AN ABSTRACT OF THE THESIS OF

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Abstract approved:

The aim of this study was to gain more information about the interactions between DNA and the histone octamer during the process of transcription. This work used a pUC8 plasmid derivative that contained the core promoter region of the RNA polymerase I of *Acanthamoeba castellanii*, placed upstream of four repeats of the 5S rDNA nucleosome positioning sequence from the sea urchin, *Lytechinus variegatus*. The plasmid was reconstituted into chromatin via addition of chicken erythrocyte histone octamers, using polyglutamic acid as a nucleosome assembly factor. The positioning of nucleosomes on the insert was monitored by restriction enzyme digestion. Proper nucleosome positioning was shown to be dependent on the presence of preassembled transcription complexes on the promoter region. The absence of preformed transcription complexes on the promoter region prior to nucleosome reconstitution perturbed the distribution of histone octamers on the repeats of the 5S rDNA.

This "mispositioning" effect was related to the location of the RNA polymerase I promoter region upstream of the four repeats of the 5S rDNA fragment. Band shift assays in polyacrylamide gel electrophoresis were used to determine the relative efficiency of

nucleosome formation on the promoter-containing fragment, on 5S rDNA and finally on nucleosome core particle DNA. The results indicate that the promoter fragment forms a nucleoprotein complex at lower concentration of histone than the 5S positioning sequence. This complex may not be a nucleosomal structure.

The reconstituted plasmid was then used to investigate the transcriptional elongation by RNA polymerase I using the chromatin-like template containing positioned nucleosomes as compared to transcription on improperly positioned nucleosomes and on free DNA. The efficiency of transcription was related to the proper positioning of nucleosomes with regard to the tandemly repeated 208-bp 5S rDNA. The presence of phased nucleosomes in the path of the transcription complex seemed not to inhibit nor to significantly slow down the elongation as compared to free DNA. Furthermore, nucleosome positioning, as assayed by restriction endonuclease digestion, did not change after passage of the polymerase I transcription complex.

THE EFFECTS OF NUCLEOSOMES ON TRANSCRIPTION BY POLYMERASE I IN A RECONSTITUTED SYSTEM

by

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The Effects of Nucleosomes on Transcription by Polymerase I in a Reconstituted System

CHAPTER 1

Introduction.

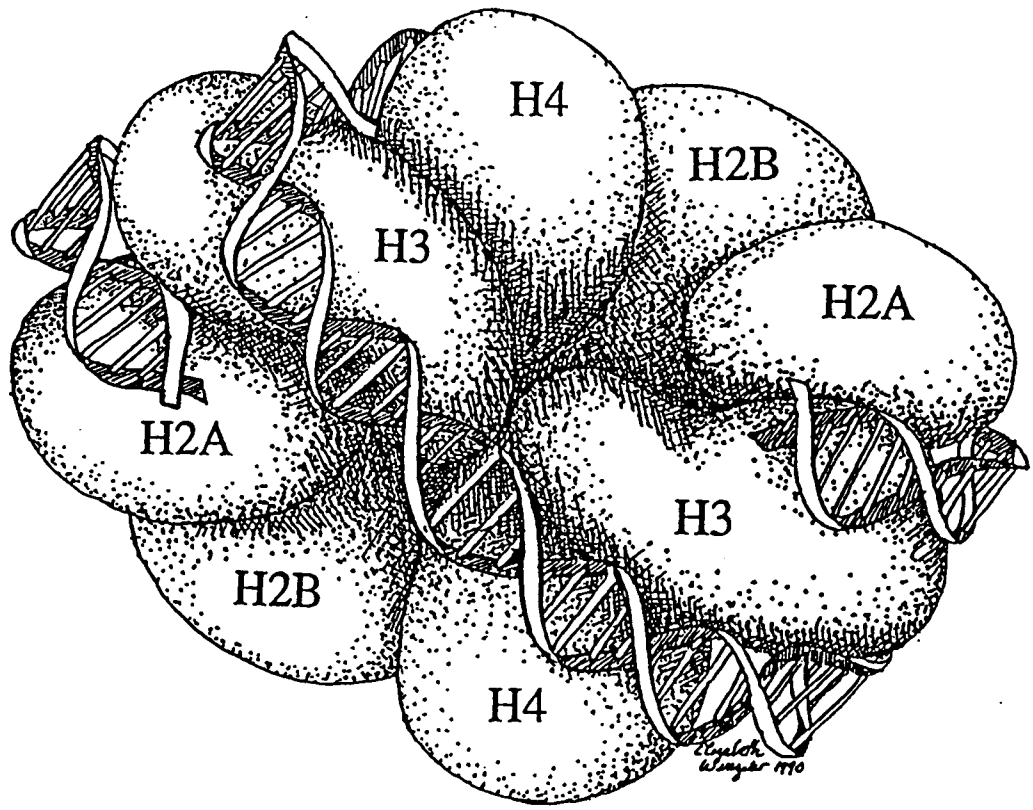
a) Chromatin and Nucleosomes

The DNA in eukaryotic cells is tightly packaged with an equivalent mass of proteins, most of which are basic. These proteins can be subdivided into two classes: histone and nonhistone chromosomal proteins. The structure generated by this combination of DNA, histones and nonhistone chromosomal proteins is referred to as chromatin.

The building block of chromatin is the nucleosome core particle (histone octamer together with 146 base pairs (bp) of DNA). It can be released from native chromatin by mild micrococcal nuclease digestion. It contains approximately 146 base pairs of DNA wound into 1.75 turns of a left-handed helix around the histone octamer. The histone octamer is composed of two dimers of histones H2A-H2B, and a centrally located tetramer of H3-H4 (see Figure 1. 1). To attain the actual chromosome structure, the string of nucleosomes, also called the 10 nm fiber, will be further condensed (at least in most regions) into a 30 nm fiber. This involves the additional binding

Figure 1. 1

Nucleosome Core Particle (Data courtesy of Elisabeth Winzeler and Dr Enoch Small).



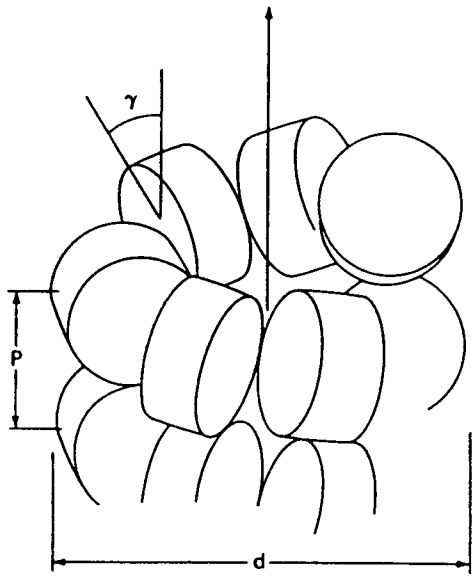
of lysine-rich histones (H1 and its variants) to the linker DNA (DNA between neighboring core particles) and interaction of H1 with the histone core of the nucleosome (Boulikas et al., 1980). The lysine-rich histones promote the coiling of the polynucleosomal chain into a more compact structure, the 30 nm fiber. The chromatosome (nucleoprotein complex containing "about 160 bp" of DNA, a histone octamer, and no more than one molecule of lysine-rich histone H1) can be condensed to be the building block of the 30 nm fiber. The higher order structure or 30 nm fiber can be further compacted into highly coiled interphase heterochromatin and metaphasic chromosomes.

The actual process of chromatin compaction is still under investigation. It is regarded as critical for explaining the mechanism of gene regulation. The structure of the 30 nm fiber has been described as a solenoid of nucleosomes (Finch and Klug, 1976, Worcel et al., 1981, Mc Ghee et al., 1983) but the orientation of nucleosomes relative to each other and the location of the linker DNA remains unresolved. For example, an alternative compaction model proposed by Woodcock (Woodcock et al., 1984) describes a zig-zag arrangement of dinucleosomes compacted to form an helical ribbon which then folds into the 30 nm fiber (see Figure 1. 2). The chromatin fiber will itself fold to pack the 30 nm chromatin into chromosomes during metaphase. The chromosomal DNA will fold, forming supercoiled loops attached to a "scaffold" structure composed of nonhistone chromosomal proteins.

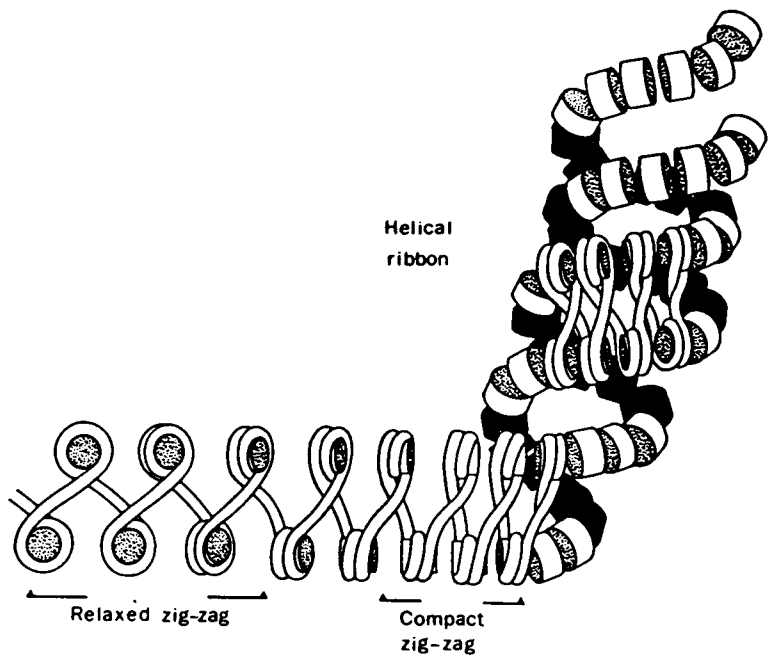
Other workers have demonstrated that nonhistone chromosomal proteins interact with the linker DNA and the histone

Figure 1. 2

Possible structures adopted by the 30 nm fiber (top: solenoid of nucleosomes and bottom: zig-zag arrangement). Data from: van Holde, K. E(1988) *Chromatin*, Verlag, New York, Berlin.



The parameters that will be used in describing a solenoid of nucleosomes. The solenoid has n nucleosomes per turn (not necessarily integral), a pitch P, and a diameter d. The nucleosome faces are tilted at an angle γ with respect to the solenoid axis.



octamer. The distribution of HMG (High Mobility Group proteins) 1, 2, 14 and 17 was indicative of a nonrandom pattern in the localization of their interactions with nucleosomes. Mild nuclease digestion experiments showed that HMG proteins could extend the protection of the DNA from the nuclease to more than 200 bp (Jackson et al., 1979). The ability of these proteins to crosslink to the linker DNA and to the core particle itself, in the case of HMG 14 and 17, are further indications of a specificity of the interactions (Mardian et al., 1980, Espel et al., 1985). The role of the nonhistone chromosomal proteins is yet unclear but seems to correlate with transcriptional activity (for review, see Goodwin and Mathews, 1982). However, the relationship of these aspects of chromatin structure to the unresolved regulation of chromatin transcription in eukaryotes remains.

b) Nucleosome positioning

It has recently been recognized that nucleosomes themselves can potentially act as regulators of transcription, replication, DNA-repair and recombination. Here, the question of the presence and location of arrays of nucleosomes or occurrence of DNA sequences with particularly high affinity for nucleosomes becomes a matter of interest (for review see Thoma, 1992). The basic question is whether nucleosomes position randomly or exhibit a specific relationship of nucleosomes to DNA sequence, and what effects such arrangements may have on regulation.

There is no general arrangement of nucleosomes. If one examines the spacing of nucleosomes, it becomes obvious, from the diversity of the length of the linker DNA, that nucleosome positioning cannot be identical in different organisms or even within the same organism in different tissues. Nonetheless, the positioning of nucleosomes on certain DNA sequences has been unequivocally demonstrated. Many examples of this are now known, among which the 5S rDNA (ribosomal DNA) is probably the most extensively studied. The presence of such a specifically positioned structure raises the question of how those complexes are generated, and what their roles may be in the regulation of gene expression.

The mechanism of nucleosome positioning may be different whether one examines the *in vitro* reconstitution (regeneration of chromatin structure) of nucleosomes or the *in vivo* process. To assess the physiological significance of the mechanism, the emphasis should be on the *in vivo* observations. Valuable information has been gathered from the TRP1 ARS1 circle of yeast *Saccharomyces cerevisiae* (Thoma et al., 1984), from *in vivo* and *in vitro* reconstitution of 5S genes from *Xenopus laevis* and *L. variegatus*, the MMTV-LTR promoter, the NFI binding site and hsp26 gene from *Drosophila melanogester.*

Several possible mechanisms can be envisaged to explain the positioning of nucleosomes:

1) During replication, a nucleosome could form as soon as enough DNA is replicated. As a direct consequence, the positioning of nucleosomes might be determined by the origins of replication.

2) The presence of sequence-specific interactions may be a strong determinant. The histone octamer might recognize specific features of the DNA to generate a precisely positioned nucleosome. These features may correspond to specific DNA sequences, or to sequences producing a given structured feature, such as DNA bending.

3) The presence of non-histone proteins could help the positioning of the histone octamer on adjacent DNA sequences.

4) The presence of flanking structures -perhaps proteins- might define boundaries between which packing could determine nucleosome positioning.

The 5S rDNA positioning sequence from *Lytechinus variegatus* apparently falls into the second category. This sequence has been extensively studied, to define the exact position of the nucleosome and the special features causing the sequence specificity(Simpson and Stafford, 1983, Dong et al., 1990, Pennings et al., 1991). It appears that in this case, as a number of others, it is the production of intrinsic DNA curvature by the base sequence that causes positioning.

Nucleosome positioning on this sequence was first reported to be very precisely located (Simpson and Stafford, 1983). More recent restriction enzyme mapping indicates that histone octamers assembled *in vitro* are located in one clearly dominant position, however there also exists a number of minor positions spaced 10 bp apart (Dong et al., 1990, Pennings et al., 1991). Such positioning, which involves always facing the same face of the DNA toward the nucleosome, is referred to as rotational positioning (Drew and Travers, 1985)

Tandemly repeated 5S rDNA sequences can be used to generate phased nucleosome arrays, which therefore can be used as models for a succession of positioned nucleosomes (Simpson and Stafford, 1983). Numerous plasmids containing variable numbers of repeats of the 5S gene have been constructed for different purposes (O'Neill et al., 1992, Pennings et al., 1991). One of these goals is to elucidate the mechanism by which nucleosomes are displaced or unfolded during transcriptional events.

c) Nucleosomes and Transcription: RNA polymerase I as a model for *in vitro* transcription using an eukaryotic RNA polymerase

The presence of well defined nucleosome structure has been convincingly demonstrated on transcribed genes, both before and after transcription (Nachevaet al., 1989, Walker et al., 1990). *In vitro* experiments have shown that, in most of the cases, nucleosomes on a DNA template inhibit transcription. Most of these studies have been performed using viral RNA polymerases either from bacteriophage T7 or SP6 (Kirov et al., 1992), or the RNA polymerase from *Escherichia coli* (Lorch et al., 1987). In a few cases eukaryotic RNA polymerases II or III have been used (Izban and Luse, 1991, Morse, 1989). These studies converged to the conclusion that the transcription process could occur through short stretches of nucleosomes, although sometimes with very low efficiency (see Freeman and Garrard, 1992 and Felsenfeld, 1992 for reviews).

Surprisingly, RNA polymerase I has been somewhat neglected by the different groups studying the mechanism of transcription at the nucleosome level. However, it presents some significant advantages over viral RNA polymerases as well as eukaryotic RNA pol II or III. The primary interest is that RNA polymerase I is of eukaryotic origin, so it is more relevant to the transcriptional mechanism in the presence of chromatin structure to use RNA pol I rather than phage or bacterial polymerases.

One other important feature of RNA polymerase I is its ability to efficiently initiate transcription *in vitro* in the presence of only one or two transcription factors (depending on the organism it has been isolated from). This is in marked contrast to RNA pol II, for which a large and still not fully defined group of factors is needed. In the case of *Acanthamoeba castellanii*, the only absolute requirement for RNA pol I is the presence of TIF-IB (transcription and initiation factor). This is in contrast with RNA pol I obtained from higher eukaryotes where there are a minimum of two transcription factors required (Learned,et al., 1986, Schnapp et al., 1990 and Pikaard et al., 1989). TIF-IB has proven difficult to purify, probably because of its very low concentration in the cell. Partial purification of transcription factors from *A castellanii* yields a mixture of two proteins. The first major component is TIF-IB. The second, predominant in quantity, is a protein of similar molecular weight to the human UBF (Upstream Binding Factor) called aUBF (Iida and Paule, 1992).

When this partially purified mixture of transcription factors is mixed with a promoter-containing DNA template, a stable

DNA/transcription factor complex is formed (Bateman and Paule, 1986). The association of TIF-IB with the promoter region generates the preinitiation complex. DNase I or MPE (Methidiumpropyl-EDTA-Fe (II)) digestion gives a footprint of the stable complex. The bound TIF-IB and aUBF protect a region from -69 to +12 relative to the transcription initiation site. When RNA pol I is subsequently added and bound, forming the complete initiation complex, the footprint extends to position +20. TIF-IB directs the binding of RNA pol I to the template via protein-protein contacts (Bateman and Paule, 1986 and Paule et al., 1991).

d) Design of our experimental system

The design of a simple system to study *in vitro* transcription through chromatin uses a plasmid that contains several repeats of a nucleosome positioning sequence downstream of an RNA polymerase I promoter region. The number of positioning sequences was to be sufficient to permit studying the behavior of phased nucleosomes. The best studied positioning sequence being the 5S rDNA, we decided to clone the 208 bp fragment from *L. variegatus* downstream of the core promoter region of RNA pol I from *A. castellanii*. Again, using RNA pol I, requiring only one transcription factor, helps us limit the number of elements in the system. Note that the partially purified TIF-IB utilized for all the experiments described here contains aUBF as a copurification product.

One more problem remained. From studies on other systems, it appeared now obvious that the particular means to reconstitute a

nucleosomal structure does not seem to matter as much as the order of the incubation of the various protein components with DNA in regenerating transcriptionally active chromatin. The presence of a preassembled transcription complex prior to nucleosome reconstitution was known to be critical for the ability of the transcription system to perform optimally. However, the 2M-salt systems used previously in our laboratory for reconstituting nucleosomes would cause dissociation of a transcriptional initiation complex. Therefore, a new method for reconstitution was needed, in which the ionic strength remained as close as possible to the physiological value to prevent the transcription complexes from falling apart. Different *in vitro* assembly systems have been generated using various assembly factors such as nucleoplasmin (a nuclear protein found in the eggs and oocytes of *Xenopus laevis*), cell-free *Drosophila melanogaster* embryo extract (Nelson et al., 1979 and Becker and Wu, 1992) or polyglutamic acid as a carrier (Retief et al., 1984). All of these probably function by helping to stabilize the histone octamer under low ionic strength conditions.

With the goal of keeping our *in vitro* transcription system simple, we chose to utilize polyglutamate (PGA) as a chromatin assembly agent. The efficiency in reconstituting chromatin under physiological conditions in the presence of PGA was demonstrated by Retief et al (1984). The optimal ratio of PGA to histone (weight/weight) has been reported to lie within a range of 2 to 5, depending on the specific laboratory. Although its mechanism of action is not totally understood, PGA probably acts as a carrier by coating the histones, thus rendering them more stable as multimer

building blocks at low ionic strength. Without PGA, at 150 mM NaCl, the nucleosome histone core structure would not be stable.

e) Format

The following two chapters are the results of my thesis research. Each of the following chapters have been submitted for publication or will be in the near future. Chapter 2 concerns the definition of the conditions of reconstitution to be used to generate *in vitro* chromatin-like system that is transcriptionaly active. Chapter 3 describes the results of *in vitro* transcription experiments using RNA polymerase I to transcribe the plasmid pPol I 208-4 as a naked DNA template or as a chromatin-like template. The appendix deals with the relative affinities of the RNA polymerase I core promoter region and the 5S rDNA positioning sequence for histones.

The figures and figure legends are grouped at the end of each chapter. The references are compiled at the end of the thesis.

CHAPTER 2

Binding of the RNA Polymerase I Transcription Complex to its Promoter can Modify Positioning of Downstream Nucleosomes Assembled *in vitro*

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a) Summary.

We have studied the reconstitution of chromatin-like structures *in vitro*, using purified RNA polymerase I transcription complexes and histone octamers. The plasmid construct used in these studies is a pUC8 derivative in which we have inserted a RNA polymerase I core promoter region of *Acanthamoeba castellanii* upstream of four repeats of the 5S rDNA nucleosome positioning sequence (208 bp) from *Lytechinus variegatus*. When histone octamers were reconstituted onto the naked DNA template, the expected nucleosome positioning (as assayed by restriction enzyme digestion mapping of the inserted region of the plasmid) previously observed using tandem repeats of the same 208 bp fragment was not obtained. We show that the location of the RNA polymerase I core promoter region, with regard to the tandemly repeated 208 bp positioning sequence, is a major determinant in the positioning of the histone octamers. Reconstituting first with the stalled transcription complex excluded octamers from the promoter region and restored the expected nucleosome positioning downstream on the 4 repeats of the 5S positioning sequence. The observed competition between histone octamers and the transcription complex for the promoter region suggest is very similar to results of *in vitro* studies with RNA polymerase II and III transcription systems. The observed results may be related to the mechanism of regulation of transcription for the RNA polymerase I.

b) Introduction:

A major problem in the field of eukaryotic transcription is the role and behavior of nucleosomes occupying the transcribed regions (see van Holde et al., 1992 for a recent review). Most attempts to study this behavior have utilized linear templates (Lorch et al., 1987, 1988; Losa and Brown, 1987, Izban and Luse., 1991, for examples). One study employed a circular template, in order to investigate effects of DNA supercoiling (Pfaffle et al., 1990); another recent study used tandemly repeated 5S genes inserted in a closed circular plasmid (O'Neil et al., 1992). However both of these works utilized prokaryotic promoter and polymerases.

In order to study *in vitro* transcription using a eukaryotic polymerase, we have constructed a plasmid used as a template for the RNA polymerase I transcription complex from *Acanthamoeba castellanii.* The plasmid designated pPol I 208-4, contains a RNA polymerase I promoter region immediately upstream of 4 repeats of the 5S rDNA nucleosome positioning sequence from *Lytechinus variegatus* (Simpson and Stafford, 1983). This repeated sequence has been used extensively in our laboratory and elsewhere in studies of nucleosome positioning (Simpson and Stafford, 1983, Simpson, 1986, Hansen et al., 1989, Dong et al., 1990, Pennings et al., 1991; see Thoma, 1992 for a review).

In order to keep the promoter site free of nucleosomes, which would interfere with initiation, we have reconstituted the RNA polymerase I plus its transcription factors on the plasmid before the nucleosome structure is formed. The step-dialysis method commonly

used to deposit histone octamers onto their target sequences cannot be applied to this system. The salt concentration (NaCl) is a critical parameter for the stability of the polymerase and its factors and has to be kept close to the physiological 150 mM of NaCl. When the reconstitution is carried out using the step dialysis method, the mixture of plasmid plus histone octamers is stepwise dialyzed from 2.2 M NaCl down to the required salt concentration; the high salt concentration employed would dissociate the transcription complex. Therefore, reconstitution of chromatin-like structure was carried out using polyglutamic acid (PGA) as carrier for the deposition of the nucleosomes onto the DNA. The polyglutamic acid method has been shown to give consistent results in reconstituting the DNA and histones into a chromatin-like structure (Retief et al., 1984).

Before beginning transcription studies, we felt it important to first determine whether normal nucleosome positioning was obtained after this kind of reconstitution, particularly in the repeated region downstream from the promoter. The positioning was investigated by restriction enzyme digestions. Surprisingly, no defined positioning was observed when the circular template was reconstituted with histone octamers by this technique. However, we observed a recovery of positioning on the repeated 5S genes when the reconstitution was performed in the presence of transcription factors TIF-IB and aUBF plus the RNA polymerase I stalled at position +8. No recovery was observed when the transcription factors were provided in the absence of the RNA polymerase I.

We further compared reconstitution using DNA that had been linearized by cleavage at different sites with regard to the core

promoter region to reconstitution using covalently closed circular plasmid, on the assumption that the integrity of specific regions might be necessary for the nucleation of positioning. Indeed, we find that the polymerase I promoter contains a strong positioning sequence which competes with the 5S rDNA signals and leads to randomization of nucleosome positioning.

The complete complex has been demonstrated to be transcriptionally active. This activity will be the subject of chapter 4.

c) Materials and methods

1) Construction of the pPol I 208-4 plasmid

A 94 bp fragment (-75 to +19) from the RNA polymerase I promoter sequence from *Acanthamoeba castellanii* was amplified by Polymerase Chain Reaction (PCR). The template used for amplification was the plasmid vector pEBH10 (Kownin et al., 1985) harboring a 200 bp sequence from the promoter region. The primers were designed to contain a *PstI* (*PstI* restriction endonuclease) restriction site on the 5' terminus of the product and to incorporate a *XbaI* (*XbaI* restriction endonuclease) restriction site on the 3' terminus. A 4 bp extension at the end of the primer sequence was included to assure a satisfactory digestion of the PCR product with restriction endonucleases. After purification by gel electrophoresis, the PCR product was digested with *PstI* and *XbaI* and then inserted into pUC19. After amplification the sequence of the product was verified by sequencing.

The 5S ribosomal sea urchin DNA was obtained from the plasmid pAT153, amplified by polymerase chain reaction and

sequenced, generating a 259 bp fragment. The primers were also designed to contain a *XbaI* restriction site at the 3' terminus and a *PstI* restriction site at the 5' terminus. The purified product was digested with *PstI* and *XbaI* endonucleases and ligated into pUC19. The polymerase I promoter region was then ligated to the 208-5S sequence.

The fragment containing the promoter and the 5S positioning sequence was amplified by polymerase chain reaction and the monomer of the 5S rDNA positioning sequence was excised with *AvaI* (*AvaI* restriction endonuclease), which cuts once on each repeat and at the 3' end of the construct, opening up an *AvaI* insertion site. Individual *AvaI* fragments were polymerized by ligation and inserted into the *AvaI* site. The asymmetry of the *AvaI* site allows only head to tail ligation, forcing the orientation of the monomeric fragments. Series of plasmids were prepared containing the RNA polymerase I promoter region upstream of up to 35 repeats of 208-5S. These plasmids were called pPol I 208-n (where n is the number of repeats).

2) Preparation of histone octamers.

Histone octamers were obtained from purified nucleosome monomers isolated from chicken erythrocytes according to the method of Yager et al. (1989). Nuclei isolated from White Leghorn rooster blood were digested for 5 minutes with 14 units of micrococccal nuclease (Worthington Biochemical) per mg of DNA. The long chromatin fraction, generated by mild digestion with micrococccal nuclease, was centrifuged at 6900g for 20 minutes and

the pellet was resuspended in 10 mM Tris-HCl, 0.25 mM EDTA and 0.35M NaCl pH 8.0. Removal of histone H1/H5 was accomplished by incubating the chromatin with 30 µg/ml carboxymethyl-Sephadex for 3 hours at 4 °C, followed by centrifugation at 7700g for 30 minutes and dialysis of the supernatant against TE (10mM Tris-HCl, 1mM EDTA pH 8.0). A 4 minute micrococcal nuclease digestion of the long chromatin free of histone H1/H5 with 5 units of micrococcal nuclease per µl of DNA reduced the long chromatin to monomers, which were then concentrated by ultrafiltration using an Amicon XM-50 ultrafiltration membrane.

The concentrated nucleosome monomer solution was made 2.2M in NaCl and 0.1 M in potassium phosphate at pH 6.7 and chromatographed, on a hydroxylapatite column equilibrated with the same buffer, to remove the DNA (Simon and Felsenfeld, 1979). The collected fractions were electrophoresed to check the histone content and stoichiometry. The concentration was determined from measurements of absorbance at 230nm (A_{230}) (Stein, 1979).

