

Nuclear matrix: application to diagnosis of cancer and role in transcription and modulation of chromatin structure

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Summary

The nuclear matrix is involved in the processing of the genetic information and in the organization of chromatin. In recent years we have come to appreciate the organization of functional domains within the nucleus (e.g., transcript domains, RNA processing sites, sites of replication). The nuclear matrix is the foundation from which this organization is built, providing a scaffold upon which nuclear processes such as DNA replication and transcription occur. Chromatin is arranged into loop domains through the attachment of matrix associated regions (MARs) at the base of the loop to nuclear matrix proteins. Considering the role of the nuclear matrix in the organization and processing of the genetic information, it is not surprising to find that nuclear matrix proteins are informative in distinguishing cell types and disease states. For example, nuclear matrix proteins informative in the diagnosis of cancer, including breast cancer have been identified. Typically nuclear matrices are obtained following the salt extraction of nuclease-digested nuclei. However, recent studies show that cisplatin preferentially crosslinks MAR DNA to nuclear matrix proteins *in situ*, providing a complimentary method to identify informative nuclear matrix proteins.

Transcribed, but not repressed chromatin, is associated with the nuclear matrix. Regions of a chromatin loop engaged in transcription are associated with the nuclear matrix through multiple dynamic interactions with nuclear matrix proteins. Nuclear matrix bound transcription factors, the transcription machinery and histone modifying enzymes are thought to mediate these dynamic attachments between the nuclear matrix and transcriptionally active chromatin. Core histones of transcriptionally active chromatin are dynamically acetylated, with histone acetyltransferases (HATs) and deacetylases (HDACs) catalyzing this reaction. Both of these enzymes, which are now known to be transcriptional coactivators and modulators, are associated with the nuclear matrix. We have proposed that these enzymes participate in the dynamic attachment of transcriptionally active chromatin with the nuclear matrix. Our recent studies show that cisplatin crosslinks nuclear matrix-bound transcription factors and transcription modulators to nuclear DNA *in situ*. This suggests that cisplatin will be most useful in the discovery of nuclear matrix MAR binding proteins involved in the organization of DNA and nuclear matrix bound transcription factors/modulators participating in the nuclear matrix association of transcriptionally active chromatin. (Supported by Medical Research Council of Canada)

I. Nuclear matrix isolation and structure

The nuclear matrix has a role in the organization and function of nuclear DNA. The structure of the nuclear

matrix consisting of residual nucleoli, surrounding nuclear pore-lamina complex, and internal matrix is revealed when nuclease-digested nuclei are extracted with salt (e.g., 0.25 M ammonium sulfate). The protocol that we use to isolate nuclear matrices is shown in **Fig. 1**. Briefly, nuclei are digested with DNAase I followed by extraction with 0.25

M ammonium sulfate, yielding NM1-IF [nuclear matrices (NM) with attached intermediate filaments (IF)] (Sun et al., 1994; Chen et al., 1996). Further extraction of the NM1-IF nuclear matrices with 2 M NaCl yields NM2-IF. The internal matrix of NM1-IF preparations has a fibrogranular appearance (Chen et al., 1996). Extraction of the NM1-IF with high salt removes proteins that decorate core filaments of the internal matrix (Penman, 1995; Nickerson et al., 1995). The core filament fiber network is also seen when nuclear DNA is removed from nuclease-digested cells by electroelution in solutions of physiological ionic strength (Jackson and Cook, 1988). Core filaments, composition of which is currently unknown, have a diameter of 10-13 nm. These filaments appear to be the underlying structure onto which other nuclear components are bound.

The nuclear matrix is composed of protein and RNA. The nuclear pore-lamina consists of lamins and pore proteins. The internal matrix has a complex protein composition, with heterogeneous nuclear ribonuclear proteins (hnRNP) being major components (Mattern et al., 1996). Most nuclear RNA is associated with the nuclear matrix and contributes to the structural integrity of the nuclear matrix (Nickerson et al., 1995). The absence of nuclear RNA may weaken nuclear matrix internal structures. For example, nuclear matrices isolated from chicken mature erythrocytes lack nuclear RNA and internal structures, while nuclear matrices from immature erythrocytes of anemic adult birds have internal structures and nuclear RNA (Chen et al., 1996).

II. Nuclear matrix proteins and the diagnosis of cancer

The protein composition of the nuclear matrix is both tissue and cell type specific, and undergoes changes with differentiation and transformation (Fey and Penman, 1988; Stuurman et al., 1990; Dworetzky et al., 1990; Cupo, 1991). Pathologists have long appreciated that irregular nuclear appearance is the signature of a malignant cell (Miller et al., 1992; Nickerson et al., 1995). Changes in the composition of nuclear matrix proteins in malignant cells may contribute to alterations in nuclear structure. Nuclear matrix proteins are informative markers of disease states (Khanuja et al., 1993; Keese et al., 1994). Informative nuclear matrix proteins have been identified for bladder, breast, colon, prostate, head, and neck cancers (Getzenberg et al., 1991a;1996; Khanuja et al., 1993; Keese et al., 1994; Donat et al., 1996). For example, the nuclear matrix protein PC-1 is found in the nuclear matrix proteins from prostate cancer but not in the nuclear matrix from normal prostate or benign prostatic hyperplasia (Getzenberg et al., 1991a). Recently, we reported that the nuclear matrix protein composition was radically altered in highly metastatic oncogene transformed mouse fibroblasts (Samuel et al., 1997b). Interestingly, highly

metastatic *ras*-transformed 10T1/2 cells and highly metastatic *fes*-transformed NIH 3T3 cells had a similar set of nuclear matrix proteins that were not seen in poorly metastatic or non-tumorigenic parental mouse fibroblast cell lines. Clearly, this study shows a correlation between the nuclear matrix protein profile and the metastatic potential of the cell. Of potential importance is the demonstration that nuclear matrix proteins can be detected in the serum and urine of cancer patients, thus suggesting that the detection of specific nuclear matrix proteins may be of value in breast cancer diagnosis (Miller et al., 1992; Replogle-Schwab et al., 1996; Carpinito et al., 1996).

