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Cell Cycle-Dependent Transduction of Cell-Permeant Cre Recombinase Proteins

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Abstract Protein transduction has been widely used to analyze biochemical processes in living cells quantitatively and under non-steady-state conditions. The present study analyzed the effects of cell cycle on the uptake and activity of cell-permeant Cre recombinase proteins. Previous studies had suggested that the efficiency of recombination and/or protein transduction varied among individual cells, even within a clonal population. We report here that cells in the G1 phase of the cell cycle undergo recombination at a lower rate than cells at other phases of the cell cycle, and that this variation results largely from differences in protein uptake, associated with differences in cell size. These results have implications regarding the mechanism of protein transduction and identify a source of heterogeneity that can influence the response of individual cells to cell-permeant proteins. J. Cell. Biochem. 89: 674–687, 2003. © 2003 Wiley-Liss, Inc.

Key words: protein transduction; cell cycle; membrane transport; proteomics

The plasma membrane normally acts as a barrier, preventing proteins and other macromolecules from entering cells. However, short protein sequences [known as membrane translocation sequences (MTSs)] have been identified that function as carriers to deliver macromolecules—including peptides, proteins, DNA fragments, and drugs—into cells [Hawiger, 1999; Schwarze and Dowdy, 2000; Dunican and Doherty, 2001]. With additional subcellular trafficking signals, transduced macromolecules can be guided to specific cellular locations, providing an effective way to modulate biochemical processes within cells. Two major types of MTSs have been characterized: (i) basic sequences including simple cationic polymers, and regions from the HIV TAT, *Drosophila* Antennapedia, and Herpes Simplex Virus VP22 proteins and (ii) hydrophobic peptides derived from the leader sequences of secreted and transmembrane proteins. While the mechanism of transduction is unknown, MTSs appear to exploit physical properties common to mammalian plasma membranes and do not require specific receptor or transporter systems. Systemic delivery of peptides and proteins has also been demonstrated in mice [Schwarze et al., 1999; Fujihara et al., 2000; Yan Liu et al., 2000; Jo et al., 2001]. Cell-permeant proteins disseminate widely throughout animals and have been reported to cross the blood–brain barrier [Schwarze et al., 1999; Jo et al., 2001].

Protein transduction provides a new paradigm for the analysis of mammalian gene functions in living cells. Cell-permeant proteins and peptides have been used to block intracellular protein trafficking [Torgerson et al., 1998], to interfere with molecular interactions involved in signal tranduction [Rojas et al., 1996; Williams et al., 1997; Derossi et al., 1998; May et al., 2000; Cunningham et al., 2001; Datta et al., 2001; Hall et al., 2001], transcription [Phelan et al., 1998], and cell cycle control [Ezhevsky et al., 1997, 2001; Gius et al., 1999;

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Zezula et al., 2001], to deliver functional enzymes [Schwarze et al., 1999; Wu et al., 2000; Jo et al., 2001] and substrates [Vocero-Akbani et al., 1999] into cells, and to characterize biologically active protein surfaces [Liu et al., 1996; Vastrik et al., 1999; Yao et al., 1999; Yigzaw et al., 2001], post-translational modifications [Hall et al., 1996; Dunican and Doherty, 2001], and protein–protein interactions [May et al., 2000]. Unlike gene-based approaches, protein transduction permits direct and quantitative analysis of protein functions under non-steady state conditions. These advantages are particularly important for analyzing biochemical processes in living cells since protein functions are typically tightly regulated and highly interconnected [Huang, 2000].

To date, most studies involving protein transduction have examined the net effects of cell-permeant proteins or peptides on populations of treated cells. However, as many biochemical processes are directly or indirectly influenced by factors such as cell-cycle stage or microenvironment, it will be important to monitor individual cells for their responses to cell-permeant proteins. Cell-to-cell variation could also result from differences in the efficiency of protein transduction itself, rather than from the intracellular activity of transduced proteins.

We [Jo et al., 2001] and others [Joshi et al., 2002; Peitz et al., 2002; Will et al., 2002] have characterized cell-permeant Cre DNA site-specific recombinases, providing an efficient means to regulate gene structure and function in living cells. Since recombination provides a stable record of protein transduction, cell-permeant Cre can also be used for quantitative studies of *cis*- and *trans*-acting factors that influence enzyme uptake or activity. For example, Cre activity was significantly enhanced by the addition of a nuclear localization signal to the protein [Jo et al., 2001; Peitz et al., 2002]. Based on the kinetics and dose-response of Cre-mediated recombination, the efficiency of protein transduction and/or recombination appears to vary among individual cells, even within the same clonal population. The present study assessed the contribution of cell cycle to this variation. We report that cells in the G1 phase of the cell cycle undergo recombination at approximately 50% of the rate of cells at other phases of the cell cycle, and that this variation results largely from differences in protein uptake associated with differences in cell size.

MATERIALS AND METHODS

Cell Culture and Protein Transduction

Tex.loxp.EG clones 74 and 31 were derived by infecting Tex cells with the pBABE.lox.stp.EGFP retrovirus as described previously [Jo et al., 2001]. Protein transduction was performed in serum-free RPMI 1640 medium, since fetal bovine serum inhibits transduction of recombinant Cre proteins. HisNLSCreMTS, HisNLSCre, and HisCre proteins were expressed in *E. coli* BL21 cells and purified as described previously [Jo et al., 2001]. Cre-mediated recombination in Tex.loxp.EG cells, which induces the expression of an enhanced green fluorescence protein (EGFP), was measured by flow cytometry using a FACSort instrument (Becton Dickinson Immunocytometry system, Franklin Lakes, NJ). Cellular DNA content was determined by FACS analysis of cells stained with propidium iodide solution (70 μM propidium iodide, 38 mM sodium citrate, 20 μg/ml RNaseA).

Centrifugal Elutriation and Southern Blot Analysis

Cells were fractionated according to cell cycle stage by centrifugal elutriation in a Beckman JE-5.0 rotor and model J6-MC centrifuge. Media flow rate was controlled with a Masterflex pump and model 7518-12 pump head (Cole-Parmer, Vernon Hills, IL). The elutriation medium consisted of equal parts of PBS and RPMI 1640 media supplemented with 1% fetal bovine serum. Approximately 2.0×10^8 cells were introduced into the elutriation chamber at 23 ml/min for 30 min. The chamber was maintained at 2,750 rpm and 4°C. The cell gradient was allowed to form for 30 min with the pump set 70 ml/min. The initial fraction was collected at a flow rate of 83 ml/min with an increase of 8 ml/min for successive fractions.