3) Purification of the transcription factors and RNA polymerase I

RNA polymerase I was purified by a modification of the method of Iida and Paule (1992). A 1.6 M to 3.0 M ammonium sulfate fraction from a nuclear extract of *Acanthamoeba castellanii* (Zwick et al., 1991) was used as starting material. This was dialyzed down to 100 mM KCl in buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 mM phenylmethane sulfonyl fluoride [PMSF]) and loaded onto a 11 x 1.5 cm BioRex 70 column in place of a phosphocellulose column, and the step-eluted

fraction between 450 and 650 mM KCl was collected. The DE52 column was step-eluted (75 to 250 mM fractions) and the heparin-Sepharose column was likewise step-eluted (300 to 500 mM KCl) instead of running gradients. The TIF-IB/aUBF fraction was obtained from the 0.5 M to 1.6 M ammonium sulfate fraction of the nuclear extract, which was chromatographed through 14 x 1.5 cm DEAE fast flow (Pharmacia) by loading it at 75 mM KCl in buffer A and, after a wash in the same buffer, eluting with a linear gradient of KCl in buffer A from 75 mM to 500 mM. The TIF-IB/aUBF-containing fractions (at approximately 300 mM KCl) were pooled, diluted to 150 mM KCl and chromatographed through a 9.5 x 0.9 cm BioRex-70 column using a KCl gradient in buffer A from 150 to 900 mM. The fractions containing TIF-IB and aUBF, eluted at approximately 430 mM KCl, were dialyzed down to 100 mM KCl in buffer A and stored at -70 °C.

4) Reconstitution of octamers onto the plasmid

The pPol I 208-4 plasmid DNA was reconstituted with histone octamers according to the method of Retief et al., (1984), which was modified according to the requirements of our system. Polyglutamic acid (PGA) (Miles laboratory) was used as a carrier for the deposition of the histones onto the circular DNA template. The salt concentration of the medium was kept at 150 mM of monovalent cations in order to prevent displacement of the transcription factors.

Reconstitution was carried out using histone octamers purified from chicken erythrocytes as described above. Twenty-five µg of plasmid were first relaxed with 0.6 units of topoisomerase I (BRL)

per µg of DNA for 90 min at 37 °C. The 2.2 M NaCl concentration of the octamer solution was reduced to 150 mM NaCl by diluting in TE; the octamers were then incubated at room temperature for 60 min in presence of a 10 mg/ml solution of polyglutamic acid at a PGA:histone ratio of 2:1 (w/w). The relaxed plasmid was then added to the mixture. The final DNA concentration was 0.05 mg/ml. Input ratios of histone to DNA from 0.6 to 2.3 (gm histone/gm DNA) were tested in order to optimize the conditions of reconstitution for the generation of phased nucleosome on the tandemly repeated positioning sequences. The 500 µl reaction mixture was reconstituted at 37 °C, overnight, under constant shaking to avoid aggregation and precipitation. The reaction mixture was centrifuged on a IEC centra-M centrifuge at top speed for 5 minutes to verify that no material had aggregated. The reconstituted plasmid was electrophoresed on a 0.8% agarose gel in 0.5X e-buffer (e-buffer contains 20mM Tris HCl, 0.5 mM EDTA and 15 mM NaOAc at pH8.0) to monitor the formation of nucleoprotein complex (see Figure 4. 1).

To generate a complex with the potential for transcriptional activity, the appropriate amount of partially purified transcription initiation factor TIF-IB, upstream binding factor (aUBF) and RNA polymerase I were incubated for 15 minutes at 25 °C, in 500 µl final volume, in the presence of 12.5 µg of pPol I 208-4 and 0.5 mM each of ATP and GTP, before regenaration of chromatin structure with histones. The transcription complex will bind to the promoter region, start to transcribe and stop at position +8, because of lack of CTP needed at +8, making the complex more stable and less likely to fall off the DNA template. The complex was then reconstituted with

histone octamers plus PGA at an input ratio of 2.05 histone to DNA and 2 to 1 PGA to histone, according to the protocol previously described. The same protocol was used for reconstituting with histone octamers, TIF-IB and aUBF, in the absence of RNA polymerase I.

5) Sedimentation velocity analysis

The plasmid and histone complexes were submitted to sedimentation velocity analysis on a Beckman model-E analytical ultracentrifuge to verify the homogeneity of the system and to monitor the efficiency of the reconstitution. The centrifugations were performed utilizing 12 mm double sector cells in a four-hole, AN-F rotor. The temperature was kept constant to within 0.1 °C. The solutions used for the sedimentation velocity studies had an A265 =0.8 to 1.0. The rotor speed, in different runs, was between 18000 rpm and 22000 rpm. The scans were analyzed by the method of van Holde and Weischet (1978) using the "UltraScan" ultracentrifuge data collection and analysis program. All data were corrected to standard conditions.

6) Micrococcal nuclease digestion

Micrococcal nuclease digestions of reconstitutes were performed in 100 µl volumes, using 5 µg of chromatin, at 0 units, 25 units and 50 units of micrococcal nuclease per µg of DNA. The reaction mixtures were incubated for 30 seconds at 37 °C, and then the reactions were stopped by making the mixture 40 mM EGTA ([Ethylenebis (oxyethylenenitrilo)] Tetraacetic acid). The products

were treated with 20 µl of 10 mg/ml proteinase K for 1 hour at 37 °C and then phenol-chloroform extracted and ethanol precipitated. The final products were resuspended in 10 µl of H_2O. The material was electrophoresed in a 1.5% agarose gel in 1X Tris Borate EDTA for 3 hours and 30 minutes at 5 volts/cm, ethidium bromide stained and photographed.

7) Restriction digestion

To attempt to map the positions of the nucleosomes on the insert region, cleavages with *PstI*, *XbaI* and *EcoRI* (*EcoRI* restriction endonuclease) were performed. All digestions were performed under the same low Mg^{+2} buffer conditions, whether naked or chromatin-like, circular or linear DNA was used. Amounts of 0.5 to 1 µg of the different DNA templates were digested for 60 minutes at 37 °C with *EcoRI*, *PstI*, *AvaI* or *XbaI* at 10u/µg of DNA. The buffer used for *EcoRI* digestion is: 50 mM Tris HCl pH 8.0, 2.5 mM $MgCl_2$ and 50 mM NaCl. The buffer used for *PstI*, *AvaI* and *XbaI* digestion is: 50 mM Tris HCl pH8.0, 2.5 mM $MgCl_2$ and 100 mM NaCl. The fragments were electrophoresed in a 1% agarose gel in 0.5X e-buffer. After the restriction digestion, half of the reconstituted DNA was phenol extracted or loaded directly in the gel with 0.5% SDS (sodium dodecyl sulfate) loading dye to deproteinize the DNA.

To linearize the plasmid with *XbaI* or *SspI* (*SspI* restriction endonuclease), before attempting reconstitution, the restriction digestions were done under the conditions indicated by the manufacturers of the enzymes (New England Biolab), using 10 mM $MgCl_2$ instead of 2.5 mM. The digestions of pPol I 208-4 for the

binding competition assay using *PvuII* (*PvuII*: restriction endonuclease) and *XbaI* were also done according to the manufacturer's reaction conditions (New England Biolab).

After restriction digestion with *EcoRI* and *PstI* of the plasmid pPol I 208-4 previously linearized with *XbaI* and then reconstituted, and restriction digestion with *EcoRI*, *PstI* and *XbaI* of the same plasmid previously linearized with *SspI* and reconstituted, the reaction products were electrophoresed in an agarose gel. To quantify the availability of the restriction sites, the gel was scanned using a Zeineh scanning densitometer SL-504-XL. Peak heights were measured and normalized so that the total amount of DNA per lane is 100%. The results were plotted to compare the efficiency of cutting by the restriction endonucleases utilized in both cases.

d) Results

1) Reconstitution of nucleosomes onto the pPol I 208-4 plasmid DNA.

The plasmid pPol I 208-4 was designed to contain a ribosomal RNA (rRNA) core promoter region directly upstream of four repeats of a sequence containing the 5S rDNA from *Lytechinus variegatus* (Figure 2. 1). This latter sequence is known for its ability to define the binding positions of histone octamers on a linear DNA template *in vitro* (Simpson and Stafford, 1983, Simpson et al., 1985, Simpson, 1986). Our initial studies were aimed at testing the efficiency of the reconstitution, which was carried out at 150 mM NaCl using the polyglutamic acid method. After overnight incubation at 37 °C, the reconstituted plasmids were submitted to sedimentation velocity analysis. The integral distribution of $S_{20,w}$, obtained for reconstituted

material at input ratios from 0.6 to 2.0 gm of histone per gm of DNA, showed an under-reconstitution (generation of chromatin structure with less than one histone octamer per repeat length of DNA) demonstrated by the presence of heterogeneous material with sedimentation coefficients ranging from about 19 S, (corresponding to the supercoiled naked DNA) up to about 85 S (see Figures 2. 2. 1 and 2. 2. 2). When the histone/DNA input ratio was increased up to 2.2 the template appeared to be over-reconstituted (regenerated chromatin structure with a greater than normaly compact spacing of octamers), exhibiting the presence of heterogeneous material with an S-value over 80 and up to 120 (see Figure 2. 9).

The optimal input ratio was found to be R=2.05, at which ratio the distribution of the S-values obtained covers a narrow range (between 76 and 79 S), as shown in Figures 2. 2. 1 and 2. 2. 2. It should be noted that input ratios are almost certainly higher than the stoichiometry of the complex because histones are lost on surfaces when working with such small volumes.

To determine the average spacing between the nucleosomes, a mild micrococcal nuclease digestion was performed, digestion products analyzed by electrophoresis in a 1.5% agarose in 0.5 X e-buffer (Figure 2. 3). The expected ladder pattern, due to the periodicity of the location of the histone octamers, was obtained, but the spacing between the fragments appearing in the gel was found to range between 123 bp and 159 bp, suggesting a more compact spacing of histone octamers than expected. For the region containing the 5S gene repeats, the spacing is expected to be about 200 bp. Although our results indicate a compact spacing under these

conditions, these data cannot describe precisely the positioning of the nucleosomes on the insert region.

2) Nucleosomes are incorrectly positioned on the 5S RNA genes when the plasmid is reconstituted in the absence of the pol I transcription complex.

To investigate nucleosome positioning after reconstitution of the DNA, the reconstituted material was digested with several restriction endonucleases and results were compared to the patterns obtained with naked DNA. The rationale of this experiment is to determine the percentage of correct positioning of histone octamers on the 5S positioning sequences by monitoring the availability of the restriction sites. If the nucleosomes are positioned as described by Dong et al. (1990) and Meersseman et al. (1991), the *EcoRI*, *PstI* and *XbaI* sites should be fully available for cutting, on the reconstituted plasmid as well as on the naked template (see Figures 2. 1 and 2. 4). The digestion patterns showed that the expected cutting was *not* observed when the reconstitution had been carried out using this circular DNA template by the polyglutamate method. For example, on a template with properly positioned nucleosomes, the *PstI*, *AvaI* sites and the most upstream *EcoRI* site of each 5S gene should be available for restriction. The extra bands in lanes 5 (*EcoRI*-digestion), 8 (*PstI*-digestion) and 11 (*XbaI*-digestion), indicate that only partial digestion of the plasmid had occured, due to the obstruction of some of the sites by nucleosomes. Lanes 6, 9 and 12 show the same pattern as lanes 5, 8 and 11, the only difference is that the bands are shifted downwards after proteinase K treatment. This band shift

confirms the presence of nucleosome structures on the DNA templates. Lanes 8 and 9 showed about 50% of complete digestion suggesting that either only one of the two PstI sites is accessible, or both are accessible 50% of the time. The *XbaI*-site seemed to be more open, but still displayed some protection (lanes 11 and 12). Neither does the pattern obtained with the plasmid pPol I 208-4 reconstituted at a ratio of histone/DNA of 2.05 and digested with *EcoRI* match the naked plasmid digestion pattern (lanes 4, 5 and 6). The partial protection of the *EcoRI* sites again indicates a mispositioning of the histone octamers. Although it is possible to see some partial digestion for some of the enzymes used in this study, the overall significance of the patterns is to demonstrate that the positioning of the histone octamers onto the DNA does not match what was expected from studies made on linear arrays of tandem repeats of 5 S genes reconstituted by salt gradient dialysis (Dong et al., 1990, Meersseman et al., 1991).

There are several possible explanations for such results.

(1) The topological constraints of a circular plasmid might have a major effect in determining the positioning or displacement of histone octamers (see Freeman and Garrard, 1992 for review). However (see below) simple linearization of the plasmid does not, in itself, assure correct positioning.

(2) It is also possible that some feature of the reconstitution protocol -PGA or low ionic strength- could be interfering with proper positioning, as has been shown on short linear DNA templates (Pennings et al., 1989). However a step-dialysis reconstitution was attempted using pPol I 208-4 at a histone/DNA input ratio of R=2.05:

this resulted in restriction digestion patterns very similar to those observed when PGA was used to reconstitute (data not shown). This result indicates that it is not the method of reconstitution but some other feature of the plasmid that produces the irregular positioning.
(3) Finally, the plasmid sequence or the RNA polymerase I promoter region might contain regions with high affinity for histone octamers, which would in turn influence nucleosome positioning in the adjacent 5S gene region.

3) Incorrect positioning in the repeat region results from the proximity of the RNA polymerase I promoter.

To assess the relative importance of DNA topology versus the effect of the proximity of the RNA polymerase I promoter region, the plasmid was linearized in two different ways prior to reconstitution; cleavage was by either restriction digestion with *XbaI* or *SspI*. The *XbaI*-linearized plasmid does not contain the polymerase I promoter region upstream of the stretch of 5S genes; rather, it is moved to a far downstream position. Thus, any possible interference from that region should disappear. On the other hand the SspI-linearized plasmid will still contain the promoter in its normal position upstream of the 5S genes and therefore will give information about the effect of that sequence on the mispositioning effect. After reconstitution, the complexes were chromatographed on an HPLC C8 column to assure the absence of free DNA before submitting the reconstituted sample to digestion. The elution gradient consists of a two buffer system. Buffer A is 20mM ammonium acetate and bufferB

is 50% of buffer A plus 50% acetonitrile. The samples showed no free DNA (see Figure 2. 9).

The efficiency with which different restriction endonucleases cut linearized pPol I 208-4 before and after reconstitution was determined by comparing the amount of digested products obtained from naked DNA and reconstituted DNA (see Figure 2. 5). It was found that reconstituted *XbaI*-digested plasmid treated with *EcoRI* displays 90% of the efficiency of cutting at the *EcoRI* restriction sites observed in the case of naked pPol I 208-4. On the other hand, when the *SspI*-linearized plasmid was digested, the relative amount of the 208 bp fragment produced drops to about 50%, showing more protection of the *EcoRI* sites and therefore reflecting a less accurate positioning (Figure 2. 5 cf lanes 4 and 5).

A similar analysis was performed on the *SspI* and the *XbaI*-linearized plasmids utilizing *PstI*. The relative efficiency of cutting was again higher in the case of the *XbaI*-treated plasmid (75%) compared to the 45% obtained for the SspI-treated pPol I 208-4.
In short, in every case the cutting was found to be more efficient when the plasmid was linearized with *XbaI*. These results demonstrated that positioning was less regular when the *SspI*-linearized DNA was provided as a DNA template for the reconstitution than when the plasmid had been linearized with *XbaI*. Thus, the proximity of the promoter region to the tandem repeat region inhibits proper reconstitution in the latter. A possible explanation is that the binding of one nucleosome on the promoter region may be changing the phasing (regular positioning of

nucleosomes on a repeating DNA sequence) of histone octamers on the adjacent tandem-repeat region (see Figure 2. 6).

4) The RNA polymerase I promoter region competes strongly with other sequences for histone octamers.

The above results imply that sequences from the RNA polymerase I promoter region might have a higher affinity for histone octamers than do the tandemly repeated 5S gene sequences. This was investigated directly by allowing three regions of the plasmid to compete for histone octamers under conditions in which histones were limiting. The pPol I 208-4 was digested with *PvuII* and *XbaI* generating 3 fragments: (1) a linear fragment (199 bp) containing the Pol I (RNA polymerase I) promoter region, (2) a 1080 bp fragment containing four copies of the 208 bp positioning sequence and (3) a fragment containing 2320 bp of the pUC8 sequence (See Figure 2. 7). A mixture of these three DNA fragments was used for the competition studies. Reconstitution was via our usual PGA technique, however the histone:DNA ratio was varied from 0.6 to 2.05 in order to assay competition. The material obtained after over night reconstitution was analyzed by band shift assay on a 3.5% acrylamide gel. This analysis showed that as the histone:DNA ratio is increased, the 199 bp fragment containing the RNA polymerase I promoter region plus 104 bp competes efficiently for the binding of histone octamers in a titration experiment with the 1080 bp fragment containing four copies of the positioning sequences or with the entire 2320 bp pUC8 fragment. This argues that the polymerase I promoter region has a

nucleosome binding affinity in the same range of magnitude as do four copies of the 5S gene DNA.

These results may also explain why nucleosomes reconstituted on DNAs containing this promoter sequence upstream from the repeat were not correctly positioned on the repeats of the 5S gene (Figures 2. 4 and 2. 5). It seems likely that the tight binding of histone octamers to the promoter region disrupts the regular nucleosome phasing across the region which contains the four repeated 5S genes.

One factor which may be important in the high affinity of the Pol I promoter for histone octamers is DNA bending. It was recently shown that the bent DNA of trypanosome kinetoplast minicircles bound nucleosomes 6-7 fold more tightly than bulk sequences. Especially significant for our studies was the observation that the location of a bend affected the position of neighboring octamers (Trifonov, 1980, Shrader and Crothers, 1989, Constanzo et al., 1990). Recently, intrinsically bent DNA has been found near the promoter of the transcription initiation site of the *Physarum* rDNA (Schroth et al., 1992). We analyzed the *Acanthamoeba* Pol I promoter region used in these experiments by computer modelling in the manner of Schroth et al. (1992), and detected a 35° bend centered at about 23 bp from the positioning sequence and +8 bp from the transcription start site. If, as has been observed for the positioning sequence itself, a favored nucleosome position puts this bend at the dyad axis, this would overlap the 5S rDNA sequence as shown in Figure 2. 6 (top). This could then disturb subsequent positions in the repeat region.

5) A stalled transcription complex restores correct nucleosome positioning on the 5S rRNA genes.

If a nucleosome bound to the promoter region causes changes in positioning of adjacent nucleosomes, what will be the effect of the binding of the transcription complex? To test for effects of transcription factors TIF-IB and aUBF and RNA polymerase I on the nucleosome positioning, we first assembled these proteins onto pPol I 208-4, then reconstituted with nucleosomes and probed restriction site availability. The plasmid was first incubated in presence of the two transcription factors, TIF-IB and aUBF and the RNA polymerase I. The transcription complex was then initiated by addition of ATP and GTP and stalled at position +8 by starving it for UTP and CTP. Once the transcription complex was engaged, reconstitution was carried out. The reconstituted plasmid was then digested with *XbaI*, *PstI*, *AvaI* or *EcoRI*; each preparation was then phenol extracted. The digestion products were electrophoresed in a 1% agarose gel in 0.5x e-buffer, next to similar digests of naked pPol I 208-4 (Figures 2. 8. 1 and 2. 8. 2).

The restriction endonuclease sites in these constructs exhibit availability consistent with correct or nearly correct nucleosome positioning. All digestions went to completion with 5 units of restriction enzyme per µg of DNA whereas some dimer and trimer were visible in the *AvaI* and *EcoRI*-digestions digested with only 1 unit of enzyme per µg of DNA.

We conclude that positioning on the 5S rDNA downstream from the promoter was rescued by the addition of the transcription factors plus the RNA polymerase I. On the other hand, the presence of the

transcription factors TIF-IB and aUBF, in the absence of polymerase I, did not rescue the positioning (data not shown). A possible reason for this is shown in Figure 2. 6 (bottom). The polymerase may prevent deposition on the promoter, and yet not interfere with adjacent nucleosomes. Why the factors themselves do not rescue is entirely unclear; at this point we cannot exclude the possibility that a nucleosome can displace the factors, but not the factors plus the polymerase.

e) Discussion

We have shown that the expected positioning of reconstituted nucleosomes on a tandemly repeated array of 5S genes is not seen when the array is placed adjacent to the *Acanthamoeba castellanii* RNA polymerase I core promoter on a circular plasmid. When the template was linearized by restriction endonuclease cutting before the reconstitution, the subsequent position pattern depended upon where the cut had been made. Retention of the promoter sequence upstream from the 5S gene repeats resulted in incorrect positioning, whereas more regular positioning was observed if the region was moved away from the 5S gene repeat. This argues that the promoter region somehow interferes with "proper" positioning. Reconstitution competition assays showed the unexpected result that the Pol I promoter has an affinity for nucleosomes comparable to that of the 208 bp positioning sequences. This may be explained by modeling studies, which predict a bent sequence in the promoter region. Such a sequence might strongly bind a nucleosome which would overlap the first 5S gene repeat, and might then interfere with further

positioning by the 5S RNA repeats. The recovery of the positioning, upon prior formation of a stalled transcription complex suggests that the presence of the complex prevents deposition of a nucleosome at this site. This event would then prevent interference with positioning of an octamer on the first 5S gene and allow the subsequent nucleosomes to adopt the expected positions (see Figure 2. 6).

The positioning on the pUC 8 portion of the plasmid was not examined but the micrococcal ladder indicates a compact spacing. The input ratio of 2.05 histone/DNA appears high and while it may not correspond to the actual stoichiometry of the complex, it may also imply a compact spacing of the nucleosomes onto most of the plasmid.

The fact that the promoter region contains a site of high affinity for nucleosomes may have wider significance. Indeed, this may be related to the proposed mechanism of regulation of transcription involving the binding of nucleosomes onto the promoter regions of RNA polymerases (Wasylyk and Chambon, 1979, Morse, 1989, Almouzni et al.,1990 and Grunstein,1990 for a review).

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Figures 2. 1. 1, 2. 1. 2 and 2. 1. 3:

Construction of the 208 bp insert and map of the plasmid DNA pPol I 208-4:

1) The figure shows the complete sequence for the polymerase chain reaction of the 208 bp sequence repeat. Both primer sets and their position of hybridization are shown. The 208 bp of the positioning sequence are underlined.

2) Details on the construction of the insert.

The desired product contained the promoter region of the RNA polymerase I from *Acanthamoeba castellanii* ligated upstream of the 208 bp fragment from *Lytechinus variegatus.* The products of the ligation of the two fragments (RNA polymerase I core promoter region and the 5S rDNA positioning sequence) were amplified using polymerase chain reaction and sequenced prior to ligation. The fragment was then inserted in the plasmid pUC8.

3)Map of the plasmid pPol I 208-4:

The plasmid constructed contains the RNA polymerase I core promoter region followed by 4 repeats of the 208 bp 5S rDNA inserted into the *PstI* site of the poly cloning region of the plasmid pUC8. The upper portion of the schematic shows the major position found for the histone octamer (grey box) on the 5S sequence, in salt-gradient reconstitution onto linear templates (see Dong et al., 1990). The *XbaI* restriction site within the insert and the *SspI* restriction site of the plasmid outside the insert are labeled and indicated by

arrows. These two separates were used to linearize the circular plasmid.