We have identified informative breast cancer nuclear matrix proteins (Samuel et al., 1997a). Typically we prepare NM2-IF nuclear matrices from breast cancer cell lines or breast tumours. To remove IFs from these preparations we disrupt nuclear matrices and attached IFs with urea (**Fig. 1**). The IFs are then allowed to reassemble and are removed from the soluble nuclear matrix proteins (Fey and Penman, 1988). Over a broad protein concentration range, this process is independent of protein concentration, but it is dependent upon temperature (**Fig. 2**). Performing the reconstitution at room temperature is recommended. About 8-10% of the nuclear protein is recovered in the nuclear matrix protein fraction.

In the search for informative breast cancer nuclear matrix proteins, we used human breast cancer cell lines T47D, MCF-7 and ZR-75 (ER+/hormone dependent), MDA MB231, and BT-20 (ER-/hormone independent), and T5-PRF (ER+/hormone independent). A non-tumorigenic, spontaneously immortalized human breast epithelial cell line known as MCF-10A1 (ER-/hormone independent) obtained from reduction mammoplasty was chosen as the closest representative of normal breast epithelia. Typically we isolate proteins from at least three nuclear matrix preparations of each cell line, and these proteins are electrophoretically separated on two dimensional gels. Comparative analysis of the two dimensional gel patterns identified nuclear matrix proteins of estrogen receptor (ER) positive breast cancer cells that were not found in ER- breast cancer cells or normal breast epithelial cells (Samuel et al., 1997a). Our criteria for designating a nuclear matrix protein as being informative in breast cancer was that the protein had to be present in each of the relevant preparations (either ER+ and/or ER-breast cancer cell nuclear matrix proteins), but not in the preparations of

Figure 1. Method to Isolate Nuclear Matrix and Nuclear Matrix Proteins.

Figure 2. Effect of Temperature on the Separation of Intermediate Filament (IF) Proteins from Nuclear Matrix Proteins (NMP). NM2-IF from human breast cancer cells was disrupted in urea and then made to different protein concentrations prior to removal of IF proteins as described (Samuel et al., 1997b).

nuclear matrix proteins from "normal" breast epithelial cells. Using the nomenclature proposed by Khanuja et al. (1993), we refer to these proteins as NMBCs (nuclear matrix proteins in breast cancer). Five NMBCs (1-5) exclusive to the ER+ cell lines and one NMBC (6)

exclusive to the ER- cell lines were identified (Samuel et al., 1997a).

The extracellular environment can alter the cellular morphology as well as the protein composition of the cytoskeletal and nuclear matrix compartments (Getzenberg et al., 1991b; Pienta et al., 1991; Fallaux et al., 1996). Thus, it was important to find out whether the changes in nuclear matrix proteins we observed with cancer cells grown on plastic were observed with cancer cells present in a breast tumour. In the preparation of nuclear matrices from breast tumours, we found that it was necessary to remove the adipose tissue surrounding the tumour. We found all NMBCs (1-5) exclusive to ER+ status in the human breast cancer cell lines as being present in the ER+ breast tumours, while NMBC-6 was not detectable (see **Fig. 3**, tumour 12797). NMBC-6, but not NMBCs 1-5, were present in ER- tumour nuclear matrix proteins.

The effect of cellular transformation on nuclear matrix protein composition is illustrated in the following study. Nuclear matrix proteins were isolated from MCF10A1 breast cancer cells that were transformed with the human T-24 mutated *Ha-ras* oncogene (MCF10AneoT) or with wild type human ER (cell line 139B6). MCF10AneoT cells are transformed and show anchorage independent growth (Basolo et al., 1991). The cell line 139B6 expresses ER at a similar level to that of MCF7 breast cancer cells (Pilat et al., 1996). In the presence of estradiol, this cell line has a slight inhibition in growth. This is typical of results of studies in which the ER is expressed in a ER- breast epithelial cell line or ER- breast cancer cell line (Pilat et al., 1996; Lundholt et al., 1996). Estradiol activated ER failed to elevate the expression of endogenous estrogen responsive genes but did induce the transient expression of an estrogen responsive element-regulated reporter gene in the 139B6 cell line (Pilat et al., 1996). Analysis of the two dimensional gel patterns of the nuclear matrix proteins from these cell lines revealed several alterations in nuclear matrix protein composition when MCF10A1 cells were transformed with *ras* or expressing ER. These differences were seen against a pattern of proteins found in all cell lines, for example hnRNP K (hk in **Fig. 3**). With the MCF10AneoT (*Ha-ras* transformed) cells, nuclear matrix proteins with a molecular mass of 47 kDa and pI range of 5.8-6.2 (constellation C in Fig. 3) were found to be exclusive to this cell line. Similarly, nuclear matrix proteins with molecular masses 50-57 kDa and pIs 5.5-5.7 (constellation B in **Fig. 3**) and proteins with molecular masses of 30-36

Figure 3. Human Breast Cancer Nuclear Matrix Proteins

Nuclear matrix proteins were isolated from MCF10A1 (parent, ER-, human breast epithelial cells), MCF10A-139B6 (parent transfected with human wild type ER), MCF10AneoT (parent transformed by T-24 Ha-*ras*), and human breast tumour 12797 (ER+). Protein (40 ug) was electrophoretically resolved on two-dimension gels. The gels were stained with silver. The position of the molecular weight standards (in thousands) is shown on the left side of each gel pattern. LA and LC are lamin A and C, respectively. The circles in MCF10A1 (parent) show the absence or decreased amount of nuclear matrix proteins highlighted in other gel patterns.

Figure 4. Sites of Post-Synthetic Modifications on the Histones.

