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In Vivo Chromatin Structure of the Murine Interleukin-5 Gene Region: A New Intact Cell System

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ABSTRACT

The study of chromatin involvement in the regulation of gene expression has traditionally required the isolation of nuclei. However, cell fractionation techniques are subject to redistribution of proteins during the isolation procedure, which prevents rigorous physiologically relevant analysis. To eliminate the need to isolate nuclei and to analyze chromatin structures in vivo in response to agents regulating murine interleukin-5 (IL-5) gene activation, we have established a novel lysolecithin permeabilized intact cell system for suspension cell types, in this case T cells. Nuclear integrity of permeabilized cells is demonstrated by nuclear transport assays using confocal laser scanning microscopy. Results are identical in unstimulated and stimulated T cells, indicating that the chromatin structure after activation is not the result of gross alterations in nuclear protein transport properties. Potential new IL-5 gene regulatory regions are identified by DNase I hypersensitivity mapping. Our lysolecithin permeabilized intact cell system is amenable to physiologically relevant analysis of responses to signaling pathways at the level of chromatin, nuclear protein translocation and possibly other cellular functions in a variety of suspension and adherent cell types.

INTRODUCTION

Eukaryotic chromatin is packaged into nucleosomes and higher-order structures that restrict the entry of RNA polymerases and transcription factors. In actively transcribed regions, the chromatin is less densely packed, being accessible for transcription and, experimentally, to nonspecific nucleases like DNase I. These so-called DNase I hypersensitive (DH) sites can be mapped by DNase I treatment of isolated nuclei and subsequent analysis by Southern blotting. This procedure allows the monitoring of chromatin structures

associated with gene activation (4) over large regions, which is not readily achieved by other techniques.

Isolation of nuclei has been particularly important for the analysis of chromatin involvement in the regulation of gene expression, although a major concern with the use of isolated nuclei is the loss of chromatin components. Other in vitro methods involve the reconstitution of the DNA-protein interaction of interest from cloned naked DNA and appropriate nuclear extracts. A number of studies (for example, References 12,15 and 17) have shown that in vitro binding data often do not reflect the in vivo binding patterns of nuclear factors within the cell of interest. Whole-cell systems avoid the problems associated with cell fractionation, permitting physiologically relevant analysis. This is particularly pertinent to certain cell types that yield fragile nuclei, such as lymphocytes (5), in which gene regulation studies can be difficult. Since nucleases such as DNase I are too large to enter cells, cellular permeabilization is required. The nonionic detergent Nonidet® P-40 (NP40) has been tried in DH mapping experiments with adherent cells (18), but NP40 permeabilization appears to result in both cell lysis and detachment of nuclei (18), and nuclear

Figure 1. Visualization of passive and active nuclear transport properties of non-permeabilized and lysolecithin-permeabilized D10.G4.1 cells. Incubations were for 40 min at room temperature. Panel A shows non-permeabilized cells with 20-kDa dextran (cellular exclusion). Permeabilized cells are shown with 20-kDa dextran (B) (fluorescence throughout cytoplasm and nucleus) or 70-kDa dextran (C) (nuclear exclusion). Panel D shows nuclear uptake of an IAF-labeled β -gal (480-kDa) fusion protein containing the T-ag NLS. The arrows in Panels A and B indicate the perimeter of the cells and in Panels C and D the perimeter of the nucleus. Results were identical for the EL4.23 cell line (results not shown).

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integrity is not retained (10). Both adherent and suspension cells permeabilized by lysolecithin have been shown to display efficient DNA replication and transcription activities (3,11), indicating that normal nuclear processes are better maintained than in isolated nuclei, which yielded poor results in these experiments. Zhang and Gralla (20) showed that lysolecithin can be used to allow DNase I to enter adherent cells for analysis of chromatin by *in vivo* footprinting (12), demonstrating that DNase I remains active in the presence of this cellular permeabilization agent.

