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Several levels of eukaryotic chromatin structure have been observed: the nucleosome, the 10 nm and 30 nm fibers and loop domains, apparently attached to the nuclear matrix. In this research, the structure and function of chromatin at two of these levels was investigated, with studies on both nucleosome positioning and chromatin interaction with the nuclear matrix.

In some instances, it seems that nucleosome positioning on genes is not random. Although no simple, definitive "nucleosome binding" sequences can be found which explicitly determine nucleosome positions, it is of interest to note that periodicity of some degenerate groupings of dinucleotides and of maximum bendability are correlated in nucleosomal DNA sequences. This research supports the proposition that nucleosome positioning on DNA may depend on the existence of periodic regularities in DNA bendability. It also indicates that information contained within local sequences, determine properties which affect the differential propensity for positioning of nucleosomes. Bendability seems to represent at least one of the major sequence-directed structural constraints on the ability of any particular stretch of DNA to form nucleosomes.

Studies of the nucleosome spacing in the 5' flanking region of the chicken beta globin gene and coding sequence of the chicken thymidine kinase gene in chicken erythrocyte cells and chicken embryo myoblast cells demonstrate that the nucleosome spacing in these regions is most likely cell type-dependent, rather than gene dependent; and probably reflects a general effect of the special histone, H5, carried in erythrocyte cells.

DNA loops are proposed to be anchored to the nuclear matrix, which may be involved in DNA replication and repair, RNA transcription and processing, hormone action, virus infection, and carcinogenesis. Studies of the relationship between gene activity and nuclear matrix association, have given both positive and negative results with the chicken thymidine kinase gene, the beta-globin gene and the mouse dihydrofolate reductase gene. There appears to be no simple correlation between nuclear matrix association and gene transcriptional activity. The working hypothesis developed here is that the apparent association of specific genes with the nuclear matrix is mainly caused by specific DNA binding proteins which partition in the nuclear matrix fraction.

Adenovirus was used as a model to investigate the role which the nuclear matrix may play in virus infection and viral DNA replication. The origins of replication of adenovirus DNA are found to be strongly associated with the nuclear matrix. One of the nuclear matrix proteins, of mass 140 KD, has been found from a UV cross-linking experiment to be able to bind specifically with the origins of replication of adenovirus. However, whether these proteins are <u>in vivo</u> components of the nuclear matrix or that their association is an artifact of the isolation, could not be determined with certainty.

Structure and Function of Chromatin: Studies at the Nucleosome and Nuclear Matrix Levels

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DEDICATION

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STRUCTURE AND FUNCTION OF CHROMATIN:

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CHAPTER 1: Introduction

All life begins with cells. The central, governing entity in a eukaryotic cell is the nucleus. If we wish to understand the structure and the function of the nucleus, it is important to know its substructure. An important part of this is the nuclear scaffold or nuclear skeleton, also known as the nuclear matrix. Many attempts have been made to isolate and characterize this structure. The nuclear matrix is defined by Berezney and Coffey (1974) as the residuum that remains after the DNA and RNA are digested, the lipids are dissolved with a nonionic detergent, and the histones and many nonhistone chromosomal proteins are solubilized by high salt. The nature and properties of the nuclear matrix will be discussed in detail in a later section.

The nuclear matrix has been variously suggested as the biochemical site for DNA replication and repair, RNA transcription and processing, hormone action, and as an important cellular target site for the transforming proteins and oncogene products, such as the myc protein, SV40 large T antigen and the E1A protein of adenovirus.

In the following review, data from the recent literature are summarized in an attempt to sort out the facts and artifacts of our information about the structure and function of the nuclear matrix. I also will briefly

review the field on nucleosome positioning and nucleosome organization to complement our understanding of the structure and function of chromatin which is used as the concrete, water and cement, together with the scaffold, to build the whole building of nucleus.

I. The Nuclear Matrix Structure and Its Isolation

A. Overall Structure and Composition

DNA is the material carrying the messages of heredity. In every eukaryotic cell, DNA is packaged with histone proteins to form a complex called chromatin. The basic repeating structure of chromatin is the nucleosome, which contains about 146 bp DNA wrapped around a histone octamer, containing two H2A-H2B dimers and one H3-H4 tetramer (for a review see van Holde, 1988). Higher order structure above the nucleosome level is exhibited in the 10 nm fiber and the 30 nm " solenoid " (Jackson,1986). A still higher order structure of chromatin has been characterized as involving DNA loops which are proposed to be anchored to the nuclear matrix or nuclear scaffold.

The nuclear matrix contains three major components: (1) the residual nuclear envelope, lamina and nuclear pore complex; (2) residual fibrillar and granular components of nucleoli and (3) residual fibrous internal network of the remaining portions of the nucleus(Smith et al., 1987; Brachet, 1985; Stalder, 1980). Many methods have been used in characterizing the structure of the nuclear matrix, including electron microscopy (EM) (De Boni,1988; Whytock and Stewart,1988; Maraldi et al., 1986; Gerace et al., 1984; Lewis et al.,1984; Ierardi et al., 1983; Kaufmann et al., 1981; Capco et al., 1982; Pouchelet et al., 1986), 2D Sodium Dodecyl Sulfate (SDS) protein gel analysis (Gerace et al., 1984; Kaufmann et al., 1983; Macfarlane, 1986;

Capco et al., 1982; Perters and Comings, 1980; Nakayasu and Ueda, 1986; Milavetz and Edwards, 1986; Simmen et al., 1984; Fey and Penman, 1987; Feuerstein and Mond, 1987; Dessev et al., 1988), and immunofluorescence techniques (Mckeon et al., 1984; Schindler and Jiang, 1986; Schimdt and Franke, 1988).

By using different isolation methods, which will be described later, different structures have been seen. Since the composition and properties of the nuclear matrix vary with different isolation methods, it may be said that there are currently as many kinds of "nuclear matrix" as there are methods for its isolation (Pogo and Procyk, 1985).

In the following section are described the components of the entire matrix, beginning with the residual nuclear envelope.

The Nuclear Envelope

Gerace and Bensen (1984) have defined the nuclear lamina as a protein meshwork associated with the nucleoplasmic surface of the inner nuclear membrane. It is suggested to be very important in organizing the nuclear envelope and the chromatin structure. In most nuclei, the lamina is composed of three well- defined proteins, lamins A, B, and C which migrate at approximately 70, 67, and 60 KD on SDS gels (Pogo and Procyk, 1985; Kaufmann et al., 1983). Lamins A and B, but not lamin C, can form a series of oligomers by cross-linking with each other through disulfide bonds (Kaufmann et al., 1983). Lamin B has been proposed to be attached to the inner nuclear membrane (Gerace et al., 1984) by binding with its receptor, a 158 KD protein (Worman et al., 1988).

By examining the synthesis and assembly of the lamins during the cell cycle, Gerace and Bensen (1984) have proposed a model by which depolymerization, a process that may be mediated by enzymatic phosphorylation, and reassembly of the lamina can regulate the reversible disassembly of the nuclear envelope during mitosis.

Although the nuclear envelope proteins consist mainly of lamins A, B, and C from the nuclear lamina, it seems clear that the whole envelope is a more complex structure, with other distinguishable parts. Both spermatocytes and spermatids possess nuclear envelopes, but not lamina (Pogo and Procyk, 1985). One of the highly conserved proteins of the nuclear envelope, 33 KD (perichromin), appears to be directly or indirectly bound to the chromosomal DNA and has been suggested to be involved in chromosome organization and condensation since this protein appears to remain attached to metaphase chromosomes after the envelope disintegrates (Mckeon et al., 1984).

The nuclear pore complex is associated with the nuclear envelope at the point of fusion between the outer and inner membrane of the envelope (Park et al., 1987). Although the precise role of the nuclear pore complex is not completely understood, these pores are apparently the morphological routes of communication between the cytoplasm and the nucleus (Park et al., 1987). By using immunofluorescence methods (Schindler and Jiang, 1986), actin and myosin have been suggested to be involved in an ATP-dependent process that alters the effective transport rate across the nuclear pores. One of the main envelope glycoproteins, a 62 KD protein named nucleoporin, has been reported (Davis and Blobel, 1987) to be localized to the nuclear pore complex. This protein, which can bind to wheat germ agglutinin, has been suggested to be involved

in protein transport. Another enzyme, tRNA ligase, also has been reported to associate with nuclear pores (Clark and Abelson, 1987), implying that this portion of the nuclear matrix may be involved in RNA processing. This possibility will be discussed in a later section.

Residual Nucleolar Structure

Isolation of nucleoli, and removal of DNA and most protein leaves a residual nucleolar structure. This structure is stabilized by disulfide bonds (Fields et al., 1986). One major polypeptide, 38 KD/ PI 5.3, isolated from rat liver nuclei, has been localized to the nucleolus by the indirect immunofluorescence method.

A protein of the same molecular weight, isolated from Xenopus laevis, has been cloned and shown to be located specifically in the nucleolus (Schimdt and Franke, 1988; Probst and Herzog, 1985). This protein may be identical or similar to another nuclear phosphoprotein B23, also named numatrin (Feuerstein and Mond, 1987) from HL60 cells, and has an important role in regulation of cellular growth in normal and malignant cells (Feuerstein et al., 1988). In humans, one component of nucleolar material, perichromonucleolin (PCN) (of mass 90 KD), was shown to be located in the nucleolus-organizing regions (Shi et al., 1987). The nucleolar skeletal complex also has been claimed to be the site of attachment of that portion of the chromosomal DNA which bears ribosomal genes (Bureau et al., 1986; Olson et al., 1986; Jackson, 1986).

The Fibrillar Network of The Nucleus

The major portion of the nuclear matrix can be characterized as a fibrillar intranuclear network. This has been shown to contain the attachment sites for DNA loop domains (Lebkowski and Laemmli, 1982; Mirkovitch et al.,

1984). From 2D SDS gel analysis it is found that actin is strongly associated to the nuclear proteinaceous network, and may play a role in the contractility of the matrix (Nakayasu and Ueda, 1986; Peters and Comings, 1980). Since Wunderlich and Herlan (1977) have shown that the matrix contractility does not depend on ATP, an actin/myosin system is apparently not involved; rather, a mechanism based on actin polymerization and depolymerization may be responsible. Many studies (Peters and Comings, 1980; Capco et al., 1982; Fey and Penman, 1987; Pouchelet et al., 1986) have shown that the HnRNP proteins P34 and P36 are also associated with the nuclear proteinaceous network. This may imply a role for the nuclear matrix in RNA processing, which will be discussed more in a later section. The protein composition of the nuclear matrix is not only complex, but susceptible modification. Some of the matrix proteins are phosphorylated, such as P65 (Nose, 1986) and topoisomerase II, and are major substrates for those protein kinases which also are associated with the nuclear matrix (Dessev et al., 1988; Simmen et al., 1984). Furthermore, the nuclear matrix of rat liver possesses thiol, serine and metal protease activity, which suggests that significant protein turnover may occur in the nuclear matrix (Kutsyl et al., 1987).

B. Isolation Methods

The structure and the protein composition of the nuclear matrix is not only cell type-dependent (CHAPTER 2, Sturman et al., 1988), but its observed composition also varies with the isolation method (Smith et al., 1987). In 1942, Mayer and Gulick first isolated a subfraction of nuclear proteins which resisted extraction with buffers of high ionic strength. Since then, many workers have published results from modifications of that isolation

method (Berezney and Coffey, 1974; Wunderlinch and Herlan, 1977; Kaufmann et al., 1981; Comerford et al., 1986; Lammeli, 1984). Because such methods may use detergent, high salt extraction, nuclease digestion and may possibly involve oxidative cross-linking and metal binding, the validity of the isolation procedure and even the physiological existence of the matrix have been controversial issues (Hadlaczky et al., 1981; Cook, 1988). Lammeli et al. (1984) developed a milder extraction method, using 25 mM lithium 3,5-diioiodosalicylate (LIS), and with a less stringent detergent, digitonin, to isolate the nuclear scaffold from Drosophila melanogaster kc cells. However, by comparing the EM ultrastructure, protein composition and distribution of replicating DNA, Smith et al.. (1987) concluded that products of the low salt method lacked morphologically distinct residual nucleoli and were markedly depleted in internal structure; furthermore, these properties were found to be LIS-concentration dependent.

As a consequence of these uncertainties, the research described in this thesis has used both high salt and low salt methods to isolate the nuclear matrix, and the results from the two techniques are compared (see CHAPTERS 2, 3).

II. Proteins Reported to Be Part of the Nuclear Matrix, and Their Functions

In this section are described various claims for the existence and function of nuclear matrix proteins. For completeness, many are listed, but it must be understood that some results may be artificial, for the reasons given above.