The structures of H2A-H2B dimers and (H3-H4)₂ tetramers and the sites of modification are shown. Ac, acetylation; Ub, ubiquitination. The enzymes catalyzing reversible histone acetylation are shown.

kDa and pIs 4.5 (constellation A in **Fig. 3**) were determined to be exclusive to the ER expressing cell line MCF10A-139B6. However, within constellation B, a 48 kDa (pI 5.5) nuclear matrix protein (denoted by * in **Fig. 3**) was observed in the MCF10A parent cell line as well as in the *ras*-transformed and ER transfected cell lines. Relative to the parent cell line, the level of this protein in MCF10AneoT and MCF10A-139B6 was higher. NMBC1 present in the ER⁺ breast tumor nuclear matrix proteins was also detected in the ER expressing cell line (**Fig. 3**). The presence of NMBC1 in the ER transfected cell line suggests that ER expression has a role in the association of NMBC1 with the nuclear matrix. These results illustrate how nuclear matrix protein profiles reflect alterations in a cell's physiological state.

III. Nuclear matrix and organization of nuclear DNA

Nuclear DNA is packaged into nucleosomes, the repeating structural units in chromatin (Van Holde, 1988). The nucleosome consists of an histone octamer core around which DNA is wrapped. The four core histones of the octamer are arranged as a (H3-H4)₂ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N terminal unstructured domain, a globular domain organized by the histone fold, and a C terminal unstructured tail (Arents and Moudrianakis, 1995) (**Fig.**

4). Histone H1 binds to the linker DNA, which joins nucleosomes together, and to core histones (Boulikas et al., 1980; Banères et al., 1994). H1 has a tripartite structure with a basic N terminal domain, a basic C terminal tail domain, and a central globular core (Ramakrishnan, 1994).

In low ionic strength, chromatin fibers depleted of H1 have a "beads-on-a-string" structure, but with H1, folding of the fiber is evident (Leuba et al., 1994). At physiological ionic strength chromatin is folded into a 30 nm fiber. H1 stabilizes the folding of the chromatin fiber (Shen et al., 1995). The native 30 nm chromatin fiber has an irregular structure (an irregular three dimension zigzag) *in vitro* (Woodcock and Horowitz, 1995). Woodcock and colleagues show that the irregularities of the 30 nm chromatin fiber can be accurately reflected in a model that accounts for variability in linker DNA length and angle of trajectory that the linker DNA has as it enters and leaves the nucleosome. Thirty nm fibers are usually not seen inside nuclei (Woodcock and Horowitz, 1995). The chromatin is observed as matted patches. It appears that neighboring zigzags interdigitate, preventing individual chromatin fibers from being seen in nuclei. The core histone tails contribute to the condensation of the chromatin fiber (Garcia-Ramirez et al., 1995; Schwarz et al., 1996; Krajewski and Ausiό, 1996). H3 and H4 tails are needed for fiber-fiber interactions (Schwarz et al., 1996).

The chromatin fiber is organized into loop domains, with an average size of 86 kb (Jackson et al., 1990; Gerdes et al., 1994) (**Fig. 5**). Transcriptionally active genes are found in DNAase I-sensitive, presumably decondensed chromatin loops that are accessible to transcription factors and transcription machinery (Davie, 1995). Transcriptionally inactive genes are in higher order, interdigitated chromatin patches, being essentially invisible to transcription factors and the transcription machinery. At the base of the loop there are DNA sequences called MARs (matrix associated regions) that bind to nuclear matrix proteins (Bode et al., 1995). MARs tend to be AT-rich, but do not have a consensus sequence (Bode et al., 1995; Mielke et al., 1996). MAR-DNA binds to both internal matrix and nuclear pore-lamina, suggesting that proteins of the nuclear pore-lamina and internal matrix are involved in the organization of chroma-

Figure 5. A Model for Transcriptionally Active and Repressed Chromatin Domains

At the base of the loop are nuclear matrix associated regions (MARs).

HET (SAF-B) is a nuclear matrix protein that binds MARs. The repressed chromatin loop has a condensed chromatin structure.

Multiple dynamic attachment sites between the transcriptionally active domain and the internal nuclear matrix are presented in the box outline.

Histone acetyltransferase (HAT A), histone deacetylase (HDAC), transcription machinery and transcription factors are shown associated with the internal nuclear matrix, mediating a dynamic attachment between transcriptionally active chromatin and the nuclear matrix. HAT A and HDAC are shown as multiprotein complexes.

tin (Zini et al., 1989). MAR-binding proteins include lamins, which are found in the nuclear pore-lamina and internal matrix (Hozák et al., 1995), topoisomerase II, SATB1, HET (SAF-B), and attachment region binding protein which is an internal matrix protein or nuclear matrix (Pommier et al., 1990; Nakayasu and Berezney, 1991; von Kries et al., 1991; Luderus et al., 1992; Nakagomi et al., 1994; Buhrmester et al., 1995; Oesterreich et al., 1997).

Alterations in MAR-binding proteins have been reported in cancer cells. In Southwestern blotting experiments with a radiolabelled mouse IgH MAR sequence, Yanagisawa et al. (1996) detected a 114-kDa MAR binding protein expressed in breast carcinomas but not normal or benign breast tissue. Further, the levels of this MAR-binding protein were elevated in poorly differentiated breast ductal carcinomas. A recent study shows that mutant, but not wild type, p53 binds to MARs (Müller et al., 1996). Changes in nuclear matrix, MAR-binding proteins could result in reorganization of nuclear DNA.

