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PROSPECT

New Insights into the Mechanisms of Nuclear Segmentation in Human Neutrophils

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Abstract During human neutrophil differentiation, large portions of the genome condense and associate with the nuclear envelope to form filament-like structures. As a result, the nucleus of the mature neutrophil typically consists of a linear array of three or four lobes joined by thin, DNA-containing filaments. Despite the medical significance of neutrophil nuclear morphology, little is known about the events regulating neutrophil nuclear differentiation and its pathological states. This work presents a new model of the mechanisms governing nuclear filament formation in human neutrophils. This model is based on recent chromosome mapping studies in human neutrophils and on studies of genetic and pathological conditions affecting neutrophil nuclear shape. According to this model, filament assembly is initiated by factors that interact with specific regions of the genome in a hierarchical and dose-dependent manner. In this regard, the strategies governing the molecular interactions responsible for filament formation appear to resemble those involved in transcriptional silencing, a phenomenon that also affects the properties of extended chromosomal regions. According to the silencing paradigm, bound filament control Factors must recruit additional Filament Foehn factors which spread along adjacent DNA to mediate filament formation. A better understanding of the factors that shape the neutrophil nucleus may lead to new clinical tools for the diagnosis and manipulation of abnormal neutrophil differentiation. J. Cell. Biochem. 73:1–10, 1999. © 1999 Wiley-Liss, Inc.

Key words: nuclear filaments; nuclear maturation; neutrophil cell differentiation; transcriptional silencing; megaloblastic anemia; Pelger-Huët anomaly

Eukaryotic cells have mechanisms that downregulate the genetic activity of chromosomal domains ten to thousands of kilobases long. Examples include X-chromosome inactivation [Rastan, 1994], transcriptional silencing [Pirrotta, 1997, 1998; Grunstein, 1998], downregulated recombination activity of chromosome III arms in yeast [Wu and Haber, 1996], and establishment of late replication domains in eukaryotic chromosomes [Friedman et al., 1996]. In each of these instances, small DNA elements (termed X-chromosome inactivation center, silencers, recombination control elements, late replication control sequences) determine the availability and function of chromosomal DNA over large distances. The best-understood long-range chromosomal event is transcriptional silencing in the yeast *Saccharomyces cerevisiae* [reviewed by Sherman and Pillus, 1997; Grunstein, 1998]. Transcriptional silencing occurs via a two-step nucleation/spreading mechanism. DNA-binding proteins first target specific regions of the genome for silencing. The bound factors then act as "seeds" for binding silencing complexes which proceed to polymerize cooperatively along the chromatin fiber over a distance of several kilobases. The polymerized silencing complexes form a heterochromatin-like structure that hinders access of the transcriptional machinery to DNA. Transcriptional silencing in *Drosophila* seems to follow a similar nucleation/spreading mechanism, but the specific molecular details are not as well understood [reviewed in Pirrotta, 1997, 1998; see also Strutt et al., 1997].

Human neutrophils exhibit another example of a long-range chromosomal event that has profound effects on nuclear morphology. Neutrophils are white blood cells that serve as the body's first line of defense against invading bacteria and fungi. During neutrophil differentiation, most chromatin condenses into heterochromatin; some of these heterochromatic re-

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gions form filament-like structures. As a result, the mature neutrophil nucleus typically consists of a linear array of three or four pyknotic lobes joined by thin chromatin filaments (Fig. 1).

Neutrophil nuclear segmentation is also a clinically important phenomenon because variations in neutrophil nuclear morphology serve as useful diagnostic indicators for an array of pathological and genetic conditions (Fig. 2). These conditions fall into three categories. The first category consists of conditions in which neutrophil nuclear maturation does not take place. Each year, thousands of people are diagnosed with various forms of myelocytic leukemias, diseases in which normal differentiation of neutrophils is arrested. The nuclei of these cells fail to condense and segment and the cells do not exit the cell cycle (Fig. 2b). The second category includes conditions in which neutrophils are released from the bone marrow before completing nuclear maturation. This occurs during chronic bacterial infections (when the demand for neutrophils is high) or at times of bone marrow stress. In these cases, afflicted individuals exhibit increased numbers of circulating neutrophils with indented nuclei (band cells; Fig. 2c). The third category consists of cases of abnormal neutrophil nuclear maturation, as it occurs in megaloblastic anemias or certain genetic conditions (Pelger-Huët anomaly). In these cases, afflicted individuals exhibit increased numbers of circulating neutrophils with either hypersegmented nuclei (megaloblastic anemias; Fig. 2d) or hyposegmented nuclei (Pelger-Huët anomaly; Fig. 2e).

