Intracellular partitioning of cell organelles and extraneous nanoparticles during mitosis

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Abstract

The nucleocytoplasmic partitioning of nanoparticles as a result of cell division is highly relevant for the field of nonviral gene delivery. Therefore, we reviewed the literature on intracellular distribution of cell organelles (endosomal vesicles, golgi apparatus, endoplasmic reticulum and nucleus), foreign macromolecules (dextrans, plasmid DNA) and inorganic nanoparticles (gold, quantum dots, iron oxide) during mitosis. For nonviral gene delivery particles (lipid- or polymer-based) indirect proof of nuclear entry during mitosis is given. Further, we describe how retroviruses and latent DNA viruses take advantage of mitosis to transfer their viral genome and to segregate their episomes into host daughter nuclei, respectively. Finally, based on this knowledge we propose strategies to improve nonviral gene delivery in dividing cells with the ultimate goal to design nonviral gene delivery systems which are as efficient as their viral counterparts, but which are non immunogenic, not oncogenic and easy and inexpensive to prepare.

Keywords

nonviral gene therapy or delivery particles, quantum dots, gold particles, retroviruses, latent viruses, dextran, plasmid DNA, cell division, cell cycle, nucleus

Abbreviations

ASV	avian sarcoma virus
AT	adenosine monophosphate thymidine monophosphate
BPV	bovine papillomavirus
Brd4	double bromodomain protein 4
CDK	cyclin-dependent kinase

EBNA1	Epstein Barr virus nuclear antigen 1
EBV	Epstein Barr virus
ER	endoplasmic reticulum
FV	foamy virus
H2A-H2B	histon 2A – histon 2B pocket
HHV	human herpesvirus
HIV	human immunodeficiency virus
HPV	human papillomavirus
HVS	herpesvirus saimiri
INM	inner nuclear membrane
IONP	iron oxide nanoparticles
KSHV	Kaposi sarcoma associated herpesvirus
LANA	latency associated nuclear antigen
LEDGF/p75	endogenous lens epithelium derived growth factor/p75
LEM	Lap2, Emerin, Man1
MuLV	murine leukemia virus
NE	nuclear envelope
NEBD	nuclear envelope breakdown
NLS	nuclear localization signal
NPC	nuclear pore complex
ONM	outer nuclear membrane
PEI	polyethyleneimine
PIC	pre-integration complex
QD	quantum dot
SNV	spleen necrosis virus
SV40	Simian vacuolating virus 40
XNER	*Xenopus* nuclear envelope reassembly

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1. General introduction

Gene therapy using plasmid DNA (pDNA) aims at inducing protein production by the administration of pDNA that encodes for the desired protein. To date, several hurdles still need to be overcome to allow highly efficient delivery of drugs or genes at the desired intracellular location. For gene therapy to be successful, nucleic acids should reach the transcription machinery in the nucleus of the target cells. Nucleic acids should also remain intact as their sequence ensures their biological activity. In recent years the notion that for maximal transfection the pDNA not just needs to reach the nucleus, but rather gain access to the right intranuclear compartment has been gaining increasing recognition [1-3]. Moreover, transfection efficiency can differ substantially between cell types, and within one cell type, between various phases of the cell cycle [4, 5].

The cell cycle can be divided into 4 phases: growth or G_1 phase, DNA synthesis or S phase, gap 2 or G_2 phase and mitosis or M phase. G_1/S/G_2 phases together are named the interphase, in which the cells prepare themselves for mitosis. During each cell cycle, the nuclear genome is doubled in S phase and the two identical genomic copies are subsequently separated into both daughter cells during M phase. The typical length of the cell cycle can differ substantially between species, but generally mitosis is completed within 1 - 1.5 hours and cells spend most of their time in interphase (e.g. approximately 8, 6 and 5 hours for G_1/S/G_2 in human beings). Cells which do not divide are

considered to be in a postmitotic rest phase or G_0. Mitosis itself is divided into several distinct subphases: i) prophase, in which the chromatin condenses into chromosomes and the nuclear envelope breaks down (NEBD), ii) metaphase, in which the chromosomes align themselves along the metaphase plate iii) anaphase, in which the sister chromatids are separated by moving to the opposite poles and iv) telophase/cytokinesis in which the nuclear envelope (NE) is reassembled around the segregated chromatids and the cell divides into two daughter cells. The different stages of the cell cycle are controlled by subsequent phosphorylation and dephosphorylation reactions governed by cyclins and cyclin-dependent kinase (CDK) complexes, which are tightly controlled both in space and in time [6, 7]. Also, several checkpoints exist, which verify the completion of the processes at each phase of the cell cycle before progression into the next phase is allowed.

It has been shown that cells which do not go through M phase are difficult to transfect [5, 8]. The main reason for this is the nuclear envelope, which consists mainly of two phospholipid bilayers [9-11]. Occasionally, the outer nuclear membrane (ONM) and the inner nuclear membrane (INM) fuse at the nuclear pore complexes (NPCs). In interphase, pDNA can only gain access to the nuclear interior through the small central channel of the NPCs, which is a very inefficient process. Although some gene delivery systems are cell cycle-independent [12], most of them profit from the process of cell division [5, 8, 13-18]. The majority of higher eukaryotes undergo 'open mitosis', characterized by the complete disassembly of the NE allowing the intermixing of nucleoplasmic and cytoplasmic components. At the end of mitosis, the NE reconstitutes and the cytoplasm and nucleoplasm 'unmix' again. It is likely that certain cytoplasmic components can gain access to the nucleoplasm during these mitotic mixing and unmixing events. In this review, we will emphasize the therapeutic potential of nonviral gene delivery particles brought to the nucleus during cell division. In that regard, we will first focus on the cellular machinery involved in NE breakdown and reassembly during mitosis. Secondly, we will review the fate of cell organelles, macromolecules and nonviral nanoparticles during mitosis. Further, we will focus on the mechanisms applied by certain viral particles to deliver their genome into the cell nucleus or segregate their latent episomes to progeny cells during mitosis (Table 1). Based on this knowledge we will propose experimental approaches which could enhance the intranuclear delivery of nonviral gene delivery complexes during cell division. This in turn might lead to an improvement of the efficacy of therapeutic strategies relying on nonviral carriers.

2. Breakdown and reassembly of the nuclear envelope structure during mitosis

2.1. Nuclear envelope structure

In eukaryotic cells, the genetic material is kept inside the nucleus, surrounded by the NE which physically separates the nucleoplasm from the cytoplasm. It consists of two phospholipid bilayers

composed of the ONM, which is continuous with the endoplasmic reticulum (ER), and the INM which interacts with the underlying lamina and chromatin through several integral and associated membrane proteins (Fig. 1) [19, 20]. The NE is equipped with NPCs which regulate traffic between the cytoplasm and the nucleus. These 125 kDa large multi-protein complexes contain an aqueous channel that permits passive transport of molecules up to 10 nm in size. Larger molecules require a nuclear localization signal (NLS) that can enlarge the central channel of the NPCs up to 40 nm [21]. NPCs are strongly anchored in the NE and are composed of strongly associated nucleoporin complexes [22]. NPCs are assumed to play a role in gene regulation since activated genes associate with the NPCs. Furthermore, they can maintain a 'transcriptional memory': previously transcribed genes are retained at the NPCs and show an accelerated gene expression following reactivation. Interestingly, this transcriptional memory can be maintained even after cell division [23].

The 30 – 100 nm thick nuclear lamina is located in-between the chromatin and the INM. It provides mechanical strength and transcriptional regulation to the cell nucleus and is composed of type A and type B lamins and lamin-associated proteins which are often integral proteins of the INM [24]. Many of these INM proteins contain the LEM-domain (Lap2, Emerin, Man1), through which they bind the lamins, as well as other components such as the barrier-to-autointegration factor (BAF). BAF on its turn can bind dsDNA, chromatin and transcription activators, thereby connecting the nuclear lamina to the peripheral chromatin [25, 26]. Due to multiple interactions between the lamina and the chromatin, the lamins play an important role in the regulation of transcription in response to a chemical or mechanical stimulus [23]. If the nuclear lamina is affected in so-called laminopathies, this can drastically alter the expression profile of the cell [27]. The most striking example is probably molecular aging in Hutchinson-Gilford progeria syndrome, which is the result of a single mutation in lamin A [28].

In the cell nucleus, DNA is packed with histones into chromatin. In interphase, at least two types of chromatin can be distinguished: a lightly packed form of chromatin (euchromatin) that has greater potential to be transcribed and highly condensed chromatin (heterochromatin) that is generally transcriptionally inactive. When the chromosomes are formed during mitosis, the chromatin is arranged into its most compact form. Euchromatin is generally found in the core of the nucleus, while heterochromatin is located closer to the lamina, suggesting that the nuclear interior promotes gene expression, whereas the nuclear periphery promotes gene silencing, unless in the areas close to NPCs [23, 29].

2.2. Nuclear envelope breakdown

In the process called ‘open mitosis’, the NE completely disassembles, resulting in the intermixing of nucleoplasmic and cytoplasmic components. The stepwise process of NEBD is initialized at late prophase by a series of CDK1-dependent phosphorylations of nucleoporins, lamins and integral membrane proteins from the INM [30]. The initial step of NEBD is the partial disassembly of the NPCs. This increases the passive permeability of the NPCs to molecules up to about 40 nm, thereby promoting the nuclear import of larger cytoplasmic molecules such as tubulin, a major component of the mitotic spindle [31]. Subsequently, the core nucleoporins are released, resulting in the complete disassembly of the NPCs. The complete disassembly of the NPCs is a rapid process, which is completed in minutes. Initially it was thought that the NE elements are distributed in the cytoplasm in vesicular form. Currently it is becoming more and more accepted that most NE membranes and their associated proteins are resorbed in the ER [19]. As the final step in NEBD, the nuclear lamina depolymerizes and INM proteins dissociate from the lamins and the chromatin.

2.3. Nuclear envelope reassembly

During anaphase, the sister chromatids are moving to opposite poles by shortening of the microtubuli [32]. This results in the physical separation of two sets of identical chromosomes, around which a new NE is formed. This NE starts to reassemble in late anaphase and NE formation is completed at telophase/cytokinesis, when two daughter cells are formed. To prevent nonnuclear material to end up in the nucleus, it is important to securely unmix the cytoplasmic and nucleoplasmic elements. Also, all chromatin should be included in only one nucleus, avoiding the formation of a second so-called ‘micronucleus’ around chromosomes which got separated from the rest. To facilitate this process, chromatin is at its most condensed state when the NE has to reform [33]. The different phases of the NE reassembly include targeting of proteins to chromosomes, assembly of the NPCs, recruitment and fusion of membrane elements and nuclear import of lamins. In contrast to NEBD, which is triggered by a series of phosphorylation reactions, a series of dephoshorylation events coordinates the NE assembly.

Two different models exist for the NE assembly. In the ‘insertion model’ NPCs are inserted in the already formed NE. In the ‘prepore’ model, first the so-called prepores or pre-NPCs are formed, which is followed by the formation of the NE in-between these pores. It was previously thought that membrane elements could bind to chromatin in form of membrane vesicles, which would finally fuse to form the NE [34-36]. Currently, however, it is thought that the NE reappears from the ER, where it was redistributed during NEBD. Very recently, Lu *et al*. [37] presented evidence that is in favor of the insertion model. The authors found that the NE reassembly primarily occurs by coordinated direct contact of ER cisternae with the chromosomes and that a functional NPC was only formed when NE formation at that site was completed. Both in the insertion and the prepore model, the final step in

NE formation is the import of lamins through the rebuilt NPCs and the assembly of the nuclear lamina through dephosphorylation of B-type lamins [7, 33]. It is not clear whether the nuclear lamina already binds to specific parts of the chromatin when it assembles or that it initially binds at random and rearranges according to the gene expression needs by detachment and rebinding afterwards [38].