Figure 2. 1. 1

Sequence of the 208 bp fragment

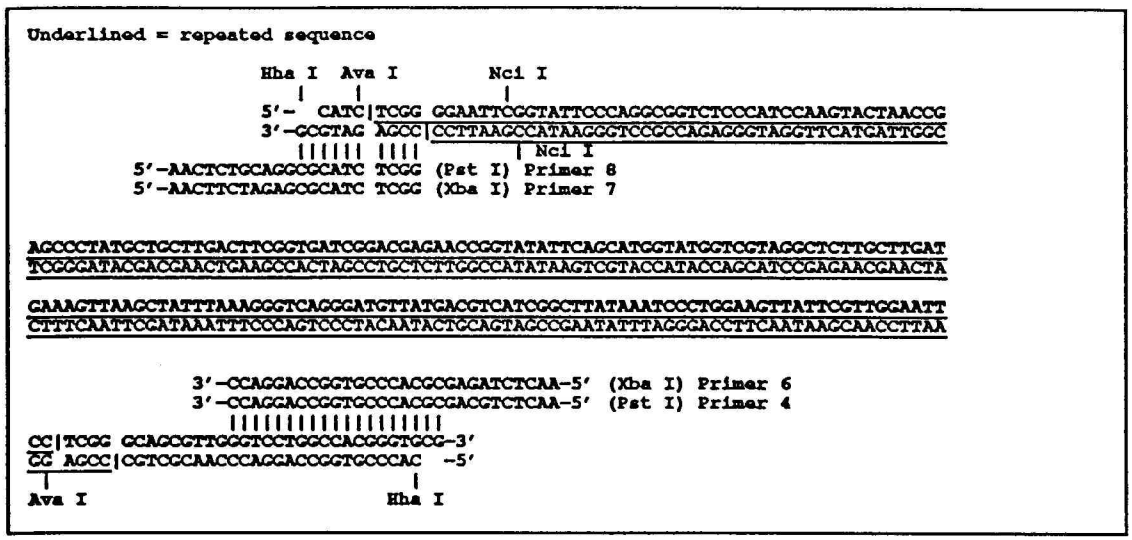


Figure 2. 1. 2

Details on the construction of the insert

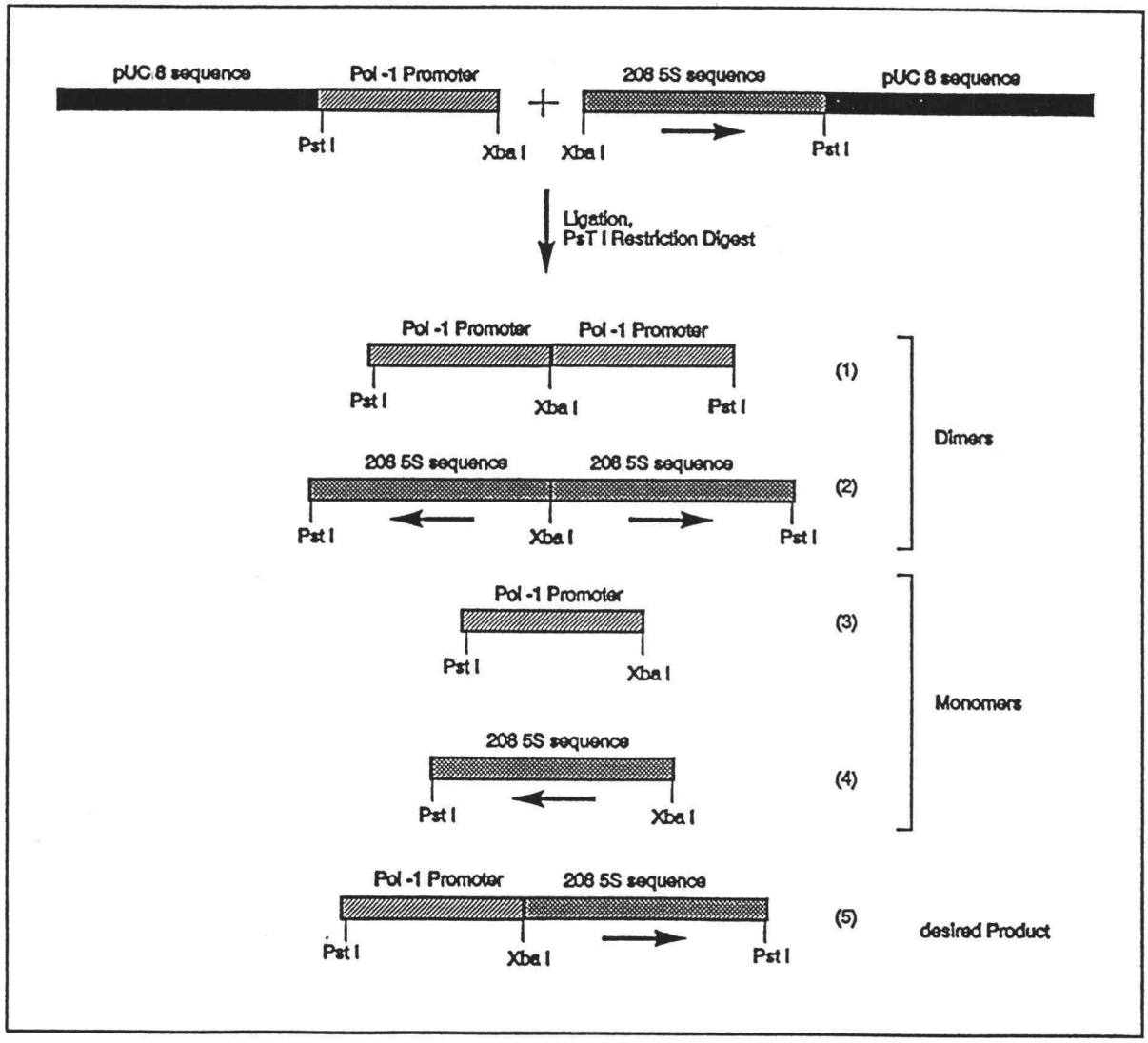
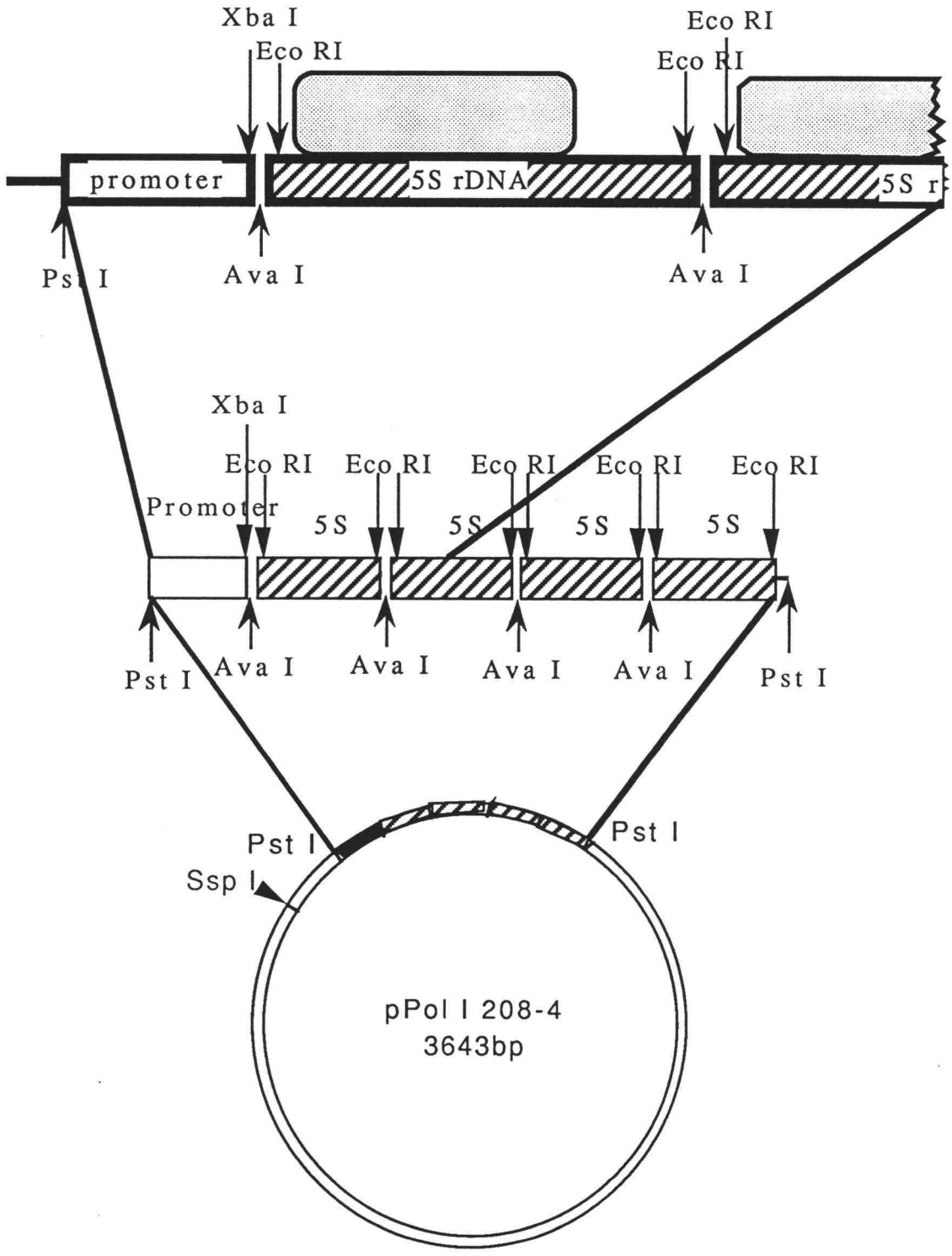


Figure 2. 1. 3

Map of the plasmid DNA pPol I 208-4



Figures 2. 2. 1 and 2. 2. 2:

Sedimentation velocity analysis of the reconstituted pPol I 208-4 chromatin at increasing histone/DNA input ratios in 150 mM NaCl. The figure illustrates the integral distribution of S-values. The y-axis measures the fraction (percentage) of material with $S_{20,w}$ values less or equal to value given on the abcissa. The vertical line at ratio of 2.05 indicates the presence of homogeneous material.

Figure 2. 2. 1

Sedimentation velocity analysis of the reconstituted pPol I 208-4 chromatin at increasing histone/DNA input ratios in 150 mM NaCl.(R= 1.4 to R= 2.15). The figure illustrates the integral distribution of S-values.

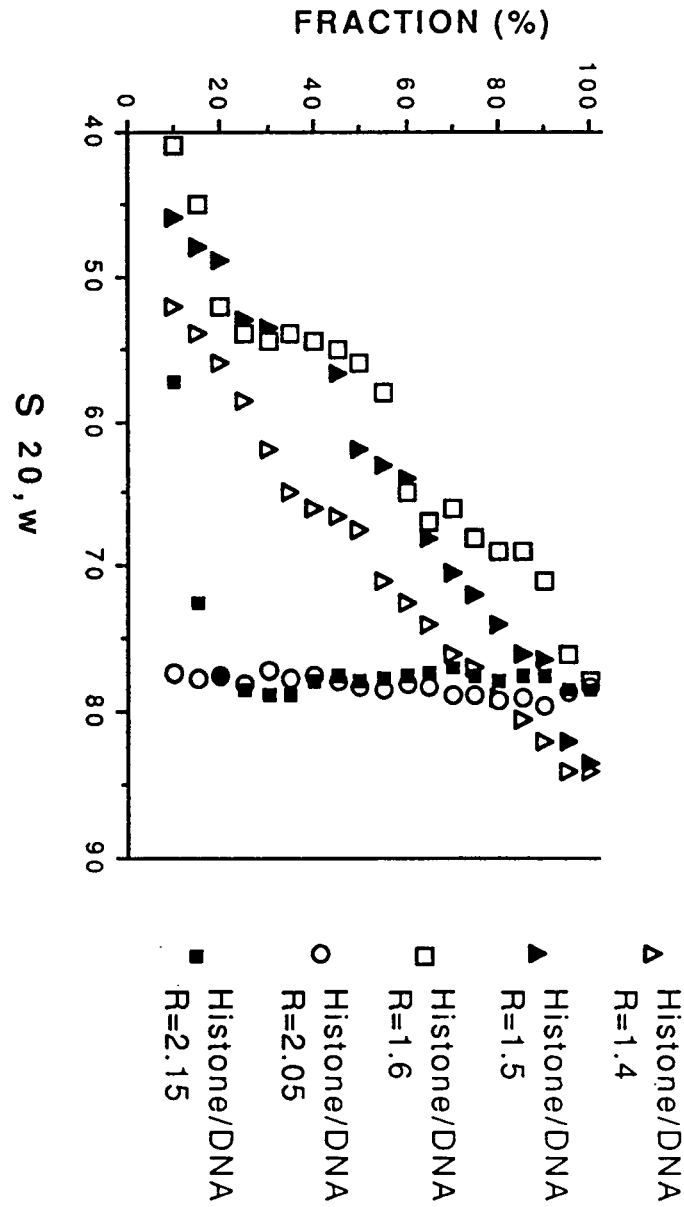


Figure 2. 2. 2

Sedimentation velocity analysis of the reconstituted pPol I 208-4 chromatin at increasing histone/DNA input ratios in 150 mM NaCl.(R= 2.05 to R= 2.2).

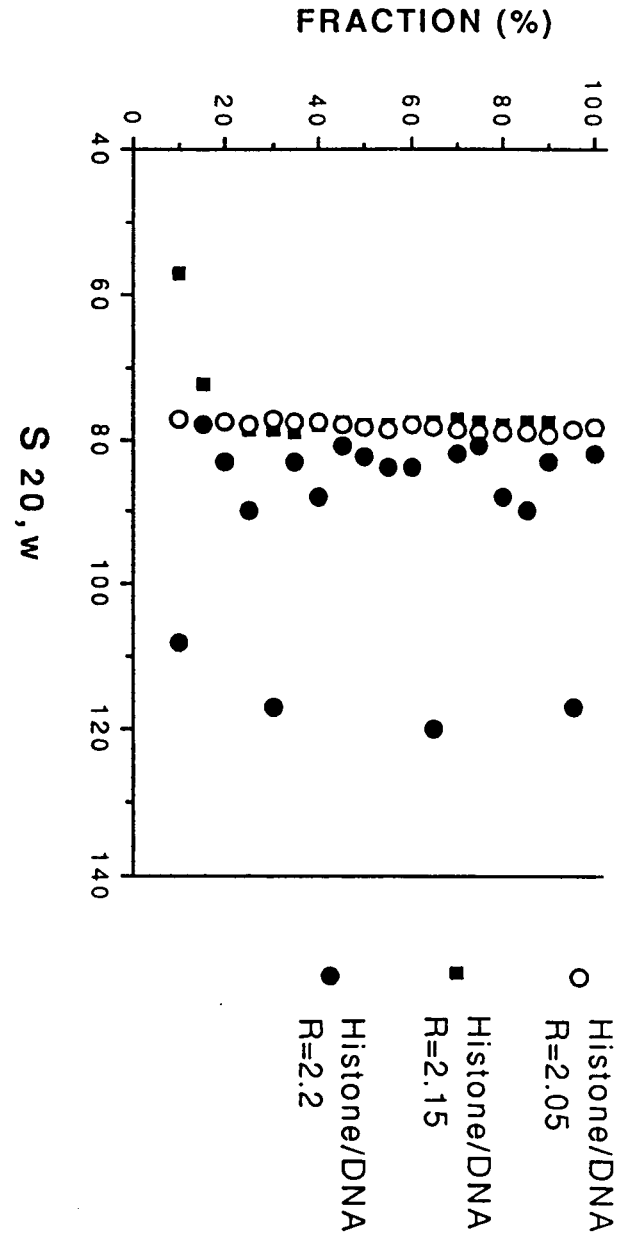


Figure 2. 3: Partial micrococcal nuclease digestion of reconstituted pPol I 208-4 DNA.

The chromatin structure was reconstituted at a ratio of histone/plasmid DNA of 2.05 and the products were electrophoresed on a 1.5% agarose gel in 0.5 x e-buffer.

lane 1: ϕX 174 DNA digested with *HhaIII* (*HhaIII* restriction endonuclease).

lane 2: pBR 322 DNA digested with *MspI* (*MspI* restriction endonuclease).

lane 3: pPol I 208-4 DNA incubated with a ratio of histone/DNA of 2.05.

lane 4: pPol I 208-4 DNA incubated with a ratio of histone/DNA of 2.05 and then digested with 5 units of MNase (micrococcal nuclease) per µg of DNA.

lane 5: pPol I 208-4 DNA incubated with a ratio of histone/DNA of 2.05 and then digested with 10 units of MNase per µg of plasmid.

Both MNase digestions were incubated for 30 seconds.

The numbers to the left correspond to the length of the different fragments in the markers lanes.

Figure 2. 3

Partial micrococcal nuclease digestion of reconstituted pPol I 208-4 DNA.

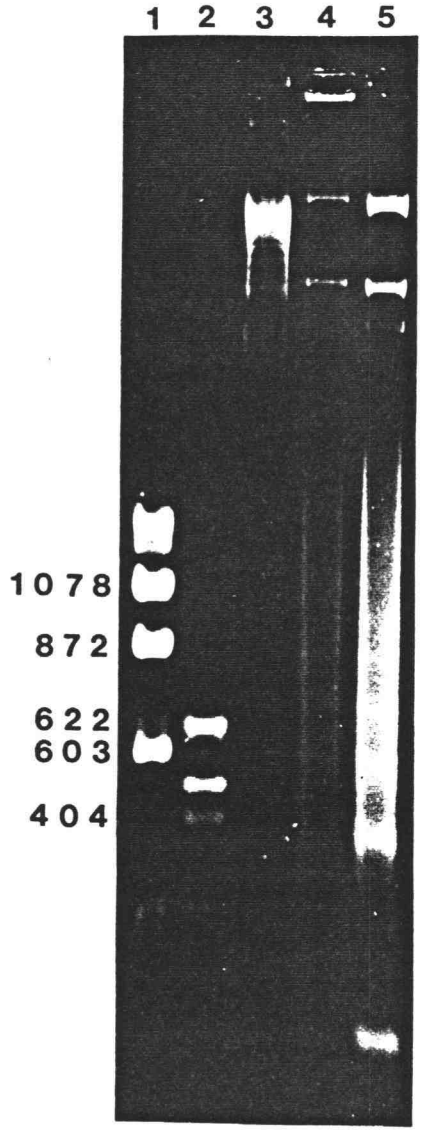


Figure 2. 4: Restriction digestion of circular pPol I 208-4 plasmid DNA

Naked and reconstituted plasmid DNA incubated with a ratio of histone/DNA of 2.05, were digested with *EcoRI*, *XbaI* and *PstI* to define the position of the nucleosomes on the 5S rDNA. Half of the reconstituted material was treated with proteinase K after digestion in order to remove the proteins from the DNA and was electrophoresed next to the naked plasmid DNA digested with the similar restriction endonuclease to compare the digestion patterns. The extra bands seen on lanes 2, 3, 11 and 12 could correspond to nicked and linear forms of the plasmid.

lane 1: Lambda phage DNA digested with *BstEII*.

lane 2: naked circular pPol I 208-4 plasmid DNA.

lane 3: reconstituted circular pPol I 208-4 plasmid DNA.

lane 4: naked pPol I 208-4 plasmid DNA digested with *EcoRI*.

lane 5: reconstituted pPol I 208-4 plasmid DNA digested with *EcoRI*.

lane 6: reconstituted pPol I 208-4 plasmid DNA digested with *EcoRI* and treated with proteinase K.

lane 7: naked pPol I 208-4 plasmid DNA digested with *PstI*.

lane 8: reconstituted pPol I 208-4 plasmid DNA digested with *PstI*.

lane 9: reconstituted pPol I 208-4 plasmid DNA digested with *PstI* and proteinase K treated.

lane 10: naked pPol I 208-4 plasmid DNA digested with *XbaI*.

lane 11: reconstituted pPol I 208-4 plasmid DNA digested with *XbaI*.

lane 12: reconstituted pPol I 208-4 plasmid DNA digested with *XbaI* and proteinase K treated.

Figure 2. 4

Restriction digestion of circular pPol I 208-4 plasmid DNA

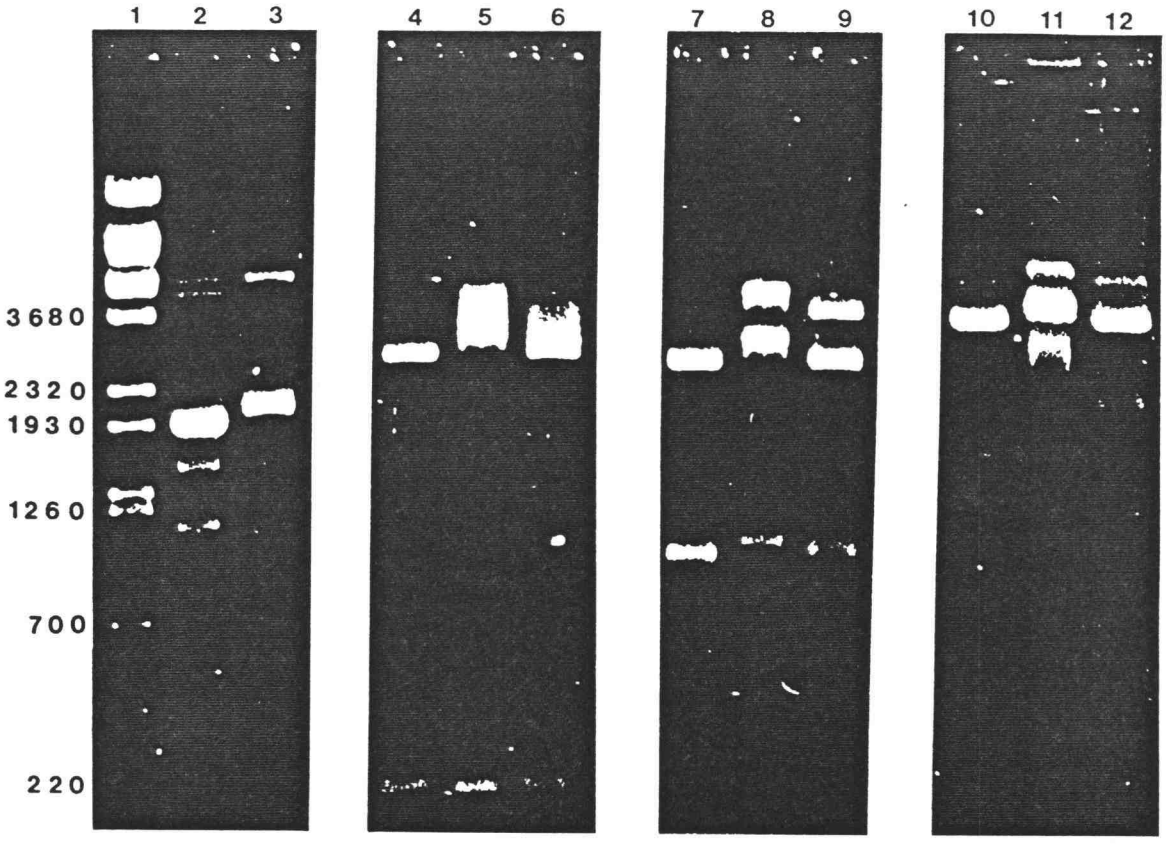


Figure 2. 5

Restriction digestion of and of pPol I 208-4 plasmid DNA linearized with *Xba*I or with *Ssp*I after incubation with histones at a ratio histone/DNA of 2.05.

lane 1: Lambda phage DNA digested with *BstEII*.

lane 2: naked pPol I 208-4 plasmid DNA digested with *Xba*I and *EcoRI*.

lane 3: naked pPol I 208-4 plasmid DNA digested with *Ssp*I and *EcoRI*.

lane 4: pPol I 208-4 plasmid DNA linearized with *Xba*I and reconstituted, then digested with *EcoRI*.

lane 5: pPol I 208-4 plasmid DNA linearized with *Ssp*I and reconstituted, then digested with *EcoRI*.

lane 6: naked pPol I 208-4 plasmid DNA digested with *Xba*I and *Pst*I.

lane 7: naked pPol I 208-4 plasmid DNA digested with *Ssp*I and *Pst*I.

lane 8: pPol I 208-4 plasmid DNA linearized with *Xba*I and reconstituted, then digested with *Pst*I.

lane 9: pPol I 208-4 plasmid DNA linearized with *Ssp*I and reconstituted, then digested with *Pst*I.

lane 10: naked pPol I 208-4 digested with *Xba*I and *Ava*I.

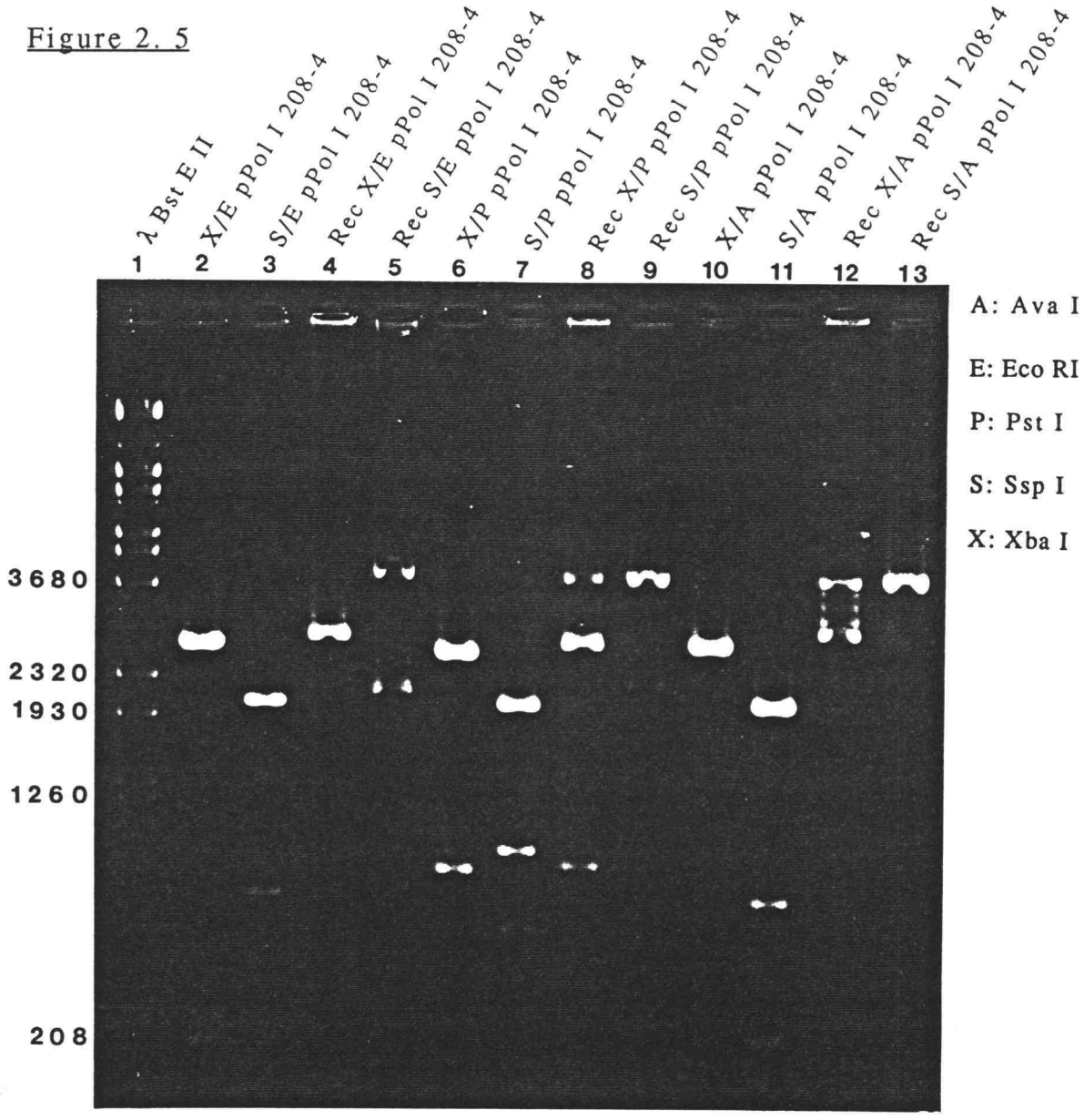
lane 11: naked pPol I 208-4 digested with *Ssp*I and *Ava*I.

lane 12: pPol I 208-4 plasmid DNA linearized with *Xba*I and reconstituted, then digested with *Ava*I.

lane 13: pPol I 208-4 plasmid DNA linearized with *Ssp*I and reconstituted, then digested with *Ava*I.

The presence of partially digested 208 bp fragments in the case of the plasmid DNA linearized with *XbaI* is consistent with the idea of nucleosomes positioned at a minor positioning site. The presence of these incompletely digested products could be explained by a slightly different position of the nucleosomes. A shift in positioning of nucleosomes of at least 6 base pairs toward the 5' end of the 208 bp fragment would be consistent with the digestion patterns observed. Such a shift would leave the *EcoRI* site available and partially protect the *AvaI* site. The change in location could also be due to a preference for one of the minor positioning sequences as observed previously on the 5S rDNA (Dong et al., 1990).

Figure 2. 5



Relative cutting efficiencies

Template	Naked	Reconstituted
Xba I/ Eco RI	100%	90%
Ssp I/ Eco RI	100%	50%
Xba I/ Pst I	100%	75%
Ssp I/ Pst I	100%	45%
Xba I/ Ava I	100%	70%
Ssp I/ Ava I	100%	0%

Figure 2. 6: Schematic of the predicted position of nucleosomes

Top: Position of nucleosome in absence of TIF-IB, aUBF and RNA polymerase I. The first nucleosome is bond to the promoter region with its dyad axis at position +8 (predicted center of the bend). We have drawn the figures to suggest an alternate phasing (with same spacing), but we cannot exclude the possibility of compact spacing on the 208-4 region.

Bottom: Predicted binding of the transcription complex onto the promoter region and nucleosome position recovery on the 5S gene.

Figure 2. 6
Schematic of the predicted position of nucleosomes

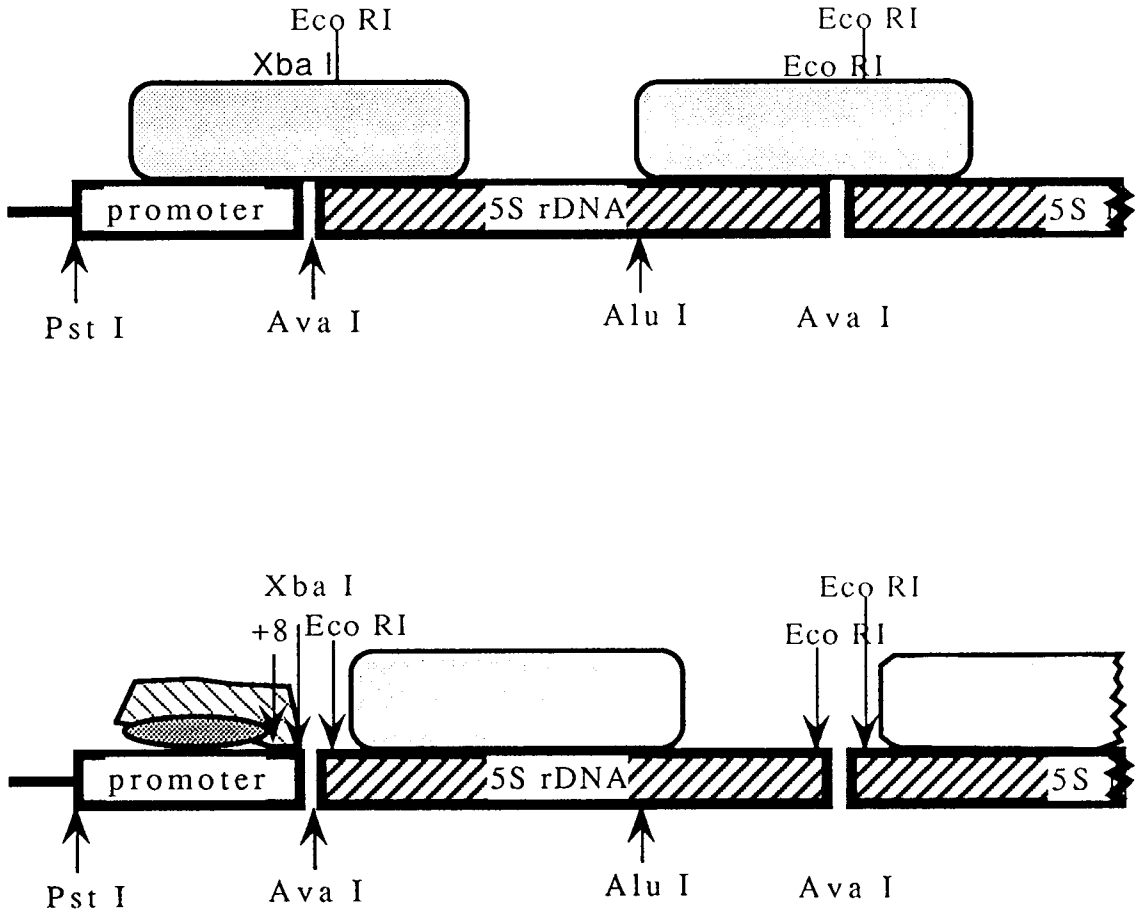


Figure 2. 7: Binding competition assay monitored by band shift assay

The three fragments obtained from the double digestion with *PvuII* and *XbaI* were reconstituted in presence of increasing ratios of histone to DNA. After reconstitution the DNA was electrophoresed in a 3.5% acrylamide gel to monitor band shifts due to the binding of histone octamer(s) onto the DNA templates. The arrow indicates the position of the 199 bp fragment after binding of the octamer.

