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Potential Roles for Chromatin Structures in the Differential Regulation of the 5S rRNA Genes in *Xenopus laevis*

by

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B.Sc., University of Victoria, 1992

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

We accept this dissertation as conforming
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I. ABSTRACT

In 1871, a unique substance was isolated from the white blood cells of pus. This substance, which later became known as chromatin, was shown to be a nucleoprotein complex which encompasses the majority of genomic DNA in all eukaryotes. Although chromatin was once viewed as primarily a structural component of the nucleus, it is now accepted that it also plays an important role in the modulation of transcription of individual genes. In this study, the 5S rRNA genes in *Xenopus laevis* were used as a system to investigate potential roles for chromatin structures in transcription regulation. *X. laevis* produces two major classes of 5S rRNA: the somatic type is present in most cells whereas the oocyte type is produced only during oogenesis and the early stages of embryogenesis. These two gene families share a very similar coding region and employ identical transcription machinery, leading researchers to believe that it is how these genes are packed into chromatin which is responsible for the differential developmental regulation.

Initially, this study focused on the binding constraints placed on the RNA polymerase III basal transcription factor, transcription factor IIIA (TFIIIA), by a histone octamer. Five overlapping fragments of the *X. laevis* oocyte and somatic 5S rRNA genes were reconstituted into nucleosomes and it was shown that each fragment positions a histone octamer at unique translational sites. Using these nucleosomes it was demonstrated that nucleosome translational positioning is the major determinant of the binding of TFIIIA to the 5S rRNA genes.

The relationship between core histone acetylation and transcription of the *X. laevis* 5S rRNA genes was also investigated. By immunoprecipitating chromatin fragments from a *X. laevis* kidney cell line with an antibody specific for hyperacetylated histone H4, it was shown that the oocyte 5S rRNA genes are packaged with hypoacetylated histone H4 when transcriptionally repressed. This taken together with the results of others, suggests a link between histone acetylation and RNA polymerase III transcription. However this study was unable to shed light on the basis for this relationship as it was found that histone acetylation did not affect the binding of TFIIIA to nucleosomal DNA.

In an attempt to understand the mechanism by which transcription factors compete with histone octamers for cognate binding sites in chromatin, the effect of the histone binding protein nucleoplasmin on the binding of TFIIIA to nucleosomal 5S rRNA genes was tested. It was shown that, despite the previously reported nucleosome remodeling ability of nucleoplasmin, the binding of TFIIIA to nucleosomal DNA cannot be facilitated by this protein. Furthermore it was demonstrated that nucleoplasmin cannot overcome nucleosome mediated repression of transcription of reconstituted 5S rRNA genes. In contrast to earlier work, this study used a homologous system composed of the 5S rRNA gene, nucleoplasmin and TFIIIA from *Xenopus laevis.*

Finally, it has long been proposed that selective binding of histone H1 is, in part, responsible for the differential developmental regulation of the oocyte and somatic 5S rRNA genes in *Xenopus laevis.* In this study it was shown that histone H1 bound both oocyte and somatic genes equally after reconstitution into mononucleosomes or oligonucleosome arrays. Furthermore it was shown that the binding of histone H1 selectively repressed only oocyte gene transcription, and that a RNA polymerase III

selectively repressed only oocyte gene transcription, and that a RNA polymerase III transcription complex was able to initiate transcription of nucleosomal somatic templates regardless of whether histone H1 was present. These results support a model in which the differential regulation of the 5S rRNA genes is not due simply to the prevention of histone H1 binding by transcription complexes on the somatic genes, but rather a difference in the interaction of histone H1 with the somatic and oocyte genes.

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II. TABLE OF CONTENTS

I. Abstract ii

II. Table of Contents v

III. List of Figures ix

IV. Acknowledgment xiii

V. Dedication xiii

VI. List of Abbreviations xv

1. INTRODUCTION 1

1.1. Chromatin Structure 4

1.2. Transcriptionally Active Chromatin 7

1.3. Nucleosome Position 9

1.4. Histone Acetylation 15

1.5. Linker Histones 19

1.6. Chromatin Remodeling Factors 22

1.7. The 5S rRNA Genes of *Xenopus laevis* 23

1.8. 5S rRNA Transcription Machinery 25

1.9. 5S rRNA Transcription During Development 28

1.10. Role of Chromatin Structures in the Differential Expression of the 5S rRNA Genes in *X. laevis* 30

1.11 Objectives 33

2. MATERIALS AND METHODS 34

2.1. DNA Fragments 34

2.2. Labeling of DNA 37

2.2.1. Labeling of DNA for electrophoretic mobility shift analysis or use as radiolabeled probes 37

2.2.2. Labeling of DNA for micrococcal nuclease digestion 38

2.2.3. Labeling DNA for DNase I footprinting 39

2.3. Protein Purification 39

2.4. Protein Electrophoresis 41

2.5. Nucleosome Reconstitution 42

2.5.1. The octamer transfer method of reconstitution 42

2.5.2. Salt-gradient dialysis reconstitution 42

2.5.3. Salt-gradient dialysis reconstitution with incorporation of histone H1 43

2.6. Determination of Nucleosome Translational Position 43

2.7. Electrophoretic Mobility Shift Assays 44

2.7.1. TFIIIA electrophoretic mobility shift assays 44

2.7.2. Histone H1 electrophoretic mobility shift assays 45

2.8. DNase I Footprinting Analysis 45

2.9. Micrococcal Nuclease Digestion of Chromatosomes and Southern Blot Hybridization 46

2.10. *In vitro* Transcription of Reconstituted 5S rRNA Genes 47

2.11. Cell Culture and Northern Blot Analysis 49

2.12. Western Blot Analysis 49

2.13. Control Immunoprecipitation 50

2.14. Chromatin Isolation, Immunoprecipitation and Dot Blot Analysis 50

2.15. Chromatin Isolation, Micrococcal Nuclease Digestion and Southern Blot Analysis 52

2.16. *In vitro* Nucleosome Assembly 53

3. RESULTS 55

3.1. Differential Transcription of *X. laevis* Oocyte and Somatic 5S rRNA Genes After Reconstitution with Histone Octamers 55

3.2. Role of Translational Position in the Differential Transcription of the 5S rRNA genes in *X. laevis* 58

3.2.1. Reconstitution of complete mononucleosome core particles 58

3.2.2. Determination of the translational position of reconstituted nucleosome core particles 63

3.2.3. DNase I footprint analysis of reconstituted nucleosomes 72

3.2.4. Binding of TFIIIA to *X. laevis* 5S rRNA gene fragments after reconstitution with histone octamers 74

3.2.5. DNase I footprint analysis of the TFIIIA, 5S rRNA gene, nucleosome complex 79

3.3. Role of Histone Acetylation in the Differential Transcription of the 5S rRNA genes in *X. laevis* 82
3.3.1. Binding of TFIIIA to *X. laevis* 5S rRNA gene fragments after reconstitution with hyperacetylated histone octamers 82
3.3.2. Northern blot analysis of 5S rRNA transcription 83
3.3.3. Western blot analysis and control immunoprecipitation 88
3.3.4. Mapping histone H4 acetylation of oocyte 5S rRNA genes 90
3.3.5. Micrococcal nuclease digestion of oocyte 5S rRNA genes 93
3.4. Role of Nucleoplasmin in the Transcription of the 5S rRNA genes in *X. laevis* 93
3.4.1. Integrity of nucleoplasmin 95
3.4.2. The effect of nucleoplasmin on the binding of TFIIIA to mononucleosomal DNA 95
3.4.3. Effect of nucleoplasmin on 5S rRNA transcription from nucleosomal templates 99
3.5. Role of Histone H1 in the Differential Transcription of the 5S rRNA Genes in *X. laevis* 101
3.5.1. The binding of histone H1 to free and nucleosomal 5S rRNA genes 101
3.5.2. DNase I footprinting of nucleosomes containing histone H1 105
3.5.3. The effect of histone acetylation on the binding of histone H1 109
3.5.4. The binding of histone H1 to nucleosomal arrays 110
3.5.5. The effect of histone H1 on transcription of the 5S rRNA genes 112
3.5.6. Micrococcal nuclease digestion of nucleosomes on the somatic 5S rRNA gene 114
3.5.7. Determination of nucleosome core particle/chromatosome positioning on reconstituted 208-12 116
3.5.8. *In vitro* transcription of reconstituted 208-12 DNA 118

4. DISCUSSION 122
4.1. The Relationship Between Translational Position and the Binding of TFIIIA to Nucleosomal DNA 123
4.2. Understanding the Binding of TFIIIA to Nucleosomal DNA 124
4.3. Nucleosome Position on the 5S rRNA Genes *in vivo* 126
4.4. The Role of Nucleosome Translational Position in the Differential Regulation of the 5S rRNA Genes 129

4.5. The Role of Histone Acetylation in the Differential Regulation of the 5S rRNA Genes 131
4.6. The Role of Nucleoplasmin in the Differential Regulation of the 5S rRNA Genes 134
4.7. The Role of Histone H1 in the Differential Regulation of the 5S rRNA Genes 135
4.8. Conclusions 139

5. LITERATURE CITED **140**

III. LIST OF FIGURES

Figure 1. Schematic representation of a nucleosome. 5

Figure 2. Nomenclature and components of intermediates produced by digestion of chromatin with micrococcal nuclease. 6

Figure 3. Schematic representation of the 30 nm fiber. 8

Figure 4. Schematic representation of the effects of nucleosome translational position on the binding of transcription factors to nucleosomal DNA. 12

Figure 5. Schematic representation of the effects of nucleosome rotational position on the binding of transcription factors to nucleosomal DNA. 14

Figure 6A. Schematic representation of nucleosome core histone tails and acetylation sites. 16

Figure 6B. Model for the possible influence of histone acetylation on the histone-DNA contacts at the mononucleosome level. 16

Figure 7. Three proposed models for the location of linker histones within the nucleosome. 20

Figure 8. Schematic representation of the oocyte and somatic 5S rRNA genes in *X. laevis*. 24

Figure 9. Model for the assembly of RNAP III pre-initiation complexes on the 5S rRNA genes of *X. laevis*. 26

Figure 10. The relative amounts of oocyte and somatic 5S rRNA present during development. 29

Figure 11. The relationship between TFIIIA/histone H1 levels and the transcription of the oocyte and somatic 5S rRNA genes in *X. laevis*. 31

Figure 12. Schematic representation of the gene fragments used in this study. 35

Figure 13. The effect of nucleosome reconstitution on 5S rRNA transcription. 56

Figure 14A. Schematic representation of the octamer transfer method of nucleosome reconstitution. 60

Figure 14B. Agarose gel electrophoresis of DNA before and after reconstitution by the octamer transfer method. 60

Figure 15. Characterization of nucleosomes reconstituted by the octamer transfer method. 62

Figure 16. Schematic representation of the method used to determine histone octamer translational position. 66

Figure 17. Determination of the translational positioning of a nucleosome reconstituted onto a *L. variegatus* 5S rRNA gene fragment. 67

Figure 18. Determination of nucleosome translational position on reconstituted *Xenopus laevis* 5S rRNA gene fragments. 68

Figure 19. Schematic representation of the most predominant nucleosome positions on the fragments of the *Xenopus laevis* oocyte and somatic 5S rRNA genes. 71

Figure 20. DNase I digestion analysis of nucleosomes reconstituted on fragments of the *Xenopus laevis* 5S rRNA genes. 73

Figure 21. Analysis of the binding of TFIIIA to nucleosomes reconstituted onto the *Xenopus laevis* 5S rRNA gene fragments. 75

Figure 22. The relationship between nucleosome translational position and TFIIIA binding. 78

Figure 23A. Representation of the translational positioning of a nucleosome reconstituted onto the *X. borealis* 5S rRNA gene fragment XP-10. 80

Figure 23B. Analysis of the binding of TFIIIA to nucleosomes reconstituted onto the *Xenopus borealis* 5S rRNA gene fragment. 80

Figure 24. DNase I footprint analysis of the complex formed by nucleosomes and/or TFIIIA on the Xlo(-83→+136) 5S rRNA gene fragment. 81

Figure 25. Triton, urea, acetic acid electrophoretic analysis of histones. 84

Figure 26. Analysis of the binding of TFIIIA to nucleosomes containing hyperacetylated HeLa histones. 85

Figure 27. The oocyte 5S rRNA genes are repressed in the *X. laevis* K2 kidney cell line. 87

Figure 28A. Western blot analysis of hyperacetylated histones. 89

Figure 28B. Control immunoprecipitation of hyperacetylated histones. 89

Figure 29. A schematic representation of the immunofractionation technique used to analyze the acetylation state of the *Xenopus laevis* oocyte 5S rRNA genes. 91

Figure 30A. The oocyte genes in the *X. laevis* somatic cells are packaged with hypoacetylated histone H4. 92

Figure 30B. Southern blot hybridization of micrococcal nuclease digested nuclei from *X. laevis* K2 kidney cell line. 92

Figure 31A. SDS PAGE analysis of the purified proteins used in the nucleoplasmin study. 96

Figure 31B. *In vitro* chromatin assembly capability of *Xenopus* nucleoplasmin. 96

Figure 32. Agarose gel electrophoresis analysis of the effect of nucleoplasmin on the binding of TFIIIA to nucleosomal *Xenopus laevis* 5S rRNA gene fragments. 98

Figure 33. The effect of nucleoplasmin on the transcription of reconstituted *Xenopus laevis* oocyte 5S rRNA genes. 100

Figure 34. The binding of histone H1 to naked and nucleosomal 5S rRNA gene fragments 102

Figure 35. SDS PAGE analysis of HeLa cell histone H1. 103

Figure 36. Histone H1 binds equally to the oocyte and somatic *Xenopus laevis* 5S rRNA gene fragments Xlo(-38→+149) and Xls(-52→+147) after reconstitution with histone octamers. 106

Figure 37. DNase I footprint analysis of the complexes formed by nucleosomes with histone H1 on the Xls (-51→+147) and Xlo(-38→+149) fragments. 107

Figure 38. Histone H1 binds to both oocyte and somatic 5S rRNA full length gene repeats after reconstitution with histone octamers. 111

Figure 39. Histone H1 represses 5S rRNA transcription of oocyte but not somatic genes reconstituted with histone octamers. 113

Figure 40. The 5S rRNA coding sequence is in contact with both the histone octamer and histone H1. 115

Figure 41. Determination of translational position of oligonucleosomes reconstituted on the 208-12 template with histone H1. 117

Figure 42. Schematic representation of the most predominant nucleosome position on the reconstituted 208-12 DNA. 119

Figure 43. The effect of oligonucleosome reconstitution, with or without histone H1, on RNA polymerase III transcription. 121

Figure 44. Crystal structure of an 86 amino acid residue fragment of the glucocorticoid complexed with its hormone response element. 125

Figure 45. Schematic representation of a proposed model for the binding of TFIIIA to the 5S rRNA gene intragenic control region 127

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V. DEDICATION

I dedicate this thesis to my Mother (and Father) Caroline (Betty) Elizabeth Howe. She has supported every decision I have made, both logical and very illogical, and taught me that the only person really worth impressing is myself. The best I could ever achieve would be to live by her example.

VI. LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bHLH	basic helix-loop-helix - a *trans*-acting factor DNA binding domain
bp	base pair
BRF	TFIIB related factor - a subunit of TFIIIB
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cpm	counts per minute
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)-tetraacetic acid
Fos	subunit of AP-1 - a family of *trans*-acting factors which can be induced in response to serum and phorbol esters
GAL4	a *trans*-acting factor required for transcription of genes involved in galactose catabolism
HD	histone deacetylase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
ICR	intragenic control region
IE	intermediate element
Jun	subunit of AP-1 - a family of *trans*-acting factors which can be induced in response to serum and phorbol esters
Kda	kilodalton
Kb	kilobase pair
MES	2-(N-morpholino)ethanesulfonic acid
NP-40	nonidet P-40
NTP	nucleoside triphosphate

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	1,4-piperazinediethanesulfonic acid
PMSF	phenylmethanesulfonyl fluoride
RNA	ribonucleic acid
RNAP	RNA polymerase
SDS	sodium dodecyl sulfate
Sp1	specificity protein - a *trans*-acting factor which binds GC boxes and activates a wide variety of vertebrate genes
TAF	TATA binding protein associated factor
TB	Tris-borate buffer
TBE	Tris-borate-EDTA buffer
TFC5	one of the three components of yeast TFIIIB
TFIIIA	transcription factor IIIA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC

1. INTRODUCTION

In 1871, Friedrich Miescher isolated a unique substance from the white blood cells of pus. This substance, found to have a high phosphorous content, probably represented the very first chromatin preparation (Miescher, 1871). The actual term "chromatin" was first used eight years after Miescher reported his findings. Walter Flemming, after observing through a microscope the presence of banded objects in the nuclei of stained cells (Flemming, 1880), called the material "chromatin" after the Greek word *chroma*, for color. In 1884, Albert Kossel extracted the basic proteins, which he named *histon*, from the nuclei of geese erythrocytes (Kossel, 1884). Thus, it was determined more than one hundred years ago, that the eukaryotic nuclei contained both a phosphorous containing acidic component and a basic proteinaceous component. Following these early observations, the study of chromatin structure was delayed somewhat by the debate over which component, the protein or the nucleic acid, was the actual genetic material. During the 1940s and 50s, the idea that nucleic acids served as the genetic blueprint gradually gained acceptance. In 1952 it was demonstrated that chromatin particles were less extended than DNA alone (Steiner, 1952), giving the first hint of one of the major functions of this nucleoprotein complex. It was also postulated around this time that the histone proteins may serve as specific transcriptional regulators for particular genes (Stedman and Stedman, 1951). This theory declined in acceptance as it was realized that there were only a small number of histone types which were highly conserved between different species and cell types. Consequently, histones came to be viewed primarily as structural components of the nucleus (van Holde, 1988).

The light microscope was responsible for the many of the early findings in the study of chromatin, including establishing a relationship between gene transcription and the extent of DNA packaging. During interphase, chromosomal DNA exists as two forms: a compact form known as heterochromatin or a "swollen" form known as euchromatin (Heitz, 1934). The ratio of euchromatin to heterochromatin increases with increasing protein activity, and pulse-labeling with RNA precursors labels euchromatin but not heterochromatin (Brown, 1966). These results were interpreted to suggest that it is euchromatin which serves as the template for transcription while heterochromatin is transcriptionally silent. Although the euchromatin state is necessary for a high state of transcription, it alone is not sufficient to determine whether transcription occurs. In contrast to euchromatin, the DNA in heterochromatin is replicated very late in the S phase of each cell cycle, probably due to unusually condensed state (local decondensation of chromatin strands is required in the vicinity of any DNA replication fork) (Lima-de-Faria *et al.*, 1968).

There exists two sub-types of heterochromatin: constitutive and facultative. Constitutive heterochromatin is thought to contain DNA which is transcriptionally silent in all cell types such as satellite DNAs (Jones *et al.*, 1970). In contrast, whether a region of DNA is condensed into facultative heterochromatic DNA is dependent on the cell type and/or the stage of the cell cycle. The best characterized example of facultative heterochromatin is the X chromosomes in female animals. Presumably, in order to prevent expression of a double dose of X chromosomal gene products, one of the two X chromosomes in a female animal is permanently inactivated. These inactive chromosomes, termed Barr bodies, can be visualized under the light microscope as existing in a condensed heterochromatic state. In females, the decision as to which X chromosome of the female is inactivated is random (termed lyonization) and is made at the multi-cellular stage in the embryo. Once this decision is made, this chromosome remains inactive through successive cell divisions demonstrating that the a heterochromatic state can be faithfully inherited in progeny cells. As a result of this, every female exists as a mosaic in which equal portions of the cells express only the maternal or paternal X chromosome (Lyon, 1992 and references therein).

Long before chromatin structure was understood, studies of *Drosophila* polytene chromosomes revealed that major changes in DNA packaging accompanied gene transcription. These unusually large chromosomes, are produced by the repeated replication of interphase chromosomes without separation, resulting in a large number of chromosomes that remain laterally aligned (Beermann, 1972). The transcription of polytene chromosomes is usually associated with local swelling (puffs) of chromosomal bands with the size of the puff relative to the amount of ribonucleotide incorporation (Pelling, 1950). It was later shown that certain puffs could be artificially induced by hormones or by the gene products of other induced puffs (inhibition of protein synthesis prevented formation of these secondary puffs) (Ashburner *et al.*, 1973).

A second example in which the light microscope was used to detect a relationship between transcription and changes in chromatin structure came in the study of the "lampbrush loops" seen in the oocytes of amphibians. These loops, like the "puffs" on the polytene chromosomes, are regions of DNA that are decondensed when compared to other regions, and transcriptionally active (Sommerville *et al.*, 1978).

Although these early studies of were unable to discern the actual structure of chromatin, the importance of these results remained in the fact that they suggest that at least some of transcriptional regulation may be due to altered DNA packaging patterns. The first step into the study of the molecular structure of chromatin came with the onset of use of the electron microscope. Using this tool, the 30 nm euchromatic fiber (Wilkins *et al.*, 1959) could be visualized as a condensation of a "beads-on-a-string" nucleoprotein complex (Olins and Olins, 1973; Woodcock, 1973). This, together with nuclease digestion (Noll, 1974), X ray diffraction (Richmond *et al.*, 1984) and neutron scattering studies (Pardon *et al.*, 1975), demonstrated for the first time that the basic unit of structure of chromatin consisted of DNA wrapped around a histone complex spaced fairly regularly along the chromosome fiber. These "beads", originally termed "nu" bodies, are now termed nucleosomes and are still, to this day, not yet fully characterized.

1.1. Chromatin Structure

The basic unit of chromatin is the nucleosome, which consists of approximately 200 bp of DNA (depending on the organism and cell type), wrapped in two negative superhelical turns, around an octamer of core histone proteins and bound by a linker histone (Figure 1). Each of the core histones within the octamer has a basic, unstructured N-terminal tail linked to an α-helical C-terminal domain known as the "histone fold". Nucleosomes can be liberated from chromatin by digestion with the enzyme, micrococcal nuclease (Figure 2). Further nuclease digestion of this particle generates a structure

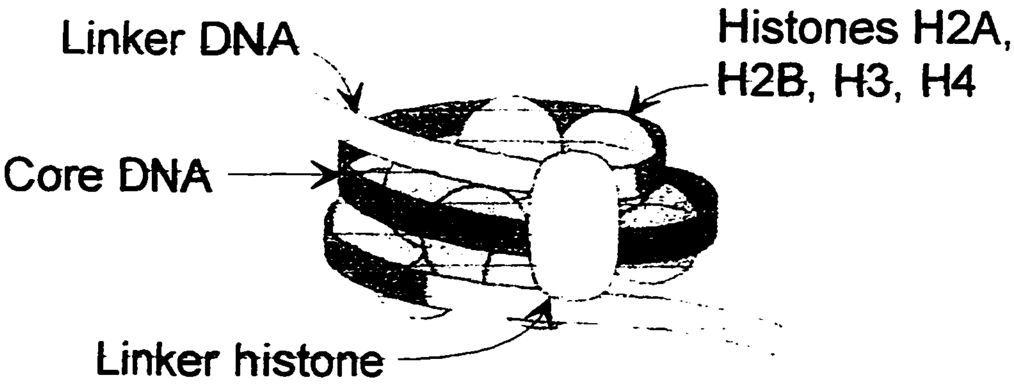

Figure 1. Schematic representation of a nucleosome.
Biochemistry, ed. L. Stryer, 1988.

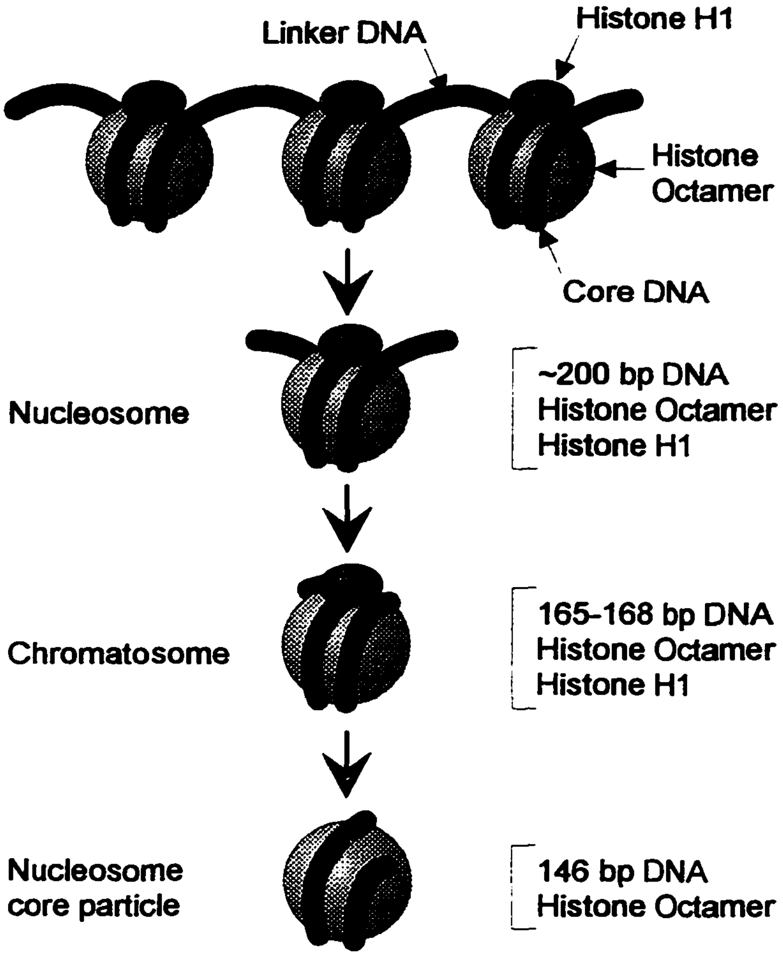

Figure 2. Nomenclature and components of intermediates produced by digestion of chromatin with micrococcal nuclease.

known as a chromatosome which is comprised of 165-168 bp of DNA, the histone octamer, and the linker histone (Simpson, 1978). Subsequent digestion of a chromatosome, produces a nucleosome core particle which is composed of 146 bp of DNA and the histone octamer. Using the digestion intermediates from micrococcal nuclease digestion, nucleosomal DNA can be classified into two groups: the core DNA and the linker DNA (Figure 1). The core DNA is defined as the 146 bp of DNA which is resistant to micrococcal nuclease digestion in the nucleosome core particle, and the linker DNA is the remaining DNA (Figure 1) responsible for joining adjacent nucleosomes.

The packaging of DNA into nucleosomes results in a seven-fold compaction. Nucleosome arrays are condensed a further seven-fold into a higher order structure known as the 30 nm fiber (Figure 3). This fiber is the form taken by the majority of chromatin during interphase (Paranjape *et al.*, 1994) but the actual arrangement of nucleosomes within this fiber has not been agreed upon. There is a general consensus that condensation into the 30 nm fiber is dependent on intranucleosome interactions, possibly requiring histone H1 [although compaction can be induced *in vivo* by specific monovalent or divalent ion concentrations (Hansen and Wolffe, 1992)]. The 30 nm fiber is further condensed into coils and loops in an undefined manner.

1.2. Transcriptionally Active Chromatin

Originally, it was postulated that the role of chromatin in transcription was strictly repressive by preventing the transcription machinery from gaining access to the DNA. This was supported by data showing that the assembly of genes into chromatin

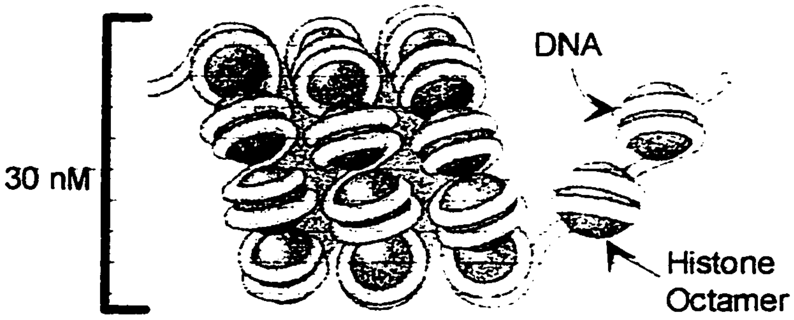

Figure 3. Schematic representation of the 30 nm fiber. *Molecular and Cellular Biology*, ed. S.L. Wolfe, 1993.

structures *in vitro* was largely repressive to transcription (Laybourn and Kadonaga, 1991; Ura *et al.*, 1995). Despite this, it has been demonstrated that most transcriptionally active genes are packaged into chromatin, although these structures differ somewhat from those of transcriptionally silent regions of the genome. Features of transcriptionally active chromatin include: histone acetylation; a generalized sensitivity (by one order of magnitude) of large regions to digestion by nucleases such as micrococcal nuclease, DNase I and DNase II (Stadler *et al.*, 1980); short regions of hypersensitivity (typically two orders of magnitude) to DNase 1 (Gross and Garrard, 1988); the absence of methylation of specific CpG dinucleotides (Bird, 1986); reduced levels or altered binding of linker histones (Zlatanova and van Holde, 1992 and references therein); the presence of HMG (high mobility group protein) 14/HMG 17 (Dorbic and Wittig, 1986; Dorbic and Wittig, 1987; Postnikov *et al.*, 1991) and ubiquitinated H2A/H2B (Levinger and Varshasky, 1982; Nickel *et al.*, 1989). Contrary to what was originally postulated, there is no evidence suggesting that the core histones H2A and H2B are depleted from chromatin that is actively being transcribed.

1.3. Nucleosome Position

Although nucleosomes can assemble on most DNA sequences, the position of the histone octamer relative to DNA sequence is not random (Drew and Travers, 1985). Although the exact mechanisms by which nucleosomes position themselves on certain sequences are not well understood, it has been suggested that nucleosomes prefer sequences which already contain an inherent bend (Drew and Travers, 1985). The position of a histone octamer relative to a DNA sequence can be described using two parameters: the translational and the rotational position. The translational position is defined as the location of a nucleosome along a gene. This is generally determined by analyzing the sequence contained in the 146 bp core particle micrococcal nuclease-resistant DNA fragment. The rotational position describes which face of the DNA double helix is in contact with the histone octamer. This is generally characterized by identifying the individual nucleotides within a nucleosome that are sensitive to DNase I or hydroxyl radical cleavage due to their position away from the surface of the histone octamer.

The packaging of DNA into nucleosomes results in one face of the DNA contacting the surface of the histone octamer. These protein-DNA contacts are so close that nucleases such as DNase I, micrococcal nuclease, and exonuclease III are impeded from accessing the nucleotides in direct contact with the surface of the histone octamer. The majority of DNA in eukaryotes is packaged with nucleosomes and thus, in order for transcription initiation to occur, some transcription factors must be able to interact with their response elements regardless of DNA interactions with the histone octamer. To date, researchers have shown that several *trans*-acting factors, including the glucocorticoid (Perlmann and Wrange, 1988, Piña *et al.*, 1990) and progesterone receptors (Piña *et al.*, 1990, Pham *et al.*, 1992), USF, (Juan *et al.*, 1994, Chen *et al.*, 1994), Sp1 (Chen *et al.*, 1994, Li *et al.*, 1994), GAL4 (Taylor *et al*, 1991), and Fos/Jun (Ng *et al.*, 1997) can bind their cognate binding sites within nucleosomal DNA, albeit with lower affinity than binding to free DNA. It has been suggested that the accessibility of nucleosomal response elements to *trans*-acting factors is dependent on both the translational and rotational position of the response element with respect to the core histones, as well as the arrangement of the nucleotide contacts required for binding. In terms of translational position, it has been observed in some instances that the accessibility of a binding site decreases as the site is moved closer to the dyad axis (center of the 146 bp core DNA fragment) of the nucleosome (Pham *et al.*, 1992, Li *et al.*, 1994, Li *et al.*, 1995) (Figure 4). As for the rotational position of the DNA helix with respect to the histone octamer, it has been suggested that the major groove of the cognate sequence must be oriented outward from the surface of the octamer, exposing the critical DNA sequences, required for factor binding, to the solvent (Piña *et al.*, 1990, Li *et al.*, 1995) (Figure 5). Despite these postulated guidelines, several unexpected results have been obtained. Li and Wrange, (1995) observed the binding of the glucocorticoid receptor to a response element which was rotationally positioned to face in toward the histone octamer. This binding occurred in the absence of any significant distortions of histone-DNA contacts as determined by DNase I footprinting. Li *et al.*, (1994) found that the constitutive *trans*-acting factor, Sp1 was able to bind a GC box after nucleosome reconstitution. Sp1 is a three zinc finger protein which, in binding to naked DNA, makes contact with the major groove for almost one complete turn of the DNA helix (Courey and Tjian, 1992) - a binding which would presumably be obstructed by the presence of a histone octamer. Finally, Taylor *et al.*, (1991) demonstrated that the yeast *trans*-acting factor GAL4 is able to bind to the 17 bp GAL4 site within nucleosomal DNA regardless of the rotational position of the bindings site. Thus it is becoming increasingly apparent that nucleosomes are dynamic structures which can permit the binding of factors to DNA sequences that would normally be unexpected when considering only the structure of the nucleosome and the DNA binding domains of the *trans*-acting factors.

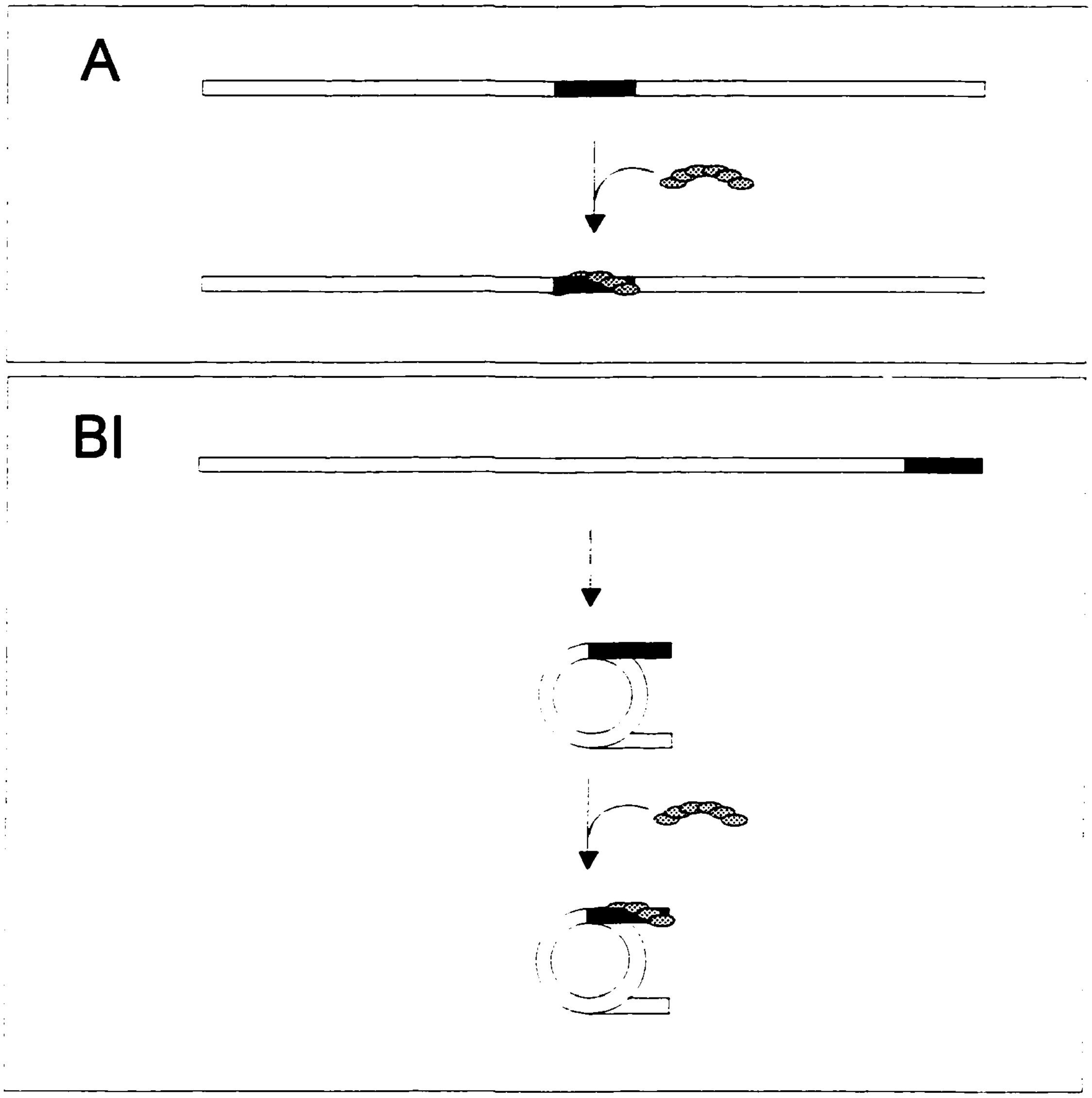

Figure 4. Schematic representation of the effects of nucleosome translational position on the binding of *trans*-acting factors to nucleosomal DNA. The open box represents the DNA double helix, the black box represents a binding site for a *trans*-acting factor with the protein contacts distributed throughout the whole circumference of the DNA helix, the gray shape represents a *trans*-acting factor and the open circle represents a histone octamer. (A) The binding of the *trans*-acting factor to naked DNA. (B) The binding of the *trans*-acting factor to nucleosomal DNA. (I) If the nucleotides within the binding site are positioned outside the 146 bp nucleosome core DNA, then the *trans*-acting factor can interact with this region allowing binding to the DNA. (II) If the nucleotides within the binding site are positioned within the core DNA, this prevents the *trans*-acting factor from binding.