3. Mitotic partitioning of cell organelles

As described above, the predominant function of cell division is to deliver a complete set of chromosomes to the two daughter cells. In addition, it should ensure appropriate distribution of key organelles located in the cytosol and prevent nonnuclear material to be included in the daughter nuclei. The latter might occur through exclusion from condensed chromatin throughout mitosis and completion of NE assembly before nuclear expansion [39]. Vesicular particles such as early or late endosomes and lysosomes are distributed in large numbers throughout the cytoplasm. On the other hand, the endoplasmic reticulum (ER) and Golgi apparatus exist as single or low-copy organelles. Therefore, it is conceivable that their partitioning during mitosis will display significant differences. Distinctive pathways of endocytosis serve to assure efficient uptake of macromolecules and (gene delivery) particulate formulations by cells. Early or late endosomes and lysosomes are prime players in this process. The *trans*-Golgi network is responsible for sorting and transport of proteins to endosomes and lysosomes.

Bergeland *et al.* [40] studied mitotic partitioning of early and late endosomes and observed that none of the endocytic vesicles fragmented or fused during cell division. Even though the numbers of both types of endosomes in the two daughter cells were roughly the same, no evidence for a strict mechanism guaranteeing their equal distribution was found. This observation was confirmed by others [41], leading to a model in which the partitioning of vesicles in the cytoplasm occurs passively but equally between the two daughter cells. In this model, it is assumed that the more vesicles are present in the cytoplasm, the more accurate the partitioning effect becomes.

On the other hand, Dunster *et al.* [41] found that late endosomes and lysosomes were inherited by daughter cells in a different manner than early endosomes. In contrast to early endosomes, late endosomes and lysosomes hardly associated with the spindle array, from prophase throughout anaphase. By telophase, the distribution of late endosomes and lysosomes mirrors that of early endosomes [41]. It was shown that clustering of endosomal vehicles occurs in a directed manner, which is in favor of the second model, that involves active partitioning of the organelles over the daughter cells, most likely dependent on the mitotic spindle and interphase microtubule scaffold [40,

41]. This was confirmed by the observation that soon after cytokinesis endosomal vesicles accumulate in the area of the microtubule organization center [40].

In mammalian interphase cells, the Golgi apparatus is typically positioned close to the centrosome, at one side of the nucleus [41, 42]. It consists of a stack of disk-shaped membranes (cisternae) surrounded on each side by dense tubular-reticular networks called the *cis*- and *trans*-Golgi network. Interestingly, it was observed already more than 100 years ago that the very characteristic stack-like organization of the Golgi apparatus changes remarkably at the onset of mitosis [43]. Electron microscopy studies published in the late 1980's clearly demonstrated that the number of Golgi stacks decreases considerably at the onset of cell division [44, 45]. This is accompanied by the appearance of large numbers of tubular and vesicular structures, which were proven to derive from the Golgi apparatus [45, 46]. The fragmentation of the Golgi apparatus into hundreds of tubular and vesicular structures (between 50 and 250 nm in size) is considered to facilitate its uniform distribution between the daughter cells [45, 47]. As for the partitioning of endocytic vesicles, the partitioning of Golgi fragments is believed to be random and from a statistical point of view its accuracy depends on two factors: the number of organelle units and their distribution in the cytosol. The larger the number of units and the more evenly they are allocated in the cytosol, the more accurate is their partitioning [47]. This random partitioning has been challenged by Shima *et al.* [48] who demonstrated that the Golgi distribution between two daughter cells is too precise for a stochastic process. Later reports demonstrated the involvement of the mitotic spindle in the organization of at least a part of Golgi-derived structures during cell division, suggesting some sort of active segregation mechanism for these cell organelles [41, 49].

Apart from the passive or active model of segregation of cell organelles, for the Golgi apparatus a third possible segregation mechanism has been proposed [50, 51]. This model assumes that the Golgi apparatus behaves more like the NE and during cell division temporary disappears and redistributes to the ER, from which it reappears when the daughter cells are formed. The ER can thus be considered as the storage box for NE and Golgi apparatus elements during mitosis. The formation of the NE around the sister chromatids assures the equal partitioning of ER and NE elements between the two daughter cells. When apart from the NE elements, also the Golgi apparatus is resorbed into the ER, it can reappear from the ER in the daughter cells resulting in its efficient partitioning.

4. Mitotic partitioning of free macromolecules such as dextrans and free plasmid DNA

4.1. Dextrans

Dextrans are electroneutral hydrophilic D-glucose polymers. Therefore, they behave as relatively inert molecules inside cells: self aggregation or binding to other cellular structures is negligible and

they show no toxicity even at high concentrations and over prolonged periods. Furthermore, dextrans are commercially available in practically any molecular weight [52]. With increasing molecular weight, neither shape nor surface properties of dextrans change. Consequently, dextrans are perfectly suited as molecular rulers to measure the permeability of pores and channels [31].

At first glance, nuclear entry during mitosis would appear to be a passive process, as NEBD would allow (exogenous) macromolecules to enter compartments which will be included in the nuclei when they reassemble. However, distribution of cellular components between the cytoplasm and the nucleoplasm is tightly controlled both during interphase and mitosis. As a result, there is only a small effect of cell division on the partitioning of exogenous macromolecules. Swanson & McNeil [39] and Benavente *et al*. [53] shortly after them, were the first to report that it appears that only the chromosomes themselves and the macromolecules physically associated with them (e.g. the perichromosomal layer [54-56]) are included in newly formed nuclei, while organelles (as described in section 3) and other large macromolecules which cannot pass the NPCs are excluded. Swanson & McNeil [39] have reported that after scratch-loading of the cells, the fluorescently labeled large dextrans (> 25 kDa) did not appear in the nuclei, even when cells went through mitosis. Dextrans smaller than 20 kDa, however, were observed in the nuclei of interphase cells regardless of cell division as they can enter the nucleus through the NPCs of the intact NE. These observations were confirmed by Ludtke *et al*. [57] (Fig. 2) who found that after cytoplasmic injection, the labeled large dextrans remained cytoplasmic regardless of mitosis. After nuclear injection, the labeled large dextrans were localized in the nucleus of cells which did not divide and in the cytosol of cells which divided. Also Lenart *et al*. [31] found that labeled large dextrans were excluded from reassembled nuclei after mitosis. Thus, cellular compartmentalization at telophase appears to be a two-step process. First, the NE tightly encloses the condensed chromosomes, excluding non-selectively all macromolecules not associated with the chromosomes. Then, selective NPC-mediated import of nuclear proteins from the cytoplasm leads together with the decondensation of the chromosomes to nuclear expansion and the interphase nuclear organization [39, 53]. It should be noted that Miyawaki *et al*. [58] identified that the NE barrier is more permeable during a short period directly after cytokinesis as a large molecule (210 kDa) diffused across the NE. They saw a subtle but substantial decrease in the diameter of the aqueous channels through the NE formation: it was ~10 nm immediately after cytokinesis and reduced to ~8 nm in 30 minutes. They do not know whether the passive diffusion is due to incompleteness of the NE sealing or altered NPC functions after nuclear reassembly. According to their findings, at least a fraction of macromolecules which are normally not able to pass the NPCs should be able to reach the cell nucleus shortly after cytokinesis.

4.2. Free plasmid DNA

The use of nonviral vectors for gene therapy is hampered by poor transfection efficiencies, largely because of the metabolic instability of pDNA in the cytosol, the inability of pDNA to diffuse freely through the dense cytoplasm and to travel through the NPC [10, 59-61]. It has been reported that as little as 1 in 10^4 pDNA molecules is able to translocate from the cytoplasm to the nucleus [62]. Several attempts to improve the entry of pDNA into the nucleus have been reported. These include the use of electrostatic binding of DNA to cationic nuclear localization signal (NLS)-containing proteins or peptides [63], as well as covalent attachments of NLS peptides to DNA [64]. Additionally, the effect of inclusion of DNA nuclear targeting sequences (DTSs) like the Simian vacuolating virus 40 (SV40) enhancer sequence in the pDNA was investigated [65]. Another strategy was to increase the permeability of the NPCs with *trans*-cyclohexane-1,2-diol [66]. All these attempts have met with limited success.

A few studies have focused on the fate of pDNA during cell division. Dowty *et al*. [67] reported an interesting study where electron microscopy was used to determine the precise intracellular localization of gold-labeled pDNA which was microinjected into the cytoplasm. The authors observed that the distance between the cytoplasmic site of delivery of pDNA and the nucleus is a key determinant for nuclear incorporation of DNA. Only when pDNA was injected close to the nucleus, nuclear translocation was observed, regardless of the cell cycle status. They have seen that pDNA entered postmitotic nuclei of primary rat myotubes and concluded that cell division-dependency for pDNA to enter the nucleus is also cell type-dependent. Remaut *et al*. [68] injected unlabeled pDNA directly in the nucleus or the cytoplasm of the cells and found that only upon nuclear injection either open circular, supercoiled and linear pDNA were equally able to be expressed, even at low injection concentrations. When injected in the cytoplasm, however, cell division and a high concentration of supercoiled pDNA were required to reach comparable transfection efficiencies. More detailed studies on the intracellular behaviour of pDNA were performed by Ludtke *et al*. [57] and Gasiorowski & Dean [69]. Next to labeled dextrans (as described in section 4.1), Ludtke *et al*. [57] also microinjected naked labeled pDNA into the cyto- or nucleoplasm. Randomly labeled pDNA resembles the intracellular localization pattern of large dextrans as described in section 4.1 (Fig. 2). Following mitosis, it was always localized in the cytosol, regardless of prior cyto- or nucleoplasmic microinjection. Gasiorowski & Dean [69], have shown that naked unlabeled plasmids microinjected directly into nuclei and later detected by FISH were found almost exclusively and in equal amounts within the nuclei of the daughter cells after mitosis. On the other hand, when plasmids were randomly labeled with several commercially available fluorescent DNA labeling kits and afterwards injected into HeLa cell nuclei, the randomly modified plasmids were excluded from the daughter

nuclei after cell division. Around the same time Shimizu *et al*. [70] reported similar findings, being that randomly labeled pDNA microinjected into the nucleus was found in the cytoplasm after mitosis. However, they concluded that unlabeled pDNA microinjected into the nucleus rapidly forms intranuclear aggregates and that unlabeled pDNA was like labeled pDNA detected (by FISH) in the cytoplasm 24h after nuclear injection. The aggregates left in the cytoplasm resembled micronuclei, which suggests its similarity to the nuclei formed by purified DNA and the proteins and membranes present in *Xenopus* egg extracts [71, 72].

The results of Gasiorowski & Dean [69] suggest that naked, unmodified plasmids are retained in the nucleus following cell division and likely continue to be expressed in the daughter cells. The episomal pDNA localizes in the region of the cell which will be enclosed by the reforming NE at the end of mitosis, even in the absence of viral chromatin associating systems (see 6.2). Alternatively, the dividing cell can simply recognize the pDNA as a marker for NE reassembly as previous experiments in chromosome-free cellular extracts have shown that functional NEs are formed around extrachromosomal DNA [71, 72]. Consequently, unlabeled pDNA can end up in the nuclei or in the cytoplasm as micronuclei. A pDNA with randomly attached fluorophores, however, is likely to be recognized as a large, foreign molecule and not as a DNA molecule. Therefore, it will be no longer highly interactive with a range of cellular DNA-binding proteins, which most likely interferes with the enclosure of the modified pDNA within the new nuclei or micronuclei [69, 73]. A solution to this problem could be the fluorescent tagging of pDNA only at a specific sequence, leaving the rest of the pDNA sequence accessible to cellular proteins. This can be achieved with quantum dots as described by Srinivasan *et al*. [74]. However, the long term follow up of the intracellular fate and nuclear uptake of these plasmids was not studied in detail.