A. DNA Polymerase and DNA replication

In animal cells, the existence of a multienzyme replicative complex, termed the replisome, has been

suggested (Nelson et al., 1986). Some components of that complex have been found in the nuclear matrix, such as DNA polymerase alpha (Bezlepkin et al., 1986; Klinge and Liu, 1986; Smith et al., 1984; Smith and Berezney, 1983; Foster and Collins, 1985; Collins and Chu, 1987), DNA polymerase beta (Bezlepkin et al., 1986; Nishizawa et al., 1984; Smith et al., 1984), DNA primase (Wood and Collins, 1985; Berrios, 1985; Tubo and Berezeny, 1987), DNA methylase (Burdon, 1985), topoisomerase I (Nishizawa, 1984) and topoisomerase II (Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986; Eissenberg et al., 1985).

Not only may the replication machinery be associated with the nuclear matrix (Jackson and Cook, 1986; Chang et al., 1978), but also the DNA origins of replication (Dijkwel et al., 1986; Todorova and Russev, 1984; Aelen et al., 1983; Razin et al., 1986; Cook and Lang, 1984; Carri et al., 1986; Jackson et al., 1984; van der Velden et al., 1984; Anachkova et al., 1984; CHAPTER 3). Newly synthesized DNA has been found to be enriched in a nuclear matrix-associated fraction (Pardoll et al., 1980; Hunt and Volgelstein, 1981; Berezney and Buchholtz, 1981; Smith et al., 1984; Djondjurov et al., 1986). Together, these results imply, but do not prove, that the nuclear matrix may play an important role in DNA replication.

B. Poly (ADP-Ribose) Synthetase and Repair

As noted above, both DNA polymerase alpha and the repair polymerase beta are associated with the nuclear matrix. Another DNA repair enzyme, poly (ADP-ribose) synthetase (Alvarez and Ringer, 1988; Cardenas et al., 1987) is also associated with the nuclear matrix which may imply that the machinery for DNA repair of UV damage (Mullenders et al., 1984; Mullenders et al., 1988) and

carcinogen adducts (Mironov, 1987) is preferentially located in the nuclear matrix (Mullenders et al., 1987).

C. Gene Activity and Nuclear Matrix Association

The possible relation between gene activity and nuclear matrix association is a controversial issue. Some of the data have suggested positive results (Mironov, 1987; Ciejek et al., 1983; Jackson and Cook, 1985; Chiu et al., 1986). Specific examples include the conalbumin and ovalbumin genes (Robinson et al., 1983; Robinson et al., 1982), heat shock genes (Small et al., 1985), ribosomal RNA genes (Keppel, 1986; Bandyopadhyay et al., 1986), chicken beta globin gene, and mouse dihydrofolate reductase (DHFR) gene (CHAPTER 2). On the other hand, some of the data have suggested negative results (Razin et al., 1985; Basler et al., 1981, Bandyopadhyay et al., 1986). Specific examples here include the chicken beta globin gene (Greenstein, 1988; CHAPTER 2), murine alpha globin gene (Kirov and Tsanev, 1986) and chicken thymidine kinase gene (CHAPTER 2). It is striking and disturbing that in some instances transcription of a specific gene (i.e. the chicken beta globin) gives different results in the hands of different workers. Possible reasons for the discrepancy between results from different laboratories are described in CHAPTER 2.

The DNA loop model for transcriptional regulation (Pienta and Coffey, 1984; Nelson et al., 1986) predicts that 5-200 kb DNA domains contain nuclear matrix attachment sites (Blasquez et al., 1989). Many genes have been found to be closely associated with the matrix associated region (MAR), sometimes called the scaffold associated region (SAR); these include Drosophila histone genes (Mirkovitch et al., 1984), the heat shock protein 70 gene (Mirkovitch et al., 1984), the chicken lysozyme gene (Phi-Van and

Stratling, 1988), the mouse immunoglobulin k gene (Cockerill and Garrard, 1986; Blasquez et al., 1989), the human beta globin gene (Jarman and Higgs, 1988; Greenstein, 1988), rat alpha 2-macroglobulin gene (Ito and Sakaki, 1987; Murty et al., 1988), and human interferon-beta gene (Bode and Mass, 1988).

MAR or SAR sequences (Blasquez et al., 1989) are about 200 bp long, and AT-rich (ca 70%). They contain topoisomerase II consensus sequences and often reside near cis-acting regulatory sequences. These sites are abundant (>10,000 per mammalian nucleus). Some evidence suggest that nuclear matrix associated DNA is enriched in repetitive sequences (Goldberg et al., 1983; Endoh and Okada, 1986), such as the KpnI family (Chimera and Musich, 1985), mouse satellite, the human Alu family (Small et al., 1982) and contains single strand sites that are sensitive to S1 nuclease digestion (Probst and Herzog, 1985).

The transcription machinery (mainly RNA polymerase II) has been studied and it was shown that neither the active nor inactive forms of the enzyme are nuclear matrix proteins (Roberge et al., 1988). This evidence has suggested a model in which the transcriptional machinery moves along DNA loops during transcription, removed from direct association with the matrix.

If this is correct, the role, if any, of the nuclear matrix in transcription must be restricted to one in regulation. It leaves open the possibility that close association of genes with the matrix-associated region may be a characteristic of either the active or inactive state, or that such association may be inconsequential in this process.

D. RNA Splicing and Transport

Gallinano et al. (1983) and Bouvier et al. (1985) studied high molecular weight nuclear ribonucleoprotein (HnRNP), a fibrillo-granular structure, and have shown that the nuclear matrix and HnRNP share a common structural constituent associated with premessenger RNA. The precursors of mRNA were found to be quantitatively associated with the nuclear matrix and mature mRNA is selectively released from the nuclear matrix by an ATP/dATP-dependent mechanism (Agutter, 1985) sensitive to topoisomerase II inhibitors (Friese et al., 1987). By using in vitro complementation reaction studies, Zeitlin et al. (1987) demonstrated that the splicing process, including both endonucleolytic cuts and branching, could proceed when the nuclear matrix is supplemented with soluble factors present in the HeLa cell nuclear extract. Furthermore, splicing intermediates have been found in the nuclear matrix fraction. The low molecular weight small nuclear ribonucleoprotein (snRNP) has been suggested to be involved in RNA splicing (Mattaj, 1988; Grabowski and Sharp, 1986; Maniatis and Reed, 1987; Lamond et al., 1988). From the electron microscopic and immunobiochemical data, the latter using polyclonal antibodies to P107, P28, Sm Antigen and a monoclonal antibody to D protein (16 KD, which is a protein core common to U1, U2, U5, U4, U6, and snRNP), Smith et al. (1986; Harris and Smith, 1988) and Reuter et al. (1984) are able to support the idea that the residual RNP network of the nuclear matrix is an isolate of a pre-existing structure and suggest that RNA splicing may take place at the nuclear matrix (Suh et al., 1987; Poznanovic and Sevaljevic, 1986; Verheijen et al., 1986).

Furthermore, poly (A) polymerase, a glycoprotein of molecular mass 64 KD, has been isolated from rat hepatic

nuclear envelope. This may imply that the peripheral portion of the nuclear matrix may be involved in the poly (A) tail polymerization (Kurl et al., 1988), a last step in the processing and export of mRNA.

E. Hormone Action

There have been a number of reports purporting to show that hormone receptors are nuclear matrix proteins. For example much evidence has been presented to show that the glucocorticoid receptor (Kaufman et al., 1986; Kirch et al., 1986), estrogen receptor (Swaneck and Alvarez, 1985), 3, 5, 3'-triiodo-L-thyronine receptor (Kumara-Siri et al., 1986; 1988), steroid receptor (Buttyan et al., 1983; Barrack, 1987) and androgen receptor (Mowszowics et al., 1988) bind to the nuclear matrix. This may suggest that the nuclear matrix may be the site of hormone action; alternatively, one must consider the possibility that these proteins are bound to the matrix during isolation procedure.

From studies of vitelloginin mRNA synthesis, Saluz et al. (1986) have shown that there is a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid receptor binding sites and the rate of avian vitelloginin mRNA synthesis. DNA methylase and steroid hormone receptor are both claimed to be associated with the nuclear matrix, which suggests that the nuclear matrix plays a direct role in hormone gene regulation. The chicken lysozyme gene contains a nuclear matrix associated steroid receptor binding site in the promoter region which regulates the gene expression (Phi-Van and Stratling, 1988).

F. Virus Pathogenesis

The nuclear matrix plays a role in virus replication (Ciampor, 1988) and maturation (Zhai et al., 1988) which is suggested from evidence for association of viral DNA with the nuclear matrix (Wilson and Price, 1988; Jones and Su, 1987; Courad and Zakin, 1988; Watson and Gralla, 1987; Ciampor, 1988; Rzeszowska et al., 1987; Smith et al., 1985; Younghusband and Maundrell, 1982). Furthermore, it has been reported that virus proteins may be matrix associated (MacLean et al., 1987; Humphrey and Pigiet, 1987; Slamon et al., 1988; Barbosa and Wellstein, 1988; Hinzpeter and Deppert, 1987; Schirmbeck and Deppert, 1987; Chapter 3 in this thesis).

By using electron microscopy and cell extraction, Zhoughe et al. (1987) have examined the nuclear matrix and cytoskeleton at 6, 13, 28, 44 hrs after adenovirus infection. They found that as infection progressed, chromatin condensed onto the cell nuceoli and the nuclear lamina. The nuclear lamina became increasingly crenated and the perinuclear cytoskeleton became rearranged after infection. In summary, they suggested that adenovirus rearranges the nuclear matrix structure of the host cells and cytoskeleton to support its own replication (Zhoughe et al., 1987).

It was also reported by Jackson et al. (1984) that during viral infection, all of cellular vimentin (57KD) is degraded and phosphorylated keratin is increased. They found two new uncharacterized peptides appearing in the nuclear matrix. These facts also indicate that the nuclear matrix-intermediate filament structure may play a role in adenovirus replication.

Another study from Khittoo et al. (1986) reported that late in the lytic cycle, the adenoviral core polypeptides V, PVII, 11 KDa were enriched in the nuclear matrix fraction and most of the virus maturation endoproteinase activity copurified with the nuclear matrix. Their results suggest that the nuclear matrix is the site of adenovirus assembly and maturation.

In CHAPTER 3, it is shown that the results of this research, both from in vivo and in vitro experiments, also confirm that the adenovirus origin of replication as well as the viral DNA are strongly associated with the nuclear matrix 24 hrs after infection.

G. Carcinogenesis

It is of interest to note that some oncogene products, such as the myc-encoded protein, are bound to the nuclear matrix (Eisenman et al., 1985; Green and Chambon, 1986; Sap et al., 1986; Weiberger et al., 1986). Tumor promoters, such as phobol 12-tetradecanoate 13-acetate (TPA) also induce a specific morphological signature in the nuclear matrix intermediate filament scaffold of Median-Darby Canine Kidney cell colonies (Fey and Penman, 1984; Nakayasu and Ueda, 1986). Furthermore, Obi (1986) and others reported the preferential binding of carcinogens, such as 2-acetyl aminofluorene, benzo[a]pyrene, and dibenzo[a, e]fluoranthene (Perin-Russel et al., 1988), to DNA in the active chromatin portion and the nuclear matrix associated fraction (Mirinov et al., 1988; Mirell et al., 1985; Gupta et al., 1985; Bresnick et al., 1977; Vaught and Bresnick, 1976). This evidence suggests that the matrix associated DNA is more accesible to carcinogens.

The activation and amplification of oncogenes can be induced by carcinogens (Marx,1984; Alitalo, 1985).

Examples include aflatoxin B1 activation of c-Ki-ras (McMahon et al., 1986). Carcinogens may directly cause the point mutation, substitution, deletion, insertion, gene translocation and DNA structure conversion (Rabbitts et al., 1980; Nordheim et al., 1983; Reddy, 1983; Lancillotti et al., 1987; Zarbol et al., 1985). It has been suggested that these modifications may induce the oncogene to be associated with the nuclear matrix and activate the gene expression (Low et al., 1986; Siebenlist et al., 1984). In summary, this evidence suggests but by no means proves that the nuclear matrix plays an important role in carcinogenesis.

H. Summary

Results from different laboratories described in the above section suggest a major role for the nuclear matrix in many cellular functions. Those reported functional proteins whether are the components of the matrix or just associated or even attached during the isolation, at present time, are difficult to distinguish. In many cases the data only indirectly support the function suggested. In the future, new techniques and more experiments are need to clarify and get more insight to those mechanisms.

III. Nucleosome Positioning and Nucleosome Organization

A. Nucleosome Positioning

In eukaryotic nuclei, the nucleosomal core particle is the basic subunit of the tandemly repeated organization of chromatin and consists of 146 bp of DNA wrapped in 1.75 turns of a left superhelix around a core of eight histones. Core particles are connected to one another by linker DNA which varies from 15 to over 100 bp in length (see van Holde, 1988). In some instances, it seems that the nucleosome positioning on DNA is not random; at least a

fraction of nucleosomes is clearly positioned in a precise way with respect to the DNA sequence. To determine the nucleosome positioning on a specific gene has become a matter of interest for understanding the structure of the eukaryotic chromosome and the relationship of that structure to the regulation of such fundamental processes as transcription, DNA repair and replication (Travers, 1987).

