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Protein Targeting to Subnuclear Higher Order Structures: A New Level of Regulation and Coordination of Nuclear Processes

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Abstract Though there are no separating membranes within the nucleus, different factors are often concentrated at sites where their respective function is required, a phenomenum referred to as functional organization of the nucleus. How is then this organization achieved and how are the different metabolic processes integrated in the nucleus? One emerging principle was revealed by the identification of protein domains that, though not involved in catalysis, regulate enzyme activity at a higher order level by targeting enzymes to the right place at the right time. These targeting sequences constitute an assembly code for nuclear 'protein factories,' which ensure the extremely high efficiency and accuracy needed in a complex and competitive environment as the living mammalian cell. J. Cell. Biochem. 70:222–230, 1998. © 1998 Wiley-Liss, Inc.

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For over a century, cytological studies had identified compartments within the interphase eukaryotic nucleus, the most notable example being the nucleolus. These morphological observations had to wait till the 1960s and the development of labeling and detection techniques to be finally linked to a function in rRNA metabolism and ribosome biogenesis [Granboulan and Granboulan, 1965].

In the following decades, a combination of electron microscopy, fluorescence detection of proteins and incorporated nucleotides, and in situ hybridization techniques allowed the identification of several other compartments in the nucleus. These analyses led to the assignment of particular biochemical processes to some of these subnuclear compartments. Among the best studied are: the speckled compartment which is proposed to play a role in pre-mRNA splicing and it is composed of a network of interchromatin granules and perichromatin fibrils [reviewed in Spector, 1993; Xing and Lawrence, 1993]; the replication foci which are visible in the S phase of the cell cycle and are the sites within the nucleus where DNA synthesis occurs [reviewed in Berezney et al., 1995; Hozak and Cook, 1994]; and the nucleolus [reviewed in Scheer and Weisenberger, 1994].

Concomitantly, the notion of an underlying skeletal framework in the nucleus was materialized in the mid-70s by the identification of a nuclear matrix composed of 98% protein (essentially free of histones), 1.2% RNA, 0.1% DNA, and 0.5% phospholipids [Berezney and Coffey, 1974]. These results were obtained by sequential extraction of isolated nuclei from regenerating rat liver with salts, detergents, and enzymatic treatments (removing essentially all the chromatin, DNA, RNA, and phospholipids) followed by biochemical analyses and electron microscopy. The remaining nuclear protein matrix maintains the spherical shape of the nucleus and extends from the nuclear envelope to the interior of the nucleus forming a network which connects to the nucleolus. Furthermore, newly synthesized DNA was found to be mostly associated with this protein matrix [Berezney and Coffey, 1975] suggesting an important role of this matrix in DNA replication.

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In the years to follow and throughout the next decade, the work on a skeletal framework within the nucleus was hampered by the argumentation that these substructures were merely artefacts generated during the unphysiological isolation conditions. In fact, that was a very old controversy that can be traced back to the end of the last century. At those days, a net-like framework in the nucleus had already been observed but only under conditions causing "coagulation" [E.B. Wilson cell biology review quoted and discussed in Cook, 1988]. Two major technical developments in the last decade contributed to the increasing acceptance of the concept of a nucleoskeleton nowadays. On one hand, the use of electron microscopy techniques providing increased depth (whole mount and thick resinless sections) thereby allowing the visualization of this filamentous structure in three dimensions [Capco et al., 1982; Fey et al., 1986; He et al., 1990; reviewed in Nickerson et al., 1995]. On the other hand, the use of isotonic salt conditions avoiding the aggregation problems by first encapsulating cells in agarose microbeads followed by lysis of the cells and enzymatic or other treatments [Jackson and Cook, 1985]. The combined use of these two advances permitted the recognition of a nucleoskeleton composed of filaments with the characteristics of intermediate filaments [Jackson and Cook, 1988].

