeloBAC11 vector. Insertion of the lac-operator repeat used Tn5 transposition. A Tn5 transposon was constructed using the pSP2 plasmid (15). The pSP2 polylinker was modified by insertion of an adaptor (AATTGACAGCTGTCGATC) containing a PshAI/PyuII site between the EcoR1 and BamH1 sites, creating pPvuII. The transposon ⟨Kan-2⟩ (Epicentre Technologies, Madison, WI) was inserted into pPvuII using the PshAI/PyuII site, creating p[Kan]. The 8.32 lac-operator repeat was cut out of pSV2-DHFR-8.32 (14) using a Sal I and XhoI digest and inserted into p[Kan] linearized with Sal I to create p[Kan-8.32].

Transposons were generated by digestion of p[Kan-8.32] with PyuII and purification from an agarose gel using Gelase (Epicentre Technologies). Transposition into the DHFR-BAC used the EZ:TN Tn5 transposition system (Epicentre Technologies) according to the manufacturer's instructions. Screening of transposition insertions by restriction enzyme digest were done to identify clones containing the full lac-operator insertion, followed by DNA sequencing into the BAC from both ends of the transposon to verify that no BAC sequence was lost during transposon insertion and to identify the insertion location.

The 057-K-8.32-C29 BAC clone we identified contained a transposon insertion 75 kb 5′ to the DHFR locus and was used for subsequent CHO cell transformation. CHO DG44 cells with a double deletion of the endogenous DHFR locus were used (16). CHO DG44 cells stably expressing EGFP-lac repressor (dimeric form) were transfected using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Stable transformants were cloned by serial dilution and selected for cell clones containing multiple, closely located fluorescent dots within interphase nuclei. The B9 subclone we identified contained a maximum of 7–10 fluorescent dots cointegrated at a single mitotic chromosome location.

Cell culture and preparation for microscopy measurements

CHO cells were cultured as described (14). For the tracking experiments, cells were plated for 2–3 days in fibronectin-coated dishes with an optic glass in the bottom. During the tracking experiments the temperature was kept at 37°C using a ΔT3 system (Bioptechs, Butler, CA), with a coverslip seal to maintain constant pH.

ATP depletion was carried out by incubating cells at 37°C for 1 h in the presence of 50 mM deoxyglucose (ICN Biomedicals, Irvine, CA) and 20 mM sodium azide (Acros Organics, Belgium) before the tracking experiment (17).

To fix the cells, they were incubated at room temperature with 1.6% formaldehyde in Dulbecco's phosphate-buffered saline for 10 min and washed several times with the phosphate-buffered saline.

Microscope setup

The tracking experiments were carried out with the Olympus IX70 microscope previously described (12). The two-photon excitation source was a mode-locked titanium-sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by an argon ion laser (Innova 300, Coherent) tuned to 920 nm. The laser power at the sample was in the 1- to 10-mW range. The light is directed into the microscope by two galvomotor-driven scanning mirrors (Cambridge Technologies, Watertown, MA) through a scanning lens. During the tracking procedure, the two scanning mirrors are moved independently by two synchronized voltage sine waves shifted 90° relative to each other and generated in a computer card (three-axis card, ISS, Champaign, IL). As a consequence, the laser moves in a circular path. The frequency of the circular orbit (f_{orbit}) was 250 Hz. The position of the scanning center is determined by the offset values of the sine waves.

The laser light is reflected with a low-pass dichroic mirror (transmission between 370 and 630 nm, Chroma Technology, Brattleboro, VT) and focused on the sample with a 63× (dry) 0.8-NA objective. Fluorescence emission collected by the objective passes through the dichroic and a short-pass filter, exiting the microscope through the side port. A Hamamatsu H7422P-40 photomultiplier tube was used as a detector with its output amplified, passed through a photon counting discriminator (PX01 Photon Counting Electronics, ISS), and counted with a data acquisition card (ISS). The experiments are controlled by a data acquisition program (SimFCS, Laboratory for Fluorescence Dynamics, Champaign, IL).

A cMOS camera (Pixelink, Ottawa, ON) was placed on the top port of the microscope to take differential interference contrast (DIC) images of the cells. In the microscope, the emission filter is placed immediately before the detector so that the near-infrared laser used for the two-photon excitation is not blocked in the camera path and can be imaged.

Tracking procedure

The particle-tracking method used in this work was similar to that described previously (12) and adapted to tracking in two dimensions. Briefly, with a fast raster scan, the fluorescence image of a large area of the sample is obtained and the particle of interest is chosen by clicking it on the image. This directs the laser beam to the particle by changing the DC offset values of the x and y outputs of the three-axis driver card. This point is considered as the initial coordinates for the tracking.

During each cycle of the tracking routine, the excitation beam does eight circular orbits with a radius of 250 nm, equal to half the x,y width of the point spread function, and centered at the initial coordinates set before. The fluorescence intensity is collected at high frequency (f_{data} = 32,000 Hz) as the laser moves around the particle (Fig. 1). Thus, in a typical experiment with f_{orbit} = 250 Hz, we measure the fluorescence intensity at 128 different points of the orbit. By performing several orbits in each cycle, we improved the signal/noise ratio.

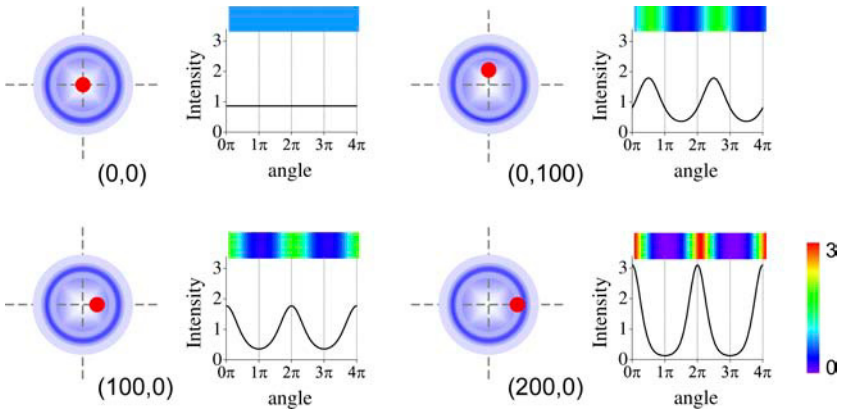

FIGURE 1 Schematic representation of the tracking procedure. The diagrams depict the laser beam (*blue*) orbiting around the particle (*red*). The simulated intensity collected during two circular orbits is represented as a function of the orbit angle for four different positions of the particle with respect to the center of scanning. Note the changes on the intensity profile (*top part* of the plots) with the particle position.