Several examples have been reported that preferentially positioned core particles can form in in vitro reconstitutions on short DNA fragments such as the sea urchin 5S rRNA gene (Drew and Calladine, 1987; Simpson et al., 1985), mouse satellite DNA (Linxweiler et al., 1985; Boeck et al., 1984), E. coli DNA fragments (Ramsay et al., 1984), plasmid DNA (Drew and McCall, 1987), the chicken beta-globin gene promoter (Ketalas et al., 1988) and the SV40 enhancer and termination regions (Clarke et al., 1985). Nucleosome positioning in vivo also has been reported by many investigators with using a method called " indirect end labelling" (Wu, 1980) which will be described in CHAPTER 4.

Zhang et al. (1984) have found that nucleosomes are positioned on mouse satellite DNA in multiple highly specific frames. Shiomada et al. (1986) have determined the chromatin structure of the human DHFR gene promoter and found that it contains positioned nucleosomes and multiple protein-binding sites.

The DNA properties of nucleosome positioning sequences have been studied by a number of researchers (Travers, 1987; Satchwell et al., 1986; Zang and Hoerz, 1984). The general conclusion is that nucleosome positioning may be largely determined by the sequence dependent variation in the mechanical properties, such as bendability of the DNA

molecules. Further by studying the assembly and polarity of nucleosomes in chicken erythrocyte chromatin, Satchwell and Travers suggested that torsional, as well as axial, flexibility of DNA may be a determinant of nucleosome positioning (Satchwell and Travers, 1989).

Despite the numerous examples and studies of nucleosome positioning, there is still little understanding of the specific DNA features that make a histone core choose a particular position on a DNA sequence. No "concensus" sequences have been identified, and it seems doubtful that any will be, considering the fact that nucleosomes are positioned on many, very different sequences. The results of satchwell et al. (1986), and Sachwell and Travers (1989) suggest the existence of periodic determinants in nucleosomal DNA. However, as will be pointed out in Chapter 4, their methods are flawed, and a part of this thesis research is devoted to a further investigation of this problem.

B. Nucleosome Organization

The nucleosome has been suggested to be involved in generating the higher orders of structure that fold DNA into the extremely compact form found in the nucleus (see reviews by Felsenfeld, 1978; Pederson et al., 1986; van Holde, 1988). There are at least four models to explain how the 10 nm filament folds to form the 30 nm fiber. (a) A single start contact solenoidal model proposed by Finch and Klug (1976), Thoma et al. (1979) and McGhee et al. (1983). This structure has a helical repeat of 6 nucleosomes, a diameter of 30 nm, and a pitch of 11 nm. (b) A twisted ribbon model proposed by Worcel et al. (1981) and Woodcock et al. (1984). This structure has a helical repeat of 18 nucleosomes, a diameter of 30 nm and a pitch of 32 nm. (c) A cross-linker model proposed by Williams et

al. (1986). This structure has a helical repeat of 18 nucleosomes, a diameter of 31 nm, and a pitch of 26 nm. (d) A superbead model proposed by Zentgraf & Franke (1984). This structure might be formed by dislocations of a model such as (c) .

Even though it is too early to draw a general picture of chromatin folding, it is clear that H1/H5 plays an important role in stabilizing the 30 nm fiber (see Felsenfeld, 1978, Thomas et al., 1985; Pederson et al., 1986). By carrying out the reconstitution of chromatin with different H1 histone types, Stein and Mitchell (1988) were able to generate different nucleosome spacing periodicities. In CHAPTER 4, *in vivo* data are presented to show that nucleosome spacing periodicities are cell type-dependent, possibly as a consequence of the existence of different H1 histone variants, such as the H5 in erythrocyte. Similar results also have been demonstrated in sea urchin sperm chromatin after fertilization (Poccia et al., 1984).

The contact between H5 and nucleosomal DNA has been studied *in vitro* by Drew and McCall (1987). They suggested that a long carboxyl-terminal domain (or "tail") of each H5 protein associates with the DNA between nucleosome cores, while its central globular domain (or "head") lies close to the dyad axis of the nucleosome. This is confirmed by a footprinting study (Staynov and Crane-Robinson, 1988), in which complete protection was found for site S7 on the dyad axis and the globular domain of H1/H5 was shown to be responsible for that protection.

The core histone "tails" have also been shown to participate in the stabilization of the 30 nm solenoid structure (Allan et al., 1982). The refolding of the 10 nm filament into the 30 nm fiber also requires the presence of

cations, such as sodium ion and magnesium ion, which act as general DNA counterions (Widom, 1986). The concentration of any cation required to induce refolding is greatly dependent on the valence of that cation. A higher concentration of cations also induces the 30 nm fiber to aggregate and precipitate *in vitro*.

It is of interest to note that there is a correlation between nucleosome organization and gene expression. Cohen and Sheffery (1985) report that chromatin insolubility and nucleosome disruption are characteristic of the transcribed domain. Nontranscribed, flanking sequences are soluble and clearly organized into nucleosomes. Prior et al. (1983) also reported reversible changes in nucleosome structure between transcriptionally active and inactive states of *Physarum sp.* rDNA chromatin. Delcuve et al. (1988) have shown that the vitellogen and ovalbumin genes (inactive) form a canonical nucleosome repeat pattern in mature and immature chicken erythrocyte cells. In contrast, the beta-globin gene and histone H5 genes (potentially active) lack a distinct nucleosomal repeat pattern in these cells. Similar changes in nucleosome arrangement have also been found to accompany the program of gene expression in murine fibroblast protooncogenes c-fos and c-myc, in which nucleosome unfolding reflects the timing and extent of transcription of associated DNA sequences (Drew and McCall, 1987). Nucleosome loss or depletion by protein factors also has been suggested to be involved in transcription initiation and subsequent elongation *in vivo* (Han and Grounstein, 1988; Lewis et al., 1988). This conversion from a

positioned nucleosome array to a more accessible conformation, often characterized by nuclease hypersensitivity, was found to depend on the DNA sequence, protein factors and the nature of the inducing agents (Fedor and Konberg, 1989; Pavlovic et al., 1989).

Experiments also have been done to show that nucleosome structure changes during the cell cycle (Moreno et al., 1986), embryogenesis (Wu and Simpson, 1985), differentiation (Chou et al., 1986), and replication (Cusick et al., 1984). All evidence seems to suggest that nucleosome structure is dynamic and varies among different physiological conditions.

IV. Scope of Thesis

The research described in this thesis is aimed at trying to further our understanding of the structure and function of chromatin at the nucleosome and nuclear matrix levels.

CHAPTER 2 describes a study of the relationship between gene activity and nuclear matrix association. Several tissues from different species have been used, and DNA from nuclear matrix bound and unbound fraction has been isolated, using two different methods, namely high salt (2 M NaCl) and low salt (15 mM LIS) extraction buffer. The matrix-enriched fraction of specific gene sequences was checked by Southern blotting. Both positive and negative results with the chicken TK gene, beta-globin gene and mouse DHFR gene have been obtained. The working hypothesis is that the association of a specific gene with the nuclear matrix is mainly caused by specific DNA binding proteins which partition to the nuclear matrix fraction. But whether these proteins are true <u>in vivo</u> components of the nuclear matrix or the association is an artifact of the

isolation, cannot be determined with certainty at this point.

CHAPTER 3 describes a study using adenovirus as a model to investigate the role which the nuclear matrix may play in virus infection and replication. In this section it is demonstrated that the origins of replication of adenovirus are strongly associated with the nuclear matrix and that matrix proteins are involved in this association. Possible chromatin structure around the origin of replication of adenovirus also has been studied by using *in vitro* reconstitution; this region is found to be capable of forming the nucleosome structure.

CHAPTER 4 describes results from analyzing a number of randomly selected nucleosomal DNA sequences and from determining the nucleosome spacing around chicken the beta-globin 5' flanking region. By modifying the "indirect end labeling method" (Wu, 1980), some features of the *in vivo* chromatin structure in the 5' flanking region of the chicken beta-globin gene were investigated . In a separate study, randomly picked nucleosomal DNA sequences were cloned into either M13 mp18 or pUC19 vectors. From this, whether any sequence preference, regular sequence periodicities, or specific DNA secondary structure observed in nucleosomal DNA will be determined.

CHAPTER 2: Nuclear Matrix Association and Gene Transcriptional Activity

Introduction

It has been suggested that one of the functions of the nuclear matrix may involve the regulation of eukaryotic gene expression (for recent reviews see Huckaby et al., 1985; Nelson et al., 1986). There are three lines of evidence that support the above hypothesis. First, DNA sequences of many actively transcribed genes appear to be associated with the nuclear matrix (Ciejek et al., 1983; Razin et al., 1985; Robinson et al., 1983; Small et al., 1985; Chiu et al., 1986; Keppel, 1986). Second, the nuclear matrix contains in its associated materials, most portions of newly synthesized pre-mRNA (Jackson and Cook, 1985; Friese et al., 1987; Gallinano et al., 1983). Third, some protein components of the transcriptional machinery are strongly associated with the nuclear matrix (Earnshaw et al., 1985; Berrios et al., 1985; Jackson and Cook, 1985; Adhya et al., 1986; Kaufmann et al., 1986; Kumara-Siri et al., 1986). Nevertheless, there is also a considerable amount of literature to indicate that some active genes are not closely associated with the matrix (Cockerill and Garrard, 1986; Hentzen et al., 1984; Kirov and Tsanev, 1986; Mirkovitch et al., 1984; Phi-van and Stratling, 1988). Furthermore, it can be argued that apparent association of pre-mRNA or components of the transcriptional machinery with the matrix are artifacts of isolation.

Since the discrepancy of the results from different laboratories may be caused by the differences in isolation

methods, animal systems, tissues and genes, it is important to investigate the problem systematically to further examine the relation between nuclear matrix association and gene transcriptional activity, using different conditions of isolation on a variety of systems and genes.

The chicken thymidine kinase gene (TK) and mouse dihydrofolate reductase gene (DHFR) are so called "housekeeping" genes, expressed in most tissues. The chicken beta-globin gene, on the other hand, is a tissue specific gene. By studying these genes in tissues from different developmental stages and cells of different differentiation states in cell culture, we have found that there is in fact no simple relation between nuclear matrix association and gene transcriptional activity. In this study, it was found that by simply changing some experimental factors, one can get opposite results even in the same system and with the same gene. This may explain why so many seemingly contradictory data have been reported in the literature.

Materials and Methods

Animals and cell culture

Adult chickens and 15 day chicken embryos were obtained from the Department of Poultry Science, Oregon State University. Mouse erythroleukemia (MEL) cells were grown in suspension cultures with FD medium supplemented with 10% (v/v) fetal bovine serum. Differentiated MEL cells were induced by adding 2% (v/v) dimethyl sulfoxide (DMSO) and 50 mg/ml bovine serum albumin (fraction V, Sigma) for 5-6 days.

Isolation of nuclei and nuclear matrix

Chicken erythrocyte (RBC) nuclei were isolated as described (Ausio et al., 1989); and a similar method was employed for embryo myoblast cells nuclei, MEL cells nuclei and HeLa cells nuclei (Mirkovitch et al., 1984). The methods used to isolate the nuclear matrix were modified from Mirkovitch et al. (1984). For high salt buffer extraction, 10 OD unit (A_{260}) of nuclei were suspended in 300 ml digestion buffer, and then digested with 250 units of a specific restriction enzyme at 37 °C for 4 hrs. After digestion the sample was extracted with the same volume of 2x high salt buffer, washed with digestion buffer three times, then redigested with 250 units of the same restriction enzyme for another 2 hrs, reextracted with high salt buffer and washed with digestion buffer. The residue was termed <u>the nuclear matrix associated DNA fraction</u>. All the supernatant was pooled from the above preparation for further DNA purification. The nuclear matrix associated DNA and <u>the supernatant fraction DNA</u> were digested with proteinase K and RNase ($A\&T_1$), and then phenol/ chloroform extracted and alcohol precipitated. DNA concentration was determined by using A_{260} nm reading or

reacting with Hochest dye 33258 for fluorescence measurement. For the low salt buffer extraction method with lithium di-iodosalicylate, either the method described as Mirkovitch (1984) was employed, using only one restriction enzyme digestion, or the same method as used in the high salt buffer method was employed, but using low salt buffer instead.

Southern blotting

DNA, 5-20 μg, was taken from each fraction, redigested with specific restriction enzymes, run on 0.8% or 1% agarose gel, stained with ethidium bromide, transferred to nitrocellulose paper, baked, prehybridized, and then hybridized with nick-translated probe as described by Maniatis et al. (1982). The blots were exposed to X-ray film in -80 °C freezer overnight.