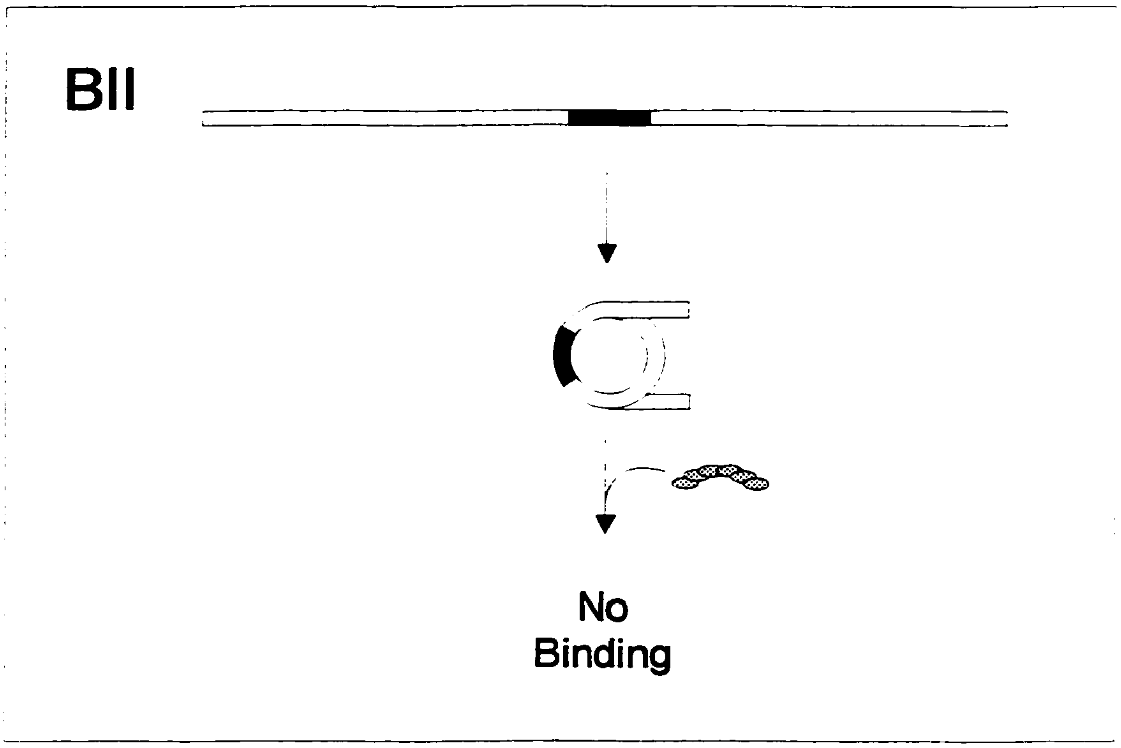

Figure 4. (continued).

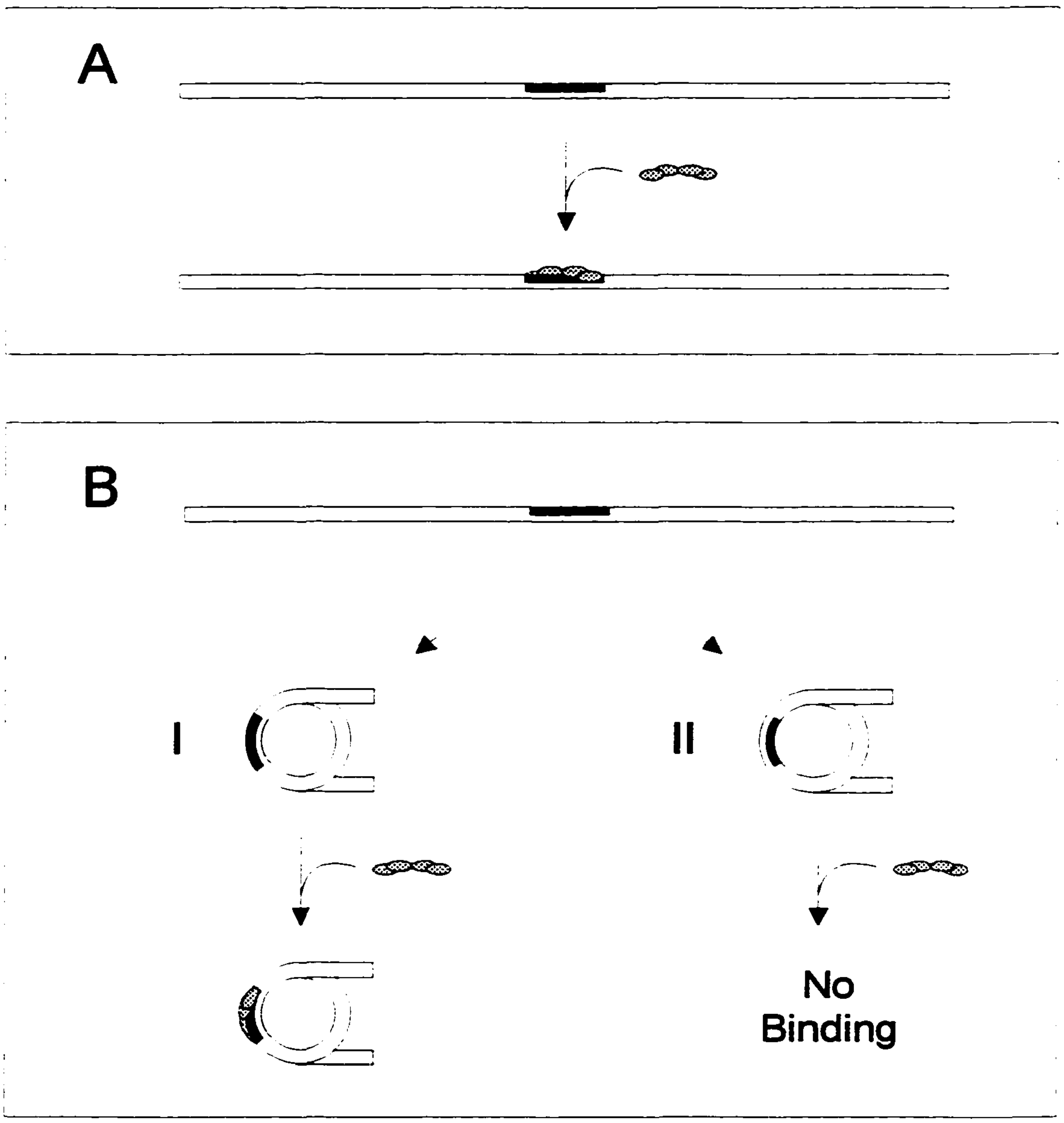

Figure 5. Schematic representation of the effects of nucleosome rotational position on the binding of a *trans*-acting factor to nucleosomal DNA. The open box represents the DNA double helix, the black box represents a *trans*-acting factor binding site with the protein contacts clustered on one side of the double helix, the gray shape represents a *trans*-acting factor and the open circle represents a histone octamer. (A) The binding of the *trans*-acting factor to naked DNA. (B) The binding of the *trans*-acting factor to nucleosomal DNA. (I) If the nucleotides within the binding site are positioned away from the histone octamer then the *trans*-acting factor can interact with this region, allowing binding to the DNA. (II) If the nucleotides within the binding site are positioned toward the histone octamer, this prevents the *trans*-acting factor from binding.

1.4. Histone Acetylation

Histones are subject to five post-translational modifications: acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation. Of these, histone acetylation has generated the most recent interest. The reversible acetylation of specific lysine residues within the unstructured, amino terminal "tails" of core histones (Figure 6A) is known to be associated with transcriptionally active genes, although the mechanistic basis of this relationship is not well understood. The early evidence for a link between histone acetylation and transcription was largely indirect (Csordas, 1990 and references therein). First, both transcriptionally active genes and acetylated chromatin show increased sensitivity to nucleases such as micrococcal nuclease, DNase I, and DNase II. Second, in *Saccharomyces cerevisiae*, in which all genes are active or potentially active during growth, the entire genome is highly acetylated. Finally, both highly acetylated histones and transcriptionally active genes co-purify by Hg-agarose affinity chromatography.

The first direct link between histone acetylation and transcription was established using antibodies, specific for hyperacetylated histones, to map core histone acetylation of active and inactive genes. For example, using this technique it was demonstrated that in chicken embryonic erythrocytes, the β globin genes are packaged with acetylated histones prior to, during and after activation during development (Hebbes *et al.*, 1992). Furthermore, the platelet derived growth factor B chain gene is also found packaged with hyperacetylated histones prior to gene induction in a human hematopoietic stem cell line (Clayton *et al.*, 1993). Therefore, in the case of these RNA polymerase II transcribed

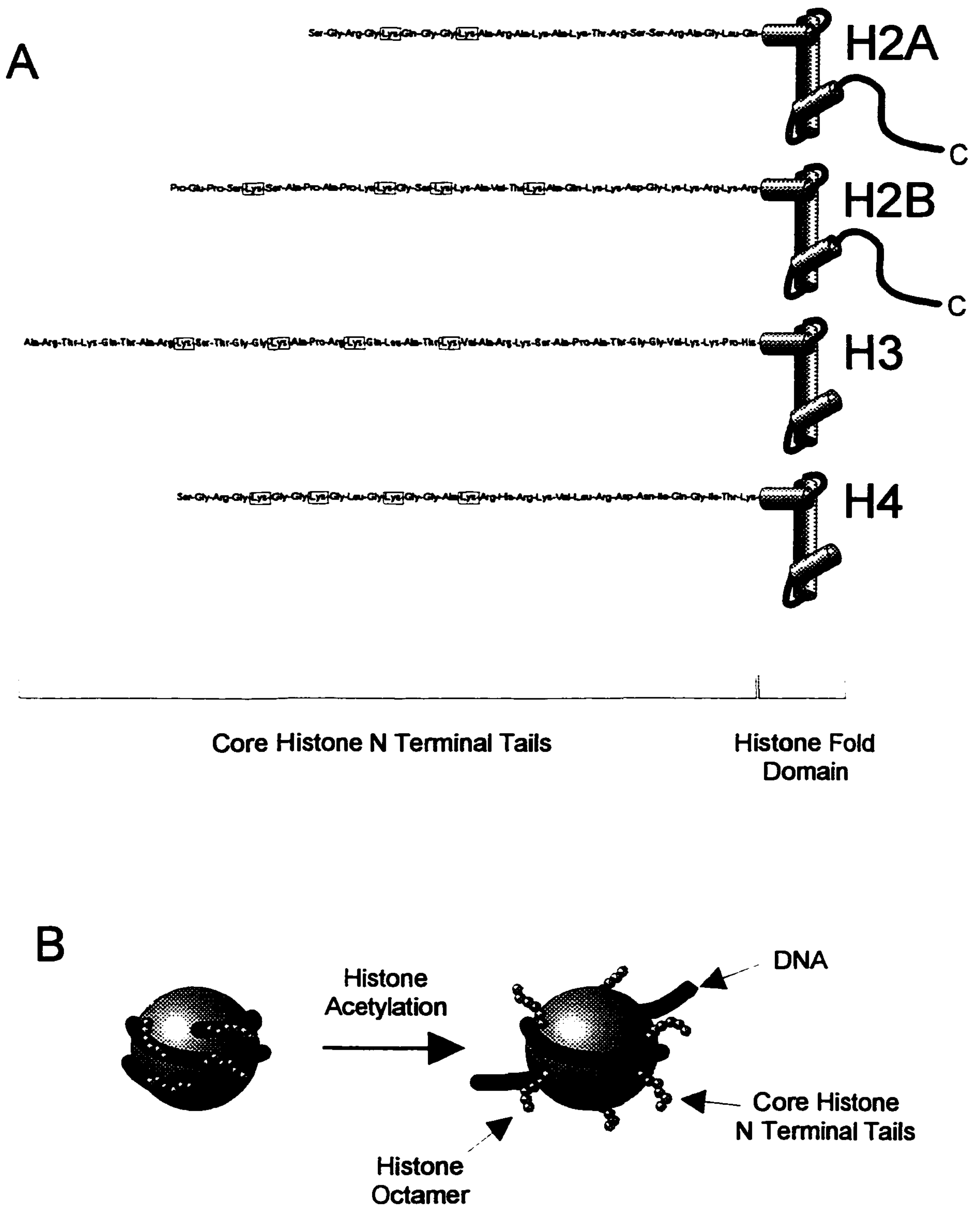

Figure 6. **(A) Schematic representation of the histone tails and acetylation sites of human core histones. (B) Model for the possible influence of histone acetylation on the histone-DNA contacts at the mononucleosome level.**

genes, histone acetylation is not a consequence but rather a precondition of transcription. The RNA polymerase I transcribed 45S genes in rat tissue culture cells are packaged with both acetylated and non-acetylated histones. It is not known whether all, or just a subset, of the genes packaged with acetylated histones are transcribed, but it was shown that those genes packaged with non-acetylated histone are transcriptionally silent (Mutskov *et al.*, 1996). It was also demonstrated that, in a *X. laevis* kidney cell line transcribing low levels of oocyte 5S rRNA, a small portion of oocyte genes are packaged with hyperacetylated histones (Reynolds *et al.*, 1982) but again it is not know whether the active oocyte genes are the subset packaged with acetylated histones in this cell line.

A breakthrough in the study of histone acetylation came in the identification of the yeast protein, Gcn5p as a histone acetyltransferases (Brownell *et al.*, 1996). Gcn5p is a transcriptional co-activator required for maximal activation of transcription by certain acidic *trans*-acting factors. Since then, several other transcription co-activators have been identified as having histone acetyltransferase activity including CBP (cAMP response element binding protein binding protein) (Bannister and Kouzarides, 1996), p300 (Ogryzko *et al.*, 1996), P/CAF (CBP associated factor) (Yang *et al.*, 1996) and TAF$_{II}$250 (Mizzen *et al.*, 1996). These proteins factors appear to be recruited by, and act as co-factors for, a large number of sequence-specific transcription factors such as members of the nuclear receptor superfamily (Hanstein *et al.*, 1996, Kamei *et al.*, 1996; Heinzel *et al.*, 1997; Nagy *et al.*, 1997), the myb proto-oncogene (Dai *et al.*, 1996), cAMP response element binding protein (Kwok *et al.*, 1994), the proto-oncogene-encoded proteins c-jun and c-fos (Bannister and Kouzarides, 1995; Kamei *et al.*, 1996), bHLH proteins (Eckner *et al.*, 1996; Sartorelli *et al.*, 1997), the p53 proto-oncogene protein (Gu *et al.*, 1997; Lill *et al.*, 1997), the Rel proto-oncogene protein (Gerritsen *et al.*, 1997), and E2F1 (Trouche and Kouzarides, 1996). In addition, several proteins recruited by factors involved in transcriptional silencing have been shown to have histone deacetylase activity including Rpd3p, HD1 (histone deacetylase 1) (Taunton *et al.*, 1996), N-CoR (nuclear receptor co-repressor) (Alland *et al.*, 1997), and components of the mSin3A multimeric complexes (Hassig *et al.*, 1997).

The charge neutralization associated with lysine acetylation is generally thought to result in a weakening of the histone DNA contacts (Figure 6B) although there is little biophysical experimental evidence to this effect (Ausió, 1992). It has been shown that acetylation loosens the interaction of the DNA with the histone octamer especially at the regions of DNA flanking the 146 bp DNA region of the core particle (Norton *et al.*, 1988; Libertini *et al.*, 1988; Garcia-Ramirez *et al.*, 1995). This in turn could allow the nucleosomal DNA to partially unwind, facilitating transcription factor binding in certain instances. Recently, the crystal structure of a nucleosome was solved to a 2.8 Å resolution, which showed that the N-terminal tails of the core histones pass over and between the DNA strands wrapping the histone octamer, and contact neighbouring nucleosome particles (Luger *et al.*, 1997). This is suggestive of a possible role for histone acetylation in the decondensation of the 30 nm fiber, which is confirmed by studies showing that, at physiological ionic strength, and in the absence of linker histones, acetylated nucleosome arrays remain in an extended conformation relative to non-acetylated counterparts (Garcia-Ramirez *et al.*, 1995). Further, studies have shown that histone acetylation can enhance the mechanics of transcription, including transcription factor binding to mononucleosomal DNA (Lee *et al.*, 1993, Vettese-Dadey *et al.*, 1996), and transcription of nucleosomal templates *in vitro* (Ura *et al.*, 1997). Exactly how acetylation mediates these effects on these less organized structures is unknown.

1.5. Linker Histones

Linker histones have been defined as abundant basic proteins which interact with the linker DNA between nucleosome cores (Wolffe *et al.*, 1997). These proteins generally, but not exclusively, have a tripartite structure consisting of a central globular domain flanked by unstructured N and C terminal domains (Wolffe *et al.*, 1997 and references therein). The exception to this are the linker histones in protozoa such as *Tetrahymena* (Wu *et al.*, 1986) and Trypanosomes (Toro *et al.*, 1993), which lack a central globular domain. It is believed that linker histones are involved in the packaging of nucleosomes into the 30 nm fiber (Renz *et al.*, 1977; Thoma *et al.*, 1979, Shen *et al.*, 1995) by either neutralizing the negative charge of the linker DNA and stabilizing the compaction of nucleosome arrays (Clark and Kimura, 1990), or through mediating the interaction of adjacent nucleosomes (Thoma *et al.*, 1979; Mamam *et al.*, 1994).

Although the function of linker histones in chromatin is apparent, there is considerable debate as to where on the nucleosome these histones bind. Two models have been proposed: a symmetrical arrangement and an "off-axis" location. The symmetrical model (Figure 7A) largely arises from the fact that a nucleosome core particle contains neither a linker histone nor linker DNA (Figure 1) whereas the chromatosome contains a linker histone and 166-168 bp of DNA. The histone octamer has a dyad-axis of rotational symmetry; this led researchers to believe that the linker histone binds in a symmetrical

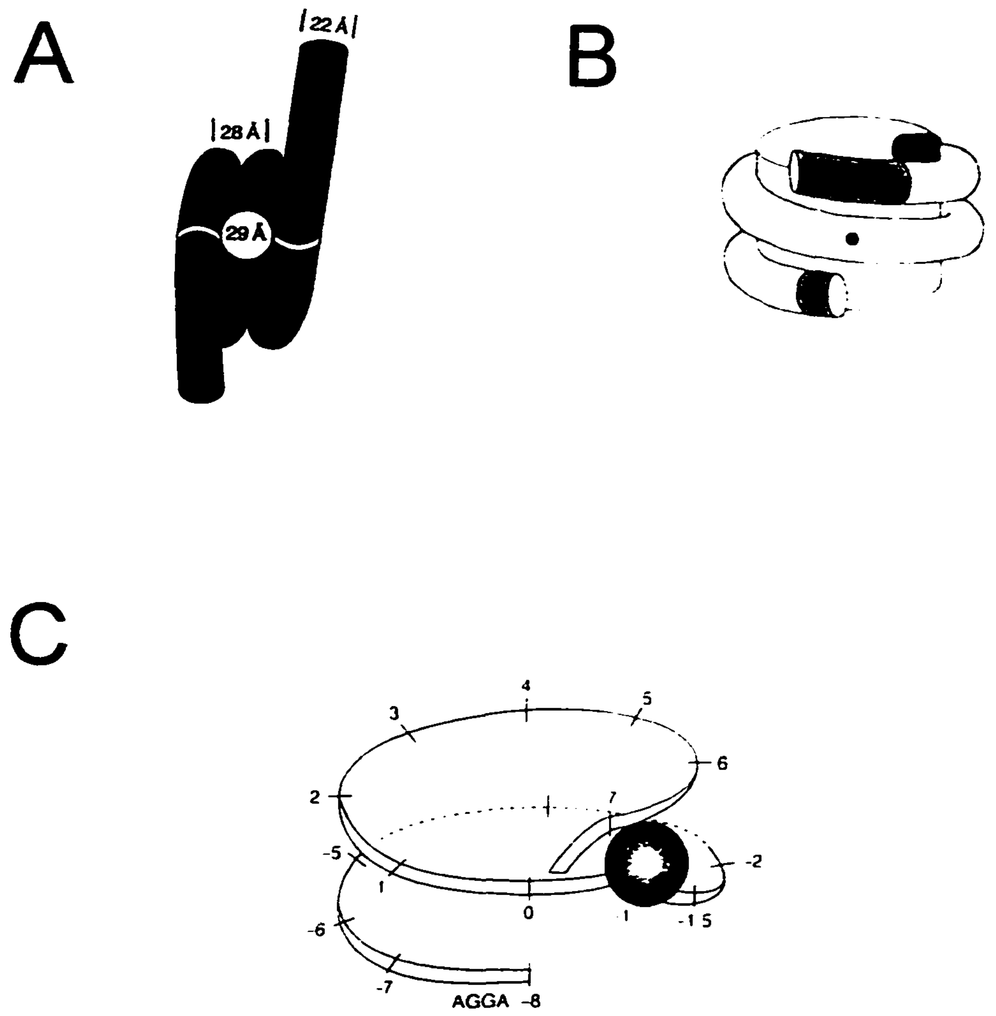

Figure 7. Three proposed models for the location of linker histones within the nucleosome. (**A**) the symmetrical arrangement model (Allan *et al.*, 1980) in which the linker histone binds to the DNA entering an exiting the nucleosome, making equal contacts with both strands. (**B**) an "off-axis" location for linker histones described by Hayes and Wolffe, 1993 in which the globular domain of the linker histone (solid black) binds to the major groove on the inside of the DNA superhelix (open tube). The cylinder represents the histone octamer, the shaded tube the DNA protected from micrococcal nuclease digestion in the chromatosome and the dot the dyad axis of the nucleosome. (**C**) The model of Travers and Muyldermans, (1996) in which the globular domain of the linker histone straddles two DNA strands at the point just within the boundary (site 7) of the core particle DNA.

fashion, making equal contacts with the DNA strands entering and exiting the nucleosome. Evidence to this effect is that linker histones preferentially bind four-way junctioned (Varga-Weisz *et al.*, 1993) and supercoiled DNA (Ivanchenko *et al.*, 1997) relative to linear DNA. The chief argument against this model is based on the fact that DNase I digestion has failed to conclusively show protection in these regions

There are currently two "off axis" models (Figure 7B and C) for the binding of linker histones to nucleosomes (Hayes and Wolffe, 1993; Travers and Muyldermans, 1996). Both models imply that the linker histone binds to either the entering or exiting strand, but not both. It must be noted that the model shown in Figure 7B relies on experiments performed exclusively with the *Xenopus borealis* somatic 5S rRNA gene (Hayes and Wolffe, 1993; Hayes *et al.*, 1994; Pruss *et al.*, 1996) which may not represent a typical nucleosome structure.

The most predominant linker histone in somatic cells is histone H1. Due to the results of several *in vitro* transcription studies (Schlissel and Brown, 1988; Wolffe, 1989, Laybourn and Kadonaga, 1991; Ura *et al.*, 1995), the incorporation of histone H1 into chromatin has long been described as a generally repressive toward transcription by all three RNA polymerases. This is further supported by studies which show that histone H1 inhibits transcription factor binding to nucleosomal DNA (Juan *et al.*, 1994, 1997) as well as reduces the mobility of nucleosomes on DNA templates (Pennings, *et al.*, 1994; Ura *et al.*, 1995). Finally, it was shown that transcriptionally active genes have reduced levels of histone H1 relative to inactive genes (Zlatanova and van Holde, 1992 and references therein). Recent lines of evidence have shown that, in some cases, the presence of histone H1 does not affect the assembly of transcription machinery. It has been demonstrated that the RNA polymerase I basal transcription factor, UBF, is able to displace histone H1 when binding nucleosomal DNA (Kermekchiev *et al.*, 1997). Furthermore, the RNA polymerase II *trans*-acting factor, NF-1, has been shown to bind linker DNA regardless of the presence of histone H1 (Eisfeld *et al.*, 1997). These results suggest that although histone H1 can repress transcription in some instances, it may be a rather selective repressor of transcription initiation.

1.6. Chromatin Remodeling Factors

The presence of chromatin structures on promoter elements is largely repressive toward transcription initiation, and thus these structures need to be disrupted to allow DNA-binding proteins access to their target sequences. In many organisms, protein complexes have been identified which are capable of remodeling chromatin structures and/or stimulating *trans*-acting factor binding to nucleosomal DNA. These "chromatin remodeling factors" can be divided into two groups: 1) histone binding chaperones involved in nucleosome assembly/disassembly and 2) energy-consuming, multi-enzyme complexes which modify nucleosome structures. The former category includes nucleoplasmin in *Xenopus* (Chen *et al.*, 1994; Walter *et al.*, 1995), and NAP-1 (nucleosome assembly protein) in mammals (Walter *et al.*, 1995), yeast (Ishimi and Kikuchi, 1991), plants (Yoon *et al.*, 1995) and *Drosophila* (Ito *et al.*, 1996b). Both proteins are highly acidic, bind preferentially to histones H2A and H2B, and stabilize *trans*-acting factor binding to nucleosomal DNA by removing H2A/H2B subsequent to transcription factor binding (Chen *et al.*, 1994; Walter *et al.*, 1995). The second category of energy dependent remodelling factors, include the SWI/SNF (switch of the mating type/sucrose nonfermenting) (Côte *et al.*, 1994) and RSC (remodel the structure of chromatin) (Cairns *et al.*, 1996) complexes in yeast, the NURF (nucleosome remodeling factor) (Tsukiyama and Wu, 1995), CHRAC (chromatin accessibility complex) (Varga-Weisz *et al.*, 1997), ACF (ATP utilizing chromatin assembly factor) (Ito *et al.*, 1997) and BRM (brahma) (Tamkum *et al.*, 1992) complexes in *Drosophila*, and the BRG1 (brm/*SWI2* - related gene 1) (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993) complex in mammals. Each of these multi-subunit complexes contains a closely related ribonucleotide triphosphate (NTP) binding subunit and is able to disrupt chromatin structures *in vivo* and *in vitro* as detected by alteration in nuclease digestion patterns. It must be noted that the specific nature of the alterations in chromatin structure made by these complexes is unknown.

1.7. The 5S rRNA Genes of Xenopus laevis

Xenopus laevis produces two major types of 5S rRNA, an essential component of ribosomes: the somatic type and the oocyte type. Each 5S rRNA type is transcribed from a distinct multigene family. The oocyte genes exist as 20 000 copies per haploid genome (Brown *et al.*, 1977) in clusters of ~1000 copies spread through most of the *Xenopus* chromosomes (Callan *et al.*, 1987). The oocyte genes can range in size from between 650 to 850 bp, and contain the 5S rRNA transcribed sequence, a pseudogene, and extremely AT rich, non-transcribed spacers (Federoff and Brown, 1978) (Figure 8). The somatic genes are 880 bp in length, and are present in 400 copies per haploid genome (Peterson *et*

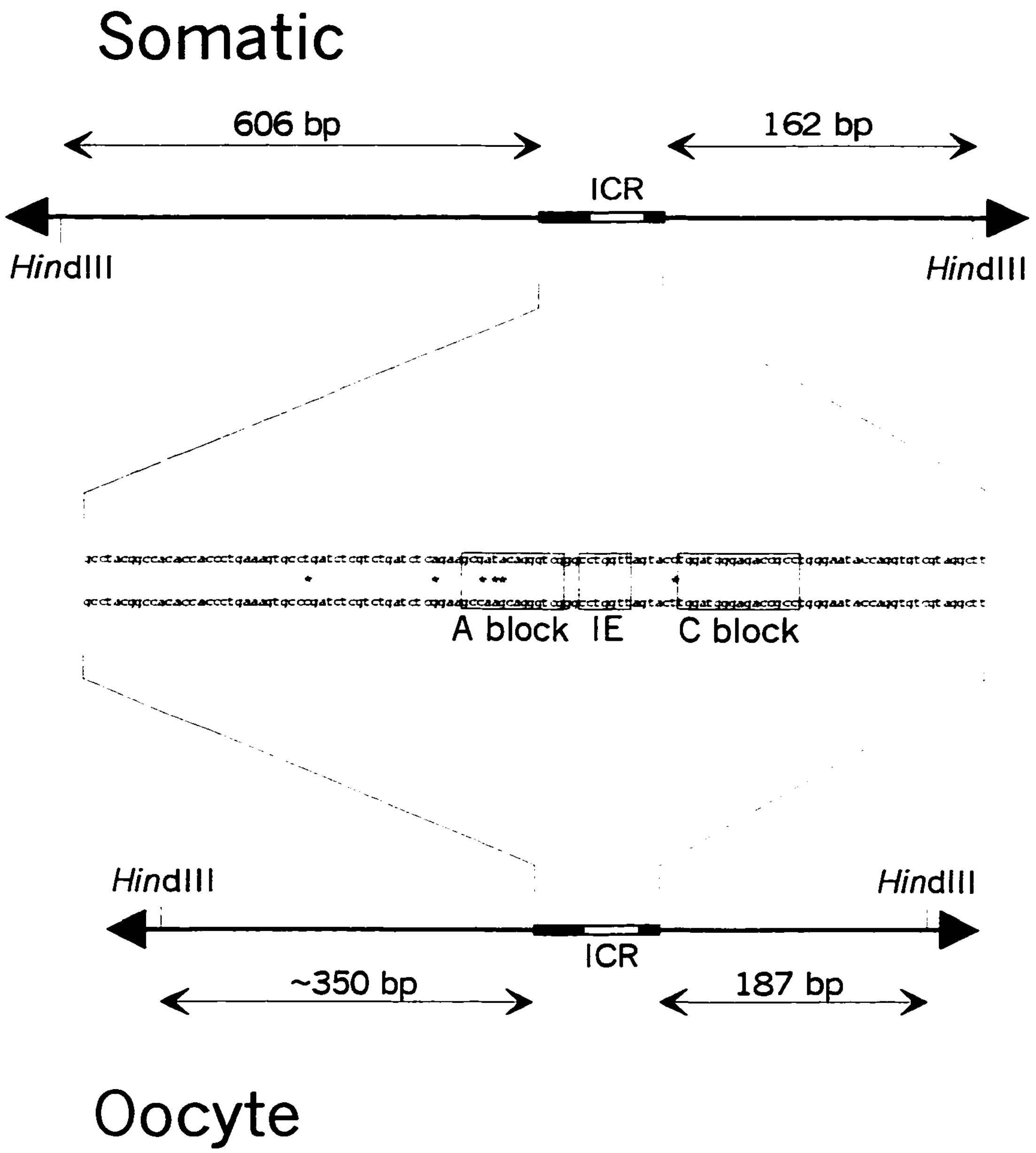

Figure 8. Schematic representation of the oocyte and somatic 5S rRNA genes in *X. laevis*. The heavy line represents the 5S rRNA coding sequence and the open box the intragenic promoter containing the A block, the intermediate element (IE) and the C block. Differences in nucleotide sequence between the oocyte and somatic genes are indicated by an *.

al., 1980), mostly clustered at one loci on a single chromosome (Callan *et al.*, 1987). In contrast to the oocyte genes, the somatic genes contain relatively GC rich non-transcribed spacers. Both the oocyte and the somatic 5S rRNAs are 120 nucleotides in length differing at 6 nucleotide positions (Figure 8).

1.8. 5S rRNA Transcription Machinery

The 5S rRNA genes have type 1 RNAP III promoters. These promoters are characterized by an intragenic control region (ICR) comprised of three sequence elements: the A block (nucleotides +50 to +64 with respect to the transcriptional start site), the intermediate element (IE) (+67 to +73) and the C block (+80 to +97) as shown in Figure 9 (Geiduschek and Kassavetis, 1992 and references therein). Three transcription factor complexes are required for assembly of a pre-initiation complex on the 5S rRNA gene: transcription factors TFIIIA, TFIIIB and TFIIIC. Originally, the terms TFIIIA, TFIIIB. and TFIIIC represented the different fractions of nuclear extracts required for reconstitution of 5S rRNA transcription, and the actual protein compositions of these fractions were unknown. Currently researchers have characterized the individual proteins in these fractions well in yeast, but in higher eukaryotes the compositions of TFIIIB and TFIIIC have not yet been agreed upon.

The first factor involved in the assembly of a transcription initiation complex on the 5S rRNA promoter is a single polypeptide known as transcription factor IIIA (TFIIIA). This protein binds DNA as a monomer and interacts with DNA through nine zinc fingers. TFIIIA has been best characterized in *Xenopus laevis* where it has been

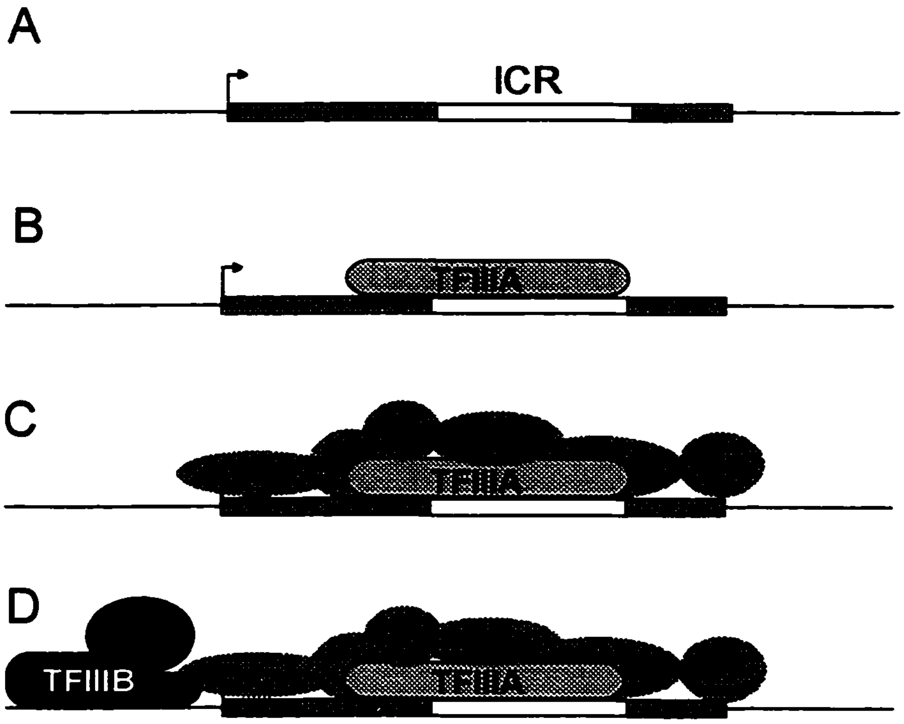

Figure 9. Model for the assembly of an RNA polymerase III pre-initiation complex on a 5S rRNA gene of *X. laevis*. The first step is the sequence specific recognition of the intragenic control region (ICR) by TFIIIA. TFIIIC binds to the TFIIIA/promoter complex through protein-protein interactions and DNA contacts. The final step in pre-initiation complex assembly is the positioning of TFIIIB upstream of the transcriptional start site. This figure is adapted from a similar Figure in Braun *et. al.*, 1992.

shown to interact most strongly and specifically with the C block and to form weaker interactions with the A block and the intermediate element (IE) (You *et al.*, 1991).