Lane 1: Lambda phage DNA digested with *BstEII*

Lane 2: pBR 322 DNA digested with *MspI*

Lane 3: naked pPol I 208-4 plasmid DNA digested with *PvuII* and *XbaI*

Lane 4: pPol I 208-4 plasmid DNA digested with *PvuII* and *XbaI* and incubated with histones at a ratio of histone/DNA (R) of 0.2.

Lane 5: same as lane 4 except the ratio of histone/DNA R=0.4.

Lane 6: same as lane 5 except the ratio of histone/DNA R=0.6

Lane 7: same as lane 6 except the ratio of histone/DNA R=0.8

Lane 8: same as lane 7 except the ratio of histone/DNA R=1.0

Lane 9: same as lane 8 except the ratio of histone/DNA R=1.2

Lane 10: same as lane 9 except the ratio of histone/DNA R=1.4

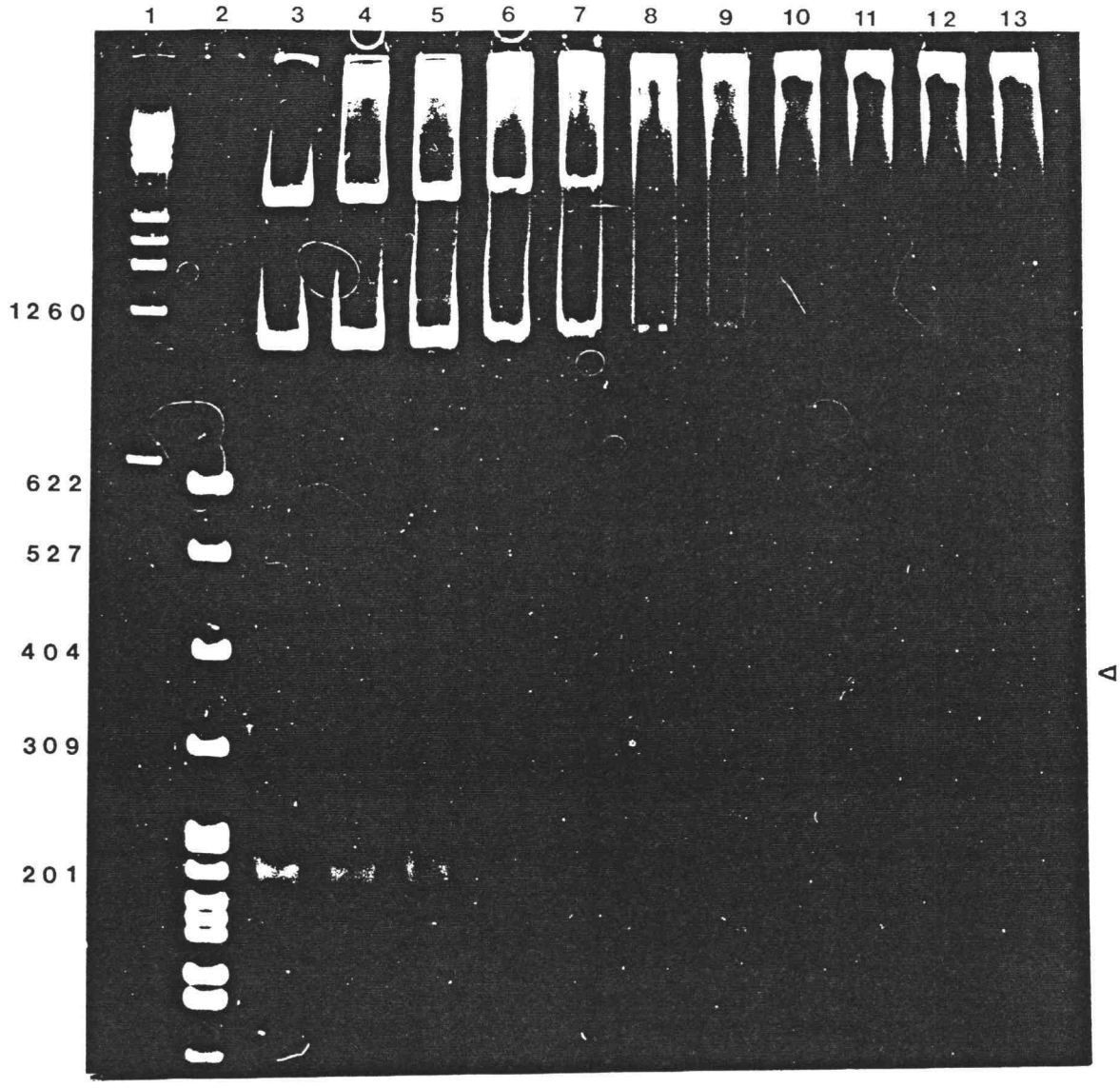
Lane 11: same as lane 10 except the ratio of histone/DNA R=1.6

Lane 12: same as lane 11 except the ratio of histone/DNA R=1.8

Lane 13: same as lane 12 except the ratio of histone/DNA R=2.05

Figure 2. 7

Binding competition assay monitored by band shift assay



Figures 2. 8. 1 and 2. 8. 2: *XbaI*, *PstI*, *EcoRI* and *AvaI* digestions of pPol I 208-4 plasmid DNA naked and incubated with histones at input ratio R =2.05 in presence of TIF-IB and aUBF + RNA pol I

1) *XbaI*, *PstI* and *EcoRI* restriction digestions of pPol I 208-4 plasmid DNA naked and reconstituted after preincubation in presence of TIF-IB, aUBF and RNA pol I, electrophoresed in a 1% agarose gel.

Lane 1: Naked pPol I 208-4 plasmid DNA digested with 1 unit of *XbaI*.

Lane 2: pPol I 208-4 plasmid DNA + TIF-IB and aUBF + RNA pol I reconstituted digested with 10 units of *XbaI*, treated with proteinase K and extracted with phenol/ chloroform.

Lane 3: Same as lane 2 digested with 5 units of *XbaI*.

Lane 4: Same as lane 2 digested with 1 unit of *XbaI*.

Lane 5: Naked pPol I 208-4 plasmid DNA digested with 1 unit of *PstI*.

Lane 6: pPol I 208-4 plasmid DNA + TIF-IB and aUBF + RNA pol I reconstituted digested with 10 units of *PstI*, treated with proteinase K and extracted with phenol/ chloroform.

Lane 7: Same as lane 6 digested with 5 units of *PstI*.

Lane 8: Same as lane 6 digested with 1 unit of *PstI*.

Lane 9: Naked pPol I 208-4 plasmid DNA digested with 1 unit of *EcoRI*.

Lane 10: pPol I 208-4 plasmid DNA + TIF-IB and aUBF + RNA pol I reconstituted digested with 10 units of *EcoRI*, treated with proteinase K and extracted with phenol/ chloroform.

Lane 11: Same as lane 10 digested with 5 units of *EcoRI*.

Lane 12: Same as lane 10 digested with 1 unit of *EcoRI*.

Lane 13: Uncut pPol I 208-4 plasmid DNA.

Lane 14: Lambda phage DNA digested with *BstEII*.

2) *AvaI* restriction digestions of pPol I 208-4 plasmid DNA naked and incubated with histones at input ratio R =2.05 after preincubation in presence of TIF-IB, aUBF and RNA pol I, electrophoresed in a 1% agarose gel.

Lane 1: Naked pPol I 208-4 plasmid DNA digested with 1 unit of *AvaI*.

Lane 2: pPol I 208-4 plasmid DNA + TIF-IB and aUBF + RNA pol I reconstituted digested with 10 units of *AvaI*, treated with proteinase K and extracted with phenol/ chloroform.

Lane 3: Same as lane 2 digested with 5 units of *AvaI*.

Lane 4 Same as lane 2 digested with 1 unit of *AvaI*.

Lane 5: Uncut pPol I 208-4 plasmid DNA.

Lane 6: λ Lambda phage DNA digested with *BstEII*.

Figure 2. 8. 1

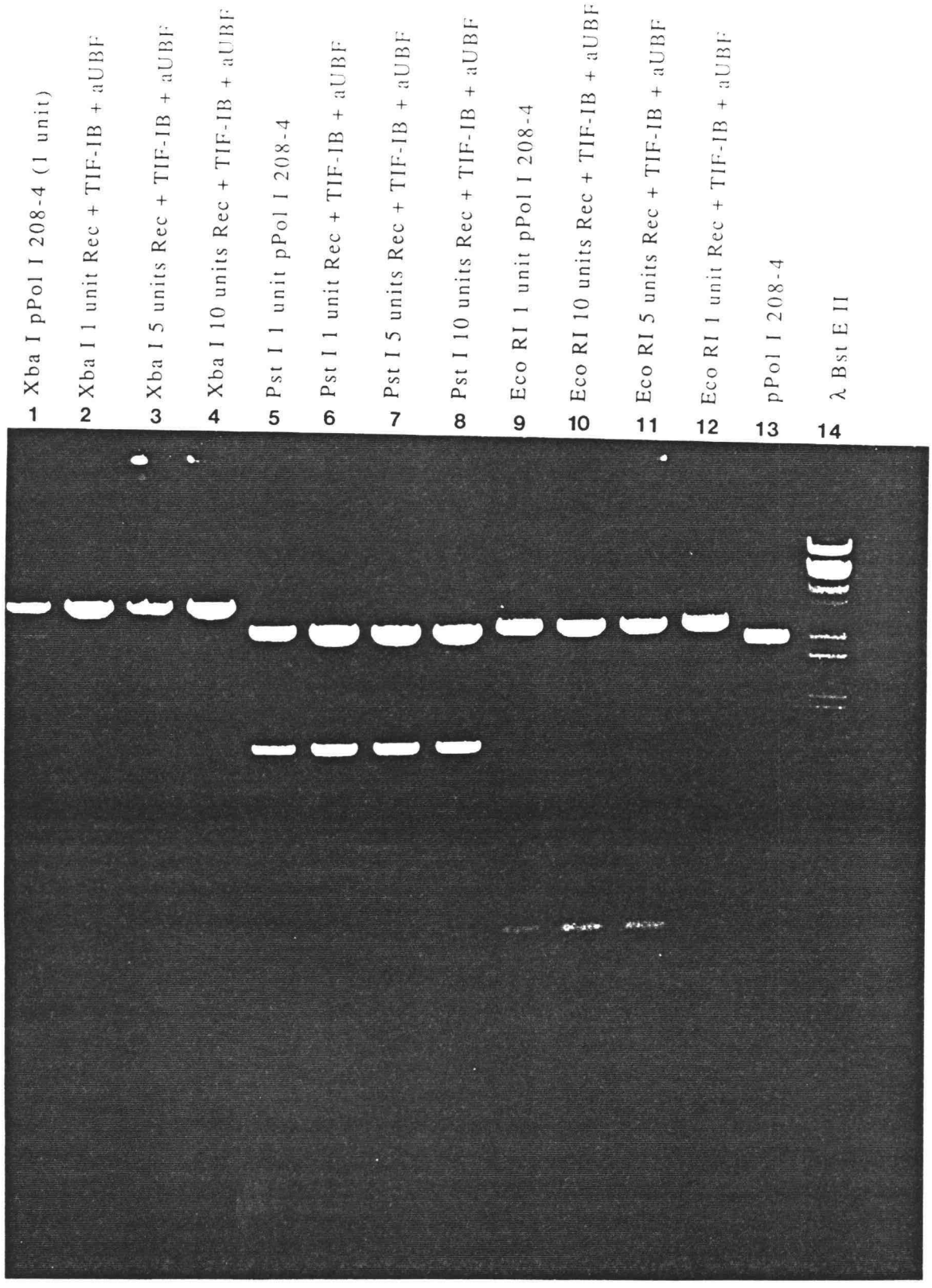

1 Xba I pPol I 208-4 (1 unit)
2 Xba I 1 unit Rec + TIF-IB + aUBF
3 Xba I 5 units Rec + TIF-IB + aUBF
4 Xba I 10 units Rec + TIF-IB + aUBF
5 Pst I 1 unit pPol I 208-4
6 Pst I 1 unit Rec + TIF-IB + aUBF
7 Pst I 5 units Rec + TIF-IB + aUBF
8 Pst I 10 units Rec + TIF-IB + aUBF
9 Eco RI 1 unit pPol I 208-4
10 Eco RI 10 units Rec + TIF-IB + aUBF
11 Eco RI 5 units Rec + TIF-IB + aUBF
12 Eco RI 1 unit Rec + TIF-IB + aUBF
13 pPol I 208-4
14 λ Bst E II

Figure 2. 8. 2

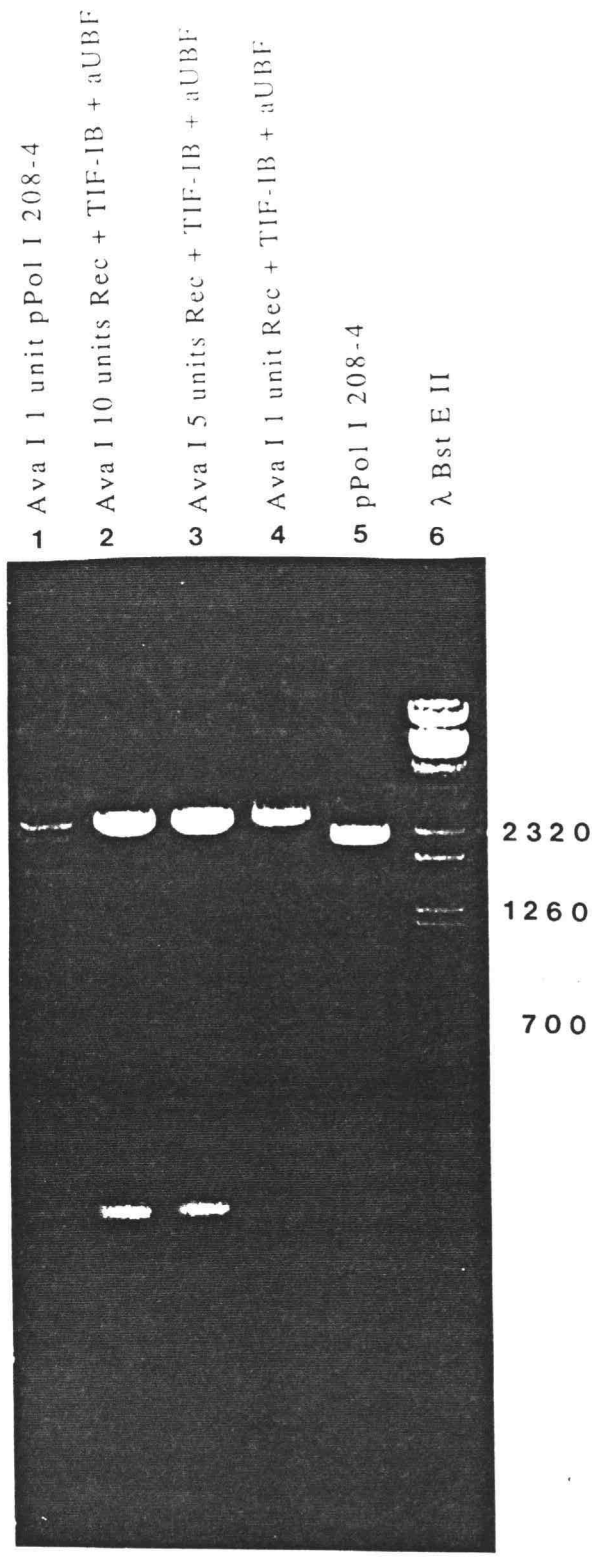

1 Ava I 1 unit pPol I 208-4
2 Ava I 10 units Rec + TIF-IB + aUBF
3 Ava I 5 units Rec + TIF-IB + aUBF
4 Ava I 1 unit Rec + TIF-IB + aUBF
5 pPol I 208-4
6 λ Bst E II
2320
1260
700

Figure 2. 9

Elution profiles of:

the plasmid DNA pPol I 208-4 naked or preincubated with TIF-IB, aUBF and RNA polymerase I, then incubated with histone at a ratio of histone/DNA of 2.05. The absorbance at 260.4 nm in milli-Absorbance Units (mAU) is plotted versus time (in minutes).

The elution gradient is expressed in percentage of buffer B.

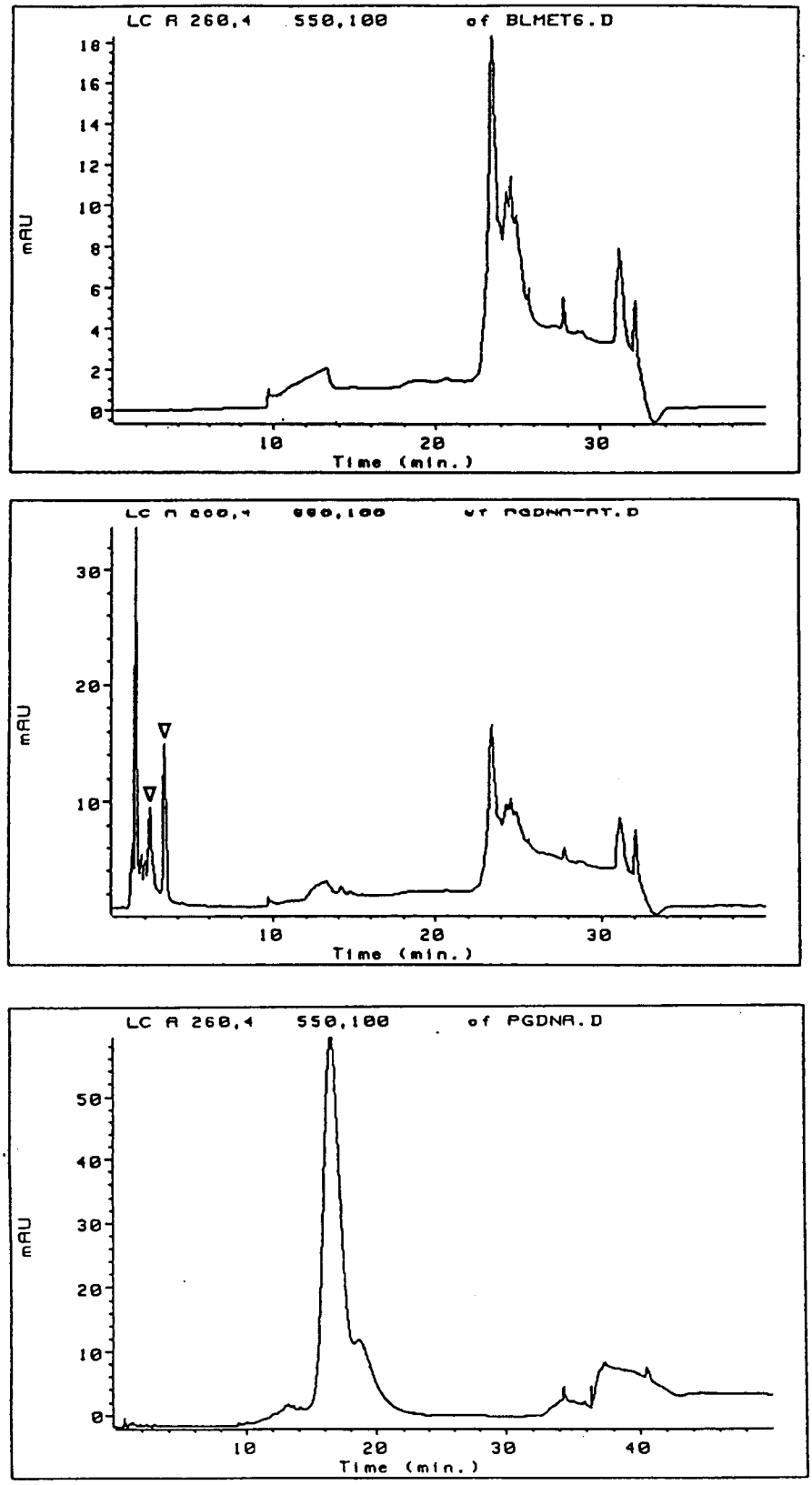
Top: Blank.

Middle: pPol I 208-4 plasmid DNA preincubated with TIF-IB, aUBF and RNA polymerase I, then incubated with histone at a ratio of histone/DNA of 2.05 (see arrow heads for peak appearances at a retention time of 2.3 to 3.2 minutes).

Bottom: pPol I 208-4 plasmid DNA naked (retention time: 17 minutes).

Figure 2. 9

Elution profiles



The abbreviations used are: rDNA, ribosomal DNA; PGA, polyglutamic acid; TIF-IB, transcription and initiation factor also called SL-1; aUBF, upstream binding factor also called SF-1; bp, base pair; PCR, polymerase chain reaction; EGTA, [Ethylenebis (oxyethylenenitrilo)] Tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethane sulfonyl fluoride; HPLC, high performance liquid chromatography; Pol I, RNA polymerase I; MNase, microccocal nuclease.

CHAPTER 3

RNA Polymerase I Transcribes Through Phased Array of Nucleosomes as Well as Free DNA.

Georgel, Philippe and van Holde, Kensal E.

To be submitted.

Coauthor contribution: K.vH: Research Director.

a) Summary

We have designed a DNA plasmid template (designated pPol I 208-4) for *in vitro* transcription experiments. It contains an RNA polymerase I core promoter region from *Acanthamoeba castellanii* placed upstream of four repeats of the 5S rDNA positioning sequence of *Lytechinus variegatus*. When this plasmid is preincubated with its cognate transcription factors and RNA polymerase I and then incubated with histones at a ratio (w/w) of histone to DNA of 2.05, we observed the formation of phased nucleosomes on the repeat of the 5S positioning sequence. When the transcription complex is not formed prior to the incubation with histones, the positioning of nucleosomes becomes more "random". Transcription efficiency was assayed for the naked plasmid DNA, the more "randomly" positioned plasmid DNA template and for the plasmid DNA containing the phased nucleosomes. The results show that the presence of nucleosomes in the path of the transcription complex does not seem to impede transcription, when comparing the naked DNA to the plasmid DNA with phased nucleosomes. On the other hand, the plasmid with "randomly" positioned nucleosomes was shown to be a poor template for transcription. The explanation for the poor efficiency of this DNA template is most likely the occupancy of the promoter region by histones. Such bound histones would compete with TIF-IB for the binding site on the promoter, therefore preventing initiation of transcription. By monitoring the availability of certain restriction sites, we also observed that the passage of the transcription complex through the nucleosome array does not remove nucleosomes from the positioning sequences.

b) Introduction

The nucleosome is a central component of the eukaryotic chromatin structure and, as observed in many recent studies (Lorch et al., 1987, Losa and Brown, 1989, Morse, R.H, 19892, and O'Neill et al., 1992), seems to be a major regulator in transcription. The transcription mechanism in eukaryotic cells has been extensively investigated in the last few years, and these studies, using both *in vitro* and *in vivo* systems, have given rise to somewhat conflicting results (see van Holde et al., 1992, Garrard, 1992 and Felsenfeld, 1992 for reviews). Some experiments seem to indicate that the efficiency of transcription is decreased by the presence of nucleosomes, whereas in other cases the conclusion is that the rate of transcription is unaffected. The differences between *in vitro* and *in vivo* systems as well as the different methods used in those experiments might explain part of the discrepancies observed. For example, a critical step in the transcription process is initiation; the sequence of events during the formation or the reconstitution of chromatin templates seems to be crucial for the generation of chromatin competent for transcriptional initiation (Wasylyk and Chambon, 1979, Batson et al., 1992). In order to obtain maximum transcriptional activity, the transcription complex has to be formed onto the core promoter region *before* nucleosome reconstitution. The presence of nucleosomes on the RNA polymerase promoter region will, at least, partially inhibit the formation of a proper transcription complex (Grunstein, 1990, Alzoumi et al., 1990, Morse, 1992). Insofar as elongation is concerned, various RNA polymerases, such as *Escherichia coli*, bacteriophage SP6, T7 and eukaryotic RNA

polymerases II and III, have been studied and the general conclusion is that the transcription process can occur through short stretches of nucleosomes although sometimes with very low efficiency (Wasylyk and Chambon, 1979, Lorch et al, 1987, Losa and Brown, 1987, Morse, 1989, Felts et al., 1990, Izban and Luse, 1991, Batson et al.,1992, , Kirov et al., 1992 and O'Neill et al., 1992).

In order to investigate this problem using a defined system incorporating only eukaryotic components, we have constructed a plasmid called pPol I 208-4, which contains a core promoter region for the RNA polymerase I of *Acanthamoeba castellanii* inserted upstream of a stretch of 4 repeats of the 5S rRNA gene of *Lytechinus variegatus* (see chapter 2). The 5S DNA has been shown to display a nucleosome positioning sequence which has been thoroughly defined. The positioning sequence specifies one clearly dominant position plus several minor positions spaced 10 bp apart.

One interesting aspect of the system, as demonstrated in our earlier studies, is that we could obtain either "randomly" or properly positioned nucleosomes on the four tandemly repeated 5S rDNA positioning sequences depending whether or not transcription factors and RNA polymerase I were preincubated with the DNA before the reconstitution with polyglutamate. The positioning was investigated by restriction digestions and shown to be, as expected, one histone octamer per 5S rDNA sequence (see chapter 2), when the DNA plasmid was preincubated with transcription factors and RNA polymerase I prior to polyglutamate reconstitution. On the other hand if the histones were added first, there was strong reconstitution

onto the promoter fragment, which apparently disrupted the phasing of nucleosomes on DNA downstream from that region.

In the present study, the reconstituted pPol I 208-4 plasmid DNA has been used to investigate the elongation process by RNA polymerase I on the chromatin-like template containing positioned nucleosomes as compared to transcription on free plasmid and on unproperly positioned pPol I 208-4 plasmid DNA.

c) Materials and methods

1) Preparation of histone octamers

Octamers were made either from long chromatin or alternatively, if core particles were available, the octamers were made directly from the particles. Long chromatin was digested for 20 minutes with micrococcal nuclease in the nuclei, based on the method of Simon and Felsenfeld (1979.). After the chromatin was stripped of H1, H5, and HMGs, and dialyzed vs 10 mM Tris pH 8.0, 1 mM EDTA, it was concentrated in an Amicon stirred cell with an XM 50 membrane, and then further concentrated in a Centricon 30 down to 1000 A_{260} units per 1.8 ml. An equal volume of 4.4 M NaCl, 0.2 M KPO_4 pH 6.7 was added to the chromatin and it was loaded onto a hydroxylapatite column equilibrated with 2.2 M NaCl, 0.1 M KPO_4 pH 6.7. Absorbance at 230 nm (A_{230}) monitoring indicated where peak fractions of histones eluted, and their quality was checked by electrophoresis on a Laemmli 15% SDS polyacrylamide gel. The first few fractions from the peaks gave the best integrity and were stored on ice at 4 °C.

The core particles were treated in a similar way to remove the DNA, and the octamers so obtained were also used in some reconstitution experiments.