We have adapted the technique of lysolecithin permeabilization for T lymphocyte clones and T cell lines in order to study *in vivo* chromatin structures of the murine interleukin-5 (IL-5) gene region by DH mapping. The blood cell growth factor IL-5 is expressed after T lymphocytes are activated either through the T cell receptor or mimicked in culture by Concanavalin A or phorbol esters such as phorbol myristate acetate (PMA). Results are presented here for nuclear transport assays and *in vivo* chromatin structure analysis in lyso-

lecithin permeabilized T cells, with or without activation of IL-5 gene expression. Nuclear integrity was also confirmed by electron microscopy (results not shown).

MATERIALS AND METHODS

Cell Culture

The murine T cell clone D10.G4.1 (9) and the murine thymoma subline EL4.23 (from Dr. C.J. Sanderson, Western Australian Institute for Child Health Research, Perth, WA, Australia) are used in this study. Culture conditions are described in Reference 1. Where indicated, D10.G4.1 cells are stimulated in culture with 5 μ g/mL Concanavalin A (Con A; Pharmacia Biotech, Uppsala, Sweden) and EL4.23 cells with 10 ng/mL phorbol 12-myris-

tate 13-acetate (PMA; Sigma Chemical, St Louis, MO, USA), both for 6 h, which is sufficient to induce IL-5 mRNA expression (13,14).

Cell Permeabilization

Cells are harvested and resuspended at 1.0×10^7 cells in 1 mL permeabilizing buffer (PB: 15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 300 mM sucrose, 0.5 mM β -mercaptoethanol). An equal volume of 0.01% (wt/vol) lysolecithin (L4129; Sigma Chemical) in PB is added, and cells are immediately centrifuged at 1500 rpm, for 5 min at room temperature. The pellet of permeabilized cells is used for further studies.

Nuclear Transport Assays

Bacterially expressed β -galactosidase (β -gal) fusion proteins containing

Figure 2. NLS-dependent nuclear import of fluorescein-labeled SV40 T-ag β -gal fusion proteins in lysolecithin-permeabilized EL4.23 cells. Each data point represents the average of >3 separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence) and autofluorescence, with Fn/c being the ratio of Fn to Fc after subtraction of autofluorescence. Data are fitted for the function $F_n/c(t) = F_n/c_{max} \cdot (1 - e^{-kt})$ (7,8). Results were identical for the D10.G4.1 cell line (results not shown).

Figure 3. DH analysis of the murine IL-5 gene region in unstimulated (lanes 1–4) or Con A-stimulated (lanes 5–8) D10.G4.1 cells. Southern blotting analysis of *Hind*III-digested DNA showing additional bands generated from permeabilized cells treated with increasing amounts of DNase I (no DNase I in lanes 1 and 5, increasing DNase I in lanes 2–4 and 6–8; 50, 100 and 200 U/mL, respectively). Longer exposures of lanes 4 and 8 are presented on the right. Below is a schematic showing location of the hybridization probe and DH sites within the 10-kb *Hind*III fragment encompassing the IL-5 gene.

the simian virus 40 (SV40) large tumor antigen (T-ag) wild-type or mutant nuclear localization signals (NLS) (7,8,16) are fluorescently labeled with 5-iodo-acetamidofluorescein (IAF; Molecular Probes, Eugene, OR, USA) as previously described (7,16). Fluorescein isothiocyanate (FITC)-labeled 20- and 70-kDa dextrans are from Sigma Chemical (FD-20 and FD-70).

Nuclear transport assays are essentially as described (7,16), except that pellets of lyssolecithin-treated D10.G4.1 or EL4.23 cells resuspended in 100 μ L intracellular buffer (IB: 110 mM KCl, 5 mM NaHCO₃, 5 mM MgCl₂, 1 mM EGTA, 0.1 mM CaCl₂, 1 mM dithiothreitol [DTT], 10 μ g/mL leupeptin, 20 mM HEPES [pH 7.4]) are used. The cells can be kept at room temperature for 4–5 h with no apparent loss of active or passive nuclear transport properties. The following is added to 1.5 μ L of permeabilized cell suspension: 1.5 μ L of reticulocyte lysate (ca. 150 mg protein/mL; Catalog No. L415A; Promega, Madison, WI, USA), 1.0 μ L of an ATP regenerating cocktail (0.625 mg/mL creatine kinase, 150 mM creatine-phosphate, 10 mM ATP in 2 $\times$ IB) and 1 μ L of FITC-labeled dextran or IAF-labeled protein (1–5 mg/mL). Nuclear transport is quantified using confocal laser scanning microscopy (CLSM; Model MRC[®]-600; Bio-Rad, Hercules, CA, USA) and image analysis using the NIH Image Version 1.49 public domain software.