After transduction of HisNLSCreMTS, pre-elutriated and elutriated cells were washed three times with PBS and half of the cells were cultured for one more day before analyzing EGFP expression. The remaining half was used to prepare genomic DNA for Southern blot analysis. Following hybridization to a ^{32}P-labeled EGFP probe, the extent of recombina-

tion was determined by an Hitachi phosphorimager (MiraiBio Inc., Alameda, CA).

Immunoassay of Cell-Associated Cre Protein

Tex.loxp.EG cells treated with HisNLSCreMTS were washed three times with ice-cold PBS and then treated with 0.25% trypsin–EDTA solution (Invitrogen, Rockville, MD) for 1 min at room temperature and with 1 × trypsin inhibitor solution (Sigma–Aldrich, St. Louis, MO) for 2 min. The cells were washed twice with ice-cold PBS, and lysed in 200 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, and 1 μg/μl each of leupeptin, aprotinin, pepstatin, chymostatin, and antipain) on ice for 5 min. Nuclei were pelleted by centrifugation, and the supernatant was saved as the cytoplasmic extract. The nuclear pellet was washed in 1 ml buffer A and resuspended in 100 μl of buffer B (20 mM HEPES, pH.7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 μg/μl each of protease inhibitors). Samples were vortexed for 15 min at 4°C, centrifuged at 10,000*g* for 15 min, and the supernatant was saved as the nuclear extract. The concentrations of proteins in cytoplasmic and nuclear extracts were determined by using the Lowry method and were normalized with buffers A and B, respectively. Extracts (40 or 4 μg/ml) were absorbed on 96 well plates for 24 h at room temperature. Various concentrations of HisNLSCreMTS in buffers A or B were used as standards. All wells were washed with PBS + 0.05% Tween 20, blocked with 1% bovine serum albumin, and incubated with anti-Cre rabbit polyclonal antibody (1:10,000; Novagen, Madison, WI) for 1 h at room temperature. Bound anti-rabbit IgG conjugated-HRP (1:8,000; Santa Cruz Biotechnology, Santa Cruz, CA) was quantified by using a microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

Preparation of FITC-Conjugated HisNLSCreMTS Protein and FACS Analysis

A total of 250 μg fluorescein isothiocyanate (FITC; Pierce Chemical, Rockford, IL) were conjugated to 5 mg HisNLSCreMTS protein according to the "standard protocol" provided by the manufacturer. FITC-conjugated SN50 peptide was provided by Dr. Jacek Hawiger, Vanderbilt University. Cells were treated with 5 μM FITC, 2.5 μM FITC-conjugated HisNLSCreMTS, or 5 μM FITC-conjugated SN50 for indicated time and temperature, were washed five times with ice-cold PBS, and were resuspended in PBS containing 2% serum for flow cytometry.

RESULTS

Cre-Mediated Recombination in Cultured Cells

Three recombinant fusion proteins (Fig. 1A) were used to deliver enzymatically active Cre proteins directly into mammalian cells. HisCre consists of the native enzyme expressed by bacteriophage P1 to which an amino-terminal 6-histidine tag (6xHis) has been added while HisNLS Cre contains an additional nuclear localization signal from SV40 large T antigen (Lin et al., submitted). HisNLSCreMTS is similar to HisNLSCre but has a FGF-4 MTS positioned on the C-terminus [Jo et al., 2001]. Previous studies have shown that the native enzyme has a low, but intrinsic ability to enter mammalian cells [Will, 2002 #62; Lin et al., submitted]. Uptake and/or activity are separately increased by the 6xHis and NLS elements, whereas the MTS interferes with Cre activity in cultured cells [Joshi et al., 2002; Peitz et al., 2002; Will et al., 2002; Lin et al., submitted].

The proteins were transduced into Tex.loxp.EG.74 cells, a T lymphoctye line in which Cre-mediated recombination activates the expression of an enhanced green fluorescent protein (EGFP) reporter gene [Jo et al., 2001]. The EGFP reporter, introduced by a retrovirus vector (Fig. 1B), contains a stop cassette flanked by loxP sites that prevents EGFP expression. Tex.loxp.EG.74 cells were exposed either to a range of concentrations of HisCre, HisNLSCre, and HisNLSCreMTS for 2 h (Fig. 1C) or to 5 μM HisCre, 2 μM HisNLSCre, or 10 μM HisNLSCreMTS for different lengths of time (Fig. 1D). The cells were then washed extensively with PBS, were cultured for 24 h to provide time for EGFP expression, and the percentage of EGFP-expressing cells was determined by flow cytometry. Southern blot analysis confirmed that expression of the EGFP reporter accurately reflected the extent of template recombination (data not shown). All three proteins entered cells and induced recombination in a dose- and time-dependent manner, with HisNLSCre displaying the greatest activity.