The second transcription factor required for the assembly of the 5S rRNA gene pre-initiation complex is TFIIIC. TFIIIC has been extensively purified in yeast and found to contain six distinct, tightly associated polypeptides (Braun *et al.*, 1992). Without the prior binding of TFIIIA, TFIIIC is unable to bind the 5S rRNA gene indicating that the binding of TFIIIC is mediated, at least in part, by protein to protein interactions with TFIIIA (Geiduschek and Kassavetis, 1992). This is not unexpected as TFIIIA and TFIIIC have been shown to associate in solution in the absence of DNA (Geiduschek and Kassavetis, 1992). Cross-linking studies in yeast (Braun *et al.*, 1992) and DNase I footprinting analysis in *Xenopus* (Stuges *et al*, 1995) have shown that TFIIIC also makes direct DNA contacts both upstream and downstream of the ICR. This indicates that DNA binding is also involved in the association of TFIIIC with the TFIIIA/5S rRNA gene complex.

Once TFIIIA and TFIIIC are bound to the 5S rRNA gene, this then allows the positioning of a final transcription factor complex, TFIIIB, upstream of the transcriptional start site (Geiduschek and Kassavetis, 1992). Like TFIIIC, TFIIIB cannot bind the 5S rRNA gene alone and requires prior binding of TFIIIC and TFIIIA. The components of yeast TFIIIB have been cloned and functional recombinant TFIIIB has been reconstituted (Kassavetis *et al.*, 1995). This complex contains three distinct proteins one of which is the TATA binding protein (TBP), although evidence suggests that the TBP in TFIIIB does not bind DNA but rather the remaining two subunits, termed BRF and TFC5, serve this function (Braun *et al.*, 1992).

Once the three transcription factors are positioned on the 5S rRNA gene, they form an extremely stable transcription complex which requires only the addition of RNA polymerase III for transcription to be initiated (Schlissel and Brown, 1984). TFIIIA and TFIIIC can be removed from the complex without disrupting TFIIIB and this factor can remain tightly bound to the DNA despite the fact that it has no affinity for the 5S rRNA promoter without TFIIIA and TFIIIC (Geiduschek and Kassavetis, 1992). This phenomenon has led researchers to conclude that TFIIIA and TFIIIC serve only to position TFIIIB on the 5S rRNA gene and the recruitment of RNAP III resides solely with TFIIIB.

1.9. 5S rRNA Transcription During Development

The somatic 5S rRNA genes are transcribed in most cell types whereas the oocyte genes are transcribed during early oogenesis, early embryogenesis and in certain tissue culture cell lines (Wormington and Brown, 1983; Ford and Mathieson, 1976). During the embryogenesis of a female frog, primordial germ cells migrate to the ovaries and proliferate by mitosis. The oogonia then enter meiosis and begin to differentiate into oocytes, but are frozen in prophase. At this point these primary oocytes begin accumulating large amounts of 5S rRNA and approximately 95% of 5S rRNA present at this point is of the oocyte type (Figure 10). When the animal becomes sexually mature and undergoes treatment with hormones, some of the eggs re-enter meiosis and develop into mature eggs. At this point transcription ceases possibly due to phosphorylation of

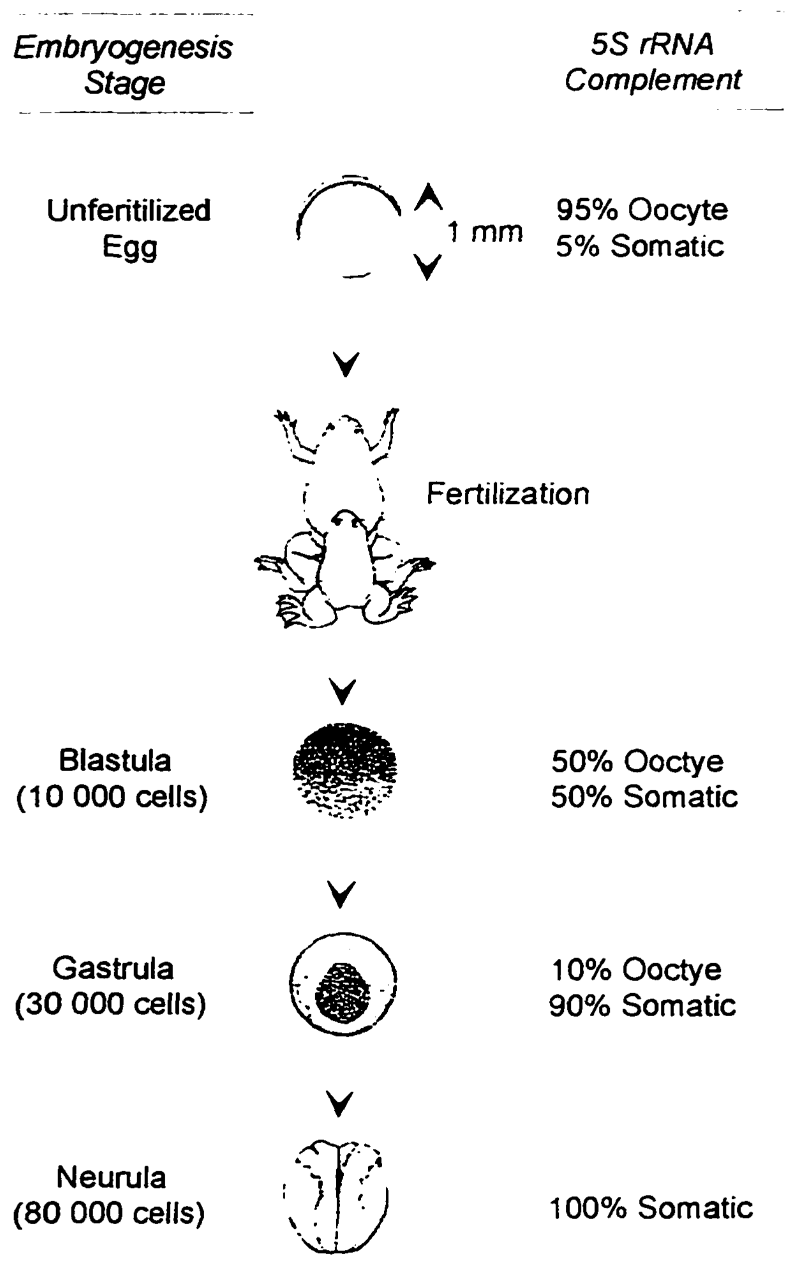

Figure 10. The relative amounts of oocyte and somatic 5S rRNA present during development (Wormington and Brown, 1986). Illustration from *Molecular Biology of the Cell*, ed. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson, 1983.

transcription factors (Hartl *et al.*, 1993; Gottesfeld *et al.*, 1994). Once the mature egg is fertilized, the embryo begins to divide rapidly. Again transcription is absent, presumably due to the fact that the cells division rate is greater than that required for transcription complex formation. When the embryo enters the blastula stage, transcription begins at a point designated the mid-blastula transition. At this point the 5S rRNA transcripts present, albeit at very low levels, are 50% oocyte and 50% somatic type. When the embryo reaches the gastrula stage, only 10% of the 5S RNA produced is from the oocyte gene. By the time the neurula stage of embryogenesis is reached, the oocyte genes are transcriptionally silent and all the 5S rRNA present is the somatic type.

1.10. Role of Chromatin Structures in the Differential Expression of the 5S rRNA Genes in X. laevis

Considerable research has focused on understanding the differential expression of the 5S rRNA genes in *Xenopus*. One hypothesis suggests that the transcription complexes which form on the oocyte genes are relatively unstable when compared to those of the somatic counterparts. As a result of this, the oocyte genes are transcribed only when TFIIIA levels are relatively high as is the case during oogenesis. In somatic cells, in which TFIIIA levels are much lower, the transcription complexes dissociate from the oocyte gene allowing the subsequent assembly of repressive nucleosome structures which preclude further factor binding (Wolffe, 1994 and references therein). This is seemingly confirmed by the fact that transcription of oocyte 5S rRNA genes in *Xenopus* embryos can be prolonged by injection of mRNA encoding TFIIIA (Bouvet *et al.*, 1994). Figure 11A demonstrates the relationship between TFIIIA levels and transcription of the oocyte 5S

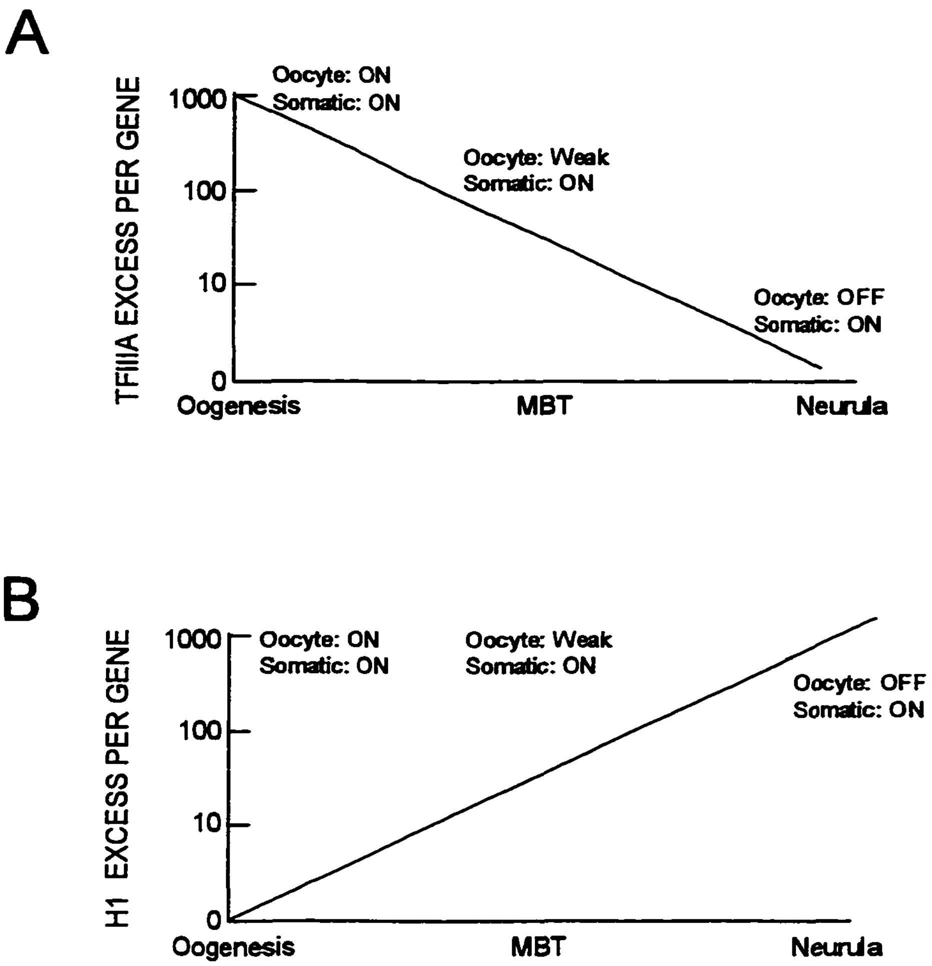

Figure 11. The relationship between TFIIIA/histone H1 levels and the transcription of the oocyte and somatic 5S rRNA genes in *X. laevis* (Wolffe, 1994). **(A)** The number of molecules of TFIIIA per 5S rRNA gene at the different stages of development and the transcriptional activity of the oocyte and somatic 5S rRNA genes at these stages. MBT is the mid-blastula transition. **(B)** The molar excess of histone H1 per 5S rRNA gene during development compared to the transcriptional activity of the oocyte and somatic 5S rRNA genes at the different stages.

rRNA genes during development.

A second hypothesis for the differential transcription of the 5S rRNA genes came from a series of *in vitro* 5S rRNA transcription studies. One study demonstrated that native chromatin from a *Xenopus* kidney cell line can serve as a template for transcription of the oocyte gene only after the selective release of histone H1 (Schlissel and Brown, 1984). A second study showed that histone H1 specifically represses transcription of an oocyte gene in chromatin reconstituted *in vitro*, while leaving a corresponding somatic gene unaffected (Tomaszewski and Jermanowski, 1997). These results suggest that it is the presence of the linker histone H1 which is responsible for the repression of oocyte transcription in somatic cells. In accordance with this hypothesis, the oocyte genes are transcribed during oogenesis simply because *Xenopus* oocytes are devoid of H1. Further evidence to support this is the fact that incorporation of a somatic histone H1 variant into chromatin during embryogenesis results in specific repression of TFIIIA-activated oocyte 5S rRNA transcription (Bouvet *et al.*, 1992). Figure 11B demonstrates the relationship between histone H1 levels and oocyte 5S rRNA transcription during embryogenesis.

One final hypothesis is that the differential regulation of the 5S rRNA genes is due to different levels of acetylation of histones packaging the two gene families. Histone acetylation has been shown to enhance the binding of TFIIIA to nucleosomal DNA (Lee *et al.*, 1993) and the transcription of 5S rRNA from dinucleosomal templates (Ura *et al.*, 1997).

1.11. Objectives

The work in this thesis elaborates on the above mentioned studies. The role of chromatin structures on the differential regulation of 5S rRNA transcription in *Xenopus laevis* was addressed in four different areas. First, the effect of histone octamer translational position on the binding of TFIIIA to nucleosomal DNA was studied. Second, the relationship between histone acetylation and the transcriptional silencing of the *Xenopus laevis* oocyte 5S rRNA genes in somatic cells was investigated. Third, it was determined whether histone H1 preferentially interacts with nucleosomal oocyte 5S rRNA genes when compared to the somatic counterparts. Finally, the effect of the histone binding protein, nucleoplasmin, on the binding of TFIIIA to nucleosomal 5S rRNA genes was tested.

2. MATERIALS AND METHODS

2.1. DNA Fragments

The plasmids used in this work were generous gifts of Dr. Paul Romaniuk (University of Victoria, Victoria, B.C.), Dr. Don Brown (Carnegie Institution of Washington, Baltimore, MD) and Dr. Robert Simpson (Pennsylvania State University, State College, PA) and are schematically represented in Figure 12. The *X. laevis* oocyte and somatic 5S rRNA genes exist in clustered, tandemly repeating copies which can be isolated as individual units by digestion with the restriction enzyme *Hin*d III. Plasmid pXls11 (Peterson *et al.*, 1980) contains a single copy of the 880 bp *Hin*d III fragment of the *X. laevis* somatic 5S rRNA gene cloned into the *Hin*d III site of the plasmid vector pUc19. The plasmid pXlo(Δ3'+176) (Bogenhagen and Brown, 1981) contains a 529 bp copy of the *X. laevis* oocyte gene truncated 56 bp downstream of the 5S rRNA coding sequence and cloned into the *Hin*d III and *Eco*R I sites of the plasmid vector Bluescript. Plasmid pXlo720 contains a single copy of the oocyte gene cloned into the *Hin*d III site of pBluescript. Plasmids pXlo and pXls each contain a copy of the oocyte and somatic 5S rRNA coding sequences respectively, cloned into pUC vectors immediately downstream of a T7 RNA polymerase promoter (Romaniuk *et al.*, 1987).

Five, ~200 bp, overlapping fragments of the *X. laevis* somatic and oocyte 5S rRNA genes were subcloned from plasmids pXls11 and pXlo(Δ3'+176). The nomenclature used to describe these new plasmids is based on the source of the gene fragment (Xlo for *X. laevis* oocyte 5S rRNA gene and Xls for the somatic) and the position of the new gene fragment termini in relation to the transcriptional start site. Due

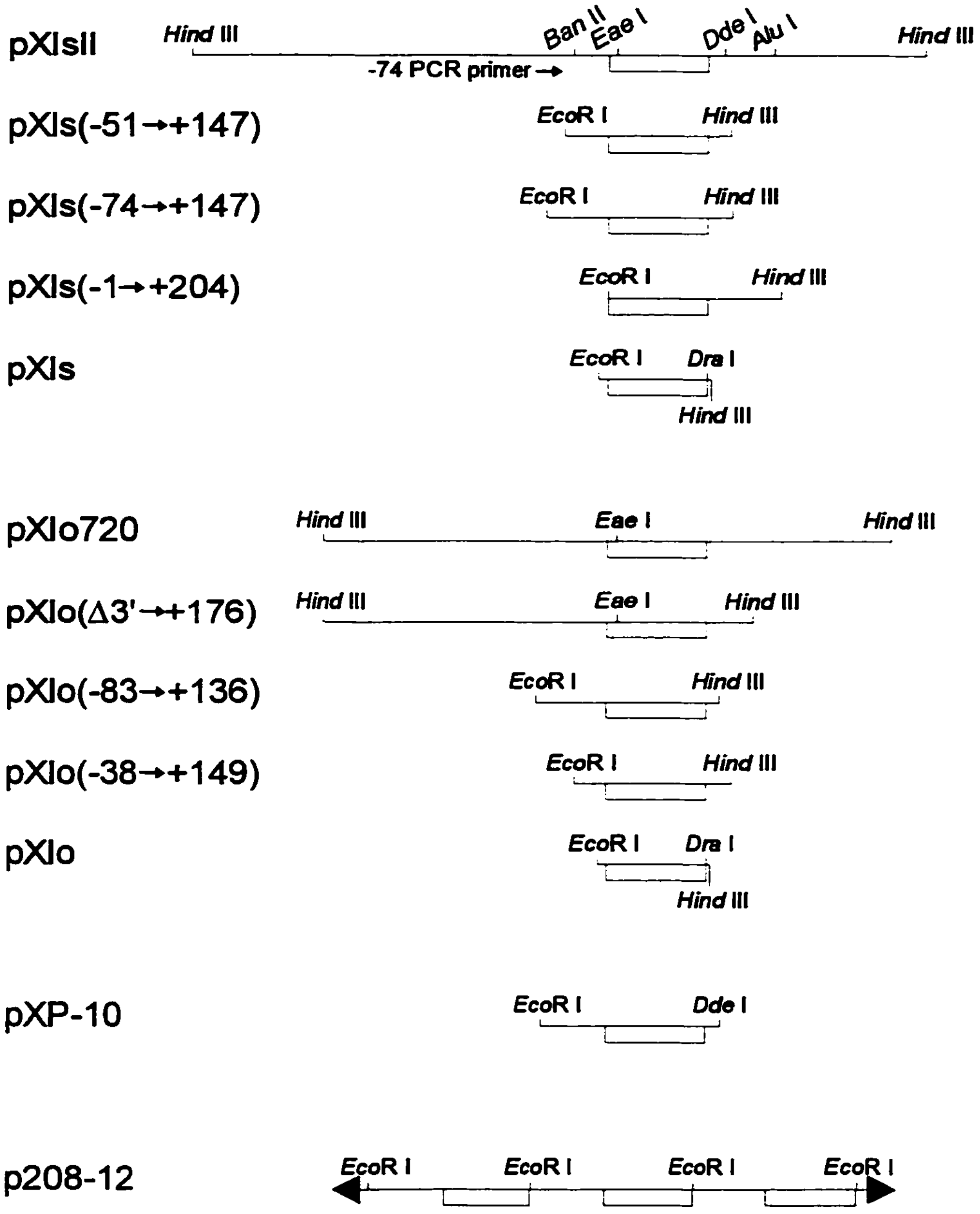

Figure 12. Schematic representation of the gene fragments used in this study. The open box represents the 5S rRNA coding sequence and the restriction enzyme sites employed are indicated.

to the lack of restriction sites in the oocyte gene, exonuclease III was used to create deletions of pXlo(Δ3'+176) to generate plasmids pXlo(-83→+136) and pXlo(-38→+149). The exonuclease III digestions were performed independently on each termini as described by Sambrook *et al*, (1989). The plasmids containing the *X. laevis* somatic 5S rRNA gene fragments designated pXls(-51→+147) and pXls(-51→+204) were created by digesting pXls11 with *Ban* II/*Dde* I and *Eae* I/*Alu* I respectively, removing the protruding 3' termini or filling in the protruding 5' termini with Klenow (Sambrook *et al*, 1989), and ligating the restriction fragments into the *Eco*R V site of Bluescript. A third plasmid containing the somatic 5S rRNA gene fragment designated pXls(-74→+147) was created by polymerase chain reaction amplification of a gene fragment from pXls11 with the primers $^{5'}$GTAAAACGACGGCCAGT$^{3'}$ and $^{5'}$CTTGGGAATTCAGCCCTGC$^{3'}$. The former primer anneals to the plasmid vector downstream of the 5S rRNA coding sequence and the latter primer to a sequence within the 5S gene upstream untranscribed region creating and *Eco*R I site 74 bp upstream of the transcription start site. Following PCR, the amplified product was *Eco*R I/*Hin*d III digested and ligated into Bluescript. The resulting plasmid was digested with *Dde* I, treated with Klenow to remove the 3' protruding termini (Sambrook *et al*, 1989), and the fragment containing the somatic gene ligated into the *Eco*R V site of Bluescript. As a result of the above sub-cloning steps, each 5S rRNA gene fragment was flanked by an *Eco*R I site upstream and a *Hin*d III site downstream of the 5S rRNA coding sequence. A 214 bp *Eco*R I-*Dde* I fragment derived from the plasmid pXP-10 (Wolffe *et al.*, 1986) was used for the experiments involving the *Xenopus borealis* 5S rRNA gene. This fragment can be described as Xbs(-75→+147) using our nomenclature.

For experiments requiring the full-length 5S rRNA gene repeat, 1 mg of plasmids pXls11 and pXlo720 were digested with 4000 units of *Hin*d III in 10 mL of 10 mM Tris-HCl, (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT for 4 hours at 37°C. The 720 bp oocyte and 880 bp somatic full-length 5S rRNA genes were isolated by repeatedly passing the *Hin*d III digests over a 3 × 70 cm Sephacryl S-1000 column (Garcia-Ramirez *et al.*, 1992) equilibrated with 10 mM Tris-HCl (pH 8), 1 mM EDTA at 60 mL/hour.

The 208-12 DNA construct consists of twelve, tandemly arranged fragments of the *Lytechinus variegatus* 5S rRNA gene (Simpson *et al.*, 1985) cloned into the *Eco*R I site of a pUC vector. The 208-12 fragment was liberated from the plasmid by digesting the plasmid sequence with the restriction enzyme *Hha* I (there are seventeen *Hha* I sites in pUC vectors and no *Hha* I sites within the 208-12 fragment). The 2.5 Kb 208-12 fragment was isolated from vector sequences by Sephacryl S-1000 chromatography as described earlier.

2.2. *Labeling of DNA*

2.2.1. *Labeling of DNA for electrophoretic mobility shift analysis or use as radiolabeled probes*

Plasmid inserts were excised by restriction enzyme digestion and 3' end-labeled by adjusting the digestion reaction to 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, 33 nM $[\alpha\text{-}^{32}P]$dATP (3000 μCi/mmol), and adding 4 units of Klenow. The reactions were incubated at room temperature for 30 minutes and the labeled fragments were purified from plasmid DNA and unincorporated label by electrophoresis on a 4% acrylamide gel

(30:0.8, acrylamide:bis-acrylamide) in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA). After autoradiography of the gel, the band containing the labeled DNA was excised and the DNA eluted from the gel slice by rotation for 16 hours in 400 µL of 0.6 M ammonium acetate, 0.1% SDS, 1 mM EDTA. The DNA was ethanol precipitated and resuspended in dH_2O.

2.2.2. Labeling of DNA for micrococcal nuclease digestion

For the micrococcal nuclease digestions, for which internally labeled DNA was required, approximately 2 µg of plasmid DNA was denatured in 10 µL of 0.2 M NaOH, 0.2 mM EDTA for 30 minutes at 37°C. The denatured plasmid was ethanol precipitated and annealed to 20 pmoles of the M13 sequencing primer $^{5'}$GTAAAACGACGGCCAGT$^{3'}$ in 10 µL dH_2O by incubation at 37°C for 30 minutes. The labeling was performed in 20 µL of 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM dCTP, dGTP, dTTP, 10 µM dATP, 50 nM [$\alpha^{32}P$] dATP (3000 µCi/mmol) with 4 units of Klenow and incubation at room temperature for 30 minutes. The Klenow was heat inactivated at 65°C for 15 minutes and the labeled DNA was digested with 10 units each of *Eco*R I and *Hin*d III for 90 minutes at 37°C. The labeled DNA fragments were purified by gel electrophoresis as described above. Using this technique a DNA fragment internally labeled on only the coding strand was produced which facilitated the interpretation of results of the nucleosome translational position analysis.

2.2.3. Labeling DNA for DNase I footprinting

For DNase I footprinting analysis, which required DNA end-labeled on only one strand, approximately 2 µg of plasmid DNA was digested in 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, with *Eco*R I for labeling of the non-coding strand and with *Hin*d III for labeling of the coding strand. Labeling was initiated by adjusting the solution to 100 µM dCTP, dGTP, dTTP and 33 nM [$\alpha^{32}P$] dATP (3000 µCi/mmol), adding 4 units of Klenow and incubating for 30 minutes at room temperature. Following this the Klenow was heat inactivated, and the DNA was restriction digested at the remaining flanking site and purified by gel electrophoresis as described above.

2.3. Protein Purification

The purification of recombinant TFIIIA from *Escherichia coli* cells harboring the expression plasmid pTF3 was carried out as described previously (Veldhoen *et al.*, 1994). The technique of Ausio *et al.*, (1989) was used for the isolation of nucleosome core particles from chicken erythrocytes. Briefly, nuclei from red blood cells were isolated by repeatedly washing chicken erythrocytes with 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5% Triton X-100 until the pellet was completely white. The nuclei were resuspended in 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and digested with 12 units of micrococcal nuclease per microgram of DNA for 7 minutes at 37°C. The digestion was stopped by adjusting the solution to 5 mM EDTA and the nuclei were pelleted by centrifugation at 5000×g. The chromatin was released by lysis of the nuclei in 0.25 mM EDTA (pH 7.5) with stirring for two hours at 4°C. The lysate was centrifuged at 8000×g for 20 minutes, the supernatant was adjusted to 0.35 M NaCl and linker histones and non-histone proteins were removed by shaking the solution with 12 mg of CM C-25 Sephadex/mg of DNA for two hours at 4°C. The resin was removed by centrifugation and the supernatant was dialyzed overnight against 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$. Following dialysis, the chromatin was digested again with 40 units of micrococcal nuclease per µg of DNA for 7 minutes at 37°C to generate nucleosome core particles. Final purification of the core particles was achieved by centrifugation in a 5 to 20% sucrose gradient [in 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA] at 25 000 rpm for 24 h in a Beckman SW-41 rotor at 4°C.

Nucleosome core particles with low or high levels of core histone acetylation were obtained, as described by Ausio and Van Holde, (1986), from HeLa cells grown in the absence or presence of 10 mM sodium butyrate respectively. Briefly, HeLa cells (S3 strain) were grown, to a cell count of 5×10^5 cells/ml, in Joklik modified minimal essential media (Gibco) supplemented with 2 g/L sodium bicarbonate, 1× PSN antibiotic (Gibco), and 10% new born calf serum. After harvesting the cells by centrifugation, nuclei were isolated by washing the cells three times in 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES (pH 6.5), 10 mM sodium butyrate, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 % Triton X-100 and 0.1 mM PMSF. The nuclei were pelleted at 3000×g, resuspended in 10 mM PIPES (pH 6.8), 10 mM sodium butyrate, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF and digested with 30 units of micrococcal nuclease per µg of DNA, for 7 minutes at 37°C. The digestion was stopped by adjusting the solution to 5 mM EGTA and the nuclei were pelleted at 3000×g. The supernatant, which contained approximately 17% of the initial DNA, was concentrated using a centricon 30 microconcentrator (AMICON Inc., Beverly, MA) and loaded onto a 5-20% linear sucrose gradient in 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.1 mM PMSF and centrifuged at 27 000 rpm for 20 h in a Beckman SW-41 rotor at 4°C.

The purification of histone H1 from HeLa cells was carried out as described previously (Garcia-Ramirez *et al.*, 1990). Purified histone octamers were purified by others from chicken erythrocytes (Hansen *et al.*, 1989). Nucleoplasmin was purified by others from *Xenopus* eggs (Rice *et al.*, 1995).

2.4. Protein Electrophoresis

The purity of the proteins used in this study was verified by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). To analyze the levels of core histone acetylation, purified HeLa histones were electrophoresed on a 6% acetic acid, 8 M Urea, 8 mM Triton X-100 gel as described by Bonner *et al.*, (1980). Analysis of the histone composition of nucleosomes reconstituted by salt gradient dialysis was carried out by two dimensional electrophoresis as follows. Approximately 10 μg of reconstituted nucleosomes were resolved on a 4% acrylamide gel (30:0.8 acrylamide:bis-acrylamide) at 10 V/cm in 0.5× TBE buffer. Following staining with ethidium bromide, the nucleosome band was excised and the gel slice equilibrated at room temperature in 1× SDS sample buffer (Laemmli, 1970) for 30 minutes. The gel slice was then loaded onto a 15% SDS-PAGE (Laemmli, 1970) and run in the second dimension. The gel was stained with Coomassie blue and scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

2.5. Nucleosome Reconstitution.

2.5.1. The octamer transfer method of reconstitution

The octamer transfer method (Rhodes and Laskey, 1989) was used to reconstitute mononucleosomes containing the ~200 bp radiolabeled fragments of the oocyte and somatic 5S rRNA genes. Initially, several different octamer transfer methods were used but finally a modification of the method described by Bresnick *et al.*, (1991) was employed as it gave the maximum incorporation of DNA into nucleosomes with the minimal amount of nucleosome aggregation. Using this technique, approximately 200 fmol of labeled DNA and 3 μg of unlabeled nucleosome cores (isolated from either chicken erythrocytes, non-butyrate treated HeLa cells or butyrate treated HeLa cells as indicated) were mixed in 25 μL of 0.8 M NaCl, 50 mM Tris-HCl (pH 8), 1 mM β mercaptoethanol, 0.1 mM PMSF. Following incubation for 30 minutes at 37°C, the nucleosomes were incubated at 4°C for 16 hours. The reconstitution reactions were then serial diluted to 0.6 and 0.1 M NaCl by addition of 50 mM Tris-HCl (pH 8), 0.1 mM PMSF after 30 minutes intervals at 4°C.

2.5.2. Salt-gradient dialysis reconstitution

The salt gradient dialysis method (Tatchell and van Holde, 1977) was used in situations where large amounts of reconstituted nucleosomes were required. To this end, 100 μg of DNA was mixed with 75 μg of chicken erythrocyte histone octamers (one octamer/217 bp) in 1 mL of 2 M NaCl, 10 mM Tris-HCl (pH 7.5). The mixture was incubated overnight at 4°C before dialyzing for 2.5 hours intervals against 1.5, 1.2, 1, 0.6

and 0.3 M NaCl, in 10 mM Tris-HCl (pH 7.5). The samples were then dialyzed overnight against 100 mM NaCl, 10 mM Tris-HCl (pH 7.5).

2.5.3. Salt-gradient dialysis reconstitution with incorporation of histone H1

The 208-12 construct and full-length 5S rRNA gene repeats, at a concentration of 100 μg/mL, were mixed with a 3-4 fold molar excess of histone octamers (one octamer/220 bp) in 2 M NaCl and dialyzed by step gradient to 0.5 M NaCl as described above. The reconstitution reactions were diluted six fold in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5) and concentration to the original volume using a centricon 30 microconcentrator (AMICON Inc., Beverly, MA). Following concentration, histone H1 [dissolved in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA] was added at 1 mole of H1 per mole of histone octamers and the reactions were dialyzed against 10 mM NaCl, 10 mM Tris-HCl (pH 7.5) overnight at 4°C.

2.6. Determination of Nucleosome Translational Position

When native, H1 depleted chromatin is digested with micrococcal nuclease, 146 bp nuclease resistant fragments are produced due to interaction of the DNA with the histone octamer. Nucleosome translational position analysis was performed by mapping the positions of these micrococcal nuclease fragments with restriction enzymes. The reconstituted nucleosomes, at a concentration of 15 ng/μL DNA, were adjusted to 1 mM $CaCl_2$ and incubated at 37°C for 5 minutes. Following this, 0.2 units of micrococcal nuclease were added for every μg of DNA and incubation was continued for a further 15 minutes at the same temperature. The time of digestion was established from a previous time course of digestion analysis carried out under the same conditions. The digestion was stopped and the DNA deproteinized by adjusting the solution to 5 mM EDTA, 0.25% SDS and extracting with phenol/chloroform/isoamyl alcohol (25:24:1). The approximately 146 bp micrococcal nuclease resistant DNA fragments were isolated by gel electrophoresis on a 6% acrylamide gel in 0.5× TBE. After elution from the acrylamide as described above, the DNA was precipitated and cut with 5 units of restriction enzyme in 20 μL of appropriate restriction enzyme buffer for 3 hours at 37°C. Following this, the digested fragments were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, and resuspended in 95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 1 mM EDTA. The fragments were denatured at 90°C for 2 minutes and resolved at 20 volt/cm on a 8% acrylamide (19:1 acrylamide:bisacrylamide) and 8.3 M urea gel in 1× TBE (90 mM Tris-borate, 2 mM EDTA). Following electrophoresis the gel was dried under vacuum at 90°C and autoradiographed.

2.7. *Electrophoretic Mobility Shift Assays*

2.7.1. *TFIIIA electrophoretic mobility shift assays*

Approximately 1 fmol of labeled reconstituted nucleosomes were incubated in 10 μL of 20 mM Tris-HCl (pH 7.5), 70 mM NaCl, 10 μM $ZnCl_2$, 6% glycerol, 0.1 mg/mL BSA (bovine serum albumin), 2.5 mM DTT, 0.07% NP-40, 40 ng/μL poly(dI-dC)·poly(dI-dC) for 20 minutes at room temperature. The amount of nucleoplasmin and/or TFIIIA was as indicated. The binding reactions were loaded onto 0.75% agarose gels containing in 0.5× TB (45 mM Tris-borate) and run at 3.5 volts/cm at room temperature. The gels were dried at 50°C and autoradiographed. TFIIIA shifts of uncomplexed DNA were carried out in a similar manner with 100 ng/µL poly(dI-dC)·poly(dI-dC) added to the binding reactions.

2.7.2. *Histone H1 electrophoretic mobility shift assays*

Histone H1 binding reactions were performed with approximately 1-2 fmoles of labeled nucleosomes or free DNA in 10 µL of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6% glycerol, 0.1 mg/mL BSA, for 20 minutes at room temperature. The amount of histone H1 was as indicated in the figure legends. The binding reactions were electrophoresed on 0.75% agarose gels in 0.5× TBE at 3.5 volts/cm at room temperature. The gels were dried at 50°C and autoradiographed.

2.8. DNase I Footprinting Analysis

Ten femtomoles of labeled reconstituted nucleosomes were incubated with approximately a 50 fold molar excess of TFIIIA in 20 µL of 20 mM Tris-HCl (pH 7.5), 70 mM NaCl, 10 µM $ZnCl_2$, 6% glycerol, 0.1 mg/mL BSA, 2.5 mM DTT, 0.07% NP-40 for 20 minutes at room temperature. Immediately thereafter, 1 ng of bovine pancreatic DNase I was added and digestion was allowed to proceed for 1 minute at room temperature. Reactions were immediately loaded onto a 4% acrylamide gel (30:0.8

acrylamide:bis-acrylamide) in 0.5× TBE buffer and run at 10 volts/cm at room temperature. The gel was autoradiographed wet and bands containing the TFIIIA shifted and control nucleosomes were excised. The digested DNA was eluted as described earlier, ethanol precipitated and resuspended in 95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 1 mM EDTA. The DNA fragments were denatured at 90° C for 2 minutes and resolved on a 8% acrylamide (19:1 acrylamide:bisacrylamide) and 8.3 M urea gel in 1× TBE. Naked DNA, digested as above, was used as a control but in this latter case, the gel purification step was omitted. Instead the DNase 1 digestions were heat inactivated at 90°C for 5 minutes and the resulting DNA fragments were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. Maxim Gilbert reactions of free DNA were performed using the technique described in Sambrook *et al.*, (1988) and used as markers.