Nuclear matrix proteins isolation and Sodium Dodecyl Sulfate (SDS) protein gel analysis

Nuclear matrix proteins were isolated by the method described by Cockerill and Garrard (1986). Briefly, 20 A_{260} of nuclei from either chicken erythrocyte or HeLa cells were suspended in RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, 0.5 mM phenyl methyl sulfonyl flouide, pH 7.4) with 0.25 M sucrose and 1 mM $CaCl_2$, and digested with 200 μg/ml DNase I, 250 μg/ml RNase ($A\&T_1$) for 1 hour at 37 °C. After digestion, nuclei were extracted twice with high salt buffer or low salt buffer, washed once with RSB buffer, and then resuspended in RSB buffer. Samples were mixed with one volume of 2x SDS gel loading buffer, boiled for 5 min, chilled on ice and loaded into 12% SDS polyacrylamide gel (PAGE) as described in Ausio et al. 1989.

Results

(1) The composition of nuclear matrix proteins varied between two different cell types and two different isolation methods

Nuclear matrix proteins isolated from HeLa cells and chicken erythrocytes were analyzed on a 12% SDS PAGE. Results in Figure I-5 show that the composition of nuclear matrix proteins is different from these two cell types. The intensity of the proteins is also different from the two different isolation methods. Histone proteins are not completely depleted by the low salt buffer when compared with the high salt buffer. Some common protein patterns, around 43KD-70KD which possibly represents Lamin A, B, C, and other matrix proteins, are shown in both cases. The protein patterns shown in this gel are in accord with the results obtained using similar methods in other laboratories (Lebkowski and Laemmli,1982) .

(2) The chicken beta-globin gene and the thymidine kinase (TK) gene are equally distributed in both the nuclear matrix and the supernatant fractions of red blood cell and embryo myoblasts

Nuclear matrix associated DNA and supernatant DNA were isolated with either high salt buffer or low salt buffer. For comparison, two different tissues were used: chicken RBC and 15 day embryo myoblast cells, and two kinds of genes: the beta-globin gene (a tissue specific gene expressed only in erythroid cells) and the TK gene (a "housekeeping" gene expressed in all cell types), to hybridize the same blot. Figure I-1A, 1B, shows that the chicken beta-globin gene and TK gene were equally distributed in both nuclear matrix DNA and supernatant fraction DNA isolated from erythrocytes with either high

salt buffer or low salt buffer extraction. The LS lane appears more intense but this is because of heavier loading, as can be seen from the ethidium bromide staining. The same result was seen in Figure I-1C and 1D, except high salt buffer extracted nuclear matrix associated DNA was less intense with both gene hybridizations. The chicken TK gene hybridized with two bands in the erythrocyte digests, but with only one band in embryo myoblast. This is because of the existence of methylation at one of the BamH I sites in erythrocytes. This methylation has been confirmed by partial digestion of the BamH I-digested total DNA with Hind III (data not shown).

(3) The mouse dihydrofolate reductase (DHFR) gene was equally distributed in both nuclear matrix and supernatant fractions in MEL cells as determined with 3.4 Kb EcoR I fragment probe; however, different results were obtained when a cDNA probe was used.

The mouse DHFR gene is a house-keeping gene. It is highly expressed in growing MEL cells, but not in cells which could be induced to differentiate by treatment with 2% (v/v) DMSO and 50 mg/ml BSA (fraction V, Sigma).

Figure I-2A, shows that DHFR as probed by the 3.4 Kb fragment appears to be equally distributed in both nuclear matrix and supernatant fractions. However when a DHFR cDNA was used for the probe, two bands were found, at 2.9 Kb and 2.7 Kb. Both of these were highly associated with the nuclear matrix of growing MEL cells, but not with the nuclear matrix of differentiated MEL cells. This was true in the high salt buffer extraction case, even when different restriction enzyme were used. For example, results with BamH I are shown in Figure I-3 instead of the data from EcoR I digestion shown in Figure I-2.

The results are summarized in Table I-1. In the 'gene activity' column (reference for each gene see Discussion section), +/_ stands for cases where gene expression could be detected by a nuclei run-off experiment but not by Northern blotting. + stands for a situation in which the gene is sufficiently active to be detected by Northern blotting. +++ indicates that gene is highly expressed. In the other four columns, +, ++, +++ stands for the relative intensity on the Southern blot.

Discussion

This study demonstrates that many experimental factors could affect the results of tests for correlation between nuclear matrix association and gene activity. In Figure I-4 some of the factors, which could cause the discrepancy among different laboratory results, are listed.

From the summary of the results in Table 1, we may draw the following conclusions: First, most results from the two different buffer isolation methods were the same except in two cases: MEL cells extracted with low salt buffer and chicken embryo myoblast extracted with high salt buffer. A possible reason for the difference is that intracellular endonuclease could digest the DNA unequally during the different periods required for isolation in the two methods. Another possible reason may be due to different affinity of DNA binding proteins between the two methods.

Next, no good correlation could be found between nuclear matrix association and gene transcriptional activity. For example, the chicken TK gene was highly expressed in embryo myoblast cells (Merrill et al., 1984), but not in adult erythrocyte cells. Yet the TK gene appears to be equally distributed in both nuclear matrix and supernatant fractions in these two cell types. The same was also true with the beta-globin gene and the mouse DHFR gene.

Third, probe size does seem to affect the results in the mouse DHFR case, in which a cDNA probe gave positive results for nuclear matrix association, but use of a 3.4 Kb fragment for the probe gave negative results. Such opposite results may be caused when a small probe is used which is far from the sequence region bound to nuclear

matrix proteins, as contrasted to a long probe which may cover these regions as well. This may indicate a basic ambiguity in all studies of this kind- "association" of a gene with matrix may be only through a long sequence of flanking DNA.

Finally, it is of interest to note that in many cases a gene sequence was equally distributed in both the nuclear matrix and the supernatant fraction. A possible explanation of these results is that DNA is randomly partitioned to both fractions unless it is bound to some nuclear matrix associated proteins which would induce unequal distribution. Specific DNA sequences can be bound by many nuclear matrix associated proteins, such as topoisomerase II (Berrios et al., 1985; Earnshaw et al., 1985; Cockerill and Garrard, 1986), glucocorticoid and steroid hormone receptor (Kaufmann et al., 1986; Buttyan et al., 1983), DNA methylase (Burdon et al., 1985), DNA polymerase (Tubo et al., 1987) and primase (Collins and Chu, 1987).

The mouse DHFR gene genomic structure contains more than 150 Kb which is likely to include the so-called MAR (matrix associated region) or SAR (scaffold associated region) either of which could be bound by nuclear matrix associated proteins as described above. The MAR or SAR sequences (Blasquez et al., 1989) are about 200 bp long, AT-rich (ca 70%), contain topoisomerase II consensus sequences, and often reside near cis-acting regulatory sequences. Their binding sites are abundant (>10,000 per mammalian nucleus). One important aim of future experiments would be to more precisely define the specific binding site and determine what proteins cause this association.

Since DNA composition can affect the fluorescence reading and nuclear matrix associated DNA contains many repetitive sequences (Goldberg et al., 1983; Endoh and Okada, 1986), such as the KpnI family (Chimera and Musich, 1985), the mouse satellite and human Alu family (Small et al., 1982), a possible error will be induced with using Hochest 33258 for DNA concentration determination. Indeed, we have found that determination of DNA concentration from fluorescence readings usually results in unequal loading, as can be seen from the ethidium bromide staining.

Figure I-1. Chicken beta-globin gene and thymidine kinase gene in nuclear matrix associated fraction and supernatant fraction.

A, B-Nuclear matrix associated DNA and supernatant DNA isolated from chicken RBC after digestion with BamH I. Each lane is loaded with 10 µg DNA
C, D-Nuclear matrix associated DNA and supernatant DNA isolated from chicken embryo myoblast cells after digestion with BamH I. Each lane is loaded with 20 µg DNA
A, C-Hybridized with nick-translated probe of chicken beta-globin gene 6.2 Kb EcoR I fragment
B, D-Hybridized with nick-translated probe of chicken TK gene Kpn I-Hind III fragment
T-Total DNA
H, L-Nuclear matrix associated DNA isolated by high salt buffer extraction and low salt buffer extraction
HS, LS-Supernatant DNA isolated by high salt buffer extraction and low salt buffer extraction

Figure I-1

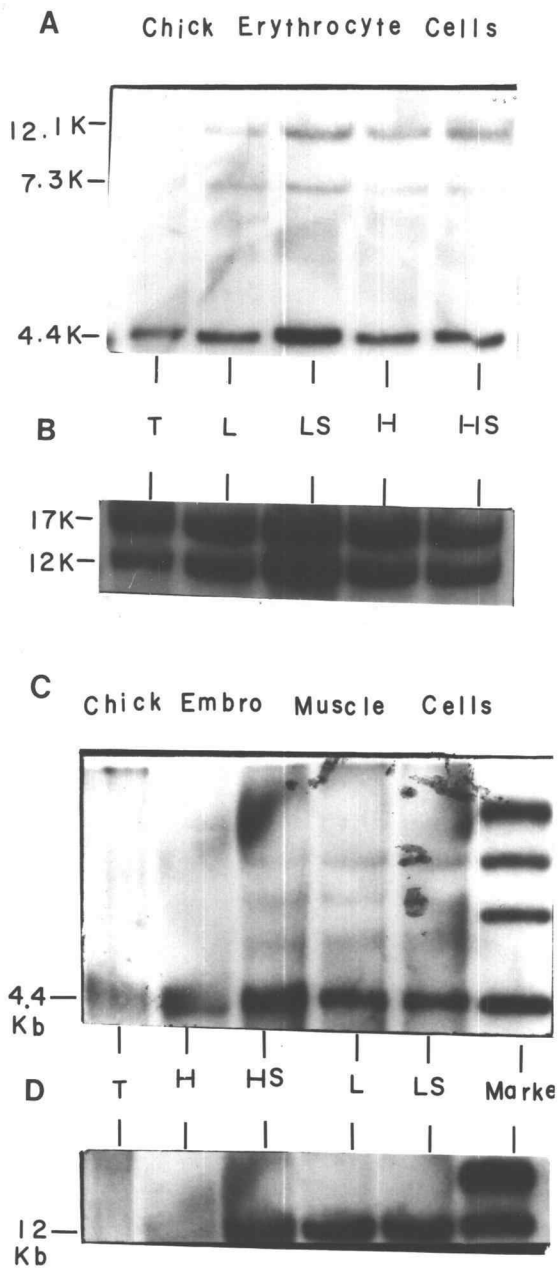

A
Chick Erythrocyte Cells
12.1K
7.3K
4.4K
T
L
LS
H
HS
B
17K
12K
C
Chick Embro Muscle Cells
4.4
Kb
T
H
HS
L
LS
Marke
D
12
Kb

Figure I-2. DHFR gene in nuclear matrix associated fraction and supernatant fraction isolated from MEL cells and DMSO induced MEL cells.

A, B-Nuclear matrix associated DNA and supernatant DNA isolated from MEL cells and DMSO induced MEL cells after digestion with EcoR I. Each lane is loaded with 10 µg DNA
A-Hybridized with nick-translated probe of mouse DHFR 3.4 Kb EcoR I fragment
B-Hybridized with nick-translated probe of mouse DHFR cDNA plasmid pSV 2
H, L-Nuclear matrix associated DNA isolated by high salt buffer extraction and low salt buffer extraction
HS, LS-Supernatant DNA isolated by high salt buffer extraction and low salt buffer extraction

Figure I-2

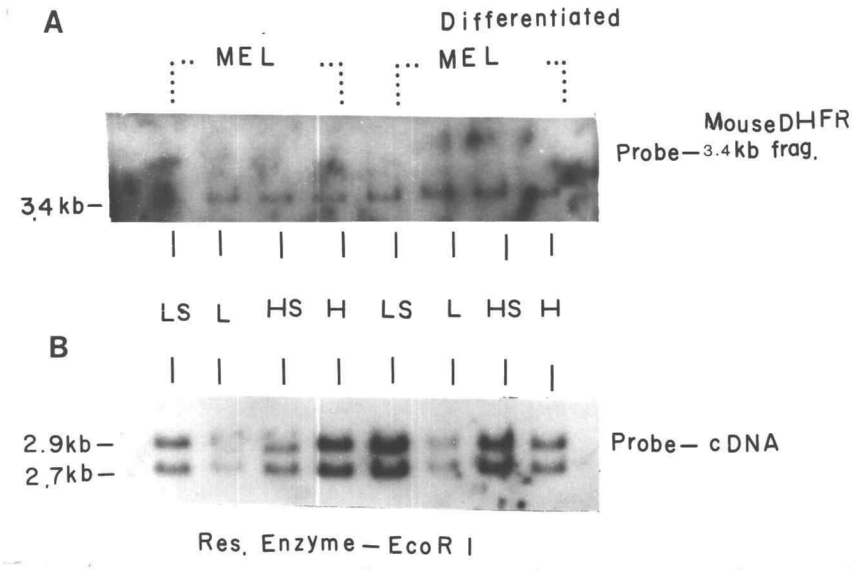
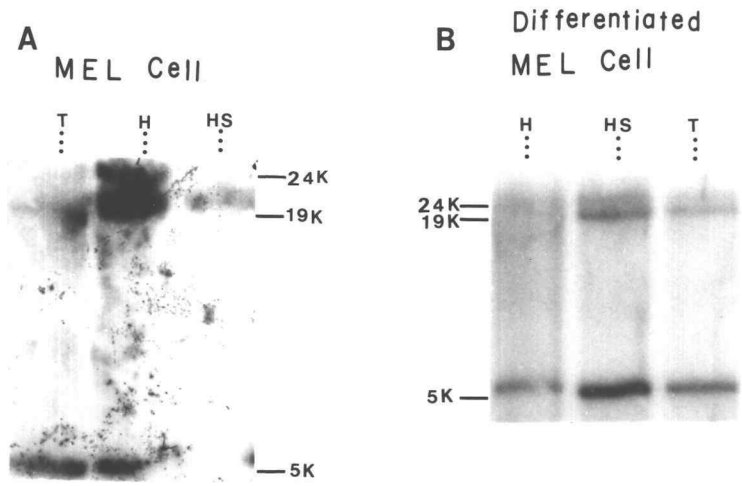

A
Differentiated
MEL
MEL
Mouse DHFR
Probe—3.4 kb frag.
3.4 kb—
LS L HS H LS L HS H
B
2.9kb—
2.7kb—
Probe— c DNA
Res. Enzyme — Eco R I

Figure I-3. DHFR gene in nuclear matrix associated fraction and supernatant fraction isolated from MEL cells and DMSO induced MEL cells.

