

COMMENTARY

Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons

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SUMMARY

Mammals and amphibians can be cloned following the transfer of embryonic nuclei into enucleated eggs or oocytes. As nuclear functions become more specialized in the differentiated cells of an adult, successful cloning using these nuclei as donors becomes more difficult. Differentiation involves the assembly of specialized forms of repressive chromatin including linker histones, Polycomb group proteins and methyl-CpG-binding proteins. These structures compartmentalize chromatin into functional domains and maintain the stability of the differentiated state through successive cell divisions. Efficient cloning requires the erasure of these structures.

The erasure can be accomplished through use of molecular chaperones and enzymatic activities present in the oocyte, egg or zygote. We discuss the mechanisms involved in reprogramming nuclei after nuclear transfer and compare them with those that occur during remodeling of somatic nuclei after heterokaryon formation. Finally we discuss how one might alter the properties of adult nuclei to improve the efficiency of cloning.

Key words: Cloning, Nucleus, Cell cycle, Chromatin, Histone, Transcriptional control

INTRODUCTION

A fundamental question in cell and developmental biology concerns how nuclei progressively acquire differentiated functions. Although the nucleus of a fertilized egg is totipotent in that all of the differentiated cell types found in the adult organism can be derived from it, this is not the case for the vast majority of somatic nuclei in the adult animal. This limitation of the genomic potential of nuclei is progressively acquired during embryonic and post-embryonic development. Although in most cells the DNA sequence content of nuclei remains unchanged as development proceeds, the repertoire of genes that are expressed in a given cell type becomes limited. It also becomes more difficult to reactivate genes that are silenced in that cell type. This limitation is now known to reflect the imposition of epigenetic regulatory mechanisms on genes, especially through the assembly of stable repressive nucleoprotein complexes in the differentiated cell nucleus. The molecular mechanisms necessary to stably repress genes are gradually established as embryogenesis and post-embryonic development proceed. Remarkably, the egg and oocyte can reverse this process of repression, disassembling repressive features of nuclear organization and, in particular circumstances, recreating a state of pluripotency and even totipotency.

The economic and medical implications of widespread cloning of domestic animals by nuclear transfer from donor embryos (Campbell et al., 1996; First and Pevather, 1991; Wolf et al., 1998), together with the potential for successful cloning of mammals using adult cell nuclei as donors (Wilmut et al., 1997; Wakayama et al., 1998; Kato et al., 1998; Wakayama and Yanagimachi, 1999), have stimulated interest in the basic molecular mechanisms involved in reprogramming the developmental fate of nuclei introduced into eggs and oocytes considerably. An understanding of these mechanisms not only will potentially provide insight into the significance of epigenetic events in establishing a developmental and differentiative program, but also might suggest new approaches towards improving the efficiency and success of nuclear transfer procedures.

RESTRICTIONS ON GENOMIC POTENTIAL ESTABLISHED DURING DEVELOPMENT

Amphibian development is characterized by multiple (>10) rapid cell divisions (30 minute cell cycles) in the absence of zygotic transcription (Graham and Morgan, 1966). Over a short period encompassing a single division cycle the cell cycle lengthens, and transcription of the embryonic nuclei