2.9. Micrococcal Nuclease Digestion of Chromatosomes and Southern Blot Hybridization.

Approximately 5 µg of nucleosomes reconstituted on full-length 5S rRNA gene repeats were adjusted to 1 mM $CaCl_2$ and digested with 1 unit of micrococcal nuclease. At time intervals, 1 µg aliquots were removed, and the digestion was stopped and the DNA deproteinized by adjusting the solution to 5 mM EDTA, 0.25% SDS and phenol/chloroform/isoamyl alcohol (25:24:1) extracting. The digestion products were ethanol precipitated and resolved on a 4% acrylamide gel (19:1 acrylamide:bis-acrylamide)

(Yager and van Holde, 1984). The gel was stained with ethidium bromide and the bands visualized under UV light.

Micrococcal nuclease digestion fragments resolved on 4% acrylamide gels were blotted onto Zeta-Probe membrane (Bio-Rad, Hercules CA) by applying the membrane dry to the gel, and vacuum drying at room temperature for 60 minutes (the gel detached once the membrane was rehydrated in 2× SSC [0.3 M NaCl, 30 mM trisodium citrate, (pH 7)]). After transfer, the membrane was placed DNA surface up for 10 minutes on three sheets of Whatman paper saturated with 0.4 M NaOH. The membrane was rinsed in 2× SSC, air dried and vacuum dried at 80°C for 30 minutes. For prehybridization, the membrane was rehydrated in 2× SSC and blocked in 5 mL of 1 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS at 65°C for 30 minutes. The prehybridization solution was replaced with the same buffer containing a denatured, 180 bp, [α-^{32}P] dATP end-labeled, *Hind* III/*Eco*R I fragment from plasmid pXls (Romaniuk *et al.*, 1987) and hybridization proceeded for 16 hours at 65°C. The membrane was washed at 65°C for 45 minutes each, 2× with 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 5% SDS and 2× with 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 1% SDS. The membrane was dried and autoradiographed.

2.10. In vitro Transcription of Reconstituted 5S rRNA Genes.

HeLa nuclear *in vitro* transcription extracts were purchased from Promega Corporation (Madison, WI) and *Xenopus* oocyte nuclear extracts were generous gifts of Dr. Chiaki Katagiri (Hokkaido University, Sapporo, Japan). When using HeLa extracts, transcriptions were performed on 250 ng of template (either reconstituted nucleosomes or uncomplexed DNA) with 8 units of transcription extract in 25 µL of 20 mM NaCl, 44 mM KCl, 9.1% glycerol, 88 nM EDTA, 0.22 mM DTT, 2 mM $MgCl_2$, 0.4 mM ATP, CTP, UTP, 7.4 mM HEPES, 2.9 mM Tris-HCl (pH 7.8) and 1 µM [α-^{32}P] GTP (400 µCi/mmol) at 30°C for 60 minutes. The extracts were supplemented with 150 nM recombinant *Xenopus* TFIIIA and for each transcription reaction an internal control of 50 ng CMV DNA was included. The reactions were terminated by addition of 175 µL of 0.3 M Tris-HCl (pH 7.4), 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA and 3 µg/mL tRNA, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and ethanol precipitated. When using the *Xenopus* oocyte nuclear extracts, 40 ng of template DNA was transcribed with 8.2 µL of transcription extract in 10 µL of 8.2 mM HEPES (pH 7.5), 1.6 mM DTT, 80 nM EDTA, 41 mM KCl, 0.4 mM ATP, CTP and UTP, 1 µM [α-^{32}P] GTP (400 µCi/mmol) and 4.1% glycerol at 30°C for 60 minutes. The reactions were terminated by addition of 90 µL of 0.3 M Tris-HCl (pH 7.4), 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA and 3 µg/mL tRNA, deproteinized and purified as described above. Precipitated transcripts were resuspended in 98% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, heated at 90°C for 10 minutes and electrophoresed at 20 volts/cm on a pre-electrophoresed 8% acrylamide (19:1 acrylamide:bisacrylamide) and 8.3 M urea gel in 1× TBE. The gel was then dried and autoradiographed.

2.11. Cell Culture and Northern Blot Analysis

Xenopus laevis K2 cells were a generous gift of Paul Labhart at the Scripps Research Institute in La Jolla, California. Cells were cultured at room temperature, without CO_2, in 0.5× Leibovitz L-15 media (Gibco) supplemented with 10 mM HEPES (pH 7.5), 1× PSN antibiotic (Gibco), 2 mM L-glutamine and 10% fetal calf serum. Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction technique (Chomczynski and Sacchi, 1987) and electrophoresed for 16 hours at 10 v/cm on a pre-electrophoresed (at 25 v/cm for 4 hours), 12.5% acrylamide (29:1 acrylamide:bis-acrylamide) and 2.5 M urea gel in 1× TBE. Following electrophoresis, the RNA was blotted onto a Zeta-Probe membrane as described above. The membrane was alkaline fixed, blocked and hybridized to a 180 bp, [α-^{32}P] dATP end-labeled, *Hin*d III/*Eco*R I fragment from plasmid pXlo (Romaniuk *et al.*, 1987). *In vitro* T7 RNA polymerase transcribed *X. laevis* oocyte and somatic 5S rRNA were prepared by digesting 2 µg of plasmids pXlo and pXls respectively (Romaniuk *et al.*, 1987) with *Dra* I, and transcribing with 50 units of T7 RNA polymerase in 50 µL of 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.1 mg/mL BSA and 0.5 mM NTPs. Reactions were terminated by addition of 98% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene blue.

2.12. Western Blot Analysis

Polyclonal antibodies specific for acetylated histone H4, prepared as previously described (Lin *et al.*, 1989), were a generous gift of Dr. David Allis (University of

Rochester, Rochester, NY). Western blot analysis (Perry *et al.*, 1993) was performed with histones isolated from HeLa cells grown in the presence of sodium butyrate (Ausió and Van Holde, 1986).

2.13. Control Immunoprecipitation

Two hundred nanograms of HeLa mononucleosome cores, either non-acetylated or hyperacetylated (fraction "c" from butyrate treated cells, Ausió *et al.*, 1989), were 5' end-labeled with 10 units of polynucleotide kinase in 20 μL of 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA and 40 nM [γ-^{32}P] ATP (3000 μCi/mmol). The labeling reaction was stopped by adjusting the solution to 10 mM EDTA and the labeled cores were diluted with a 1000 molar excess of unlabeled chicken erythrocyte mononucleosome cores to produce solution with a final mononucleosome concentration of 1 mg/mL. The samples were treated with 1% formaldehyde at room temperature for 20 minutes, dialyzed against 10 mM Tris-HCl (pH 7.5), 10 mM EDTA to remove the formaldehyde and immunoprecipitated as described in the following section.

2.14. Chromatin Isolation, Immunoprecipitation and Dot Blot Analysis

Chromatin fragments were isolated from *X. laevis* tissue culture cells fixed with 1% formaldehyde for 20 minutes at room temperature. The cells were harvested by centrifugation at 2000×g and resuspended sequentially, to a concentration of approximately 5.5×10^7cells/mL, in ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl (pH 7.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl (pH 7.5)]. The final pellet was resuspended in a one half volume of buffer III [1% SDS, 10 mM EDTA, 50 mM Tris-HCl, (pH 7.5), Complete Protease Inhibitor Cocktail (Boehringer Mannheim)] and sonicated 10 times for 10 sec each on setting 3.5 and 50% duty on a W-385 ultrasonic processor (Heat Systems Ultrasonics Inc.). Immunoprecipitation of hyperacetylated nucleosomes was performed by diluting the sonicated chromatin in buffer IV [1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 7.5)] to a final DNA concentration of 100 ng/µl, mixing 100 µg with 7.5 µL of undiluted polysera and rotating at 4°C for 16 hours. Immunocomplexes were precipitated by addition of 2 µg of chicken erythrocyte core particle DNA and 45 µL of protein A-Sepharose CL 4B beads [125 mg of resin equilibrated overnight in 1 mL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA], rotation was continued for one hour followed by centrifugation at 2000×g for 2 minutes. The beads were sequentially washed for 10 minutes each in 1 mL of buffer V [150 mM NaCl, 0.1% SDS, % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)], buffer VI [500 mM NaCl, 0.1% SDS, % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)] and buffer VII [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8)]. The beads were then washed 3× in 1 mL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA before extracting 3× with 133 µl of 1% SDS, 0.1 M $NaHCO_3$. The pooled eluates were heated at 65°C for 4 hours to reverse the formaldehyde cross-links and the released DNA was ethanol precipitated. The pelleted DNA was resuspended in dH_2O and deproteinized by digestion with 50 µg/mL proteinase K for 30 minutes at 37°C and extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was ethanol precipitated, resuspended in dH_2O and dot blotted onto Zeta Probe membranes using a Bio-Dot dot blot apparatus (Bio-Rad, Hercules CA). The membrane was blocked and hybridized, as described earlier, to a ~360 bp, [α-^{32}P] dATP end-labeled, *Hin*d III/*Eae* I fragment derived from plasmid pXlo720. This labeled fragment contains the 5' non-transcribed spacer of the *X. laevis* oocyte 5S rRNA gene. The 720 bp oocyte and 880 bp somatic full-length 5S rRNA gene repeats were used as oocyte and somatic 5S rRNA gene controls.

2.15. Chromatin isolation, micrococcal nuclease digestion and Southern blot analysis

Nuclei isolated from the *X. laevis* K2 kidney cell line (Ausió and Van Holde, 1986) were suspended in 10 mM PIPES (pH 6.8), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF and digested with 4 units/µg micrococcal nuclease at 37°C for various timed intervals. The digestions were terminated and the nuclei lysed by adjusting the solution to 10 mM EDTA, 0.5% SDS. The DNA was deproteinized by digestion with 0.2 mg/mL Proteinase K for 3 hours at 37°C followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The samples were ethanol precipitated, electrophoresed on a 1% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA), Southern blotted (Sambrook *et al*, 1989), and probed with the ~360 bp, [α-^{32}P] dATP end-labeled, *Hin*d III/*Eae* I fragment from plasmid pXlo720.

2.16. In vitro Nucleosome Assembly.

The ability of nucleoplasmin to assemble nucleosomes *in vitro* was tested by a supercoiling assay. Five hundred ng of histone octamers were incubated with or without 1 µg nucleoplasmin in 100 µL of 15 mM Tris-HCl (pH 7.5), 160 mM NaCl, 0.35 mM EDTA, 1 mM DTT, 1 mM β mercaptoethanol and 0.01% Nonidet P-40 for 45 minutes at room temperature. Following this, 0.5 µg of SV40 DNA in 5 µL of 50 mM Tris-HCl (pH

8), 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 20% glycerol and 7 units/μL of topoisomerase I was added and incubation proceeded for 90 minutes at 37°C. The reactions were centrifuged for 10 minutes at 15000×g and the supernatants were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. The different DNA topoisomers produced by deposition of histone octamers and subsequent supercoil relaxation were resolved by electrophoresis on a 2% agarose gel in 1× TPE (90 mM Tris-phosphate, 2 mM EDTA) at 4 volts/cm for 16 hours with buffer recirculation. The gel was ethidium bromide stained and the bands visualized under UV light.

3. RESULTS

3.1. Differential transcription of X. laevis oocyte and somatic 5S rRNA genes after reconstitution with histone octamers

To test whether the differential transcription of the oocyte and somatic 5S rRNA genes could be reproduced *in vitro*, histone octamers were reconstituted onto full length 5S rRNA genes (880 and 720 bp in length for the somatic and oocyte genes respectively - see Figure 12) by a salt-gradient dialysis method (Tatchell and van Holde, 1977). This reconstitution technique involves mixing DNA with purified histone octamers in 2M NaCl, followed by reduction of the salt concentration in a stepwise manner allowing formation of nucleosome core particles. This procedure has been shown to produce nucleosome core particles identical to those found in native chromatin after analysis by several criteria including histone content, electron microscopy, DNase I digestion, X ray crystallography and micrococcal nuclease digestion (Rhodes and Laskey, 1989).

When native, H1-depleted chromatin is digested with micrococcal nuclease, 146 bp nuclease-resistant fragments are produced due to the interaction of DNA with the histone octamer (see Figure 2). Thus a simple and highly informative assay for determining the quality of reconstituted chromatin, is the micrococcal nuclease digestion assay. A micrococcal nuclease digestion time course (Figure 13A), demonstrates that digestion of both the reconstituted oocyte and somatic 5S rRNA genes produced ~146 bp nuclease resistant fragments characteristic of packaging with histone octamers. These reconstituted templates were transcribed in HeLa cell nuclear extracts and the results (Figure 13B) indicate that octamers reconstituted on the oocyte 5S rRNA gene repressed

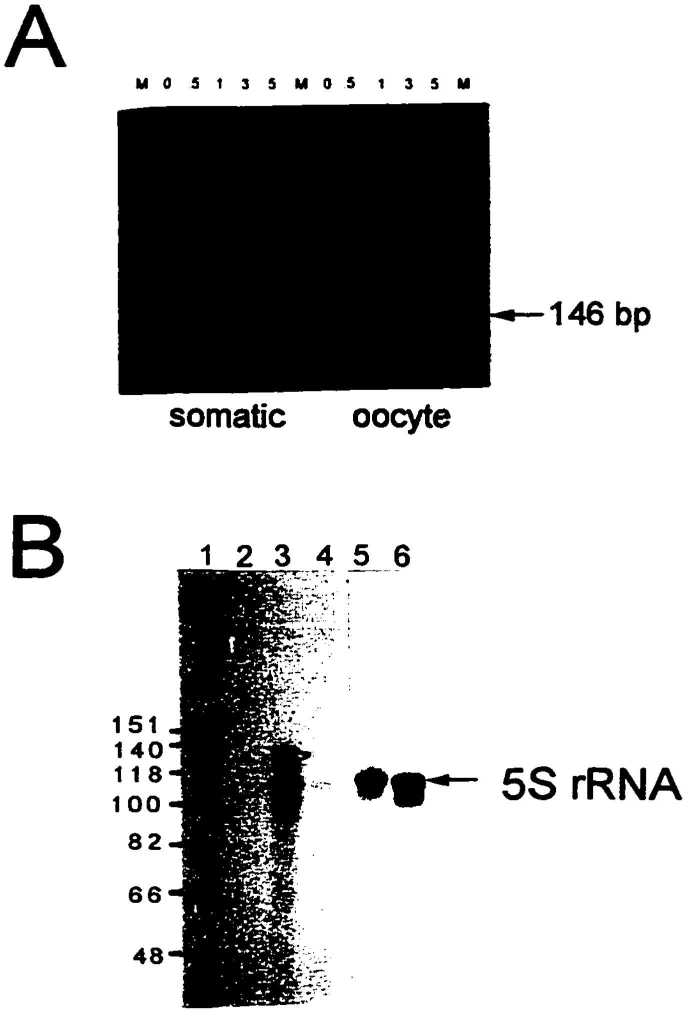

Figure 13. The effect of nucleosome reconstitution on 5S rRNA transcription. (**A**) Micrococcal nuclease digestion of nucleosomes reconstituted on full length *Xenopus laevis* oocyte and somatic genes. Digestions were carried out at a nucleosome concentration of 0.1 mg/ml (DNA weight) and an enzyme concentration of 20 units/ml for the minutes indicated on the top of each lane. The resulting DNA fragments were deproteinized and electrophoresed on a 4% non-denaturing gel. (M) *Hha* I cut pBR322. (**B**) Approximately 250 ng of the oocyte (lanes 3 and 4) or somatic (lanes 5 and 6) 5S rRNA genes, either uncomplexed (lanes 3 and 5) or reconstituted with chicken erythrocyte core histones (lanes 4 and 6) were transcribed in HeLa cell nuclear extracts. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (8% acrylamide and 8.3 M urea in 1× TBE). Lane 1, Klenow end-labeled *Hinf* I cut ϕX174 DNA (sizes of marker fragments shown as number of nucleotides); lane 2, transcription in the absence of template DNA.

transcription (compare lanes 3 and 4). This result is similar to the work of others demonstrating that transcription of cloned oocyte 5S rRNA genes after reconstitution with histone octamers can occur only if, prior to reconstitution, a transcription complex or minimally TFIIIA is assembled on the intragenic promoter (Gottesfeld and Bloomer, 1982; Stunkel *et al.*, 1995). In contrast to the transcriptional repression of the oocyte gene, octamers on the somatic gene did not decrease the level of transcription from this template (compare lanes 5 and 6). This differential transcription of the oocyte and somatic 5S rRNA genes is not observed when the genes exist as uncomplexed DNA (lanes 3 and 5), suggesting that it is a difference in the packaging of the DNA which is responsible for the effect. There are a number of possible explanations for how reconstitution with histone octamers could result in differential transcription of these constructs. First, it is possible that this effect is a reflection of a differential affinity of transcription complexes for the two genes. A transcription complex with higher affinity for the somatic gene compared to the oocyte, could disrupt a nucleosome on the somatic gene while, at the same time, be blocked from accessing the oocyte gene. This repression could potentially be reversed by altering the transcription conditions to more closely match those during oogenesis, such as including higher concentrations of TFIIIA or histone chaperones such as nucleoplasmin. Conversely, the oocyte gene specific repression could be due to the presence of low levels of histone H1 in the transcription extracts. Histone H1 has been shown to selectively bind the oocyte gene over the somatic (Jermanoski and Cole, 1990) and thus this could result in selective repression of oocyte gene transcription. Finally, because the translational position of a histone octamer is sequence dependent (Drew and Travers, 1985), it is expected that the oocyte and somatic genes would position nucleosomes differently due to differences in the non-transcribed flanking sequences. This may indicate a possible role for the translational position of histone octamers in the differential regulation of 5S rRNA transcription.

3.2. Role of Translational Position in the Differential Transcription of the 5S rRNA genes in X. laevis

3.2.1. Reconstitution of complete mononucleosome core particles

Immediately following DNA replication, there is a competition between histones and transcription factors for DNA binding sites. In *X. laevis* somatic cells, it is believed that as a result of this competition, the somatic genes acquire a complete transcription complex while the oocyte genes are packaged into nucleosomes (Wolffe and Brown, 1988, Wolffe, 1994). The differing fates of these genes have been attributed to a faster rate of assembly of transcription complexes on the somatic gene compared to the oocyte (Seidel and Peck, 1992). The first objective of this study was to determine whether nucleosome translational position could also play a role in this event. Translational position has been shown to be a major determinant in the binding of transcription factors to nucleosomal DNA (Pham *et al.*, 1992; Li *et al.*, 1994; Li *et al.*, 1995). To determine whether this factor affects the binding of TFIIIA to nucleosomal DNA, it was necessary to develop a method for varying the position of nucleosomes on the 5S rRNA genes. The disadvantage of using the salt-gradient dialysis method is it fails to space nucleosomes at the regular intervals found *in vivo*. Instead this technique creates arrays in which the nucleosomes can become very tightly packed without any linker DNA. This can be seen in Figure 13A which shows the presence of micrococcal nuclease digestion intermediates of ~300 and ~450 bp. Other methods have been developed to produce nucleosome arrays with physiological spacing, but these techniques involve the use of histone binding components such as polyglutamic acid, nucleoplasmin, and NAP-1, the latter two of which have been shown to enhance transcription factor binding to nucleosomal DNA (Chen *et al.*, 1994; Walter *et al.*, 1995). To overcome the problem of compacted histone octamers, reconstitutions were performed on DNA fragments long enough to allow reconstitution of one histone octamer but not two (~160-270 bp). The product generated from reconstitution of a single histone octamer onto a fragment of DNA greater than 146 bp does not have an official definition in the literature. It should not be classified as a nucleosome due to the absence of histone H1, nor as a nucleosome core particle due to the presence of greater than 146 bp of DNA. Often it has also been designated in the literature, albeit incorrectly, a nucleosome. In the absence of a better nomenclature, this will be the term used to describe these reconstituted products in this study.

Histone octamers were reconstituted onto radiolabeled fragments of the *X. laevis* 5S rRNA genes using the octamer transfer method (Rhodes and Laskey, 1989). In this method, a radiolabeled DNA fragment is mixed with a large molar excess of unlabeled nucleosome core particles in 0.8 M NaCl (Figure 14A). Under these conditions, the histone octamers dissociate from the DNA, and nucleosome core particles are formed on the radiolabeled DNA as the salt concentration is lowered to 0.1 M NaCl by step-wise addition of aliquots of low-salt buffer. The benefit of the octamer transfer method over the salt-gradient dialysis technique, is it can be performed on a very small scale, requiring

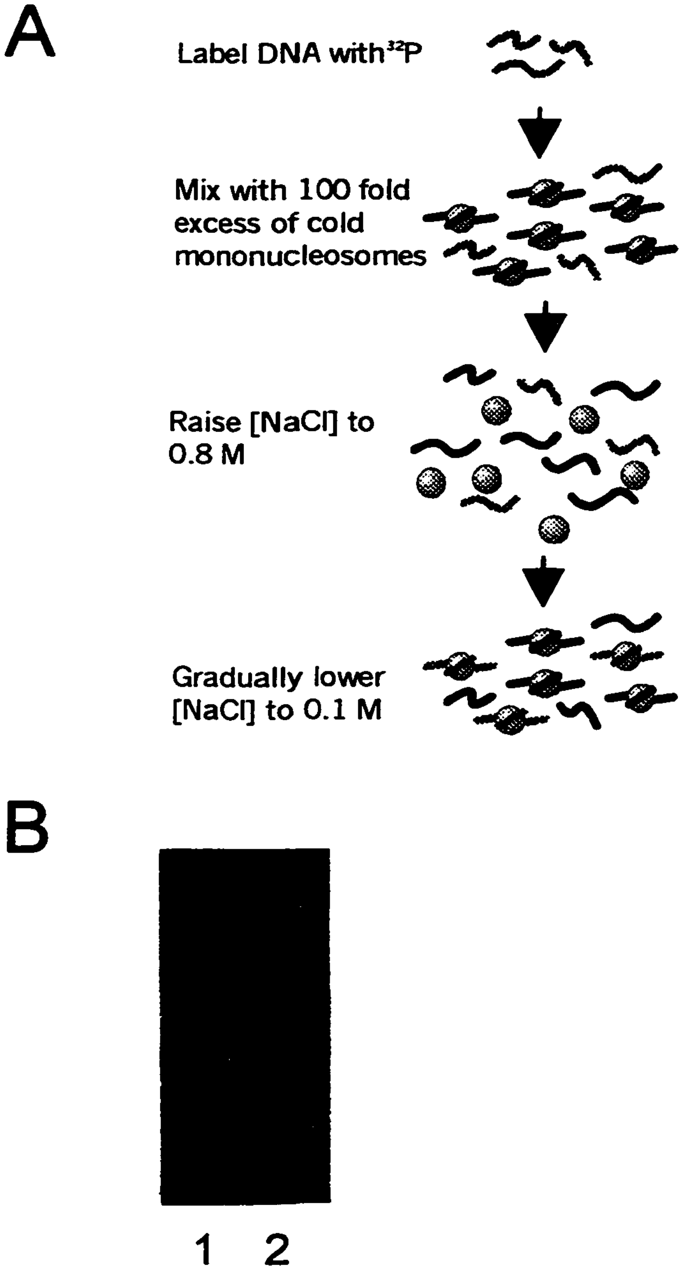

Figure 14. **(A)** Schematic representation of the octamer transfer method of nucleosome reconstitution. The shaded circle represent the histone octamer, the black line unlabelled DNA and the gray line ^{32}P labeled DNA. **(B)** Agarose gel electrophoresis of DNA before (lane 1) and after (lane 2) reconstitution by the octamer transfer method.

less than 50 ng of the labeled DNA. The drawback to the octamer transfer method is the difficulty in determining whether complete nucleosomes are formed, as it is not possible to ascertain under these conditions if H2A, H2B, H3 and H4 have reassociated on the labeled DNA in equal molar amounts as found in the histone octamer. The results of this work rely heavily on the octamer transfer reconstitution technique, and thus it was important to determine the integrity of the nucleosomes reconstituted by this technique. It has been shown that the presence of only histone H3/H4 tetramers on labeled DNA can enhance transcription factor binding relative to that of a histone octamer (Hayes and Wolffe, 1992). To address this concern, one of the oocyte 5S rRNA gene fragments used in this work, [Xlo(-83→+136)], was reconstituted with histone octamers using the salt-gradient dialysis technique which produces enough reconstituted product to allow analysis of protein content by SDS PAGE. The nucleosomes reconstituted this way were subjected to native gel electrophoresis and the gel slice containing the reconstituted nucleosomes was resolved in a second dimension, by SDS PAGE, to determine the protein composition of these nucleosomes (Figure 15A). The results show that nucleosomes reconstituted by the salt-gradient dialysis method (lane 3) contained all four of the core histone proteins in amounts (Figure 15B) similar to the nucleosome core particles employed for the octamer transfer method (lane 1) and the histone octamers used for the salt-gradient dialysis reconstitution (lane 2). The integrity of nucleosomes reconstituted by the octamer transfer method was assessed indirectly by comparing the electrophoretic and hydrodynamic properties of these reconstitutes with those of the salt-gradient dialysis reconstituted nucleosomes. Figure 15C shows a native polyacrylamide gel comparing labeled salt-gradient dialysis reconstituted nucleosomes (lane 1) with nucleosomes prepared by the

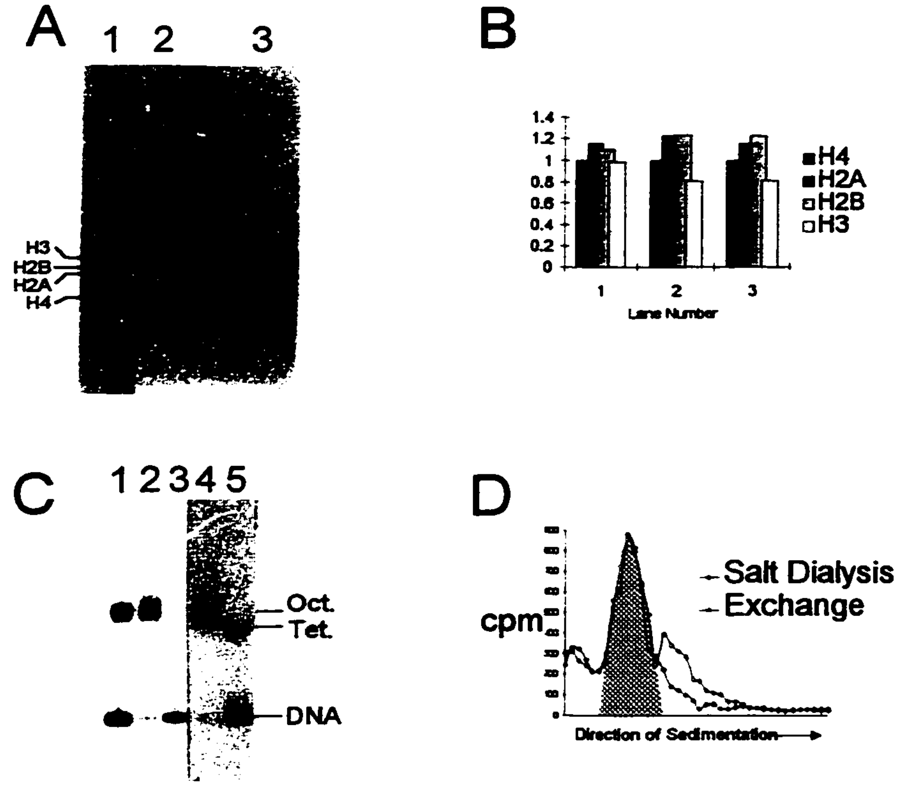

Figure 15. Characterization of the reconstituted nucleosomes. **(A)** SDS PAGE analysis of the proteins associated with the nucleosomes reconstituted by salt gradient dialysis. Lane 1, histones from the nucleosome core particles used in the octamer transfer method; lane 2, input histones used for the salt gradient dialysis reconstitution; lane 3, histones of the band migrating in the nucleosome region (see 2C) excised from a native gel and run on a second dimension SDS PAGE. **(B)** Densitometric scans of the gel shown in 1A. **(C)** Non-denaturing PAGE analysis of the electrophoretic mobility of nucleosomes reconstituted by the salt gradient dialysis and octamer transfer methods. Lane 1, α^{32}P end-labeled nucleosomes reconstituted by salt gradient dialysis; lane 2, nucleosomes reconstituted by the octamer transfer method; lane 3, α^{32}P Klenow labeled DNA used in the octamer transfer reconstitution, lanes 4,5, nucleosome reconstituted by the exchange method before (4) and after (5) treatment with 4M Urea, 0.35 M NaCl. This treatment induces the dissociation of H2A-H2B leading to a subnucleosome particle containing only H3-H4 (Sibbet and Carpenter, 1983). **(D)** Sucrose gradient fractionation of nucleosomes reconstituted by the salt gradient dialysis method and the octamer transfer method. The gradient was linear from 5-20% sucrose in 10 mM Tris-HCl (pH 7.5), 25 mM NaCl and was run in a Beckman SW-41 rotor at 24 000 rpm at 4°C for 20 hours. The solid line represents the DNA distribution as measured with a scintillation counter. The shaded region represents the peak corresponding to the reconstituted nucleosomes.

octamer transfer method (lane 2). As can be seen, nucleosomes reconstituted with the two methods showed an identical electrophoretic mobility. To verify that this gel system can indeed separate nucleosomes containing a histone octamer from those containing only a H3/H4 tetramer, nucleosomes deficient in histone H2A and H2B were run on this gel system as well (lane 4). Figure 15D compares the sedimentation behavior of the octamer transfer method and salt-gradient dialysis reconstituted nucleosomes in a sucrose gradient. As can be seen, the two types of reconstitution produced nucleosomes with identical hydrodynamic characteristics. These results clearly indicate that reconstitution by the octamer transfer method produced nucleosomes identical to those reconstituted by the salt-gradient dialysis method which, as shown in Figures 15A and B, contained complete histone octamers.

3.2.2. Determination of the translational position of reconstituted nucleosome core particles

To determine whether nucleosome translational position can mediate TFIIIA binding, it was necessary to create several mononucleosome particles in which the position of the nucleosome relative to the intragenic promoter was varied. In previous investigations designed to study the effect of nucleosome position, synthetic DNA-bending sequences were used to position *trans*-acting factor binding sites at different locations with respect to the histone octamer (Taylor *et al*, 1991, Li and Wrange, 1993, Li and Wrange, 1995, Blomquist *et al.*, 1996). The TFIIIA binding site is relatively large (~46 bp), and thus it would be difficult to introduce this site into a DNA sequence designed to position a nucleosome without altering the artificial positioning signals. For this reason, the natural nucleosome positioning signals within the 5S rRNA genes were used and the position of nucleosomes on the 5S rRNA genes were varied by altering the fragments used for nucleosome reconstitution. Fragments of both the oocyte and somatic 5S rRNA genes were used as the two genes would be expected to position nucleosomes differently due to their differing 5' and 3' flanking sequences and yet bind TFIIIA with a similar affinity.

Previous to this work, the position of nucleosomes reconstituted on *X. laevis* oocyte gene fragments had not been published and that of the somatic gene had been studied extensively albeit with conflicting results. According to Gottesfeld, (1987), a nucleosome reconstituted onto different fragments of the somatic 5S rRNA gene occupies a region spanning nucleotides +20 to +200 with respect to the transcriptional start site. In contrast, when Lee *et al*, (1993) reconstituted a nucleosome on a different *X. laevis* somatic 5S rRNA gene fragment [Xls(-43→+139)] they described the nucleosome position as further upstream, spanning nucleotides -41 to +105. The gene fragments used in this study were Xlo(-83→+136), Xlo(-38→+143), Xls(-74→+147), Xls(-51→+147) and Xls(-1→+204) with the prefix indicating the source of the gene (Xlo referring to oocyte and Xls somatic) and numbers within the brackets representing the 5' and 3' ends of the DNA fragments in relation to the site of transcription initiation (see Figure 12).

Using a similar approach as Dong *et al.*, (1990), the positions of a nucleosome, reconstituted by the octamer transfer method, on the 5S rRNA gene fragments was determined. Briefly, this technique involved digestion of the nucleosomes with micrococcal nuclease to generate the ~146 bp of core particle DNA. These micrococcal nuclease resistant fragments were subsequently digested with one or more restriction enzymes to map the position of the core particle DNA relative to the restriction enzyme sites (Figure 16). Using this approach, Dong *et al.*, (1990) determined the nucleosome position on *Lytechinus variegatus* 5S rRNA gene fragments. As a control experiment, this work was repeated for this study using a 208 bp fragment of the same *L. variegatus* 5S rRNA gene. Figure 17A, lane 4 shows the products of an *Alu* I restriction digestion of the core particle DNA fragment. This lane shows only two bands of 65 and 81 bp, indicating that the nucleosome was positioned at only one location on this DNA fragment. Digestion of the micrococcal nuclease resistant band with *Bst*N I produced a 130 bp fragment (lane 3) and *Msp* I digestion produced a product of 114 bp (lane 2). Presumably only one observable band was present in these two cases due to the small size of the second fragments and the resulting difficulty in detecting the band. From this analysis, it was determined that the position of a nucleosome on the 208 bp DNA fragment of the *L. variegatus* 5S rRNA gene was from -86 to +60 bp (Figure 17B). This is a 4 bp difference from the nucleosome position of -90 to +56 reported by Dong *et al.*, (1990).

The above mentioned analysis was repeated with the 5S rRNA gene fragments from *X. laevis* and an example of the electrophoresis patterns obtained is shown (Figure 18). At first glance it can be seen that, unlike the *L. variegatus* 5S rRNA gene, the majority of the *X. laevis* 5S rRNA gene fragments tested, positioned a nucleosome at more than one predominant location. This is made evident by the presence of more than one pair of restriction digestion products for each gene fragment tested [with the exception of Xls(-1→+204)]. In the case of the somatic gene, this result conflicts previously published results which state that different fragments of this gene position a

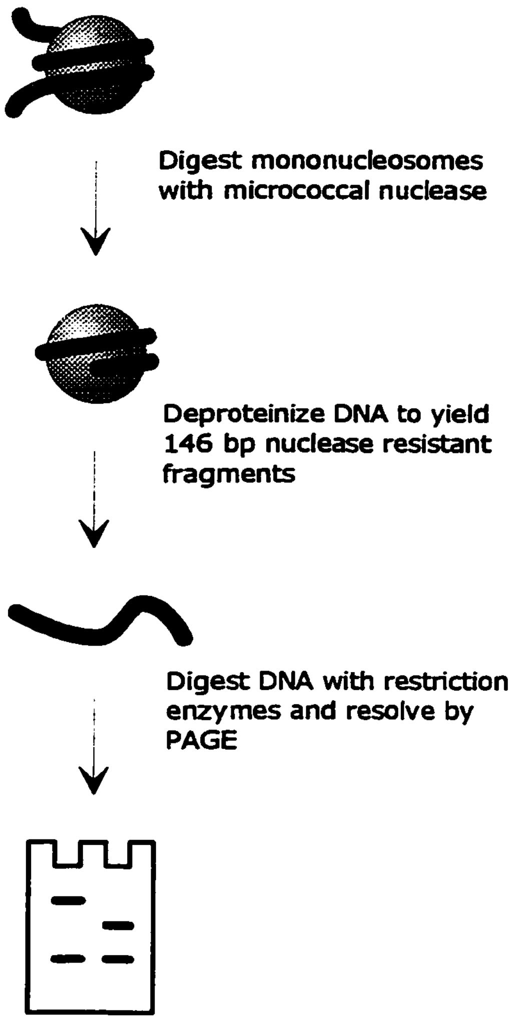

Figure 16. Schematic representation of the method used to determine histone octamer translational position. The shaded circle represent the histone octamer, and the black line the DNA.