A, B-Nuclear matrix associated DNA and supernatant DNA isolated from MEL cells (A) and DMSO-induced MEL (B) cells after digestion with BamH I, each lane loaded 10 μg DNA, hybridized with nick-translated probe of mouse DHFR cDNA plasmid

Figure I-3



Probe — Nick-Translated cDNA
Mouse DHFR Gene
Res. Enzyme — Bam H I

Figure I-4. Experimental factors that could cause the discrepancy among various laboratory results

Figure I-4

Experimental Factors That May Cause the Discrepancy Among Various Laboratory Results:

(1). Cell types from different tissues, species, cell cycles.

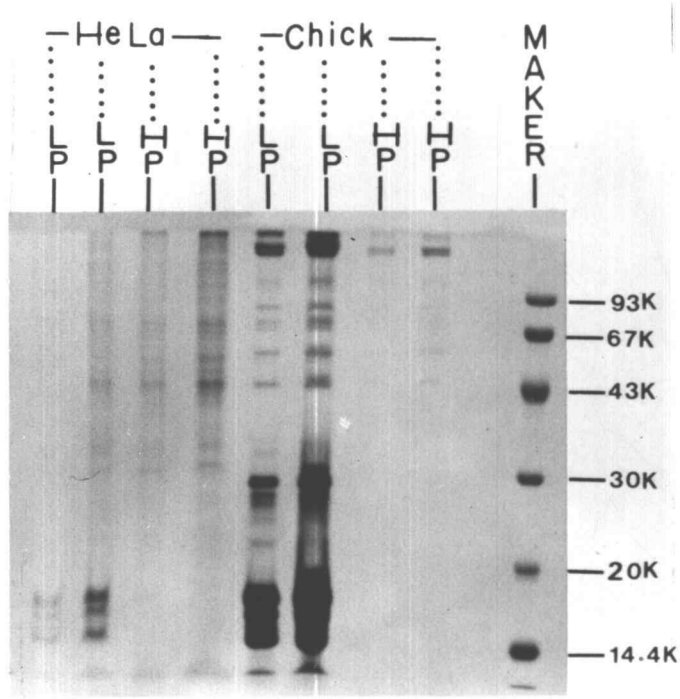
(2). Variation of the isolation method.

(3). Probe size and transfer efficiency.

(4). Method for DNA concentration determination and unequal loading.

Figure I-5. 12% SDS protein PAGE of the nuclear matrix proteins from chicken erythrocyte and HeLa cells.
HP=nuclear matrix protens isolated from high salt extraction method; LP= nuclear matrix proteins isolated from low salt extraction method.

Figure I-5



Nuclear Matrix Protein
12% SDS PAGE

Table I-1. The summary of the results from Figure I- 1 to 3

Nuclear Matrix Association and Gene Transcription Activity						
Gene	Cell Type	Gene Activity	High Salt Extraction Method		Low Salt Extraction Method	
			Nuclear Matrix Fraction	Supernatant Fraction	Nuclear Matrix Fraction	Supernatant Fraction
TK	Chicken Erythrocyte	+/-	+	+	+	+
	Chicken Embryo Muscle	+++	+	++	+	+
Globin	Chicken Erythrocyte	+	+	+	+	+
	Chicken Embryo Muscle	+/-	+	++	+	+
DHFR	MEL c DNA Probe	+++	+++	+	+	++
	Differentiated MEL	+/-	+	+++	+	+++
	MEL Eco R I 3.4 Kb	+++	+	+	+	+
	Differentiated MEL	+/-	+	+	+	+

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CHAPTER 3: Adenovirus Origins of Replication Are Associated with the Nuclear Matrix

Introduction

Much evidence has been presented to support the hypothesis that the nuclear matrix may be involved in eukaryotic DNA replication (Farnham and Schimke, 1985; Jones and Su, 1987; Buongiorne-Nardelli et al., 1982; Volgelstein et al., 1980; Jackson et al., 1984; van der Velden et al., 1984; Pardoll et al., 1980; Hunt and Volgelstein, 1981; Berezney and Buchholtz, 1981; Aelen et al., 1983; Smith et al., 1984; Foster and Collins, 1986; Carri et al., 1986; Razin et al., 1986; Dijkwel et al., 1986). However, there are also reports in opposition to this hypothesis (Djondjurov et al., 1986; Todorova and Russev, 1984). The main criticism is that the isolation of nuclear matrix using very harsh treatment, such as high salt buffer and strong detergent, may disrupt the nuclear structure and cause experimental artifacts. The research described in this thesis has used both high salt and low salt buffer for the extraction in an attempt to circumvent problems caused by hypertonic buffer.

The replication of adenovirus has been studied *in vivo* and *in vitro* (for a recent review, see Campbell, 1986). Six proteins are required for adenovirus replication *in vitro*: the 59 KD adenovirus DNA binding protein, the 80 KD adenovirus preterminal protein, the 140 KD adenovirus DNA polymerase, host nuclear factor I, host nuclear factor II and host nuclear factor III (see references in Campbell, 1986). Another requirement is the viral origin of replication, a sequence of about 50 bp, found in both ends of the inverted repeat (see references in Cambell, 1986). An aim of the present research is to investigate whether

the adenovirus origin of replication is associated with the nuclear matrix, and if so, to determine which proteins might cause the association.

It has been reported that 10-25% of SV40 minichromosomes contain a sequence around the origin of replication that is nucleosome-depleted (Saragosti et al., 1980; Li et al., 1986). This result suggests that sequences near the adenovirus origin of replication might also specifically exclude nucleosomes. In this research, two techniques have been used to examine this question: isolation of nucleosomes from *in vivo* and nucleosome reconstitution *in vitro* on this region.

Materials and Methods

Cell culture and virus infection

HeLa S3 cells were grown in suspension culture using medium F-13 supplemented with 7% fetal calf serum and infected with type 5 adenovirus as previously described (Bodnar and Pearson, 1980).

Nuclei and nuclear matrix isolation

Nuclei and nuclear matrix isolation was carried out following a method modified from that in Mirkovitch et al. (1984). For high salt buffer extraction, 10 A_{260} units of nuclei were suspended in 300 µl digestion buffer and digested with 250 units of specific restriction enzyme at 37 °C for 4 hrs. After digestion, the nuclei were extracted with the same volume of 2x high salt buffer, washed with digestion buffer three times, then redigested with 250 units of a specific restriction enzyme for another 2 hrs. The nuclei were then extracted again with high salt buffer and washed with digestion buffer. The pellet was termed the nuclear matrix associated DNA fraction. The supernatants were pooled from the above preparation for purification (see below) of a non-matrix-associated supernatant fraction. The nuclear matrix associated fraction and the supernatant fraction were each digested with proteinase K and RNase ($A\&T_1$), extracted with phenol/ chloroform /isoamyl alcohol (25:24:1), and precipitated with ethanol. DNA concentration was determined by using absorbance at 260 nm or by fluoresence after reacting with Hochest dye 33258. The low salt buffer extraction method with lithium di-iodosalicylate salt, followed either the method described by Mirkovitch et al. (1984) using only one restriction enzyme digestion or simply followed the high salt buffer method substituting low salt buffer instead.

Restriction enzymes were from Bethesda Research Laboratories (BRL) or New England BioLabs and used as suggested by manufacturers. Nuclear matrix proteins were isolated by the method described by Cockerill and Garrard (1986). Briefly, 20 A_{260} of nuclei were suspended in RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, 0.5 mM phenyl methyl sulfonyl flouide, pH 7.4) with 0.25 M sucrose and 1 mM $CaCl_2$, and digested with 200 µg/ml DNase I, 250 µg/ml RNase (A&T_1) for 1 hour at 37 °C. After digestion, nuclei were extracted twice with high salt buffer, washed once with RSB buffer, and then resuspended in RSB buffer.

Band shift experiment

A plasmid, pT4, obtained from Dr. George D. Pearson, containing the adenovirus origin of replication, was digested with EcoR I, filled-in with [a-^{32}P] dATP using Klenow fragment of E.coli DNA polymerase I, and then redigested with Ssp I after heat denaturing the polymerase. The EcoR I-Ssp I fragment (338 bp) was incubated either with 1.5 µg nuclear matrix proteins isolated from different cells, or with histone octamer proteins for the control. Lamda DNA (1.5 µg) or unlabeled probe was also included for competition. The reaction mixture was run on native 4% polyacrylamide gel (PAGE) (prepared and run as described in Ausio et al., 1989) and exposed to X-ray film for 3 hrs at -70 °C.

UV cross-linking experiment

The EcoR I-Ssp I 338 bp fragment, which was labelled with [a-^{32}P] dTTP by a random primer labelling method (Boehringer Mannheim), was incubated on ice for 30 minutes with 1.5 µg nuclear matrix proteins (isolated from adenovirus-infected HeLa cells with high salt buffer), and then UV cross-linked under 280 nm light (11 cm light path, 25 µl light exposure volume) at 10 °C for 0, 0.5, 2, or 5

minutes. After exposure, the mixture was digested with 2 units DNase I at 37 °C for 50 minutes, washed with the digestion buffer three times, resuspended with sodium dodecyl sulfate (SDS) sample buffer, heated at 95 °C for 5 minutes, cooled with ice, then loaded on 12% SDS PAGE (prepared and run as Ausio et al., 1989), and exposed to X-ray film after electrophoresis.

Nucleosome isolation and characterization

Nucleosomes were isolated from HeLa S3 cells and adenovirus- infected HeLa S3 cells as described (Sturman et al., 1988, also see CHAPTER 4). Native particle gels and SDS protein gels were run using the method described in Sturman et al. (1988). Histone octamers were prepared as described in Bode and Mass (1988).

In vitro nucleosome reconstitution

The adenovirus EcoR I-Ssp I fragment was labeled by filling-in with [a-^{32}P] dATP using Klenow fragment of *E.coli* DNA polymerase I (BRL) as described in Kurl et al. (1988) and in the previous section. Nucleosome reconstitution *in vitro* was carried out by the histone octamer exchange method described by Moyer (1988). Specifically, labelled DNA was incubated with chicken erythrocyte nucleosomes (1 molecules:100 molecules) in 0.8 M NaCl/ TE buffer, dialysed against 0.6 M NaCl/TE buffer overnight, then against 0.1 M NaCl/TE buffer for at least 4 hrs.

Southern blotting

Southern blotting was performed as described in Chapter 2. All the probes were prepared by using a nick-translation kit (BRL).

Results

The origin of replication of adenovirus is associated with the nuclear matrix

To avoid artifacts which might be caused by the use of a particular isolation method, nuclear matrix associated DNA and supernatant fraction DNA were isolated by using both high salt and low salt buffer (25 mM lithium di-iodo salicylate salt) for the extraction. Results are shown in Figure II-1. Both methods show that the origin of replication of adenovirus is partitioned to the nuclear matrix fraction. This is true for 24 hrs or 48 hrs of postinfection.

It is of interest to note that there are three bands on the Southern blot. A major band was 451 bp in length and two minor bands were 1120 bp and 1300 bp. From the restriction map of type 5 adenovirus digested with Pvu II, two bands (451 bp and 1120 bp) were expected to hybridize with the probe. These fragments contain the inverted repeat located at both ends of linear adenovirus DNA. The source of the third band is at present unclear. Nevertheless, the existence of the third band does not compromise the major conclusion: the origin of adenovirus replication in infected cells is closely associated with the nuclear matrix.