Though a passive role of the nuclear matrix in organizing chromatin loops became generally accepted already in the 80s, a functional role of this framework in the major nuclear processes (transcription, mRNA splicing, DNA replication, DNA methylation, and DNA repair) has been, until recently, ignored. One of the reasons stems from the development of cell-free systems for replication, repair, transcription, and splicing which give credit to the teleological argument that attachment is not required for any of these metabolic processes to occur. Though it is true that soluble systems do work, there are several lines of evidence that this is not the situation in vivo. First, the polymerizing efficiency of even the best of this systems is orders of magnitude lower than the in vivo rates [reviewed in Cook, 1988]. Second, after nucleoskeleton preparation by the combined method mentioned above followed by digestion and removal of most of the unattached chromatin, most polymerizing activity is retained at these skeleton [reviewed in Jackson and Cook, 1995]. Third, pulse labeling using nucleotide analogs followed by detection of the incorporated nucleotides by fluorescence microscopy shows that these different processes occur at discrete sites within the nucleus [reviewed in Leonhardt and Cardoso, 1995; Spector, 1993]. Fourth, several of the enzymes involved in these processes show also a discrete subnuclear pattern and have been found to be associated with the different compartments in a dynamic mode [reviewed in Leonhardt and Cardoso, 1995]. From these observations stems the concept of "functional organization of the nucleus."

Functional Organization of the Nucleus

The functional organization or architecture of the nucleus is based on the dynamic interplay of nucleic acids and protein components. The notion that the nucleus is a relatively unstructured subcellular compartment and that besides the chromatin most nuclear proteins are floating in the nucleoplasm has in the last years finally declined.

In Figure 1, we show, in a diagram, the interdependency of major nuclear processes and their interrelations with the genome and nuclear architecture. The three activities depicted, methylation, replication, and transcription, change in a concerted way during development and in disease. Approximately 50–80% of all CpG dinucleotides in both strands of the mammalian genome are methylated and the pattern of methylated sites is precisely propagated over cell division cycles. Each time DNA is replicated during the cell cycle, an unmethylated strand is generated that has to be recognized and methylated by DNA methyltransferase (DNA MTase) requiring tight coordination of DNA replication and DNA methylation [reviewed in Leonhardt and Bestor, 1993]. This epigenetic process is essential in mammalian development since even partial inactivation of DNA MTase causes embryonic lethality [Li et al., 1992]. On the other hand, there are literally hundreds of reports correlating DNA methylation and transcription, with highly methylated regions being poorly transcribed and, in many cases, corresponding to heterochromatin. As a corollary, changes in the methylation of promoter regions have been found in many tumors and have similar effect on shutting off gene expression as inactivating genetic mutations [reviewed in Cardoso and Leonhardt, in press]. Also replicational timing is dependent

on chromosomal localization and gene activity, with actively transcribed genes usually replicated earlier in S phase and inactive ones later [Calza et al., 1984; Hatton et al., 1988]. At the same time, all these metabolic processes are dependent on and also influence the structural organization of the genome. For example, transcribed genes, in contrast to inactive genes, are preferentially associated with subnuclear compartments enriched in splicing factors (speckled compartment) and transcription and splicing of these genes occurs at the borders of the speckles [Xing et al., 1995]. Also, highly methylated chromatin, such as centromeric heterochromatin, replicates late in S phase [Leonhardt et al., 1992]. Finally, as discussed above, all these processes and components are integrated into a structural nuclear framework.

How is nuclear metabolism or the corresponding metabolic enzymes and regulatory factors organized? In the previous section, we argued that the major metabolic processes in the nucleus, though not separated by membranes, are organized in discrete subnuclear domains attached to an underlying structure which provides the active sites where enzymes and nucleic acids function. These arguments were born from immunofluorescence and immunoelectron microscopy analyses of the localization of nuclear factors together with their respective metabolic compartments visualized by detection of incorporated nucleotides or in situ hybridization techniques. In that way, several proteins directly or indirectly involved in the different biochemical processes were localized to the respective compartments providing several interesting clues about the organization and regulation of these structures [reviewed in Leonhardt and Cardoso, 1995]. In the case of replication foci, these proteins include not only replication factors (PCNA, DNA polymerase α, replication protein A and DNA ligase I) but also other proteins