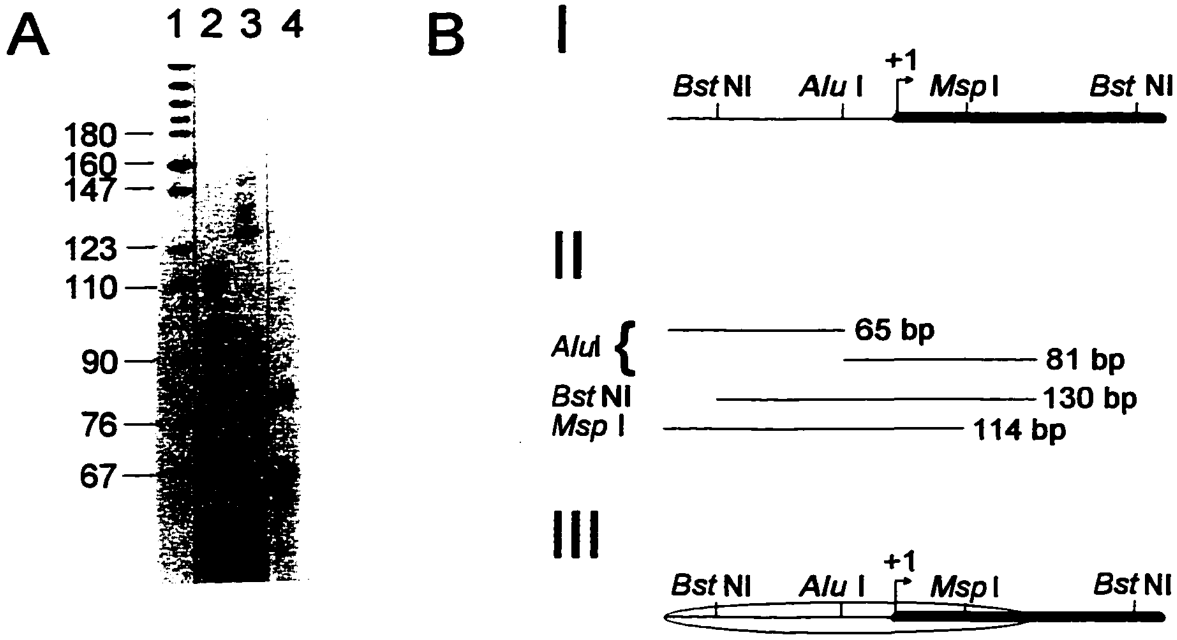

Figure 17. Determination of the translational positioning of a nucleosome reconstituted onto a *L. variegatus* 5S rRNA gene fragment. The region of DNA in direct association with the histone octamer was determined by digestion of the ~146 bp micrococcal nuclease resistant fragment with different restriction enzymes. **(A)** Denaturing (8% acrylamide, 8.3 M urea in 1x TBE) PAGE of the fragments obtained by digestion with the restriction enzymes *Msp* I (lane 2); *Bst*N I (lane 3); and *Alu* I (lane 4). Lane 1 is an *Msp* I digest of pBR322 with the sizes of the marker fragments shown as number of nucleotides. **(B)** **(I)** Schematic representation of the *L. variegatus* 5S rRNA gene showing the restriction sites used to determine the nucleosome translational position. The thick line represents the coding sequence of the 5S rRNA gene, and the transcription start site is indicated by +1. **(II)** Representation of the restriction fragments produced by digestion of the micrococcal nuclease resistant fragment, and probable alignment with respect to each other as determined by the position of the restriction sites. **(III)** Representation of the nucleosome position (open ellipsoid) on the 5S rRNA gene as determined by this analysis.

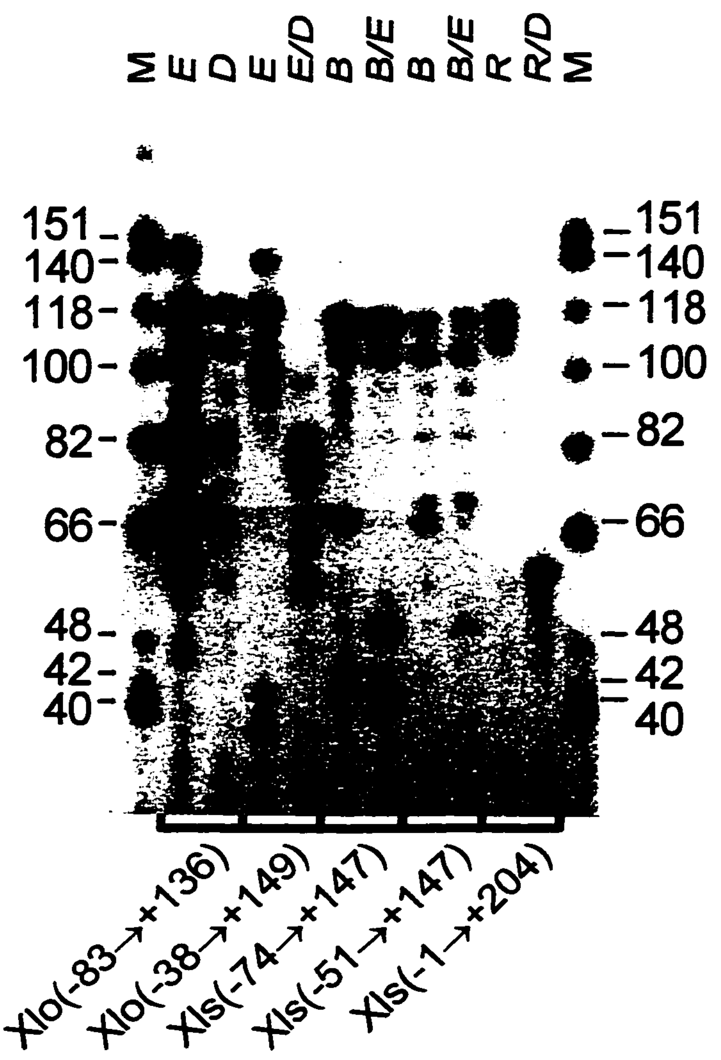

Figure 18. Determination of nucleosome translational position on reconstituted *X. laevis* 5S rRNA gene fragments. The region of DNA in direct association with the histone octamer was determined by digestion of ~146 bp, internally labeled, micrococcal nuclease resistant fragments with different combinations of restriction enzymes as indicated. The resulting restriction fragments were resolved by denaturing PAGE (8% acrylamide, 8.3 M urea in 1× TBE). (M) Klenow end-labeled *Hinf* I cut ϕX174 DNA (sizes of marker fragments shown as number of nucleotides). The restriction enzymes used are indicated on the top of the gel as: *E* (*Eae* I), *D* (*Dde* I), *B* (*Bsp* 1286) and *R* (*Rsa* I).

nucleosome at predominantly one location (Gottesfeld, 1987; Lee *et al.*, 1993). The results also show that, as expected, altering the amount of 5' and 3' flanking sequence rearranges the translational positions of nucleosomes reconstituted on the gene fragments [for example compare the *Eae* I restriction fragments produced by digestion of Xlo(-83→+136) with Xlo (-38→+149)]. This later observation could presumably resolve the conflict existing between the two different nucleosome positions on the somatic gene reported by Gottesfeld, (1987) and Lee *et al.*, (1993), as each study used different gene fragments. Due to the fact that the gene fragments used in this study exhibited multiple translational positions, it was necessary to perform double digests on some of the micrococcal nuclease fragments to ensure proper assignment of the 5` and 3` termini. After analysis of the restriction digestion patterns of several trials (summarized in Table 1), the most abundant nucleosome positions on each fragment were determined (Figure 19). Varying the conditions of nucleosome reconstitution, or the temperature or micrococcal nuclease digestion had no effect on the translational positioning results (data not shown). Of the three somatic gene fragments tested, one [Xls(-51→+147)] positioned a nucleosome at a similar site as that seen by Lee, *et al.*, (1993) spanning nucleotides -42 to +104. Once again, it is important to note however, that unlike the fragment used by Lee, *et al*, (1993), Xls(-51→+147) positioned a nucleosome at more than one predominant position. One explanation for this difference is the slightly increased length of the fragment used in this study [Xls(-51→+147) compared to Xls(-43→+139) used by Lee *et al.*, 1993]. The somatic gene fragment, Xls(-1→+204), positioned a nucleosome at one position, spanning nucleotides +49→+195, which is within the region described by Gottesfeld, (1987) of +20→+200. It is important to note that these positions

Table 1. Summary of the restriction fragments lengths used to determine nucleosome translational position on reconstituted *X. laevis* 5S rRNA gene fragments. The restriction fragments were generated by digestion of the ~146 bp, internally labeled, micrococcal nuclease resistant fragments with different combinations of restriction enzymes as indicated.

Gene Fragment	Restriction Enzyme	Fragment Size	Nucleosome Position
Xlo(-83→+136)	*Dde* I	59, 83	-16→+130
		109	-66→+80
		122	-79→+67
	Eae I	62, 85	-79→+67
		72, 74	-66→+80
		124	-16→+130
	Rsa I	52/93	-16→+130
		140	-66→+80
		146	-79→+67
Xlo(-38→+149)	*Dde* I	62, 84	-15→+131
		64, 81	-35→+111
	Eae I	105	-35→+111
		125	-15→+131
	Rsa I	36, 110	-35→+111
		56, 90	-15→+131
	Eae I/*Rsa* I	67	-35→+111
		86/87	-15→+131
Xls(-74→+147)	*Bsp* 1286	44, 47, 71	-71→+75
		110	-12→+134
		121	+1→+146
	Eae I	63, 83	-71→+75
		76	-12→+134
		141	+1→+146
	Rsa I	69, 77	+1→+146
		146	-71→+75
	Bsp 1286/*Eae* II	44, 47, 54	-71→+75
		110	-12→+134
		121	+1→+146
Xls(-51→+147)	*Bsp* 1286	71, 75	-42→+104
		110	-12→+134
		121	+1→+146
	Eae I	44, 102	-42→+104
		114	-12→+134
		136	+1→+146
	Rsa I	58, 88	-12→+134
		69, 77	+1→+146
		118	-42→+104
	Bsp 1286/*Eae* I	54, 75	-42→+104
		110	-12→+134
		121	+1→+146
Xls(-1→+204)	*Rsa* I	124	+52→+198
	Dde I/*Rsa* I	62	+52→+198

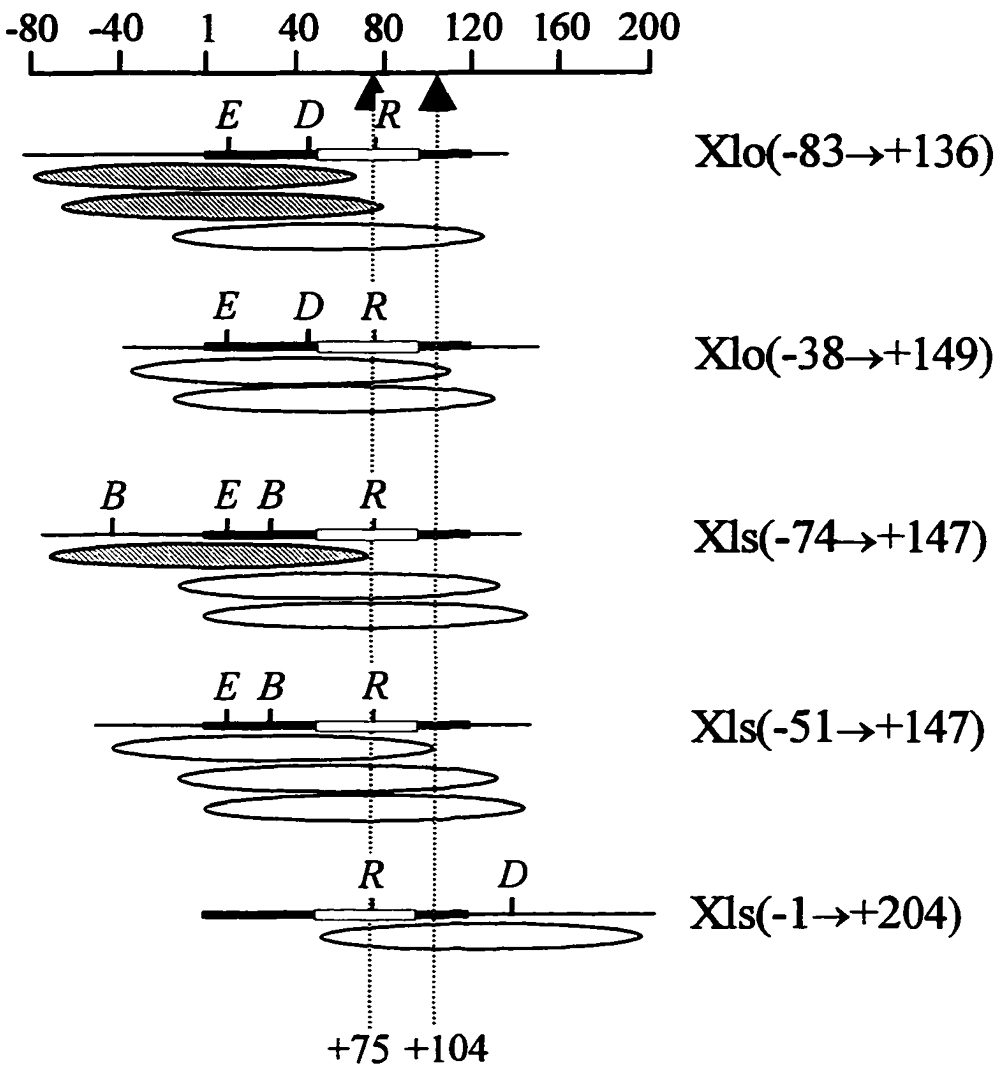

Figure 19. Schematic representation of the most predominant nucleosome positions on the five different fragments of the *X. laevis* oocyte and somatic 5S rRNA genes resulting from the electrophoretic analysis shown in **Figure 18.**. Due to variations in base composition and thus radioactive label, the intensity of the bands in relation to dATP content of the fragments was considered in these calculations. The ellipsoids indicate the most abundant positions of the ~146 bp micrococcal nuclease resistant fragments. The thick black line represents the 5S rRNA transcribed sequence and the open box indicates the intragenic TFIIIA binding site. Nucleotide positions relative to the transcription start site are indicated on the scale on top, and the dashed vertical lines indicate the positions of nucleotides +75 and +104. The hatched ellipsoids are those nucleosome positions postulated to allow TFIIIA binding whereas open ellipsoids are those which are thought to be repressive to TFIIIA binding based on the results of You *et al.*, (1991).

are intrinsic to the DNA fragments used and not meant to represent the position nucleosomes occupy *in vivo*.

3.2.3. DNase I footprint analysis of reconstituted nucleosomes

In the past, it has been common practice to determine nucleosome translational position by DNase I footprinting. DNase I binds across the minor groove of DNA cutting either strand. After reconstitution of DNA into nucleosomes, steric hindrance allows DNase I to digest only those minor grooves which are directed away from the nucleosome (Lutter, 1978). Due to the periodicity of DNA, a uniquely positioned nucleosome produces a digestion pattern with an approximately 10 bp interval between cleavage sites. It is clear from the preceding section that it would be very difficult to use DNase I footprinting to determine the translational position of a histone octamer on the *X. laevis* 5S rRNA gene fragments considering the number of overlapping nucleosome positions present. However, DNase I digestion still provides a useful tool for determining the rotational position of a nucleosome on a piece of DNA by determining which nucleotides face away from the histone octamer.

Each of the nucleosomes used for this study was labeled on the 3' end of the coding strand and digested with DNase I. The patterns of digestion are shown in Figure 20 with the most predominant translational positions from Figure 16 indicated. As can be seen, all five of the fragments produced short stretches of 10 bp "ladders", as shown by the arrows on the right, characteristic of nucleosomes with unique rotational positions. This 10 bp interval of DNase I cleavage sites was not always seen in regions where

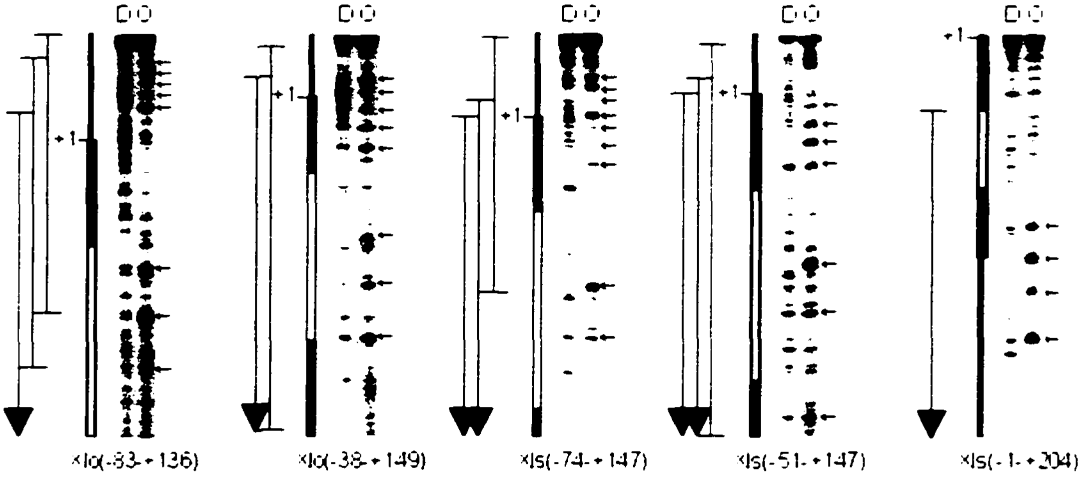

Figure 20. DNase I digestion analysis of nucleosomes reconstituted on fragments of the *X. laevis* 5S rRNA genes. The coding strands of the five *X. laevis* 5S rRNA gene fragments were labeled at the 3' end, reconstituted with chicken erythrocyte histones and digested with DNase I. The DNase I digestion patterns were resolved by denaturing PAGE (8% acrylamide, 8.3 M urea in 1× TBE). (D), uncomplexed DNA; (N), nucleosomal DNA. The arrows to the right show the positions of the more distinct periodic cleavage sites within the nucleosomes. The lines on the left show the translational positioning of nucleosomes on these fragments, relative to the 5S rRNA gene with the transcriptional start site indicated as +1.

different nucleosome translational positions overlapped suggesting that rotational position varies with translational position. Several nucleosomes, with multiple translational positions but the same rotational position, would be expected to produce a continuous 10 bp "ladder" which extends over a greater range than that of a single nucleosome.

3.2.4. Binding of TFIIIA to X. laevis 5S rRNA gene fragments after reconstitution with histone octamers

Previous work reported that TFIIIA cannot bind *X. laevis* somatic 5S rRNA gene fragments after reconstitution with a complete histone octamer (Gottesfeld, 1987; Lee, *et al.*, 1993). When a similar experiment was performed with the *X. borealis* somatic 5S rRNA gene, which has been shown to position nucleosomes differently from the *laevis* (Gottesfeld, 1987; Rhodes, 1985), the results were conflicting. Rhodes, (1985) was able to demonstrate TFIIIA binding to a nucleosomal *X. borealis* somatic 5S rRNA gene fragment while Hayes and Wolffe, (1992) found the TFIIIA binding site blocked after nucleosome reconstitution. This is despite the fact that both studies were in agreement regarding nucleosome translational positioning on *X. borealis* gene fragments. Lee *et al.*, (1993) found that reconstitution with tetramers rather than complete octamers permits TFIIIA binding to the *X. borealis* gene but not the *X. laevis*. This difference was attributed to the differential translational positions of nucleosomes on the two somatic genes thus suggesting that nucleosome position can affect transcription factor binding.

To test whether any of the nucleosomes reconstituted for this study could bind TFIIIA, electrophoretic mobility shift assays were performed (Figure 21). The molar ratio

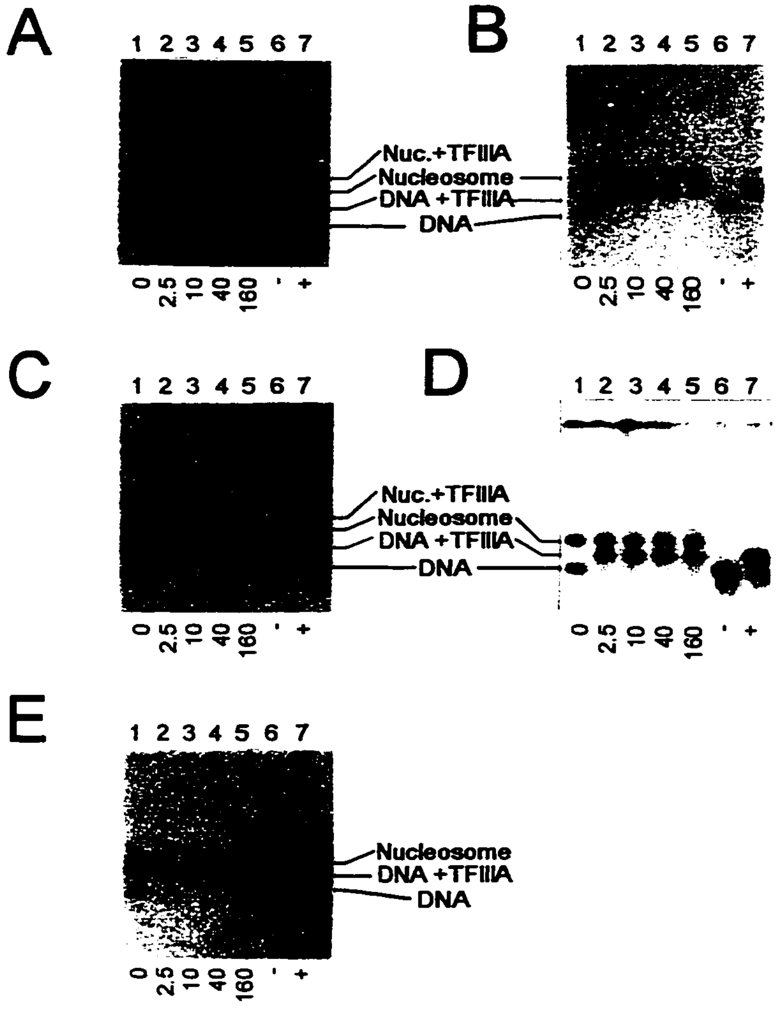

Figure 21. Analysis, by agarose gel electrophoresis, of the binding of TFIIIA to nucleosomes reconstituted onto the *X. laevis* 5S rRNA gene fragments. (**A**) Xlo(-83→+136); (**B**) Xlo(-38→+149); (**C**) Xls(-74→+147); (**D**) Xls(-51→+147); (**E**) Xls(-1→+204). Nucleosomes (lanes 1-5) reconstituted with histones isolated from non-butyrate treated HeLa cells in the absence (lane 1) or presence (lanes 2-5) of increasing amounts of TFIIIA (molar ratios of TFIIIA to 5S rRNA genes is indicated beneath the gels). Lanes 6 and 7, the corresponding DNA templates in the absence (-) or presence (+) of TFIIIA. The samples were incubated for 20 minutes at room temperature before loading on 0.75% agarose gels.

of TFIIIA to 5S rRNA genes is as indicated at the bottom the gels. It must be noted that the TFIIIA concentrations used in all these studies were far less than what is present *in vivo* because, during early oogenesis, TFIIIA concentrations can reach 10^7 times the number of oocyte 5S rRNA genes (Shastry *et al.*, 1984), although much of this becomes complexed as the 7S particle with 5S rRNA. The higher concentrations used in this study were sufficient to completely shift the corresponding uncomplexed DNA alone (see Figure 21, lanes 6 and 7). Under these conditions, TFIIIA would be expected to saturate all the available binding sites.

Of the five gene fragments tested, only one oocyte [Xlo(-83→+136)], and one somatic [Xls(-74→+147)] gene fragment were able to bind TFIIIA after nucleosome reconstitution (Figure 21). However, in none of these cases could a complete shift of all nucleosomes be obtained suggesting that either only a fraction of the nucleosomes are capable of binding TFIIIA, or that insufficient TFIIIA is present. The remaining 5S rRNA gene fragments, Xlo(-38→+143), Xls(-51→+147) and Xls(-1→+204) were unable to bind TFIIIA after nucleosome reconstitution even though in every instance the amount of transcription factor present was enough to completely shift the corresponding DNA alone (see Figure 21).

Due to the fact that the fragments used in this study exhibited multiple nucleosome positions, it was difficult to determine exactly which positions facilitated TFIIIA binding. The one exception was fragment Xls(-1→+204). This fragment positioned a histone octamer at a unique site (spanning nucleotides +49 to +195), the 5' termini of which overlapped the "A block". This nucleosome position blocked TFIIIA binding. The fragment Xlo(-83→+136) positioned a nucleosome at three major locations (Figure 19) with 3' boundaries at nucleotides +67, +80 and +130 with respect to the transcriptional start site. TFIIIA bound to an uncomplexed *X. borealis* somatic 5S rRNA gene has been shown to occupy nucleotides +45 to +97 (Rhodes, 1985) with residues +81 to +91 forming the minimal requirements for TFIIIA binding (You *et al.*, 1991) (Figure 22A). Thus presumably, the fraction of nucleosomes reconstituted on Xlo(-83→+136) which bound TFIIIA would be that with the TFIIIA binding site partially exposed (nucleosomes with 3' boundaries of +67 and +80). More evidence to support this conclusion is that fragment Xlo(-38→+143), which positioned nucleosomes with boundaries at approximately +111 and +131, did not bind TFIIIA. Of the three somatic gene fragments tested, only one, Xls(-74→+147), bound TFIIIA after nucleosome reconstitution. This fragment positioned a nucleosome at three major sites with downstream boundaries of +75, +134 and +146. The latter two positions, +134 and +146, were shared by nucleosomes reconstituted on Xls(-51→+147). This later construct did not bind TFIIIA when existing in a nucleosomal form suggesting that it was the nucleosome with the +75 downstream boundary on Xls(-74→+147) which permitted TFIIIA binding. This data is summarized in Figure 22B which shows only those positions which do not overlap nucleotides +78→+86 and the correlation with TFIIIA binding.

Although from this data it is not possible to conclude exactly which nucleotides must be free in order for TFIIIA binding to occur, these results suggest that nucleosomes positioned with their 3' boundary upstream of +75 are capable of binding TFIIIA whereas nucleosomes with their 3' boundary downstream of +104 cannot bind TFIIIA. To further

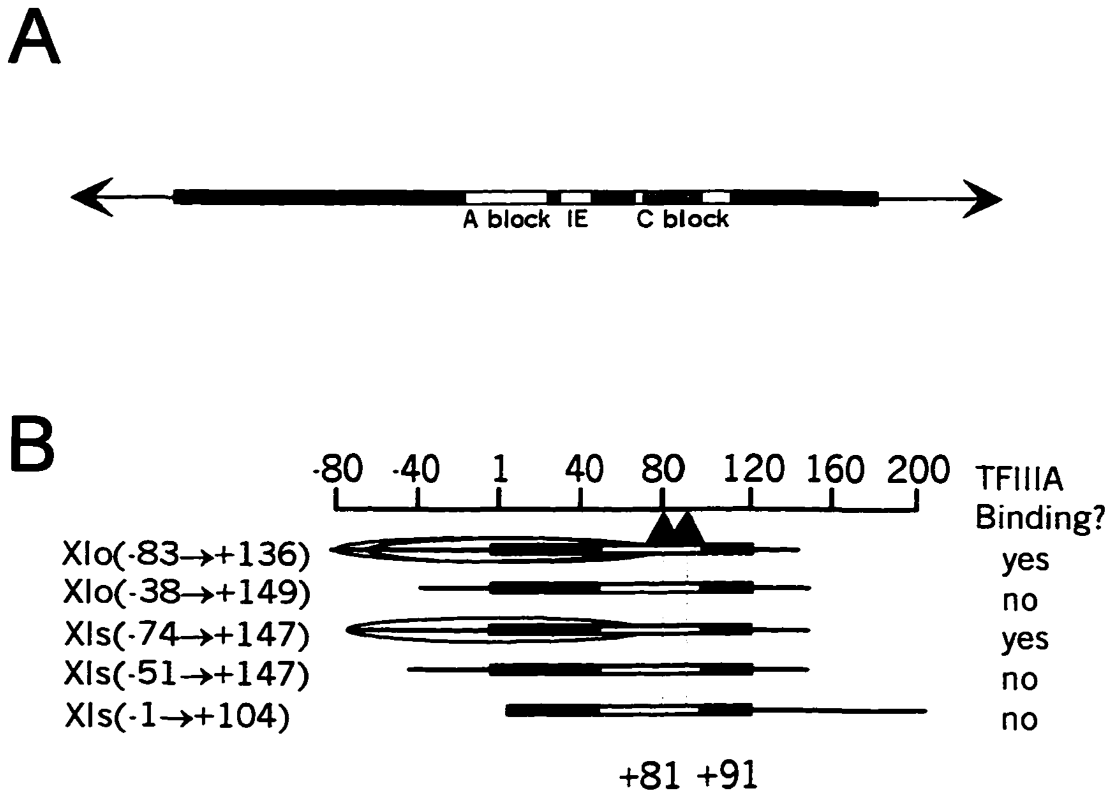

Figure 22. The relationship between nucleosome translational position and TFIIIA binding. (**A**) The structure of the *X. laevis* 5S rRNA gene and intragenic promoter. The heavy black line represents the 5S rRNA coding sequence and the open boxes the promoter elements as indicated. The shaded represents nucleotides +81 to +91 described by You *et al.*, 1991. (**B**) Nucleosome translational positions (open ellipsoids) on the five *X. laevis* 5S rRNA gene fragments which do not overlap nucleotides +81 to +91. Whether or not these constructs bind TFIIIA after nucleosome reconstitution is indicated.

narrow this region, an electrophoretic mobility shift assay was performed using a fragment of the *X. borealis* 5S rRNA gene. This fragment contains one of the strongest nucleosome positioning signals described and positions a nucleosome at approximately -70 to +79 (Rhodes, 1985) (Figure 23A). The results (Figure 23B), indicate that this gene fragment was almost completely shifted by TFIIIA suggesting that even if the "A block" and "IE" are within the core DNA, TFIIIA can still bind nucleosomal DNA. The above results demonstrate that nucleosome translational positioning is a major determinant of the binding of TFIIIA to nucleosomal DNA

3.2.5. DNase I footprint analysis of the TFIIIA, 5S rRNA gene, nucleosome complex

To rule out non-specific binding in the mobility shift assays seen in the Figure 21, it was necessary to show correct contacts between TFIIIA and the DNA in a nucleosome. To this end, DNase I footprinting of the TFIIIA-nucleosome complex was performed using the oocyte 5S rRNA gene fragment, Xlo(-83→+136). To ensure this footprint represented the true TFIIIA-nucleosome complex, the TFIIIA-nucleosome complex was purified by native gel electrophoresis following nuclease digestion. The DNase I digestion patterns of both the coding and non-coding strands are shown (Figure 24). By comparing lane 2 with 3 and 7 with 8, the protection pattern of TFIIIA on the naked DNA could be established. The transcription factor protected a region extending from approximately +45→+91. Comparison between lanes 3 and 5 as well as 8 and 10 shows the altered DNase I digestion pattern characteristic of a several overlapping nucleosome positions. The resolved digestion pattern of the TFIIIA-nucleosome complex seen in lanes 5 and 10

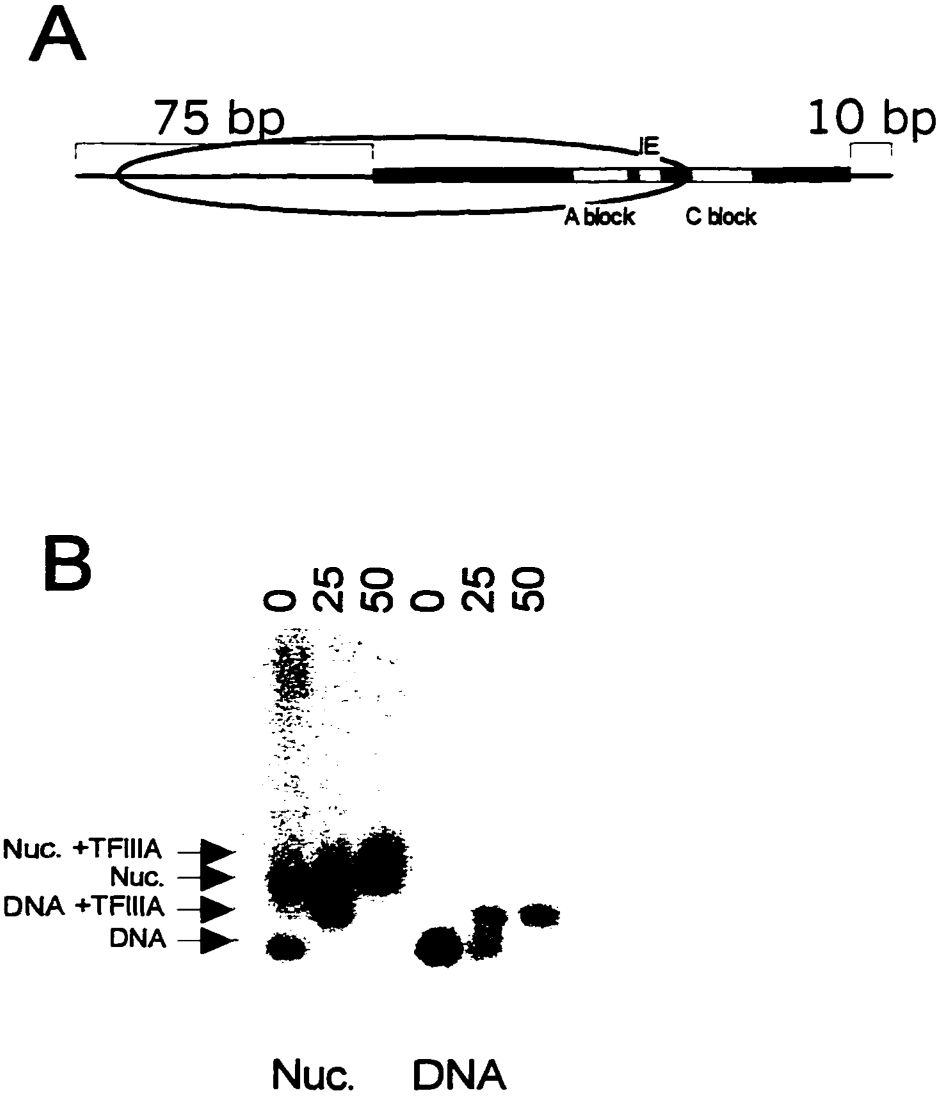

Figure 23. (A) Representation of the translational position of a nucleosome reconstituted onto a *X. borealis* 5S rRNA gene fragment (Rhodes, 1985). The thick line represents the transcribed sequence of the 5S rRNA gene, the open box the intragenic promoter and the open ellipsoid the nucleosome translational position. The shaded box represents the nucleotides +81 to +91 described by You *et al.*, 1991. (**B**). Analysis of the binding of TFIIIA to nucleosomes reconstituted onto the *X. borealis* 5S rRNA gene fragment by agarose gel electrophoresis. Nucleosomes (lanes 1-3) or uncomplexed DNA lanes (4-6) in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5 and 6) of increasing amounts of TFIIIA. The nucleosomes used for the reconstitution were isolated from chicken erythrocytes and the relative molar ratios of TFIIIA is indicated at the top of the gel.