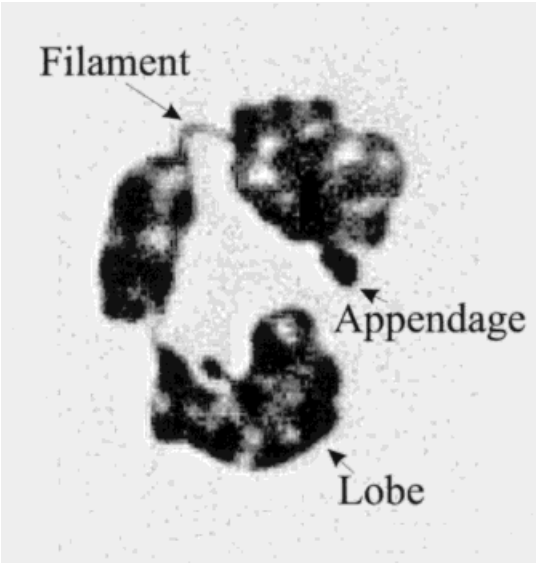

Fig. 1. Structure of a typical neutrophil nucleus from a blood smear after methanol fixation and DNA staining with 4,6-diamidino-2-phenylindole (DAPI). This image was obtained by collecting optical slices of a neutrophil nucleus at 0.25-μm intervals along the Z-plane and then processing and integrating the resulting images using the CELLscan software (Scanalytics, CSPI Inc., Billerica MA; see Sanchez et al. [1997] for a description of this image analysis software). Note the organization of the nucleus into filaments and lobes, the extent of chromatin condensation and heterochromatization. Scale bar = 3 μm.

This work describes new insights into the mechanisms of filament formation in differentiating human neutrophil nuclei based on our research [Sanchez et al., 1997], the classic literature on neutrophil nuclear shape, and recent advances in the study of chromosomal inactivation over large distances. We first examine current models of transcriptional silencing and then describe nuclear morphogenesis during neutrophil cell differentiation. We then discuss the relationship between filaments and specific chromosomes and review the work on genetic and pathological conditions that alter neutrophil nuclear morphogenesis. Consideration of these phenomena suggests a two-step model of filament formation that resembles the prevailing view of the mechanisms of transcriptional silencing. According to this model, chromatin condensation during filament formation is initiated through specific DNA-protein interactions at particular chromosomal locations. Once the initiator protein is bound to DNA, a cooperative process of protein-protein and protein-nuclear envelope interactions functions to expand the region of condensed chromatin and package it into a filamentous structure.

Transcriptional Silencing in Budding Yeast and Other Organisms

In *Saccharomyces cerevisiae*, a multiprotein complex mediates silencing at the inactive mating type loci and at the telomere-proximal genes. At the silent mating type (HM) loci, the proteins Rap1p, Abf1p, and the origin recognition complex (ORC) first bind to silencer sequences and then recruit a multisubunit Sir protein complex consisting of Sir2p, Sir3p, and Sir4p. After this nucleation step, the silencing effect propagates along the chromatin fiber via cooperative binding of Sir complexes and via interaction between Sir complexes and the N-terminal tails of histones H3 and H4 in nucleosomes. The resulting Sir/histones complexes restrict access of the transcriptional machinery to the DNA. Under normal conditions, transcriptional