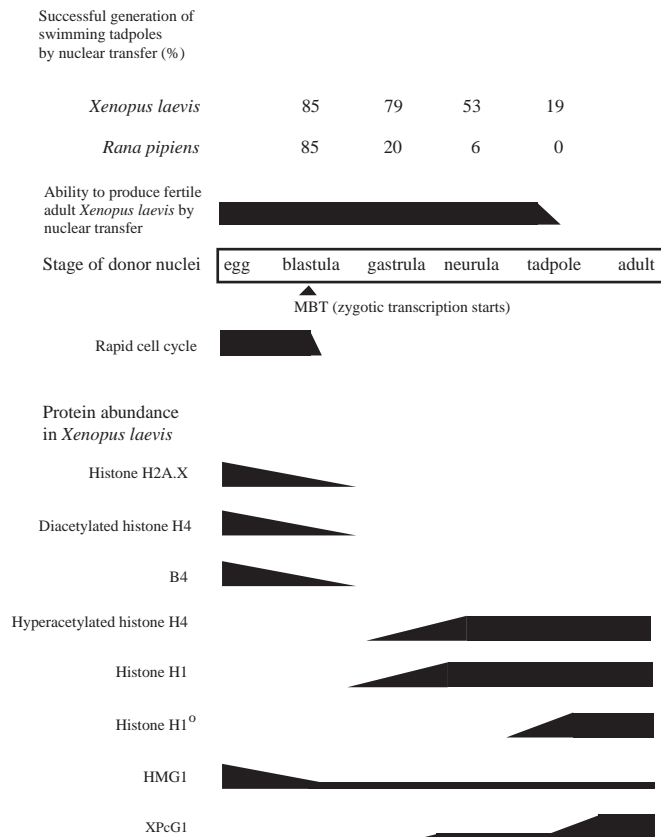


Fig. 1. Developmental success and chromatin remodeling in early amphibian development. The developmental success of nuclear-transplant embryos, as assayed by the generation of tadpoles (Gurdon, 1960, 1962b; Briggs and King, 1957), relative to changes in chromatin composition and DNA replication rates in donor nuclei. Data for H2AX (Kleinschmidt et al., 1991), diacetylated H4 (Woodland, 1979), hyperacetylated histone H4 (Dimitrov et al., 1993), histone B4 (Smith et al., 1988), histone H1 (Hook et al., 1993), histone H1^o (Grunwald et al., 1995), HMG₁ (Kleinschmidt et al., 1983) and *Xenopus* Polycomb (Strouboulis et al., 1999) are shown.

begins (Fig. 1). This mid-blastula transition (MBT) occurs when the embryo consists of several thousand cells (4,000 in *Xenopus laevis*). At this time, cell fate is beginning to be determined owing to inductive interactions between ectodermal and endodermal regions (Davidson, 1986). Briggs and King (1952, 1960) systematically assessed the capacity of nuclei from *Rana pipiens* blastulae (8,000 cells) and gastrulae (16,000 cells) to support development after transfer to enucleated eggs. Nuclei from such cells, and those of other amphibian embryos (Gurdon, 1962a) at comparable embryonic stages, were totipotent, as defined by the cloning of fertile females: a single fertile female for McKinnell (1962) and 150 adult frogs, both male and female for Gurdon (1962a). The genomic potential of more-advanced embryonic nuclei declined dramatically (Briggs, 1979; Fig. 1); however, the use of endodermal nuclei from the intestines of feeding tadpoles as donors still produced fertile females with a 2% success rate (Gurdon and Uehlinger, 1966). Nevertheless, nuclei from adults or from cells in tissue culture could not generate such animals, although development progressed through

metamorphosis, leading to the appearance of many adult cell types (Gurdon and Laskey, 1970; Gurdon et al., 1979; Di Bernardino et al., 1986). The nuclei of adult amphibian cells have not yet been shown to be totipotent (Di Bernardino, 1987, 1997; Gurdon, 1999). Mammalian and amphibian embryos regulate gene expression during early development differently. The cell division cycles are much more protracted, the first cell cycle taking >20 hours to complete. Transcriptional activation of embryonic nuclei occurs at the 1- to 2-cell stage in the mouse (Flach et al., 1982; Latham et al., 1992; Christians et al., 1995), the four-cell stage in pigs (Jarell et al., 1991), and the 8- to 16-cell stage in sheep (Crosby et al., 1988) and cattle (Barnes and Eyestone, 1990; Table 1). In the mouse and in other mammals, nuclei at the two-cell stage are totipotent: the separation of blastomeres leads to the development of identical twins (Tsunoda and McLaren, 1983). Nuclear-transfer experiments using four-cell embryonic nuclei as donors can lead to the development of fertile mice (Kono et al., 1991), and eight-cell embryonic nuclei allow development to term, which suggests that they are pluripotent (reviewed by Kato and Tsunoda, 1993). However, systematic analysis by McGrath and Solter (1984) shows a major decline in the developmental potential of donor nuclei (as assayed by blastocyst formation) between the one-cell (90% success) and two-cell stages (12.6% success). It should be noted that, in these experiments, the transfer of nuclei into oocytes or zygotes will have a different outcome. The oocyte is a developing egg cell, and most transfer experiments in mammalian systems use secondary oocytes that have passed through the first meiotic division to split off the first polar body. The egg represents the fully developed gamete that is expelled from the tissues of the female. A zygote represents the cell that results from the union of the male and female gametes after fertilization. Oocytes, eggs and zygotes have distinct biological properties. The experiments carried out by McGrath and Solter (1984) used zygotes as recipients. This gives substantially poorer results, compared with when oocytes are used (reviewed by Di Bernardino, 1997). Given that development to the blastocyst stages occurs at a high frequency when nuclei are transferred to recipient oocytes, this may reflect the greater time available in the parthenogenetically activated oocyte for successful nuclear remodeling before cell division (see later). In the embryos of domestic animals in which transcriptional activation of embryonic nuclei occurs at a later time than in the mouse, donor nuclei from the inner cell mass (in sheep; Smith and Wilmut, 1989) and the 48-cell stage (in cattle; Bondioli et al., 1990) are totipotent. Rapid progress in this research area has allowed successful pregnancies using donor nuclei from cultured embryonic cells (Sims and First, 1994; Sun and Moor, 1995; Campbell et al., 1995, 1996; Cibelli et al., 1998) and from adult cells (Wilmut et al., 1997).