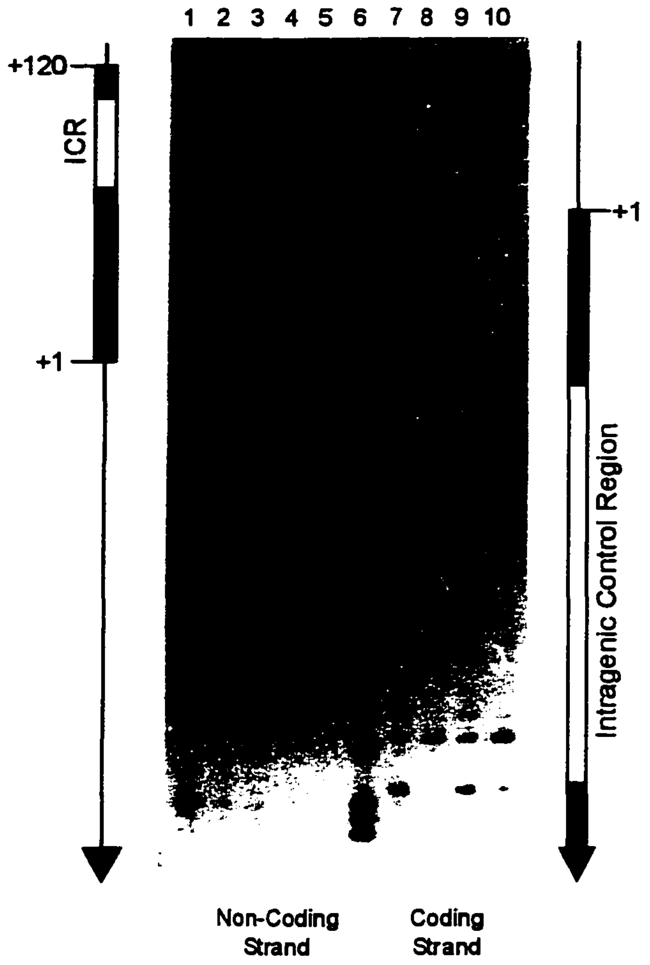

Figure 24. DNase I footprint analysis of the complexes formed by nucleosomes and/or TFIIIA on the Xlo(-83→+136) 5S rRNA gene fragment (see Figure 3B). Nucleosomes labeled at the 3' end of the coding and non-coding strands were incubated in the presence of TFIIIA and subsequently digested with DNase I. The partially digested TFIIIA-nucleosome complexes were purified from free DNA and unbound nucleosomes by native gel electrophoresis (see materials and methods). The footprints for naked DNA (lanes 2 and 7), TFIIIA bound DNA (lanes 3 and 8), reconstituted nucleosome (lanes 4 and 9) and TFIIIA-nucleosome complexes (lanes 5 and 10) are shown. Also shown are the Maxim and Gilbert reactions of the labeled DNA (lanes 1 and 6). The 5S rRNA gene is indicated by the thick black line and the TFIIIA binding site by an open box.

shows characteristics of both TFIIIA and nucleosome binding. The fact that there was "TFIIIA-like" protection over the entire TFIIIA binding site of the non-coding strand and not just nucleotides +78→+86 indicates that all nine zinc fingers of TFIIIA were in contact with the DNA and not just fingers 1-3 which bind to nucleotides +78→+86. This would seemingly suggest that TFIIIA unwrapped the DNA from the nucleosome in the process of binding.

3.3. Role of Histone Acetylation in the Differential Transcription of the 5S rRNA genes in X. laevis

This study investigates a possible relationship between histone acetylation and the transcriptional regulation of the *X. laevis* oocyte genes by addressing two questions. First, does histone acetylation facilitate the binding of TFIIIA to nucleosomal 5S rRNA genes *in vitro*, and second, does the presence or absence of histone acetylation correlate with transcription of 5S rRNA genes *in vivo.*

3.3.1. Binding of TFIIIA to X. laevis 5S rRNA gene fragments after reconstitution with hyperacetylated histone octamers

Recently, it was reported that histone acetylation enhances RNA polymerase III transcription of dinucleosomal 5S rRNA gene templates (Ura *et al.*, 1997), suggesting a possible role for histone acetylation in 5S rRNA transcription. Lee *et al.*, (1993) demonstrated that a blockage of TFIIIA binding by nucleosomes could be overcome by reconstitution with acetylated histones. Thus for this study, the TFIIIA binding experiments were repeated using histones from sodium butyrate treated HeLa cells to determine whether histone acetylation could circumvent blockage of transcription factor binding. Treatment with sodium butyrate causes inhibition of histone deacetylases resulting in an increase in the net amount of acetylated histones in a cell. This together with selective micrococcal nuclease digestion and monovalent ion extraction of nucleosomes from nuclease treated nuclei, results in a nucleosome preparation with very highly acetylated histones. Triton - urea - acetic acid gels can be used to resolve the different levels of histone acetylation due to differences, between the isoforms, in the binding of Triton X-100. Using this gel system (Figure 25) shows that the majority of histone H4 isolated from the sodium butyrate treated cells existed in the tri- and tetra-acetylated forms. When these nucleosomes were used for the octamer transfer reactions, the results of electrophoretic mobility shift assays did not show any effect of histone acetylation on the binding of TFIIIA to nucleosomal DNA. Histone acetylation did not facilitate TFIIIA binding in the case of Xlo(-38→+149), Xls(-51→+147) and Xls(-1→+204) (Figure 26) nor enhance the binding to Xlo(-83→+136) and Xls(-74→+147) (compare Figures 21 and 26).

3.3.2. *Northern blot analysis of 5S rRNA transcription*

It was demonstrated that, in a *X. laevis* kidney cell line transcribing low levels of oocyte 5S rRNA, a small portion of the oocyte genes were packaged with hyperacetylated histones (Reynolds *et al.*, 1982). Although this seemingly indicates a direct relationship between histone acetylation and 5S rRNA transcription, it is not known whether the active oocyte genes are the subset packaged with acetylated histones in this cell line. To examine

Figure 25. Acetic acid (6%) - Urea (8M) - Triton X-100 (8 mM) electrophoretic analysis of the histones from the nucleosome core particles used for the octamer transfer reconstitutions. Lane 1, chicken erythrocyte core particles; lane 2, HeLa cell nucleosome core particles; lane 3, nucleosome core particles from butyrate treated HeLa cells. The number of acetyl groups on histone H4 is indicated by the numbers to the right of the gel lanes.

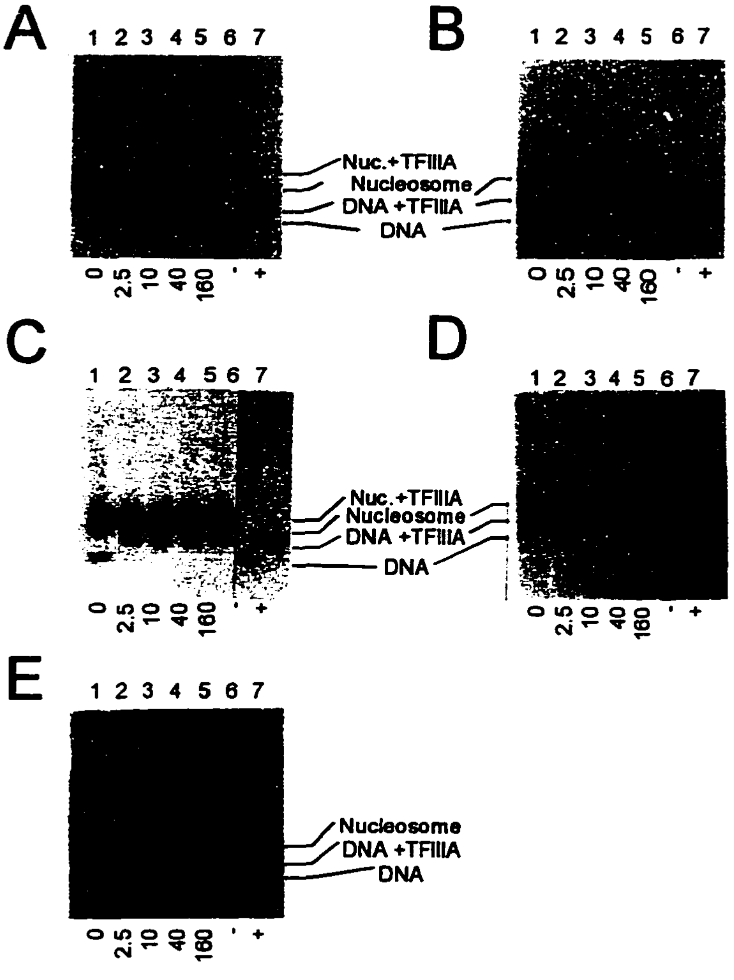

Figure 26. Analysis of the binding of TFIIIA to nucleosomes containing hyperacetylated HeLa histones. The five *X. laevis* 5S rRNA gene fragments were tested for binding after reconstitution with nucleosome core particles isolated from sodium butyrate treated HeLa cells. (A) Xlo(-83→+136); (B) Xlo(-38→+149); (C) Xls(-74→+147); (D) Xls(-51→+147); and (E) Xls(-1→+204). Lane 1, no TFIIIA; lanes 2-5, increasing amounts of TFIIIA (molar ratios indicated beneath the gel); lanes 6 and 7, the corresponding DNA templates in the absence (-) or presence (+) of TFIIIA. The samples were incubated for 20 minutes at room temperature before loading on 0.75% agarose gels.

a possible relationship between histone hypoacetylation and the transcriptional silencing of the oocyte 5S rRNA genes, we sought to determine whether these genes are packaged with hypoacetylated histone H4 when transcriptionally repressed in somatic cells. Certain *Xenopus* tissue culture cell lines express low levels of oocyte 5S rRNA (Ford and Mathieson, 1976) and thus it was necessary to determine whether the oocyte genes were truly repressed in the somatic cell line used in this investigation. To this end, total cellular RNA was isolated from a *Xenopus* kidney cell line, resolved by partially denaturing PAGE, blotted and probed with the oocyte 5S rRNA coding sequence. Due to a six nucleotide difference between the oocyte and somatic 5S rRNAs, the two molecules migrate differently in this partially denaturing gel system. This can be seen by comparing lanes 1 and 2 (Figure 27) which show that the T7 RNA polymerase transcribed somatic 5S rRNA migrated faster than its oocyte counterpart. Lane 3 shows the results of Northern blot analysis of the total cellular RNA from the *Xenopus* tissue culture cell line. The results show only one band, the migration of which closely resembled that of the somatic 5S rRNA (lane 1). No oocyte-like transcripts were observed. The lowest limit of detection of this analysis is ~0.5 pg of 5S rRNA. This, taken together with the fact that there are 50 times more oocyte than somatic genes, suggests that the oocyte genes are transcribed at a rate at least 5000 fold less than that of the somatic. It must be noted that the T7 RNA polymerase transcribed somatic 5S rRNA migrated slightly slower than that isolated from the tissue culture cells. This is due to the fact that the T7 transcribed 5S rRNAs were 121 nucleotides in length due to run-off transcription of *Dra* I digested plasmids, whereas native 5S rRNA is only 120 nucleotides. This extra nucleotide has been shown not to participate in the secondary structure of 5S rRNA. The conclusion of these

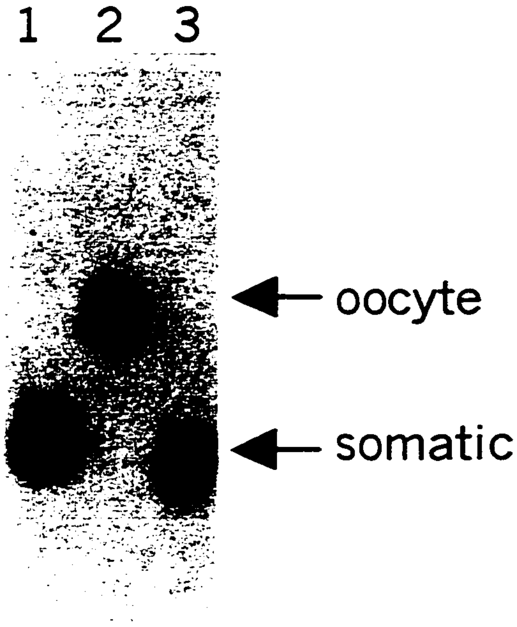

Figure 27. The oocyte 5S rRNA genes are repressed in the *X. laevis* K2 kidney cell line. Total *X laevis* K2 kidney cell line RNA was resolved by partially denaturing PAGE (12.5% acrylamide and 2.5 M urea in 1× TBE,), Northern blotted and probed with a 180 bp fragment containing a single copy of the oocyte 5S rRNA transcribed sequence. **Lane 1**, T7 RNA polymerase transcribed somatic 5S rRNA (~25 ng); **lane 2**, T7 RNA polymerase transcribed oocyte 5S rRNA (~25 ng); **lane 3**, total cellular *X. laevis* K2 kidney cell line RNA (from ~2 × 10^5 cells).

results is that, in the *Xenopus* tissue culture cell line used in this study, the oocyte genes were repressed.

3.3.3. Western blot analysis and control immunoprecipitation

In this investigation, an antibody specific for hyperacetylated histone H4 was used to immunoprecipitate chromatin fragments. This antibody, prepared as described previously (Lin *et al.*, 1989), was raised against the first 20 amino acids of tetra-acetylated histone H4 from *Tetrahymena*, which differs significantly from that of *Xenopus*. To determine whether this antibody showed the same specificity for hyperacetylated *Xenopus* histone H4, a western blot analysis (Figure 28A) was performed using histones from butyrate treated HeLa cells (*X. laevis* histone H4 shares an identical first 20 amino acids with human H4). By comparing the coomassie stained histones (lane 1), with the immunodetected histones (lane 2), it can be seen that the antibody used in this study, strongly reacted with the di- to tetra- acetylated H4 but did not recognize non-acetylated H4. Thus, this antibody would be expected to only immunoprecipitate nucleosomes packaged with acetylated histone H4. To verify this, a control immunoprecipitation was performed. The starting samples for this immunoprecipitation consisted of unlabeled, non-acetylated nucleosome cores (chicken erythrocyte), containing a 0.1% fraction of either radiolabeled acetylated, or radiolabeled hypoacetylated HeLa cell nucleosome core particles. These samples were used to demonstrate that the antibody used in this study could selectively fractionate the hyperacetylated cores from the non-acetylated cores (Figure 28B).

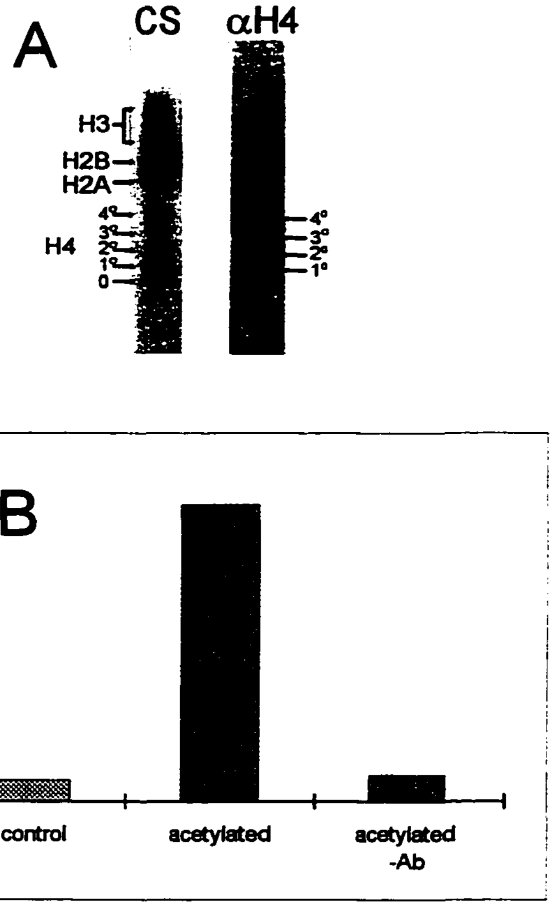

Figure 28. **(A)** Western blot analysis of hyperacetylated histones. Approximately 10 μg of histones isolated from butyrate treated HeLa cells (fraction "c", Ausió *et al.*, 1989) were resolved on an acetic acid/urea polyacrylamide gel, blotted and immunodetected with an antibody against *Tetrahymena* acetylated histone H4. Lane 1, coomassie stained gel; lane 2, Immunostained blot. **(B)** Control immunoprecipitation of hyperacetylated histones. Approximately 15 ng of $[\gamma\text{-}^{32}P]$ ATP labeled, non-acetylated (**control**) or hyperacetylated (**acetylated**) HeLa cell mononucleosome cores were mixed with 100 μg of unlabeled chicken erythrocyte mononucleosome cores and subjected to immunoprecipitation with an antibody specific for hyperacetylated histone H4. The total counts per minute of a one ten volume of the total immunoprecipitate was determined. A negative control immunoprecipitation of hyperacetylated nucleosomes was performed in the absence of antibody (**acetylated - Ab**).

3.3.4. Mapping histone H4 acetylation of oocyte 5S rRNA genes

Chromatin fragments isolated from the *X. laevis* kidney cell line were immunoprecipitated with the anti-acetylated histone H4 antibody using the experimental scheme shown (Figure 29). The resulting nucleosomal DNA was deproteinized and probed by dot blot analysis for the presence of the oocyte 5S rRNA gene. A DNA fragment corresponding to the upstream non-transcribed sequence of the oocyte gene was used as a probe and Figure 30A shows that this probe did not anneal to any portion of the somatic 5S rRNA gene (S). Identical A_{260}'s of DNA was applied to each dot in Figure 30A and thus, if the oocyte 5S rRNA genes were packaged with acetylated histone H4, there would be an enrichment for this sequence in the immunoprecipitate, and a more intense signal for the bound fraction (AB) than that of the unbound (UB). The results show that, although there was some signal (αH4), there was not an enrichment for the oocyte genes in the bound fraction. The fact that the "bound" signal is considerably less intense than that of the unbound, suggests the oocyte genes were selectively not precipitated. This demonstrates that the upstream, non-transcribed regions of the oocyte 5S rRNA genes were not packaged with hyperacetylated histone H4. Assuming that the acetylation of the spacer nucleosome reflects that of the nucleosomes on the 5S rRNA coding sequence, these results indicate that the transcriptionally silent oocyte genes in *X. laevis* are not packaged with acetylated histones. Immunoprecipitations performed in the absence of antibody (-Ab), or with non-specific polysera (PS), did not show any signal.

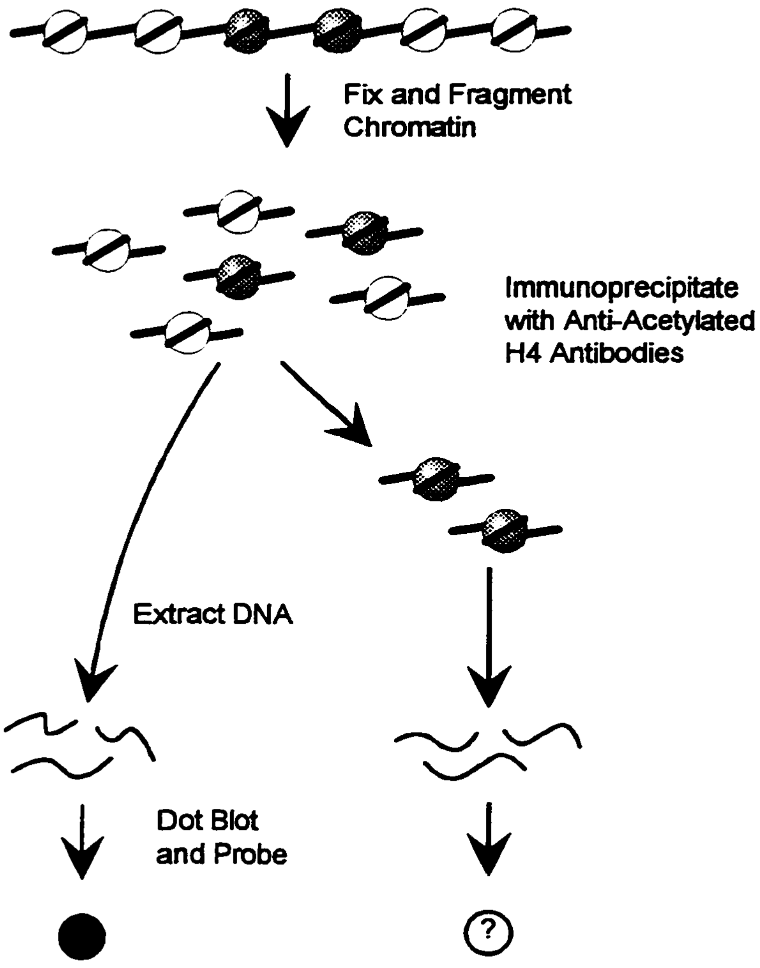

Figure 29. A schematic representation of the immunofractionation technique used to analyze the acetylation state of the *X. laevis* oocyte 5S rRNA genes. The circles represent the histone octamer, either acetylated (shaded) or non-acetylated (open) and the black lines represent the DNA.

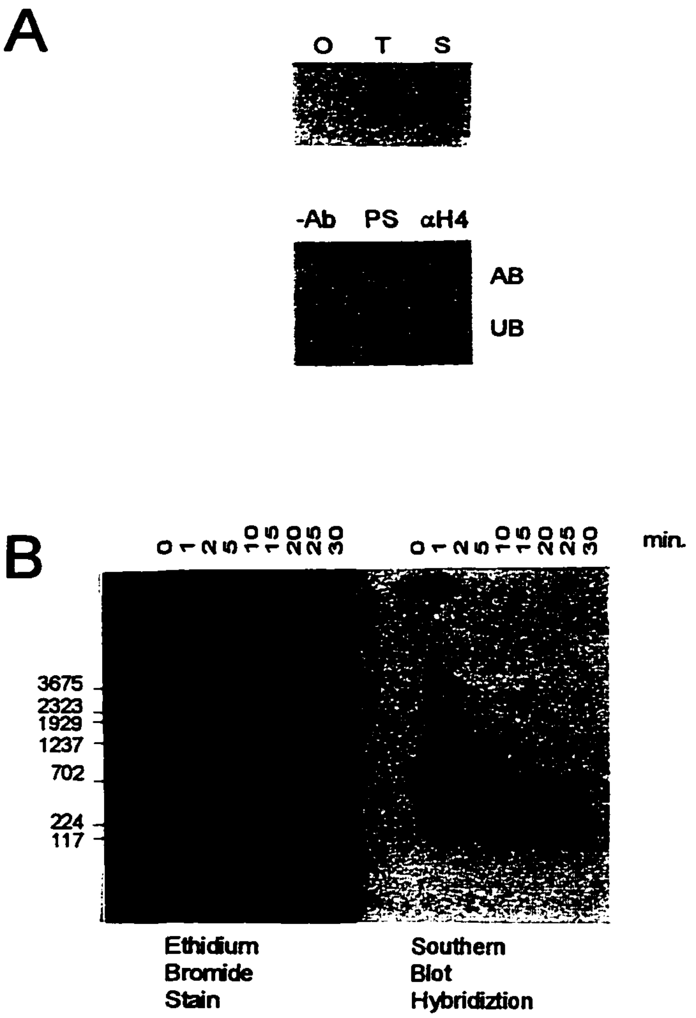

Figure 30. The oocyte genes in the *X. laevis* somatic cells are packaged with hypoacetylated histone H4. (**A**) Fragmented chromatin isolated from *Xenopus* tissue culture cells was immunoprecipitated with an anti-acetylated histone H4 antibody (αH4); non-specific polysera (**PS**) or without antibody (-**Ab**). Forty nanograms of the precipitated DNA was applied to a Zeta-Probe membrane and probed with a ~360 bp, upstream, non-transcribed sequence of an oocyte 5S rRNA gene. **O**, 50 pg of a full length oocyte 5S rRNA gene isolated from pXlo8; S, 50 pg of a full length somatic 5S rRNA gene isolated from pXls11; **T**, ~300 ng total chromatin; **AB**, immunoprecipitated chromatin; **UB**, unbound chromatin. (**B**) Southern blot hybridization of micrococcal nuclease digested nuclei from *X. laevis* K2 kidney cell line. Nuclei were digested for the times indicated with 4 units of micrococcal nuclease per μg of DNA. Following nuclei lysis and DNA deproteinization, approximately 6 μg aliquots of DNA were resolved on a 1% agarose gel, ethidium bromide stained, Southern blotted and probed with the ~360 bp upstream non-transcribed sequence of the oocyte 5S rRNA gene.

3.3.5. Micrococcal nuclease digestion of oocyte 5S rRNA genes

The above results suggest that transcriptionally silent oocyte 5S rRNA genes are not packaged with hyperacetylated histone H4. This would seemingly suggest the presence of non-acetylated histones on these genes. To verify the presence of nucleosomes on the oocyte genes, tissue culture cell chromatin was digested with micrococcal nuclease and Southern blotted. If the upstream, non-transcribed sequence of the oocyte gene had a low abundance of nucleosomes, this region of DNA would be sensitive to micrococcal nuclease digestion. Upon electrophoresis (Figure 30B), the resulting DNA from the digested chromatin gives a characteristic "ladder" of fragments derived from multiple units of the nucleosome repeat. This was seen for both the bulk chromatin (ethidium bromide stained) and the oocyte genes (Southern blot hybridized). These results also show that the oocyte genes in this cell line were less sensitive to micrococcal digestion than the bulk of the chromatin, as more time was required to digest the oocyte genes to mononucleosomes (compare the digestion profiles of the bulk chromatin and oocyte genes after 1 min of digestion).

3.4. Role of Nucleoplasmin in the Transcription of the 5S rRNA genes in X. laevis

The phosphoprotein nucleoplasmin is the most abundant protein in *Xenopus* oocyte nuclei (Mills *et al.*, 1980, Krone and Franke, 1980). This protein is extremely acidic, consisting of 23% glutamate and aspartate residues (Burglin *et al.*, 1987, Dingwall *et al.*, 1987). Initially the function of nucleoplasmin was postulated to be two-fold: post-fertilization decondensation of sperm chromatin by removal of the basic sperm protamines, and assembly of nucleosomes by binding histones H2A and H2B and transferring them to DNA (Philpott and Leno, 1992, Laskey *et al.*, 1993, Katagiri and Ohsumi, 1994). Evidence suggests that nucleoplasmin may serve other functions as well. First, nucleoplasmin is present throughout oogenesis and not simply in the mature, unfertilized egg as would be expected if only required post-fertilization (Burglin *et al.*, 1987, Litvin and King, 1988). Second, a nucleoplasmin-like protein has been identified in *Drosophila* somatic cells (Ito *et al.*, 1996a), suggesting these highly acidic proteins are required for some function other than the decondensation of sperm chromatin as originally proposed. Finally, in the amphibian *Pleurodeles waltlii*, nucleoplasmin is found associated with lampbrush loops (Moreau *et al.*, 1986), which are regions of transcriptional activity, suggesting a role for nucleoplasmin in transcription. It has been demonstrated that nucleoplasmin stimulates the binding of the *trans*-acting factors GAL-4, USF and Sp1 to their cognate binding sites within nucleosomal DNA *in vitro* (Chen *et al.*, 1994, Walter *et al.*, 1995), and that nucleoplasmin functions in this capacity by removing the core histones H2A and H2B subsequent to transcription factor binding. In these studies however, a heterologous system was used which mixed yeast and mammalian *trans*-acting factors, *Xenopus* egg nucleoplasmin, and HeLa cell histones. Although such a system may provide a suitable model for examining the nucleosome remodeling effects of nucleoplasmin, a more suitable homologous system would be required to determine whether this effect has any *in vivo* relevance.

3.4.1. Integrity of nucleoplasmin

The proteins used for the nucleoplasmin study are shown on a SDS PAGE (Figure 31A). It must be noted that, chicken erythrocyte histones and not *Xenopus* histones were used for this study. It is unlikely however, that this affected the results due to the highly conserved nature of core histones (histones H3 and H4 share identical sequences in *Xenopus* and chicken). Furthermore, studies mapping nucleosome positions on reconstituted *X. laevis* 5S rRNA gene fragments, showed similar results with both *Xenopus* and chicken erythrocyte histones (Gottesfeld, 1987). To demonstrate that the nucleoplasmin was functional with respect to nucleosome assembly activity, a supercoiling assay was performed (Figure 31B). In this assay, nucleosomes were mixed with closed circular SV40 DNA (one octamer for each 165 bp of DNA) at physiological concentrations of salt. Under these conditions, in the absence of nucleoplasmin, an insoluble complex formed (compare lanes 3 and 4) due to the electrostatic interactions between the DNA and histones. Nucleoplasmin serves to circumvent this aggregation by binding histones and regulating the formation of a soluble complex (compare lanes 5 and 6) containing nucleosomes. The deposition of nucleosomes in the presence of topoisomerase I caused the DNA to become highly supercoiled as can be seen in lane 6.

3.4.2. The effect of nucleoplasmin on the binding of TFIIIA to mononucleosomal DNA

Nucleoplasmin is the most abundant protein in the nucleus of *Xenopus* oocytes and thus, if it does play a role in enhancing transcription factor accessibility, this should also be observed with transcription factors present in *Xenopus* oocytes. Previous to this work it

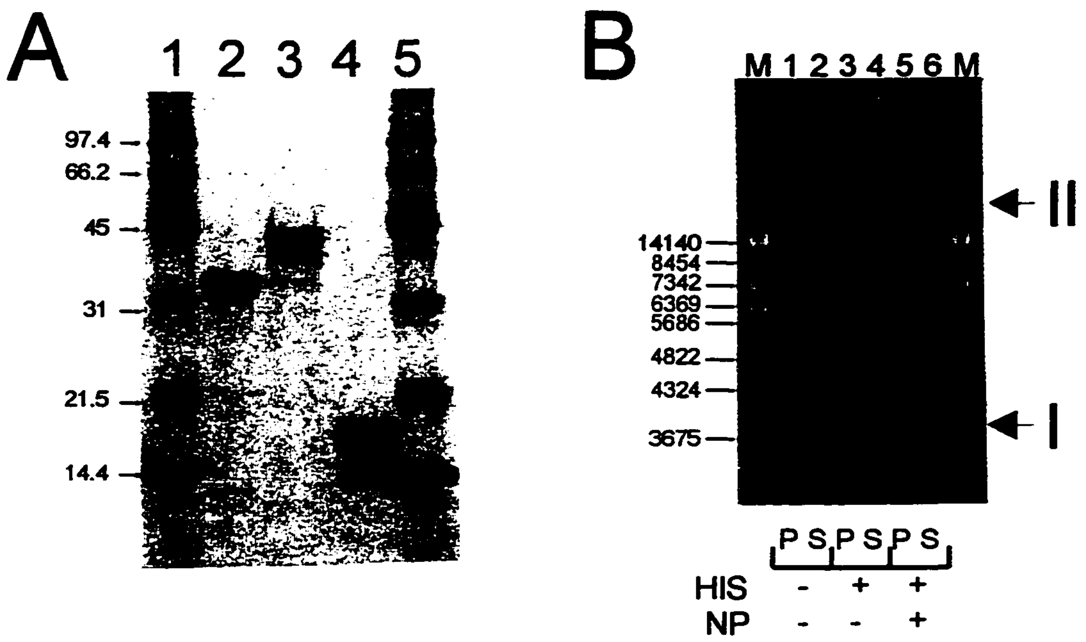

Figure 31. **(A)** SDS PAGE analysis of the purified proteins used in the nucleoplasmin study. Lanes 1 and 5, molecular weight protein markers (sizes shown in kDa); lane 2, nucleoplasmin purified from unfertilized *Xenopus* eggs; lane 3, chicken erythrocyte core particle histones; lane 4, recombinant *Xenopus* TFIIIA. **(B)** *In vitro* chromatin assembly capability of *Xenopus* nucleoplasmin. Five hundred ng of topoisomerase I treated, closed circular SV40 DNA (lane 2) was incubated with 0.5 μg of histone octamers previously mixed without (lanes 3 and 4) or with (lanes 5 and 6) 1 μg nucleoplasmin. After 90 minutes at 37°C , the assembly reactions were centrifuged for 10 minutes at 15000×g and both the pelleted (P) and supernatant (S) material were deproteinized and electrophoresed on a 2% agarose 1× TPE gel to resolve the relaxed (II) or supercoiled (I) DNA forms.

was demonstrated that removal of histones H2A and H2B overcomes the blockage of TFIIIA binding by nucleosomes (Hayes and Wolffe, 1992). The initial objective of this study was to determine whether nucleoplasmin, which preferentially removes histones H2A and H2B from nucleosomes (Chen *et al.*, 1994), could facilitate the binding of TFIIIA to a nucleosomal 5S rRNA gene fragment which normally will not bind TFIIIA. Figure 32A shows a TFIIIA electrophoretic mobility shift assay of the oocyte 5S rRNA gene fragment Xlo(-38→+149) complexed (lanes 1 - 10) or uncomplexed (lanes 11 and 12) into nucleosomes. The concentration of TFIIIA is indicated at the bottom the gel and the higher concentrations used in this study were sufficient to completely shift the corresponding uncomplexed DNA alone. As shown before, a nucleosome on this gene fragment blocked TFIIIA binding (lanes 1 through 5). Upon addition of nucleoplasmin to the binding reaction, a nucleosomal shift by TFIIIA was still absent (lanes 6 through 10) indicating nucleoplasmin was unable to circumvent the nucleosomal blockage of TFIIIA binding to this gene fragment. The results suggest that, despite the previously reported nucleosome remodeling ability of nucleoplasmin, the binding of TFIIIA to nucleosomal DNA could not be facilitated by this protein.

One possible explanation for the discrepancy between our results and those of Chen *et al.*, (1994), is in their study, a small amount of transcription factor binding was already evident in the absence of nucleoplasmin, and the addition of nucleoplasmin served to greatly increase this binding. In our study however, no binding of TFIIIA to nucleosomal Xlo(-38→+149) DNA was evident, and thus we repeated the TFIIIA electrophoretic mobility shift assay using the 5S rRNA gene fragment Xlo(-83→+136) instead. As shown earlier, this fragment positions nucleosomes in a manner which permits

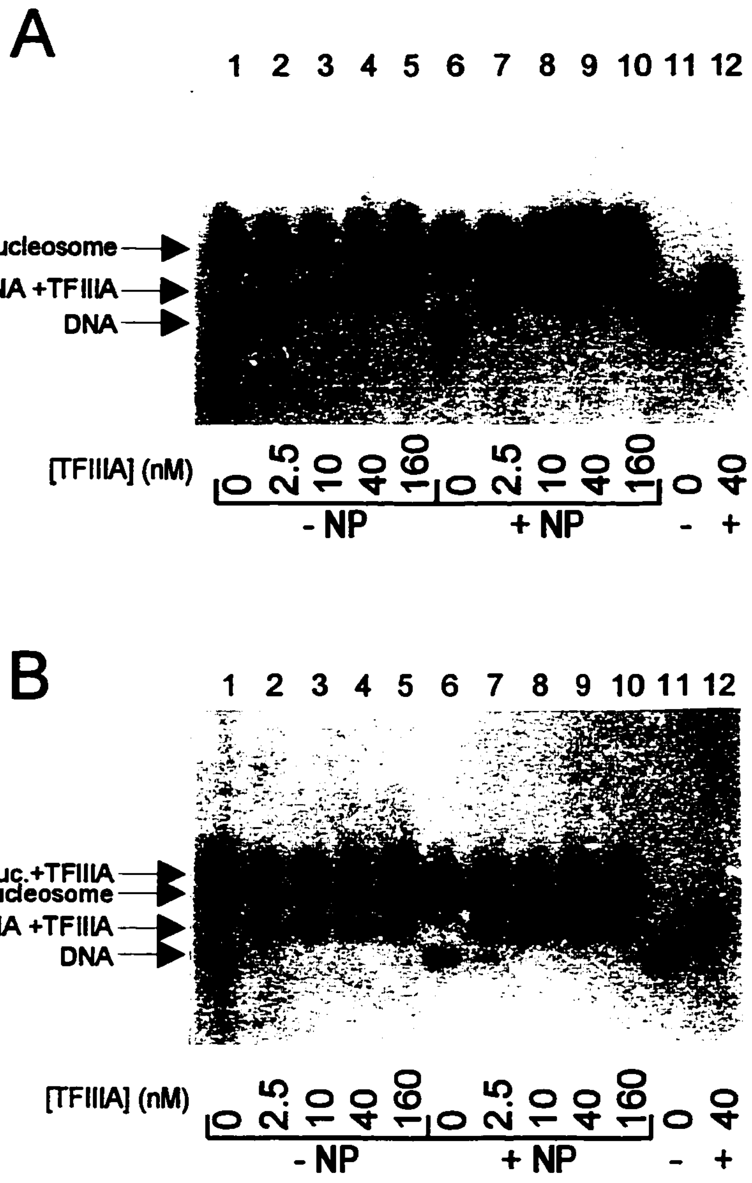

Figure 32. Agarose gel electrophoretic analysis of the effect of nucleoplasmin on the binding of TFIIIA to nucleosomal *X. laevis* 5S rRNA gene fragments. (A) and (B) Nucleosomes (1 fmole) reconstituted with chicken erythrocyte histones on Xlo(-38→+149) (A) or Xlo(-83→+136) (B) in the absence (lanes 1 - 5) or presence (lanes 6 - 10) of 150 ng of *Xenopus* nucleoplasmin and increasing amounts of TFIIIA (molar ratios indicated beneath the gels). Lanes 11 and 12, the corresponding uncomplexed DNA in the absence (-) or presence (+) of TFIIIA. The samples were incubated for 20 minutes at room temperature before loading on the 0.75% agarose gels.