2) Purification of the transcription factors and RNA polymerase I

RNA polymerase I was purified by a modification of the method of Iida and Paule (1992). A 1.6 M to 3.0 M ammonium sulfate fraction from a nuclear extract of *Acanthamoeba castellanii* (Zwick et al., 1991) was used as starting material. This was dialyzed down to 100 mM KCl in buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 mM phenylmethane sulfonyl fluoride [PMSF]) and loaded onto a 11 x 1.5 cm BioRex 70 column in place of a phosphocellulose column, and the step-eluted fraction between 450 and 650 mM KCl was collected. The DE52 column was step-eluted (75 to 250 mM fractions) and the heparin-Sepharose column was likewise step-eluted (300 to 500 mM KCl) instead of running gradients. The TIF-IB/aUBF fraction was obtained from the 0.5 M to 1.6 M ammonium sulfate fraction of the nuclear extract, which was chromatographed through 14 x 1.5 cm DEAE fast flow (Pharmacia) by loading it at 75 mM KCl in buffer A and, after a wash in the same buffer, eluting with a linear gradient of KCl in buffer A from 75 mM to 500 mM. The TIF-IB/aUBF-containing fractions (at approximately 300 mM KCl) were pooled, diluted to 150 mM KCl and chromatographed through a 9.5 x 0.9 cm BioRex 70 column using a KCl gradient from 150 to 900 mM in buffer A. The fractions containing TIF-IB and aUBF, eluted at approximately 430

mM KCl, were dialyzed down to 100 mM KCl in buffer A and stored at -70 °C.

3) Reconstitution of histone octamers onto the plasmid

To study the importance of the proper positioning on the 5 S rDNA, the system was first reconstituted in absence of transcription factors and RNA polymerase I. The reconstitution was carried out using histone octamers purified from chicken erythrocytes as described above. Twenty-five µg of plasmid DNA were first relaxed with 0.6 units of topoisomerase I (BRL) per µg of DNA for 90 min at 37 °C. The 2.2 M Na Cl concentration of the octamer solution was reduced to 150 mM Na Cl by diluting in TE; the octamers were then incubated at room temperature for 60 min in presence of a 10 mg/ml solution of polyglutamic acid at a ratio PGA:histone of 2:1 (w/w). The relaxed plasmid DNA was then added to the mixture. The final DNA concentration was 0.05 mg/ml. The optimal input ratio of histone to DNA was defined to be R=2.05 (gm histone/gm DNA). The 500 µl reaction mixture was reconstituted at 37 °C, overnight, under constant shaking to avoid aggregation and precipitation. The reaction mixture was centrifuged on a IEC centra-M centrifuge at top speed for 5 minutes to verify that no material had aggregated.

To generate a system with maximized potential for transcriptional activity another procedure was used. The appropriate amount of partially purified transcription initiation factor TIF-IB, upstream binding factor (aUBF) and RNA polymerase I were incubated for 15 minutes at 25 °C, in 500 µl final volume, in the presence of 12.5 µg of pPol I 208-4 and 0.5 mM each of ATP and GTP, *before reconstitution with histone octamers.* The transcription

complex will bind to the promoter region, start to transcribe and stall at position +8, making the complex more stable and less likely to fall off the DNA template. The pre-incubated complex was then reconstituted with histone octamers, according to the method of Retief et al.(1984), which was modified according to the requirements of our system. Polyglutamic acid (PGA) was used as a carrier for the deposition of the histones onto the circular template. The salt concentration of the medium was kept at 150 mM of monovalent cations in order to prevent displacement of the transcription factors.

4) Control of the formation of nucleoprotein complexes.

The reconstituted plasmid DNA, preincubated with transcription factors and RNA pol I was checked for the presence of free DNA by gel electrophoresis. Four hundred nanograms of reconstituted material were electrophoresed in a 0.8% agarose gel in 0.5 x e-buffer for 3 hours at 40 volts (see Figure 3. 1). The gel showed a band shifted upward (compared to the free DNA), characteristic of reconstituted pPol I 208-4. No uncomplexed DNA was detectable (lane 2). Lanes 3 to 8 correspond to a step dilution of the free plasmid DNA, from 0.5 µg down to 15 ng.

The same material was chromatographed by HPLC using a C8 column (see Figure 2. 9). No free DNA was detectable by these method. Previous reconstitutions using plasmid DNA treated under the same conditions, were analyzed by ultra centrifugation techniques, showing the presence of homogeneous complexes, but no trace of free DNA in the samples.

The positioning of the nucleosomes onto the tandemly repeated 5S rDNA sequences was checked by restriction endonucleases digestions (*AvaI* and *EcoRI*), showing 85 to 100% availability of the previously cited restriction sites. On the other hand the plasmid reconstituted without TIF-IB, aUBF and RNA pol I showed no regular positioning on the 5S sequences (see chapter 2).

5) Transcription: analysis by primer extension

The reconstituted chromatin (pPol I 208-4 plasmid DNA), after pre-incubation with transcription factors and pol I, was provided with the missing nucleotides CTP and UTP and transcription was allowed to proceed for 30 min at 25 °C. The RNA products were analyzed by primer extension. The 21 mer primer used for the extension starts 68 base pairs downstream from the first AvaI site of the 5S gene, and has the sequence 5'CGGTGATCGGACGAGAACCGG3'. To reverse transcribe the 5S RNA products, which display strong secondary structure, retrotherm reverse transcriptase (Epicentre) was used at 75 °C. The DNA was labeled using [α^{32}P] dATP. Fifty ng of reconstituted pPol I 208-4 plasmid DNA were transcribed in 2.5 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 500 µM final of each NTP's in presence of actinomycine D and RNasin.

The primer extension was carried out using the buffer provided by Epicentre for the retrotherm reverse transcriptase. DTT and dNTP's were provided to, respectively, the final concentration of 20 mM and 2.5 mM in presence of [α^{32}P] dATP (10 mCi/mol). The molar ratio of primer to pPol I 208-4 plasmid DNA was varied from 0.05 up to 0.5.

6) Analysis of elongation

The time course of the elongation was monitored by determining the percentage of incorporation of the total [α^{32}P] UTP input in the nascent RNA species. The assay was performed in a final volume of 120 µl, using 2 µg of each DNA template, in 15 mM Tris HCl pH7.5, 150 mM Na Cl, 4 mM $MgCl_2$, 0.8 mM of ATP, CTP, GTP, 0.2 mM UTP, 1 unit of RNase inhibitor per µg of DNA and 5 µCi of [α^{32}P] UTP. The course of transcription was determined for the following templates: (1) naked pPol I 208-4 plasmid DNA, (2) PGA reconstituted pPol I 208-4 plasmid DNA at a ratio of histone to DNA of R=2.05, with polymerase and factors added subsequently -see below- (referred to as Rec 2.05) and (3) pPol I 208-4 plasmid DNA preincubated with transcription factors and RNA polymerase I and then reconstituted using the PGA protocol at R=2.05 (this reconstitute was named Rec TIF pol I).

The naked plasmid DNA and the "pPol I 208-4 Rec 2.05" were preincubated with TIF-IB, aUBF and RNA pol I in presence of 0.5 mM of ATP and GTP for 30 minutes at room temperature, prior to adding the transcription mix. Time points were taken between 30 seconds and 4 hours (see Figures 3. 2. 1 and 3. 2. 2). The reactions were stopped by making the mixture 25 mM EDTA and by immediately putting it on ice. Aliquots of 5 µl were taken for each time point. The aliquots were phenol/ chloroform extracted, ethanol precipitated, and then resuspended in 15 µl of loading buffer. The products of transcription were electrophoresed in a 4% acrylamide denaturing gel in 0.5X e-buffer at 150 volts for 2 to 3 hours. The gel was then exposed to X-AR Kodak film. To measure the incorporation of [α^{32}P] UTP versus time, the gels were scanned on a flat bed scanner and

integrated by use of the program Image (NIH software). The values were normalized with 100% representing the efficiency of incorporation of [α^{32}P] UTP using the free DNA template for transcription after 240 minutes of incubation.

7) Positioning of the nucleosomes before and after transcription.

The DNA was reconstituted under the same conditions as described previously, including the preincubation with transcription factors and RNA polymerase I. Transcription was performed as described in the preceding paragraph, with 0.8 mM of each nucleotides and no labeled UTP. The reconstitute was incubated (allowing transcription) at room temperature for 1 hour. The positioning was monitored by *EcoRI* restriction digestion, as described previously.

d) Results

1) Transcription efficiency is decreased by the presence of nucleosomes on the pol I promoter region.

The efficiency of transcription for the 3 different samples was assessed by plotting the normalized intensities (aftre integration of the scanns of the autoradiograms) versus time (see Figures 3. 2. 1 and 3. 2. 2). Both naked DNA template and "Rec TIF pol I" showed a very similar efficiency of incorporation, while "Rec 2.05", reconstituted with histone octamers before polymerase I and the transcription factors were added, displayed a much lower efficiency of transcription and levelled out much earlier. The incorporation of ^{32}P between 30 sec and 60 minutes goes up to 85% +/- 5% of the maximum value obtained using the free DNA as template for

transcription, for both the naked plasmid DNA and the "Rec TIF pol I".

If we analyze in more detail the data obtained using "Rec TIF pol I" and the naked plasmid DNA as templates for transcription, we see very little difference in the progress of transcription on the two templates. The presence of properly positioned nucleosomes seemed neither to inhibit nor to even significantly slow down the elongation. For all three saples transcribed, the efficiency of incorporation seems to be in thesame range during the first 10 minutes. In other words, the transcription complexes appear to behave in a similar manner immediately after initiation. It indicates that at least some TIF-IB and aUBF plus RNA pol I were able to bind to the core promoter region and initiate transcription even in the case of "Rec 2.05".

2) Products of transcription

A direct examination of the transcription products was conducted by gel electrophoresis analysis. An overexposed gel (Figure 3. 3. 1) demonstrates that a band at about 220 nucleotides is barely visible when "Rec 2.05" is transcribed, but is present in the case of free pPol I 208-4 plasmid DNA and is significantly enhanced when "Rec TIF-pol I" is used as transcription template.

In the case of the "Rec 2.05", the size of the RNA products indicated no real pattern, and only very short products, on the order of 100 nucleotides or less, were obtained (see Figure 3. 3. 2, lanes 11 to 15 gel #1 and lanes 2 to 6 gel#2). In contrast, the gels displaying transcripts of the free DNA and the "Rec TIF pol I" templates showed an increase of both the concentration and the length of the transcripts with time. Banding patterns are recorded for the naked

plasmid DNA (Figure 3. 3. 2, lane 7 to 16 gel#2) and the "Rec TIF pol I" (Figure 3. 3. 2, lane 2 to 10 gel#1). More distinct bands are visible in the case of the chromatin-like template (lanes 6 to 10 gel#1). The periodicity seems to indicate either pausing or the presence of a termination signal at the end of each nucleosome positioning sequence (band at 213 +/- 10 bp and at 390 +/- 20 bp). The data indicate that a pausing signal resides in the DNA sequence itself but is apparently amplified by the changes generated in the DNA structure by the binding of nucleosomes.

The band seen at about 93 (+/- 5) nucleotides could correspond to a pause at the site of strong bending (position 40 to 45) of the DNA on the nucleosome (see map Figure 3. 4. 2). It has been observed before that the RNA polymerase will pause after or during the process of transcribing through a nucleosome (O' Neill et al., 1992, Izban and Luse, 1991). The pausing we observe seems to be of different nature, more related to the direct interaction between the DNA and the polymerase rather than to the interaction between the nucleosome and the polymerase. It is conceivable that the bend is directly involved in the pausing process. Additional discrete shorter bands are also visible in the case of naked DNA.

3) Primer extension and position of the transcripts with regard to the nucleosome structure.

The primer extension technique used to analyse the transcripts obtained from a 30 minute incubation of the plasmid generated fragments of DNA of 58, 63, 94, 102, 115, 138, and 217 base pairs (see Figure 3. 4. 1). The strongest signal was detected at 94 bp and is consistent with the RNA fragment of about 93 nucleotides previously

described. Position 94 lies close to the dyad axis of the first nucleosome and close to the bending of the DNA (see map of interactions, Figure 3. 4. 2). It is one more piece of evidence for a pausing signal within the positioning sequence. One should expect to see another pause at position 94 + 208, but the conditions under which the extension was performed did not permit the appearance of well defined bands longer than 217 bp. The 217 bp fragment is much fainter than the shorter ones.

The positioning sequence is repeated four times and therefore the primer can hybridize at 4 different loci (one per 5S rDNA monomer). The presence of double stranded RNA/DNA complex will present an obstacle for the polymerase and will hinder the extension process. At ratios of 0.1 to 0.25, the RNA loci seem not to be saturated, therefore, extension may proceed through more than one positioning unit. However the efficiency at these low ratios would be diminished. At a primer to plasmid molar ratio of 0.1, a 400 bp fragment begins to be apparent but is very faint. At higher ratios the shorter bands become the major component of the primer extension. When the ratio of primer to plasmid is greater than 0.1, the number of primer molecules bound to the 5S rRNA increases. When the DNA polymerase encounters such DNA/RNA hybrid molecules, it will stop extending. At a ratio of 0.5 statistically every other site is occupied, therefore preventing the generation of DNA fragments longer than about 400 bp.

Another potential explanation for the presence of short fragments is the high level of secondary structure of the 5S RNA. Even though a thermostable polymerase was used, the temperature

at which the reaction is run may have to be raised in order to get extension of the longer transcripts. Melting point calculations performed using the PC gene melting temperature algorithm indicate that 75 °C should melt a RNA of the overall base composition of the 5S rRNA. However, this does not exclude the possibility of local region of higher melting temperature, which could block the polymerase.

4) The positioning of the nucleosomes is unchanged by transcription.

The efficiency of formation of nucleosome structure on the positioning sequence was tested by a gel shift assay. The "Rec TIF-pol I" was digested with *Eco R I*. The products of digestion were electrophoresed in a 1% agarose and 0.5% NuSieve gel to test for the presence of free 5S rDNA, comigrating with the 220 base pair fragment from the λ phage DNA *BstEII* marker (Figure 3. 5). The band corresponding to the reconstituted 196 bp fragment complexed with histones is shifted upward in the gel, so any unoccupied sites would appear at the 196 bp position. No free 196 bp fragment was detectable, showing that all the positioning sequences were present in a nucleosomal form (see lane 6, Figure 3. 5).

Both before and after transcription at room temperature for 1 hour, aliquots of the "Rec TIF-Pol I" were submitted to *EcoRI* restriction digestion as above and electrophoresed in a 0.8% agarose gel. The restriction patterns obtained were scanned (see Figure 3. 6). The profiles of the scans indicate the presence of about 10% of incompletely digested pPol I 208-4 plasmid DNA (see shoulders on the downside of the highest peaks on the two profiles to the right side of Figure 3. 6). Incomplete digestion is most likely a

consequence of the presence of nucleosomes at minor positioning sites interfering with *EcoRI* digestion. The percentage of 196 bp fragments is virtually identical in the two cases (before and after passage of the transcription complex). The difference in absolute size of the corresponding peaks is simply due to different amounts of material loaded.

e) Discussion.

Figures 3. 2. 1 and 3. 2. 2 show that the plasmid pPol I 208-4 can be transcribed by RNA polymerase I using a reconstituted chromatin template, with nearly the same efficiency as is a free pPol I 208-4 DNA template. The similarity in the two cases was at first sight a little surprising. One would expect to see a lower efficiency of transcription in the case of the chromatin template, due to the presence of nucleosomes in the path of the RNA pol I. Indeed, such behavior has been reported in a number of different systems, including T7 RNA polymerase (Kirov et al., 1992), RNA pol II (Izban and Luze, 1991, Becker and Wu, 1992 and O'Neill and al., 1992) and RNA polymerase III (Morse, 1989). On the other hand behavior similar to that found here has been observed with SP6 RNA polymerase and RNA pol II (Lorch et al., 1987, Losa and Brown, 1987).

Monitoring the incorporation of [α^{32}P] UTP in the transcription products by the mispositioned plasmid (Rec 2.05), demonstrated that the efficiency of the transcription reaction was reduced when nucleosomes were mispositioned on the DNA template, a situation that could include octamers bound next to or on the core promoter

region. Under such conditions, the efficiency of transcription dropped to about half of that observed with free DNA. The RNA polymerase I needs the presence of the transcription factors on the promoter region to be able to bind and therefore to initiate transcription. From the low efficiency of elongation observed with the DNA template "Rec 2.05", it is obvious that the presence of histone octamers mispositioned on the insert containing the core promoter region and the four tandem repeats of the 5S rDNA seriously impedes transcription. In previous experiments, restriction analysis of Rec 2.05 template showed that 70 to 90% of the time, the *Xbal* site (forming the boundary of the 5' end of the RNA polymerase I core promoter region) was protected by a histone octamer (see chapter 2). In addition, a band shift assay performed on a mixture of 3 fragments generated by *PvuII* and *Xbal* digestion of pPol I 208-4 plasmid DNA indicated that the affinity of the histone octamers for the core promoter region was comparable to that observed for the 5S rDNA positioning sequence. In such a situation, when reconstitution is carried out before the polymerase and transcription factors have been fixed in place, the likelihood of a nucleosome covering the promoter region is very high. If the promoter region is in a nucleosomal form, the transcription factors will not be able to bind to their target sequence and therefore will not direct the deposition of the RNA pol I at its specific site.

The difference in the subsequent progress of radioisotopes incorporation could result from the inability for the "Rec 2.05" template to reinitiate transcription after a few cycles. The amount of RNA generated by the "Rec 2.05" might be related to the percentage

of promoter region free of nucleosomes, therefore available for TIF to bind. Also, when we examine the size of the transcripts for the "Rec 2.05", we note that only very short products can be observed.

A second element that might also interfere with an efficient transcription of the "Rec 2.05" template is the presence of closely packed nucleosomes along the DNA on the region downstream from the promoter. Although we know little about the interaction of polymerase with nucleosomes, it seems likely that the topological constraints in such a situation will slow down the elongation, even to the point of stopping it.

The free DNA and the "Rec TIF-Pol I" DNA templates display very similar patterns in terms of incorporation of radioactive tracer. The efficiencies of elongation observed with free DNA and "Rec TIF pol I" remain virtually identical for the next 4 hours. The small difference in efficiency of transcription in favor of the "Rec TIF-Pol I" is within experimental error.

The gel patterns (Fig 3. 3. 1) show a strong band at about 220 nucleotides indicative of the presence of transcripts of defined length in the free DNA and the "Rec TIF-Pol I" lanes, but in larger quantity in the case of "Rec TIF-Pol I". This could indicate the presence of a pausing signal for the RNA polymerase I after the transcription of the first 5S rDNA positioning sequence (confirmed by the band at 217 in the primer extension experiment). The difference in quantity could be thought of as a function of nucleosome occupancy. When nucleosomes are present in the path of the transcription complex, then the pausing effect might be enhanced. The pausing effect at 220 bp was not observed with the "Rec 2.05" DNA template with its

apparently randomly positioned nucleosomes. A possible explanation is that random "noise" in pausing produced by randomly located nucleosomes masks the inherent pausing signal.

The absence of change in the nucleosome pattern (Fig 3. 6) observed after passage of the transcription complex can be interpreted in several ways. First it could mean that the histone octamers are not displaced by polymerase passage. For example, the nucleosomes might be temporarily unfolded by the transcription bubble. A second alternative is that only H2A and H2B are released during polymerase passage, and these then rebind (van Holde et al., 1992). Finally, it is possible that each octamer sitting on a positioning sequence is displaced and then returns to its original location. Recent results by Clark and Felsenfeld (1992) indicated that a nucleosome was transferred from the polymerase path to a different locus on the same plasmid. In our system, it would be difficult to record such a phenomenon since the plasmid is fully reconstituted, leading one to wonder if there is enough free DNA in a fully reconsituted system for such a mechanism to occur, unless transfer is onto an existing nucleosome.

From previous observations of histone affinity for the RNA polymerase I promoter region, we might expect the first histone octamer to bind to the promoter region if displaced (or transferred) from another location during the transcription. The promoter region is located immediately upstream from the four tandemly repeated 5S rDNA sequences and was reported to efficiently compete with the 5S rDNA for the formation of a nucleosome structure (see chapter 2). Such a complex on the promoter region would block the availability

for the first *EcoRI* site next to the major position and subsequently modify the restriction digestion pattern in a manner not observed (see Figure 3. 6).

From the electrophoresis data, the ways nucleosomes are dealt with (at least for our *in vitro* system) by RNA polymerase I appear to be different from what we would have expected. It may be possible that one or two of the transcription factors remain attached to the promoter region and consequently prevent the formation of a nucleosome at that spot. It is also still conceivable that the histone octamer does not move as one unit but is only partially unfolded, keeping some contact with the DNA molecule, as proposed by van Holde et al (1992) (see Figure 3. 7).

Figure 3. 1

Formation of nucleoprotein complex

The reconstituted material was electrophoresed in a 1% agarose gel.

Lane 1: Lambda phage DNA digested with *BstEII*

Lane 2: Reconstituted pPol I 208-4 plasmid DNA + TIF-IB, aUBF and RNA pol I (0.4 μg).

Lane 3: pPol I 208-4 plasmid DNA (0.5 μg).

Lane 4: Same as lane 3, only 0.25 μg of plasmid DNA.

Lane 5: Same as lane 3, only 0.125 μg of plasmid DNA.

Lane 6: Same as lane 3, only 0.0625 μg of plasmid DNA.

Lane 7: Same as lane 3, only 0.0312 μg of plasmid DNA.

Lane 8: Same as lane 3, only 0.0156 μg of plasmid DNA.

Lane 2 contains aggregated material (top of the well)

Figure 3. 1

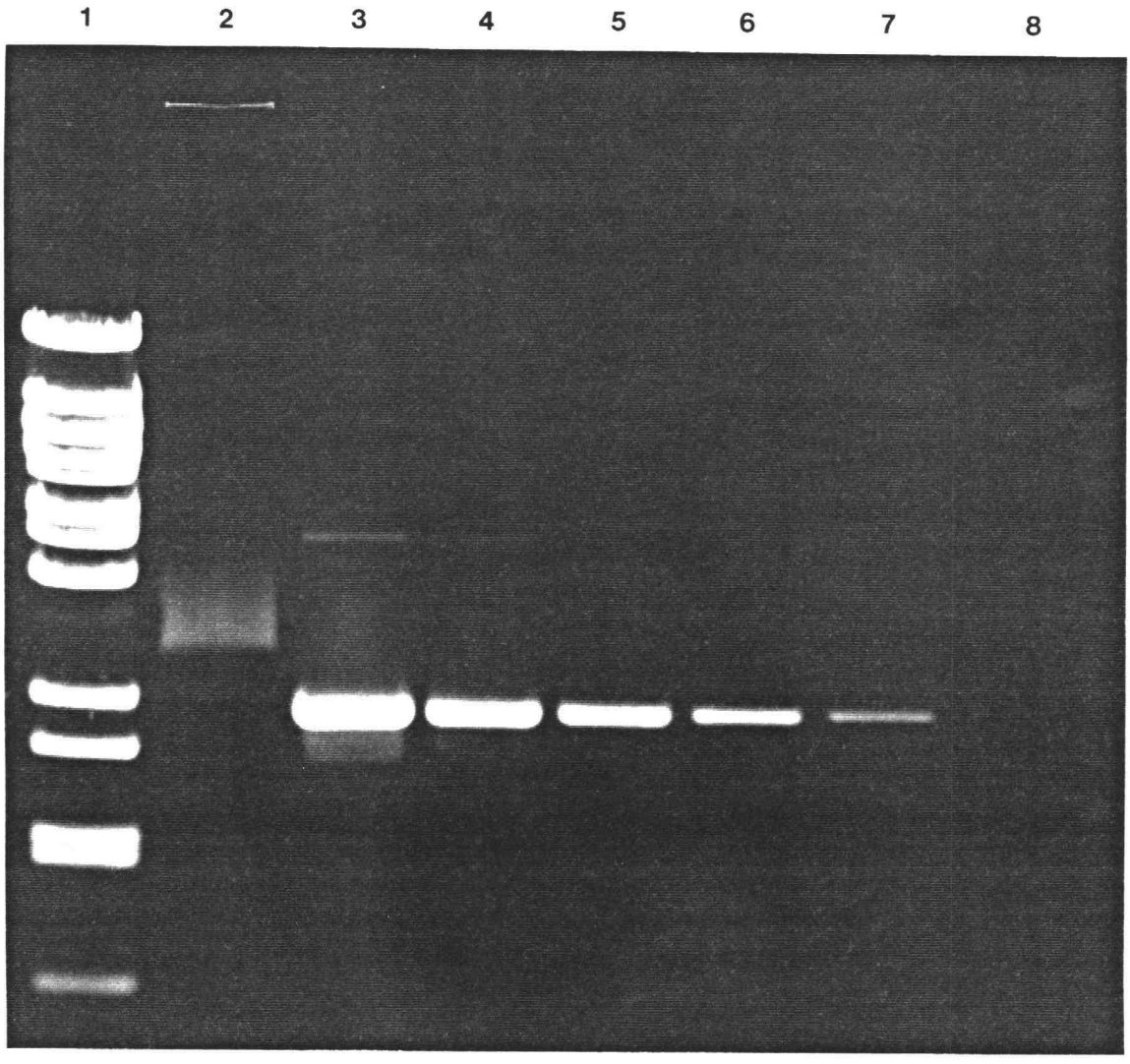


Figure 3. 2

Time course of the percentage of [α^{32}P] UTP incorporation into the transcripts.

1) The autoradiograms (Fig 3. 3) were scanned and integrated and then normalized as explained. The percentages of incorporation (normalized intensities) were plotted versus time for the 3 templates tested.

2) Initial part of the plot of elongation efficiency were dealt with by looking only at the first 60 minutes of transcription.