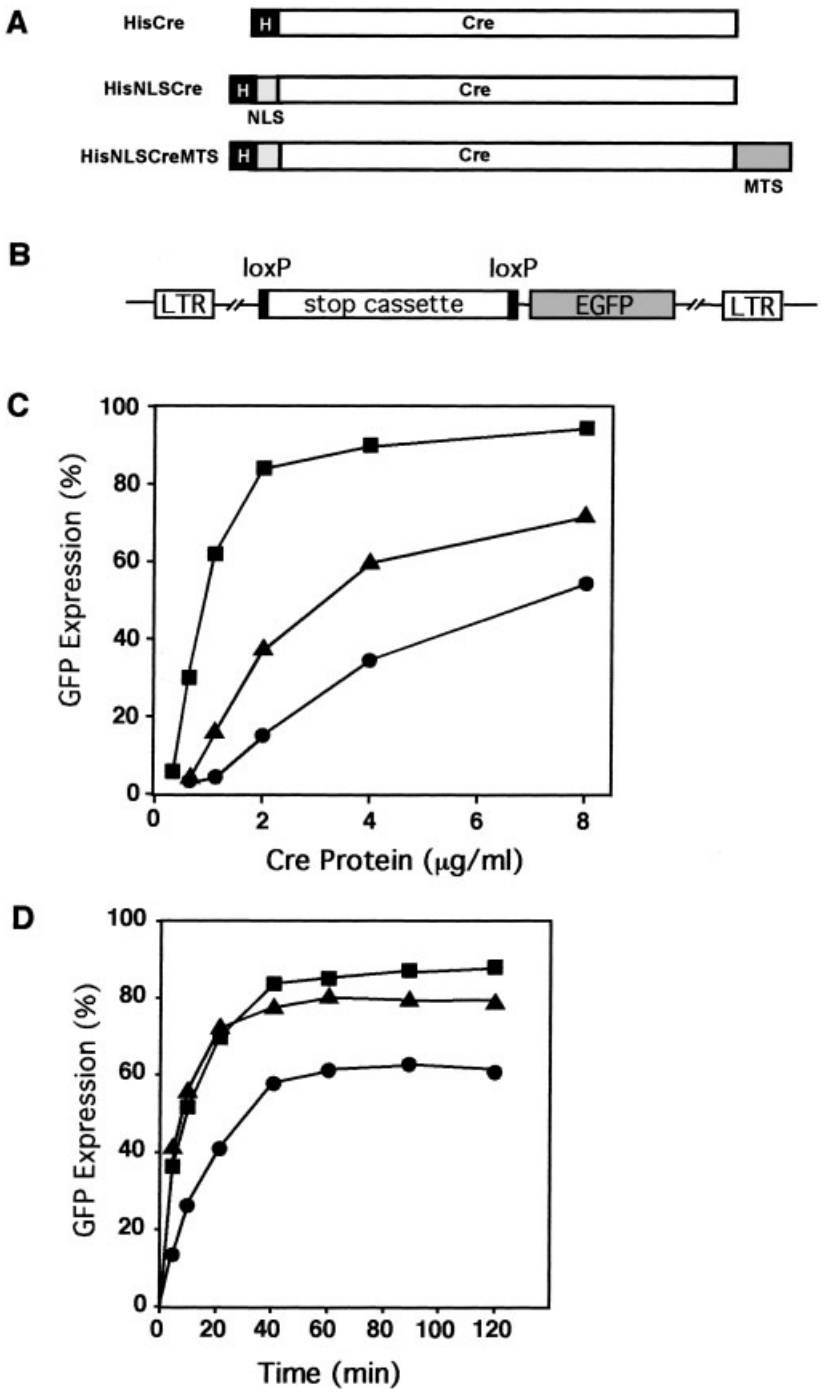

Fig. 1. In vivo Cre-mediated recombination. **A**: Structures of recombinant Cre fusion proteins. Cre sequences (not shaded) from nucleotide 484 to 1513 (GenBank X03453) were expressed as fusion proteins with one or more of the following elements: a 6xHis tag (MGSSHHHHHHSSLVPRGSH, black); an SV40 large T antigen NLS (PKKKRKV, light gray); and a FGF-4 MTS (AAVLLPVLLAP, dark gray). **B**: Structure of the recombination substrate in Tex.loxp.EG cells. The pBABE.lox.stp.EGFP retrovirus vector contains a stop cassette and loxP sites such that Cre-mediated recombination activates the expression of an enhanced green fluorescent protein (EGFP) reporter gene. **C**: Dose-response of Cre-mediated recombination. Tex.loxp.EG.74 cells, containing a single copy of the pBABE.lox.stp.EGFP provirus, were treated with increasing concentrations of HisNLSCre (squares), HisNLSCreMTS (triangles), or HisCre (circles) for 2 h at 37°C, and after 24 h, the percent of EGFP expressing cells was determined by flow cytometry. **D**: Time course of protein uptake. Tex.loxp.EG.74 cells were treated with 3 μM HisNLSCre (squares), 10 μM HisNLSCreMTS (triangles), or 5 μM HisCre (circles) for different lengths of time at 37°C, and after 24 h, the percent of EGFP expressing cells was determined by flow cytometry.

Cell Cycle Dependency of Cre-Mediated Recombination

The kinetics and dose-response of Cre-induced EGFP expression (Fig. 1) suggested some heterogeneity within the clonal population of target cells with regard to protein transduction and/or recombination. To test for potential cell cycle effects, Tex.loxp.EG.74 cells were sorted by centrifugal elutriation and analyzed for Cre-mediated recombination at different stages of the cell cycle. Centrifugal elutriation separates cells according to size [Grdina et al., 1986], which increases as cells progress through the cell cycle. A total of nine fractions were collected and analyzed for DNA content to assess the enrichment by cell cycle phase. For example, fractions 2, 4, 5, and 7 were highly enriched for G1, early S, late S, and G2/M phase cells, respectively (Fig. 2A). Pre-elutriated and elutriated cells were treated as before with 10 μM HisNLSCreMTS for 1 h and subsequently analyzed for EGFP expression. As shown in Figure 2B, cell cycle phase had a striking effect on the ability of cell-permeant Cre to induce EGFP expression. Only about 20% of G1 cells (fractions 1–2) treated with HisNLSCreMTS later expressed EGFP, as compared to over 70% of cells with G2/M DNA content (fractions 6–9). S phase enriched cells (fractions 3–5) showed an intermediate response. The percent of cells in each fraction induced to express EGFP was inversely correlated with the percent of cells with a G1 DNA content, such that the distribution of G1 phase cells accounted for essentially all of the variation ($R^2 = 0.99$) among the elutriated fractions (Fig. 2C).

Cre-induced EGFP expression does not provide a direct measure of recombination since one must allow time for EGFP expression after exposing cells to the Cre protein. To directly assess recombination in elutriated cell fractions, DNA was extracted immediately following Cre treatment and analyzed by Southern blot hybridization (Fig. 2D). As before, cell cycle phase had a striking effect, with recombination occurring in less than 20% of G1 and early S phase templates and increasing sharply during S phase. Differences between the extent of physical recombination after 1 h and EGFP expression after 24 h (Fig. 2D) suggest that up to half of the eventual recombination took place after cells were exposed to cell-permeant Cre. This difference was most pronounced in G2/M cells.

Similar cell cycle effects were observed using other Cre proteins. Fractions of elutriated Tex.loxp.EG.74 cells were treated with 1.5 μM HisNLSCre and 5 μM HisCre proteins for 30 min (Fig. 3), and for comparison, the cells were also treated for 30 min with 8 μM HisNLSCreMTS. Recombination was normalized to the highest value obtained with each protein, which for HisNLSCreMTS, HisNLSCre, and HisCre corresponded to recombination in 30, 46, and 61% of the treated cells, respectively. As before, the relative extent of recombination was inversely correlated with the percent of cells with a G1 DNA content (Fig. 3C), with R^2 values of 0.92, 0.88, and 0.93 following treatment with HisNLSCreMTS, HisNLSCre, and HisCre, respectively. G2/M may also affect recombination induced by HisNLSCre, since later fractions showed greater increases in recombination as compared to cells transduced with HisNLSCreMTS, and HisCre. Moreover, R^2 values for cells treated with HisNLSCre improve to 0.94 if the last two fractions are excluded from the analysis. Similar results were also obtained in cells treated with the same proteins for 100 min (data not shown).