TFIIIA binding, but at much higher concentrations of TFIIIA than is required for binding of naked DNA alone. We were thus testing whether nucleoplasmin could enhance the binding of TFIIIA to nucleosomal DNA. The results, shown in Figure 32B, demonstrate that a 160 nM concentration of TFIIIA partially shifted Xlo(-83→+136) after reconstitution into nucleosomes (lane 5). If nucleoplasmin was able to remodel this nucleosome in a manner which facilitated TFIIIA binding, this shift should be evident at lower concentrations of TFIIIA which is not the case (lanes 6-10). This demonstrates that nucleoplasmin was unable to enhance the binding of TFIIIA to nucleosomal DNA.

3.4.3. Effect of nucleoplasmin on 5S rRNA transcription from nucleosomal templates

Previously, it was shown that deposition of nucleosomes onto plasmids containing 5S rRNA genes, repressed 5S rRNA transcription (Gottesfeld and Bloomer, 1982, Stunkel *et al.*, 1995). Similar plasmids, reconstituted with physiological levels of histones H3 and H4 alone, are permissive to 5S rRNA transcription (Tremethick *et al.*, 1990). We took advantage of these results to test whether nucleoplasmin, by selectively removing H2A and H2B, could enhance transcription of nucleosomal 5S rRNA genes. To this end, nucleosomes were reconstituted onto a full length oocyte 5S rRNA gene repeat by the salt-gradient dialysis method, and these genes were transcribed in HeLa nuclear extracts supplemented with TFIIIA and increasing amounts of nucleoplasmin. HeLa cell nuclear extracts were chosen over *Xenopus* oocyte extracts because they lacked nucleoplasmin. The results of transcription studies (Figure 33), indicate that nucleosomes reconstituted on the oocyte gene repeat repressed 5S rRNA transcription (lane 3). Nucleoplasmin did not

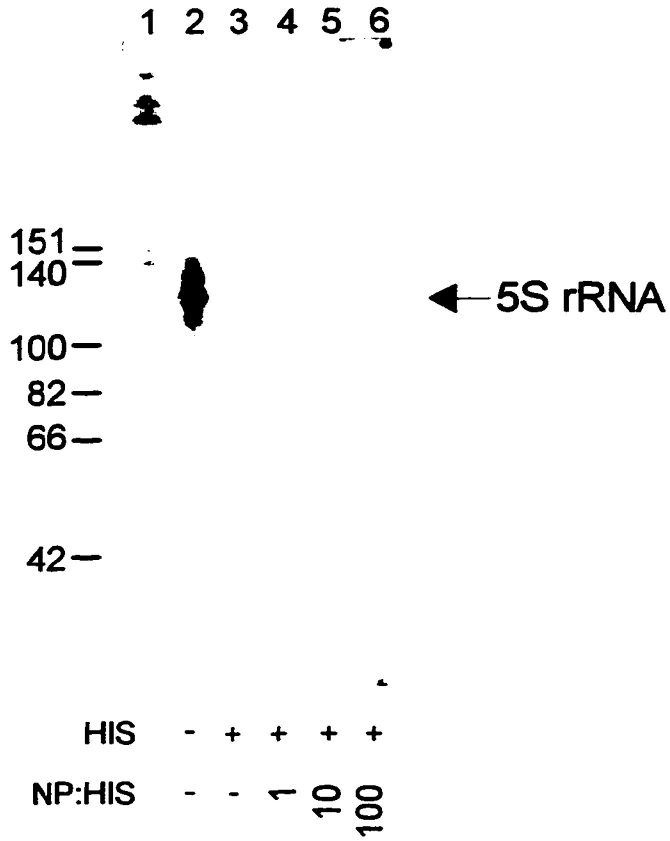

Figure 33. The effect of nucleoplasmin on the transcription of reconstituted *Xenopus laevis* oocyte 5S rRNA genes. Approximately 250 ng of oocyte 5S rRNA genes either uncomplexed (lane 2) or reconstituted with histones isolated from chicken erythrocytes (lanes 3-6) were transcribed in the absence (lanes 2 and 3), presence (lanes 4-6) of increasing amounts of nucleoplasmin as shown. The transcriptions were performed in HeLa nuclear extracts supplemented with 150 nM recombinant *Xenopus* TFIIIA and 2 mM $MgCl_2$. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (8% acrylamide, 8.3 M urea in 1× TBE). Lane 1, Klenow end-labeled *Hinf*I cut ϕX174 DNA (sizes of marker fragments shown as number of nucleotides).

circumvent this repression even at ratios of 100 molecules of nucleoplasmin for every histone octamer present (lanes 4-6).

3.5. Role of Histone H1 in the Differential Transcription of the 5S rRNA genes in X. laevis

3.5.1. The binding of histone H1 to free and nucleosomal 5S rRNA genes

In 1984, Schlissel and Brown demonstrated that, in somatic cells, repression of oocyte 5S rRNA gene transcription was due to the presence of histone H1. Later, it was shown that incorporation of histone H1 into chromatin during embryogenesis, results in specific repression of TFIIIA-activated oocyte 5S rRNA production (Bouvet *et al.*, 1994). In 1990, Jermanowski and Cole showed that histone H1 binds preferentially to the oocyte gene over the somatic, and this preference was due to differences in the 5' and 3' flanking regions of the two 5S rRNA gene families. However Jermanowski and Cole performed their experiments using naked DNA which binds H1 in a different manner than nucleosomal DNA (Hayes and Wolffe, 1993). The difference in these two modes of binding can be seen in an electrophoretic mobility shift assay (Figure 34A). Both naked and nucleosomal DNA were incubated with increasing concentrations of HeLa cell histone H1 (shown in the SDS PAGE in Figure 35), and in both cases, incubation with the higher levels of H1 produced aggregates made evident by the radioactive signal in the area of the wells. However, at lower H1 concentrations, transient complexes were formed which entered the gel but with reduced mobility when compared to the unbound substrate. In the case of naked DNA, these complexes were very sensitive to H1 concentrations in that

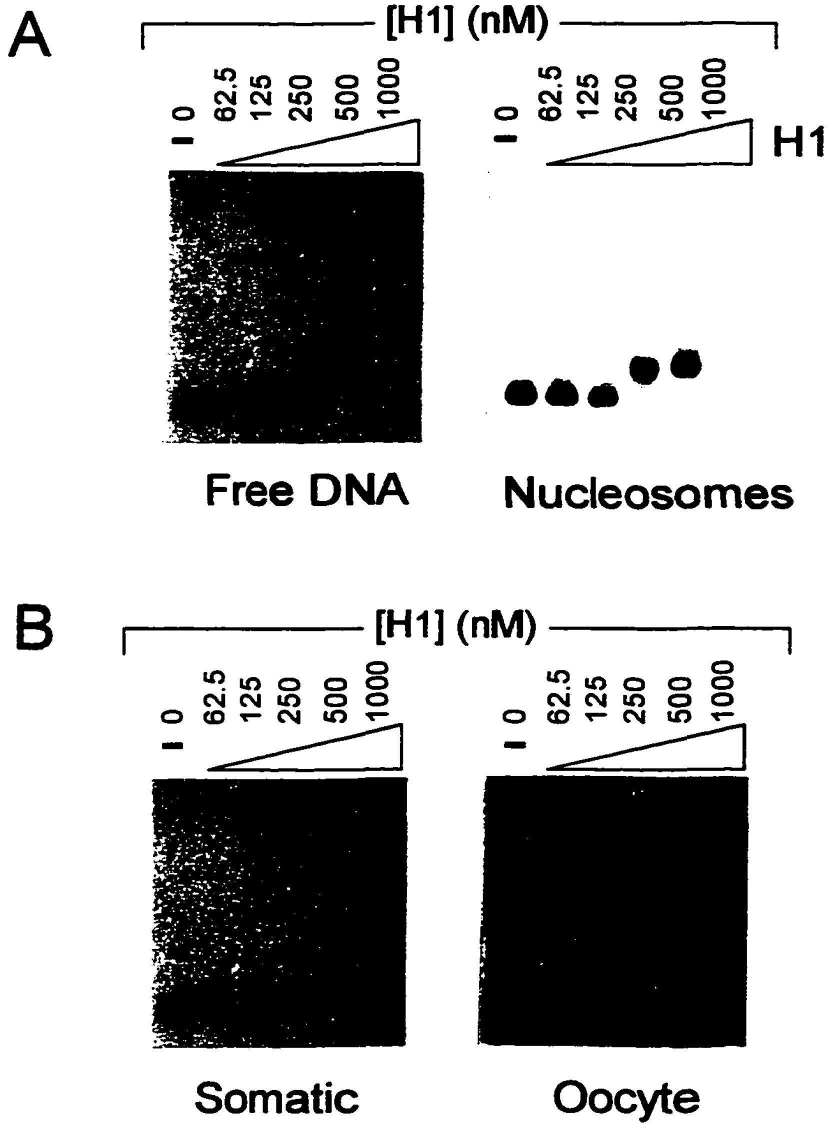

Figure 34. The binding of histone H1 to naked and nucleosomal 5S rRNA gene fragments. **(A)** A comparison of the binding of histone H1 to naked and nucleosomal Xls(-52→+147). **(B)** A similar comparison of the binding of histone H1 to oocyte and somatic *X. laevis* 5S rRNA gene fragments Xlo(-38→+149) and Xls(-52→+147) in the absence of nucleosomes. For each shift, ~0.5-1 fmoles of labeled nucleosomes were incubated with the indicated amounts of histone H1, incubated for 20 minutes at room temperature and electrophoresed on 0.75% agarose gels.

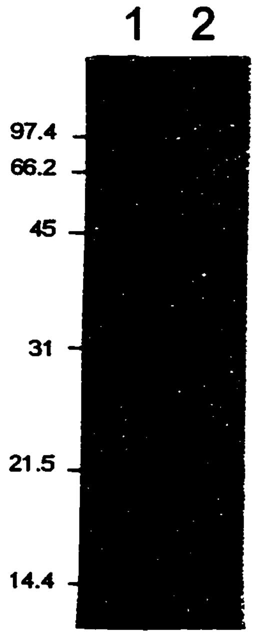

Figure 35. Coomassie stained SDS PAGE analysis of HeLa cell histone H1 used in this study. Lane 1, molecular weight protein markers (sizes shown in kDa); lane 2, histone H1 (1 μg).

they were only present with a specific amount of H1. In contrast, the binding of H1 to nucleosomal DNA appeared to be more stable since the H1-nucleosome complex was present at least two different concentrations of H1. This reaffirms previously published data showing that the binding of histone H1 to naked DNA is highly co-operative compared to that of nucleosomal DNA (Clark and Thomas, 1988). Thus these results show that the binding of H1 to a nucleosome is different than that of naked DNA.

The object of this study was to make a direct comparison between the somatic and oocyte 5S rRNA genes with respect to the binding of histone H1 to nucleosomal DNA. Initially, we reconstituted mononucleosomes onto ~200 bp fragments of each gene [Xlo(-38→+149) and Xls(-52→+147)]. These fragments were selected because they include the entire 5S rRNA intragenic promoter and coding sequence, and thus the region of DNA to which histone H1 binding would exhibit its greatest effect. As shown earlier, both fragments position nucleosomes at multiple positions (Figure 19). Although at first glance, these multiple translational positions would be expected to complicate analysis of results, because the oocyte genes have been shown to contain multiple, overlapping positions *in vivo* (Gottesfeld and Bloomer, 1980), we feel this is a better representation than a gene fragment with a strong octamer positioning signal such as that of the *X. borealis* gene (Rhodes, 1985). An H1 titration of these DNA fragments was performed to determine whether a difference in H1 binding to naked DNA could be observed. The results, shown in Figure 34B, suggest that the difference in histone H1 binding between the oocyte and somatic 5S rRNA genes was negligible. The oocyte gene fragment formed aggregates at lower H1 concentrations than the somatic counterpart, but the difference was minor.

To compare the binding of histone H1 to nucleosomal oocyte and somatic 5S rRNA genes, gene fragments were reconstituted with HeLa cell histone octamers and tested for H1 binding. The results (Figure 36A) demonstrate that histone H1 bound equally to both fragments after nucleosome reconstitution. It is interesting to note that in the case of the oocyte gene, there appeared to be a secondary shift which is suggestive of more than one molecule of H1 interacting in a stable manner with one nucleosome. Although these results do not show an increased affinity of histone H1 for the oocyte gene, they do suggest that the oocyte nucleosome is capable of binding more than one molecule of histone H1.

3.5.2. DNase I footprinting of nucleosomes containing histone H1

Several researchers have attempted to detect histone H1 binding to nucleosomal DNA by DNase I footprinting. The results thus far have been discouraging with no histone H1 DNase I protection evident. However, in the case of the *X. laevis* 5S rRNA genes, the footprints have always been performed using the somatic gene which may not strongly bind histone H1 *in vivo*. DNase I footprinting was used in this study to determine whether any difference could be observed between the binding of histone H1 to oocyte 5S rRNA genes compared to the somatic counterparts. To this end, nucleosomes reconstituted on fragments Xls(-51→+147) and Xlo(-38→+149) were incubated with increasing concentrations of histone H1 and digested with DNase I. Denaturing gels (Figures 37A and B) of the resulting fragments show that the presence of histone H1 did not affect the rate of digestion of the nucleosomal somatic gene fragment (Figure 37A). A

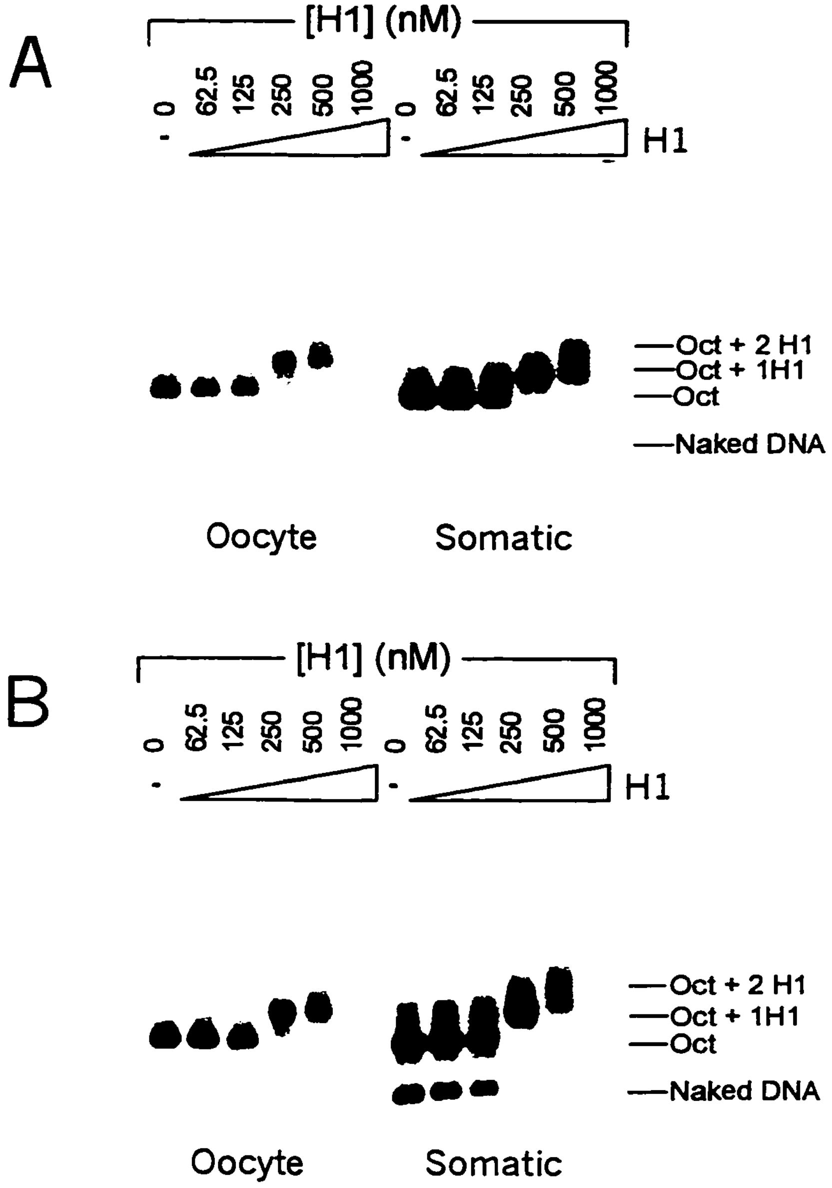

Figure 36. Histone H1 binds equally to the oocyte and somatic *Xenopus laevis* 5S rRNA gene fragments Xlo(-38→+149) and Xls(-51→+147) after reconstitution with either control (**A**), or hyperacetylated histone octamers (**B**). Approximately 0.5-1 fmoles of labeled nucleosomes were incubated with the indicated amounts of histone H1, incubated for 20 minutes at room temperature and electrophoresed on 0.75% agarose gels.

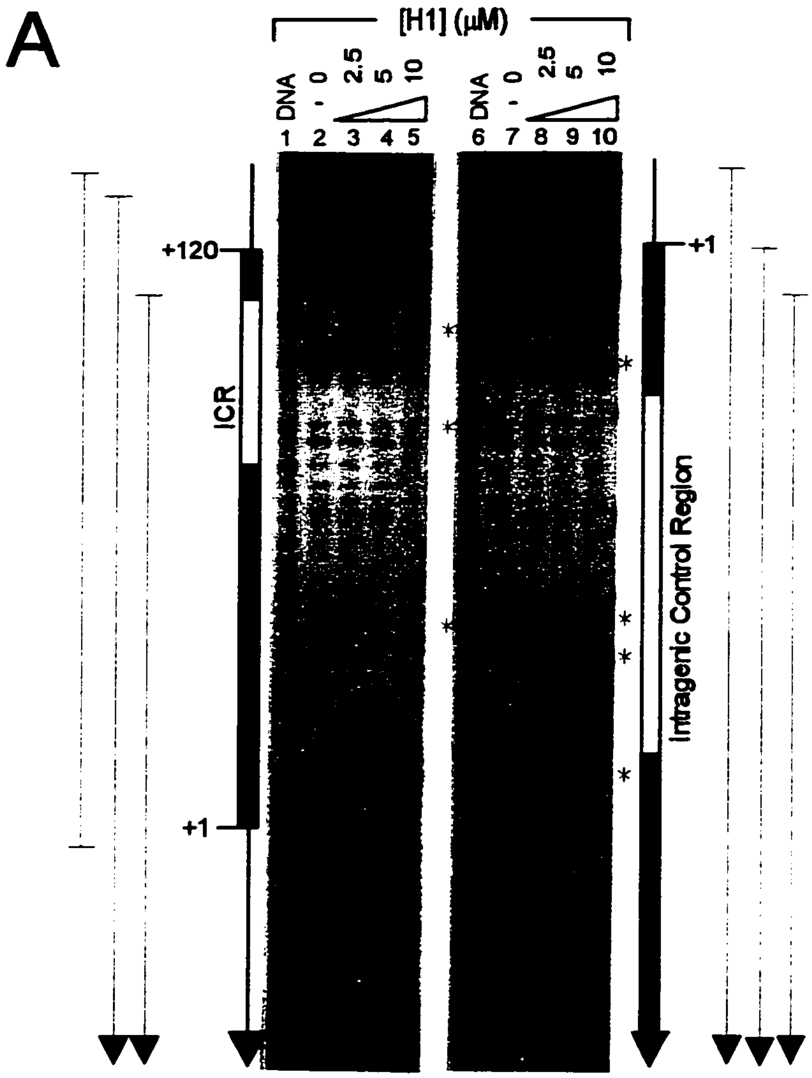

Figure 37. DNase I footprint analysis of the complexes formed by nucleosomes with increasing amounts of histone H1 on the Xls (-51→+147) (**A**) and Xlo(-38→+149) (**B**) 5S rRNA gene fragments. Ten femtomoles of nucleosomes labeled at the 3' end of the coding (lanes 2 to 5) and non-coding (lanes 7 to 10) strands were incubated in the presence of histone H1 and subsequently digested with DNase I. The 5S rRNA gene is indicated by the thick black line and the intragenic control region (ICR) by an open box. Lines to the left and the right of the footprints represent the translational position of nucleosomes on these fragments. Sites of DNase I protection by incorporation of histone H1 are indicated by an *.

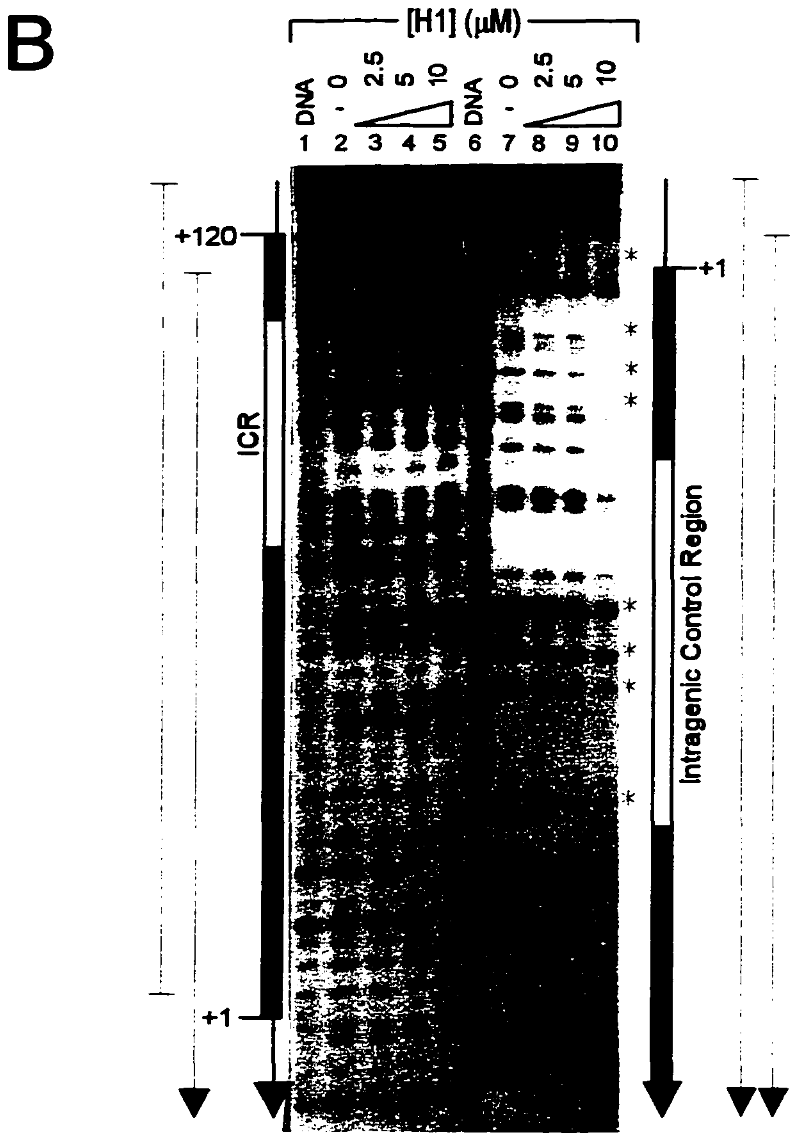

Figure 37. (continued)

few sites which become less sensitive to digestion with only the highest histone H1 concentration are indicated by an *. The footprint of the oocyte gene fragment (Figure 37B) is similar to that of the somatic in that increasing concentrations of histone H1 had no effect on the rate of digestion of the 3` end of the gene. In contrast, increasing the concentration of H1 decreased the rate of digestion of the 5` of the gene. This may indicate an "off-axis" location of histone H1 binding to this fragment. This asymmetrical protection was observed over a region extending 100 bp which is probably not attributed to protection of these nucleotides by the linker histone, but rather an alteration in the contacts between the core histones and DNA in this region.

3.5.3. The effect of histone acetylation on the binding of histone H1

Histone acetylation has long been linked to transcriptional activity and studies have shown that the histone amino termini modulate the interaction of histone H1 with nucleosomes (Juan *et al.*, 1997). Previously it was demonstrated, using a fragment of the *X. borealis* somatic 5S rRNA gene, that histone acetylation does not prevent the binding of histone H1 to nucleosomal DNA (Ura *et al.*, 1994, 1997). Again, the importance of this observation may be limited by the fact that histone H1 may have a low affinity for the somatic genes *in vivo* (Wolffe and Brown, 1988). Figure 36B shows a similar titration as in Figure 36A with the exception that the core histones were isolated from butyrate treated HeLa cells. Once again, histone H1 did not show a preference for binding to nucleosomal oocyte genes when compared to the somatic counterparts, but again a secondary shift of the oocyte nucleosome was observed at higher concentrations of histone H1.

3.5.4. The binding of histone H1 to nucleosomal arrays

The results of this work thus far suggest that histone H1 can bind both the oocyte and somatic 5S rRNA genes after nucleosome reconstitution. This is not necessarily surprising because although histone H1 is proposed to be a repressor of transcription, studies show that transcriptionally active genes are not necessarily devoid of histone H1 (Zlatanova and van Holde, 1992 and references therein). It has been suggested that it is not whether histone H1 binds which determines transcriptional repression, but rather how histone H1 binds. In this study, the repressive effects of histone H1 on the transcription of nucleosomal 5S rRNA gene templates were tested. In these experiments, full length 5S rRNA gene repeats were used rather than ~200 bp fragments. This allowed more of the 5' and 3' flanking regions to be examined, and more than one nucleosome to be reconstituted per DNA strand, allowing possible internucleosome interactions to be investigated. Figure 38, an agarose gel of the reconstituted arrays, demonstrates that the majority of DNA existed in nucleoprotein complexes. DNA reconstituted with histone octamers alone migrated predominantly as one diffuse band, although some slower migrating complexes were observed (lanes 4 and 6). This indicates that the bulk of the nucleosome arrays had a similar amount of histone octamers. After addition of histone H1, the electrophoretic mobility of the arrays were retarded indicating that histone H1 was capable of binding both genes.

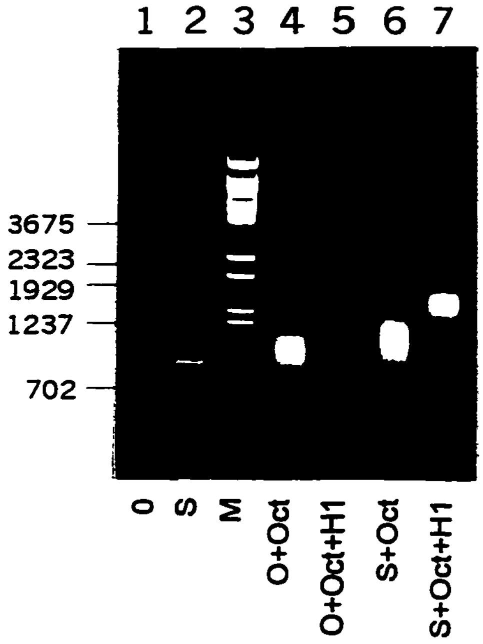

Figure 38. Histone H1 binds to both oocyte and somatic 5S rRNA full length gene repeats after reconstitution with histone octamers as demonstrated by an electrophoretic mobility shift assay on a 1% agarose gel in 1x TBE. Lanes 1 and 2, uncomplexed oocyte (O) and somatic (S) full length 5S rRNA genes; lanes 4 and 6, oocyte (O+Oct) and somatic (S+Oct) genes after reconstitution with histone octamers; lanes 5 and 7, oocyte (O+Oct+H1) and somatic (S+Oct+H1) genes after reconstitution with histone octamers and histone H1.

3.5.5. The effect of histone H1 on transcription of the 5S rRNA genes

To test the effect of histone H1 incorporation on the transcription of the 5S rRNA genes, the reconstituted arrays were transcribed in *Xenopus* oocyte extracts. Oocyte extracts were chosen instead of HeLa cell nuclear extracts because *Xenopus* oocytes lack histone H1. The results of the transcription analysis (Figure 39) demonstrate that reconstitution of an oocyte gene repeat with histone octamers once again repressed 5S rRNA transcription, but in this case, only by ~33% (compare lanes 2 and 3). The difference between this result and that seen earlier with the HeLa cell nuclear extracts (Figure 13), could be due to either an increased amount of TFIIIA in the *Xenopus* oocyte extracts or the presence of small amounts of histone H1 in the HeLa cell nuclear extracts. In contrast to the results with the oocyte gene, when a somatic gene was reconstituted with histone octamers, there did not appear to be any significant effect on gene transcription (compare lanes 5 and 6). When histone H1 was included in the oocyte and somatic reconstitutions, residual transcription of the oocyte 5S rRNA gene was decreased a further ~66% (compare lanes 3 and 4). In contrast, the presence of histone H1 on the somatic gene only decreased transcription by about 20% (compare lanes 6 and 7) indicating that RNA polymerase III was less affected by the presence of histone H1 on the somatic gene when compared to the oocyte gene.

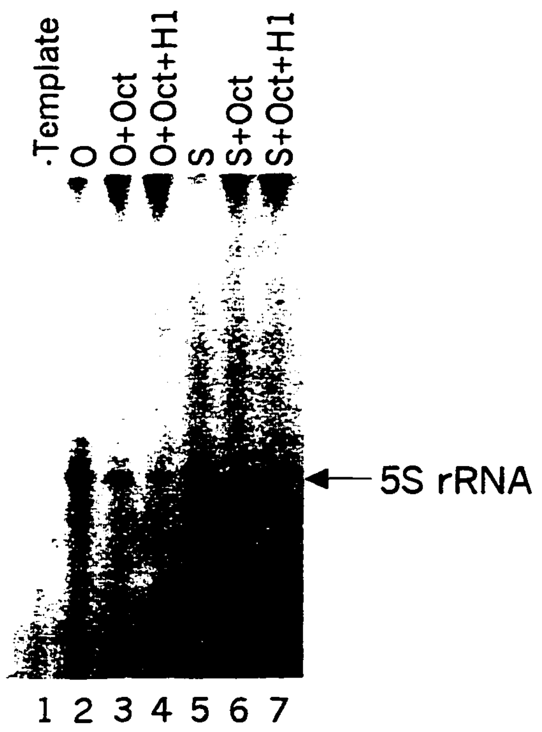

Figure 39. Histone H1 represses 5S rRNA transcription of oocyte but not somatic genes reconstituted with histone octamers. Approximately 40 ng of the oocyte (lanes 2-4) or somatic (lanes 5-7) 5S rRNA genes, either uncomplexed (lanes 2 and 5), reconstituted with chicken erythrocyte core histones (lanes 3 and 6), or reconstituted with chicken erythrocyte core histones and HeLa cell histone H1 (lanes 4 and 7) were transcribed in *Xenopus* oocyte extracts. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (8% acrylamide, 8.3 M urea in 1x TBE). Lane 1, transcription in the absence of template DNA.

3.5.6. Micrococcal nuclease digestion of nucleosomes on the somatic 5S rRNA gene.

It has been shown previously that only a partial, and not a complete loss of histone H1 coincides with transcription (Zlatanova and van Holde, 1992 and references therein). It is not known if this partial loss is observed over an entire gene or whether this represents a complete loss of linker histones from specific locations. One possible explanation as to why histone H1 incorporation did not affect transcription of the somatic 5S rRNA gene, is if histone H1 is only bound to nucleosomes packaging the non-transcribed sequences of the 5S rRNA genes and not the actual coding sequences and intragenic promoter. To test this hypothesis, nucleosome arrays, with and without histone H1, were digested with micrococcal nuclease. In native chromatin, histone H1 has been shown to protect an additional 20 bp of DNA from micrococcal nuclease compared to that of the histone octamer alone. The micrococcal nuclease digestion patterns of the reconstituted somatic 5S rRNA genes shown in Figure 40A demonstrates that, in the presence of histone H1, ~165 bp of DNA was protected, indicating that histone H1 was bound to the reconstituted somatic genes in a similar fashion as found in native chromatin. It is possible that, although some of the histone octamers were capable of binding histone H1, the octamers in direct contact with the actual 5S rRNA coding sequence excluded H1. Although the results (Figure 36) suggest this was not the case, the possibility was tested by Southern blot analysis. Nucleosome arrays, containing histone H1, were digested with micrococcal nuclease, blotted and probed for the 5S rRNA coding sequence. The results (Figure 40B) demonstrate that the 5S rRNA coding sequence was packaged both with histone octamers

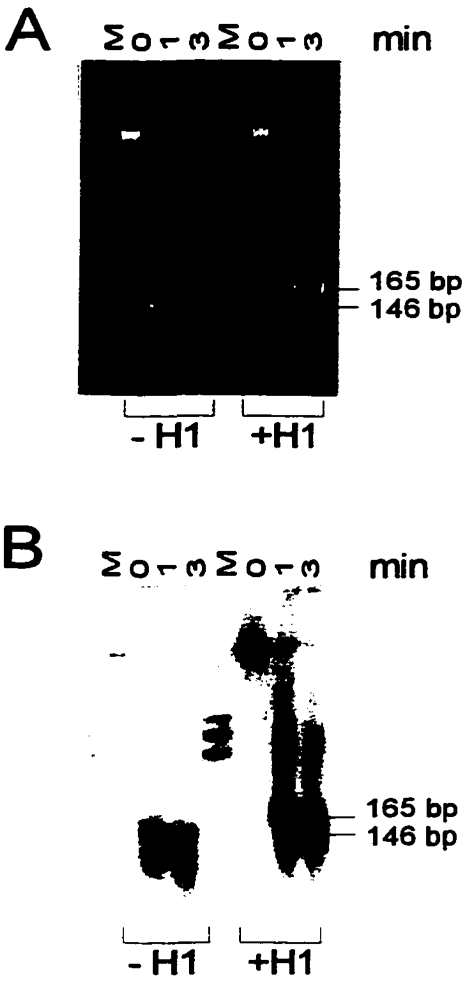

Figure 40. The somatic 5S rRNA coding sequence is in contact with both the histone octamer and histone H1 after reconstitution into nucleosomal arrays containing histone H1. **(A)** Micrococcal nuclease digestion of full length *Xenopus laevis* somatic genes. Digestions were carried out at a nucleosome concentration of 0.1 mg/ml (DNA weight) and enzyme concentration of 20 units/ml for the minutes indicated on the top of each lane. The resulting DNA fragments were deproteinized, end-labeled with [γ-^{32}P]ATP, and electrophoresed on a 4% non-denaturing gel. **(M)** *Hha* I cut pBR322. **(B)** Southern blot hybridization of the gel shown in (A) probed with the [α-^{32}P]dATP labeled 5S rRNA coding sequence.

and histone H1 as is indicated by hybridization of the 5S rRNA coding sequence with the ~165 bp micrococcal nuclease resistant fragment. This, together with the transcription studies shown in Figure 39, indicates that histone H1 binding does not inhibit transcription initiation of nucleosomal *X. laevis* somatic 5S rRNA templates.