DNase I Hypersensitivity (DH) Mapping

D10.G4.1 cells are resuspended at 1×10^7 cells/mL (typically 4×10^7 cells) of PB containing 0, 100, 200 or 400 U/mL of DNase I (Boehringer Mannheim, Mannheim, Germany). An equal volume of 0.01% lyssolecithin solution (4 mL) is then added, incubated for 4 min at room temperature, followed by addition of 0.4 vol (1.6 mL) of lysis buffer (1.25 mg/mL of proteinase K [Boehringer Mannheim], 312.5 mM Tris-HCl, pH 8.0, 125 mM EDTA, pH 8.0 and 6.25% [wt/vol] sodium dodecyl sulfate [SDS]). Incubation is overnight at 37°C with 40 rpm agitation. The solution is phenol-chloroform-extracted and ethanol-precipitated. RNA is

removed by digestion with RNase A followed by ethanol-precipitation. Genomic DNA yields are approximately 100 μ g DNA per 1×10^7 cells.

Fifty micrograms of genomic DNA from each treatment is digested with a restriction endonuclease, fractionated through a 1.0% agarose gel and subjected to Southern analysis as described (2), except that a ³²P-labeled DNA

probe is prepared by the polymerase chain reaction (PCR). The reaction mixture (50 μ L) contains: 25 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 3.2 μ M dCTP, 120 μ Ci or 0.8 μ M [α -³²P]dCTP (>3000 Ci/mmol; Amersham International, Little Chalfont, Bucks, England, UK), 0.5 μ M each of SP6 and T7

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primers (New England Biolabs, Beverly, MA, USA), 2.5 U *Taq* DNA Polymerase (Boehringer Mannheim) and 1 ng plasmid DNA, with 50 μ L mineral oil overlay. Using a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT, USA) incubations for 30 cycles are as follows: 94°C 1 min, 60°C 1 min and 72°C 1 min. The probe is separated from unincorporated nucleotides by fractionation through a NICK Column® (Pharmacia Biotech).

RESULTS

Optimization of T Cell Permeabilization

In preliminary experiments, we examined the efficiency of permeabilization on EL4.23 and D10.G4.1 cells by using various concentrations of lysolecithin for 4 min at room temperature. Permeabilization was assessed by trypan blue (M_r ca. 1000) staining. The lowest concentration that gave 100% staining was 0.005% (wt/vol). Even at the highest concentration tested (0.5%) cellular structures appear intact under phase-contrast microscopy.

Assessment of Nuclear Integrity in Lysolecithin Permeabilized T Cells

Nuclear envelope integrity of lysolecithin permeabilized cells was investigated first by electron microscopy (EM; results not shown); the nuclear envelope of permeabilized unstimulated and PMA-stimulated EL4.23 cells appearing completely intact with nuclear pore complexes (NPCs) evident.

Passive nuclear permeability properties were assessed using FITC-labeled dextrans (Figure 1), while active transport was monitored using IAF-labeled β -gal fusion proteins containing the SV40 large T-ag NLS or a NLS-deficient derivative (Figure 2). Results were identical for both cell lines (results not shown). A 70-kDa dextran entered the cytoplasm but was excluded from the nucleus (Figure 1C), while a 20-kDa dextran rapidly entered the nucleus (Figure 1B), in contrast to non-permeabilized cells (Figure 1A). These results are completely consistent with the molecular weight limit for passive diffusion of about 45 kDa (6) for the

NPC in the nuclear envelope. The nuclei were also capable of active transport as shown by their rapid accumulation ($t_{1/2}$ = ca. 11.5 min) of a SV40 large T-ag- β -gal fusion protein (480 kDa) in NLS-dependent fashion (Figures 1D and 2). This is consistent with the NLS requirement for proteins >45 kDa to be transported to the nucleus (6). Thus, passive and active nuclear transport properties indicated functional integrity of the nuclear envelope subsequent to lysolecithin treatment.