5. Mitotic partitioning of inorganic quantum dots, gold and iron oxide nanoparticles

In the past two decades, rapid developments in the field of nanotechnology have led to a steady increase of applications in the area of nanomedicine [75]. Examples of frequently used nanoparticles are gold, silver or iron oxide nanoparticles (IONPs), quantum dots (QDs) or carbon nanotubes. Also in the area of drug and gene delivery, these particles exhibit several characteristics which make them highly interesting as carrier systems for efficient delivery. Specifically, the small size (ranging between one and several tens of nms) and solid structure of the nanoparticles cause them to be avidly taken up by cultured cells, while their large surface areas enable profound surface chemistry. The particles can thus be provided with multiple functionalities and their specific characteristics allow its combined application of several different modalities (imaging, therapy and carrier systems for delivery) through one nanoparticle type.

For drug and gene delivery, IONPs, QDs and gold particles have been used most frequently. IONPs can be supplied with a cationic coating which allows DNA binding. Their cellular uptake can be strongly enhanced by applying a static magnetic field gradient over the target cells in a procedure which is known as 'magnetofection' [76]. QDs are fluorescent particles exhibiting high photostability, limited photobleaching, high fluorescence emission intensities and narrow fluorescence emission spectra, which allows multi-colour analysis of complex localization, even *in vivo* [77]. Gold nanoparticles are considered to be the most biocompatible, allow facile surface chemistry and are able to strongly adsorb or scatter incident light at a certain resonance wavelength (often in the near Infra-Red region), a phenomenon called localized surface plasmon resonance, enabling microscopy-based long term tracking of complexes in cells and tissues [78].

One of the few studies focusing on the fate of nanoparticles during cell division was performed by Feldherr *et al*. [79]. They microinjected gold particles of different sizes (3-17 nm) in the cytoplasm of amoebas and studied the cytoplasmic/nucleoplasmic distribution by electron microscopy. Cells injected in interphase contained about 2.6 times more particles in the cytoplasm than in the nucleoplasm. Interestingly, mainly small gold particles (<14 nm) were able to reach the nucleus through the NPCs. When injections were performed in prophase or shortly after mitosis, about 7.3 times more particles could be found in the nucleoplasm when compared to the cytoplasm. Furthermore, the inclusion of larger gold particles (>14 nm) was also observed. Thus, higher relative uptake and incorporation of larger gold nanoparticles were observed in nuclei of dividing cells compared to nondividing cells. According to Feldherr *et al*. [79], the gold particles enter the nuclei during and/or shortly after mitosis, what could be related to an increased permeability of the NE shortly after it was formed. Later on, Feldherr *et al*. [80] demonstrated that recently formed NEs with newly forming NPCs are more permeable than those of older cells with fully reconstituted mature NPCs. These studies indicate that the NEBD on itself is not of special importance with regard to the uptake of material into the nucleoplasm, but rather the increased permeability of the NPCs. The rates of diffusion across the NE are the highest during the first and fifth hour after the onset of anaphase [80]. This corresponds with the similar time pattern and non-uniform rate of NPC formation reported in HeLa cells [81]. These findings are consistent with electron microcopy studies of dividing cells [82-84] and the study of Swanson & McNeil [39] which have shown that the NE reforms at the surface of the chromosomes, excluding cytoplasmic substances from the daughter nuclei at the time of reassembly. Therefore, small nanoparticles such as these gold particles are probably not included in the nuclei during mitosis, but are imported shortly afterwards through the more permeable NPCs.

For IONPs or QDs, the focus has been put on the dilution of these particles due to cell division, rather than their nucleocytoplasmic distribution. It has been accepted that for these slowly or non degrading particles, cell division usually results in an exponential decrease in the intracellular concentration of the particles, as in general both daughter cells receive approximately 50% of the particles. Recently, several studies questioned the general applicability of this finding [85, 86]. Walczak *et al*. [87] observed clear differences in endosomally located IONP turnover in C17.2 neural progenitor cells which were either left to proliferate and showed a uniform dilution of the IONPs or underwent differentiation, resulting in a large difference in numbers of particles per cell. The authors found that this effect was due to asymmetric cell division, a phenomenon quite common for stem cells, which occurs during simultaneously occurring differentiation and renewal of stem cells. This results in an abrupt and faster dilution of IONPs than in continuously proliferating cells undergoing symmetric cell division (Fig. 3) [87].

Also for cells dividing symmetrically, however, the dilution of nanoparticles can differ between the two daughter cells. The dilution of endocytosed QDs upon cell division leads to an asymmetric segregation of endosomes and thus of QDs [88]. Using a statistical model, the same group further showed that partitioning of nanoparticles upon cell division is a random and asymmetric event [89]. From a biological point of view, it has been suggested that large cell populations can proceed to asymmetric cell divisions in order to be able to more effectively counteract the effects of the 'toxic' nanoparticles [90]. As one cell receives most of the particles, this cell is likely 'sacrificed' so that the remaining daughter cells with nearly no particles have a better chance to survive. Cellular responses to stress induced by nanosized materials can also lead to the induction of autophagy, a self-defense mechanism which can occur in all eukaryotic cells which contain lysosomes [91]. Typically, two membranes will be formed surrounding the region where the nanoparticles are located, to separate this region from the rest of the cytoplasm and then fuse with lysosomes in order to reduce cellular stress and degrade the particles [91]. Also gold nanoparticles, which are generally seen as quite biocompatible, induce oxidative stress upon cellular internalization which in turn activates autophagy [92].

It should be noted that in general, the above mentioned nanoparticles are actively endocytosed and remain within the endosomal compartment of the cell. This most likely explains why these nanoparticles are not frequently reported to be in the nucleus of the cells, although their small size could permit access to the nuclear interior through the NPCs.

6. Effect of cell division on nonviral gene delivery particles

As naked pDNA molecules are only poorly internalized, they need a complexation partner to help them to reach the cytoplasm or the nucleus of the cells. Ideally, this carrier should also provide protection of the nucleic acids against enzymatic degradation during the different steps of the delivery process. In the field of nonviral gene delivery, liposomal and polymeric delivery systems (known as lipoplexes and polyplexes, respectively) have a long history [93]. Compared to viral delivery systems, both lipo- and polyplexes are generally easy to prepare, not or weakly immunogenic and allow the incorporation of larger DNA chains as there is no intrinsic size limitation as with viral particles [13]. Typically, liposomal systems consist of at least two lipid types: a cationic lipid, which allows them to electrostatically interact with polyanionic DNA and facilitates cellular uptake through endocytosis, and an accompanying matrix lipid with long, unsaturated fatty acid chains, which mostly aid in the endosomal escape of these lipoplexes, resulting in the release of free pDNA in the cytoplasm of the cells [94, 95].

Polyplexes contain a polycationic polymer, such as polyethylenimine (PEI), that binds to the anionic DNA. Also in this case a surplus of cationic charges allows for binding to and uptake by the cells. Like lipoplexes, polyplexes are taken up by endocytosis and will therefore end up in the endosomal compartment from which they must be released to allow nuclear translocation of the DNA. In general, polyplexes with high buffering capacity are expected to finally escape out of the endosomes into the cell cytoplasm by an osmotic rupture according to the proton sponge hypothesis [96].

When compared to viral vectors, the overall expression efficiency of lipo- and polyplexes is rather low, commonly resulting in transgene expression in less than 50% of all treated cells [14]. This low expression efficiency can be explained by taking into account several hurdles which are intrinsically associated with lipo- or polyplex-based gene delivery: 1) cellular uptake of the complexes, 2) the translocation of the complexes from the endosomes to the cytoplasm, 3) dissociation of the DNA from the lipid or polymer carrier and 4) translocation of the DNA from the cytoplasm to the nuclear compartment [11, 97, 98]. Which one of these barriers limits the transfection efficiency in the most pronounced way is debatable. Most likely, all barriers contribute to some extent to the lowering of the obtained gene expression. For pDNA, however, the transfer of free DNA from the cytoplasm to the nucleus has been reported to be a very inefficient process. Capecchi [10] and Escriou *et al*. [61] observed transgene expression in 50-100% of the cells microinjected with naked DNA directly into the nucleus but no significant expression upon DNA microinjection in the cytoplasm. Our recent results also indicate that the NE is the main obstacle to pDNA delivery as we saw nearly no GFP-positive HepG2 cells transfected with pDNA/cyclodextrin complexes. In contrast, when the same cyclodextrin complexes were used to deliver mRNA, that only needs to reach the cytosol to display biological activity, up to 30% of GFP-positive HepG2 cells were detected [99]. An even more

pronounced difference was observed for HeLa cells [100]. Furthermore, although both lipo- and polyplexes are specifically designed to enable endosomal escape, the majority of the complexes remains entrapped within the acidic endosomes [61, 101]. The acidic environment of the endosomes and presence of degrading enzymes such as lipases will result in the degradation of most liposomes and polymers [102], where the nondegradable polymer backbones or cationic lipids are exocytosed or kept within the lysosomes. DNA molecules which do reach the cell cytoplasm are rapidly degraded by cytoplasmic nucleases and their cytoplasmic residence time should therefore be kept minimal [103].

The intracellular fate of nonviral gene delivery particles still remains obscure. As long as the lipo- or polyplexes have not escaped the endosomal compartment, they are expected to distribute within endocytic vesicles among daughter cells upon recurrent cell division. For lipoplexes, it is assumed that endosomal escape results in the delivery of naked pDNA into the cytoplasm of the cells. This dissociation seems to be necessary since it has been shown that pDNA is not transcriptionally active upon injection of the intact lipoplexes in the cytoplasm nor the nucleus [11, 62]. Therefore, upon cell division, the naked pDNA in the cytoplasm of the cells will most likely behave as described above (see 4.2). For polyplexes, however, it is still under debate whether or not pDNA has to dissociate from the polyplexes before nuclear translocation can occur. From time to time, PEI polyplexes have been observed in the nuclei of transfected cells, suggesting that nuclear uptake of the polyplexes should be possible [104]. Also, Pollard *et al*. [62] showed that PEI polyplexes injected in the nucleus were transcriptionally active, questioning the need for cytoplasmic pDNA unloading. In line with these speculations, Breuzard *et al*. [105] found evidence of nuclear polyplexes by FRET imaging. On the contrary, Bieber *et al*. [106] found no evidence of nuclear PEI polyplexes and mainly observed colocalization with lysosomes. Also, Itaka *et al*. [107] observed fast release of pDNA from (linear) PEI polyplexes in the cytoplasm, questioning the long-term existence of cytoplasmic PEI polyplexes and their subsequent delivery to the nucleus. The nuclear observed PEI polyplexes in the aforementioned studies could then result from a recomplexation of pDNA and PEI polymers, after both of them reached the nuclear interior independently. Overall, the observation of polyplexes in the nucleus of cells seems to be very rare.