IV. *In situ* crosslinking with cisplatin

Recent studies suggest that cisplatin (*cis*-diammine dichloroplatinum or *cis*-DDP) preferentially crosslink MARs to nuclear matrix proteins *in situ*. Either cells or nuclei can be incubated with cisplatin to crosslink protein to DNA. Most proteins crosslinked to DNA with cisplatin are nuclear matrix proteins, and the DNA crosslinked to protein is enriched in MAR-DNA sequences (Wedrychowski et al., 1986; 1989; Ferraro et al., 1992; 1995; Bubley et al., 1996; Olinski et al., 1987). **Fig. 6** shows the protocol to isolate proteins crosslinked to DNA

in situ. A comparison of two dimension gel patterns of nuclear matrix proteins and proteins crosslinked to DNA with cisplatin in ZR-75 human breast cancer cells shows that several abundant nuclear matrix proteins are crosslinked to DNA in the cells (**Fig. 7**). Lamins A and C, components of the nuclear pore-lamina, are crosslinked *in situ* to nuclear DNA consistent with *in vitro* data suggesting that these proteins are involved in the organization of nuclear DNA (Wedrychowski et al., 1986; 1989). Abundant nuclear matrix proteins found

Figure 6. Method to Isolate Proteins Crosslinked to DNA in Cells or Nuclei with Cisplatin

crosslinked to nuclear DNA *in situ* with cisplatin are F-actin and hnRNP K (Miller et al., 1991; Sauman and Berry, 1994) (**Fig. 7**). hnRNP K is a single-strand DNA-binding protein that is associated with the nuclear matrix and has an important role in regulating the expression of the *c-myc* gene (Michelotti et al., 1996; Mattern et al., 1996). Further, hnRNP K interacts with TATA-binding protein (Michelotti et al., 1996). This transcription factor is a prominent protein observed in both the nuclear matrix fraction and proteins crosslinked to DNA *in situ* with cisplatin in ZR-75 human breast cancer cells (**Fig. 7**).

Figure 7. Analysis of Nuclear Matrix Proteins and Proteins Crosslinked to DNA

ZR-75 human breast cancer nuclear matrix proteins (40 ug) and proteins crosslinked to DNA by *cis*-DDP *in situ* (40 ug) were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular weight standards (in thousands) is shown to the left of the gel patterns. LA and LC show lamin A and C, respectively. HnRNP K is shown as hk.

V. Transcription factors: activators of transcription

Current evidence suggest that an interaction between an enhancer or locus control region and promoter is an essential step in forming the open chromatin domain (Reitman et al., 1993). The enhancer/locus control region-promoter interaction is mediated by protein-protein associations between transcription factors bound to these *cis*-acting regulatory elements. This complex recruits the transcription initiation machinery and initiates the transcription cycle. The transcription cycle can be separated into at least four stages: initiation, promoter clearance, elongation, and termination. During the initiation stage, the pre-initiation complex (PIC) is formed at the promoter of a RNA polymerase II transcribed gene.

The ability of cisplatin to preferentially crosslink nuclear matrix proteins to nuclear DNA *in situ* has great potential in identifying nuclear matrix proteins involved in the organization and function of nuclear DNA. Several transcription factors are nuclear matrix proteins thought to interact with promoter and enhancer elements of specific genes. It has been proposed that the interaction of nuclear matrix bound transcription factors with regulatory DNA sequences has a role in attaching transcriptionally active chromatin to nuclear matrix (see below). Crosslinking with cisplatin may provide a method to find if the nuclear matrix associated transcription factor is bound to the DNA sequence of interest *in situ*. We are currently developing methods that will identify nuclear matrix associated transcription factors and their bound DNA sequences. Further, these methods are being used to find informative DNA-binding nuclear matrix proteins in the diagnosis of cancer.

The basal transcription factors TFIIA, TFIIB, TFIID, TFIIIE, TFIIIF, and TFIIH and RNA polymerase II are involved in the assembly of the PIC (for review see Orphanides et al., 1996; Pugh, 1996; Nikolov and Burley, 1997). *In vitro* studies show that there is a defined order by which the basal transcription factors are assembled into the PIC. TFIID, one of the first factors involved, binds to the TATA-box and consists of the TATA-binding protein (TBP) and several TBP-associated factors (TAFs). However, there is evidence that the PIC comes partially preassembled (Maldonado et al., 1996). In the formation of the PIC at any given promoter, TFIID binding appears to be the rate limiting step. Transcription factors bound to enhancers and upstream promoter elements interact through their activation domains with the TAFs of TFIID or with other components of the PIC (e.g., TFIIB), increasing the rate that the PIC is formed at the promoter

(for review see Tjian and Maniatis, 1994; Chiang et al., 1996; Gupta et al., 1996). TAFs are referred to as coactivators, proteins that mediate an interaction between transcription factors and PIC. Transcription factors bind to different TAFs, and these multiple contacts between the transcription factors and TAFs ensure the efficient recruitment of TFIID (Sauer et al., 1995; Chi et al., 1995). Transcription factors with multimerization domains also

have key roles in juxtapositioning enhancer and promoter elements. For example, multimers of the transcription factor Sp1 bound at DNA sites separated by 1.8 kb will interact, resulting in a looping out of the intervening sequences (Pascal and Tjian, 1991). Once these *cis*-acting elements are positioned next to each other, there will be a high local concentration of activators in the vicinity of the promoter (**Fig. 8**).

Figure 8. Model for Histone H5 Chromatin in Chicken Immature Erythrocytes.

The 3' enhancer is positioned next to the 5' promoter through protein-protein interactions. NF1 and multiprotein complexes (HAT As, HDACs, transcription machinery) are shown mediating the dynamic attachments of the histone H5 gene to the nuclear matrix at sites of transcription.