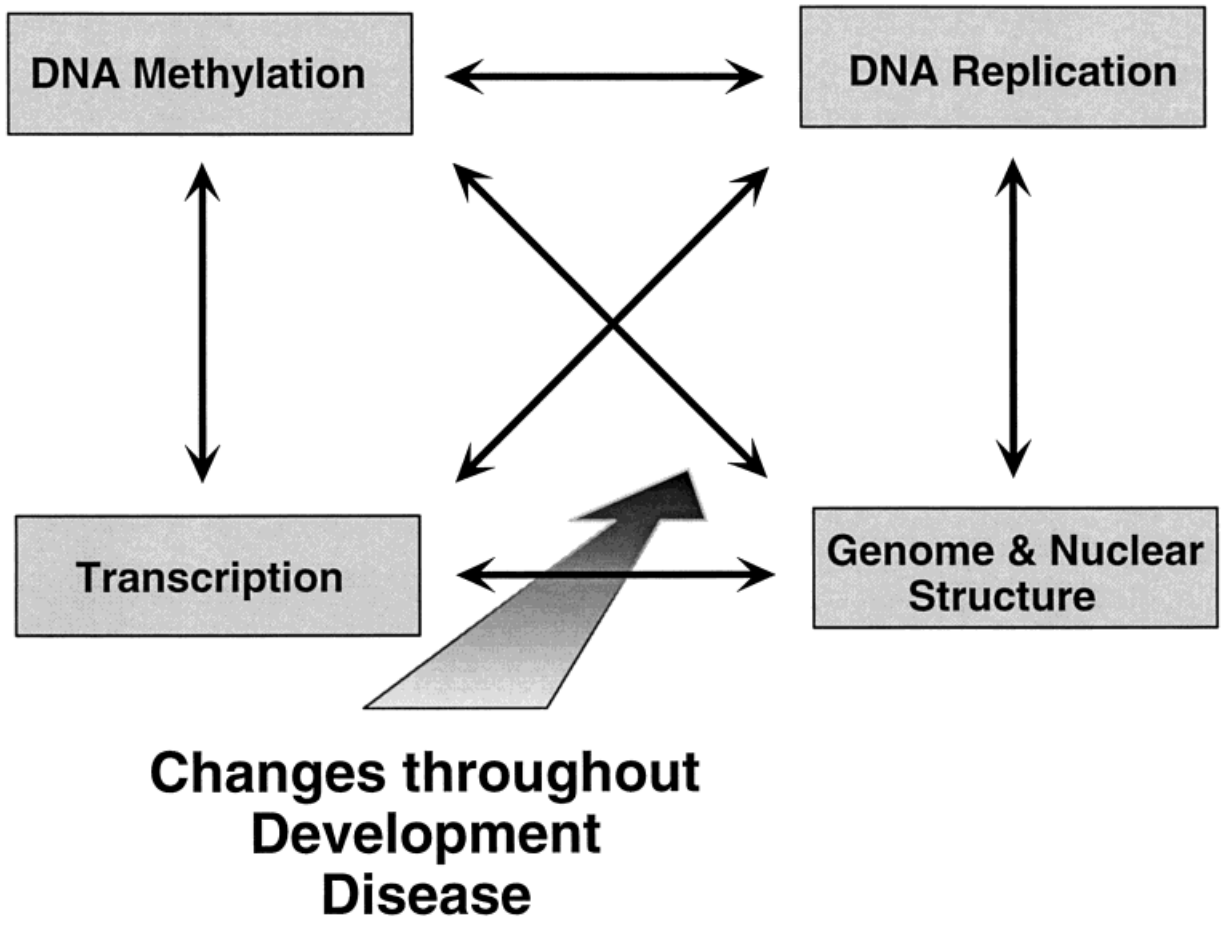

Fig. 1. Interrelations between different biochemical processes taking place in the nucleus and genome & nuclear structure. All these activities and structures change in an interdependent fashion during development and in disease.