Cell Cycle-Dependent Uptake of HisNLSCreMTS Protein

In principle, the effects of cell cycle on Cre-mediated recombination could result from a number of factors, including differences in protein transduction, nuclear translocation, substrate accessibility, and Cre stability or activity. However, the task of examining any of these factors quantitatively is complicated by difficulties of distinguishing internalized from cell-associated proteins after exposing cells to micromolar quantities of cell-permeant protein [Lundberg and Johansson, 2001]. To approach this problem, we first compared Cre-mediated recombination and protein uptake at 4 and 37°C. As shown in Figure 4A, recombination was much lower in cells treated for 1 h with 7 μM HisNLSCreMTS at 4 than at 37°C. While proteins containing the FGF-4 MTS are not expected to cross the plasma membrane at 4°C [Lin et al., 1995], residual recombination (about 5% of that observed at 37°C) apparently results from cell-associated protein, which may gain entry when cells are later cultured at 37°C. Indeed, despite extensive washing, the levels of HisNLSCreMTS in cytoplasmic and nuclear extracts from cells exposed to the protein at

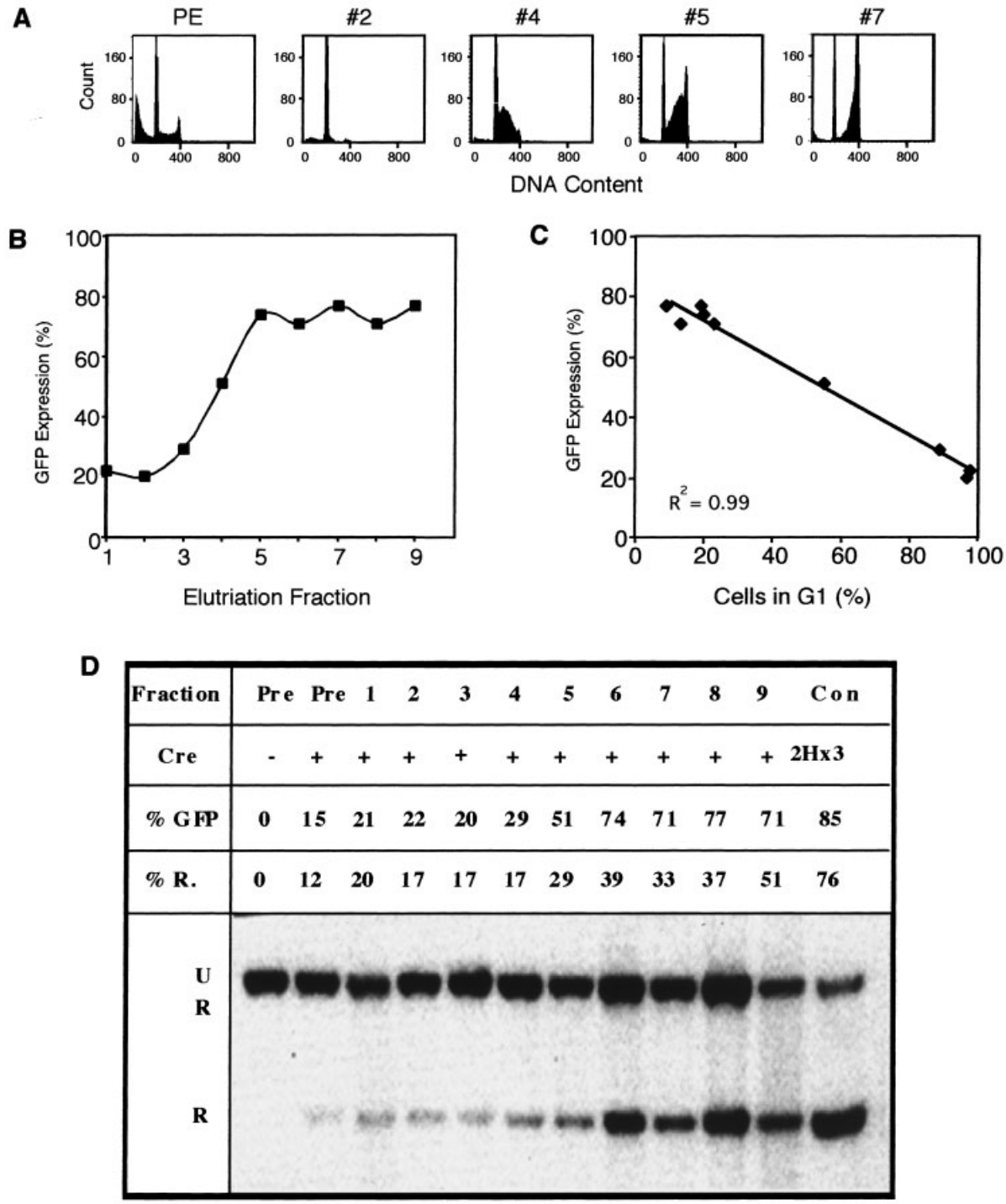

Fig. 2. Cell cycle-dependent Cre-mediated recombination. **A**: Enrichment by cell cycle stage. Tex.loxp.EG.74 (2.0×10^9) cells were fractionated by centrifugal elutriation, and aliquots of each fraction were stained with propidium iodide and analyzed for DNA content by flow cytometry. FACS profiles of pre-elutriated cells (PE) and fractions 2 (G1 phase), 4 (early S phase), 5 (late S phase), and 7 (G2/M phase) are shown. **B**: Cell cycle-dependent induction of EGFP expression. Elutriated cells were incubated with 10 μM HisNLSCreMTS for 1 h at 37°C, were washed three times with PBS, and after 24 h, the percentage of EGFP expressing cells was determined by flow cytometry. **C**: Inverse correlation between EGFP expression and the percent of cells in the G1 phase of the cell cycle. **D**: Cell cycle-dependent recombination. DNA extracted immediately after Cre treatment was digested with *Xba*I and analyzed by Southern blot hybridization, using a ^{32}P-labeled EGFP probe. The extent of recombination (%R), as quantified by phosphoimager analysis, is compared to the percent of cells expressing EGFP 24 h after Cre treatment and to the percent of G1 phase cells.