VI. Regulation of transcription

Within the DNAase I sensitive chromatin domains containing transcriptionally active genes are regions that are hypersensitive to DNAase I attack. The DNAase I hypersensitive (DH) regions of chromatin may lack nucleosomes and often mark chromatin for the presence of *cis*-acting regulatory DNA sequences and *trans*-acting factors. DH sites in human breast cancer *c-myc* chromatin and chicken erythrocyte histone H5 chromatin map with promoters and enhancers, and using *in vitro* assays we identified the transcription factors binding to these regulatory DNA elements (Penner and Davie, 1992; 1994; Sun et al., 1992; 1993; Miller et al., 1993; 1996; Murphy et al., 1996). However, *in vitro* assays can sometimes be misleading, and the most rigorous method in finding transcription factor occupancy is *in situ* footprinting (Becker et al., 1987; Mueller and Wold, 1989). We did *in situ* footprinting using a procedure called ligation-

mediated PCR to reveal the occupancy of factor binding sites in the promoter and enhancer of the H5 gene in chicken erythrocytes (Sun et al., 1996b). Some factor binding sites in the promoter and enhancer identified *in vitro* were not occupied *in situ*. Based upon our studies on the chromatin structure and transcription factors associated with the H5 promoter and enhancer, we put forth a model for the transcriptionally active H5 gene (**Fig. 8**).

VII. Nuclear matrix and processing of the genetic information

The nuclear matrix is involved in the processing of the genetic information. In recent years we have come to appreciate that functional components (e.g., transcript domains, RNA processing sites, sites of replication) of the nucleus are highly organized (Hendzel and Bazett-Jones, 1995; Penman, 1995; Xing et al., 1995). Transcribed

genes are found in discrete foci (Jackson et al., 1993; Iborra et al., 1996; Wansink et al., 1996). The nuclear matrix is the foundation from which this organization is built, providing a scaffold from which nuclear processes such as DNA replication and transcription occur (Berezney, 1991; Iborra et al., 1996). It is important to note that these functional centers in the nucleus are dynamic in their formation and dissociation. For example, sites of replication will assemble on the nuclear matrix at or near transcription foci in early S phase of the cell cycle. Once replication of these regions of the genome is complete, the replication machinery will disassemble from its site on the nuclear matrix and reassemble at other sites continuing replication of other regions of the genome (Hozák et al., 1993; Bassim Hassan et al., 1994). The process of transcription occurs at the nuclear matrix, and it has been proposed that the chromatin fiber moves through the nuclear matrix bound transcription apparatus as transcription proceeds (Cook, 1994; Hendzel and Bazett-Jones, 1995; Iborra et al., 1996). The transcription machinery is a massive multiprotein complex (Aso et al., 1995; Chao et al., 1996; Maldonado et al., 1996). Thus, it is unlikely that RNA polymerase travels along the DNA as text book models often show. Further, the nascent RNA becomes associated with the nuclear matrix. A solid state process by which DNA is driven through the nuclear matrix bound machinery and the nascent RNA is processed at the nuclear matrix would be an efficient way of dealing with these nuclear activities.

VIII. Nuclear matrix and transcriptionally active chromatin

Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus (Andreeva et al., 1992; Gerdes et al., 1994; Davie, 1995). Actively transcribed, but not inactive, chromatin regions are immobilized on the nuclear matrix by multiple dynamic attachment sites (Fig. 5 and 8). When histones are removed by high salt, loops of DNA are seen emanating from a central nuclear skeleton, forming a halo around this nuclear structure. Transcriptionally inactive genes are found in the halo, while DNA loops with transcriptionally active genes remain associated with the nuclear skeleton (Gerdes et al., 1994). The transcription machinery, specific transcription factors, and nuclear enzymes (e.g., histone acetyltransferase, histone deacetylase, see Fig. 8) are thought to mediate the dynamic attachments between transcribing chromatin and nuclear matrix (van Wijnen et al., 1993; Cook, 1994; 1995; Bagchi et al., 1995; Merriman et al., 1995).

The nuclear matrix is selective for which transcription factors it binds, and this selectivity varies with cell type (van Wijnen et al., 1993; Sun et al., 1994; 1996a). It has been postulated that the nuclear matrix has a role in the expression of genes by concentrating a subset of

transcription factors at specific nuclear sites (Stein et al., 1991; Merriman et al., 1995). Transcription factors associated with the nuclear matrix include ER, HET, GATA-1, YY1, AML-1, Sp1, Oct1, mutant p53, and Rb (Dworetzky et al., 1992; Isomura et al., 1992; Vassetzky et al., 1993; van Wijnen et al., 1993; Sun et al., 1994; Merriman et al., 1995; Guo et al., 1995; Müller et al., 1996; Mancini et al., 1996; Kim et al., 1996; Oesterreich et al., 1997). HET is a transcriptional repressor. Interestingly, sequencing of HET revealed that it was identical to SAF-B, a protein isolated by its ability to bind MARs (Renz and Fackelmayer, 1996; Oesterreich et al., 1997). Thus, HET (alias SAF-B) is a nuclear matrix protein in breast cancer cells that binds to MARs and acts as a repressor.

Protein domains involved in targeting transcription factors to the nuclear matrix have been identified. However, it is too early to know whether a consensus nuclear matrix localization signal will emerge from these studies. The N-terminal domains of the androgen and glucocorticoid receptor are involved in directing these receptors to the nuclear matrix (van Steensel et al., 1995). There are examples of where the association of transcription factors with the nuclear matrix is regulated by modifications. For example, the association of Rb with the nuclear matrix appears to be regulated by phosphorylation and is cell-cycle dependent. When Rb is in a hypophosphorylated state in G1-phase of the cell cycle, it is attached to the nuclear matrix. But the highly phosphorylated Rb of S-phase is not associated with the matrix (Mancini et al., 1994). The amino terminus of hypophosphorylated Rb binds to a p84 nuclear matrix protein (Durfee et al., 1994).

ER is associated with the nuclear matrix of estrogen responsive tissues (Metzger and Korach, 1990; Metzger et al., 1991; Thorburn and Knowland, 1993). *In vitro* reconstitution studies with nuclear matrices and hormone receptors (e.g., ER and androgen receptor) show that nuclear acceptor sites for the hormone receptors are associated with the nuclear matrix (Barrack, 1987; Metzger and Korach, 1990; Lauber et al., 1995). The binding of the ER to the nuclear matrix was saturable, of high affinity, target tissue specific, and receptor specific (Metzger and Korach, 1990). Acceptor proteins for ER have been identified in a variety of estrogen-responsive tissues (Lauber et al., 1995; Ruh et al., 1996).