which are not directly involved in DNA replication, such as the cell cycle regulators cyclin A and cdk2 [Cardoso et al., 1993; Sobczak-Thepot et al., 1993] and DNA MTase which works postreplicatively [Leonhardt et al., 1992]. In the case of the speckled compartment, in addition to small nuclear ribonucleoprotein particle (snRNP) components also non-snRNP splicing factors (e.g., SC35 and SF2/ASF) and, most interesting, nuclear matrix antigens have been identified at these nuclear speckles providing a direct link between nuclear matrix and RNA processing [reviewed in Leonhardt and Cardoso, 1995]. In the case of the nucleolus, its identification dates back to the past century and its ultrastructure has been relatively well studied. Correspondingly, a vast array of factors have been localized to it and, in some cases, information on their subnucleolar localization is also available. The list includes, besides ribosomal proteins and RNAs, RNA polymerase I and auxiliary factors, topoisomerases, rRNA processing factors, nucleolar-cytoplasmic shuttling proteins, and viral proteins [reviewed in Leonhardt and Cardoso, 1995].

Is this organization also present in living cells or are small changes in local concentration of a protein enhanced or distorted by the detection protocols? Are these compartments stable or do they change or even dissociate in a dynamic way during the cell cycle? In Figure 2, we present, in an abstract form, the two opposite views of nuclear architecture, that is to say: random diffusion versus functional domains. The former depicts a nucleus where the different groups of factors are soluble and move randomly within the nucleus coming stochastically into contact with their targets or substrates. The latter shows a nucleus in which each group of factors involved in a particular process is concentrated in a discrete compartment within the nucleus where their function is required. Are these two principles mutually exclusive or can one and the same protein be found in the both states? The evidence presented in the previous section argues against soluble and freely diffusing factors being responsible for any of the major nuclear metabolic processes. An arrangement based on random diffusion and random encounters is hard to imagine as sufficient for a complex and competitive environment as the mammalian nucleus. Indeed, the list of proteins found to be concentrated in functional compartments of the nucleus is getting longer every year. On the other hand, for example DNA replication happens only during S phase of the cell cycle. One can envisage that during the other phases at least some of the replication associated factors are randomly distributed throughout the nucleoplasm and just before S phase assemble into multiprotein assembly lines or "replication factories" (a term coined by the group of P.R. Cook) and become activated. These factories would be organized at the nuclear skeleton where the chromatin loops and metabolic complexes would be integrated. This dynamic changes between soluble and insoluble states (attachment or dettachment from the matrix) should then be a higher level of coordinating nuclear functions in a spatial and temporal order. Furthermore, depending of the metabolic pathway, at the same time some groups of factors could be randomly distributed throughout the nucleoplasm while others could be organized into functional domains. Until recently, however, it was not possible to directly study the temporal order of these events.

A true revolution in the way we can visualize proteins in living cells happened in the last years with the cloning and characterization of the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria*. Fusions between the protein of interest and GFP can be engineered and introduced into mammalian cells and their localization directly followed in living cells by fluorescence microscopy. In Figure 2, we show the patterns of nuclear distribution of such a translational fusion between GFP and a replication associated factor (DNA ligase I) in living mouse cells. DNA ligase I is the enzyme responsible for ligation of the Okasaki fragments during lagging strand DNA synthesis. The left nucleus in the micrograph shows a dispersed distribution throughout the nucleoplasm and exclusion from the nucleoli which corresponds to a non-S phase stage of the cell cycle. This pattern corresponds in our abstract view to the random diffusion state when DNA ligase I is not active in DNA replication. The other nuclei in the micrograph show a discrete pattern of multiple small (middle nucleus) or few larger (right nucleus) foci which are visualized during S phase and correspond to the association of DNA ligase I to replication factories where it exerts its function [Cardoso et al., 1997]. These discrete compartments within the nucleus would correspond to the functional domains in our abstract view. Recently, the speckled compart-