Surprisingly, the long term intracellular fate of lipo- and polyplexes during cell division has never been investigated in detail. Consequently, little is known on the nuclear entry of nanoparticles during mitosis, although the nuclear inclusion appears a very simple concept to study. To address this question, in theory only a detailed real-time analysis of the intracellular distribution of cytoplasmically microinjected nanoparticles would be needed. In living cells, however, the lack of information can partially be explained by the difficulties to inject particles which are larger than 200

nm because of problems of aggregation, needle clogging and long-term survival of the cells after injection. Therefore, most information on the nuclear delivery of pDNA to the nucleus upon lipid-based [5, 8, 13-17] or polymer-based [5, 8, 12] delivery comes from indirect evidence of nuclear entry, namely an increased gene expression, which was reported to be two- to several hundredfold higher in dividing than in nondividing cells. As mentioned in section 2, the cell cycle can be roughly subdivided into five different phases, being: G_0, G_1, S, G_2 and M phase. A common finding is that the expression efficiency for both lipo- and polyplexes is considerably higher during S, G_2 or M phases than during G_0 or G_1 phases [5, 13, 16, 18]. Generally, it is believed that during M phase and associated NEBD, the larger complexes can reach the nuclear DNA [15]. Complexes which were taken up by cells in G_0 or G_1 phase are likely not retained long enough to still be fully functional when reaching the M phase [5]. Apart from NEBD, other events such as a cell cycle dependent internalization of the complexes may play a significant role as well. Although studies have indicated that the cell cycle status has no effect on the cellular uptake of the complexes [108], other studies point to a clear link between complex uptake efficiency and cell cycle status [14, 18]. Männisto *et al*. [18] linked their observations to previous studies in which it was shown that the rate of endocytosis, in general, increases gradually during the G_1, S and G_2 phases, but drops drastically at the beginning of mitosis. Of further interest is the study by Marenzi *et al*. [109] who links reporter gene expression levels to the compaction state of transfected DNA at different cell cycle phases. For transfection of cells in G_0 or G_1 phase, the plasmid is packaged into a more compact form which inactivates reporter gene expression. This compact pDNA is not reactivated when cells are stimulated to cycle. Therefore, differences in gene expression are not directly linked to alterations in transfection efficiencies but rather to the inability of the pDNA to be transcribed when packaged into a more compact form.

Cell cycle independent gene transfer by using polyplexes composed of linear PEI has been reported as well [12]. Also, compact DNA particles were shown to transfect postmitotic cells, showing that at least some nanoparticle types are able to reach the nucleus of nondividing cells [110]. Furthermore, Escriou *et al*. [15] although confirming that mitosis is an important upregulator of expression efficiency, also observed expression in cells which had not divided, albeit to a lower extent. In line with these findings Dowty *et al*. [67] found that when pDNA was injected close to the nucleus, nuclear translocation was observed, regardless of the cell cycle status.

Overall, these data indicate a clear effect of cell cycle progression on transgene expression levels, but the variety of cell types and carrier systems used make it hard to clearly define the exact mechanism underlying this effect. Likely, a combination of factors play a role, where cells in S or G_2 phase have the highest endocytic capacity, while in M phase facilitated nuclear transport by NEBD occurs and the

pDNA is allowed to be packaged into a more open form, thus enabling high reporter gene expression levels.

7. Viral particles taking advantage of mitosis

Viral vectors remain to have the best transfection efficiencies because for millions of years they have developed mechanisms to survive in the extracellular environment, stabilize virus-cell interactions, increase internalization, hijack intracellular transport systems, deliver their genomes into the appropriate subcellular compartment (cytosol or nucleus) of dividing and nondividing cells and increase transcription. Most of these mechanisms involve design and incorporation of specific proteins into the virus. Many viruses have evolved, gaining the ability to replicate and store their genome in the cell nucleus. These viruses aim at maintaining their genome in the infected cells until the host organism dies. They usually achieve this by integrating genomic DNA into the host chromosomes or segregating episomal DNA into daughter cells [111]. Two groups of viruses which take advantage of cell division during their life cycle, will be discussed in this section: certain retroviruses which depend on mitosis to integrate their genome and thus infect the host cells and some DNA viruses which use mitosis to partition their episomes in the progeny cells of latent infected host cells.

7.1. Retroviruses

Retroviral virions are enveloped particles of about 80-100 nm in diameter [112]. In retroviruses, the viral ssRNA is reverse transcribed in the cytosol via reverse transcriptase into dsDNA. This DNA together with viral integrase is organized into the pre-integration complex (PIC) [111, 113]. Also, host proteins such as the barrier-to-autointegration factor (BAF) or high mobility group (HMG) proteins can associate with the PIC [114]. Generally, viruses deliver their genome through the NE of the interphase nucleus. This allows infection of nondividing cells and offers the opportunity to infect terminally differentiated cells. Some retroviruses, however, are dependent on mitosis for infection and their PIC waits in the cytosol until the cell undergoes mitosis. During the temporary NEBD, the PIC can associate with the chromosomes and enter a newly assembling nucleus after which the cDNA integrates into the host genome (Fig. 4 A) [113]. Many retroviruses infect cells exclusively, or more efficiently, if the host cell divides [115, 116]. These include murine leukemia virus (MuLV) [117, 118], human immunodeficiency virus (HIV) [119-121], spleen necrosis virus (SNV) [122], avian sarcoma virus (ASV) [122-128] and foamy virus (FV) [129, 130].

For example, gene transfer and expression of exogenous genes using retrovirus based vectors, such as MuLV, requires cell proliferation which limits their application to dividing cells. Why MuLV cannot work its way into nondividing cell nuclei via nuclear pores is unclear. Yamashita *et al.* [131] indicated that the MuLV capsid protein that is closely associated with MuLV PICs, prevents PICs from migrating into the nucleus of nondividing cells. MuLV PICs perhaps lack NLSs or fail to display NLSs due to MuLV capsid, preventing their NPC import. Alternatively, the MuLV capsid may sterically hinder NPC import [132]. For that reason, MuLV PICs bind the host chromosomes during mitosis and are subsequently enclosed together with them in the daughter nuclei. A particular factor with tethering activity to mitotic chromosomes has not yet been described for MuLV. However, phosphoprotein p12, a part of the MuLV PIC, bears a similar N-terminus as the histone H5 protein [133] and shows a close association with the chromatin. These together tempt to assume that p12 also functions in tethering the MuLV PIC to the chromatin [134]. It should be stressed here that the limited knowledge of PICs in general is caused by the difficulty of obtaining large amounts of material [135].

Contrary to MuLVs, lentiviruses such as HIV-1 infect dividing as well as nondividing cells, like terminally differentiated macrophages [136, 137]. HIV-1 capsid is not strongly associated with HIV-1 PICs as opposed to MuLV capsid [131]. How HIV-1 PICs move through NPCs despite their large size ~56 nm [138] exceeding the limit for active NPC transport also remains unclear. For a review about this controversial matter, the reader is referred to ref. [139]. Overall, transport of the HIV-1 PICs into the nucleus may require the combined action of three constituent proteins, namely matrix (MA), viral protein R (Vpr) and integrase (IN), in addition to specific DNA sequences. During transport, several factors such as virus uncoating, NLS signals and disruptions in NE integrity may play a significant role [111, 113]. The integration efficiency of HIV-1 is up to threefold higher in dividing cells than in nondividing cells as a result of the balance of two opposite effects. While NEBD facilitates docking of the viral genome to the host chromatin, chromosome condensation directly antagonizes and therefore delays integration [140]. Also for lentiviruses, evidence of existence of a specific chromosome binding factor is lacking but the endogenous lens epithelium derived growth factor/p75 (LEDGF/p75) is an attractive candidate chromatin tethering factor. LEDGF/p75 is a nuclear protein that binds integrase via its C-terminus and binds chromatin via its N-terminus, thereby tethering integrase and thus the PIC to the chromatin [141]. However, Katz *et al.* [142] have reported that nuclear import of HIV-1 PICs might be mitosis-independent in dividing cells. This would indicate that LEDGF/p75 as a chromatin tethering factor is not necessary for nuclear entry of HIV-1 PICs, but that it is required for viral cDNA integration in the host genome.

Aside from lentiviruses, also other retroviruses such as ASV and FV enter the nuclei of nondividing cells, albeit less efficiently than that of dividing cells [129, 132]. Recently, the FV group-specific

antigen (Gag) was found to be the essential linker between the viral DNA in the PICs via its C-terminus and host chromosomes during mitosis through interaction with H2A/H2B histones via its N-terminus [143, 144]. The substitution of this chromatin binding site with the chromatin binding site of LANA (see 6.2) allows binding of the incoming PICs onto host chromosomes but it does not restore full infectivity [143]. For ASV, the factors which translocate their PICs into the nucleus of (non)dividing cells are speculated or unknown e.g. an NLS in the ASV integrase protein may underlie PIC import [132]. In contrast to MuLV, lentiviruses and FV vectors showed a long-term persistence of a stable transduction intermediate in quiescent cells, so that they efficiently transduced G_0 fibroblasts which were later stimulated to divide [129]. A mechanism envisioned for how retroviruses might deal with the potentially restrictive environment of nondividing cells, is that the retroviruses themselves may induce cell cycle progression to create a more favorable environment [145, 146]. Studies have indicated that binding of some retroviruses to Toll-like receptors on the cell surface can stimulate the cell to enter into the cell cycle [145]. However, such stimulatory activities are unlikely to be universal [142].

7.2. Latent DNA viruses

Besides the retroviruses which make use of mitosis to integrate their genome into the host DNA, some latent dsDNA viruses associate their genome with the host mitotic chromosomes for persistence of viral episomes in infected cells and their progeny (Fig. 4 B). This is achieved by attaching the viral circular dsDNA as an extrachromosomal element to the host genome. DNA viruses which use this strategy include certain herpesviruses such as Epstein Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV) and herpesvirus saimiri (HVS) as well as latent papillomaviruses such as bovine papillomavirus (BPV-1) and human papillomavirus (HPV). The virions of herpesviruses are enveloped and rather big (180-200 nm) while papillomaviruses (53-57 nm) and polyomaviruses (40-47 nm) are very small and do not have an envelope [112].

Herpesviruses

EBV or human herpesvirus 4 (HHV-4) that is best known as the cause of infectious mononucleosis, can infect a number of cell types, including epithelial and B cells. During latency, EBV is stably maintained as a circular plasmid that is replicated once per cell cycle and partitioned at mitosis. The AT hook activity of a single viral protein named EBV nuclear antigen 1 (EBNA1) is required for both processes. AT hooks are DNA binding motifs found in a family of proteins which bind the minor groove of AT rich scaffold-associated regions on metaphase chromosomes [147, 148]. The stable circular episome carries only two EBV components: the *cis* acting origin of replication (oriP) and the

latently expressed EBNA1 in *trans*. EBNA1 binds directly to viral DNA via its C-terminal domain and tethers the EBV genome to chromatin through its N-terminal domain, initially by direct interactions via its AT hooks, or afterwards through association with the nucleolar EBNA1 binding protein 2 (EBP2). The latter is a part of the perichromosomal layer and the binding with EBNA1 may be required to stabilize the EBNA1-chromosome interaction [149].

Additionally, HVS which transforms T cells was seen to be closely related to EBV and has similarly a *cis* acting oriP that permits stable replication and the efficient persistence of episomes in HVS-infected cells [150].