Transcription factors associated with the nuclear matrix can change throughout development and differentiation (Stein et al., 1994; Davie, 1995; Merriman et al., 1995; Bagchi et al., 1995; Sun et al., 1996a). For example, transcription factors associated with the chick erythrocyte nuclear matrix change throughout development (Sun et al., 1996a). Primitive red blood cells from 5-day old embryos have high levels of nuclear matrix-bound transcription factors, including GATA-1, CACCC-binding proteins, and NF1; factors that have key

roles in erythroid-specific gene expression. In definitive red blood cells (11-day and 15-day embryos) the levels of these nuclear matrix bound transcription factors decline. Erythroid nuclear matrices preferred to bind CACCC-binding proteins and not Sp1. Promoters and enhancers of erythroid-specific genes have Sp1 binding sites that bind both CACCC-binding proteins and Sp1. It is possible that the selective nuclear matrix binding of CACCC-binding proteins gives the CACCC-binding proteins an advantage over Sp1 in binding to a Sp1/CACCC site.

Although we know that transcription factors are associated with the nuclear matrix, evidence that nuclear matrix associated transcription factors are bound to regulatory DNA elements of specific genes is currently lacking. For example, NF1 is a nuclear matrix associated transcription factor that binds to the enhancer of the chicken histone H5 gene (**Fig. 8**). *In vitro* footprinting and electrophoretic mobility shift assays show that NF1 isolated from immature erythrocyte nuclear matrices binds to the H5 enhancer (Sun et al., 1994). *In situ* footprinting shows that the NF1 binding site in the H5 enhancer is occupied in chicken immature erythrocytes (Sun et al., 1996b). We have proposed that NF1 recruits the H5 enhancer to the nuclear matrix (Davie, 1996). However, we have yet to show that nuclear matrix associated NF1 is the protein occupying the H5 enhancer NF1 binding site in erythroid cells. Cisplatin crosslinking may provide direct evidence to test this model (see above).

IX. Dynamic histone acetylation

Transcribed DNA is associated with acetylated histones (Hebbes et al., 1994; O'Neill and Turner, 1995; Mutskov et al., 1996). The core histones are reversibly modified by acetylation of lysines located in their basic N terminal domains (**Fig. 4**). Reversible histone acetylation is catalyzed by histone acetyltransferases (HATs) and deacetylases (HDACs), with the level of acetylation being decided by the net activities of these two enzymes. Histone acetylation alters nucleosome and higher order chromatin structure (Davie, 1995; 1997). For example, chromatin associated with highly acetylated histones does not undergo histone H1 mediated aggregation at physiological ionic strength, while chromatin with unacetylated histones aggregates when associated with H1 (Ridsdale et al., 1990; Davie, 1997). Besides modulating nucleosome and higher order chromatin packaging, the core histone tails bind to regulatory proteins (Ma et al., 1996; Edmondson et al., 1996). For example, yeast repressor protein Tup1 binds to the tails of H3 and H4. Acetylation of H3 and H4 prevents the binding of Tup1 (Edmondson et al., 1996). In mammalian cells and chicken erythrocytes, transcriptionally active chromatin regions have core histones undergoing high rates of acetylation and deacetylation, while in repressed chromatin regions the rate of reversible acetylation is slow

(Davie, 1996; 1997). Thus, we expect that the interaction of regulatory proteins with the histone tails and chromatin structure of transcriptionally active regions of mammalian and chicken erythrocytes is in dynamic flux.

The process of reversible histone acetylation is not dependent upon ongoing transcription (Ruiz-Carrillo et al., 1976). To date, the only histone modifications dependent upon ongoing transcription are ubiquitination of H2B (see **Fig. 4**) and phosphorylation of mouse H1b (Davie and Murphy, 1990; Chadee et al., 1997). However, interference of dynamic acetylation by inhibiting deacetylation with histone deacetylase inhibitors (e.g., sodium butyrate, trichostatin A or trapoxin) greatly affects cell cycle progression, arresting cells in G1 or G2, and may enhance or repress the expression of genes (Yoshida et al., 1995; Johnston et al., 1992; Girardot et al., 1994; Miyashita et al., 1994; Laughlin et al., 1995).

Figure 9. CBP/p300 Cointegrates Diverse Signalling Pathways

CBP and its functional/protein interaction domains are shown.

X. Histone acetyltransferase and gene activation

Histone acetylation is not limited to transcriptionally active chromatin, but also has a role in DNA replication (deposition-related acetylation) and DNA repair (for review see Davie, 1995; 1997). Deposition-related

acetylation of H4 is catalyzed by HAT B, a cytoplasmic enzyme (Kleff et al., 1995; Brownell and Allis, 1996). HAT A is responsible for transcriptionally active chromatin-associated acetylation. Nuclear HAT A is bound to chromatin and acetylates all core histones when free or within nucleosomes (Brownell and Allis, 1996). Dr. Allis and colleagues were the first to purify and clone a HAT A. Their studies showed that *Tetrahymena* HAT A (p55) is homologous to yeast Gcn5, a transcriptional adaptor, that has HAT activity (Brownell et al., 1996). This important breakthrough provided a direct link between the process of transcription activation and histone acetylation.