Band shift experiment

<u>In vitro</u> binding of nuclear matrix proteins with the origin of replication of adenovirus was studied by using the electrophoretic band shift method (Speck and Baltimore, 1987). Results are shown in Figure II-2. The 3' end-labeled origin probe (EcoR I- Ssp I 338 bp fragment) was incubated with nuclear matrix proteins, either isolated

from HeLa cells or adenovirus-infected HeLa cells with both high salt buffer and low salt buffer. Incubation with histone octamer was used as a control. Nuclear matrix proteins isolated by either method were able to induce a specific band shift which could be distinguished from the nonspecific shift caused by histone-binding.

These results and results from other laboratories (Cook, 1988) indicate that some host-coded proteins, (and possibly virus-coded proteins as well) in the nuclear matrix fraction could bind with the origin of replication of adenovirus *in vitro*. By using the cold origin-containing fragment to compete with hot probe, the band position could be specifically changed (see arrow in Figure II-2). Displacement of the band from the original position to a new position, still different from free DNA, indicates that there are multiple factors capable of binding this sequence.

UV cross-linking experiment

In an attempt to determine which proteins could bind with the origin of replication of adenovirus, the *in vitro* UV cross-linking experiments were performed. A mixture of labeled probe (EcoR I-Ssp I fragment labeled with [a-^{32}P] dTTP using the random primer method) and nuclear matrix proteins was exposed to UV light (280 nm) for different times. After digestion with DNase I and removal of noncovalent-bound probe, the nuclear matrix protein were analyzed with SDS protein PAGE. Since only the proteins containing covalently bound probe would show on the gel, it should be possible to determine their molecular weights and to characterize them.

From Figure II-3, at the exposure time 5 minutes, some nonspecifically bound proteins are shown in both the control and sample lanes. It is of interest to note that

only in the origin-containing sample lane one band of approximately 140 KD was found on the autoradiograph. This band does not appear on the line which only HeLa cell matrix proteins were used. This evidence shows that this protein is a virus-coded protein.

The chromatin structure around the origin of replication of adenovirus *in vivo* and *in vitro*

Bulk nucleosomes, isolated from HeLa cells and adenovirus infected HeLa cells, have been characterized by native particle PAGE and SDS protein PAGE; they are indistinguishable on the basis of molecular weight. The nucleosomal DNA from uninfected HeLa cells did not cross-hybridize to adenovirus DNA, as shown in Figure II-4A. However when the adenovirus infected nucleosomal DNA was used as a probe, some evidence was found for nucleosomes in adenovirus DNA (Figure II-4B).

In Figure II-4B, the lane containing Hind III digested adenovirus DNA, two very faint bands at 1004 bp and at 451 bp position are observed. In addition, a very faint band at the 338 bp position in lane pt4 is found. All this evidence shows that not only the bulk adenovirus DNA, but also the DNA close to the origin, will be able to form nucleosome in vivo. These data must be interpreted with caution. Since only a small amount of DNA is necessary for the probe to have a positive result, it may be that only small portions of adenovirus DNA can form nucleosome structure *in vivo*.

Figure II-5 shows that the probe containing the adenovirus origin of replication, the EcoR I-Ssp I 338 bp fragment of pT4, could indeed form a nucleosome structure in reconstitution experiments *in vitro*.

Adenovirus DNA is associated with the nuclear matrix

Figure II-6, shows that adenovirus DNA is strongly associated with the nuclear matrix *in vivo*. Nuclear matrix associated DNA (H) and supernatant DNA (HS) were obtained by a high salt extraction, which were subsequently run on a 1% agarose gel, and then hybridized with radioactively labeled adenovirus DNA as probe. The band of nuclear matrix DNA was more intensely labeled than the band of supernatant DNA. This result is in agreement with, and complements the results of Smith et al. (1985). They used nuclear matrix associated DNA as the probe to hybridize with adenovirus DNA immobilized on a gel to show that adenovirus DNA was associated with the nuclear matrix.

Discussion

The origin of replication of eukaryotic cells is not well understood. This research is an initial attempt to investigate the roles which the nuclear matrix may play in DNA replication. Adenovirus was used in this study because it is a well characterized eukaryotic virus. Results from two methods of nuclear matrix isolation have shown that the origins of replication of adenovirus are associated with the nuclear matrix *in vivo*. The *in vitro* band shift and UV cross-linking experiments indicate that some nuclear matrix proteins can specifically bind with the adenovirus origins of replication, and these proteins are found in both host HeLa cells and virus-infected HeLa cells. One virus-coded protein, with a mass of 140 KD, was found to bind specifically with the origin. There are many other proteins which can associate with the origin as shown by the band shift experiments, and as confirmed with UV cross-linking data.

From these data (see Figure II-6) and also other laboratory reports (Smith et al., 1985; Younghusband and Maundrell, 1982), adenovirus DNA is known to be associated with the nuclear matrix both *in vivo* and *in vitro*. After adenovirus infection, the virus-related inclusions appear in the nuclei, where they partition with the nuclear matrix. Two new proteins can be detected in the nuclear matrix (Zhai et al., 1988). What precise role the nuclear matrix plays in adenovirus infection is not completely understood, but these facts suggest that it may be involved in adenovirus maturation (Dery et al., 1986).

The chromatin structure around the origin of replication of SV40 has been extensively studied (Sergeant et al., 1979; Li et al., 1986). About 10-25% of SV40 minichromosomes contain a nucleosome-depleted region around

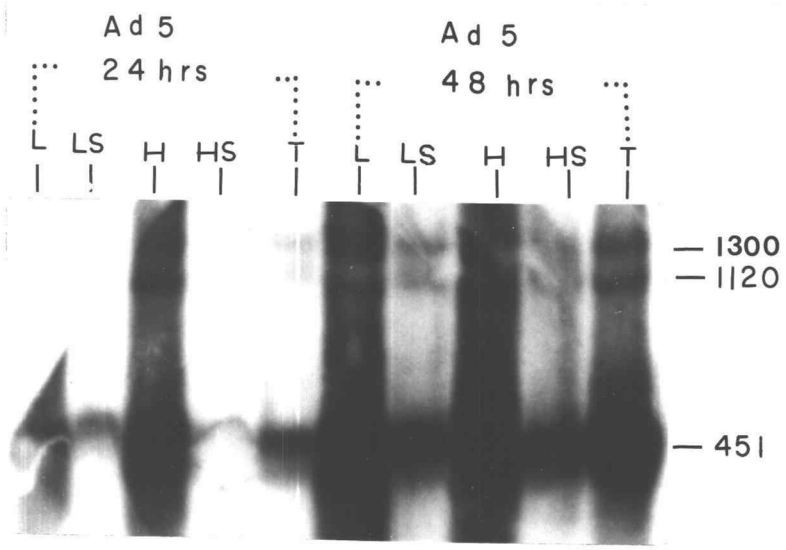
the origin of replication. It seems possible that the region near the origin of adenovirus might contain sequences that specifically exclude nucleosomes. However, it seems unlikely that this is the case. Nucleosomal DNA isolated from adenovirus-infected HeLa cells will hybridize with terminal adenovirus sequence demonstrating that this region, 338 bp, which contains the origin of replication, must form some degree of nucleosome structure *in vivo*. That such nucleosome formation can occur is confirmed by *in vitro* nucleosome reconstitution experiments. At this stage, we do not know whether this example can be extrapolated to other eukaryotic viruses.

There are many factors which have been reported to bind to the adenovirus origin of replication, such as nuclear factor I, nuclear factor III, terminal binding protein and the virus-coded DNA polymerase. It is of interest to note that a virus-coded protein found from UV cross-linking experiments has the same molecular weight as the virus-coded DNA polymerase. However, it has not been shown yet whether they are the same protein or not. The DNA and protein complex around the origin of replication plays an important role in adenovirus replication. It will be interesting to know the detailed structure of this complex which will give us more insight into adenovirus replication and gene expression.

Figure II-1. The origin of replication of adenovirus is associated with the nuclear matrix.

Nuclear matrix associated DNA and supernatant DNA were isolated from adenovirus infected HeLa cells, 24 hrs and 48 hrs after infection, using either high salt buffer or low salt buffer for the extraction. DNA was digested with Pvu II, fractioned on a 2% agarose gel, blotted onto nitrocellulose paper and probed with a nick-translated adenovirus EcoR I- Ssp I fragment which contained the origin of replication. H and L stand for nuclear matrix associated DNA isolated by high salt buffer and low salt buffer respectively. HS and LS stand for supernatant DNA isolated by high salt buffer and low salt buffer respectively. T stands for total DNA.

Figure II-1



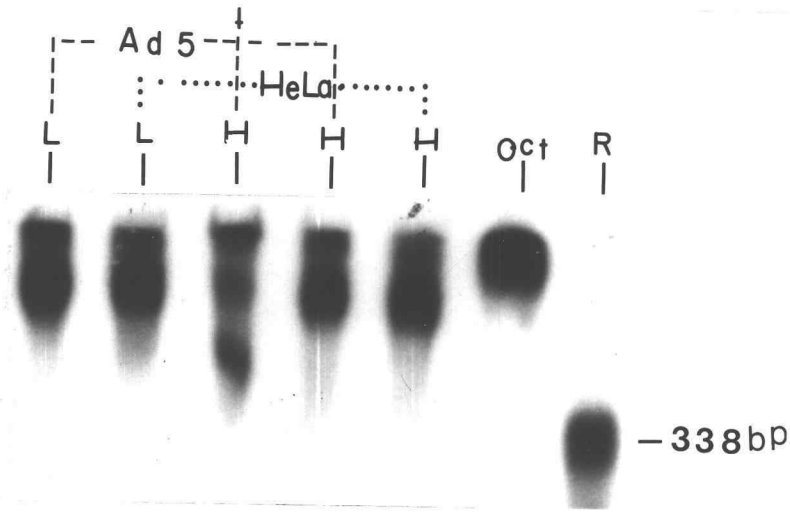
Res. Enzyme – Pvu II

Probe – Ad 5 EcoR I – Ssp I

Figure II-2. Nuclear matrix proteins cause a band shift of the origin of adenovirus DNA replication.

Adenovirus EcoR I- Ssp I fragment (338 bp) filled-in with [a-^{32}P] dATP using the Klenow fragment of E.coli DNA polymerase I (BRL), was incubated with 1.5 µg nuclear matrix protein. HeLa=HeLa cells. AD5=adenovirus infected HeLa cells. H=high salt buffer extracted. L=low salt buffer extracted. R=probe only. Oct=histone octamer control. Each sample also included with1.5 µg lamda DNA or cold probe for the competition. The reaction mixture was run on 4% native PAGE and exposed to X-ray film for 3 hrs at -70 °C.

Figure II-2



Band Shift Exp

Figure II-3. *In vitro* UV cross-linking of nuclear matrix proteins with the adenovirus origin of replication.

Random labeled EcoR I-Ssp I 338 bp fragment was incubated on ice for 30 minutes with 1.5 μg nuclear matrix proteins (isolated from HeLa cells and adenovirus infected-HeLa cells with high salt buffer), UV cross-linked under 280 nm light (11 cm light length, 25 μl light exposure volume) at 10 °C for 0, 0.5, 2, or 5 minutes. After the exposure, the mixture was digested with 2 units of DNase I at 37 °C for 50 minutes, washed with the digestion buffer three times, resuspended with SDS gel sample loading buffer, heated at 95 °C for 5 minutes, cooled on ice, then loaded on 12% SDS PAGE. After electrophoresis, the gel was exposed to X-ray film.

Figure II-3

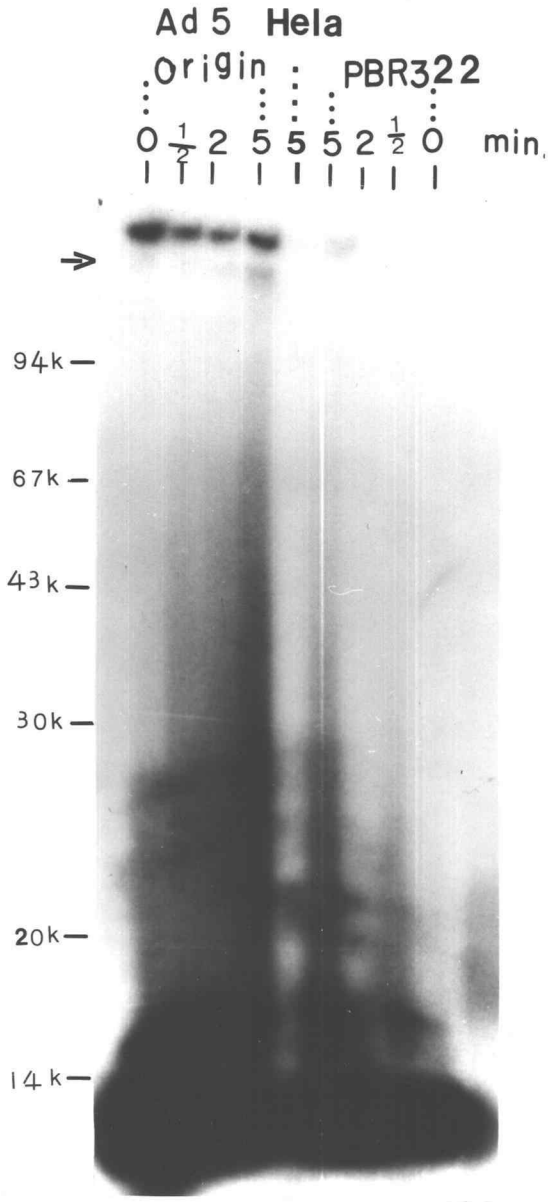


Figure II-4. Some portions of adenovirus DNA in HeLa cells can be isolated in a nucleosome structure.