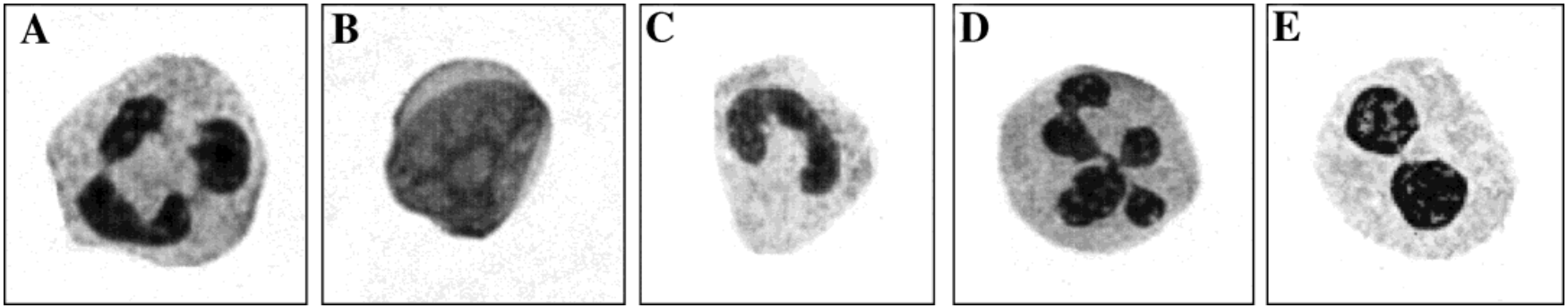

Fig. 2. Variations in neutrophil nuclear morphology associated with various clinical and genetic conditions. **A:** Typical circulating neutrophil in a healthy individual. **B:** Example of failed nuclear segmentation. Neutrophilic myeloblast found in the blood of an individual afflicted with acute myeloblastic leukemia (AML). **C:** Example of premature release of neutrophil precursor cells into the bloodstream before completion of nuclear segmentation. Band cell in circulating blood (see text for details). **D:** Example of abnormal nuclear segmentation: hypersegmented neutrophil from a megaloblastic anemia patient (see text for details). **E:** Example of abnormal nuclear segmentation. Hyposegmented neutrophil from a heterozygous Pelger-Huët individual (see text for details). These images correspond to Giemsa-stained blood smears obtained from http://www.wadsworth.org/chemheme/heme/microscope/celllist.htm.

repression spreads 2–3 kilobase pairs (kbp) from the nucleation site. Silencing at telomere proximal regions follows a similar pattern once Rap1p binds telomeric TG_{1-3} repeats [Hecht et al., 1995, 1996; Marcand et al., 1996; Triolo et al., 1996; Strahl-Bolsinger et al., 1997].

Silencing efficiency is determined in part by competition among loci for binding limiting amounts of interacting factors. As a result, silencing is both hierarchical and dose dependent. Thus, the greater affinity of Rap1p for silencers at the mating type loci causes these loci to be more tightly repressed than subtelomeric regions [reviewed in Sherman and Pillus, 1997]. Overexpression of Sir complex components extends the spreading of the silencing effect over a distance of 15–20 kilobases (kb) from the original nucleation site [Renauld et al., 1993].

Silencing in other model systems involves similar nucleation/spreading strategies with a few variations. X-chromosome inactivation starts from a single region on the X chromosome known as the X-inactivation center (Xic). The initiation event involves the synthesis and stabilization of Xist RNA encoded in the Xic. Xist RNA is not transcribed into protein, instead, Xist RNA acts *in cis* and coats the entire X-chromosome. This observation suggests that spreading of X-inactivation involves the recruitment of protein factors by the chromatin-bound Xist RNA [Herzing et al., 1997; Lee et al., 1997; Panning and Jaenisch, 1998]. In *Drosophila*, silencing of homeotic genes requires DNA sequences known as polycomb response elements (PRE). These sequences "seed" the formation of interacting Polycomb-Group (PcG) protein complexes, which spread the silencing effect from the PRE. PREs are several hundred to a few thousand base pairs (bp) in length and consist of smaller sequence elements that have poor silencing activity on their own and lack common sequence motifs. The failure to identify a nucleation factor that binds to the PRE together with the weak DNA binding activity of PcG proteins led to the proposal that PREs correspond to clusters of low-affinity and low-specificity binding sites for several PcG components. Once PcG factors reach a critical mass at the PRE, they would form a stable nucleation complex capable of catalyzing the spreading step [reviewed by Pirrotta, 1997, 1998]. Artificial targeting of PcG-Gal4 fusion proteins to Gal4-DNA binding sites can also lead to PcG-dependent silencing of neighboring reporter genes [Müller, 1995].

The above examples of transcriptional silencing illustrate the characteristics expected for a nucleation/spreading event responsible for altering the properties of extended chromosomal regions. The nucleation factors must (1) target specific regions of the genome; (2) associate with other components of the chromatin-modifying machinery once bound to DNA, and (3) act in a hierarchical and dose-dependent manner provided nucleation factors occur in limiting concentrations and share binding sites of various affinities. Spreading factors must (1) be recruited at nucleation sites; (2) polymerize along adjacent DNA via cooperative interactions; and (3) act in a dose-dependent manner. It should be noted that spreading factors are not necessarily or exclusively proteins (as in the case of the Xist RNA). As discussed below, careful examination of both the classic and recent literature on neutrophils nuclear shape led us to identify a potential nucleation event involved in nuclear filament formation.