Those studies in amphibia and mammals lead to the clear conclusion that nuclei from the cells of an early embryo are much more pluripotent and potentially totipotent than adult cells. The progressive restriction in the developmental capacity of nuclei correlates with aspects of nuclear function. For example, the time at which transcription begins to occur parallels a rapid decline in the efficiency of successful nuclear transfer (Newport and Kirshner, 1982a,b; Briggs and King, 1960; McGrath and Solter, 1984; Flach et al., 1982), but even the use of fully active nuclei does not preclude the occasional

Table 1. Comparison of the timing of zygotic gene activation in mammalian embryos and loss of capacity to generate cloned embryos efficiently from embryonic donor nuclei

Species	Initiation of zygotic transcription	Latest embryonic donor nuclei that allows efficient cloning	Donor nuclei from adult tissue capable of producing live birth
Mouse	2-cell	8-cell	Cumulus cell, cell from tail tip
Sheep	8-cell	Inner cell mass	Mammary gland cell
Cattle	8-cell	48-cell	Cumulus cell, oviduct cell granulosa cell
Rabbit	8-cell	16-cell	Not described
Pig	4-cell	4-cell	Not described

The types of adult cells that allow cloning are also listed. Data are for the mouse (Flach et al., 1982; Cheong et al., 1993; Tsunoda et al., 1987; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999); for sheep (Crosby et al., 1988; Smith and Wilmut, 1989; Wilmut et al., 1997); cattle (Barnes and Eyestone, 1990; Bondioli et al., 1990; Kato et al., 1998; Wells et al., 1999); rabbit (Manes, 1977; Collas and Robl, 1990); and pig (Jarrell et al., 1991; Prather et al., 1989).

success (Gurdon and Uehlinger, 1966; Sims and First, 1994; Wilmut et al., 1997).

CELL CYCLE INFLUENCES ON NUCLEAR REPROGRAMMING

For successful nuclear transfer and development of the resulting 'fertilized' egg, the properties of the donor nucleus have to become like those of the normal zygotic nucleus. The donor nucleus must adopt the cell cycle parameters of the zygote, including DNA replication, nuclear envelope breakdown, chromosome condensation and chromosome segregation, and, subsequently, embryonic patterns of DNA replication and transcription. The cytoplasm of the recipient oocyte, egg or blastomere has to direct this reprogramming of the donor nucleus. This requires the activities of cell cycle regulators such as the p34^{cdc2}/cyclin B kinase (also known as maturation promoting factor, MPF), which facilitate the remodeling of nuclear structure (Fulka et al., 1996). In addition maternal stores of protein that are normally used for assembly of nuclei during embryonic development (Almouzni and Wolffe, 1993a) are co-opted to replace proteins in the donor nucleus. The capacity of the recipient cytoplasm to remodel the donor nucleus will therefore influence the chromosome complement, the timing of subsequent developmental events and thus genomic potential.