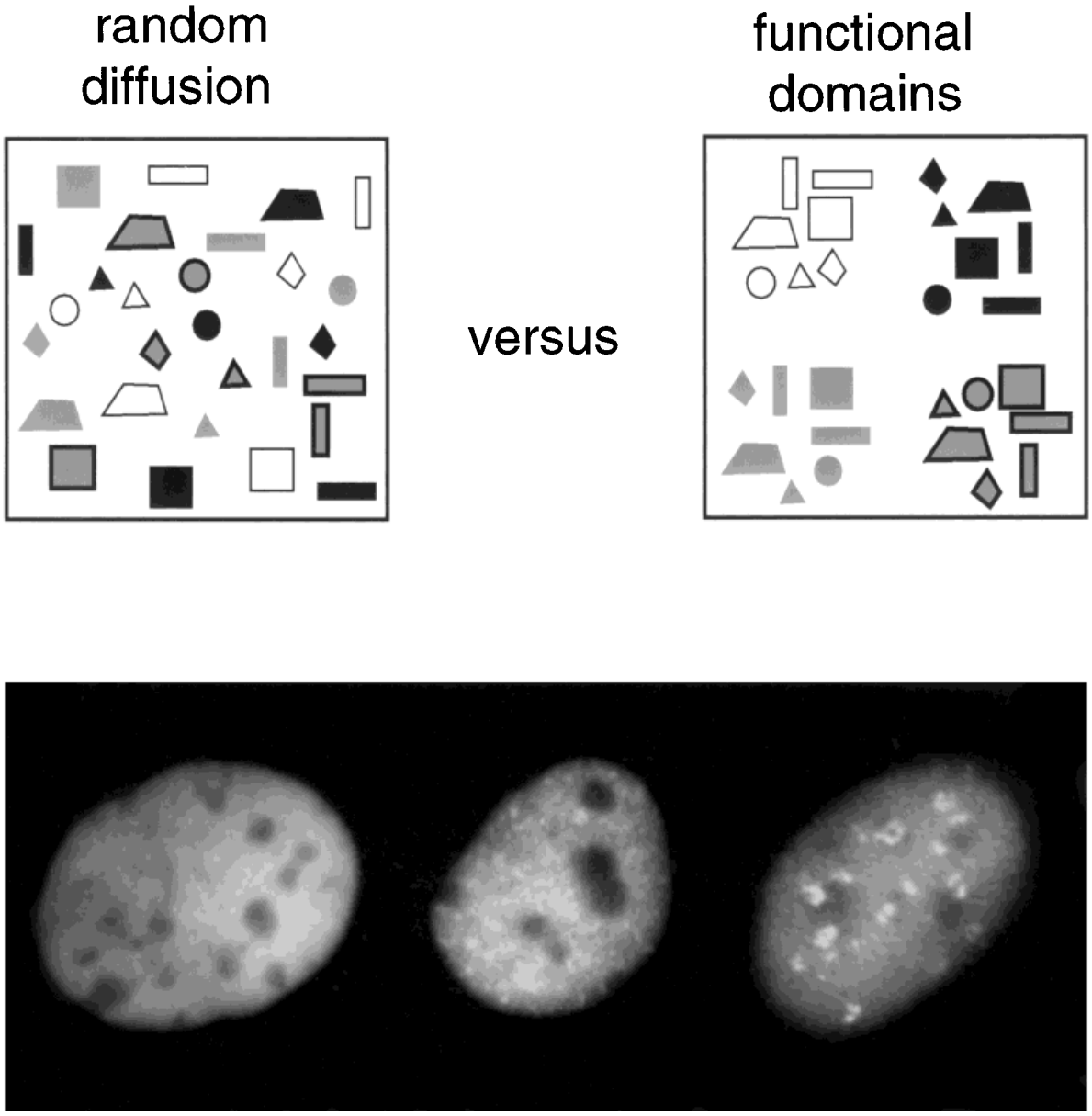

Fig. 2. Two possible models of nuclear organization. **Left**: Different nuclear factors are soluble and 'randomly diffuse' within the nucleus coming stochastically into contact with their targets or substrates. **Right**: The different nuclear factors are organized in 'functional domains' meaning that factors involved in a particular biochemical process are concentrated together in the distinct subnuclear compartment where their respective function is needed. These two models are not mutually exclusive since one and the same protein can exist in both states. Association or targeting to a specific subnuclear domain controls the formation and activation of multiprotein complexes and couples them to the chromatin. Disassembly would then mean deactivation of these complexes into individual soluble components which no longer carry out their function. This is better illustrated by the nuclear distribution of a replication enzyme (DNA ligase I) in living cells, shown in the panel of micrographs below. This panel depicts the cell cycle dependent redistribution of DNA ligase I to replication sites within the nucleus of living cells. The different subnuclear patterns of human DNA ligase I expressed in murine cells are visualized here by fusion with the green fluorescent protein. On the left side, dispersed distribution in a non S phase nucleus. The middle and right hand side nuclei show a very distinct organization of the DNA ligase I fusion protein into foci with very characteristic shapes corresponding to sites where DNA replication is taking place.

ment was also visualized in living cells and individual speckles were shown to form continuously changing patterns of foldings and extensions [Misteli et al., 1997]. These movements occurred throughout the cell cycle and responded to transcriptional activation of nearby genes but were severely disrupted by inhibition of either RNA polymerase or kinase activity. This novel technique provides the best illustration of the dynamic functional organization of the nucleus and avoids all potential artefacts derived from fixation and staining protocols. A cautionary note is mandatory at this point, one should pay attention to granular patterns which can sometimes be seen when a particular protein is highly overproduced, e.g., overexpression in the COS cell system. In addition to spatial resolution (limited only by the resolution of the light microscope) this exciting strategy provides us, for the first time, with the temporal resolution. One can in fact analyze the dynamics of these processes in 4 dimensions in living cells. In that sense, an enormous potential lies in the use and improvement of multiple, spectrally distinguishable GFP fusion proteins to visualize, e.g., different components of the replication machinery, to determine their order of assembly, and to follow them throughout the cell cycle or during differentiation.