KSHV, also known as human herpesvirus 8 (HHV-8), commonly occurs in AIDS patients and persists as a multi-copy episome in latently-infected tumor cells. KSHV latency associated nuclear antigen (LANA) is required for episome persistence in host cells during latent infection. LANA mediates viral genome attachment to mitotic chromosomes [151]. Previous studies have shown that LANA directly binds the viral episome via its C-terminal domain, nucleosomes via its N-terminal domain through the folded region of histones H2A-H2B [151, 152] and the mitotic chromosomes via its C-terminal domain through the double bromodomain protein 4 (Brd4) [152]. Brd4 is identified as the first mitotic chromosome receptor for DNA viruses [153]. Recently, however, Xiao *et al*. [154] have reported that the molecular mechanism underlying the bridge between the virus, LANA and the host chromosome is likely complex, involving numerous proteins, such as the kinetochore protein budding uninhibited by benzimidazole 1 (Bub1), with which association throughout mitosis is maintained, making the LANA-Bub1 interaction likely critical for segregation to daughter nuclei [154].

Papillomaviruses

Besides the herpesviruses discussed above, also certain papillomaviruses use mitosis to persist in latently infected host cells. For example, HPV-16, together with type 18, causes most of the HPV-associated cancers (e.g. cervical cancer). By the same token, BPV-1 infects paragenital areas in cattle and provides an excellent model for studying papillomavirus molecular biology. A common strategy for papillomaviruses, is that the viral E2 proteins tether episomes to mitotic chromosomes so that the episomes are segregated and enclosed in the daughter nuclei. For example, HPV-8 E2, interacts via its flexible linker hinge region between the N- and C-terminus, with regions of ribosomal DNA repeats located on the short arm of human acrocentric chromosomes [155] and via its C-terminus with the episome [156]. Long term genome maintenance and stable copy number require multiple E2 binding sites in the episome, which are used to tether the episomes to mitotic chromosomes [157]. Brd4 was previously identified as the mitotic chromosome anchor for BPV-1 and HPV-16 via the N-terminus of E2 [153]. Recently, Silla *et al*. [158] have reported that the formation of the BPV-1 E2

segregation complex is Brd4 independent, which is also the case for all HPVs [159]. Silla *et al*. [158] additionally concluded that the formation of segregation-competent complexes depends on a certain protein level threshold and that multiple E2 molecules are present in a single segregation complex together with cellular proteins, including certain components of the transcription machinery. Therefore, it seems that the chromosome binding action and the transcriptional regulation mediated by the E2 protein are not operating independently. The E2 proteins have been observed to be associated with condensed chromatin at all stages of mitosis, even at times when many other transcription factors have been displaced from mitotic chromatin [160]. E2 proteins are multifunctional, as they are also involved in initiating viral DNA replication and regulating viral transcription, in addition to maintaining the genome as an extrachromosomal replicating element [155]. Also interesting to add is that it has been shown that the consensus sequence RXXS in the hinge region is important for chromosome binding by the HPV-8 E2 protein. The serine residue in this region is phosphorylated by cell cycle-dependent kinases, which most likely regulates the chromosome binding. Furthermore, the RXXS motif can be found in the N-terminal domain of LANA and in the AT hooks of EBNA1, suggesting that cell cycle-dependent phosphorylation could be a common strategy to regulate chromosome tethering during cell division [155].

Polyomavirus Simian vacuolating virus 40

An additional example of a latent DNA virus which is neither herpesvirus nor papillomavirus, is the polyomavirus Simian vacuolating virus 40 (SV40). SV40 is found both in monkeys and humans and has the potential to cause cancers but most often persists as a latent infection. The circular SV40 genome has been shown to be maintained in certain intracranial tumors, but Krieg *et al.* [161] suggest that SV40 is not the cause of these human tumors. It remains unknown which protein forms the physical connection between the SV40 episome and host chromosomes [162].

8. Reflections on therapeutic strategies for the improvement of nuclear inclusion of exogenous nucleic acids or other macromolecules

We and many others are interested in cationic carrier-mediated delivery of pDNA to the nuclei of target cells. During interphase, pDNA has to access the nuclear interior via the NPCs. pDNA is however a large hydrophilic molecule which does not easily pass the hydrophobic central channel of the NPCs. Efforts to increase the nuclear import of pDNA through NPCs by the use of NLSs have met with limited success, although this strategy initially seemed very promising [163, 164]. The use of NLSs to bring pDNA into the nucleus is not within the scope of this review and has been reviewed elsewhere [1, 21]. As described above, when cells undergo mitosis the NE is temporary disassembled, to reappear at the end of mitosis, when a new NE forms around the segregated sister chromatids. It

has been shown that cell division greatly enhances the transfection efficiency of pDNA, presumably because of the intermixing of cytoplasmic and nucleoplasmic components during mitosis, followed by accidental retention of pDNA in the newly formed daughter nuclei [5, 8, 13, 14, 68]. We hypothesize that it should be possible to increase the nuclear inclusion of DNA during cell division by different strategies, which will be discussed here.

8.1. Chromatin targeting

First of all, one could think of targeting pDNA to chromatin, so that it is included in the nuclear interior as the NE is formed around it. Both the pDNA itself and the nonviral gene delivery particles containing the pDNA could be equipped with chromatin binding factors. These factors should be able to bind the chromatin directly, or by the recruitment of endogenous proteins which mediate the binding to chromatin. Also, as evidenced by viral examples, the pDNA should contain multiple chromatin binding sites, to assure a strong anchoring of the chromatin during cell division. It should be noted that during mitosis, the chromatin is surrounded by the so-called perichromosomal layer [54-56]. The function of this protein layer is not completely understood, but it is assumed to play a possible role in chromosome organization and/or compartmentalization of cells in prophase to telophase, serve as a binding site for chromosomal passenger proteins or just as a protective barrier around chromosomes. It is very likely that the presence of the perichromosomal layer prevents the attachment of exogenous pDNA to the chromatin unless correct targeting proteins are used. It should also be noted that the space between the chromatin and the newly forming NE is very narrow, raising the question whether at all it will be possible to attach large pDNA molecules or nanoparticles to the chromatin, without disturbing the formation of a smooth NE. However, as described above both retroviruses (7.1) and latent DNA viruses (7.2) frequently use the chromatin targeting strategy to bind their genome to the chromatin and assure its partitioning to the daughter cells (Fig. 4). Also, it was shown that large macromolecules such as free dextrans or pDNA which do not interact with the chromatin, are not retained into the nuclei upon cell division [57, 69]. Therefore, we believe the chromatin targeting strategy has great potential in improving both the amount of pDNA that reaches the cell nucleus during cell division, and the chance the nuclear pDNA gets inherited by daughter cells during subsequent cell divisions.

Nonviral chromatin tethering factors

All known chromosomal or DNA binding proteins contain one or more AT hooks, suggesting that it may act as a universal chromosome tethering factor. Therefore, coupling the AT hook region of proteins (consensus motif PRGRP), instead of the full length protein to pDNA or pDNA containing nanoparticles could be sufficient to mediate chromosome binding. Recently, we showed with the

Xenopus nuclear envelope reassembly (XNER) assay that the nuclear inclusion of polystyrene beads and pDNA/PEI nanoparticles could be improved about threefold upon chromatin targeting with AT hooks derived from the endogenous Mel-28 (ELYS in mammalian cells) and HMGA2β [165]. This clearly shows that chromatin targeting indeed improves the nuclear inclusion of nanoparticles which are bound to the chromatin. In living cells, however, microinjected nanoparticles seemed to be 'trapped' in the endosomal compartment, causing their equal distribution to daughter cells upon cell division, without any apparent nuclear inclusion of the complexes [165]. It should be noted that in this study the chromatin binding peptides were bound to the nanoparticles which contained the pDNA and not to the pDNA itself. Consequently, as soon as the pDNA is released from the nanoparticles, the chromatin targeting properties are lost. In future it could be interesting to attach the chromatin binding peptides directly to pDNA and observe if this results in an increased nuclear retention upon cell division.

Endogenous proteins such as members of the high mobility group proteins (HMG) and histones have also been used as pDNA delivery systems as such [166, 167] or in combination with conventional lipids and polymers [168] to increase transfection efficiency [169]. These proteins were able to enhance pDNA nuclear translocation and transfection efficiency to some extent, most likely due to their innate DNA binding and condensing properties, as well as their intrinsic NLSs. As the chromatin binding domain (N-terminus) of EBNA1 can be replaced by those of either HMG-I or histone H1, with successful maintenance of daughter cell episomes after cell division, these motifs also have potential for mediating pDNA attachment to chromatin and pDNA segregation [170].

Additionally, already more than 10 years ago, a new episomal system has been described (pEPI-1) which possesses replication and episomal retention features through the function of a scaffold/matrix attachment region (S/MAR) isolated from the human β-interferon gene. S/MARs are associated with most of the characterized mammalian origins of replication [172, 173]. The 30 nm fiber of DNA, histones and chromatin proteins appears to be organized into loops by interaction of S/MARs with the nuclear matrix [174]. S/MARs have besides their structural function, also an important role in temporal and spatial organization of gene expression [175]. Recently, Lufino *et al*. [176] showed full functional recovery of low density lipoprotein receptor (LDLR) activity in a deficient cell line. *LDLR* transgene expression retained physiological regulation and was repressed by high sterol levels. They showed also a high mitotic stability of the vector by demonstrating long-term episomal retention in the absence of selection. The mitotic stability is provided by the specific interaction of the vector with the chromosome scaffold via scaffold attachment factor A (SAF-A) from the nuclear matrix. This enables co-segregation with the chromosomes during cell division [177]. Moreover, Tessadori *et al*. [178] concluded that the regulatory mechanisms that act on episomal

genes seem to be similar to those that control host genes. The episomal chromatin undergoes the same modifications as the host chromatin e.g. histone modifications and DNA methylation. The generation of artificial chromosomes with an optimal size with respect to stability as well as transfection efficiencies, can be promising for the future of nonviral gene therapy as it may form the perfect alternative for viral episomal gene vectors (see next paragraph). This is even more interesting in the context of possible immunogenic or oncogenic properties of viral vectors [171]. Additionally, the transgenes bear strong constitutive promoters, which result in nonphysiological overexpression. To achieve physiological conditions of expression, correct alternative splicing and promoter usage, an efficient vector should be based on sequences derived from the human genome, which include the native promoter and all regulatory sequences of a gene [172, 173].

Viral chromatin tethering factors

AT hooks often also occur in the chromosome binding region of viral segregation proteins which mediate viral genome retention into the nuclei of the host cells. The most interesting features to this point are the chromosome tethering factors of the latent DNA viruses, as the information of responsible chromosome binding factors in the different retrovirus PICs is still limited. Proteins which are identified to have chromosome binding properties are EBNA1, LANA and E2. In addition to sharing the common function of genome tethering, these proteins also play an important role in viral genome replication and transcriptional regulation [152]. Structurally, all three proteins form, or are predicted to form, a similar dimeric β-barrel C-terminal DNA binding structure, regardless of a lack of sequence homology [174]. However, Sekhar *et al.* [155] have shown that the HPV-8 E2 chromosome binding sequence has similarities with chromosome binding regions in EBNA1 and LANA tethering proteins. Although these proteins may have different chromosomal targets, a common mechanism of regulation of chromosome binding function could be the phosphorylation by certain cell cycle-dependent kinases [155]. The recent discovery of the RXXS motif, that is a recognition target of several cell cycle-dependent kinases, in all three proteins is in favor of this hypothesis. As it has been shown that the segregation complex in papillomaviruses contains multiple E2 proteins and also the viral genome should contain at least 5 E2 protein recognition sites [158], it can be expected that a strong chromosomal binding is needed to assure efficient partitioning during cell division. Also, the local organization of chromatin is expected to greatly influence the ability of binding nanoparticles during mitosis. For the EBNA1 protein, for example, a possible chromosome binding place has been identified in chromosome 11, although only in 23 out of the 771 possible binding places genome-wide EBNA1-mediated binding was also actually observed [175].