Donor nuclei need to replicate their genomes. In adult amphibian and mammalian cells, the proportion of cells engaged in replication at any one time ranges from zero (mature amphibian erythrocytes) to 1% (adult brain or liver) to 20% (fibroblasts in culture) (Graham et al., 1966; De Roper et al., 1977; Di Berardino et al., 1986). There is also a selective use of replication origins in adult cells (De Pamphilis, 1993) and an S phase of eight hours or more, in which certain portions of the genome replicate before others (Wolffe, 1991). Moreover, donor nuclei should be in G₁ phase (the interval between mitosis and the initiation of DNA replication) or in G₀ phase, in which they remain metabolically active but have exited the cell cycle. If nuclei in S phase or G₂ phase are used, then the potential reduplication of the genome directed by the recipient cytoplasm will result in aberrant development. The use of donor nuclei arrested in G₁ or G₀ phase increases the efficiency of successful nuclear transfer (Collas et al., 1992; Wilmut et al., 1997). A second major problem is the initiation

of premature nuclear breakdown and chromosome condensation if DNA synthesis is not complete, which leads to chromosome loss and aneuploidy. This will occur if the cell cycle of the recipient cytoplasm enters G₂/M phase when the donor nucleus is in G₁/S phase. Synchronization of the cell cycle stage of the recipient cytoplasm with that of the donor nuclei improves the developmental capacity of the resulting embryos. Although the efficiency of successful development remains very low, prodigious efforts to coordinate the cell cycles of nucleus and cytoplasm using tissue culture nuclei improves the success rates for mammalian blastocyst development significantly (Campbell et al., 1996). Clearly, multiple parameters constrain the genomic potential of late embryonic and adult cell nuclei.

EPIGENETIC CONSTRAINTS ON GENOMIC POTENTIAL

The process of development relies on the differential expression of genes in particular cell types. Stem-cell populations renew themselves while providing cells that stably differentiate. Most cells in developing amphibian embryos continue to divide beyond gastrulation, as do most cells beyond the 64-cell stage in mammalian embryos. However, lineage-tracing studies demonstrate that, if embryogenesis is allowed to proceed, these cells have already begun to have their fate determined (Davidson, 1986). The imposition of epigenetic controls involves both the activation of the transcriptional machinery (Almouzni and Wolffe, 1995; Majumder et al., 1993, 1997; Veenstra et al., 1999) and significant alterations in chromatin organization. In general, cell cycle controls direct the reversible dissociation of the transcriptional machinery from chromosomes and constrain the function of individual transcription factors (Martinez-Balbas et al., 1995; Segil et al., 1996; Shermoen and O'Farrell, 1991; Landsberger and Wolffe, 1995). In addition, DNA replication is not impeded by the presence of transcription factors bound to DNA (Wolffe and Brown, 1986). Thus, one might anticipate that the presence of transcription pre-initiation complexes and engaged RNA polymerases does not impose significant constraints on the properties of donor nuclei in recipient cytoplasm. Chromatin structure imposes more of a problem in terms of the release of nucleoprotein complexes from the chromosome, because of the

stable association of structural proteins with DNA through both replication (Sogo et al., 1986) and chromosome condensation (Koshland and Strunnikov, 1996; Nan et al., 1996). The properties of the chromatin that is assembled are strongly influenced by methylation of CpG dinucleotides, the major covalent modification of DNA found in vertebrate embryos (Antequera et al., 1989; Keshet et al., 1986). DNA methylation states are stably maintained in somatic cells through DNA replication and cell division (Holliday, 1987).

A major function of chromatin and DNA methylation in a mammalian cell is stable repression of genes known to be imprinted; these genes display allele-specific patterns of activity that depend on whether they are derived from the paternal or maternal genome (Wolffe and Matzke, 1999). DNA methylation is also essential for X-chromosome inactivation in female mammals (Li et al., 1992). Amphibian chromosomes do not show such methylation-dependent imprints; however, DNA methylation in amphibia and mammals is also important for repressing the transcriptional activity of the many promoter sequences present in retrotransposons and bona fide genes not required for the maintenance of a particular differentiated phenotype (Lin and Riggs, 1975; Bird, 1995; Yoder et al., 1997). Methylated DNA is recognized by specific repressor proteins that are stably assembled into chromatin (Nan et al., 1996, 1997; Chandler et al., 1999). These proteins work together with transcriptional co-repressors to silence transcription through the deacetylation of the histone N-terminal tails within nucleosomes (Jones et al., 1998; Kass et al., 1997ab; Nan et al., 1998; Wade et al., 1999). DNA methylation patterns change dramatically during early mammalian development, and significant genome-wide demethylation occurs (Monk et al., 1987; Krafi et al., 1993). DNA methylation levels then increase during subsequent development. How DNA demethylation is controlled remains unknown (Wolffe et al., 1999). For efficient cloning, the important aspect is that methylation states are reversible and can be re-established *de novo* (see also Tada et al., 1997).