Figure 3. 2. 1

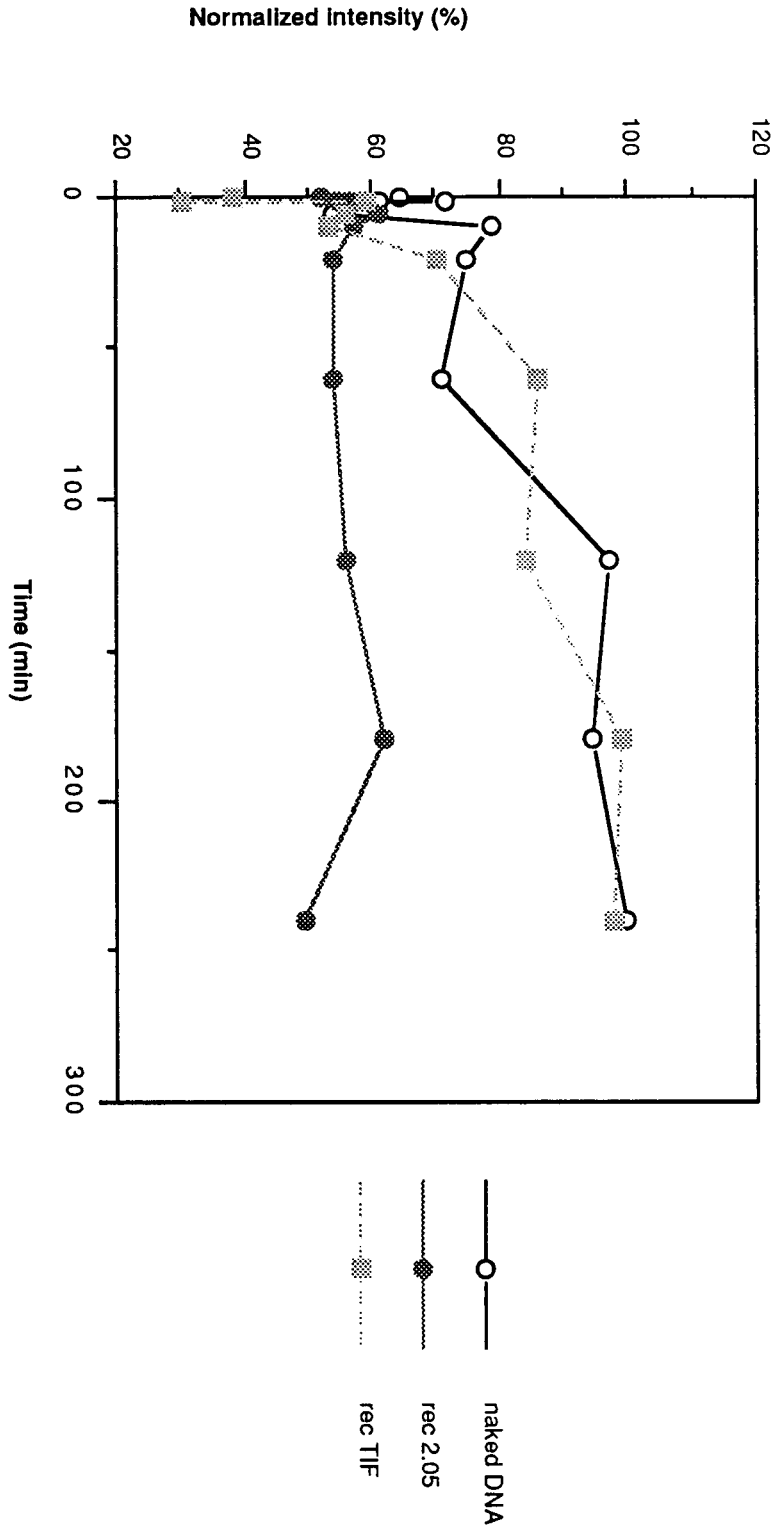

Normalized intensity (%)
20
40
60
80
100
120
0
100
200
300
Time (min)
naked DNA
rec 2.05
rec TIF

Figure 3. 2. 2

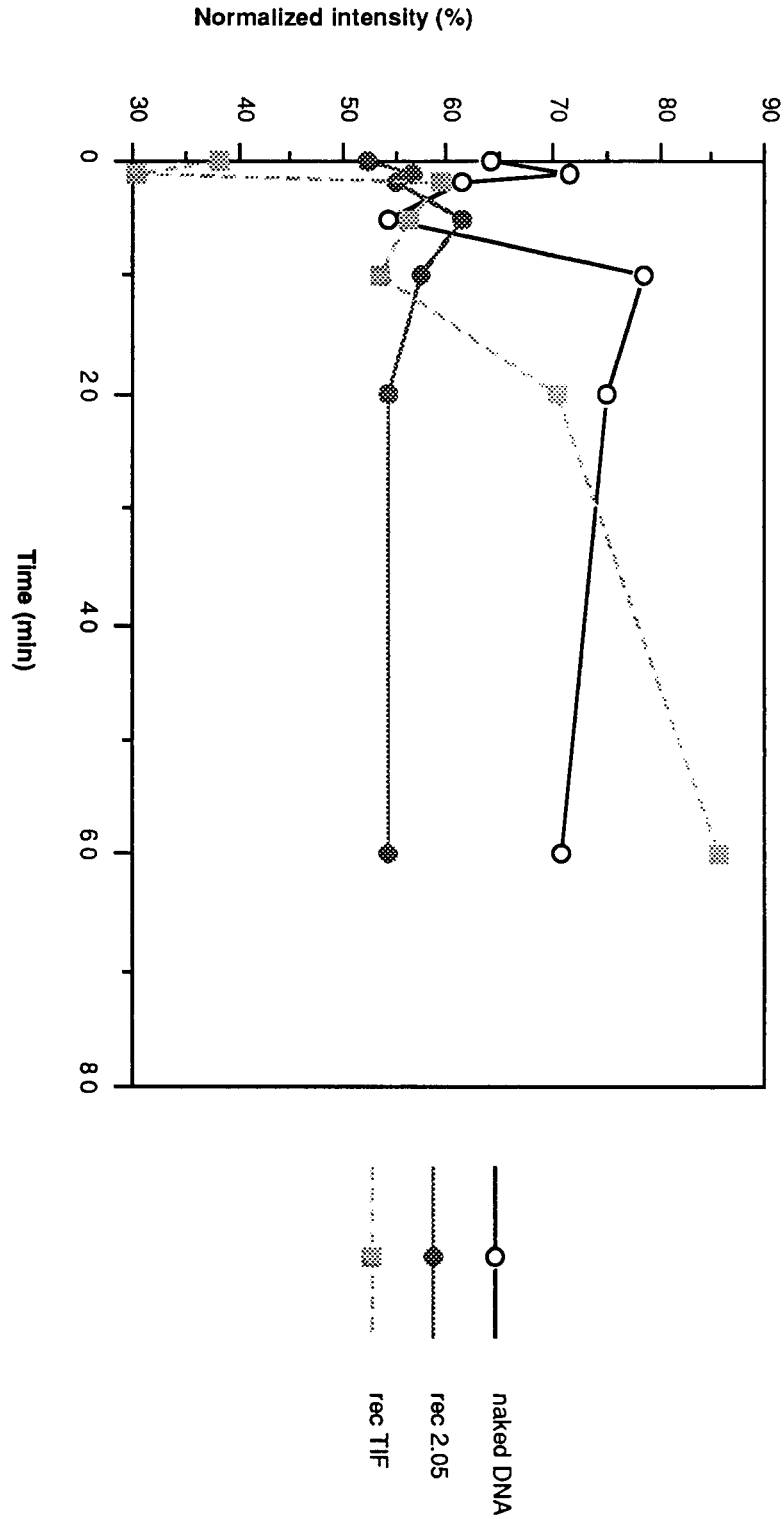

Normalized intensity (%)
30
40
50
60
70
80
90
0
20
40
60
80
Time (min)
naked DNA
rec 2.05
rec TIF

Figure 3. 3

1) Overexposed autoradiogram of a 120 minute time-course of transcription

Lane 1: Transcription products of pPol I 208-4 plasmid DNA incubated with histone at a ratio of histone/DNA=2.05 after a time of 2 min.

Lane 2: same as lane 1, time: 5 min.

Lane 3: same as lane 2, time: 10 min.

Lane 4: same as lane 3, time: 20 min.

Lane 5: same as lane 4, time: 60 min.

Lane 6: same as lane 5, time: 120 min.

Lane 7: Transcription products of naked pPol I 208-4 plasmid DNA after a time of 2 min.

Lane 8: same as lane 7, time: 5 min.

Lane 9: same as lane 8, time: 10 min.

Lane 10: same as lane 9, time: 20 min.

Lane 11: same as lane 10, time: 60 min.

Lane 12: same as lane 11, time: 120 min.

Lane 13: Transcription products of pPol I 208-4 plasmid DNA preincubated with TIF-IB, aUBF and RNA pol I and then incubated with histones at a ratio histone/DNA =2.05 after a time of 2 min.

Lane 14: same as lane 13, time: 5 min.

Lane 15: same as lane 14, time: 10 min.

Lane 16: same as lane 15, time: 20 min.

Lane 17: same as lane 16, time: 60 min.

Lane 18: same as lane 17, time: 120 min.

2) Time course of transcription by RNA polymerase I.

The transcription material was electrophoresed in a 4% acrylamide/bis acrylamide denaturing gel.

Gel #1:

Lane 1: pPol I 208-4 plasmid DNA preincubated with TIF-IB, aUBF and RNA pol I and then incubated with histones at a ratio histone/DNA =2.05. Transcription products after 0 sec.

Lane 2: same as lane 1, time: 30 sec.

Lane 3: same as lane 1, time: 2 min.

Lane 4: same as lane 1, time: 5 min.

Lane 5: same as lane 1, time: 10 min.

Lane 6: same as lane 1, time: 20 min.

Lane 7: same as lane 1, time:60 min.

Lane 8: same as lane 1, time: 120 min.

Lane 9: same as lane 1, time: 180 min.

Lane 10: same as lane 1, time: 240 min.

Lane 11: pPol I 208-4 plasmid DNA incubated with histones at a ratio histone/DNA =2.05. Transcription products after 0 sec.

Lane 12: same as lane 11, time: 30 sec.

Lane 13: same as lane 11, time: 2 min.

Lane 14: same as lane 11, time: 5 min.

Lane 15: same as lane 11, time: 10 min.

Lane 16: Lambda phage DNA digested with *BstEII*.

Gel #2

Lane 1: Lambda phage DNA digested with *BstEII*.

Lane 2: pPol I 208-4 plasmid DNA incubated with histones at a ratio histone/DNA =2.05. Transcription products after 20 min.

Lane 3: same as lane 2, time: 60 min.

Lane 4: same as lane 2, time: 120 min.

Lane 5: same as lane 2, time: 180 min.

Lane 6: same as lane 2, time: 240 min.

Lane 7: Naked pPol I 208-4 plasmid DNA. Transcription products after 0 sec.

Lane 8: same as lane 7, time: 30 sec.

Lane 9: same as lane 7, time: 2 min.

Lane 10: same as lane 7, time: 5 min.

Lane 11: same as lane 7, time: 10 min.

Lane 12: same as lane 7, time: 20 min.

Lane 13: same as lane 7, time: 60 min.

Lane 14: same as lane 7, time: 120 min.

Lane 15: same as lane 7, time: 180 min.

Lane 16: same as lane 7, time: 240 min.

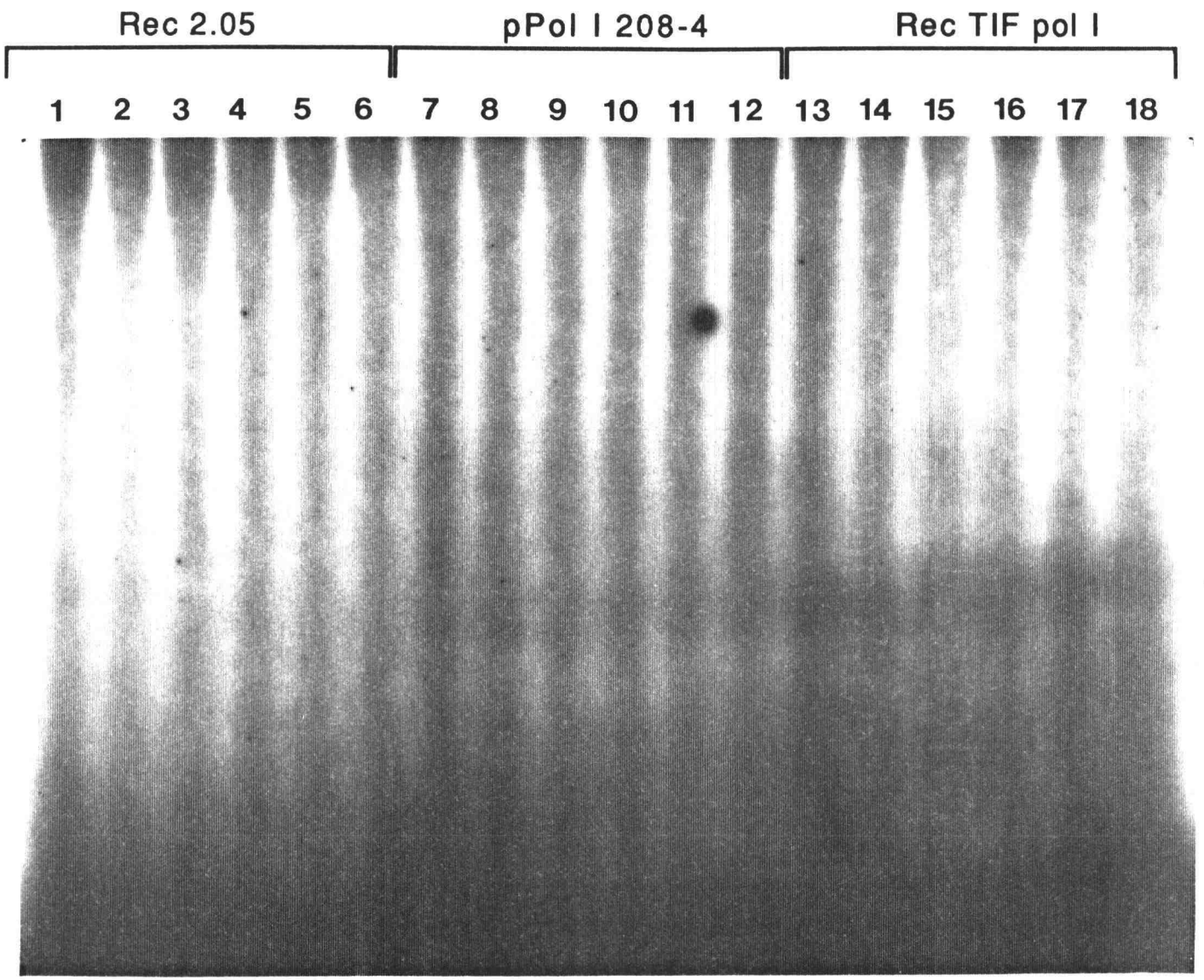

Rec 2.05
pPol I 208-4
Rec TIF pol I
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
224

Figure 3.3.1

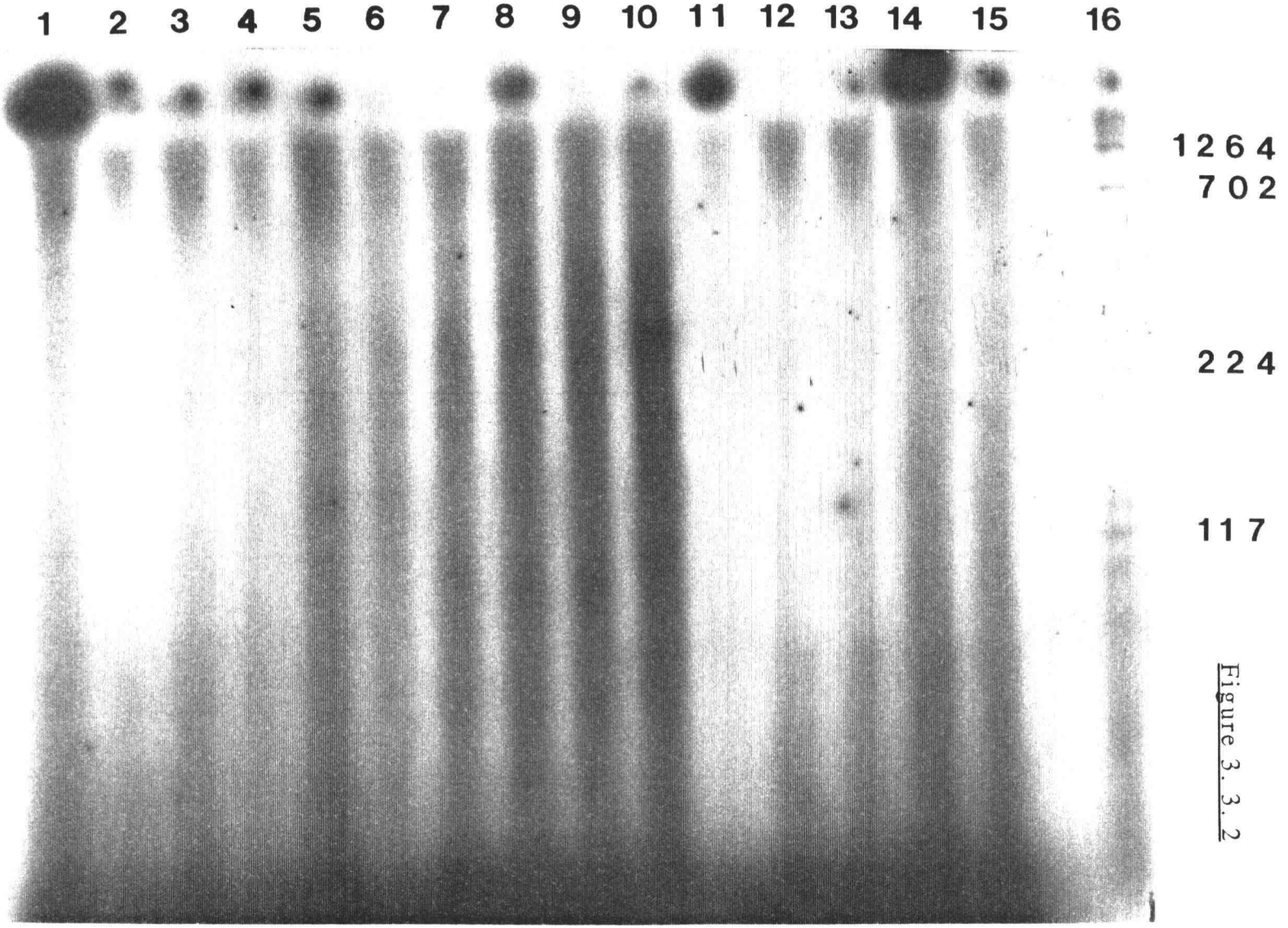


Figure 3.3.2

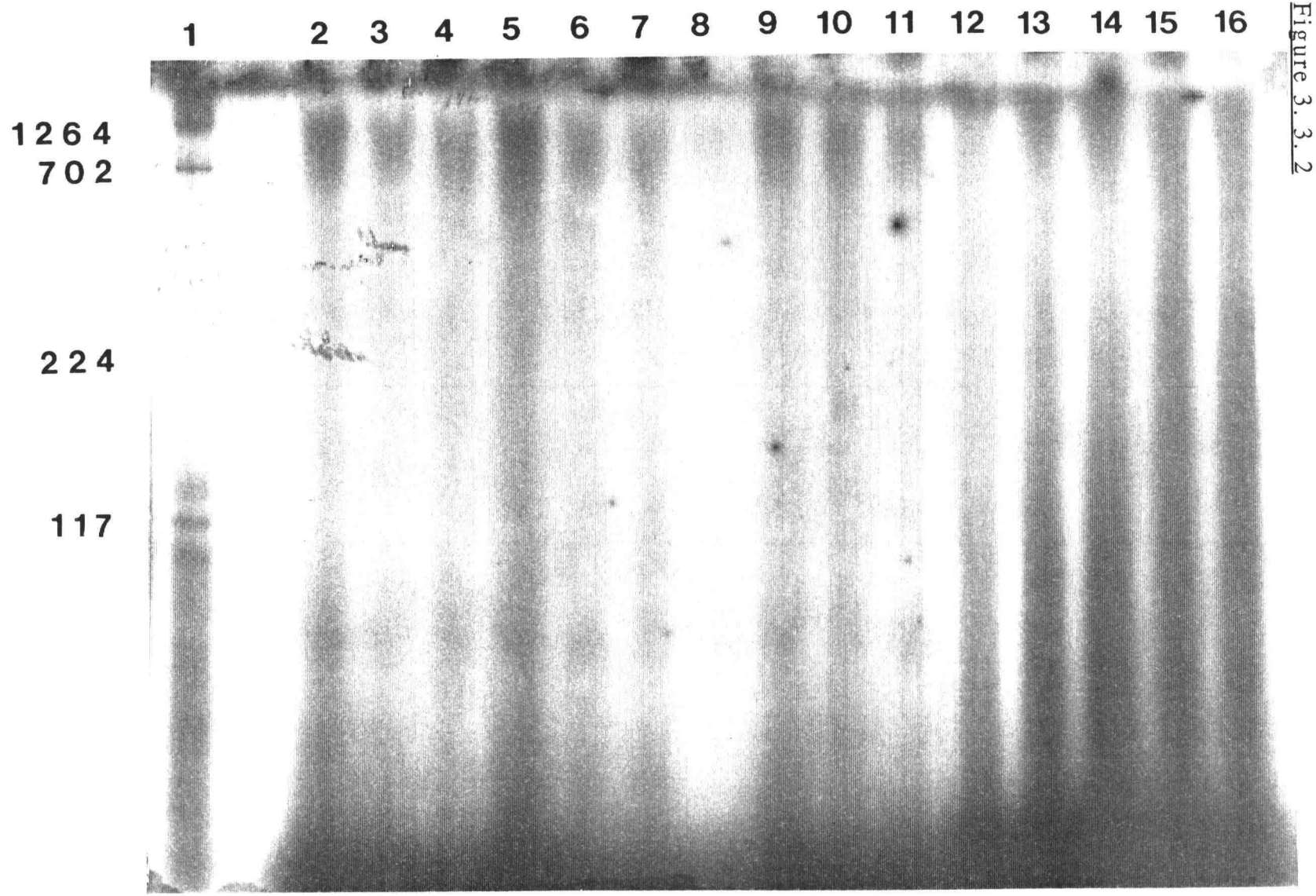


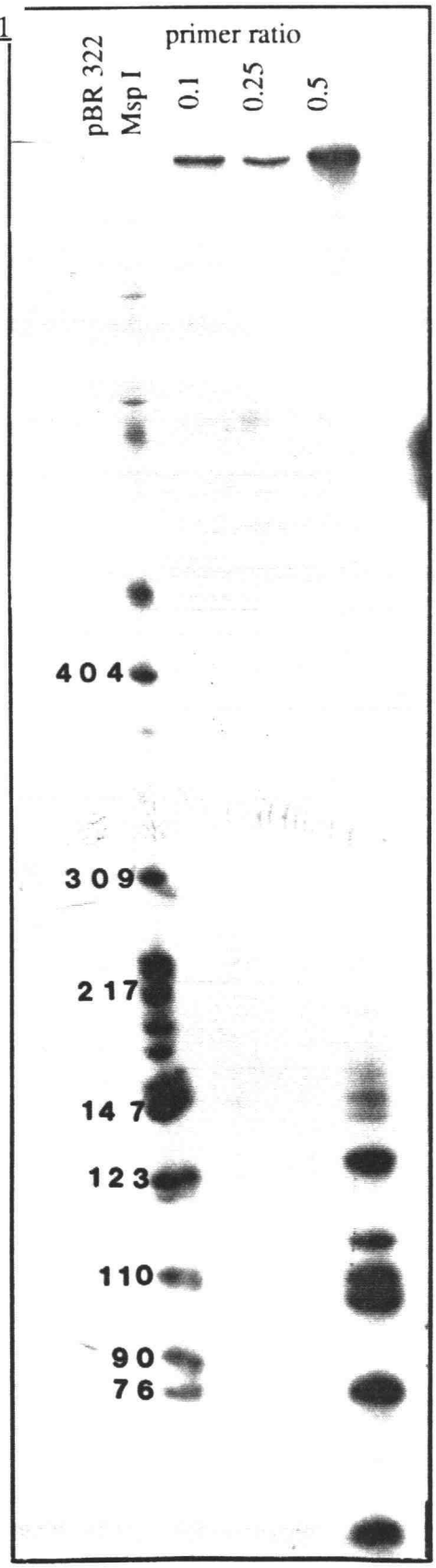
Figure 3.3.2

Figure 3. 4

1) Primer extension of the Rec TIF pol I 30 minute transcripts at increasing molar ratios of primer to plasmid (from 0.1 to 0.5). The products were electrophoresed in a 6% acrylamide denaturing gel.
The first lane contains pBR322 plasmid DNA digested with *MspI*.
Lane 2: Primer extension products at a molar ratio of 0.1 (primer to plasmid)
Lane 3: Primer extension products at a molar ratio of 0.25
Lane 4: Primer extension products at a molar ratio of 0.5

2) Superimposition of the 5S rDNA positioning sequence shows DNA/histone contacts from Mirzabekov and coworkers (Schick et al., 1980).

Figure 3. 4. 1



Primer extension of Rec TIF pol I

Figure 3. 4. 2

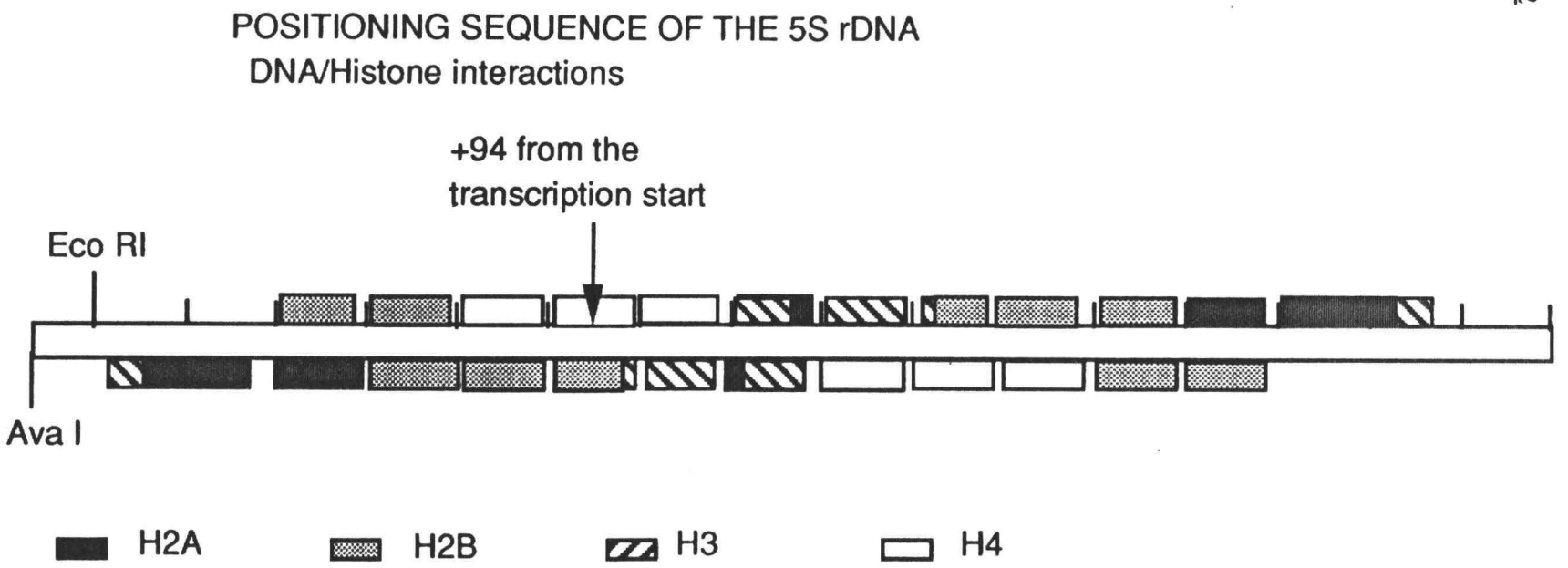

POSITIONING SEQUENCE OF THE 5S rDNA
DNA/Histone interactions
+94 from the
transcription start
Eco RI
Ava I
H2A
H2B
H3
H4

Figure 3. 5

EcoRI and *AvaI* restriction digestion of pPol I 208-4 plasmid DNA naked and reconstituted in presence of TIF and RNA pol I electrophoresed on a 1% agarose and 0.5% NuSieve

Lane 1: Lambda phage DNA digested with *BstEII*.

Lane 2: Naked circular pPol I 208-4 plasmid DNA

Lane 3: pPol I 208-4 + TIF + RNA pol I reconstituted

Lane 4: Naked pPol I 208-4 plasmid DNA digested with *EcoRI*

Lane 5: pPol I 208-4 plasmid DNA + TIF + RNA pol I reconstituted and then digested with *EcoRI* and extracted with phenol/ chloroform

Lane 6: pPol I 208-4 plasmid DNA + TIF + RNA pol I reconstituted and then digested with *EcoRI*

The arrow head indicates the shifted band corresponding to the histone/ 196 bp fragment complex.