Nuclear Segmentation in Neutrophils

The neutrophil lineage originates in the bone marrow as colony-forming unit-granulocyte (CFU-G) progenitor cells give rise to myeloblasts in response to growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). Myeloblasts, in turn, divide and differentiate into promyelocytes, and then into myelocytes. Eventually, myelocytes exit the cell cycle and differentiate into metamyelocytes, band cells, and finally mature neutrophils [Bessis, 1973; Lazlo and Rundles, 1977; Marmont et al., 1988](Fig. 3). Only fully differentiated neutrophils are normally released into the bloodstream, although band cells, and even metamyelocytes, may also appear in the circulation in response to chronic bacterial infections [Athens, 1993]. Mature neutrophils spend 7–10 h in the peripheral blood and then move to sites of infection in the surrounding tissues by attaching to blood vessels and crawling between endothelial cells through *diapedesis*.

The nucleus of the mature neutrophil typically consists of a linear array of three or four distinct lobes joined by filaments (Fig. 1). Filaments are a thin extension of heterochromatic regions ranging in width from 0.3 to 0.5 μm [Sanchez et al., 1997]; they do not appear to

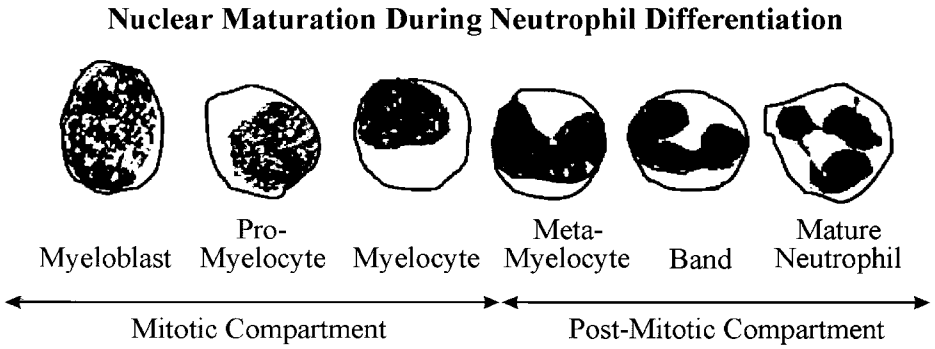

Fig. 3. Nuclear maturation during neutrophil differentiation. This diagram illustrates the process of nuclear maturation in the neutrophil cell lineage in the bone marrow (see text for details).

depend on actin or microtubule-based cytoskeletal elements [Campbell et al., 1995]. Lobes and filaments represent stable domains of the neutrophil interphase nucleus; their number and relative positions in a cell remain fixed during maturation [Campbell, 1995]. Analysis of live neutrophils reveal that nuclear shape can vary considerably in terms of the shape of lobes and the length of filaments [Campbell, 1995]. Such fluidity may facilitate passage of neutrophils between endothelial cells in blood vessels.

The function of nuclear filaments remains uncertain. Changes in nuclear morphology during neutrophil differentiation may be part of a developmental program that permanently shuts off most gene activity during terminal differentiation in this cell type [Jack et al., 1988; Beaulieu et al., 1992]. However, other cell types become genetically quiescent in the absence of nuclear segmentation. Analyses of individuals with defects in neutrophil filament formation demonstrate that nuclear segmentation is not required for normal neutrophil function [Johnson et al., 1980; Matsumoto et al., 1984; see below].

Neutrophils, like other hematopoietic cells, eventually undergo apoptosis. However, nuclear morphology of circulating neutrophils in the bloodstream does not represent an early stage of apoptosis [Payne et al., 1994]. The apoptotic neutrophil nucleus displays even more condensed chromatin (often with margination), lacks a nuclear membrane surrounding some of the chromatin masses, and occasionally exhibits chromatin spurs extending into the cytoplasm and extensive folding of the nuclear envelope [Payne et al., 1994].