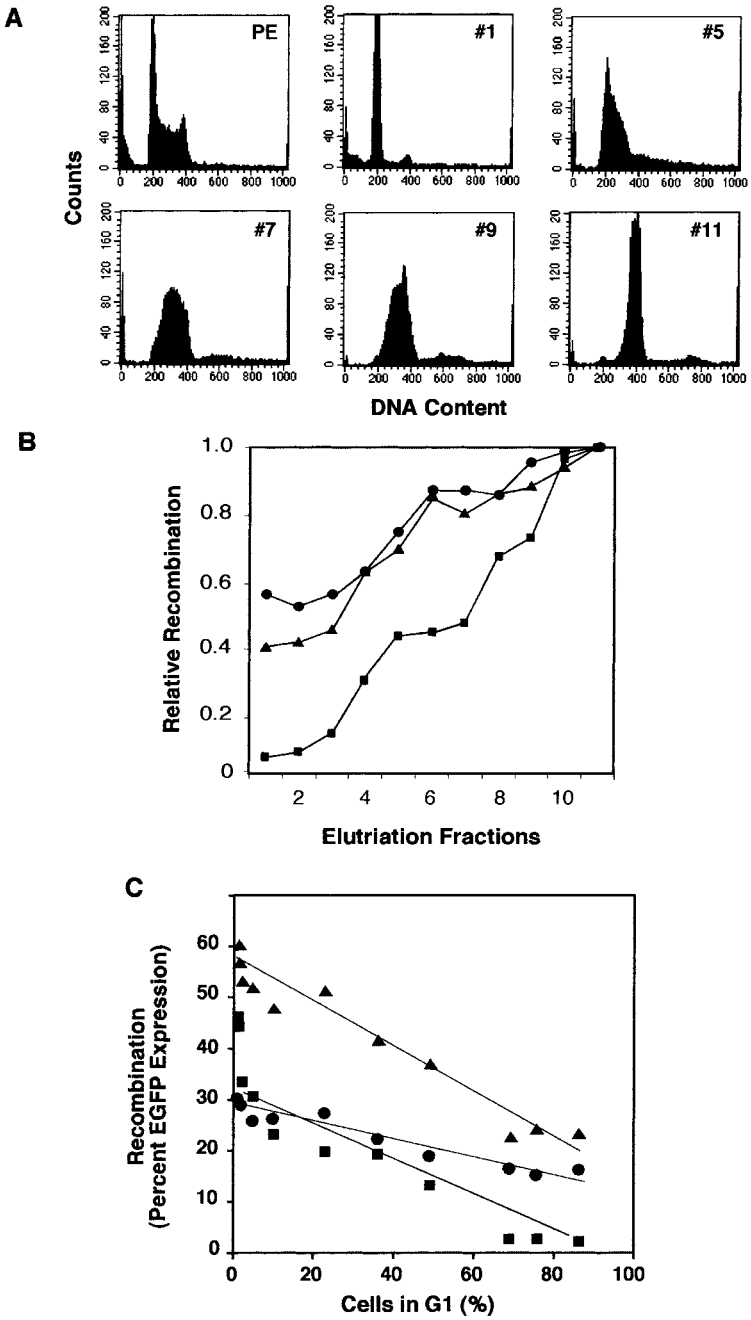

Fig. 3. Cell cycle-dependent recombination by different cell-permeant Cre proteins. **A**: Enrichment by cell cycle stage. Tex.loxp.EG.74 (2.0×10^8) cells were fractionated by centrifugal elutriation and aliquots of each fraction were stained with propidium iodide and analyzed for DNA content by FACS. FACS profiles of pre-elutriated cells (PE) and fractions 1 (G1 phase), 5 (early S phase), 7 (S phase), 9 (late S phase), and 11 (G2/M phase) are shown. **B**: Cell cycle-dependent induction of EGFP expression following treatment with different cell-permeant Cre proteins. Elutriated cells from each fraction (1.0×10^6 cells per 2 cm^2 well) were incubated with 1.5 μM HisNLSCre (●), 8 μM HisNLSCreMTS (■), and 5 μM HisCre (▲) for 30 min at 37°C, were washed three times with PBS, and after 24 h, GFP expression was determined by flow cytometry. Relative recombination is normalized to the highest recombination level of each protein. **C**: Inverse correlation between EGFP expression and the percent of G1 phase cells. R^2 values were calculated following treatment with 1.5 μM HisNLSCre (●), 8 μM HisNLSCreMTS (■), and 5 μM HisCre (▲) were 0.88, 0.92, and 0.93, respectively.