Figure 3. 5

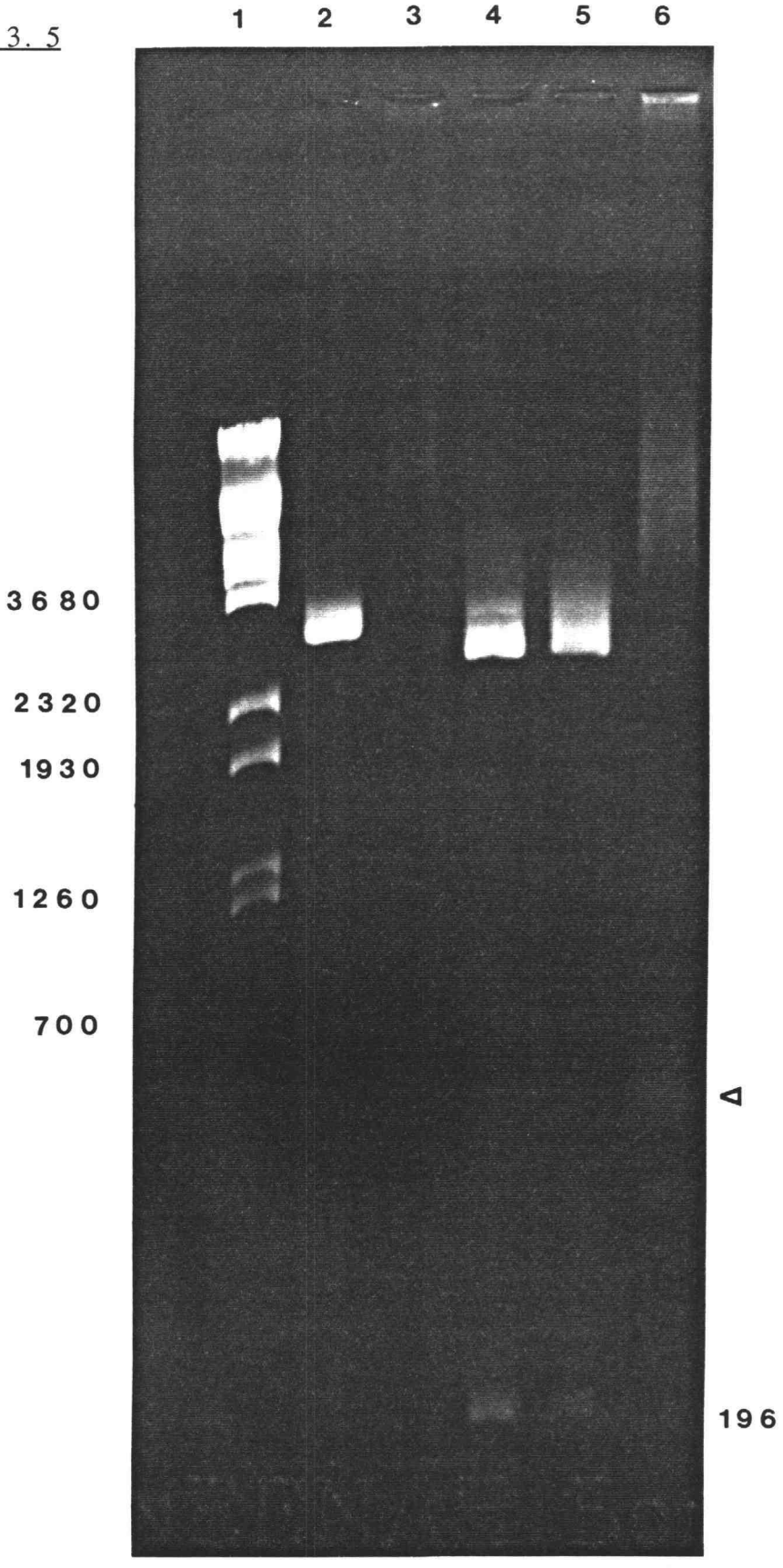


Figure 3. 6

Scans of the plasmid pPol I 208-4 digested with *EcoRI* and electrophoresed in a 0.8% agarose gel, before (top panel) and after (bottom panel) the passage of the transcription complex.

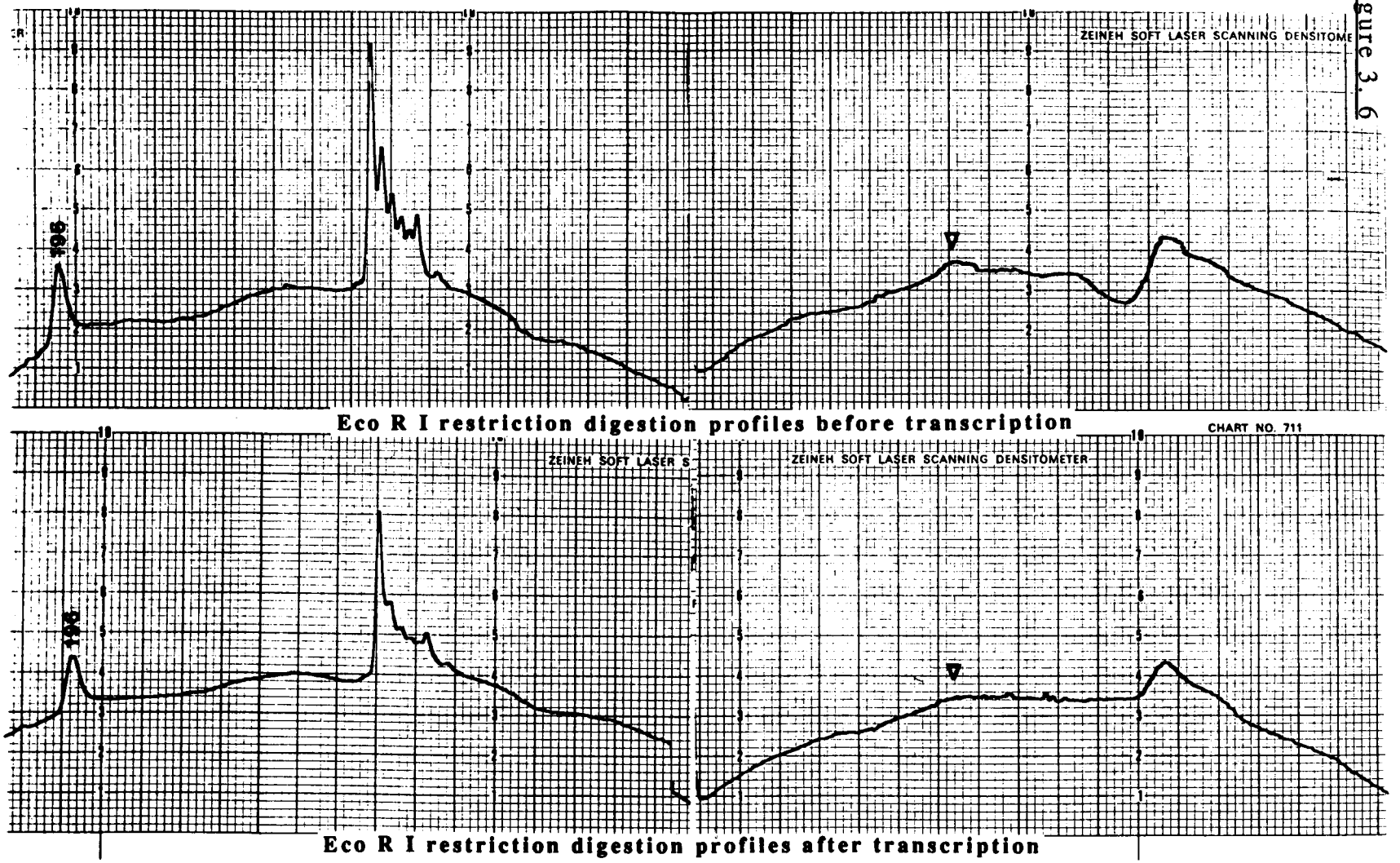
The peaks labeled 196 bp corresponds to the fragment generated by restriction digestion with *EcoRI* of the 5S rDNA positioning sequence tandemly repeated.

The highest peak corresponds to the pPol I 208-4 plasmid DNA with the four repeats of the 5S rDNA clipped out.

The shoulders visible on the downside of the highest peak correspond to the incomplete *EcoRI* digestion products of the p Pol I 208-4 plasmid DNA. The amount of incompletely digested 5S rDNA (multimer of the 196 bp fragment) is small enough not to be detectable by ethidium bromide staining of the gel.

The arrow heads indicate the position at which the 196 bp fragments complexed to histones are migrating in the gel.

Figure 3.6



Eco R I restriction digestion profiles before transcription

Eco R I restriction digestion profiles after transcription

Figure 3. 7

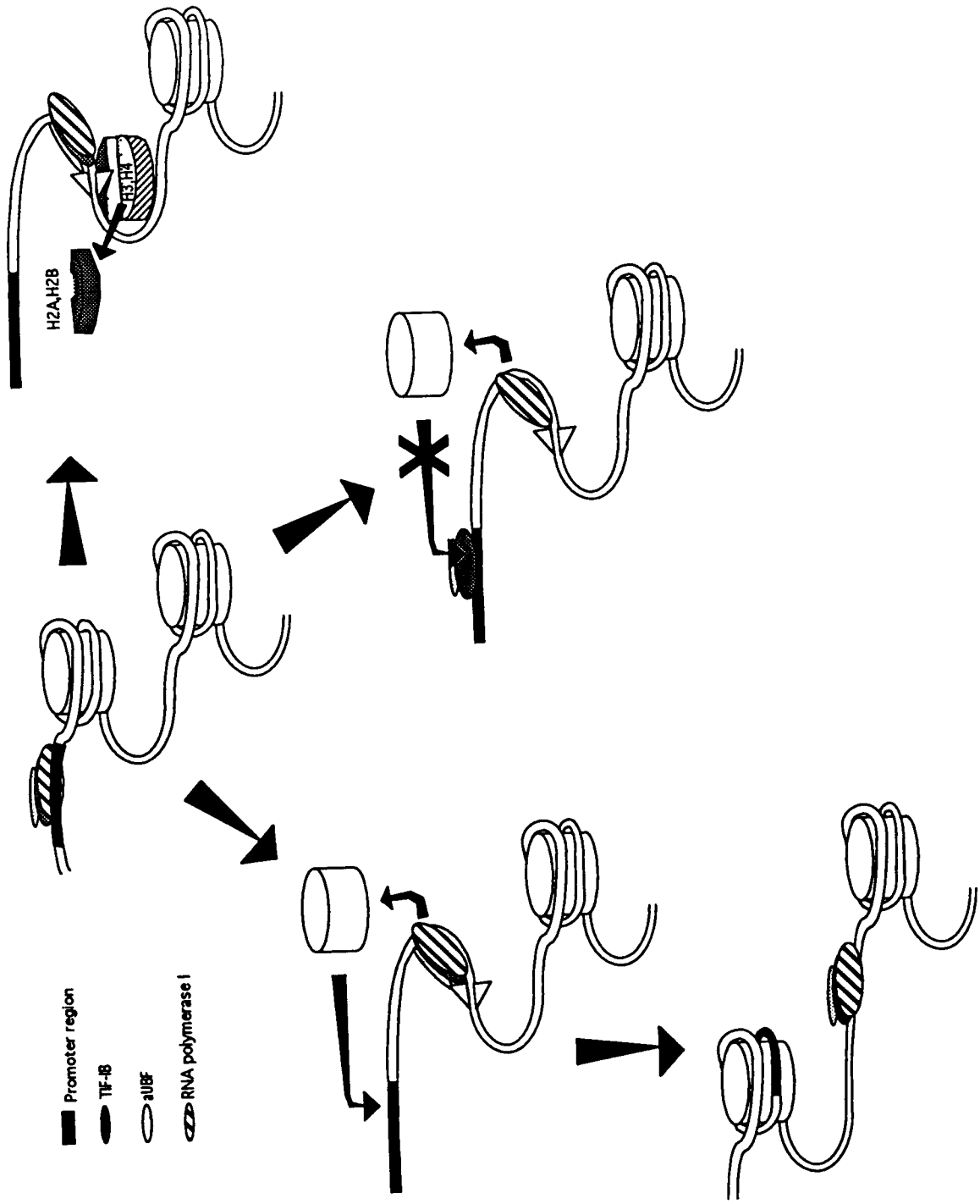
Schematic of the sequence of changes occuring at the level of the promoter region and at the first positioning sequence level. Three possible models are presented.

Left: the histone octamer is displaced as a unit and will redeposit a nucleosome structure that covers the promoter region.

Center: the nucleosome is removed from the DNA and will regenerate an identical structure at the same location. It cannot bind at the promoter region for this is still occupied by the transcription factors.

Right: a dimer of histone H2A and H2B is released, unlocking the nucleosome structure which can then be transcribed through as described in the progressive displacement model. The nucleosome structure will be regenerated after passage of the RNA pol I complex.

Figure 3. 7



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APPENDIX

Determination of the Comparative Energies of Nucleosome Formation on Isolated Sequences

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To be submitted.

Coauthor contribution: C.H.R: computer calculations and data fitting, K.E.v.H: Research director.

a) Summary.

We have studied the efficiency of formation of nucleosomes on different DNA sequences. We observed previously (see chapter 2) that the RNA polymerase I core promoter region from *Acanthamoeba castellanii* could efficiently compete with the 5S rDNA positioning sequence from *Lytechinus variegatus* for the binding of histones. The comparison was made by means of a band shift assay of a fragment containing 95 bp from the promoter region plus 104 bp from the plasmid pUC8 and a second fragment of DNA containing four repeats of the 5S gene positioning sequence. To quantify more precisely the affinity of the different fragments, we compared directly, by band shift assay, the respective affinity for histones of the monomer of the 5S positioning sequence to the 199 bp fragment containing the promoter region. Nucleosome core particle DNA was utilized under the same conditions as a reference for random nucleosomed sequence. The results suggest that the promoter region forms a complex with histone at a lower ratio of histone to DNA, but also suggest that this complex may not be a nucleosomal structure. The ability of the DNA to bend has been shown to be related to the nucleation of formation of nucleosomes. The presence of two strong bends in the DNA of the promoter sequence (stronger than the one predicted in the 5S gene) could explain the unexpected behavior of the promoter region with regard to the formation of nucleosomes, allowing the binding of two tetramers of H3-H4 too close to each other to allow formation of an octamer.

b) Introduction

Extensive studies of the structure of chromatin have shown that nucleosomes are frequently positioned in a nonrandom way on the DNA. It has been observed by several groups that some particular DNA sequences, like the 5S rDNA (Simpson, 1986) or the Heat Shock Protein 26 (hsp26) gene from *Drosophila melanogester* (Thomas and Elgin, 1988) generate specific positioning of nucleosomes. One of the most extensively studied of these sequences is the 5S rDNA from *Lytechinus variegatus* (Simpson et al.,1985, Dong et al.,1990 Hansen et al.,1989 and Pennings et al., 1992). These studies indicate one clearly dominant position and the presence of some minor positions 10 bp apart.

Our studies of the *in vitro* reconstitution of the plasmid DNA pPol I 208-4 included an investigation of the effect of the position of one RNA polymerase I core promoter region on the generation of a downstream stretch of phased nucleosomes (Georgel et al., in press 1993). Linearization of the plasmid at the *XbaI* site displaced the promoter region to the distal end of the plasmid and thus diminished the mispositioning effect on the downstream sequences by about 75 to 90% (as measured by digestion susceptibility to different restriction enzymes) compared to the circular pPol I 208-4 plasmid DNA. When *SspI* restriction endonuclease was used to linearize, which left the promoter still present directly upstream of the positioning sequences, the extent of "mispositioning" was comparable to the results obtained from circular pPol I 208-4 plasmid DNA.

The same plasmid DNA digested with *PvuII* and *XbaI* generated 3 fragments containing respectively, 1) the promoter

region plus 104 bp from pUC8 plasmid DNA , 2) the four repeats of the 5S rDNA fragment and 3) the rest of the pUC8 plasmid DNA sequence (referred to as bulk DNA) (see Figure 4. 5). When these DNA-fragments were titrated with a mixture of histone octamers and polyglutamic acid (PGA) the order of band retardation detected in a 3.5% polyacrylamide gel (i. e nucleosome formation) at increasing concentrations of histone was: first the promoter region, second the four repeats and then the bulk DNA.

The 5S rDNA positioning sequence has been described as one of the stronger positioning stretches in DNA. The deduction from the above data that the core promoter region of the RNA pol I could efficiently compete with the 5S rDNA positioning sequence for the formation of nucleosomes demanded more quantitative analysis. Competitive reconstitution on artificial and natural positioning DNA sequences (5S rDNA genes from *L variegatus* and *Xenopus laevis*) by Shrader and Crothers (1989) has tested the relationship between DNA bendability and the ability to generate nucleosomal structure. We began experiments to study the comparative energies of forming nucleosome on isolated DNA sequences, i.e the core promoter region, the monomer of the positioning sequence and bulk DNA isolated from chicken erythrocyte nucleosome core particles. It was important to carry out these experiments under the reconstitution conditions used in our earlier work on this plasmid (using polyglutamic acid as a protein carrier at moderate salt concentration). The binding affinities for the different sequences involved could give us an idea of the likelihood of the formation of a nucleosome on the promoter region or on the 5S rDNA during the formation of the transcription complex,

which might relate the formation of chromatin structure to the regulation of transcription.

c) Materials and methods

1) Construction of the plasmid pPol I 208-4.

The plasmid pPol I 208-4 was constructed by inserting a fragment of DNA containing a RNA polymerase I core promoter region from *Acanthamoeba castellanii* immediately upstream of four repeats of the 5SrDNA nucleosome positioning sequence from *Lytechinus variegatus* (Simpson and Stafford, 1983). For a more detailed description, see Chapter 2.

2) Preparation of the 208 bp fragment.

The monomer of the 5S rDNA sequence was obtained by restriction digestion by *AvaI* of the 12 tandemly repeated 5S gene sequence obtained from the pPol I 208-12 plasmid DNA The completion of the reaction was verified by gel electrophoresis of the reaction mixture using 3.5% polyacrylamide gel. The pattern obtained indicated no detectable incomplete digestion.

3) Preparation of the 199 bp promoter DNA fragment.

The pPol I 208-4 plasmid DNA was digested with *PvuII* and *XbaI* generating 3 fragments (see Georgel et al. 1992): (1) a 199 bp fragment containing the core promoter region, (2) a 1080 bp fragment containing four copies of the 208 bp positioning sequence and (3) a fragment of 2320 bp from the pUC8 plasmid DNA sequence.

The 199 bp fragment, called P/X 199 was purified by gel electrophoresis on a 1% agarose and 2% Nu Sieve gel, electroeluted and then resuspended in a 150 mM NaCl solution.

4) Preparation of the nucleosome core particle DNA.

The nucleosome core particles were obtained as described by Yager et al (1989). The isolated chromatin was digested with 14 units of micrococcal nuclease per mg of DNA, centrifuged and then resuspended in 10 mM Tris-HCl, 0.25 mM EDTA and 0.65 M Na Cl pH 8.0. After removal of histone H1/H5 by incubation with 30 µg/ml of carboxymethyl Sephadex, the stripped long chromatin (oligomers and monomers of nucleosomes, depleted of histone H1 and H5) was digested for 4 minutes with 5 units of micrococcal nuclease per µl of solution. The core particles were digested with proteinase K, extracted with phenol and the DNA precipitated with ethanol. The DNA was resuspended and the size of the fragments was measured by electrophoresis in a 3.5% polyacrylamide gel.

5) Preparation of histone octamers.

The histone octamers were purified from nucleosome monomers isolated from chicken erythrocytes, according to the method of Yager et al (1989), and then stripped of DNA by chromatography on a hydroxylapatite column (Simon et al., 1979) equilibrated with 2.2 M NaCl, 0.1 M KPO_4 pH 6.7. The peaks were collected and checked by electrophoresis on a Laemmli SDS 15% polyacrylamide gel. The histone-containing fractions were stored on ice at 4 °C.

6) Reconstitution of histone octamers on the DNA templates.

The reconstitution protocol used was described as a modified version of the method by Retief et al (1984) (see chapter 2), using polyglutamic acid (PGA) as a carrier for the deposition of the histone octamers onto the DNA. The ratio of PGA: histone was kept constant at 2:1 (w/w). For each separate experiment, a constant amount of

DNA was incubated, in a final volume of 19 µl, with histones at different ratios of histone to DNA. Depending on the nature of the sequence of the DNA reconstituted (promoter region, 5SrDNA or nucleosome core particle DNA) the amount of DNA used varied from 0.05 µg to 1.0 µg.

7) Preparation of end-labeled DNA templates.

The DNA 5' ends of the fragments were labeled with [γ^{32} P] ATP, 6000 mCi/mMol, by use of T4 polynucleotide kinase, using the reaction conditions recomended by the manufacturer (New England Biolab), and then extracted with phenol and precipitated with ethanol. The labeled DNA was resuspended in a 150 mM NaCl solution.

8) Nucleoprotein gel electrophoresis.

The analysis was performed on 3.5% polyacrylamide gels with a 29:1 acrylamide: bis (acrylamide) ratio. The electrophoresis buffer is 0.5 X e buffer (0.5 X e buffer is 10 mm Tris HCl, 0.25 mM EDTA and 7.5 mM Na OAc pH 8.0).

9) DNA quantification.

In the case of the end labeled DNA fragments, the gels were first autoradiographed and then scanned on the Ambis Radioanalytic Imaging System to estimate the amount of material.

When the DNA fragments were not end labeled, the gels were stained with ethidium bromide, photographed and the negatives were scanned on a flat bed scanner. Quantification was by use of the Image software (NIH) to determine the profile of each lane and then transfered to Mathematica for the final integration of the peaks.

d) Results

Our measurements were aimed at determinig the relative histone binding affinities of the "promoter" DNA and the 5S rDNA. Reconstitutions were performed as described above. The reconstitutes were electrophoresed on 3.5% polyacrylamide gel to investigate the formation of the expected DNA/histone complexes. The presence of a band demonstrated to comigrate with native nucleosome core particles was observed (see Figures 4. 1, 4. 2 and 4. 3). The first striking phenomenon we observed was the appearance of unexpected extra bands, called "nucleosome" complex I and II, displaying lower electrophoretic mobilities than the mononucleosome. The same bands were detectable, to a greater or lesser degree, for each monomeric fragment tested under every concentration used (arrow heads in Figures 4. 1, 4. 2 and 4. 3). We also noticed that at high enough concentration of histone octamer-PGA these more slower moving bands disappeared.

The quantification of the different bands appearing in the gels in the case of ethidium bromide stained gels and autoradiograms of ^{32}P labeled fragments have to be analyzed in two different ways. Although binding of ethidium bromide to nucleosomes has been investigated (Mc Murray and van Holde, 1991) the affinity of ethidium bromide for nucleosomal DNA is different than that for naked DNA, hence comparison of bands is hazardous. Therefore the only quantifiable material in such gels was the free DNA. After scanning, the free DNA peaks were integrated and the areas compared with those obtained from known amounts of serial dilutions of the 208 bp fragment or pBR 322 plasmid DNA digested

with *MspI*, electrophoresed in the same gel. The results are summarized in Table 1.

The experiments with ^{32}P end labeled DNA using 5 to 10 times lower DNA concentration allowed us to quantify not only the free DNA but also the amount bound to the "nucleosome" complexes I and II (higher bands in the gel) and also the complexes that stayed in the wells.

It is disconcerting to note that the sum of the radioactivity detected in the free DNA plus the two complexes (histone-DNA plus the radioactivity present in the wells) does not add up to the original amount of radiolabeled DNA loaded. It is possible that some of the DNA did not enter the gel at all. This could occur if some of the DNA fragments formed complexes with multiple copies of histones in which the overall charge of the complex was positive. Such particles would migrate towards the cathode, and not enter the gel at all.

The mass of DNA in its several forms (free DNA and complex I and II plus DNA in the wells) is plotted versus the logarithm of the mass of octamer for both end labelled fragments: the 208 5S rDNA and the promoter fragment (see Figure 4. 4).

A previous experiment comparing the disappearance of the free DNA indicated at the first sight that the promoter fragment displays a stronger propensity to form a nucleosome complex than does the 5S rDNA 208 bp fragment or the bulk DNA. When we plotted the mass of DNA versus the log of the mass of octamers (using 0.1 μg of 208 bp fragment and 0.05 μg of promoter fragment P/X199) half saturation was obtained at lower octamer concentration for the promoter fragment than for the 208 bp fragment. But, if we

consider the amount of DNA bound to histones for a molar ratio of DNA to histone-PGA of 1, the situation looks quite different. Almost all of the 199 bp promoter fragment is present as free DNA under conditions where about half of the 208 bp positioning sequence has been complexed with histones. This might indicate that, even though by this analysis the 208 bp fragment seems to form a nucleosome structure first, the actual complex generated by the promoter fragment could be of a different nature- for example, containing a tetramer of histones instead of the histone octamer.

Analysis of the data (Table 1) shows that the ratio of concentrations of octamer to DNA required to half-deplete the DNA is about 2 times as great for the promoter as for the 208 bp 5S sequence.

Since a number of studies indicated that DNA bending is important in determining position, the two sequences of interest- the promoter region and the 5S positioning sequence- were examined by a program which can model the secondary structure of the DNA. This program developed by Professor P.S Ho is based on the work by Bolshoy et al (1991) defining the sequence dependence of DNA bending. The results predicted a 30° bend centered 40 bp downstream of the TATA box in the internal control region (ICR) of the 5S gene. This presumably determines the major position on this sequence. On the other hand, the 199 bp fragment contains *two* fairly strong bends. The first is centered just upstream of the RNA pol I TATA box and the second one is present 85 bp downstream of the same TATA box (see Figure 4. 6).

As previously reported, the ability for the DNA to bend may play a key role in the nucleation of the formation of nucleosome. The explanation of the peculiar binding behavior of the promoter region may reside in the particular structure of that DNA sequence.

e) Discussion

The data we collected gave rise to a paradox. The first binding competition assay, using 3 fragments generated by *PvuII* and *XbaI* restriction digestion (see chapter 2) clearly indicated a better efficiency of forming histone-DNA complex for the fragment containing the core promoter region than for the fragment containing the four repeats of the 5S positioning sequence. In contrast we see here that the promoter region may bind histones in a more complex and unexpected manner than we anticipated. If we recapitulate the conclusions of the experiments, we notice that:

1) The binding is concentration dependent and that higher DNA concentration is less favorable to the formation of normal mononucleosomes, eventually leading to aggregation.

2) The promoter has a higher affinity for histones, but it seems to be forming *nucleosome* structure with a lower efficiency than the 208 bp fragment.

3) The predicted structure of the promoter region indicates two strong bends each of which could be eventually used for the nucleation of a nucleosome.

The exact effect of the polyglutamic acid on the association of an octamer structure is still unclear; in particular we do not know the

state of aggregation of the histones present in the reconstitution mixture.

A possible explanation for the histone binding behavior of the promoter can be derived from the sum of these pieces of information. If we consider the binding of H3-H4 tetramers on the promoter region , it is sterically possible to fit two of those units next to each other, each one of them centered on the middle of one of the bends. After the binding of the first tetramer H3-H4, two possible routes open. The complex formed can either bind two dimers of H2A-H2B and generate a nucleosome or it can bind a second H3-H4 tetramer. The formation of such a structure with two tetramers would block the formation of normal nucleosome structure due to steric interactions. The equilibrium between the two cases would explain the high molar ratio of histones to DNA at half saturation for the promoter fragment.

The appearance of nucleosome complexes I and II was also shown to be concentration dependent. The larger DNA/ histone complexes formed at higher concentration are possibly due to interaction between nucleosomes.

Table 1

Concentration in nM of the different DNA fragments at half-depletion.

C_{oct}/C_{DNA} indicates the molar ratio of histone octamers to DNA.

Table 1

DNA fragment	C_{DNA}, nM	$C_{oct}(1/2)$, nM	C_{oct}/C_{DNA}
5S rDNA	38.3	43.6	1.1
(208 bp)	414	331	0.8
Promoter	22.8	41.1	1.8
(199 bp)	108	263	2.4
Core Particle DNA (~146 bp)	358	282	0.8

Figure 4. 1

1) Band shift assay of the polyglutamic acid mediated reconstituted 208 bp fragment.

One µg of DNA was added per aliquot and incubated overnight in presence of a step dilution of histone octamer-PGA starting from 0.1mg/ml down to 0.8 µg/ml. The gel is a native 3.5% acrylamide and was stained with ethidium bromide. The nucleosome complexes and subnucleosome complexes (complex I and II) are indicated by arrow heads.

2) Autoradiogram of 208 bp fragment end-labelled reconstituted as described above (only 0.1 µg of DNA were used for the reconstitution) and electrophoresed in a native 3.5% acrylamide gel. The gel was dryed and then exposed on X-AR Kodak film for 24 hours. Lanes 1 to 5 contain a serial dilution of the 208 bp 5S rDNA fragment and were used as an internal standard for calibration. Lane 6 contains 0.1 µg of free 208 bp DNA. Lanes 7 to 18 contain 0.1 µg of 208 bp DNA fragment reconstituted into chromatin structure as described above. The disappearance of the subnucleosome complexed DNA is visible at higher ratio of histone-PGA to DNA.