In relation to gene therapy, most progress has been made with EBV plasmid vectors, which can be combined with cationic polymers. These EBV plasmid vectors contain oriP and the *EBNA1* gene. It has been shown that EBNA1 maintains and equally partitions a plasmid that contains the viral oriP region *in cis* by tethering it to endogenous chromatin [147] (reviewed by He & Zhang [176]) (Fig. 4 B). The mechanisms underlying the efficient transfection/expression are not yet fully understood, because EBNA1 exerts a variety of functions through sequence-specific binding to oriP. However, Kishida *et al.* [177] have shown that the efficient delivery and expression is predominantly due to the promotion of cytoplasm-to-nuclear recruitment as well as enhancement of transcription, while the episomal replication is not essentially involved. A major drawback of EBV vectors is the possibility of tumor development in human. Therefore, the possible oncogenicity of all viral vectors *in vivo* has to be thoroughly investigated [178]. For retroviruses for example, the insertion of the MuLV genome in the host gene promoter and its proviral enhancer activity underscores the potential dangers of MuLV as gene therapy vector [135]. Its weak bias for integration in genes might render ASV a preferable alternative vector [179]. As soon as the retroviral and cellular determinants of integration site selection are elucidated, safer and more effective gene therapy vectors may be developed [135]. Additionally, all the (new) knowledge about viral vectors, will give us in the end the opportunity to make equivalent but safer nonviral vectors.

As an alternative to the use of the whole viral vector or the viral plasmid with oriP and the *EBNA1* gene, another option is to use only the chromatin binding motifs of the EBNA1, LANA or E2 proteins (7.2). Both the direct binding motifs of these proteins, which bind the mitotic chromosomes themselves and the indirect binding motifs, which bind Brd4 or other cellular factors on mitotic chromosomes could be useful as chromosome anchor peptides on pDNA or artificial nonviral pDNA containing particles. Once inside the cytosol, these nonviral particles could target and attach to the chromosomes during mitosis and subsequently be enclosed in the newly formed daughter nuclei. Interestingly, the chromatin binding part of IL-33 was shown to have striking sequence similarities with the H2A-H2B binding motif of LANA, leading to the identification of the hexapeptide MXLRSG that was determined to be crucial for docking into the H2A-H2B acidic pocket [180]. Since IL-33 is an architectural chromatin binding nuclear factor, it has been suggested that KSHV copied the chromatin binding motif of IL-33 to establish latent infection in human cells [180]. This suggests that incorporating the minimal chromatin binding motif MXLRSG could serve to target pDNA to chromosomes during cell division.

Additionally, not the use of a viral protein, but rather that of the host cell protein Brd4, can be a strategy to target gene therapy particles to the host mitotic chromosomes too, as we learn from KSHV and papillomaviruses [152, 153]. Brd4 is a member of the BET family. Their characteristic

feature is the retention on chromosomes during mitosis which allows them to play a possible role in the transmission of epigenetic memory across the entire process of cell division [181]. Also, the possible consequences related to epigenetics of using proteins of this family, is therefore worthwhile studying intensively.

8.2. Targeting non-chromatin nuclear elements

Another strategy one could think of is targeting pDNA or pDNA nanoparticles to the mitotic spindle, the NPCs, the INM or the underlying nuclear lamina. Bausinger *et al*. [182] demonstrated that pDNA/PEI complexes are transported along the microtubules of the spindle apparatus and suggest that the concentration of polyplexes at the location of the emerging daughter nuclei strongly contributes to the transfection process. This indicates that targeting the mitotic spindle could indeed be interesting to increase transfection efficiency, e.g. by the use of Nucleolar and Spindle Associated Protein (NuSAP) [183]. INM proteins which could be used for targeting to the nuclear interior are abundant. Most of them, such as Lap2β, emerin and Man1 share a common protein domain, the so-called LEM-domain [24]. Coupling this ~40 residue motif to pDNA could ensure that pDNA or pDNA containing nanoparticles bind the INM or nuclear lamina, ensuring their presence in the interior of the forming nucleus. When the pDNA is targeted to the nuclear lamina, however, we must take into account that the nuclear lamins are only imported in the forming nuclei after the NE has been formed. Therefore, the pDNA should be able to pass the NPCs together with the lamins, which may be impossible to achieve. On the other hand, several studies confirmed that the permeability barrier of the NPCs is not so strict directly after NE formation [31, 58, 80, 184], which could be in favor of pDNA nuclear import shortly after mitosis. In yeast, tethering to NPCs was shown to promote extrachromosomal DNA partitioning during (asymmetric) cell division [185]. Whether targeting NPCs in mammalian cells could also help to increase the nuclear inclusion during or shortly after cell division is unclear. Another possible concern with the above mentioned strategies, however, is the presence of a large pool of endogenous proteins, which could compete for binding to the chromatin, the INM, the NPCs and the mitotic spindle. Also, it is not sure if the proteins will retain their chromatin-, INM-, NPC- or mitotic spindle targeting properties after coupling to pDNA or pDNA containing nanoparticles.

8.3. Cell-division responsive nanoparticles

Apart from chromatin- and/or INM-targeting, cell-division responsive pDNA containing nanoparticles might present an interesting option to increase gene expression efficiency. We may expect that free pDNA in the cytoplasm acts as a substrate for several intracellular nucleases. On the other hand, at

the moment of cell division, a fraction of free pDNA seems to be able to reach the nuclei of the daughter cells. Therefore, we hypothesize that when the amount of pDNA is the highest at the onset of cell division, this should improve gene delivery efficiency by improving the at random inclusion of pDNA in the nuclei of the daughter cells. This could be achieved by cell division-responsive pDNA nanoparticles which release their pDNA only at the onset of mitosis. In this way the time between release of the pDNA and cell division is as short as possible, which presumably avoids excessive degradation of the pDNA in the cytoplasm before nuclear enclosure can occur. The different phases of cell division are characterized by subsequent phosphorylation reactions by CDKs. By incorporating the recognition sequence of CDK1 (S/TPXK/R) [186, 187] in pDNA containing nanoparticles, one could obtain DNA nanoparticles which are specifically phosphorylated during cell division. The introduction of negatively charged phosphate groups is expected to decrease the DNA binding capacity of the nanoparticles, resulting in an enhanced release of the pDNA upon phosphorylation. This strategy has been used by Wilke *et al*. [8] who developed a pDNA carrier based on synthetic peptides. One of these peptides was derived from DNA binding proteins and contained the repeated SPKR motif, which is a substrate for CDKs. They found that a mitotic event indeed greatly improved expression efficiency with this peptide based pDNA carrier and asssumed that phosphorylation in the early phases of mitosis strongly diminished the carriers affinity towards pDNA and thus resulted in pDNA release at the time it has the best chances to enter the daughter nuclei [188]. An additional advantage is that the pDNA is well protected against nucleases in the cytosol until it has the possibility to be enclosed in the daughter nuclei [8].

Comparable strategies have been used to develop delivery systems which are responsive to protein kinase A and to cyclic AMP-dependent protein kinase [189-192]. Also here it was demonstrated that by incorporation of the appropriate kinase recognition site, specific phosphorylation could be obtained which resulted in release and an enhanced transfection efficiency of the complexed pDNA. It should be noted that the amount of free pDNA in the cytoplasm probably should not rise above a certain threshold, since it has been demonstrated that in cell-free systems pDNA competes with chromatin for binding of NE membrane fragments, thereby disturbing the formation of a smooth NE [193]. Whether this can also occur in living cells, and in this way contribute to the cytotoxicity of pDNA containing nanoparticles remains to be seen.

8.4. *Xenopus* nuclear envelope reassembly assay to study nuclear envelope dynamics

Insight in the different steps of the nuclear envelope assembly has substantially gained since the use of cell-free systems to study the reassembly of the NE *in vitro* [194, 195]. The *in vitro* assembled nuclei have a NE consisting out of two phospholipid bilayers with embedded NPCs and a peripheral

nuclear lamina, just like normal eukaryotic nuclei. Furthermore, they have a functional nuclear import and export machinery and can even go through mitosis. The XNER assay, serves as a good model to study the chromatin binding and resulting inclusion in the artificial nuclei, without the interference of other extra- and intracellular barriers. In the XNER assay, membrane fraction and cytoplasmic extract are isolated from *Xenopus* eggs, mostly in interphase [30, 196]. It is also possible to isolate mitotic extracts, by preventing the destruction of cyclin during the extraction and maintaining its phosphorylation state. When DNA is added to a mixture of these isolated cytoplasmic extract and membrane fragments, the assembly of a NE occurs spontaneously as long as an energy source is provided (Fig. 5). In the XNER assay, mostly membrane-devoid sperm chromatin is used as a DNA template. Is has been shown, however, that the NE can even form around protein-free DNA, after initial assembly of the DNA into nucleosomes and further organization into condensed spheres [197]. The ability of *Xenopus* eggs to form an intact, functional NE around added DNA most likely results from the large amounts of nuclear components which are stored in the eggs for subsequent cell divisions during embryo development. An additional advantage of these cell-free systems, is that certain protein components can be depleted from or added to the XNER assay, to assess their function in the NE assembly and disassembly processes. Furthermore, the nuclear inclusion of pDNA or pDNA containing nanoparticles can be studied by simply adding these particles to the XNER assay. The nuclear inclusion can then easily be followed using microscopy techniques such as phase contrast or fluorescence microscopy. It should be noted that although cell-free systems are very useful to isolate processes from the full complexity of the cellular environment, the results obtained with them should always be confirmed in living cells. Also, the XNER assay cannot be used to study targeting to H2A-H2B pockets, as the *Xenopus* sperm chromatin is devoid of them [151].

9. General conclusions

In nondiving cells, all cargo that goes into and out of the cell nucleus has to pass the NPCs. In dividing cells, however, the nuclear permeability barrier temporary disappears, leading to extra opportunities for macromolecules and nanoparticles to reach the nuclear interior during or shortly after mitosis. Viral particles already explored this mitotic window of opportunity, by taking advantage of mitosis to strongly anchor their genome to the chromosomes of the cells. The viral genomes than integrate (retroviruses) into the host genome, or are kept as an extrachromosomal episome (latent DNA viruses) that replicates and partitions together with the host genome. However, viruses display several properties which limit their clinical utility, such as limited DNA carrying capacity, immunogenicity and for some viral vectors, insertional mutagenesis. Therefore, the ultimate challenge is to design an artificial viral carrier which equals viral transfection efficiencies, but which is

safe to use, non immunogenic, non oncogenic and relatively inexpensive and easy to produce. Unfortunately, this is difficult to achieve as it requires the integration of many different and often counteracting features in a single particle [198]. In this review, we focused on features which could enable nuclear enclosure of pDNA containing particles during mitosis.

The partitioning of cell organelles has been reported to be a random process. The equal distribution of key organelles located in the cytosol to the daughter cells appears to be passive or active, involving transport along microtubules. For exogenous macromolecules such as pDNA and dextrans, which cannot pass the NPCs, it has been demonstrated that NEBD is not sufficient to ensure efficient uptake into the daughter nuclei. Rather, it seems that only chromosomes and macromolecules associated with them are included in newly formed nuclei. This most likely results from the fact that a closed NE is formed around tightly compacted chromatin before nuclear expansion occurs, leaving no space for neighboring molecules, cell organelles and nanoparticles to be enclosed. Shortly after mitosis, however, the NPCs in the newly formed NE are reported to be more permeable, thereby temporary shifting the size exclusion criteria to larger molecular weights.