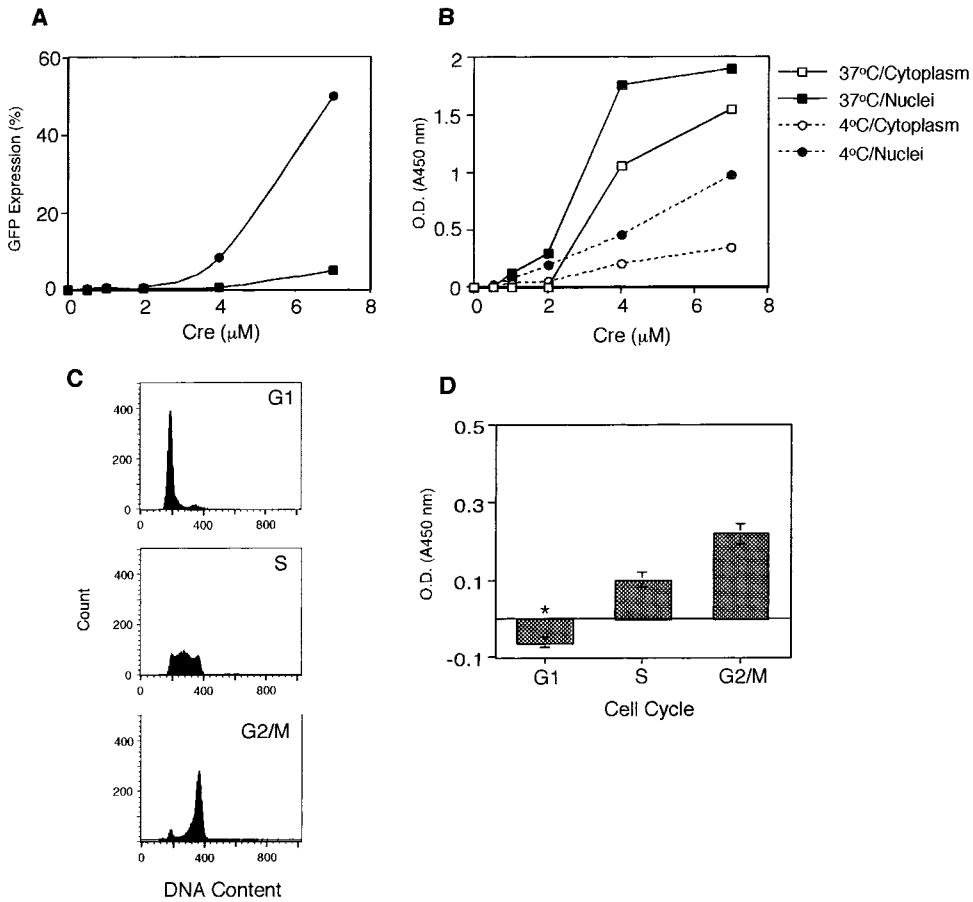

Fig. 4. Temperature- and cell cycle-dependent uptake of cell-permeant Cre protein. **A**: Temperature-dependent recombination. Tex.loxp.EG.74 cells were treated with increasing concentrations of HisNLSCreMTS for 1 h at either 37°C (circles) or 4°C (squares), and after 24 h, the percent of EGFP expressing cells was determined by flow cytometry. **B**: Temperature-conditional protein uptake. Cytoplasmic (○, □) and nuclear (■, ●) extracts were prepared from cells treated with HisNLSCreMTS at 37 (□, ■) or 4°C (○, ●), and the amount of HisNLSCreMTS protein was measured by ELISA (40 μg protein/well). **C**: Enrichment by cell cycle stage. 2.0×10^8 Tex.loxp.EG.74 cells were fractionated by centrifugal elutriatation, and aliquots of pooled fractions were stained with propidium iodide and analyzed for DNA content by flow cytometry. **D**: Cell cycle-dependent protein uptake. The amount of Cre protein internalized by elutriated cells in (C) was measured by ELISA (4 μg cytoplasmic extract/well). *The values at 4°C (non-internalized protein) were subtracted from those at 37°C. Data are mean ± SEM from two or more independent experiments, each performed in triplicate. Differences between G1 and S or G2/M phase cells are significant with $P < 0.001$.

4°C were 25 and 50% of those transduced at 37°C (Fig. 4B). We assume that most of the Cre associated with cells at 4°C was not internalized since it did not promote recombination and thus provides a measure of non-specific binding.

To examine potential cell cycle effects on protein uptake, elutriated G1, S, and G2/M phase cells (Fig. 4C) were treated with 7 μM HisNLSCreMTS for 1 h at 4 and 37°C, and cell extracts were analyzed for Cre protein. The amount of Cre associated with cells treated at 37°C increased as cells progressed through the cell cycle as shown in Figure 4D, after subtracting the levels of Cre bound at 4°C.

Kinetics of Protein Transduction at Different Stages of the Cell Cycle

To assess the relative rates of protein transduction at different stages of the cell cycle, cell-permeant proteins were labeled with a FITC tag, and protein uptake by G1, S, and G2/M phase cells (Fig. 5A) was analyzed by flow cytometry. Control experiments showed that the uptake of FITC-labeled HisNLSCreMTS at 37°C was much greater than the uptake of either FITC alone (at either temperature) or FITC-labeled HisNLSCreMTS at 4°C (Fig. 5B). As before, protein transduction was highest in G2/