Genetic Composition of Filaments

The first clear indication that filaments may contain specific portions of the genome comes from fluorescent *in situ* hybridization (FISH) analysis of the relationship between chromosome location and nuclear morphology in human neutrophils [Sanchez et al., 1997]. These studies demonstrate that chromosomes partition randomly among lobes during nuclear segmentation. As a result, similar lobes in different neutrophils vary in genetic composition. By contrast, the same studies showed that chromosomes do not segregate randomly among filaments. FISH analysis with painting probes from chromosomes X, Y, 2, 18 shows that these probes do not hybridize to filaments, even though filaments are readily stained with a total genomic FISH probe [Sanchez et al., 1997]. These observations indicate that only a subset of chromosomes participate in filament formation.

Additional indirect evidence for chromosome specificity within filaments comes from consideration of the "drumstick" appendages that appear exclusively on nuclear lobes in a fraction of neutrophil nuclei from healthy women [Davidson and Smith, 1954]. Genetic and cytological analyses demonstrate that the drumstick appendage contains the inactive X chromosome [Davidson and Smith, 1954; Tolksdorf, 1974; Hochstenbach et al., 1986; Sanchez et al., 1997](Fig. 4).

Drumstick appendages thus represent the first known case in which a specific chromosome is physically associated with a distinct nuclear structure. The frequency of drumstick formation increases with the extent of nuclear segmentation [Davidson and Smith, 1954], sug-

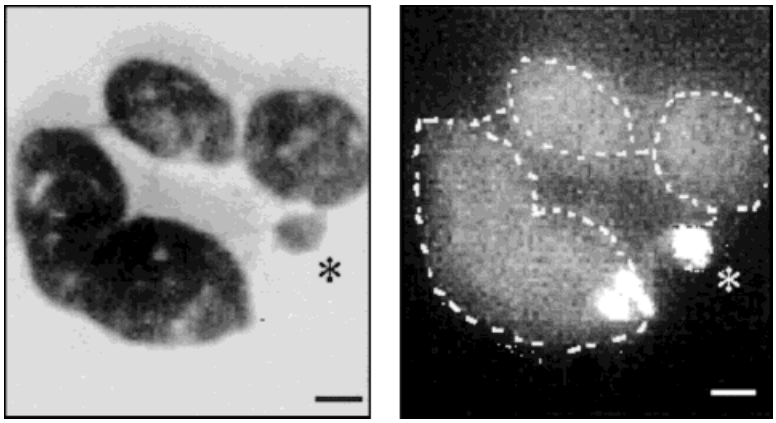

Fig. 4. Localization of X-chromosome sequences in XX female neutrophil nuclei by fluorescent *in situ* hybridization (FISH). Between 1% and 17% of neutrophil nuclei in healthy women (mean 2.6%) display a well-defined, sex-specific nuclear appendage known as a "drumstick" (next to asterisks). FISH analysis using X-chromosome centromeric DNA sequences as a hybridization probe demonstrates the inactive heterochromatic X chromosome resides in the drumstick appendage. In this nucleus, the active X chromosome (identified by its larger hybridization signal) resides in a separate lobe [see Sanchez et al., 1997, for experimental details]. Scale bar = 2.5 μm.

gesting that the same mechanisms that mediate or regulate drumstick formation may also control filament assembly during neutrophil differentiation. These findings, together with the results of the FISH studies described above, indicate that filaments, like drumsticks, contain specific chromosomes.

Insights into the Mechanisms of Filament Formation From Analyses of Pathological and Genetic Conditions Affecting Neutrophil Nuclear Shape

Pelger-Huët anomaly: hereditary hyposegmentation due to a defect in filament formation. Pelger-Huët anomaly is an autosomal dominant disorder of neutrophil filament formation [Pelger, 1928; Huët, 1931]. Individuals heterozygous for this trait have mature neutrophils with "dumbbell," or "pince-nez" -shaped nuclei that exhibit a single filament, instead of the two or three typically found in non-Pelger-Huët cases (Fig. 2e). People and animals with this condition are healthy; the condition usually goes undetected because heterozygous Pelger-Huët anomaly is a nuclear defect that does not affect cytoplasm differentiation critical to cellular function [Johnson et al., 1980; Matsumoto, 1984; Latimer et al., 1985, 1987]. Estimates of the frequency of heterozygous Pelger-Huët individuals in human populations run as high as 0.001% [Wintrobe et al., 1974]. By contrast, homozygous Pelger-Huët individuals have mature neutrophils with no filaments at all. Analysis of bone marrow from these people reveals normal morphological features in all neutrophil precursors up to the myelocytic stage. However, at later stages of cellular maturation, nuclear indentation and filament formation do not take place and the resulting nuclei are round [Bessis, 1973](Table I). The homozygous Pelger-Huët condition is rare and may be lethal [Nachtsheim, 1950; Haverkamp and van Lookeren, 1952; Stobbe and Jorke, 1965; Aznar and Vaya, 1981].