The intracellular fate of small inorganic nanoparticles such as gold, iron oxide nanoparticles and quantum dots has been reported. In general, these nanoparticles are endocytosed and remain within the endosomal compartments of the cell. They are not frequently reported to be in the nucleus of the cells, although their small size (10-20 nm) most likely permits access to the nuclear interior through the NPCs. Upon cell division, in many cases a nice and uniform dilution of the endocytosed particles has been described, but this is not a general rule. Asymmetric division of stem cells, asymmetric segregation of particles in endosomes and the possible induction of autophagy are several examples of nature's way to overcome nanoparticle-induced cellular stress and which may result in a large heterogeneity of nanoparticle numbers upon continued cell proliferation.

Studies focusing on the partitioning during cell division of larger nonviral gene delivery nanoparticles (100 – 200 nm) are very rare, although insight in the cytoplasmic versus nucleoplasmic distribution of nanoparticles during subsequent cell divisions is highly relevant. It has been assumed that NEBD aids the nanoparticles or pDNA to reach the nuclear interior, as most authors observed increased transfection efficiencies following mitosis. A direct prove of this hypothesis is up to date, however, not given. Also we share the opinion that NEBD helps nanoparticles to deliver their pDNA to the nucleus of the cells. Furthermore, we believe that targeting of pDNA or pDNA containing nanoparticles to chromatin could be an interesting feature to further increase transfection efficiency in dividing cells. Based upon the viral examples given, chromatin binding as such is not a guarantee for nuclear inclusion. Instead, a strong anchoring to chromatin is needed, involving multiple chromatin-binding protein recognition sites in the pDNA and the presence of multiple chromatin

tethering factors and endogenous cellular proteins in one segregation complex. To date, the chromatin binding properties of the viral proteins EBNA1, E2 and LANA provide the most interesting features to explore chromatin targeting. Ideally, we can incorporate some interesting viral features for chromosome binding, without the introduction of the immunogenicity, oncogenicity or insertional mutagenesis that is often associated with viral carriers. Also, development of cell responsive particles that disintegrate at the onset of mitosis seems to be a promising concept to study. It is however not realistic to believe that the various options as presented by us in the foregoing will easily lead to the ultimate goal of improving intra-nuclear delivery of exogenous genes. Only careful and systematic studies of the individual steps involved in the process, without ignoring the transfection process as a whole, can lead to success. The ideally designed nonviral particles will have to overcome many extra- and intracellular barriers to accomplish highly efficient pDNA expression, replication and segregation of the pDNA to the progeny cells.

Acknowledgments

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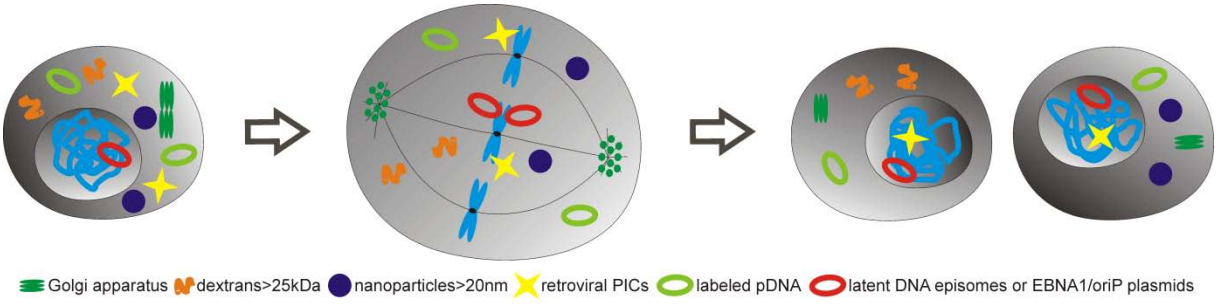
Graphical abstract



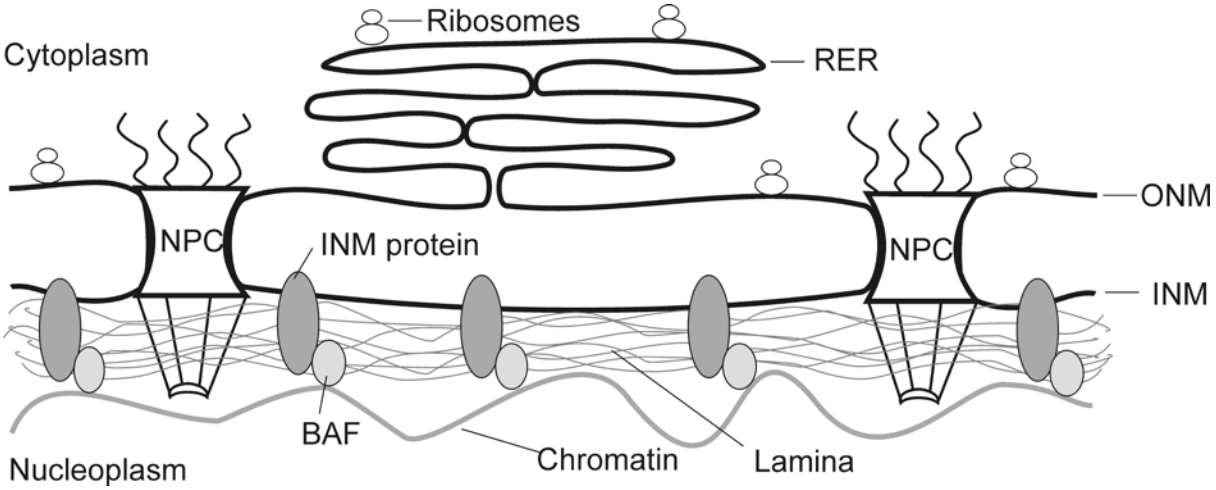


Figure 1. Structure of the nuclear envelope (NE). The NE consists of an outer nuclear membrane (ONM) continuous with the rough endoplasmic reticulum (RER), and the inner nuclear membrane (INM), which fuse at the so-called nuclear pore complexes (NPCs). Barrier-to-autointegration factor (BAF) is a chromatin-associated protein that also binds to the nuclear lamina and several nuclear envelope proteins.

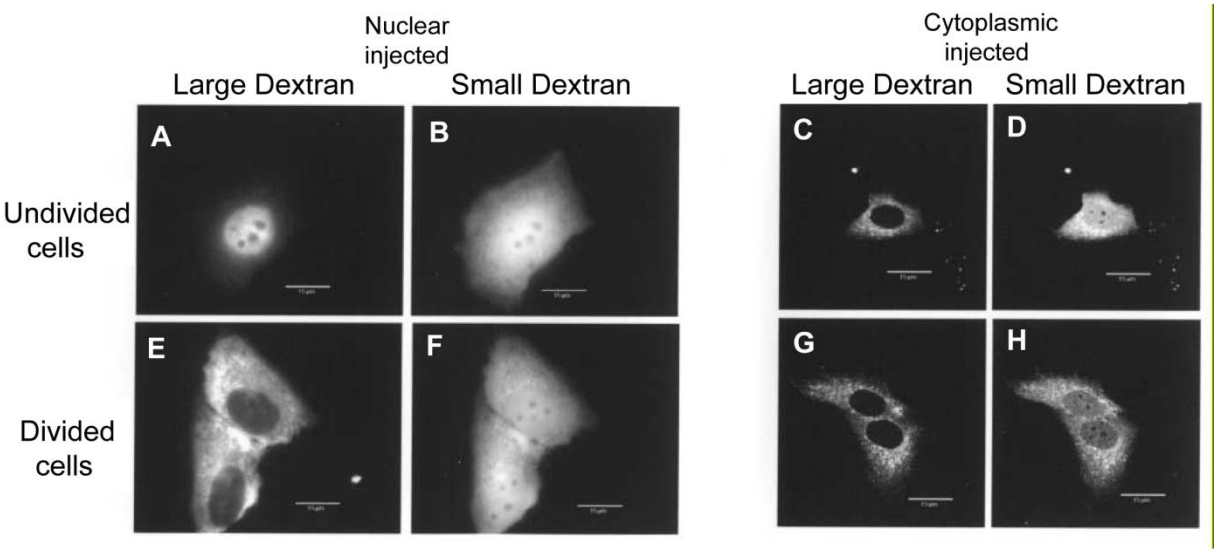


Figure 2. Subcellular distribution of dextrans. Cells were injected with rhodamine-labeled small dextran (11 kDa) and fluorescein-labeled large dextran (485 kDa). (A and B) After nuclear injection in an undivided cell. (C and D) After cytoplasmic injection in an undivided cell. (E and F) After nuclear injection in a divided cell. (G and H) After cytoplasmic injection in a divided cell. (A), (B), (E), and (F) are epifluorescent images. (C), (D), (G), and (H) are single optical sections of confocal images. Scale bars, 15 µm. (Reprinted by permission from Macmillan Publishers Ltd: [Molecular Therapy] [57], copyright (2002), DOI: 10.1006/mthe.2002.0581, http://www.nature.com/mt/journal/v5/n5/full/mt200282a.html)