Protein Targeting to Subnuclear Higher Order Structures

The molecular mechanisms underlying the formation of these functional factories are largely unknown. Since 1984 with the identification of the first nuclear localization sequence in the simian virus T antigen protein [Kalderon et al., 1984], a lot of information was gathered on how proteins enter the nucleus throught the nuclear pores. Unfortunately, most research on intracellular protein sorting actually ends at this point. The realization that the nucleus is highly organized and that probably quite different principles apply in view of the absence of separating membranes is only recently becoming accepted. How is then this organization achieved? One possibility would be that enzymes accumulate where their respective substrate concentration is higher. Which would then open the question how is the DNA or RNA organized within the nucleus providing this differential distribution of their respective metabolic enzymes. Furthermore, how does it change over the cell cycle and during differentiation? Another possibility would be that specific protein domains, independent of catalysis and therefore of local substrate concentration, "target" the respective protein to their places in the assembly-line. Obviously, that does not rule out that RNA and DNA (or better ribonucleoproteins particles (RNP) and chromatin) form separate compartments or contribute to the formation of these factories. The targeting sequences would mediate assembly of these factories and would therefore constitute the molecular determinants of a new mode of regulation of nuclear metabolism and enzyme kinetics at a higher order level. These targeting sequences would function as a module, i.e., translational fusions would be sufficient to target heterologous proteins to the particular compartment.

The first indications came from studies mapping the molecular determinants responsible for the recruitment of key regulatory proteins of human retroviruses to the nucleolus [Hatanaka, 1990]. These turned out to be clusters of basic amino acids which also mediate nuclear import. Similar work on cellular nucleolar proteins was somehow not so successful in the sense that in many nucleolar proteins several domains seemed to be required for nucleolar localization [reviewed in Leonhardt and Cardoso, 1995].