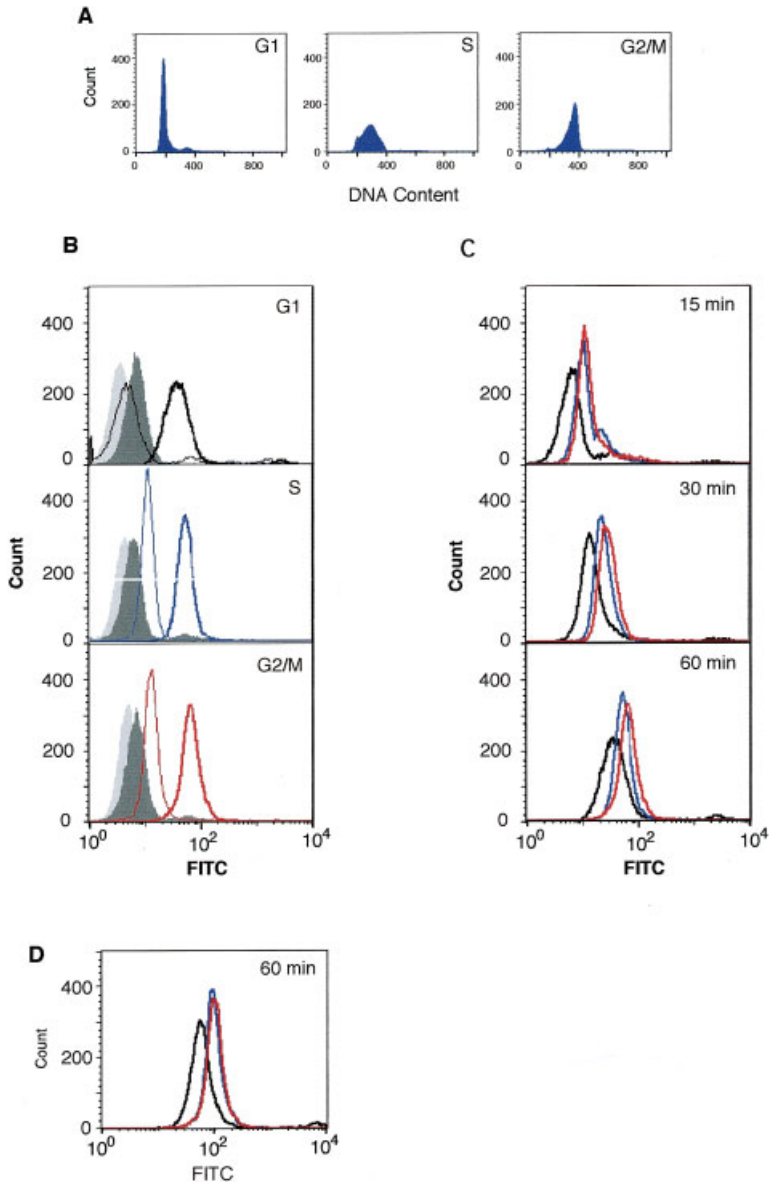

Fig. 5. Cell cycle-dependent protein transduction. **A**: Enrichment by cell cycle stage. Tex.loxp.EG.74 (2.0×10^8) cells were fractionated by centrifugal elutriation, and aliquots of pooled fractions were stained with propidium iodide and analyzed for DNA content by flow cytometry. **B**: Cell cycle-dependent protein uptake. Elutriated Tex.loxp.EG.74 cells enriched in (A) were treated with 5 μM FITC (filled) or 2.5 μM FITC-conjugated HisNLSCreMTS (lines) for 60 min either at 4°C (filled light grey or dashed lines) or at 37°C (filled dark grey or solid lines) and were analyzed by flow cytometry. **C**: Kinetics of protein uptake. Elutriated Tex.loxp.EG.74 cells enriched for G1 (black), S (blue), and G2M (red) phase cells were treated with 2.5 μM FITC-conjugated HisNLSCreMTS for the indicated times and were analyzed by flow cytometry. **D**: Cell cycle-dependent uptake of FITC-SN50 peptide. Elutriated Tex.loxp.EG.74 cells enriched for G1 (black), S (blue), and G2M (red) phase cells were treated with 5 μM FITC-conjugated SN50 peptide for 60 min and were analyzed by flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

M phase cells and lowest in G1 phase cells (Fig. 5C). For this experiment, cells were treated with 2.5 μM FITC-labeled HisNLSCreMTS for 15, 30, and 60 min. Similar results were obtained following treatment with an FITC-labeled SN50 peptide [Yan Liu et al., 2000] that consists of the FGF-4 MTS sequence coupled to the Nuclear Factor kappa B nuclear localization sequence (Fig. 5D). Overall, the level of protein transduction in G1 phase cells was approximately 50% of that observed in S and G2/M phase cells—sufficient to account for the cell cycle effects on Cre-mediated recombination.

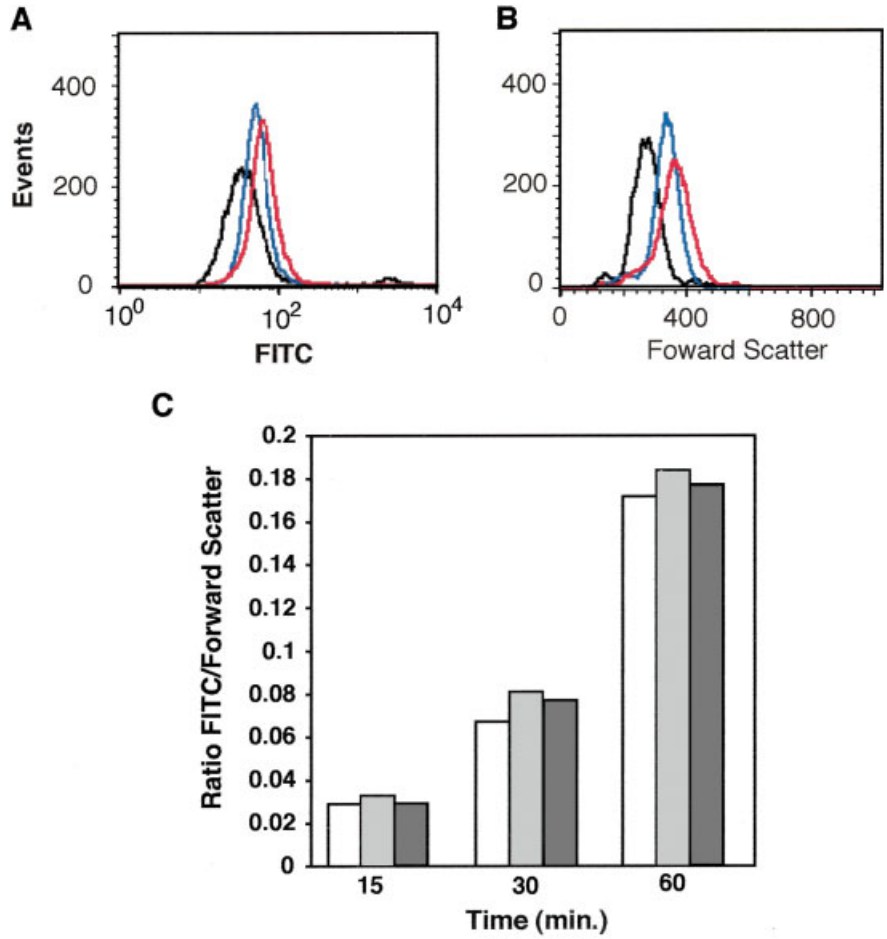

Fig. 6. Cell cycle-dependent protein uptake correlates with cell size. Elutriated Tex.loxp.EG.74 cells were treated with 2.5 μM FITC-conjugated HisNLSCreMTS for 60 min and were analyzed by flow cytometry. The distribution of FITC–HisNLSCreMTS uptake in G1 (black), S (blue), and G2M (red) phase cells (**A**) was similar to the size distributions of the cells, as measured by forward light scatter (**B**). **C**: The ratio of protein uptake (fluorescence) to forward light scatter was similar for G1 (white), S (light gray), and G2/M (dark gray) phase at each time point following exposure to FITC–HisNLSCreMTS. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Differences in the uptake of FITC-labeled Cre closely matched differences in cell size as estimated by forward light scatter (Fig. 6). Thus, fluorescence and light scatter FACS profiles of G1, S, and G2/M phase cells were nearly identical as illustrated in Figure 6A,B, respectively. Moreover, the ratio of fluorescence to forward light scatter at each time point was similar for all three cell populations, suggesting that the uptake of FITC-labeled Cre at each cell cycle stage was proportional to cell size.