Chromatin composition undergoes profound change during early vertebrate development (Patterton and Wolffe, 1996; Fig. 1). Failure to deacetylate the histones blocks amphibian development shortly after gastrulation (Almouzni et al., 1994). Core-histone acetylation changes dramatically within chromatin during the first few cell divisions of mouse embryogenesis. Chromatin that contains acetylated histone H4 becomes enriched at the nuclear periphery when the zygotic genome is strongly activated at the two-cell stage (Worrad et al., 1995). Inhibition of histone deacetylase using Trichostatin A increases the efficiency of gene expression. Acetylated chromatin localizes with RNA polymerase II, which suggests that it represents the site of active transcription. This localization of acetylated chromatin to the nuclear periphery is lost in the four-cell embryo and during subsequent development. An important conclusion from these experiments is that the functional compartmentalization of the nucleus occurs very early in mouse embryogenesis (Thompson et al., 1995).

The type of linker histone present within chromatin shows regulated changes during both amphibian and mammalian embryogenesis (Clarke et al., 1998; Hock et al., 1993; Dimitrov et al., 1993). Accumulation of the somatic type of histone H1 in *Xenopus* embryos directs the specific repression

of some oocyte-specific genes (Bouvet et al., 1994) and causes ectodermal cells to lose their competence to differentiate into mesodermal tissue (Steinbach et al., 1997; Vermaak et al., 1998). *Xenopus* Polycomb proteins, another repressive component of chromatin (van Lohuizen, 1999) accumulate even later in development than histone H1 (Strouboulis et al., 1999). H1 achieves normal abundance by gastrulation (Dimitrov et al., 1993); yet Polycomb only begins to accumulate in chromatin at this time. Methylation of the genome in the mouse embryos is also very dynamic during the early cell divisions (Razin and Shemer, 1995; Yoder et al., 1997). Methylation is essential for post-gastrulation development (Li et al., 1992), as is the methylation-specific transcriptional repressor MeCP2 (Tate et al., 1996). The role of DNA methylation in early amphibian development has not yet been investigated.

These observations demonstrate that the structural components of chromatin and the methylation status of DNA have a very significant role in establishing the developmental fate of particular cells in the embryo. This function is exerted by preventing uniform access of the transcriptional machinery to all of the promoters in the genome. The proteins that assemble chromatin not only establish but also serve to maintain stable states of gene repression (Wolffe, 1994). This is because of the stable association of histones, their modification states and associated proteins through DNA replication, chromosome condensation and segregation (Perry et al., 1993; Sogo et al., 1986). Repressive chromatin structures do not disassemble very readily, and thus the inability to express certain genes might impose a significant obstacle to the development of nuclear transfer embryos. Although the sperm chromatin that will assemble the paternal pronucleus is transcriptionally silent and highly condensed, the proteins that package DNA in sperm chromatin are highly adapted for rapid release from DNA on exposure to egg cytoplasm (Philpott et al., 1991; Philpott and Leno, 1992). The remodeling of somatic nuclei by egg cytoplasm poses a much more formidable problem.

REMODELING SOMATIC NUCLEI IN *XENOPUS* EGG AND EMBRYOS

Among the first experiments to consider specific changes in gene activity were those of Gurdon and Brown (1965), who were examining the regulation of rRNA gene transcription, using somatic nuclei transplanted into *Xenopus* eggs. Somatic nuclei actively transcribe rRNA prior to transplantation; however, once placed into an egg, the nucleoli disappear and the rRNA genes are inactivated. As development of the embryo containing the transplanted nucleus proceeds, the rRNA genes are reactivated and nucleoli reappear. This reversible inhibition of rRNA gene activity coupled to the dissolution and reassembly of a specific nuclear compartment clearly demonstrated the powerful remodeling influence egg cytoplasm can have on nuclear activity.

A considerable movement of proteins from the egg cytoplasm into the somatic nucleus occurs after transplantation (Merriam, 1969; Barry and Merriam, 1972). This movement is concomitant with nuclear swelling and a significant reduction in the amount of heterochromatin in the somatic nucleus.