Tetrahymena HAT A (p55) and yeast Gcn5 are components of large multiprotein complexes, and the substrate specificity of the catalytic subunit is regulated by the proteins binding to it (Grant et al., 1997). Yeast Gcn5 and *Tetrahymena* p55 can acetylate free histone H3 but these HAT As are unable to acetylate histones in nucleosomes. Yeast Gcn5 is a component of two high molecular mass complexes (0.8 and 1.8 megadaltons) (Grant et al., 1997). These high molecular mass, multiprotein complexes acetylated histones in nucleosomes and free histones. Both HAT A complexes contain Ada2 and Ada3. Gcn5-Ada2-Ada3 is a putative adaptor complex that connects DNA-bound transcription factors (activators) to components of the PIC (Candau et al., 1997). The HAT domain of yeast Gcn5 has been localized. Gcn5 requires both the HAT domain of Gcn5 and interaction with Ada2 for transcriptional activation (Candau et al., 1997). Human homologues of Gcn5 and Ada2 have been identified (Candau et al., 1996).

Tetrahymena HAT A and yeast Gcn5 have a bromodomain that is lacking in yeast Hat1p. The bromodomain, which is thought to be a protein-protein interaction domain, is found in the C-termini of these proteins (Haynes et al., 1992). Several other recently identified HAT As have the bromodomain, including TAF_{II}250 (a 250 kDa protein that binds to TATA-binding protein), CBP/p300 but not P/CAF (Yang et al., 1996; Bannister and Kouzarides, 1996; Mizzen et al., 1997; Ogryzko et al., 1997). It is possible that HAT As interact with other transcription factors through the bromodomain, directing HAT A to specific regions in chromatin and in the nucleus (Brownell and Allis, 1996).

Most HAT As are coactivators (e.g., Gcn5, TAF_{II}250, CBP/p300). CBP/p300 binds to hormone receptors, AP-1, c-Myb, SV40 large T antigen, and adenovirus E1a, and appears to be an integrator of multiple signalling pathways (Kamei et al., 1996; Avantaggiati et al., 1996; Oelgeschläger et al., 1996; Hanstein et al., 1996) (**Fig. 9**). Unlike Gcn5, *Tetrahymena* p55, TAF_{II}250, or P/CAF, CBP acetylates all four core histones in nucleosomes (Ogryzko et al., 1997). The discovery that several transcription modulators or coactivators have HAT activity provides a mechanism by which chromatin structure is altered in the vicinity of DNA-bound transcription activators. A variety of transcription factors including hormone receptors, CREB, and fos-jun will bind directly or indirectly to CBP, recruiting a coactivator with histone acetyltransferase activity (**Fig. 10**). The HAT activity of CBP would then acetylate surrounding histones in nucleosomes, leading to the destabilization of nucleo-

Figure 10. Role of HAT As and HDACs in Transcriptional Activation and Repression

Top panel: Fos-Jun is shown recruiting the coactivator CBP, resulting in the acetylation of nucleosomal histones. Bottom panel: Mad-Max is shown recruiting the corepressor HDAC multiprotein complex, resulting in the deacetylation of nucleosomal histones.

some and higher order chromatin structure. Such a chromatin state is thought to be facilitate the binding of other transcription factors and, in general, aid the transcription process.

XI. Histone deacetylase and gene repression

Histone deacetylases are nuclear enzymes that have been isolated from a variety of sources. Our studies have focused on the chicken erythrocyte histone deacetylase, an enzyme associated with the nuclear matrix (Hendzel et al., 1991). Chicken erythrocyte histone deacetylase is a component of a multiprotein complex that has a molecular mass in excess of 400 kDa (Li et al., 1996). The chicken histone deacetylase complex extracted from nuclei dissociates to a 66-kDa form in 1.6 M NaCl or when applied to an ion-exchange column (e.g., Q-sepharose). However, the high molecular mass histone deacetylase complex extracted from chicken erythrocyte nuclear matrices does not dissociate in 1.6 M NaCl, but this HDAC complex did dissociate to a 66-kDa form when applied to a Q-sepharose column (Li et al., 1996). These observations suggest that the solubilized nuclear matrix histone deacetylase is associated with proteins that stabilize the complex from dissociation into the 66-kDa form in a high concentration of salt.

The high molecular mass chicken erythrocyte histone deacetylase complex deacetylates the four core histones in chromatin, but has a preference for H2B (Li et al., 1996). Dissociation of the multiprotein histone deacetylase complex resulted in a change in substrate preference. The 66-kDa enzyme could not deacetylate histones in chromatin and had a preference for free H3. The data suggest that proteins important in regulating HDAC activity were lost during enzyme purification (Li et al., 1996).

Dr. Schreiber and colleagues were the first to clone a mammalian histone deacetylase (HDAC1, 55 kDa) (Tauton et al., 1996). They found that mammalian histone deacetylase was related to the yeast transcription regulator Rpd3p, providing a link between transcription regulation and histone deacetylation. At around the same time, Dr. Grunstein and colleagues purified two yeast histone deacetylase complexes, HDA (350 kDa) and HDB (600 kDa) (Carmen et al., 1996; Rundlett et al., 1996). The HDA complex consists of multiple peptides with molecular masses of approximately 70 kDa. Two peptides from the HDA complex have been sequenced, and yeast genes HDA1 (codes for p75) and HDA3 (codes for p71) isolated. HDA1 shares sequence similarity with Rpd3p, a yeast histone deacetylase. Gene disruptions of HDA1 or HDA3 resulted in the loss of the HDA, but not, HDB complex. Rpd3p is a component of HDB (Rundlett et al.,

1996). Rpd3p binds to Sin3 which in turn associates with Ume6, a DNA-binding protein required for the repression of several genes including those involved in meiosis (Kadosh and Struhl, 1997). These observations suggest that the yeast HDB complex consists of Rpd3p, Sin3 and Ume6.

Mammalian histone deacetylase HDAC1 is related to yeast transcriptional regulator Rpd3p (Tauton et al., 1996). Although HDAC1 has a reported molecular mass of 55 kDa, we have found that it migrates on our SDS polyacrylamide gels with an apparent molecular mass of 66 kDa. The mammalian homologue of Rpd3p, named HDAC2, has been cloned (Yang et al., 1996). Chicken erythrocyte histone deacetylase has been purified, and the enzyme migrates as a 66-kDa band on SDS gels (J.-M. Sun, H. Y. Chen, J. R. Davie, unpublished observations). Thus, chicken erythrocyte histone deacetylase has a molecular mass similar to that of mammalian HDAC1.