TABLE I. Extent of Nuclear Segmentation in Neutrophils from Normal Individuals, Heterozygous, and Homozygous Pelger-Huët (PH) Cases[a]

	Normal (%)	PH-heterozygous (%)	PH-homozygous (%)
Band (no lobes)	2.8 ± 2.8	31.3 ± 9.2	100
Two lobes	22.0 ± 6.3	63.8 ± 9.5	
Three lobes	54.3 ± 5.3	4.9 ± 3.7	
Four lobes	18.1 ± 6.9	0.3	
Five lobes	2.8 ± 2.1	0.0	

[a]Table was modified from Wintrobe et al. [1974].

Hereditary Pelger-Huët anomaly can be confused with pathological conditions that generate cells with a similar nuclear morphology [Dorr, 1959]. These nonhereditary conditions, known as acquired, or pseudo Pelger-Huët anomalies, are distinct from the hereditary form because only hereditary Pelger-Huët exhibits persistent hyposegmented granulocytes among closely related family members in the absence of severe infections or hematological disease. Acquired Pelger-Huët anomaly is commonly associated with myelodysplastic syndromes and other malignant hemopathies [Kuriyama et al., 1986; Greenberg, 1995].

Collectively, these findings suggest that nuclear segmentation is entirely dependent on a very small number of gene products, possibly one. Since the Pelger-Huët anomaly affects filament number but not filament length we postulate that the gene product or products missing in Pelger-Huët individuals must serve to regulate initiation of filament formation, rather than assembly of the filament itself. Thus, in homozygous Pelger-Huët neutrophils, nuclear segmentation fails because no initiation factor is present to tell the cell to start building one or more filaments. In heterozygous Pelger-Huët neutrophils the amounts of the initiation factor must be so limited that they only support initiation of a single filament, at most. The result is a "dumbbell," or "pince-nez"-shaped nucleus with a single filament of normal length and width. We further postulate that acquired Pelger Huët is due to limited synthesis or diminished function of the same initiation factor for nongenetic reasons. These arguments suggest that the postulated initiation factor acts in a dose-dependent manner.

Megaloblastic anemia and neutrophil nuclear hypersegmentation. Additional evidence for a dose-dependent factor controlling the initiation of filament formation comes from analysis of megaloblastic anemia, a condition accompanied by hypersegmentation of neutrophil nuclei. Relative to healthy individuals, patients with megaloblastic anemias have greater than 5% of their neutrophils exhibiting five or even six lobes [Wintrobe, 1974](Fig. 2d). In fact, excessive filament formation is a diagnostic indicator of megaloblastic anemia and, in many cases, may be the only clear sign of this disease [Lindenbaum and Nath, 1980].

The etiology of megaloblastic anemias suggests the increased number of filaments results from artifactual accumulation or stability of factors controlling filament formation. Megaloblastic anemia and its accompanying neutrophil nuclear hypersegmentation are caused by deficiencies in vitamin B_{12} (cobalamine) and/or folic acid, chemicals required for the normal production of deoxyribonucleotides in mammalian cells [Bills and Spatz, 1977]. Inadequate supplies of vitamin B_{12} and folic acid lead to retardation of DNA synthesis, while RNA and protein synthesis continue. As a result, cytoplasmic and nuclear components accumulate in excessive amounts, cells increase in size, and nuclei become hypersegmented. Correction of the folate or vitamin B_{12} deficiency results in the gradual reappearance of mature neutrophils with normal numbers of lobes. Inhibition of DNA synthesis by other methods, such as chemotherapy with drugs that target DNA synthesis, also results in neutrophil nuclear hypersegmentation [Wintrobe, 1974].

As in the case of Pelger-Huët anomaly, megaloblastic anemia appears to affect only the factors that specify filament number since other filament properties such as width and length remain unaltered. We postulate that these putative filament control factors must have a hierarchy of interacting sites in the genome since a higher fraction of the genome becomes packaged into filaments only when these factors appear to be overproduced.