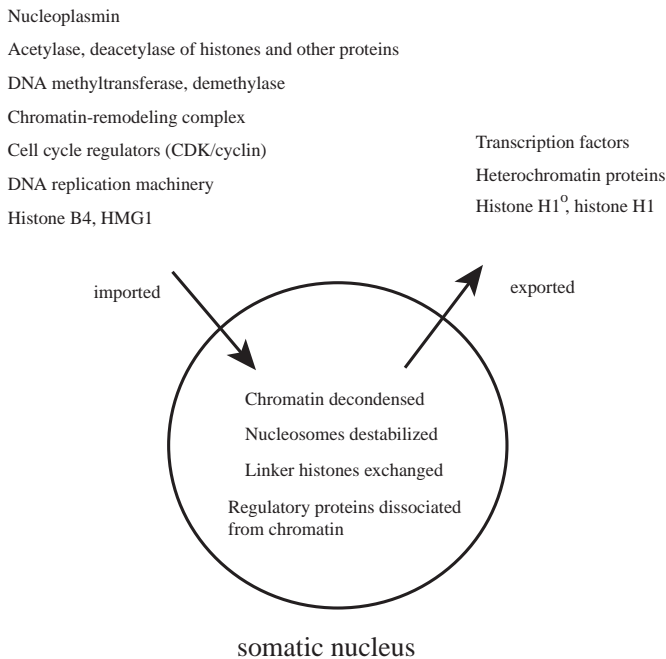


Fig. 2. Diagram showing the exchange of proteins that occurs when a somatic donor nucleus is transplanted into an amphibian egg. The exchange of nuclear proteins that is illustrated occurs without DNA replication and mitosis. These events will further facilitate nuclear reprogramming (see text for details).

Remarkably, >75% of pre-existing protein is lost from the somatic nucleus. Thus, the reprogramming of somatic nuclei following transplantation involves a tremendous exchange of chromatin components. *Xenopus* egg cytoplasm is much more effective than oocyte cytoplasm in remodeling somatic nuclei; however, if the oocyte germinal vesicle (nucleus) is first ruptured (which allows the contents to mix with the cytoplasm), then the efficiency of remodeling increases dramatically (Gurdon, 1968, 1976; Gurdon et al., 1979). This suggests that the large stores of nuclear components stored in the oocyte germinal vesicle facilitate remodeling of somatic nuclei. These stores include molecular chaperones, such as nucleoplasmin (Laskey et al., 1978) and N1/N2 (Kleinschmidt et al., 1986). Both of these chaperones can mediate the transfer of core histones to DNA and the assembly of nucleosomes. After fertilization, nucleoplasmin also mediates the removal of arginine-rich sperm-specific protamines from sperm chromatin (Hiyoshi et al., 1991) while facilitating the deposition of histones H2A and H2B into chromatin (Philpott et al., 1991; Philpott and Leno, 1992). Nucleoplasmin is phosphorylated on maturation of the oocyte to an egg (Sealy et al., 1986). The phosphorylated form is more efficient in removing the sperm protamines and decondensing the sperm chromatin to allow the assembly of the paternal pronucleus (Leno et al., 1996).

Nucleoplasmin also has a major role in remodeling somatic nuclei in *Xenopus* egg cytoplasm. A major defined change in chromatin composition that occurs is the loss of the somatic linker histone variants (H1, H1^o) and their replacement by the oocyte-specific histone variant B4 and the chromatin structural protein HMG1 (Dimitrov and Wolffe, 1996). Although various forms of linker histone and HMG1 appear to have similar

structural roles in chromatin, this replacement is surprising because histone B4 and HMG1 form complexes with chromatin that are much less stable than those involving the somatic linker histones (Nightingale et al., 1996; Ura et al., 1996). This relative instability probably reflects the fact that histone B4 is much less basic than the somatic linker histones (Doenecke and Tonjes, 1986; Smith et al., 1988; Dimitrov et al., 1993; Khochbin and Wolffe, 1994). However, nucleoplasmin much prefers to interact with arginine-rich proteins such as H1^o and H1 than with histone B4 and HMG1 (Dimitrov and Wolffe, 1996). Thus protein-protein interactions between nucleoplasmin and the somatic linker histones probably account for their selective removal from the chromatin of the somatic nucleus.