3.5.7. Determination of nucleosome core particle/chromatosome positioning on reconstituted 208-12

Currently there is a debate regarding the actual binding site for histone H1 in a nucleosome, with both a symmetrical arrangement and an "off-axis" location proposed (see Figure 7). Due to the multiple translational positions exhibited by nucleosomes on the *X. laevis* genes, presumably it would be very difficult to determine exactly which nucleotides are protected by the binding of histone H1. To approach this question from another angle, the 208-12 DNA fragment was employed. The 208-12 DNA construct consists of twelve, tandemly arranged, 208 bp fragments of the *Lytechinus variegatus* 5S rRNA gene, one of the most strongly nucleosome positioning sequences known. Reconstitution of nucleosomes onto this fragment, generates a nucleosome array with twelve evenly spaced nucleosomes. When histone H1 is added to this array, the fiber condenses in a manner which is almost indistinguishable from that of native chromatin (Howe *et al.*, 1998). In this study, the positions of nucleosome core particles and chromatosomes on the 208-12 DNA construct were mapped by restriction digestion of micrococcal nuclease resistant fragments. In Figure 41, lanes 1 and 2 show the 145 bp (nucleosome core particle) and 165 bp (chromatosome) micrococcal nuclease resistant

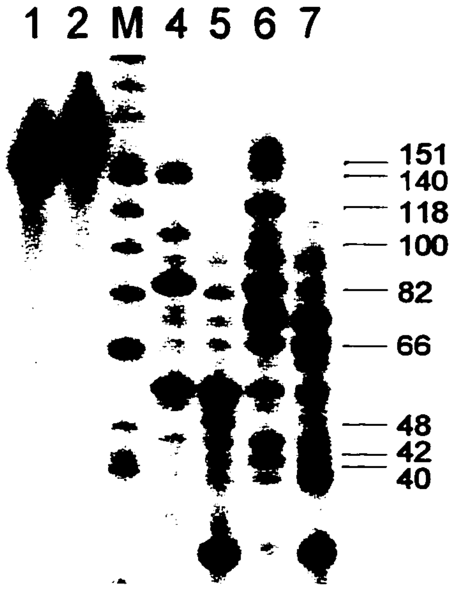

Figure 41. Determination of the translational position of oligonucleosomes reconstituted on the 208-12 template with histone H1. The region of DNA in direct association with the histone octamer and/or histone H1, was determined by digestion of an end-labeled micrococcal nuclease resistant fragment with different combinations of restriction enzymes. The resulting restriction fragments were resolved by denaturing PAGE (8% acrylamide, 8.3 M urea in 1×TBE). Lanes 1, 4 and 5 contain micrococcal nuclease resistant 146 bp fragments, either undigested (lane 1), *Dra* I digested (lane 4) or *Dra I/Msp* I double digested (lane 5). Lanes 2, 6 and 7 contain micrococcal nuclease resistant 165 bp fragments either undigested (lane 2), *Dra* I digested (lane 6) or *Dra I/Msp* I double digested (lane 7). (M) Klenow labeled *Hinf* I cut ϕX174 DNA used as a marker with the sizes of the marker fragments shown as number of nucleotides.

fragments respectively, demonstrating that there were no internal nicks within these fragments. Lane 4 shows the products of the *Dra* I restriction digestion of the 145 bp core particle fragment. This lane shows two bands of 57 and 88 bp indicating that the nucleosome core particle adopts primarily one position on the individual 208 subunits. Cleavage of these two fragments with *Msp I* (lane 5) had no effect on the 57 bp fragment, but the 88 bp band was cut to yield a 28 bp product indicating that this fragment originated from of the 3' end of the micrococcal nuclease resistant fragment. Figure 42 schematically represents the position of the nucleosome core particle on the 208 subunits determined from this analysis. When the 165 bp fragment underwent a similar treatment, three "pairs" of bands were observed. The most predominant "pair" of 88 and 77 bp is indicative of an "off-axis protection" of DNA (see Figure 7) seen by Hayes and Wolffe, (1993). A second pair of bands, with sizes 96 and 69 bp, suggest an almost symmetrical protection by histone H1. Finally, a 125 bp *Dra* I restriction fragment which was cleaved by *Msp* I, suggests a third chromatosome position which does not overlap the original core particle position. This implies that the incorporation of histone H1 caused the histone octamer to adopt a new position on the 208 fragment.

3.5.8. In vitro transcription of reconstituted 208-12 DNA

DNA reconstituted into both nucleosome core particles and chromatosomes were transcribed in HeLa cells extracts to determine the effects of histone H1 on transcription of this construct. During the construction of the 208-12 fragment, the termination site of the 5S rRNA gene was removed. Due to this, transcription of 208-12 yields a "ladder" of

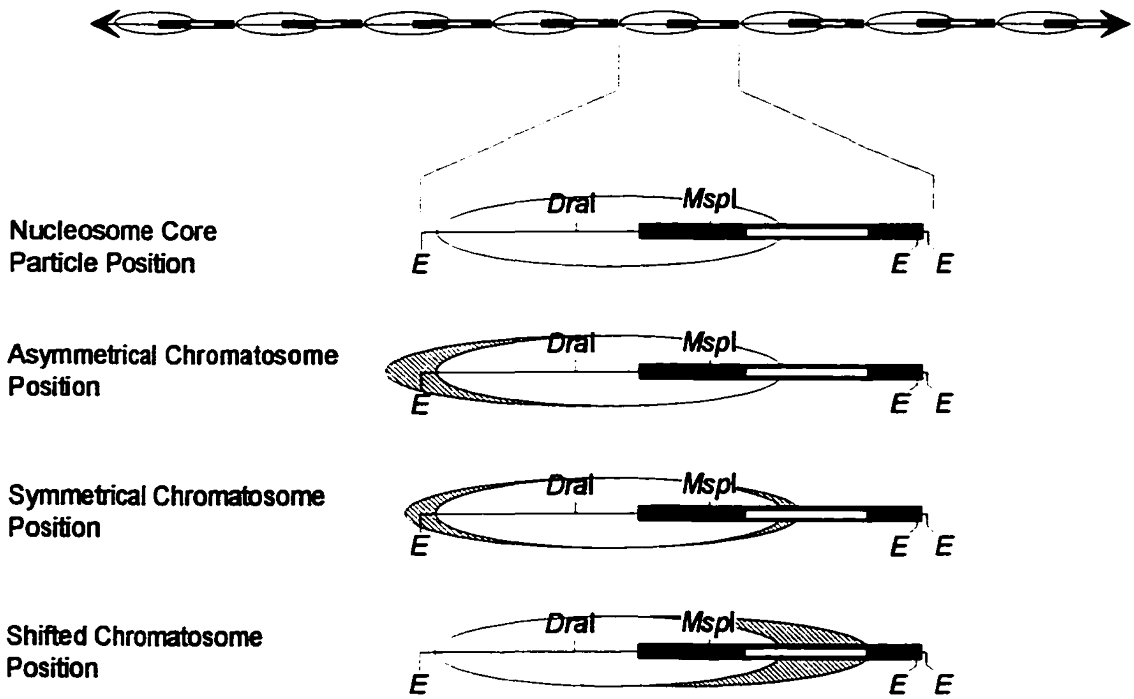

Figure 42. Schematic representation of the most predominant nucleosome position on the reconstituted 208-12 DNA. The open ellipsoids indicate the position of the nucleosome core particle on 208-12 reconstituted in the absence of histone H1. The hatched ellipsoids represent the DNA protected from micrococcal nuclease digestion upon addition of histone H1. The heavy line indicates the 5S rRNA coding sequence and the open box indicates the intragenic TFIIIA binding site. Restriction enzyme sites used for mapping are indicated as are the *Eco*R I sites which separate the 208 fragments (*E*)

transcripts resulting from run-on transcription initiating at the many different transcriptional start sites. This can be seen in Figure 43, lane 3 which shows the products generated by transcription of naked 208-12 DNA. Lanes 2, 4 and 5 contain a 120 nucleotide band due to the inclusion of the *X. laevis* oocyte 5S rRNA gene as an internal control (shown by itself in lane 2). The products generated by transcription of the 208-12 DNA after reconstitution with histone octamers are shown in lane 4. As can be seen, the presence of nucleosomes on the 208-12 DNA did not prevent transcription initiation of the 5S rRNA genes. Lane 5 shows that the addition of histone H1 to the nucleosome arrays did not affect the amount or lengths of transcripts produced.

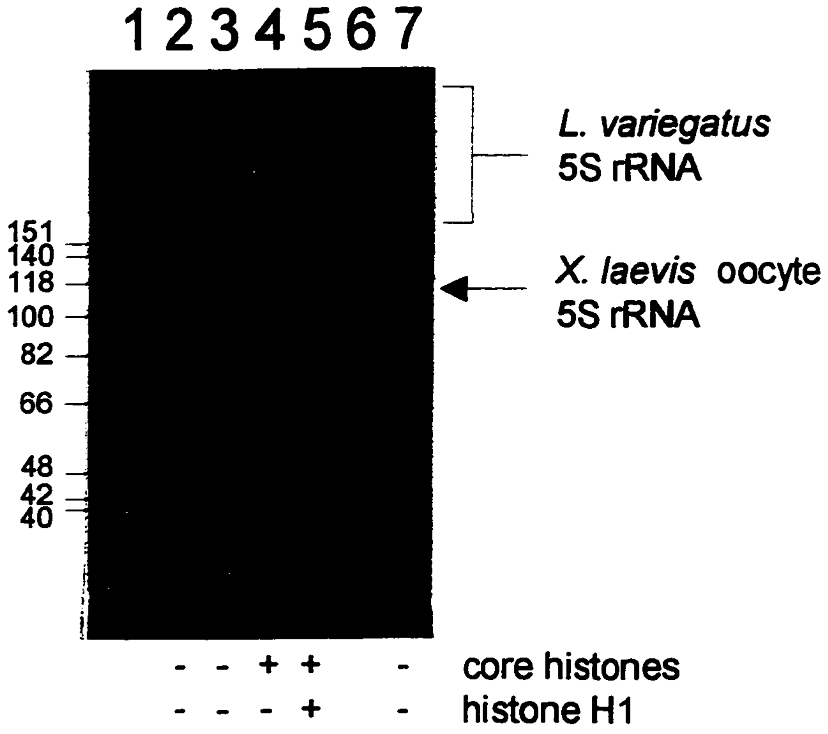

Figure 43. The effect of oligonucleosome reconstitution, with or without histone H1, on RNA polymerase III transcription. Approximately 250 ng of the 208-12 DNA, either uncomplexed (lanes 3 and 7) or reconstituted with histone octamers, without (lane 4) or with (lane5) histone H1 were transcribed with HeLa cell nuclear extracts supplemented with 150 nM recombinant *Xenopus* TFIIIA and 2 mM $MgCl_2$. Reactions in lanes 2, 4 and 5 contained 500 ng of *X. laevis* oocyte 5S rRNA gene DNA as an internal transcription control. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (8% acrylamide, 8.3 M urea in 1× TBE). Lanes 1 and 6, Klenow end-labeled *Hinf*I cut ϕX174 DNA (sizes of the marker fragments shown in number of nucleotides); lane 2, transcribed *X. laevis* oocyte 5S rRNA gene DNA alone; lane 7, a lower exposure of lane 3.

4. DISCUSSION

In eukaryotes, the template for transcription in not naked DNA but rather chromatin. Despite this, the majority of knowledge regarding the regulation of transcription has been acquired through the use of naked DNA as transcription templates *in vitro*. Using this approach however, researchers have been unable reproduce, *in vitro*, many of the characteristics of transcriptional regulation found *in vivo*, such as transcriptional repression or the large magnitude of activation induced by *trans*-acting factors. For these reasons, it is becoming increasingly apparent that chromatin structures play a role in the regulation of transcription and that transcriptional enhancers may function to counter chromatin mediated repression rather than just increase the rate of transcription complex assembly as previously thought.

This work focused primarily on the role of chromatin structures in the transcriptional regulation of somatic and oocyte 5S rRNA genes in *X. laevis*. *Xenopus* has developed a dual 5S rRNA gene system presumably to allow for a large amount of 5S rRNA to be produced early in oogenesis, for the later assembly of the large amounts of ribosomes required post-fertilization. The differential regulation of the oocyte and somatic genes in somatic cells has been the focus of considerable study, especially in the field of chromatin. Chromatin is still thought of as repressive in nature toward transcription and thus the selective repression of the 5S rRNA genes in *Xenopus* has always been quite enigmatic. Promoter specific chromatin-mediated repression has been seen before. For example, mutations in the gene encoding histone H3 increase activation of the *GAL1* but not the *PHO5* promoters in yeast (Mann and Grunstein, 1992). This specificity has been attributed to differences in nucleosome position relative to promoter elements, differences in *trans*-acting factors and differences in the interaction of chromatin remodelling machines such as the SWI/SNF complex. The fact that the two 5S rRNA gene families in *Xenopus* presumably share the identical transcription machinery suggests that other factors, such as nucleosome position, histone acetylation, histone H1 binding or the presence of histone binding proteins may play a major role in the differential expression of these genes.

4.1. The relationship between translational position and the binding of TFIIIA to nucleosomal DNA

One of the main focuses of this work was to investigate the effect of nucleosome translational position on TFIIIA binding. Each of the two oocyte fragments and three somatic fragments analyzed were tested for TFIIIA binding before and after nucleosome reconstitution. These *in vitro* results demonstrated that in cases where the nucleosome was positioned in such a manner that the C block of the 5S rRNA intragenic promoter was within the 146 bp of DNA protected from micrococcal nuclease, TFIIIA could not bind the DNA (see Figures 18 and 19). A similar result was seen by Gottesfeld, (1987) who showed that a nucleosome with the translational position of +20 to +200 on the *Xenopus laevis* 5S rRNA gene blocks TFIIIA binding. Secondly the results of this work suggest that in those instances where at least the C block or more of the TFIIIA binding site were outside this region of DNA protected by the histone core, TFIIIA could bind (Figures 18 and 19). This finding is in agreement with previous results (Rhodes, 1985) with the

Xenopus borealis 5S rRNA gene. DNase I footprinting showed (Figure 24) that in one such instance both the IE element and the A block were bound by TFIIIA. This suggests that, upon binding to the C block, TFIIIA is able to unwind the DNA from the nucleosome to gain access to DNA sequences which are intimately associated with the histone octamer. This may occur by a mechanism of cooperative binding such as that already described by others (Adams and Workman, 1995; Polach and Widom, 1995, 1996) for the binding of two or more eukaryotic regulatory proteins to nucleosomal target sites.

4.2. Understanding the binding of TFIIIA to nucleosomal DNA

Many studies have been performed to demonstrate the binding of *trans*-acting factors to sites within nucleosomal DNA (Perlmann and Wrange, 1988, Piña *et al.*, 1990, Taylor *et al*, 1991, Li and Wrange, 1993, Juan *et al.*, 1994, Li *et al.*, 1994, Chen *et al.*, 1994, Li and Wrange, 1995, Vettese-Dadey *et al.*, 1996). It was shown in the case of the glucocorticoid receptor, that binding of this receptor to sites near the dyad axis of a nucleosome was less favorable than binding to other sites (Li and Wrange, 1995). Thus, the translational position of a nucleosome affects the accessibility to response elements of the glucocorticoid receptor, but does not preclude binding as is the case with TFIIIA. In binding to DNA, the glucocorticoid receptor dimerizes placing the DNA binding domains in two adjacent major grooves along one face of the DNA (Figure 44) (Luisi *et al.*, 1991) and thus the association of a nucleosome with the other face does not necessarily preclude factor binding. The binding of TFIIIA to the 5S gene poses an additional problem. TFIIIA binds to a site which spans approximately five turns of DNA and consists of three

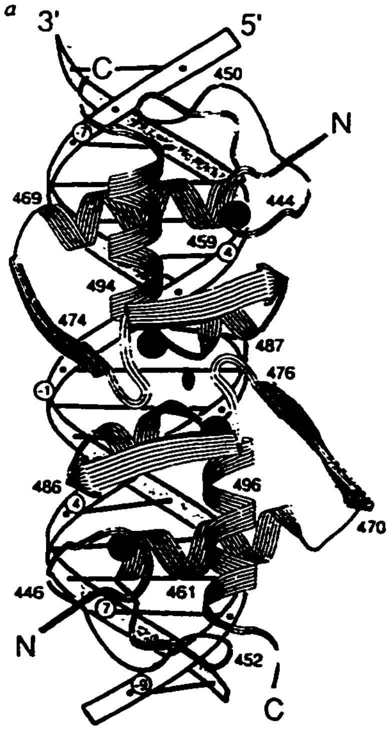

Figure 44. Crystal structure of an 86-amino-acid residue fragment of the glucocorticoid receptor complexed with its hormone response element. The black balls represent zinc ions. Luisi *et al.*, 1991.

elements: the A block (+50 to +64), the IE element (+67 to +72) and the C block (+80 to +96). Current models for TFIIIA binding (Clemens *et al.*, 1992, Hayes and Tullius, 1992) suggest that at least one complete turn of the DNA at each end of the intragenic promoter is bound on all faces of the helix by three zinc fingers in the major groove (Figure 45). Thus unlike the glucocorticoid receptor, complete binding of TFIIIA requires access to all sides of the DNA and full binding would not seem possible if the entire TFIIIA binding site is associated with a nucleosome as was found in this study.

Beato and Eisfeld, (1997) classified transcription factors into two categories based on their ability to bind nucleosomal DNA: (i) *initiator* factors which are able to bind their target sequences within nucleosomes and initiate events leading to transcriptional activation and (ii) *effector* factors which are unable to bind regular nucleosomes and depend on other factors or on a pre-set nucleosomal structure for accessing their target sequences in chromatin. *Initiator* factors have only a few DNA contacts clustered on one side of the double helix (such as the glucocorticoid receptor), whereas *effector* factors have extensive contacts distributed throughout the whole circumference of the DNA helix. Using these criteria, it is clear that TFIIIA can be defined as an *effector* factor requiring a "pre-set" nucleosome structure to bind DNA. This structure could involve specific nucleosome translational position, histone acetylation or the absence of histone H1.

4.3. Nucleosome position on the 5S rRNA genes in vivo

As mentioned earlier, the varied nucleosome positions on the 5S rRNA gene fragments were used to study transcription factor binding, and not meant to represent the actual

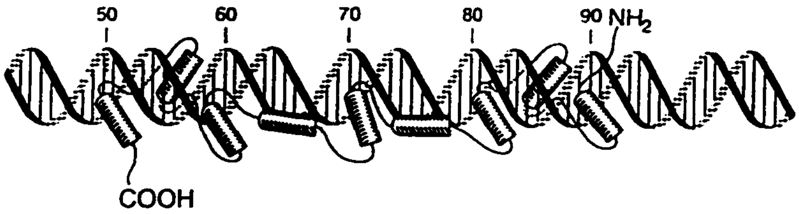

Figure 45. Schematic representation of a proposed model for the binding of TFIIIA to the 5S rRNA gene intragenic control region. The three N terminal fingers (fingers 1-3) wrap around the major groove for approximately one turn of the helix. Finger 4 crosses the minor groove. Finger 5 binds in the major groove of the IE element, followed by finger 6, which again crosses the minor groove. The three C terminal fingers (fingers 7-9), wrap around the major groove for approximately one helical turn (Clemens *et al.*, 1992)

nucleosome position *in vivo*. Nucleosome translational position is not random and nucleosomes exhibit a thermodynamic preference for positions allowing the minor groove of A/T rich DNA to face inward toward the octamer and for the minor groove of G/C rich DNA to face away (GC rich DNA has a wider minor groove than AT rich DNA). However, there is not a good correlation between the position of nucleosomes reconstituted *in vitro* on short DNA fragments and the positions nucleosomes occupy *in vivo* for a variety of reasons. First, nucleosome position on short DNA fragments is affected by boundary constraints imposed by the ends of the fragments. This can be seen in the results of this study which show that over half the nucleosome positions observed are within 10 bp of the end of a DNA fragment. A second reason for the differences between nucleosome position *in vivo* and *in vitro* is that DNA sequence alone may not be sufficient for determination of nucleosome position *in vivo*. It has already been demonstrated that specific DNA binding proteins, such as the α2 factor in yeast, serve to position nucleosomes *in vivo* (Roth *et al.*, 1992).

The position of nucleosomes *in vivo* on the oocyte genes in *Xenopus* have been characterized extensively. In instances where the oocyte genes are repressed, such as in somatic cells and erythrocyte nuclei, micrococcal nuclease digestion suggests that the genes are packaged into loosely positioned nucleosomes with downstream boundaries at nucleotides +119, +134, +180, and +198 (Gottesfeld and Bloomer, 1982; Chipev and Wolffe, 1992). According to the data presented in this work, all of these positions would be expected to restrict TFIIIA binding to the ICR. Of these positions, only one (+134) was similar to the position of a nucleosome reconstituted onto the oocyte gene fragments [+130 for Xlo(-83→+136) and +131 for Xlo(-38→+149)]. It must be noted though, that nucleosomes with downstream boundaries of +180 and +198 were not expected due to the limited sized fragments used in this study. "Genomic" DNase I footprinting of the oocyte genes produces a pattern characteristic of naked DNA which is attributed to the absence of rotational positioned nucleosomes on these genes (Engelke and Gottesfeld, 1990). A similar absence of a unique rotational position was seen on the gene fragments used in this study. The genomic footprinting of presumably active oocyte genes in immature oocytes gave puzzling results - the footprint did not resemble a nucleosome, naked DNA or TFIIIA (Engelke and Gottesfeld, 1990). The position of nucleosomes on the somatic 5S rRNA genes in *Xenopus* has not been studied extensively. The results of micrococcal nuclease and restriction enzyme digestion suggests that these genes are associated with transcription complexes and not nucleosomes (Chipev and Wolffe, 1992). It must be noted however, that these results were performed on transcriptionally quiescent *Xenopus* erythrocytes.

4.4. The role of nucleosome translational position in the differential regulation of the 5S rRNA genes

Alternate to the idea that a "pre-set" nucleosome structure is necessary for 5S gene transcription, the assembly of a transcription complex on the 5S rRNA genes could be DNA replication dependent. It has been demonstrated that DNA replication disrupts RNA polymerase III transcription complexes *in vitro* (Wolffe and Brown, 1986) but the order of events that occur afterwards is not known. Following DNA replication, chromatin is assembled in a step-wise manner with first the deposition of histones H3/H4 onto the

DNA, followed by addition of H2A and H2B. At which step during this process the RNA polymerase III transcription complex forms is unknown. One study has shown that the presence of histones H3 and H4 alone, but not a complete histone octamer, is permissive to 5S rRNA transcription (Tremethick *et al.*, 1990) suggesting that the RNAP III complex must form prior to the addition of H2A and H2B. This is seemingly confirmed by studies showing that in somatic *Xenopus* cell lines producing small amounts of oocyte 5S rRNA, a set of oocyte genes have been translocated to a region of the genome that is replicated earlier (Guinta *et al.*, 1986). It is thus possible that these genes are able to sequester the limited transcription complexes before the somatic genes are replicated. Despite this attractive hypothesis, the actual chromatin structure of the translocated genes has not been probed. It is possible that this region is located in a less condensed, region of high transcription activity or a region with a lower concentration of silencing factors. In addition, it is not known whether the translocated genes are the subset that are actively transcribed or whether the oocyte 5S rRNA produced is a result of a low level transcription of all genes. Furthermore, it has not been published whether these cell lines produce somatic cell levels of TFIIIA or histone H1. Finally, this work, and the work of others using native chromatin (Schlissel and Brown, 1984), suggest that an RNAP III transcription complex can form on 5S rRNA genes that have been packaged with histone octamers, suggesting that DNA replication is not a necessary prerequisite to oocyte 5S rRNA gene transcription.

If indeed a differential translational position of nucleosomes on the oocyte and somatic 5S rRNA genes contributes to the differential regulation of these two gene families in somatic cells, then how does one explain the high level of expression of the oocyte genes during early oogenesis. First, during oogenesis, the levels of TFIIIA are extremely high which may overcome the non-permissive translational position. Second, results already exist which strongly suggest that it is the preference of histone H1 for the oocyte gene that is responsible for the differential regulation of these genes (Schlissel and Brown, 1984, Bouvet *et al.*, 1994, Tomaszewski and Jermanowski, 1997) but the actual mechanism of H1 mediated repression is not known. Previous work has shown that histone H1 mediated reduction in nucleosome mobility is responsible for repression of transcription of a dinucleosome reconstituted onto a dimerized *Xenopus borealis* somatic 5 S rRNA gene (Ura, *et al.*, 1995). Reduced mobility would only be expected to affect transcription if certain translational positions restrict access of transcription factors to their cognate binding sites.

4.5. The role of histone acetylation in the differential regulation of the 5S rRNA genes

The acetylation of core histones has long been known to be associated with transcriptionally active genes (Csordas, 1990, Turner, 1991). Several experiments have shown that one of the roles of acetylation is to enhance the accessibility of DNA to transcription factors (Lee *et al.*, 1993, Vetesse-Dadey *et al.*, 1996). One such report used the TFIIIA - 5S rRNA system as a model (Lee *et al.*, 1993) and showed that in this case, reconstitution of the *X. borealis* and *X. laevis* somatic 5S rRNA genes with acetylated nucleosomes allows TFIIIA binding whereas reconstitution with non-acetylated nucleosomes prevents binding of this transcription factor. In this work, an attempt to reproduce these results found that in no case could histone acetylation overcome the blockage of TFIIIA binding after nucleosome reconstitution. Furthermore, it was shown in this study that TFIIIA was able to bind nucleosomal *Xenopus borealis* genes in the absence of histone hyperacetylation. Although this supports the results of Rhodes, (1985) it is in direct conflict with those of Lee *et al.* (1993). Differences between this work and the experiments of Lee *et al.* (1993) include the use of high concentrations of $MgCl_2$ and more dilute nucleosome concentrations by Lee *et al.* (1993) (in our study an approximate 100 fold excess of nucleosome cores to labeled DNA was used for exchange reconstitutions whereas Lee *et al.* (1993) used a only a five fold excess). Both of these factors have been shown to lead to disruption of histone-DNA contacts (Ausió *et al.*, 1984; Dong *et al.*, 1990; Godde and Wolffe, 1995). An additional factor which could explain the differing results is the use of a slightly higher TFIIIA concentration in this study as compared to that of Lee *et al.* (1993).

The second focus of the acetylation study was to determine the level of acetylation of histones packaging transcriptionally silent oocyte 5S rRNA genes in somatic cells. These results demonstrated that these genes are packaged with hypoacetylated nucleosomes. In addition to histone acetylation, another well defined property of transcriptionally “competent” regions is a general sensitivity to nucleases such as DNase I, DNase II and micrococcal nuclease. In *X. laevis* somatic cells, the transcriptionally silent oocyte 5S rRNA genes cannot be considered transcriptionally “competent" using these classifications as they are packaged with hypoacetylated histones and are less sensitive to micrococcal nuclease digestion than bulk chromatin. These results, taken together with those of others (Reynolds *et al.*, 1982) suggest a link between histone acetylation and oocyte 5S rRNA transcription. This is seemingly in conflict with the first part of this study suggesting that histone acetylation does not facilitate the binding of TFIIIA to nucleosomal DNA. There are however, many other ways in which acetylation could be involved, such as in decondensation of the 30 nm fiber - an effect that cannot be reproduced *in vitro* at the mononucleosome level.

Since the onset of this work, several crucial discoveries have occurred in the area of histone acetylation and transcription. First, it has been shown that histone acetyltransferases (HATs) are components of the transcriptional machinery as either co-factors for *trans*-activators, or a components of the basal transcription factors (TAF$_{II}$250 of TFIID) (Wade *et al.*, 1997 and references therein). Currently there are no identified RNAP III specific HATs, but since there are no classical *trans*-activators for transcription of the 5S rRNA genes, they must be recruited by the transcriptional machinery. Alternatively, since the repression of oocyte 5S rRNA transcription in somatic cells is reportedly due to the presence of histone H1 (Schlissel and Brown, 1984, Bouvet et al., 1994), the lack of histone acetylation in transcriptionally silent oocyte 5S rRNA chromatin could then be explained by a blockage of HAT activity by histone H1.

A second important discovery in the field of histone acetylation and transcription is that the acetylation of specific lysine residues is not random (Grunstein, 1997 and references therein). For example, lysine 12 of *Drosophila* and yeast histone H4 is acetylated in transcriptionally silent regions of the genome and may be required for this silencing (Turner *et al.*, 1992; Braustein *et al.*, 1996). For this reason, the results of this study, and those of other studies regarding enhancement of transcription factor binding by histone acetylation, must be interpreted with caution. It is quite possible that an enhancement of TFIIIA binding was not observed due to an incorrect "pattern" of lysine acetylation or an over-acetylation of the bulk of the histones used. Currently, work is being performed by other groups to characterize HATs and their substrate specificity. Once this work progresses, HATs may be effectively used , for example as restriction enzymes are today, to investigate the role of transcription factor binding and histone acetylation more thoroughly.

4.6. The role of nucleoplasmin in the differential regulation of the 5S rRNA genes

The third objective of this work was to determine whether *Xenopus* egg nucleoplasmin could facilitate the binding of TFIIIA to nucleosomal 5S rRNA genes. The rationale for these studies was driven by earlier observations using highly heterologous systems which indicated that *in vitro*, nucleoplasmin could enhance the binding of transcription factors to their cognate binding sites in chromatin (Chen *et al.*, 1994, Walter *et al.*, 1995). The *in vivo* relevance of these early *in vitro* studies, is limited by the heterologous nature of their components and by the lack of information on the existence of nucleoplasmin in their *in vivo* counterparts. In this respect the TFIIIA - nucleoplasmin system appears to be better suited for this kind of analysis especially since these two proteins have been shown to coexist in the oocyte nucleus of *Xenopus*. In addition, the nucleosome remodeling capabilities of nucleoplasmin may play a role in oocyte 5S rRNA expression since nucleoplasmin and oocyte 5S rRNA are present only in oocytes.

The results of our analysis showed that nucleoplasmin did not enhance the binding of TFIIIA to nucleosomal DNA. Furthermore, nucleoplasmin was unable to enhance transcription of 5S rRNA from nucleosomal templates. These results are not surprising considering that, although nucleoplasmin has been shown to specifically bind histones H2A and H2B, it is unable to release these histones from chromatin during chromosome remodeling in oocyte extracts (Dimitrov and Wolffe, 1996). A further explanation for why the results of this study differed from those of Chen *et al.*, 1994, is the differences in transcription factors used. The *trans*-activators used in their studies binds nucleosomes within the core DNA. This binding presumably destabilizes the interaction of DNA with the core histones. Nucleoplasmin can then conceivably serve as a sink for the destabilized H2A and H2B to bind. Removal of these histones would then enhance the protein-DNA contacts made between the trans-acting factor and the DNA. TFIIIA which is unable to bind the core DNA, would be unable to initiate this destabilization event.

4.7. The role of histone H1 in the differential regulation of the 5S rRNA genes

The final focus of this study was an attempt to demonstrate *in vitro*, a preferential binding of histone H1 for nucleosomal oocyte 5S rRNA genes compared to the somatic counterparts. This preferential interaction has long been suggested to account for the differential developmental regulation of the two gene families. Using mononucleosomes, it was demonstrated that histone H1 bound equally to both oocyte and somatic gene fragments after nucleosome reconstitution regardless of the level of acetylation of the core histones. The only difference observed was a binding of more than one molecule of H1 by the oocyte gene while the somatic gene did not appear to do so. The fragments used for these experiments were designed to include the entire 5S rRNA intragenic promoter, and thus the region of DNA to which histone H1 binding would exhibit its greatest effect. In addition, the two fragments used contained similar translational positioning signals which allowed direct comparison to be made between the H1 binding properties of the oocyte and somatic flanking regions. The disadvantage to this approach was that only the regions immediately flanking the 5S rRNA genes were included. In the case of the oocyte gene, these regions are not as AT rich compared to sequences further upstream. It has been suggested that it is the presence of extremely AT rich sequences which is responsible for the increased binding of histone H1 to the oocyte gene (Jermanowski and Cole, 1992). Therefore, it is possible that a preference of H1 binding to mononucleosomes was not detected because the fragments used excluded these sequences. It is conceivable that, by using artificially constructed sequences, a preference of histone H1 for oocyte gene might be demonstrated, but in this study, using native DNA sequences, we were unable to do so.

In the second part of the histone H1 study, nucleosomes were reconstituted onto the full length oocyte and somatic 5S rRNA gene repeats. The presence of histone octamers alone on an oocyte gene repressed transcription in *Xenopus* oocyte extracts by approximately 33%. In comparison, transcription of a somatic gene was relatively unaffected by the presence of histone octamers. Transcriptional repression by histone octamers in the absence of histone H1 has also been observed with RNA polymerase II transcribed genes (Sandaltzopoulos *et al.*, 1994). In this case, presumably this repression occurs at the level of transcription initiation as the RNAP III transcription complex overlaps the entire 5S rRNA coding region and thus would disrupt any nucleosomes present during the initiation stage of transcription. When the oocyte and somatic gene repeats were reconstituted into nucleosomes containing histone H1, residual oocyte transcription was further inhibited by ~66% while somatic 5S rRNA transcription was decreased by only ~20%. The differential effect of histone H1 on the transcription of the oocyte and somatic 5S rRNA genes has been described by others (Tomaszewski and Jermanowski, 1997), but for the first time, this study was able to show that histone H1 was actually bound to the somatic 5S rRNA coding sequence without significantly repressing transcription. It is not known how the interaction of histone H1 with one gene can cause transcriptional repression while a similar binding to another gene does not have an effect. One suggestion is based on the fact that histone H1 is capable of binding DNA though three different domains: the globular core and the unstructured C terminal and N terminal domains. Cross-linking studies have argued that interaction of the globular domain is altered upon gene activation but the histone still remains associated with the nucleosome via its tails (Zlatanova and van Holde, 1992 and references therein).

Due to the results of several *in vitro* transcription studies (Schissel and Brown, 1988, Wolffe, 1989, Laybourn and Kadonaga, 1991, Ura *et al.*, 1995), the incorporation of histone H1 into chromatin has long been described as a generally repressive toward transcription. This is further supported by studies which show that histone H1 inhibits transcription factor binding to nucleosomal DNA (Juan et al., 1994, 1997). Recent lines of evidence however, including this study, have shown that histone H1 can bind nucleosomes without affecting the assembly of the transcription machinery. It has been demonstrated that the RNA polymerase I basal transcription factor, UBF, is able to displace histone H1 when binding nucleosomal DNA (Kermekchiev *et al.*, 1997) Furthermore, the RNA polymerase II trans-acting factor, NF-1, has been shown to bind linker DNA regardless of the presence of histone H1 (Eisfeld *et al.*, 1997). These results suggest that although histone H1 can repress transcription in some instances, it is a rather selective repressor of transcription initiation.

The histone H1 transcription studies were repeated using the 208-12 DNA fragment. This construct offered the advantages of controlled nucleosome position and the capacity for enough nucleosomes for a compaction event to occur. The work of others (Howe *et al.*, 1998), demonstrates that, after incorporation of histone H1, nucleosomal arrays on the 208-12 construct exhibit the salt dependent folding of native chromatin. When transcribed in HeLa cell nuclear extracts, it was shown once again that histone H1 did not affect transcription of the sea urchin 5S rRNA gene. When analyzed to determine the position of histone H1 with respect to the histone octamer, it was shown that H1 sat in both the symmetrical and off-axis positions previously described (Allan *et al.*, 1980; Hayes and Wolffe, 1993; Travers and Muyldermans, 1996). In addition, there was a third chromatin position suggesting an apparent remodeling of the position of the histone octamer by the binding of histone H1. When Travers and Muydermans (1996) proposed their off-axis model, they defined two sequence elements common to many chromatosomes: a nucleotide quartet, (NGG$^{G}/_{A}$), located at one, but not both termini of the 166 bp chromatosome fragment and an AAA/TTT triplet located about 70 bp away (about one helical turn from the dyad axis). The *Lytechinus* 5S rRNA gene contains an AAA/TTT sequence centered 15 bp from the dyad axis and the sequence AGGA 70 bp away (4 bp from the end of the chromosome fragment). This model explains why, when asymmetrical position is seen, it consistently protects termini and not both as would be expected if there was no sequence specificity to H1 binding. Regardless of the manner to which histone H1 binds to the sea urchin gene, it does not appear to repress transcription.

4.8. Conclusions

The results of this study shed new light on the basis for the differential transcription of the oocyte and somatic 5S rRNA genes in somatic cells. Previous explanations relied heavily on the concept that histone H1 preferentially interacted with the oocyte genes preventing the formation of an RNA polymerase transcription complex (Wolffe, 1994). Only those genes which could form active transcription complexes, prior to addition of histone H1, would be programmed for transcription. In this study it was shown that histone H1 could bind both the somatic and oocyte genes equally, and could directly interact with histone octamers on the somatic 5S rRNA coding sequence without preventing transcription. This did not seem to be the case for the oocyte gene as is seen in this study and the results of others (Tomaszewski and Jermanowski, 1997). The binding of histone H1 has been shown to reduce the mobility of histone octamers on nucleosomal DNA and it was shown that translational position of nucleosomes affected the binding of TFIIIA nucleosomal DNA. Together, these results support a model in which the differential regulation of the 5S rRNA genes is not due simply to a competition of histone H1 with the transcription machinery for DNA sites, but rather a difference in the interaction of histone H1 with the somatic and oocyte genes and differences in translational position of histone octamers on these genes. In addition, these results provided support for the idea that histone hypoacetylation may serve a role in the transcriptional silencing of the oocyte genes in somatic cells.

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