Recombination Following Drug-Induced Cell Cycle Arrest

We tested the effect of artificially arresting cells at different cell cycle stages on Cre-mediated recombination. Two different clones of Tex.loxp.EG were examined to assess potential clonal variation in protein transduction/recombination. As shown in Table I, treatment of Tex.loxp.EG.74 and Tex.loxp.EG.31 cells with 10 μM HisNLSCreMTS for 2 h, induced similar levels of EGFP expression—74 and 61%, respectively. The clones, tested under normal growth conditions, contained approximately 40% G1 cells. Cells were also treated with different chemical agents to arrest cells at different points in the cell cycle as summarized in Table I. 5-Aza-2-deoxycytidine (5aCdr), a DNA methyltransferase inhibitor increased the proportion of S and G2/M phase cells. Trichostatin A (TSA), a reversible histone deacetylase inhibitor, blocked progression from G1 to S phase. Taxol, which binds and stabilizes microtubles, arrested cells at S and G2/M. Finally, mimosine, a DNA replication inhibitor, had little effect on

TABLE I. Effects of Chemotherapeutic Drugs on Cre-Mediated Recombination

Clone	Drug (concentration)	% GFP	G1/S/G2M
74	None	74	42/34/24
31		61	39/37/26
74	5-Aza-2-deoxycytidine	31	14/42/44
31	(1 μM)	38	15/49/36
74	Trichostatin A	9	61/18/24
31	(17 nM)	12	68/21/11
74	Cisplatin	23	30/40/30
31	(30 μM)	13	25/44/30
74	Taxol	56	30/30/40
31	(10 nM)	61	14/31/55
74	Minosine	62	37/35/28
31	(20 μM)	51	37/35/28

Cells from Tex.loxp.EG clones 74 and 31 were treated with the indicated drugs for 24 h, and then the percent of cells in the G1, S, and G2/M phases of the cell cycle was determined by flow cytometry, and the cells were exposed to HisNLSCre–MTS protein for one hour. After 24 h, the percent of cells expressing %EGFP was determined by flow cytometry.

Tex.loxp.EG cells. While all agents had similar effects on the two Tex.loxp.EG clones with regard to Cre-induced EGFP expression, there was little correlation ($R^2 = 0.3$) between the proportion of cells with G1 DNA content and levels of recombination (Table I). Moreover, none of the agents was able to enhance recombination even when the proportion of G1 cells was reduced.

DISCUSSION

The present study analyzed the effects of cell cycle on the uptake and activity of cell-permeant Cre recombinase proteins. Previous studies suggested that the efficiency of recombination and/or protein transduction varied among individual cells, even within a clonal population. We report here that cells in the G1 phase of the cell cycle undergo recombination at a significantly lower rate than cells at other phases of the cell cycle, and that this variation results largely from differences in protein uptake associated largely, but not entirely, with differences in cell size.

Protein transduction is thought to involve direct penetration of the plasma membrane, although mechanisms responsible for membrane translocation are not well understood. The process appears to exploit properties common to most, if not all, mammalian cell types without involving specific receptor or transporter systems [Hawiger, 1999; Schwarze and Dowdy, 2000; Dunican and Doherty, 2001]. Sequences with membrane translocating activity include basic sequences such as HIV Tat and Antennapedia and hydrophobic (or "H") sequences derived from the N-terminal signal peptides of secreted and trans-membrane proteins. Native Cre protein has a low, but intrinsic ability to enter mammalian cells, but sequences responsible for protein uptake have not been characterized [Will et al., 2002]. Sequences such as the TAT transduction domain, the SV40 NLS and sequences containing a 6xHis tag stimulate Cre uptake and/or activity in cultured cells, while the FGF-4 MTS and some tags used for affinity purification have net deleterious effects [Jo et al., 2001; Joshi et al., 2002; Peitz et al., 2002; Will et al., 2002]. These additional sequences have minimal effects on the in vitro catalytic activity of the enzyme, suggesting that membrane translocation and/or in vivo activity is influenced by structural features of the cargo. The three cell-permeant Cre proteins tested, HisNLSCreMTS, HisNLSCre, and HisCre, all showed cell cycle-dependent variation in their ability to induce recombination. The cell cycle effects were observed in elutriated fractions of naturally proliferating cells but not in cells artificially arrested with metabolic poisons or genotoxic agents.

In principle, the cell cycle could affect the process by which cell permeant proteins enter cells. For example, cell cycle variation in the composition [Pan et al., 1990], structure [Incardona and Eaton, 2000; Schlegel et al., 2000], or activity [Illinger et al., 1993] of the plasma membrane could be expected to influence protein uptake. Our results suggest that pinocytosis, which declines during mitosis [Berlin et al., 1978; Oliver et al., 1985], is not responsible for Cre uptake. Similarly, protein transduction can be distinguished from a mitosis-specific process by which normally impermeant macromolecules, including proteins coated with carrier peptides, can enter cells [Pellegrin et al., 2002]. In contrast, membrane fluidity, as measured by either microviscosity or lateral diffusion of membrane proteins, is minimal during G1 and increases during cell cycle progression [de Laat et al., 1977, 1980; Lai et al., 1980]. The number of microvilli and rates of endocytosis have also been reported to increase during cell cycle progression [Knutton et al., 1975; Illinger et al., 1993]. However, after factoring the influence of cell size, these correlative physical changes do not appear to have major effects on protein transduction.

As reported previously [Lin et al., 1995], transduction of cargoes containing the FGF-4 MTS was greatly decreased at 4°C, presumably because of reduced membrane fluidity. Similar results were obtained when protein transduction was monitored either by Cre-mediated recombination or by uptake of fluorescein-labeled proteins. These results are at odds with the reported behavior of other cell-permeant proteins, in which protein transduction appeared to be unaffected by low temperature [Mann and Frankel, 1991; Derossi et al., 1994; Elliott and O'Hare, 1997; Morris et al., 1997, 2001]. Reasons for these differences are not clear. The presence or absence of a temperature effect has been repeated in a number of laboratories and confirmed by using a variety of methods and may therefore reflect significant differences in the mechanisms of cell entry. Alternatively, it may be prudent to reexamine the question of low temperature protein transduction in light of problems in distinguishing internalized from cell-associated proteins [Lundberg and Johansson, 2001].

The rate of protein import into G1 cells was approximately half that of S and G2/M phase cells, and the levels of recombination in cells treated with Cre for 1 h were reduced by a similar extent. Cell cycle effects were even greater, approximately fourfold, when recombination was monitored by EGFP expression in Tex.loxp.EG cells. The relatively lower rate of recombination at G1 can be circumvented by exposing cells to cell-permeant Cre for longer times, with higher concentrations of protein or by using more active enzymes.

Protein transduction permits direct and quantitative analysis of protein functions under non-steady state conditions. Kinetic (time-course) studies are particularly important for understanding biochemical processes in living cells, in which proteins function in tightly regulated and highly interconnected networks [Huang, 2000]. However, differences in cell size/protein uptake are likely to influence the relative activities of cell-permeant proteins in different cell types. Cell cycle effects on protein uptake could also cause quiescent and proliferating cells to respond differently to cell permeant proteins, or alternatively, the enhanced activity of a protein in G1 cells could be masked by reduced protein uptake. Consequently, cell cycle- and size-dependent variation in protein uptake represents important factors to consider when analyzing the biochemical activities of cell-permeant proteins in mammalian cells.

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