The reduction in histone H1 content of somatic nuclei should remove one major impediment to the reprogramming of genes (Bouvet et al., 1994; Kandolf, 1994; Steinbach et al., 1997). In fact *Xenopus* erythrocyte nuclei that have packaged their DNA within heterochromatin from which the transcriptional machinery has been erased (Hentschel and Tata, 1978) can be transcriptionally activated following remodeling in *Xenopus* egg extracts (Wolffe, 1989ab; Dimitrov and Wolffe, 1996). Wangh and colleagues have also documented the reacquisition of replication competence in *Xenopus* erythrocyte nuclei incubated in egg extracts (Coppock et al., 1989; Wangh et al., 1995). The readdition of histone H1 to remodeled nuclei in the egg extract severely compromises both transcription (Dimitrov and Wolffe, 1996) and replication (Lu et al., 1997, 1998).

Aside from the activity of proteins such as nucleoplasmin, which provide a sink for sequestration of proteins that freely exchange from chromatin, such as H1 (Caron and Thomas, 1981), energy-dependent chromatin-decondensation processes will probably be required for reprogramming nuclei (Blank et al., 1992). Such energy-dependent processes might involve the engines that normally drive mitotic chromosome condensation, such as the SMC (stability and maintenance of chromosomes) ATPases (Koshland and Strunnikov, 1996) or DNA polymerases (see earlier), or dedicated chromatin-remodeling machines of the SWI2/SNF2 superfamily (Peterson and Tamkun, 1995). *Xenopus* eggs contain large amounts of SWI2/SNF2-related proteins (Wade et al., 1998a,b, 1999).

The unifying aspect of the reprogramming of somatic nuclei following their transfer into the egg is that the biochemical changes establishing constraints on genetic potential are reversed. The efficiency of this reversal most probably determines the subsequent developmental success of the nuclear transfer embryo. It is easier to reverse the constraints imposed that have been imposed on early embryonic nuclei than those present in adult nuclei. This correlates with the progressive stabilization of various repressive chromatin structures that assemble as development proceeds. Just as the assembly of repressive chromatin is an active, energy-requiring process (Almouzni and Wolffe, 1993b; Wade et al., 1999), it is clear that energy must be expended to remodel somatic nuclei following transfer to the egg (Blank et al., 1992). Understanding how both targeted and general chromatin and chromosome remodeling occur is an important area for future investigation. The *Xenopus* egg and oocyte systems offer a powerful research tool for examining these issues.

LESSONS FROM HETEROKARYONS

A conceptually related approach to the reprogramming of somatic nuclei after their introduction into the cytoplasm of a *Xenopus* egg or oocyte is the study of changes in nuclear function that occur after the fusion of two distinct somatic cells to form a single cell that contains two different nuclei in a common cytoplasm (a heterokaryon). Gene expression in the donor cells changes dramatically after formation of a heterokaryon, which suggests that specialized trans-acting factors that differentially regulate gene expression exist in eukaryotes (Ephrussi, 1972; Ringertz and Savage, 1976). A gene normally active only in a differentiated cell is often inactivated upon fusion with a different differentiated cell or an undifferentiated cell. Somatic-cell hybrids in which the two nuclei of the heterokaryon fuse often lose chromosomes in culture. This type of phenomenon led to the attribution of individual repressive effects to particular chromosomes (Ephrussi, 1972).

Very occasionally, gene activation occurs in cell fusion experiments. For example, extensive experiments in heterokaryons have clearly shown that fusion of one differentiated cell (a muscle cell) with a cell in which muscle genes are not normally expressed (a human amniocyte) leads to the activation of muscle genes in the amniocyte (Blau et al., 1983). Similar approaches show that rhabdomyosarcoma cells lack a factor required for muscle differentiation (Tapscott et al., 1993). These results suggest that factors capable of activating genes can either exchange freely between nuclei or exist in excess within the cytoplasm. Recent experiments have shown this to be true for regulatory transcription factors such as the glucocorticoid receptor (Hache et al., 1999). The activation of differentiated genes in an undifferentiated cell is rapid (within two days) and does not require cell division or DNA replication. This implies that genes can be activated (at some level) without requiring replication events.