Nucleosomes isolated from uninfected HeLa cells or HeLa cells infected for 48 hrs with adenovirus, were purified on sucrose gradients and characterized with 4% native PAGE or 15% SDS PAGE. DNA was purified after proteinase K digestion, extraction with phenol/ chloroform , ethanol precipitation, and nick-translated as probe to hybridize with the blot containing Hind III- or Pvu II-cut adenovirus DNA or the EcoR I-Ssp I 338 bp fragment. Figure II-4A: Results from blotting with HeLa cell nucleosomal DNA as a probe; Figure II-4B: Results from blotting using nucleosomal DNA isolated from adenovirus-infected HeLa cells as a probe. M=marker.

Figure II-4

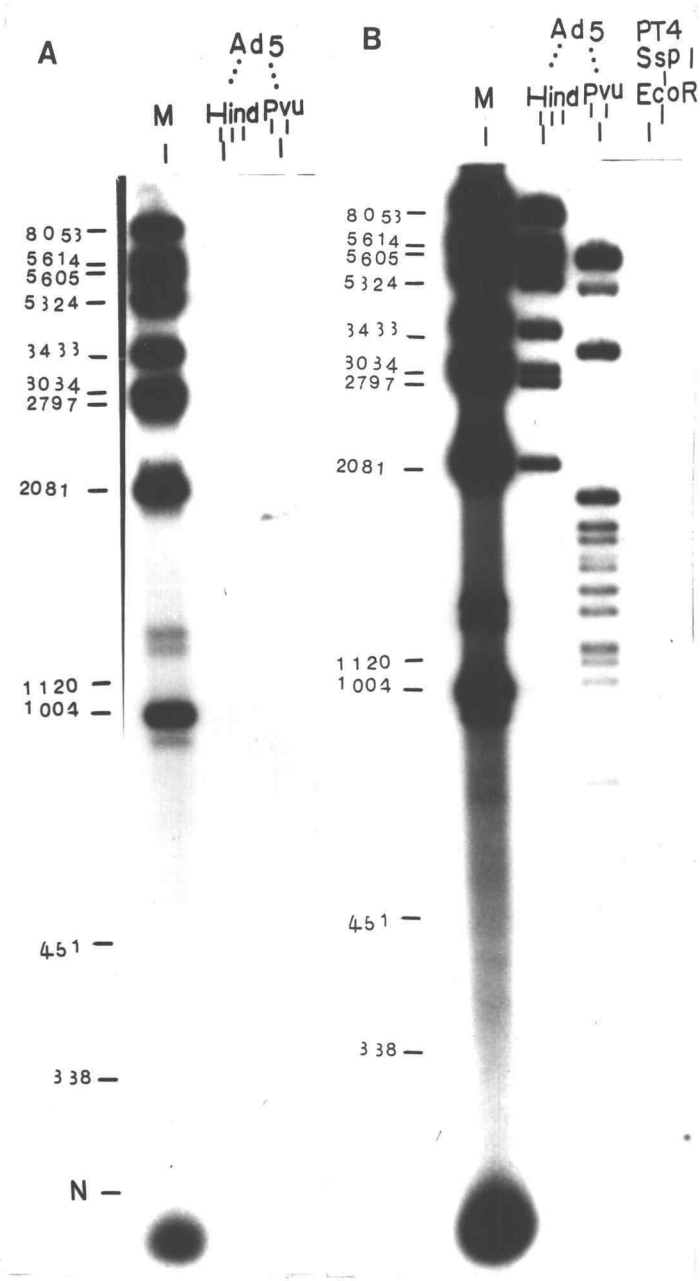

A
Ad5
M
Hind
Pvu
8053
5614
5605
5324
3433
3034
2797
2081
1120
1004
451
338
N
B
Ad5
PT4
Ssp I
M
Hind
Pvu
EcoR
8053
5614
5605
5324
3433
3034
2797
2081
1120
1004
451
338

Figure II-5. The origin of replication of adenovirus can form nucleosome structure in an *in vitro* reconstitution experiment.

Radioactively labeled EcoR I-Ssp I fragment of pT4 containing the adenovirus replication origin was incubated with 15 µg of nucleosomes isolated from chicken erythrocytes in 0.8 M NaCl/TE buffer (pH 7.5), dialyzed against 0.6 M NaCl/TE buffer (pH 7.5) overnight, then against 0.1 M NaCl/TE buffer (PH 7.5) for 5 hrs, electrophoresis on 3.5% native PAGE, dried, and exposed to X-ray film. R=probe only. N=reconstituted samples.

Figure II-5

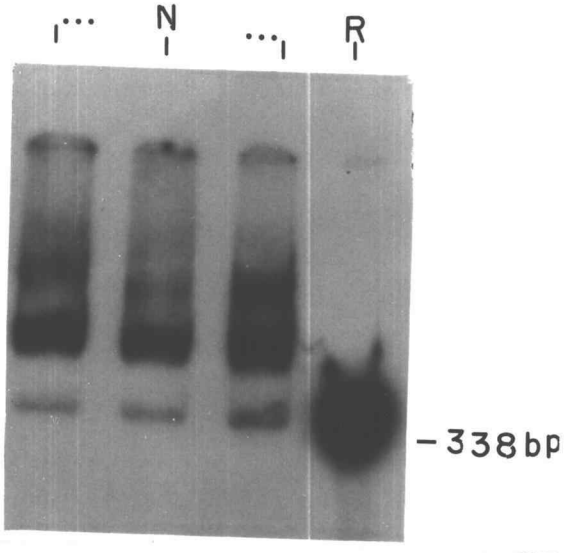
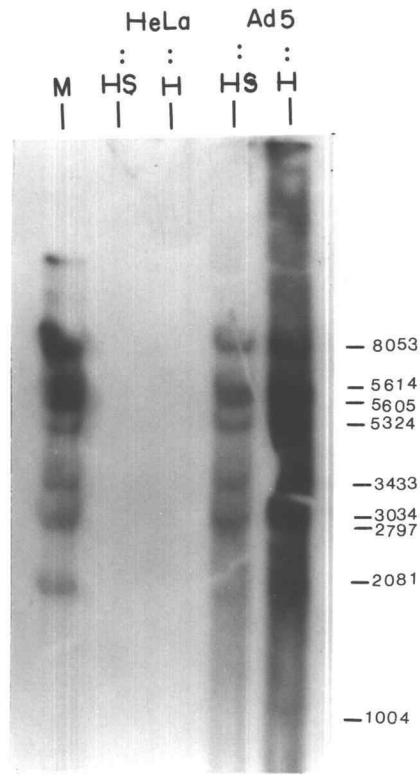


Figure II-6. Adenovirus DNA is associated with the nuclear matrix *in vivo*.

Nuclear matrix associated DNA (H) and supernatant DNA (HS) were extracted by high salt buffer from either HeLa cells (HeLa) or adenovirus 5 infected HeLa cells (AD5) after digestion with Hind III, run on a 1% agarose gel, and hybridized with nick-translated adenovirus DNA as probe. M: Hind III digested adenovirus DNA as marker.

Figure II-6


HeLa
Ad5
M
HS
H
HS
H
8053
5614
5605
5324
3433
3034
2797
2081
1004
Res. Enzyme – Hind III
Probe – Ad 5

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CHAPTER 4: Nucleosome Positioning: Placement of Nucleosomes on A Specific Gene and Analysis of Sequences of Randomly Selected Nucleosomes

Introduction

The basic repeating unit of eukaryotic chromatin is the nucleosome, a protein-DNA complex in which 146 bp of DNA bends around an octamer of histone proteins to form about 1.75 turns of a left-handed superhelix (for a recent review see "Chromatin" van Holde, 1988). Data from in vivo and in vitro experiments have shown that nucleosomes are preferentially located, or specifically positioned in some cases, at certain DNA sequences (see review in CHAPTER 1). Nevertheless, no consensus sequences have been found for nucleosome positioning. It is also clear, from many studies, that at least some nucleosomes are arranged randomly with regard to the DNA base sequence (Igo-Kermenes et al., 1982). Thus what determines which nucleosomes are specifically positioned remains a mystery.

By sequencing different DNA molecules cloned from chicken erythrocyte core particles, Satchwell et al. (1986) proposed a periodic arrangement of DNA bending sequences which correlates well with the detailed path of the DNA as it wraps around the histone octamer. In addition, they also have observed that long runs of homopolymer (dA)/(dT) prefer to occupy the ends of core DNA, five to six helix turns away from the dyad. These same sequences are apparently excluded from the near-center of core DNA, two to three helix turns from the dyad. The dinucleotide ApA/TpT is reported to be located preferentially where the minor groove faces inwards and the dinucleotides GpC and TpG/CpA are preferentially located where the minor groove faces out.

In further studies involving the cloning and sequencing of nucleosome dimers containing a single molecule of histone H5, Satchwell and Travers (1989) found that the sequences associated with H5-containing nucleosome dimers and core particles are significantly different. Those differences in sequences are suggested to be caused by removing histone H5 (or H1) from chromatin. Such removal, using certain procedures, may result in the migration of histone octamer to a new location and thus induce changes in nucleosomes spacing (D'Anna and Tobey, 1989; Bavykin et al., 1988; Pardoll et al., 1980).

In the work of Satchwell et al. (1986) on nucleosome positioning, there is a cloning selection problem caused by using Sma I in their ligation procedure. They found that Sma I has a preference to ligate a T/C at the 5' end and A/G at 3' end. Also there is a possible micrococcal nuclease overdigestion problem in their procedure for trimming down the long chromatin fragments to core particles. Finally, there is a most serious potential artifact caused by the likelihood of histone octamer translocation during the removal of H5/H1 in the method used by these workers which involved exposing the chromatin to 0.65 M NaCl for 3-6 hours. Numerous workers have shown evidence for extensive nucleosome "sliding" under such conditions (Steinmetz et al., 1978; Weischet, 1979; Spadafora et al., 1979). Because of these complications in earlier work and the importance of the question, a reexamination of the problem was deemed necessary. Accordingly, in the research described herein the procedures of both isolation and cloning have been modified in an attempt to circumvent these problems. Details are described in the Materials and Methods and Discussion section.

Any examination of specific positioning of nucleosomes on genes must recognize the fact that nucleosome arrangement has been shown to be dynamic and to vary between different physiological conditions, such as during the cell cycle (Moreno et al., 1986), embryogenesis (Wu and Simpson, 1985), differentiation (Chou et al., 1986), and replication (Cusick, 1984). For a review of the dependence of nucleosome organization on cell type and gene activity, see Igo-Kemenes et al. (1982). In another part of the research described in this thesis, two kinds of cells from different developmental stages and two different gene types were compared to investigate the relation between nucleosome spacing and gene expression. Even though it is still unclear what determines nucleosome positioning or spacing, histone H1/H5 and more recently histone H2B have been suggested to influence the spacer length (Felsenfeld, 1978; Thomas et al., 1985; Allan et al., 1982; Pederson et al., 1986). The data in this report support this spacing hypothesis.

Materials and Methods

Isolation of nuclei

Adult chickens and 15 day chicken embryos were obtained from Department of Poultry Science, Oregon State University. Animals were sacrificed and blood was collected or muscle cells were isolated and homogenized with a tissue processor. Cells were centrifuged at 4000 rpm , 4 °C for 10 minutes, with either GSA or SS34 rotor. The pellet was resuspended and washed with buffer 1 (buffer 1, 2 and 3 are the same as described in Ausio et al., 1989) , then was lysed with buffer 2, washed with buffer 3 to finally yield homogeneous nuclei as described in the Materials and Methods section of Ausio et al. (1989).

Micrococcal nuclease partial digestion and nucleosomal DNA preparation

Nuclei (100 OD/ml) were partially digested with micrococcal nuclease (45 unit/ml) for different times, and the reaction was stopped by adding 0.2 M EDTA to a final concentration of 10 mM. DNA was purified through proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. The sample was then digested with RNase (A&T_1) and repurified. This DNA was used for the nucleosome spacing analyses shown on Figure III-1 and Figure III-2.