Figure 4. 1. 1

Serial dilution of
pBR 322 Msp I

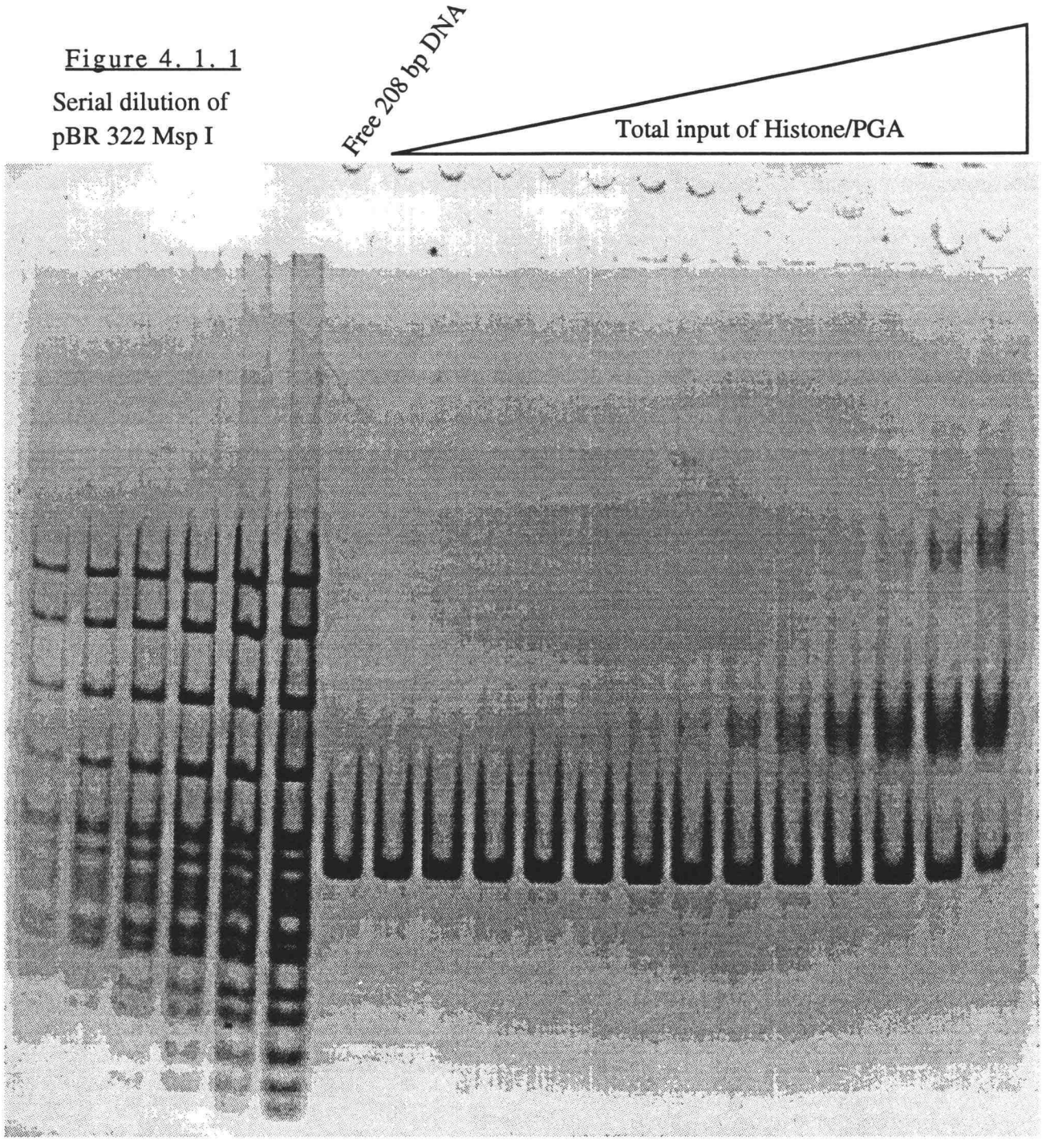


Figure 4. 1. 2

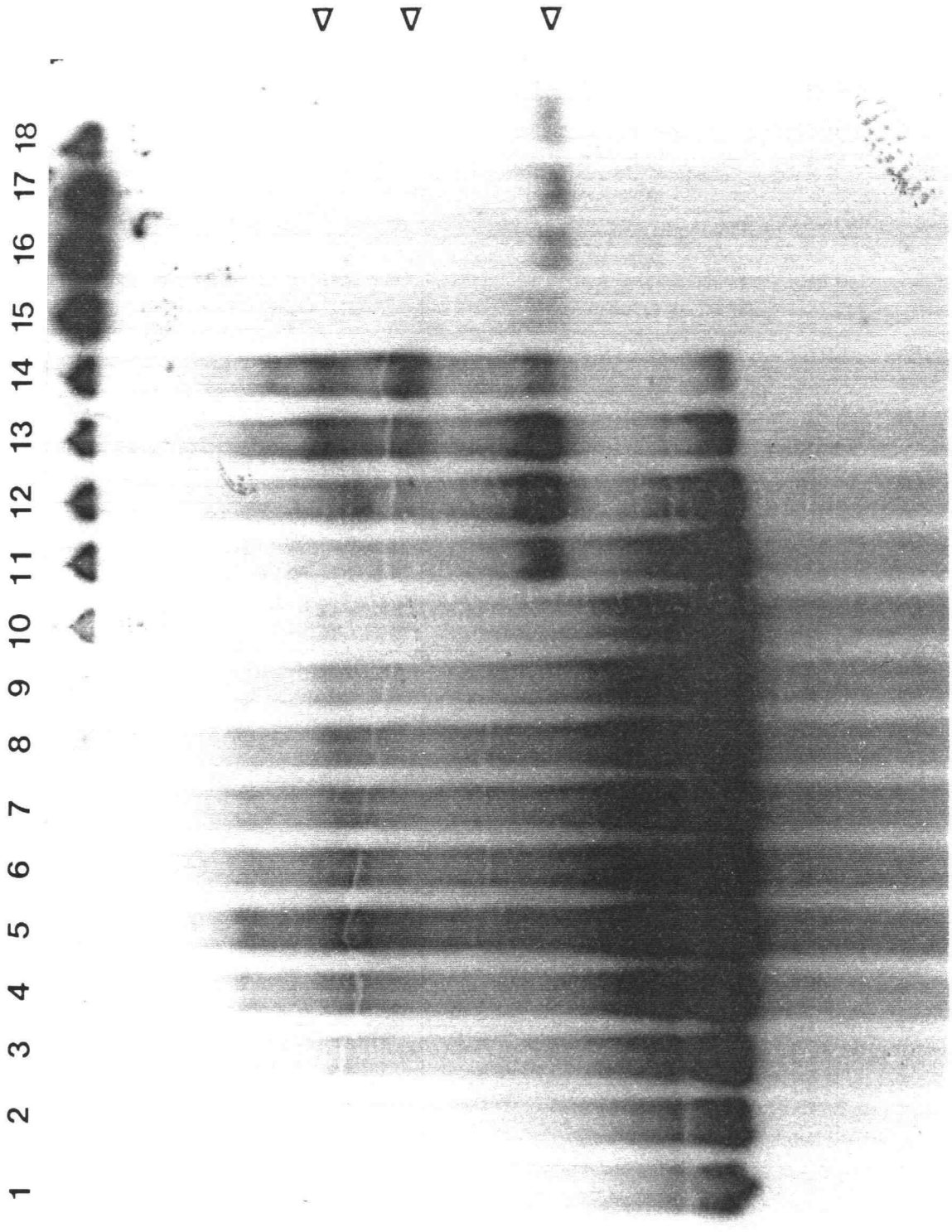


Figure 4. 2

1) Band shift assay of polyglutamic acid mediated reconstituted 199 bp promoter fragment.

0.25 µg of DNA was added per aliquot and incubated overnight in presence of a step dilution of histone octamer-PGA starting from 0.1mg/ml down to 0.8 µg/ml. The gel is a native 3.5% acrylamide and was stained with ethidium bromide. The nucleosome complexes and subnucleosome complexes (complex I and II) are indicated by arrow heads.

2) Autoradiogram of 199 bp promoter fragment end-labelled reconstituted as described above (only 0.05 µg of DNA were used for the reconstitution) and electrophoresed in a native 3.5% acrylamide gel. The gel was dryed and then exposed on X-AR Kodak film for 24 hours. Lanes 1 to 6 contain a serial dilution of the 208 bp 5S rDNA and were used as an internal standard for calibration. Lane 7 contains 0.05 µg of free 199 bp DNA fragment. Lanes 8 to 19 contain 0.05 µg of 199 bp DNA fragment reconstituted into chromatin structure as described above. The disappearance of the subnucleosome complexed DNA is visible at higher ratio of histone-PGA to DNA.

Figure 4. 2. 1

Serial dilution of
208 bp 5S rDNA

Free 199 bp promoter

Total input of Histone/PGA

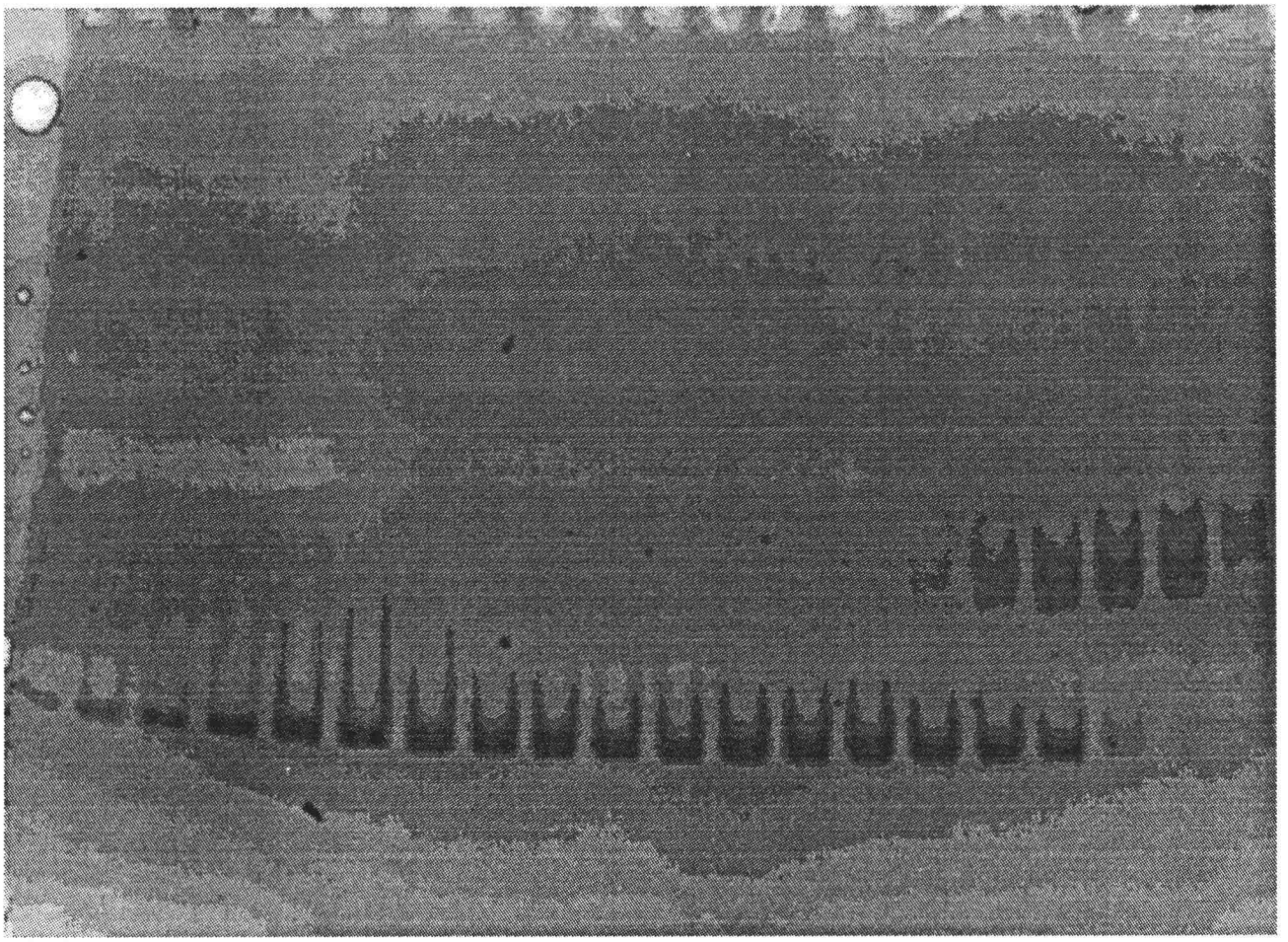


Figure 4. 2. 2

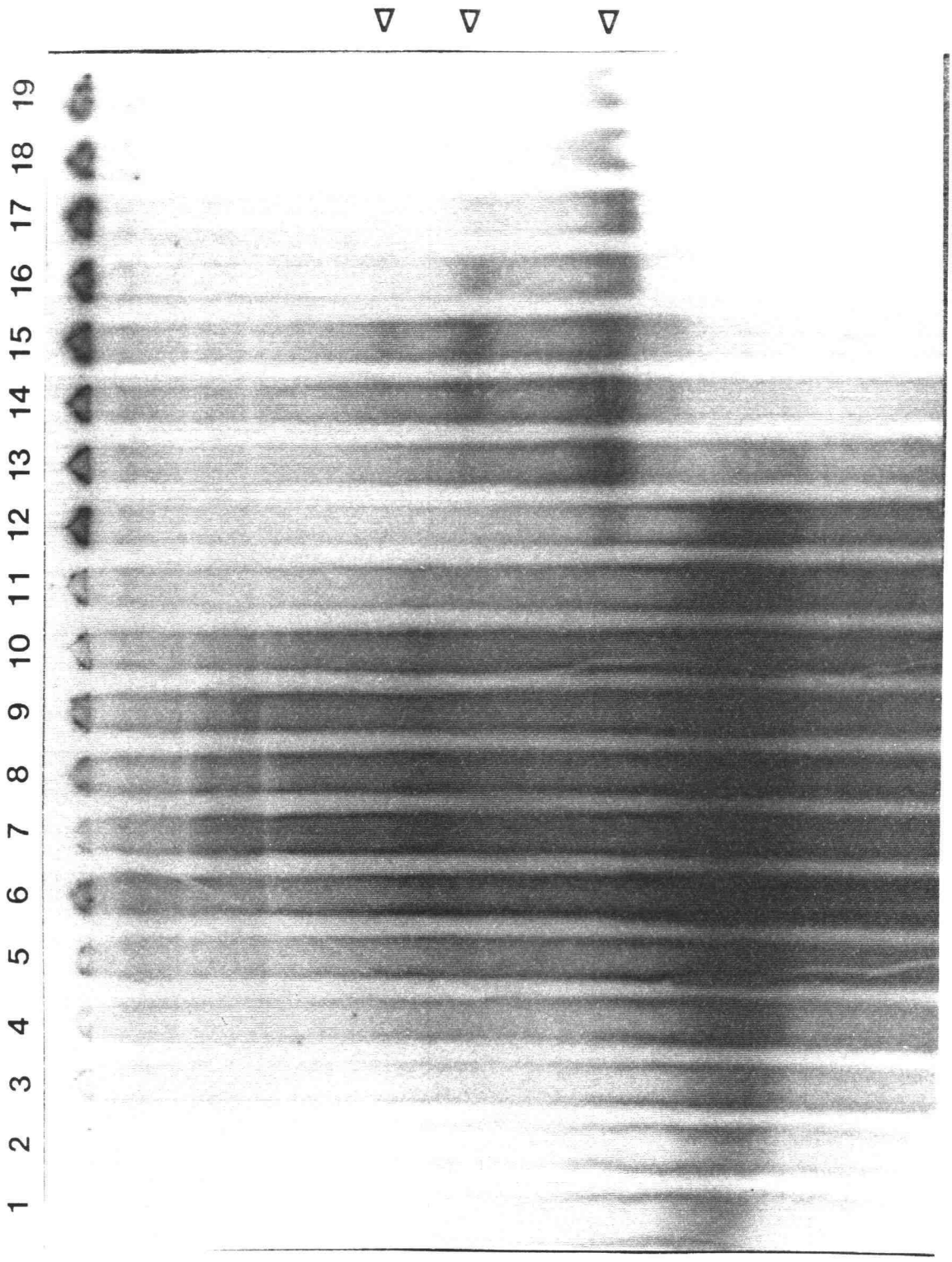


Figure 4. 3

Band shift assay of polyglutamic acid mediated reconstituted nucleosome core particle DNA (NCP).

One µg of DNA was added per aliquot and incubated overnight in presence of a step dilution of histone octamer-PGA starting from 0.1mg/ml down to 0.8 µg/ml. The gel is a native 3.5% acrylamide and was stained with ethidium bromide. The nucleosome complexes are indicated by arrow heads.

Figure 4. 3

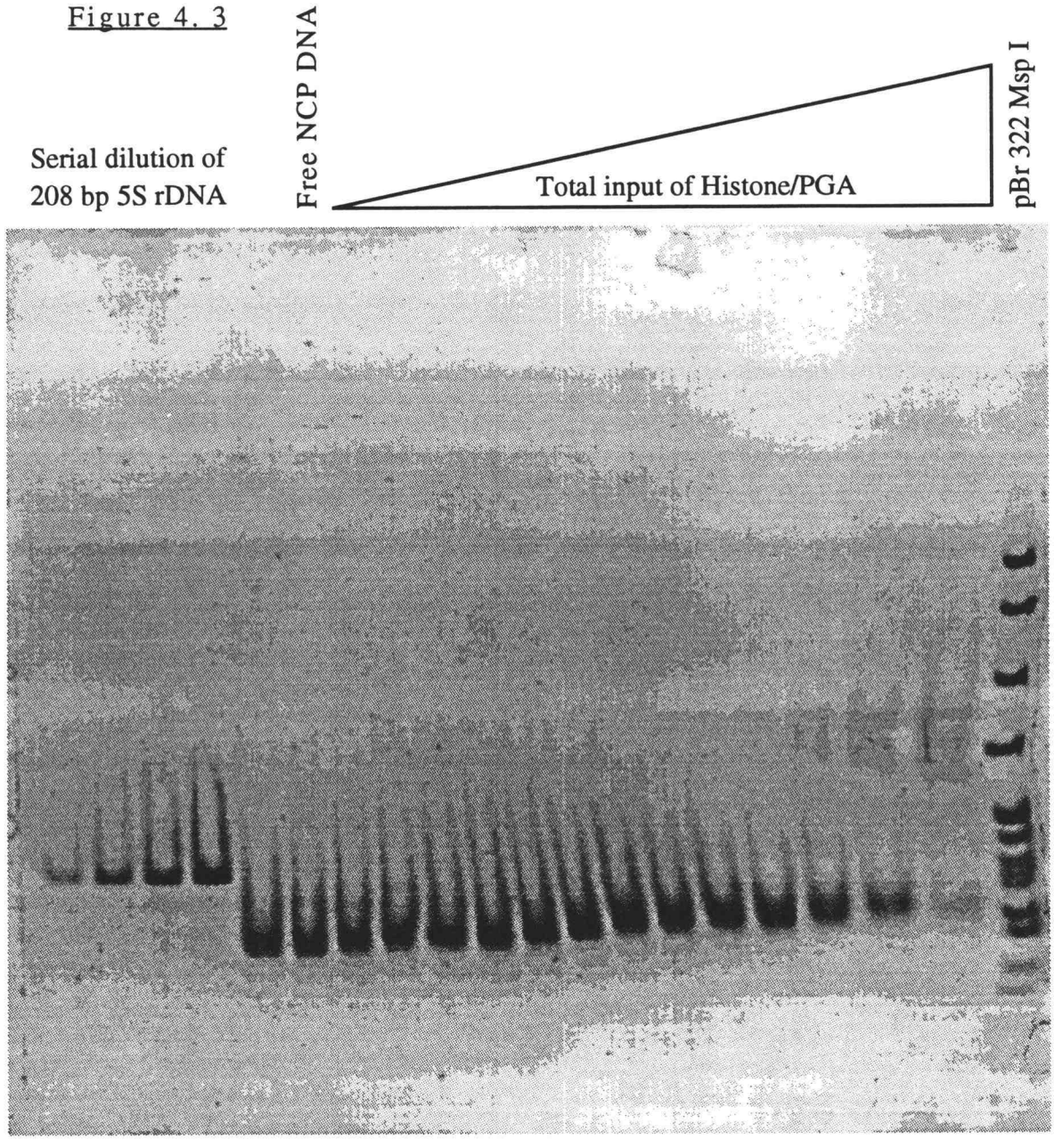

Serial dilution of
208 bp 5S rDNA
Free NCP DNA
Total input of Histone/PGA
pBr 322 Msp I

Figure 4. 4

Results of the histone titration of the 208 bp 5S rDNA and of the promoter region fragment.

The mass of DNA was plotted versus the logarithm of the mass of octamer. The values obtained at half-depletion are indicated by a solid line for the promoter fragment and by a dotted line for the 208 bp 5S rDNA.

Figure 4. 4

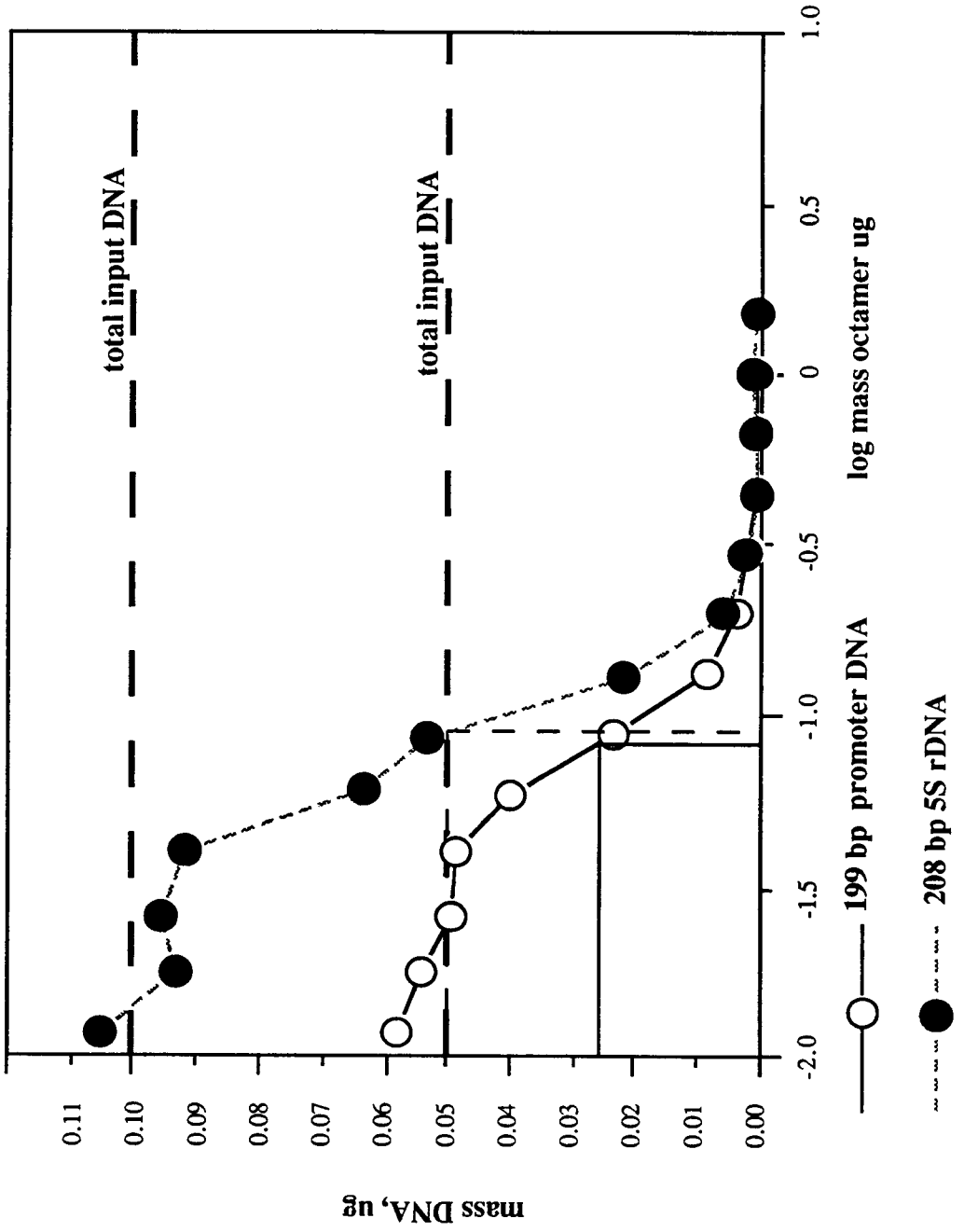

total input DNA
total input DNA
mass DNA, ug
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
log mass octamer ug
199 bp promoter DNA
208 bp 5S rDNA

Figure 4. 5

Restriction map of the plasmid pPol I 208-4.

The four repeats of the 5S rDNA sequence are displayed in black.

Figure 4. 5

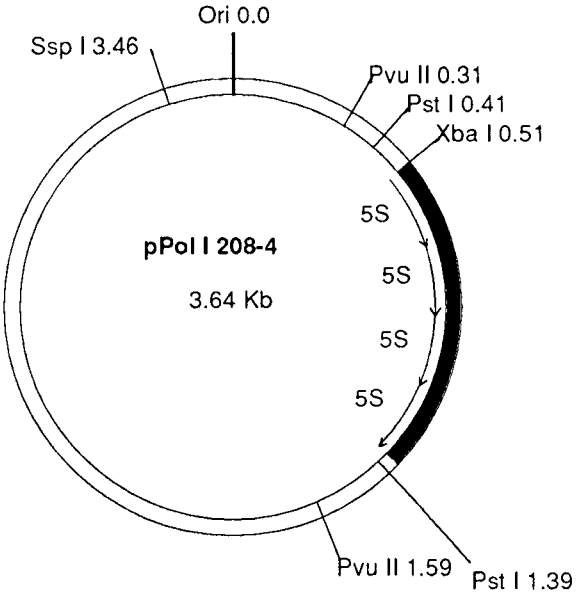
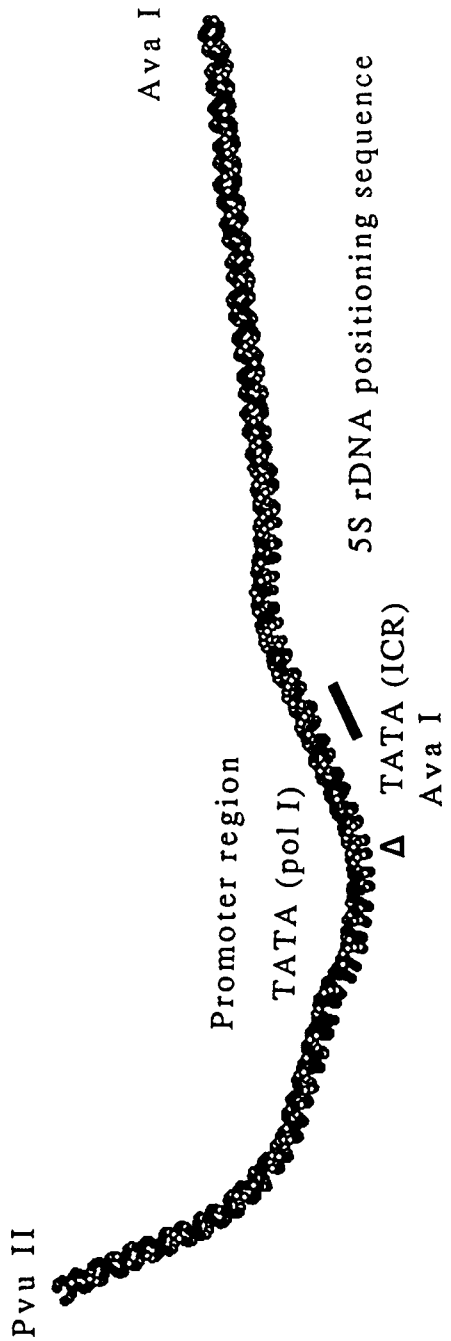

Ori 0.0
Ssp I 3.46
Pvu II 0.31
Pst I 0.41
Xba I 0.51
5S
5S
5S
5S
pPol I 208-4
3.64 Kb
Pvu II 1.59
Pst I 1.39

Figure 4. 6

Predicted structure of the 208 bp positioning sequence and of the promoter containing fragment.

The Internal Control Region of the 5S rDNA is underlined. The TATA box of the RNA polymerase I promoter region is marked by a triangle.

Figure 4. 6


Ava I
5S rDNA positioning sequence
TATA (ICR)
Ava I
Promoter region
TATA (pol I)
Pvu II