Figure 11. Regions of mSin3A Involved in Protein Interactions

N-CoR, SMRT and Mad family members interact with different paired amphipathic helix (PAH) domains in mSin3A. The HID domain which binds to HDAC 1 and 2 is shown.

As with yeast and chicken histone deacetylases, mammalian histone deacetylases, HDAC1 and HDAC2, exist as high molecular mass, multiprotein complexes (Hassig et al., 1997). HDAC1 and HDAC2 bind to a variety of proteins, including RbAp48, YY1, and mammalian (m) Sin3A and mSin3B (Tauton et al., 1996; Yang et al., 1996; Laherty et al., 1997). These HDAC-binding proteins may exist in different HDAC multiprotein complexes. For example, HDAC complexes with mSin3 do not contain YY1 (Zhang et al., 1997).

HDAC1 was purified as a complex with RbAp48, a 50 kDa Rb-binding protein that binds to the C-terminus of unphosphorylated or hypophosphorylated Rb (Tauton et al., 1996; Qian et al., 1993). RbAp48 has several partners in addition to HDAC1. RbAp48 is a component of human and *Drosophila* CAF-1 (chromatin assembly factor 1) (Verreault et al., 1996). A yeast protein similar to RbAp48, Hat2p, is component of yeast HAT B (Roth and Allis, 1996; Parthun et al., 1996).

HDAC1 and/or HDAC2 are in large multiprotein complexes that contain mSin3, N-CoR, and SMRT, proteins that are corepressors (Nagy et al., 1997; Hassig et al., 1997; Laherty et al., 1997; Heinzl et al., 1997). Mammalian Sin3A and mSin3B have four paired amphipathic helix (PAH) domains thought to be involved in protein-protein interactions (**Fig. 11**). HDAC1 and HDAC2 bind to the region between PAH3 and PAH4, referred to as HID [the histone deacetylase interaction domain (HID)] (Laherty et al., 1997). The HID region is conserved in mSin3A, mSin3B and yeast Sin3. Mammalian Sin3A (150 kDa) interacts with many other proteins, including SAP18 (mSin3 associated protein), Mad family members (Mad1, Mad3, Mad4, Mxi1) and Max-binding repressor Mnt, SMRT, and N-CoR (Laherty et al., 1997; Zhang et al., 1997; Nagy et al., 1997; Heinzl et al., 1997; Alland et al., 1997). mSin3A does not have DNA binding ability; however, several of the proteins associated with mSin3A can direct it to specific DNA regulatory regions. The N-terminal region (SID, mSin3 interaction domain) of the Mad family members and Mnt binds to PAH2 of mSin3 (**Fig. 11**). Mad family members form a dimer with Max, a DNA-binding complex that binds to E-box related DNA sequences (Laherty et al., 1997). Max and Mad proteins are members of the basic region-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors. Myc forms a heterodimer with Max which binds to the same E-box-related DNA sequences as does Mad-Max heterodimers. However, Myc-Max activates genes, while Mad-Max represses their transcription. The repressive action of Mad-Max is mediated in part by the interaction of Mad with mSin3 which in turn is associated with HDAC1 and/or HDAC2. N-CoR and SMRT bind to unliganded retinoid and thyroid hormone receptors (Nagy et al., 1997; Heinzl et al., 1997). Thus, like the bHLH-Zip repressor proteins, unliganded hormone receptors recruit the HDAC multiprotein complex. HDAC has a principal role in transcription repression. Several studies show that tethering HDAC1 or 2 to a promoter by fusing HDAC to a DNA-binding domain (e.g., Gal4 DNA-binding domain) results in transcription inhibition (Yang et al., 1996; Zhang et al., 1997; Kadosh and Struhl, 1997; Nagy et al., 1997). These studies suggest that repressors recruit histone deacetylase which would deacetylate histones in nucleosomes, leading to the condensation of chromatin (Wolffe, 1997) (**Fig. 10**).

Although these studies show that HDAC is involved in repression, HDAC is associated with transcriptionally active chromatin. Both HAT As and HDACs are needed to catalyze dynamic acetylation of histones associated with transcribed chromatin domains. The presence of both HAT As and HDACs at transcriptionally active regions allows the rapid manipulation of nucleosome and chromatin structure (Wade and Wolffe, 1997).

XII. Histone acetylation and nuclear matrix

Vertebrate histone acetyltransferase (HAT A) and histone deacetylase (HDAC) are associated with the nuclear matrix (Henzel et al., 1991; 1992; 1994; Li et al., 1996). Nuclear skeletons from chicken immature erythrocytes retain 80% of the nuclear HAT A and HDAC activities, and these enzymes catalyze reversible acetylation using as substrate the chromatin fragments associated with the nuclear skeletons (Henzel et al., 1994). These studies suggest that HAT A and HDAC are colocalized to specific sites on the nuclear matrix. However, there is no evidence that HAT A and HDAC are part of the same large complex. We proposed a model in which nuclear matrix-bound HAT A and HDAC mediate dynamic interactions between the nuclear matrix and transcriptionally active chromatin (**Fig. 8**) (Davie and Henzel, 1994; Davie, 1995). We have evidence that HDAC1 is associated with the matrix, but the identity of the nuclear matrix bound HAT A is currently unknown. Several transcription factors binding directly or indirectly with HAT A and HDAC are nuclear matrix proteins. For example, YY1 is a nuclear matrix protein that binds to HDACs (Yang et al., 1996; Guo et al., 1995). Estrogen receptors bound to the nuclear matrix could recruit CBP, an HAT A (Hanstein et al., 1996). Determining how HATs and HDACs are recruited to nuclear matrix sites engaged in transcription will be an important challenge.

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