Filament formation in Pelger-Huët individuals afflicted with megaloblastic anemias. Characterization of neutrophil nuclear morphology in heterozygous Pelger-Huët individuals with megaloblastic anemias [Ardeman et al., 1963; Taylor, 1973] provides further evidence for the dose-dependent activity of filament control factor and suggests that such a factor may be encoded by the Pelger-Huët gene. Despite their Pelger-Huët anomaly and megaloblastic condition, neutrophil nuclei from these patients are neither hypo- nor hypersegmented. Instead, these nuclei exhibit normal patterns of filament formation and segmentation characteristic of non Pelger-Huët individuals. Somehow, megaloblastic anemia counterbalances the Pelger-Huët anomaly. Once these patients recover from the megaloblastic condition, their neutrophil nuclei return to the hyposegmented morphology. These observations support our suggestion that megaloblastic anemia causes either excessive accumulation or stabilization of filament control factor. As a result, the neutrophils from genetically normal individuals with megaloblastic anemia become hypersegmented, while the neutrophils of Pelger-Huët appear normal temporarily. Thus, megaloblastic anemia and the Pelger-Huët anomaly may well exert opposite effects on the functional concentration of the same factor.

A model for filament formation in human neutrophils. The above studies suggest that filament control factor is present in limited amounts during neutrophil differentiation and interacts with specific sites in the human genome in a hierarchical, dose-dependent manner to initiate filament formation. In these

respects, filament control factor exhibits properties similar to those of nucleation factors in transcriptional silencing. If filament control factor is indeed a nucleation factor for filament formation, the silencing paradigm postulates the existence of spreading factors that must interact with bound filament control factor. These spreading factor would then build filaments by first oligomerizing with themselves along adjacent DNA sequences to create a stretch of filamentous chromatin and then promoting tight interactions between this chromatin filament and the nuclear envelope (Fig. 5). We designate these putative spreading factors filament forming factors. Given the extensive regions of chromosomal DNA present in filaments, filament forming factors must be relatively abundant proteins. The localized effect of filament forming factors in the neutrophil nucleus distinguishes these proteins from proteins implicated in overall chromatin condensation during genome inactivation, such as histone H5 in chicken erythrocytes [Sun et al., 1989] or protamines in mature sperm cells [Balhorn, 1990]. Studies in rabbits suggest that filament forming factors may represent nuclear matrix proteins whose affinity for DNA increases as neutrophils mature [Eastment et al., 1981]. Filament forming factors might therefore correspond to proteins implicated in facultative heterochromatin formation [Eissenber et al., 1994; Saunders et al., 1993].

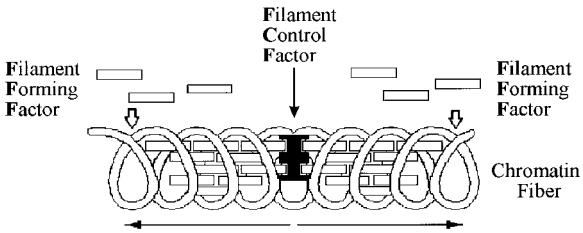

Fig. 5. Strategies governing the molecular interactions during neutrophil nuclear filament assembly may be similar to those occurring during heterochromatin assembly. During heterochromatin assembly, binding of a nucleation factor to a specific chromosomal region serves as a nucleation site for the polymerization of additional factors along large stretches of adjacent DNA (see text). The available data suggest a model whereby filament formation is initiated by binding of filament control factor, FCF, to specific DNA sequences in a hierarchical and dose-dependent manner. Bound FCF must then serve as the nucleation site for the cooperative olygomerization of an additional factor (filament-forming factor [FFF]) along adjacent DNA sequences, resulting in their packaging into an elongated chromatin filament. [Modified from Wolffe, 1998.]

The identity of filament control factor, on the other hand, is not yet clear. Several groups are attempting to map the Pelger-Huët gene by examining specific deletions in chromosome 17 that generate a Pelger-Huët-like phenotype [Lai et al., 1995; Fugazza et al., 1996]. Other groups have associated Pelger-Huët anomaly with cases of enlarged short arm of chromosome 22, trisomy 18, trisomy 13, Klinefelter's syndrome (XXY), and Down's syndrome (chromosome 21 trisomy)[Berman et al., 1983; Irken et al., 1993]. It is possible, however, that the Pelger-Huët phenotype in such aneuploid individuals simply results from titration of a particular nuclear segmentation factor by the extra chromosomes or chromosomal regions.

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