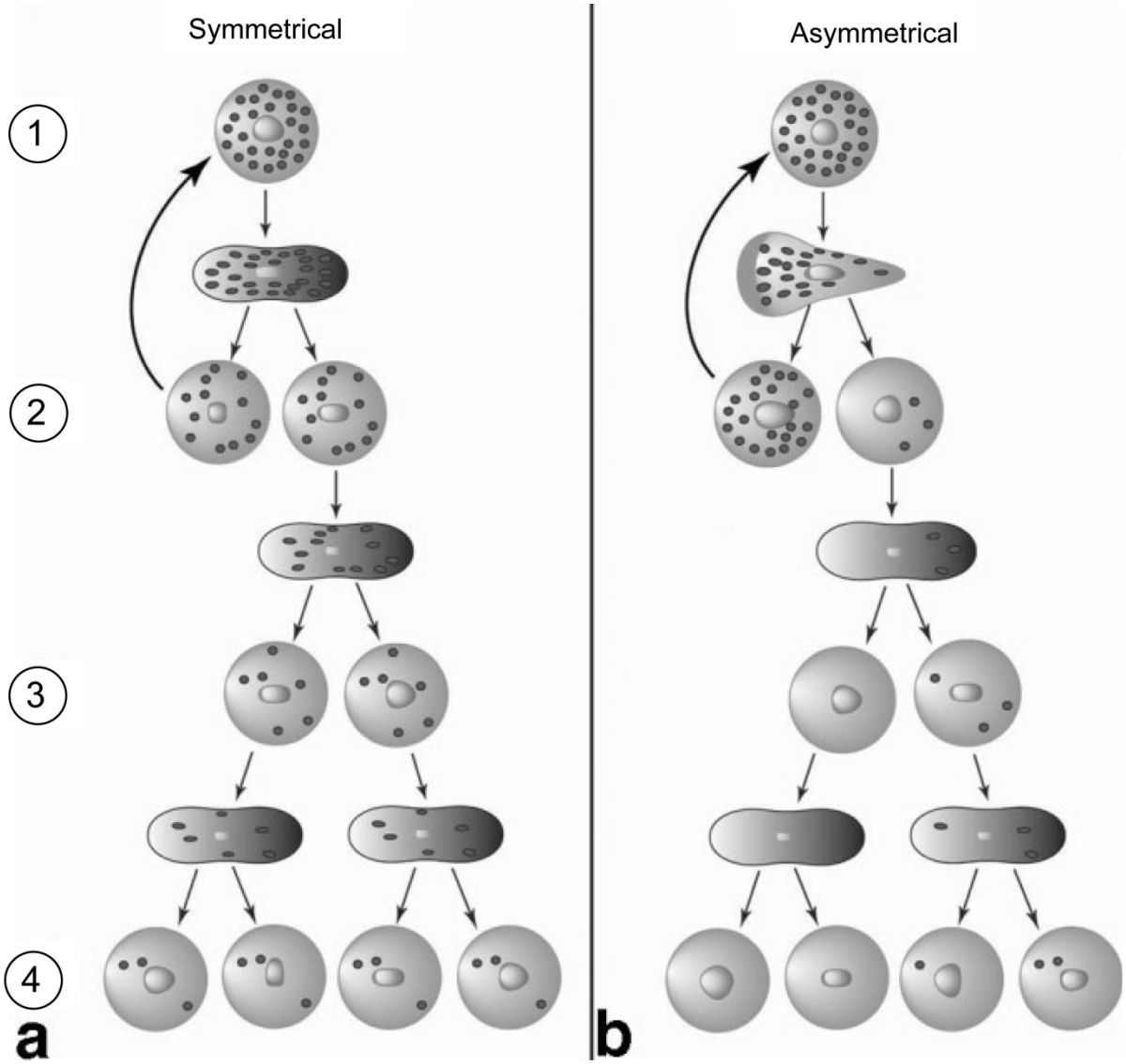


Figure 3. Schematic presentation of symmetrical vs. asymmetrical cell division and dilution of Feridex particles. In symmetrically dividing cells the contrast agent is distributed equally among daughter cells (a). Asymmetric cell division, as commonly encountered in stem cells, leads to a unequal/uneven distribution of Feridex particles. This in turn can lead to a sharp decline in cell detectability (b). (Reprinted by permission from John Wiley and Sons: [Magnetic Resonance in Medicine] [87], copyright (2007), DOI: 10.1002/mrm.21280, http://onlinelibrary.wiley.com/doi/10.1002/mrm.21280/abstract;jsessionid=D765ABF7E11FB97240148DDDE79FFA9E.d01t03)

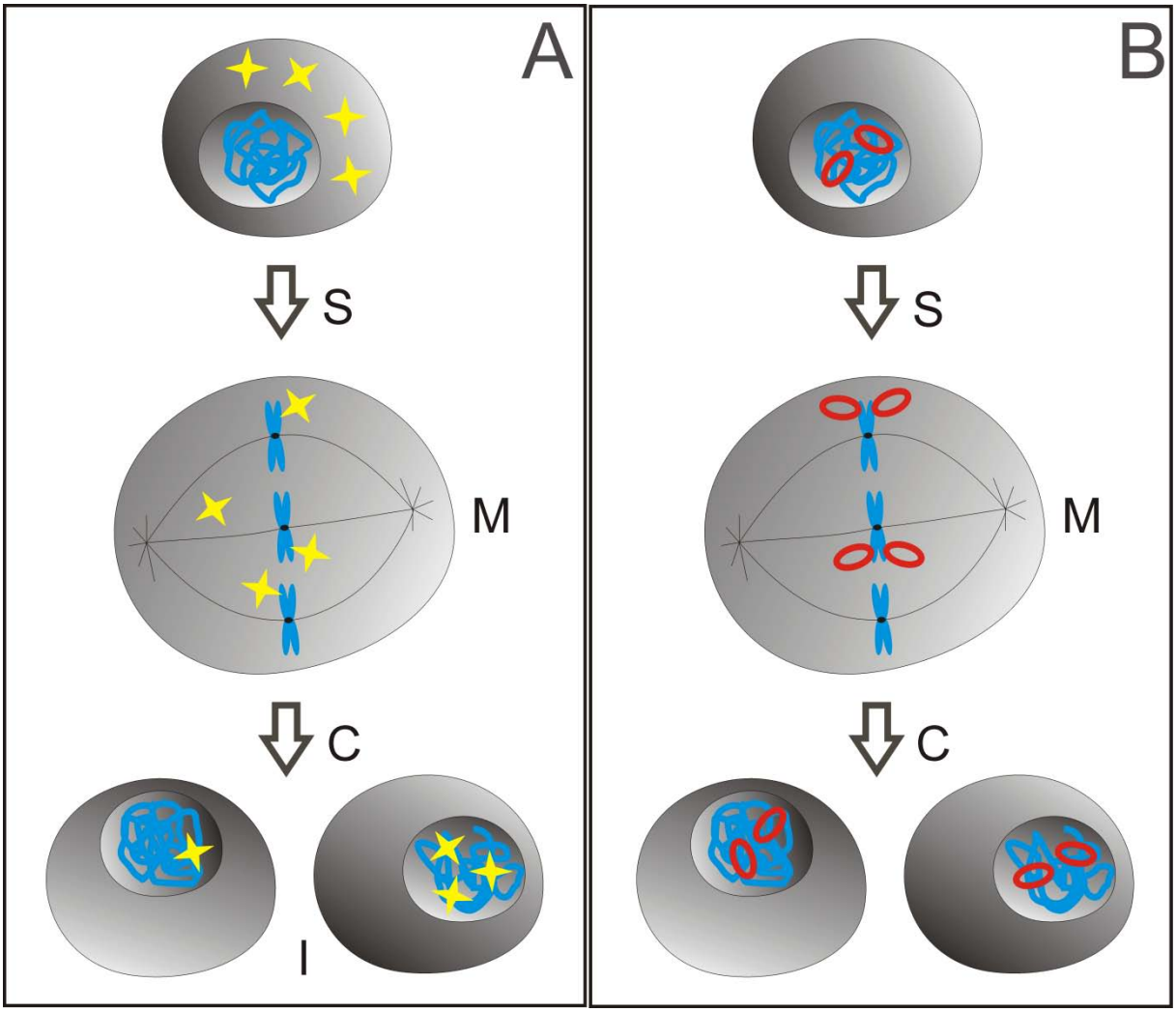


Figure 4. Schematic presentation of how retroviruses (A) and latent DNA viruses (B) take advantage of mitosis. A) Infection of retroviruses. After reverse transcription packages of viral DNA are assembled (pre-integration complexes: PICs). The PICs tether host chromosomes during methapase (M) and are following cytokinesis (C) enclosed into the daughter nuclei, where integration (I) can take place. B) Latent DNA virus episome or EBNA1/oriP plasmid maintenance and segregation. Episomes/oriP plasmids are bound to the host chromatin. Replication of host chromosomes and episomes/oriP plasmids and partitioning of episomes/oriP plasmids to sister chromatids take place during DNA synthesis (S) phase. Nuclear segregation of episomes/oriP plasmids to the daughter nuclei is achieved following mitosis (M+C).

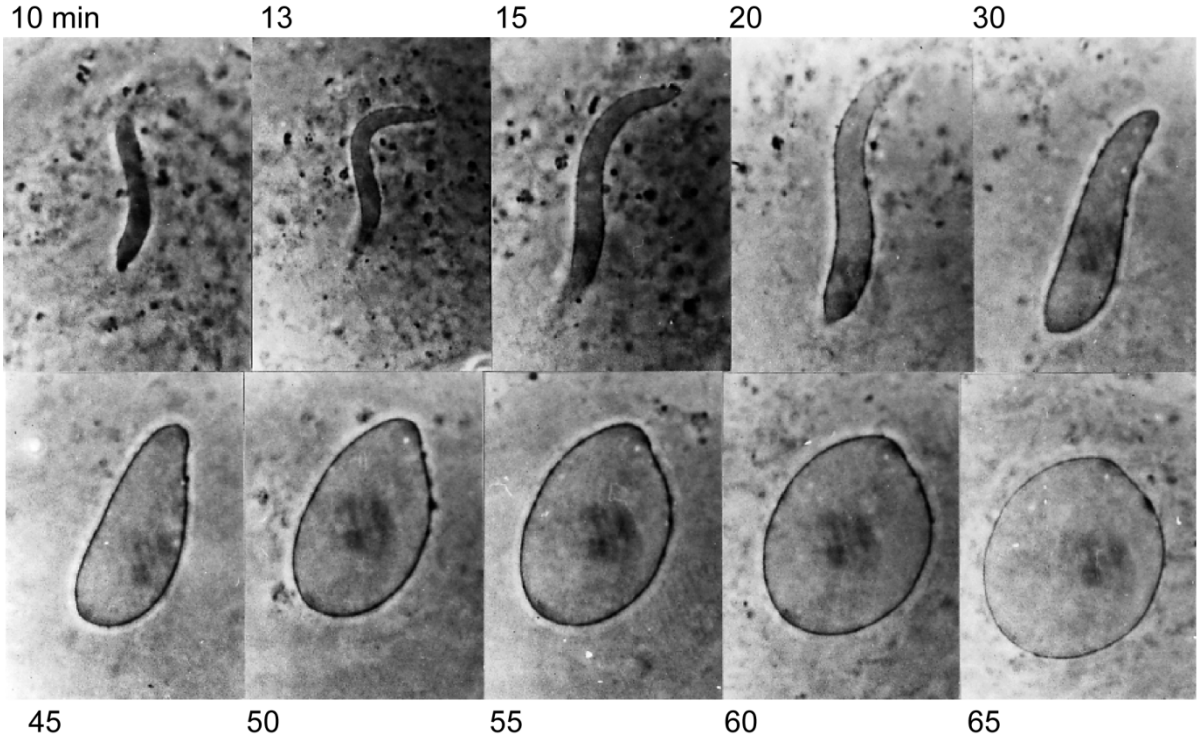


Figure 5. Different stages of the formation of an artificial nucleus around chromatin in the XNER assay. (Reproduced with permission from Journal of Cell Science [199], copyright (1997), http://jcs.biologists.org/content/110/13/1489.long)

Table 1. Intracellular partitioning and nuclear enclosure/exclusion of cell organelles, free macromolecules, inorganic nanoparticles, nonviral gene delivery particles and viruses upon mitosis.

		References
Cell organelles		
Endosomal vesicles	passive or active (through interaction	[40, 41]
Golgi apparatus	with the mitotic spindle or the	[41, 45, 47-49]
Endoplasmic reticulum /	chromosomes) partitioning;	[19, 37, 38, 50,
Nuclear envelope	nuclear exclusion	51]
Free macromolecules		
Labeled dextrans	< 25 kDa: through NPCs	[31, 39, 57, 58]
	> 25 kDa: nuclear exclusion	
plasmid DNA	labeled: nuclear exclusion	[57, 68-70]
	unlabeled: nuclear enclosure	
Inorganic nanoparticles		
Gold particles	< 10 nm: through NPCs	[88, 89]

	10-20 nm: nuclear enclosure > 20 nm: nuclear exclusion	
Quantum dots	Symmetric or asymmetric segregation	[79, 80]
Iron oxide nanoparticles	within endosomes; nuclear exclusion	[87]
Nonviral gene delivery particles		
Lipid carriers	unknown	[5, 8, 13-17]
Polymer carriers		[5, 8, 12]
Viruses	*chromatin tethering factor*	
Murine leukemia virus (MuLV)	(p12)	[133, 134]
Human immunodeficiency virus (HIV)	(LEDGF/p75)	[141]
Avian sarcoma virus (ASV)	(IN)	[132]
Foamy virus (FV)	(Gag)	[143, 144]
Epstein Barr virus (EBV)	EBNA1	[147-149]
Kaposi sarcoma-associated herpesvirus (KSHV)	LANA	[151, 152, 154]
Bovine (BPV) and human papillomavirus (HPV)	E2	[155-158]
Simian vacuolating virus 40 (SV40)	unknown	[161, 162]

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