Subsequent studies mapped the domains responsible for directing two *Drosophila* splicing regulators, *transformer (tra)*, and *suppressor-of-white-apricot (su[w^a])*, to the speckled compartment to an about 120 amino acid arginine/serine-rich (RS) domain [Li and Bingham, 1991]. This sequence works across species since the mapping experiments were performed in mammalian cells and it seems to be dispensible for enzyme activity but required for efficiency. Another RNP-containing compartment in the nucleus are the coiled bodies [reviewed in Lamond and Carmo-Fonseca, 1993]). Though the function of this compartment is unclear some of its components start to be elucidated. A major component, coilin, contains a 102-amino acid N-terminal domain which is necessary and sufficient for localization at the sphere organelles which appear to be the amphibian homologues of coiled bodies [Wu et al., 1994].

The first step towards the molecular dissection of "replication factories" was the identification of a domain in the regulatory region of DNA MTase which is necessary and sufficient to control association with subnuclear sites of

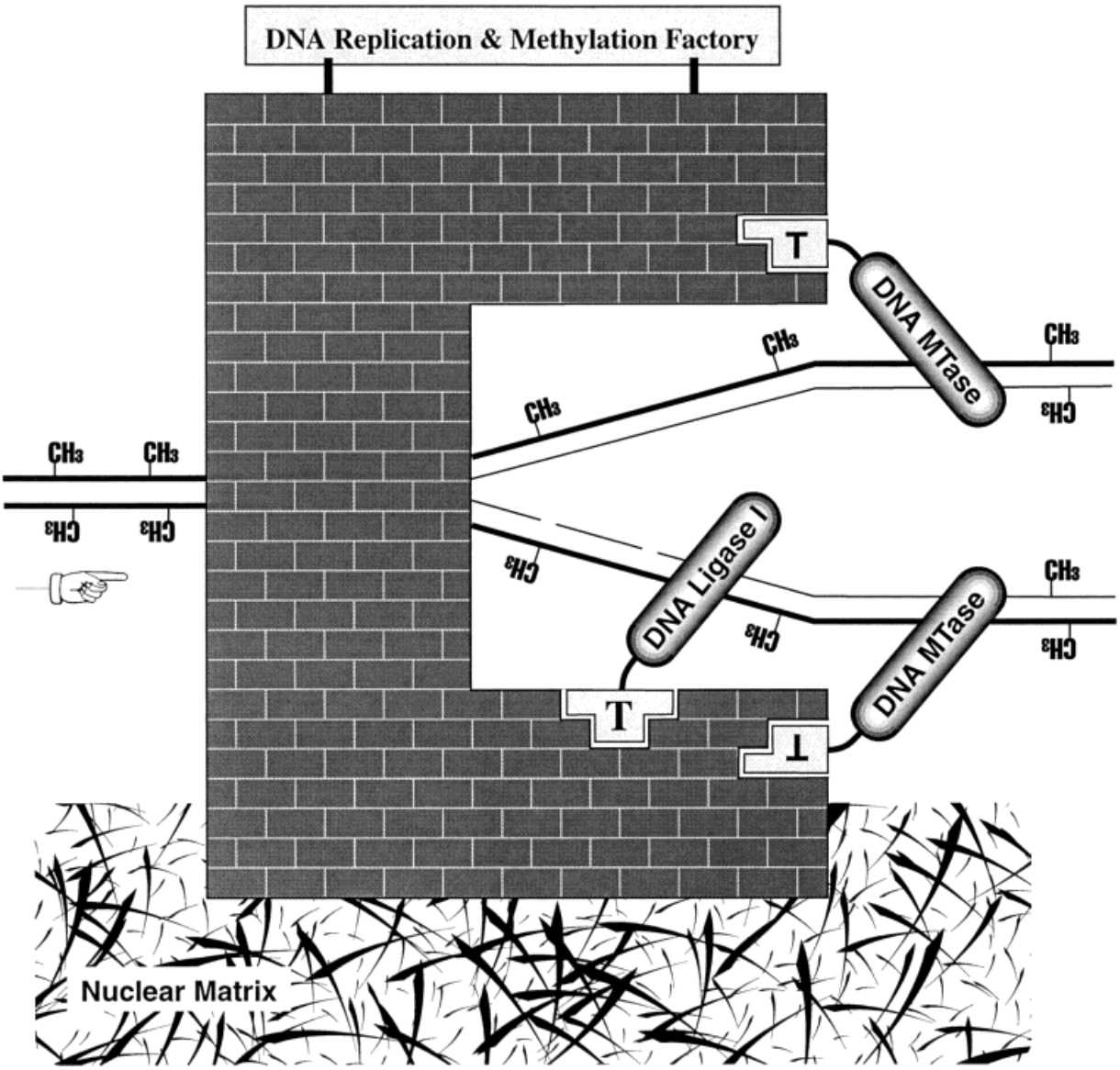

Fig. 3. Model of targeting to a DNA replication and methylation factory attached to the nuclear matrix. The DNA is reeled through the factory where the necessary enzymes and auxiliary factors are organized in an assembly line like mode. Tethering of DNA ligase I and DNA MTase via their targeting sequences to the respective sites in these factories guarantees that all Okasaki fragments generated during lagging strand DNA synthesis are ligated and all methyl (CH3) groups are added at hemimethylated sites in the newly synthesized strand with high accuracy and efficiency. Targeting sequences are shown as separate domains since in both cases they are protease sensitive domains, that are necessary and sufficient for localization at replication foci and are not required for catalysis in vitro. Targeting of enzymes to the right place at the right time during the cell cycle constitutes a higher order level of regulation of enzyme activity in the living cell nucleus.

DNA replication during S phase [Leonhardt et al., 1992]. The close association of DNA MTase with replication sites, should remind us that the exact duplication of the genome requires also the duplication of the epigenetic features such as DNA methylation. A better designation of these factories would thus be "DNA replication and methylation factories." In Figure 3, we depict how the DNA MTase targeting sequence could function to couple these two biochemical processes and, thereby, ensure the accurate maintenance of genomic methylation patterns over many cell generations. Errors in this process can lead to gene inactivation and consequently to disease as we discussed in the previous section and in Figure 1. This sequence is therefore a good target for regulatory factors controlling DNA methylation.