The maintenance of specialized cellular phenotypes through dynamic interplay between positive and negative regulatory molecules could involve either direct interactions by complementing a particular deficiency in one of the cell types in a heterokaryon (Baron, 1993; Blau, 1992), or it could involve indirect effects. Such indirect effects might occur when a positive regulatory factor induces other cell-specific transcription factors that in turn might activate a diverse group of downstream genes (Hardeman et al., 1986). This latter mechanism appears to operate when erythroid cells are fused with non-erythroid cells (Baron and Maniatis, 1986, 1991; Baron and Farrington, 1994). Certain experiments fuse erythroid cells with embryonic stem cells that lack a key transcriptional regulator of the globin genes (GATA1) (Evans and Felsenfeld, 1989, 1991); yet the nuclei of the embryonic stem cells can still be reprogrammed to express their globin genes in the heterokaryons. This indicates that erythroid cells contain the complement of factors necessary for activation of the globin genes, as well as upstream regulators such as GATA1 (Baron, 1993; Baron and Farrington, 1994).

Experiments on heterokaryons and *Xenopus* eggs have been interpreted as providing evidence for a continuous regulation of a plastic differentiated state (Blau and Baltimore, 1991). Implicit in this model is the idea that all genes are continually regulated by trans-acting factors that can either activate or

repress genes (Chiu and Blau, 1984; Blau et al., 1985; Blau and Baltimore, 1991). The process of transcription requires considerable remodeling of chromosomal structure, such as occurs in *Xenopus* egg cytoplasm. A similar, albeit less impressive, remodeling of chromosomes occurs in heterokaryons. For example, the nuclei of chicken erythrocytes consist predominantly of heterochromatin containing the specialized linker histone H5. In heterokaryons formed by fusion of chicken erythrocytes with proliferating mammalian cells, the chicken erythrocyte nuclei once again become transcriptionally active. This process is accompanied by decondensation of chromatin, enlargement of the nucleus and the appearance of nucleoli. Transcription and replication of these nuclei are activated. The enlargement of the chicken erythrocyte nucleus is due to a massive, but selective, uptake of mammalian nuclear proteins, including RNA polymerases. Histone H5 is partially lost from the chicken erythrocyte nucleus and partially taken up by the mammalian nucleus in the heterokaryon (Ringertz et al., 1985). Histones H2A and H2B also exchange under these circumstances, but histones H3 and H4 do not. These results might be expected, considering the relative affinity of the histones for DNA and their organization in the nucleosome (Pruss et al., 1995). This reorganization is independent of replication. Clearly, therefore, chromosome structure is quite dynamic, and some histones (H1, H2A, H2B) continually exchange with a free pool of proteins in the cytoplasm.

The stability of DNA methylation states has also been explored in heterokaryons. Cell fusion between mouse germ cells from female embryos and somatic thymic lymphocytes induces reprogramming of gene expression in which many lymphocyte specific genes are silenced. More interestingly, there are striking changes in methylation in the somatic nucleus, in which several imprinted and non-imprinted genes were demethylated. These changes in methylation status are heritable and lead to the reactivation of at least one normally maternally silent gene in the somatic nucleus (Tada et al., 1997). Thus the embryonic germ cell can impose the embryonic pattern of methylation on a somatic cell. Comparable events might occur in somatic nuclei transplanted into mammalian eggs.

OUTLOOK

The success of amphibian and mammalian cloning through the transfer of somatic nuclei into eggs or oocytes comes at a time when considerable progress has been made in our understanding of gene expression. Regulated changes in the nucleoprotein organization of genes, as reflected in chromatin and chromosomal structure and function, have been found to contribute to the developmental control of differential gene expression. Molecular chaperones and machines that can reverse these differentiative processes are being defined. Nucleoplasmin will remove somatic linker histones from somatic nuclei; given that these linker histones selectively repress genes in the developing embryo, their removal should facilitate reversion from the differentiated state to pluripotency and perhaps totipotency. This possibility is currently being tested. Such a simple approach is unlikely to resolve all of the complex issues concerning the cell biology of the somatic cell

nuclei chosen for nuclear transplantation. However, the choice of non-dividing cells will allow limitation of chromosome damage due to failed replication or mitotic events, and will not impede chromatin remodeling, because chaperones such as nucleoplasmin prefer to interact with the H1^o found in quiescent cells (G₀).

Research on transcriptional control has also uncovered an impressive repertoire of chromatin-remodeling engines. Most investigators have focused on gene-specific control of transcription by these regulatory complexes. However, the large stores of these complexes present in vertebrate eggs probably also drive a genome-wide remodeling process that facilitates both the exclusion and inclusion of chromatin components and regulatory transcription factors. Understanding this type of active remodeling and the possibility of selectively regulating the remodeling process could open numerous avenues towards allowing quiescent or senescent cells to reacquire useful functions in a differentiated organism.

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