Passive and active nuclear transport properties were identical in unstimulated and Con A-stimulated permeabilized D10.G4.1 cells. Values for the maximal level of accumulation (F_n/c_{max}) at 40 min in unstimulated and stimulated cells, respectively, were as follows: 20-kDa dextran, 0.79 and 0.76; 70-kDa dextran, 0.58 and 0.45; T-ag-NLS- β -gal, 1.58 and 1.63. (Note that F_n/c_{max} is concentration-dependent, see Figure 2.) This indicates that chromatin structure after Con A stimulation (see below) is not attributable to gross changes in nuclear protein transport properties. Results (not shown) were identical for EL4.23 cells.

Analysis of In Vivo Chromatin Structure of the Murine IL-5 Gene Region

DH sites were mapped in the IL-5 gene region in permeabilized D10.G4.1 cells with or without Con A stimulation. The Southern blot presented in Figure 3 shows the expected single 10-kb *Hind*III band in the no-DNase I controls. Additional bands are seen where increasing concentrations of DNase I are used, due to entry of DNase I (30 kDa) into the nuclei of lysolecithin-treated D10.G4.1 cells to reveal DH sites. The fact that DNase I concentrations of at least 50 U/mL are required for the detection of DH sites, greater than those used in isolated nuclei (19), presumably relates to the use of an intact cell system, potentially complicated by inhibitory factors (19) and limited access.

All sites mapped were constitutive, present in both unstimulated and Con A-stimulated D10.G4.1 cells. A very strong site was located at about 800 bp upstream from the start of transcription (-800 bp) as well as a site near the start

of transcription and another at about +900 bp, in either intron 1 or 2 (exon 2 being small). There were additional less prominent sites seen upstream from the start of transcription as follows: -1400, -1800, -2100, -2600 and -2900. These results in D10.G4.1 cells have been confirmed using two different probes and in unstimulated and PMA-stimulated EL4.23 cells (results not shown).

DISCUSSION

The data presented here demonstrate that lysolecithin treatment is an efficient method of permeabilizing T cells, which leaves the nuclei intact. This technique circumvents the problem of reproducibility of nuclear preparations and avoids artifacts associated with isolation techniques, such as the loss of chromatin components or transcription factors. In addition, the procedure is rapid and efficient.

Lysolecithin permeabilization is used here successfully for in vivo DH mapping in the IL-5 gene region. This is indicated by the DH sites located at -800 and at the start of transcription, both correlating to sequences required for IL-5 gene expression (1). The other DH sites identified represent putative regulatory regions not identified using other approaches. No chromatin changes were observed in response to Con A or PMA stimulation. This may imply that the IL-5 gene region in these cells is primed for activation during development, such that response to cell surface signaling in mature T cells occurs through preformed chromatin structures. Alternatively, regulatory regions and DH sites responsive to signals may be outside of the regions mapped here. Preliminary data (not shown) investigating the murine IL-3 gene show an inducible DH site upstream of the start of transcription, demonstrating that inducible chromatin changes can be detected using our whole cell approach. The role of the DH sites detected here needs to be further investigated, but DH mapping in our lysolecithin permeabilized cell system has shown the potential to identify regulatory regions not previously foreseen to provide insight into in vivo mechanisms of regulation

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of gene expression.

Our analysis shows that nuclear protein transport in our lysolecithin permeabilized cell system is not only NLS-dependent, as described here, but also dependent on ATP and cytosolic factors, as well as inhibitable by both GTP γ S and the lectin wheat germ agglutinin (results not shown). Thus, lysolecithin permeabilized cells represent a nuclear transport assay system with all the characteristics of in vivo passive and active nuclear transport (6). While the system described here is for suspension cells and is particularly useful in this regard, we have also used it for various other suspension and adherent cell types, including rat hepatoma and smooth muscle cells (manuscript in preparation). This system is also likely to be applicable to the study of other cellular activities, for example, the identification of newly transcribed RNA using nuclear run off transcription assays (5).

In summary, our lysolecithin permeabilized intact cell system is amenable to physiologically relevant analysis of responses to signaling pathways at the level of both chromatin and nuclear protein translocation in a variety of cell types.

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