More recently, we have identified a functionally similar targeting sequence in the amino terminus of DNA ligase I [Cardoso et al., 1997], which is the enzyme responsible for Okasaki fragment ligation during lagging strand DNA synthesis. Mistakes in the ligation of Okasaki fragments would lead to genome instability. The DNA MTase and the DNA ligase I targeting sequences, though both controlling the association with DNA replication sites during S phase, do not show any significant sequence homology. That suggests that they bind to different components at these sites as illustrated in our model in Figure 3 and play a regulatory role in the coordination of both processes as one processive replication-methylation machinery working like an assembly line. Both these sequences conform to the definition of a subcellular targeting sequence that is to say a protein sequence that i) is necessary and ii) sufficient for subcellular localization, iii) works position independently, iv) is separated from the catalytic domain, and v) can target heterologous proteins to the respective subcellular domain.

In evolutionary terms, the DNA ligase I targeting sequence is conserved among vertebrates but not in the lower eukaryotic counterparts, suggesting an important function that has recently developed in evolution. With increasing genome complexity the demands on the performance of these enzymes also increased and would require more efficient and better controlled systems, with rapid assembly and disassembly of these active complexes. The targeting sequences, serving as an assembly code for the complex nuclear architecture and constituting an additional level of regulation, would therefore provide a selective advantage.

With the advent of techniques allowing us to visualize these compartments in vivo, the next years should witness analyses of the four dimensional organization of chromatin and enzymes involved in DNA and RNA metabolism during cell cycle progression and differentiation in living cells. The molecular dissection of the assembly code (targeting sequences) of these compartments and the regulatory role of the sequences that mediate this process is already being actively pursued. As depicted in Figure 3, there are still many 'bricks' of the 'replication and methylation factory' to be identified and their spatiotemporal relationships to be elucidated. Together with similar studies on the other subnuclear compartments, this work will advance our understanding of the regulation of nuclear processes.

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