Nucleosomal DNA for cloning was prepared as described in Ausio et al. (1989). Briefly, after an initial light micrococcal nuclease digestion, the nuclear pellet was lysed with 0.25 mM EDTA. The long chromatin was collected by centrifugation, then H1/H5 and nonhistone proteins were removed with CM-Sephadex (C-25) after bringing the sample

to 0.35 M NaCl (use of these conditions instead of 0.65 M NaCl will prevent nucleosome sliding, see van Holde, 1988). After dialysis, the supernatant was collected following centrifugation. The nucleosomes were trimmed by a second micrococcal nuclease digestion. To find the best digestion conditions, a pilot digestion was carried out for varying lengths of time to determine the optimum time for a mass digestion. A time point was chosen at which very little dimer existed and only one band of monomer, without sub-monomer fragments was seen on a DNA gel. DNA from nucleosomes digested for the appropriate time was purified as in the above description.

Southern blotting

For each sample, 15-50 µg DNA was redigested with a restriction enzyme (shown on Figure III-1, 2 and 3-5 units/µg DNA) (BRL/ New England BioLab) and run on 2% agarose gel. The gel was blotted to nitrocellulose or "Zeta-Blot" membrane (BioRad) and hybridized with either nick-translated probe (BRL) or random primer labelled probe (Boehringer Mennheim) as suggested by the manufacturer.

Nucleosomal DNA cloning procedure

Nucleosomal DNA, about 200 µg in total, was digested with 100 units S1 nuclease (BRL) at 37 °C for 10 minutes to trim any single strand ends or filled-in with dNTP using Klenow fragment of E.coli DNA polymerase I (BRL) as described in Kurl et al. (1988) and in Chapter 3. After phenol/chloroform extraction, and ethanol precipitation, the purified sample was digested with 100 units bacterial alkaline phosphatase at 37 °C for 1 hour to remove terminal phosphate. The DNA was again purified as above and resuspended with 5 µl water, 35 µl EcoR I linker (IBI, 50 µg/ml) and 10 µl 5X ligase buffer (BRL) to which 10 units T4 polynucleotide kinase (NE BioLab) were added. After

incubation at 37 °C for 1 hour, 2 units T4 ligase (BRL) were added and reaction was incubated at room temperature overnight. The ligated sample was purified and digested with 100 units EcoR I at 37 °C for 6 hours. Subsequently, the sample was passed through a 5 ml Biogel A 0.5 (BioRad) column, and eluted with TE/ 0.3 M NaCl. The 12 fractions, 300 µl/ fraction, were characterized by running a portion of each sample on 1.5 % agarose gel. The fractions desired, those which containing the linker and insert, were pooled and mixed with 5 µg M13mp18 which had been digested with 25 units EcoR I at 37 °C for 1 hour. After phenol/chloroform extraction, and ethanol precipitation, the dried sample was resuspended with 38 µl water, then 10 µl 5X ligase buffer (BRL) and 2 units T4 ligase (BRL) were added. Ligation proceeded at 15 °C for 24 hours. In another set of experiments the nucleosomal DNA was treated with 100 units EcoR I methylase (BRL) at 37 °C for one hour before the cloning and pUC19 was used as the vector instead of M13mp18. The preparation of JM105 competent cells, transformation of the ligation mixture and growth on X-gal/ IPTG plates followed the protocols provided by BioLab. Single strand DNA template was isolated by randomly picking up a white plaque, growing in YT medium for 7 hours and purified with the polyethyleneglycol method as suggested in the protocol.

DNA sequencing and data analysis

Cloned M13 single strand DNA molecules and pUC19 plasmids have been sequenced by using a Sequenase Version 2.0 sequencing kit (United States Biochemical). The sequence data was analyzed by using the GENALIGN and PCGENE programs from Intellegenetics, and the FREQS, the BEND, the WRAP and the XBEND programs from Jack Kramer,

and the BIOCAD program from Dr. Pui Shing Ho.

RESULTS

Nucleosome spacing on chicken beta-globin and thymidine kinase (TK) gene is cell type-dependent

By modifying the "indirect end labelling" method (Wu, 1980), the nucleosome spacing has been determined in two different genes from different cell types of the same species at different developmental stages.

DNA from partial micrococcal nuclease digests of chromatin from chicken erythrocyte and embryo myoblast cells was hybridized with a Kpn I-EcoR I 2kb probe containing the chicken TK gene coding sequence. As Figure III-1 shows, the histone-protected DNA fragments from red blood cells are longer than corresponding fragments from myoblast cells. Myoblast nucleosome monomer size determined from micrococcal nuclease partial digestion is about the same size as the H1/H5 depleted and trimmed core particle size from chicken erythrocytes. Thus the apparent length differences can be attributed to the H1/H5 protection.

Figure III-2, shows similar results using a DNA probe which contains a BstN I 467 bp fragment and locates in the 5' flanking region of the chicken beta-globin gene. The finding of a similar result in both cases of two quite different genes indicates that nucleosome spacing described here is more likely cell type-dependent, than gene dependent, and probably reflects a general effect of the special histone H5 carried in erythrocyte cells.

Random nucleosomal DNA sequencing and data analysis

To seek evidence of the determinants of <u>in vivo</u> nucleosome positioning while avoiding the problems inherent in the study by Satchwell et al. (1986), nucleosomal DNA

fragments were cloned directly into M13mp18 and pUC19 vectors. 82 of the M13mp18 clones together with 24 of the pUC19 clones have been randomly selected and sequenced, Figure III-6. Among these were 23 well defined sequences from M13mp18 clones which contained the EcoR I linker region at one end, and therefore included a possible nucleosomal DNA insert. Others either contained only the vector sequence, were truncated by the EcoR I site, or were hard to read because of possible contamination. Another 12 molecules containing the correct insert were obtained from the pUC19 clones. Figure 6 shows one of the sequencing gel which was sequenced from both ends of the insert.

The base composition of the entire set of sequences was about 27.2% adenine (A), 28.4% thymidine (T) , 21.5% guanine (G) and 22.9% cytidine (C). The total content of A plus T was around 55.6% which was approximately equal to the content of A plus T in total chicken DNA (57.3% from Drew et al., 1986; 56.4% from Handbook of Biochemistry & Mol. Biol., 2nd Ed by H. E. Sobher). These results indicate that the DNA molecules were randomly selected, exhibiting no preference for AT-rich repetitive sequences.

The sequence set was aligned using the GENALIGN program (Intelligenetics) in an attempt to determine a consensus. The results are shown in Figure III-3. After analyzing the best multiple alignment generated by that program, no meaningful consensus of primary structure of those molecules was found. Initial visual analysis indicated some possible repeat patterns in some consenses, but GENALIGN produced consenses vary considerably depending principally on the clustering order of the sequences in the multiple alignment. This order is in turn extremely sensitive to arbitrary parameter settings for

the alignment algorithm. In addition, the limited number of base types increases the probability of matches occuring at repetitive positions due to chance alone. The combination of these effects precluded the use of these consenses to determine any truely significant repeat patterns.

Consequently, a new program, FREQS, was written by Jack Kramer to analyze the distribution and periodicity of dinucleotides within the sequences. This program calculates and graphically displays the relative composition of each dinucleotide at each position. Possible slight misalignments, due to missreading of sequencing gels or shifting of the nuclease digestion, were compensated for by an adjustable rolling average window.

The dinucleotide distributions along these sequences are shown in Figure III-4. In cases where visual analysis of the frequency distributions indicated any potential periodicity, a fast Fourier transformation (FFT) was performed to quantify the periodic occurrences. Frequence domain plots of the FFT results from the FREQ program are shown in Figure III-5.

Table III-1 summarizes the major periodicity of the various dinucleotides obtained from the FFT computations as depicted in Figure III-4. Comparison of these periods between the left and right dyad parts, and between each part and the whole molecule were performed to check for asymetry. Periods of each dinucleotide in this data do vary depending on the part of the molecule examined. For example, dinucleotide TT was shown to have a major period at 8 bp in the right part and in the whole molecule, which was not evident in the left part. Another period appeared at 10.7 bp in both left and right parts but only at 11.7 bp in the whole molecules. This unequal distribution between

two sides of the dyad was also true for all other dinucleotides analysed. Periods of dinucleotides of the whole molecules were more similar to one side or another indicating potential asymmetry of the molecules with one side contributing the principal component. Different kinds of dinucleotide were also found to have the same periods permitting necessary degeneracy to allow nucleosomes to form on stretches of DNA which may have other overriding structural or functional constraints. This idea will be discussed further in conjunction with the bending of the molecules.

Looking over the Figure III-4, it is difficult to recognize any specific pattern of any particular dinucleotide or grouping of dinucleotides. Considering that DNA secondary structure may play a role in nucleosome positioning, an Intelligenetics PCGENE program, DNAHELIX was executed to check the distribution of base pair structure; specifically helix twist, propeller twist, torsion angle (d) and roll. Again, no positive results were found.

Since DNA bending intuitively seems so important to nucleosome positioning, a BEND program was written by Jack Kramer to try to elucidate any such relationship. This program calculates the local maximum bend for all rotational degrees of freedom of a chosen size fragment centered at each sequence position. For each molecule the local maximum bend versus sequence position was plotted. Periodicity of the bending was calculated by FFT method. Table III-1 is a summary of this analysis listing the dinucleotide frequency periodicities which correspond to bend frequency periodicities.

It could not be demonstrated from this analysis how this local bending of the molecule correlates with

nucleosome positioning. Therefore another program, WRAP, was written to check the potential for the global axis of the DNA sequences to bend enough to wrap around the entire equator of a histone octamer. Table III-2 shows the results from the WRAP program. This potential bend was calculated from axial wedge tilt and roll angles (kindly provided by P. McNamara and R. Harrington, personal communication) and the helix twists (Kabsch et al., 1982) over all possible (0 - 360) rotational degrees of freedom. The accumulating bend is computed proceeding along the sequence at each dinucleotide position as follows:

Accumulated bend = sum of the wedge angle of each dinucleotide projected onto a rotation plane up to this position.

The projected angle added for each dinucleotide =
$\cos(\text{rotation angle} + \Sigma\text{helix twist}) * \text{roll}$
$+ \sin(\text{rotation angle} + \Sigma\text{helix twist}) * \text{tilt}$

The maximum bend of 35 molecules obtained from this program was found to have a range of approximately 380.8 to 532.1 degrees. This can be interpreted to mean that the native curvature of the DNA would permit a 146mer to wrap around the histone octomer in 1.1 to 1.5 wraps.

Other sequences from the literature were then checked to determine if this program could be used to predict potential nucleosome positioning. The maximum bend of 146 bp of the positioned nucleosome in Sea Urchin 5S RNA gene (Simpson et al. 1983) was about 411.3 degrees. The value for another known E. coli DNA fragment (Ramsay et al., 1984), which was found to be able to form a nucleosome in vitro, was 459.6 degrees. These values fall acceptably within the range shown above. Identical calculations for poly(dA).poly(dT) and poly(dG).poly(dC), where no reconstitution has succeeded, gave values around 198.9 and

253.3 degrees respectively. Similarly values of about 258.8 and 280.5 degrees for poly(dA.dT).poly(dA.dT) and poly(dG.dC).poly(dG.dC), which have been reconstituted with low efficiency, were computed. These results were encouraging and indicate that this program might be applied to the prediction of nucleosome positioning. A extension of the BEND program, XBEND, was then written by Jack Kramer to perform such predictions on longer pieces of DNA, by calculating the maximum bend of 146 bp fragments at each position along these sequences in a selected rotation plane. Four cases from the literature were used as a test. The results are shown in Figure III-7 and summarized in Table III-3. Although not conclusive yet, these results, when compared with published experimentally determined nucleosome positioning, indicate good potential for this method.

Discussion

The method of both isolation and cloning have been modified from the procedure of Satchwell et al. (1986) in an attempt to circumvent possible artifacts and selection problems present in that work. First, an EcoR I linker was used to ligate nucleosomal DNA molecules, so as to circumvent the specific selection problem with Sma I, which will lead to preferential ligation of A/G to 3' end and T/C to 5' end. In fact a nearly random distribution of nucleotides (A:11 ; T:10 ; G:14 ; C:7) was found to ligate with the linker for all molecules sequenced. One possible bias of using EcoR I linker will result from the possible missing a fraction of nucleosomal DNA sequence which contain the EcoR I site. This would be especially serious if the EcoR I site is unusually frequent in the nucleosomal DNA. Considering this possible bias caused by the EcoR I digestion, comparison experiments were performed to check the percentage of nucleosomal DNA sequences which may contain the EcoR I site. The results from the direct EcoR I digestion, before and after methylase protection, show very little DNA was digested; in fact none could be detected by PAGE (data not shown). Another set of cloning experiments in which the nucleosomal DNA was initially protected with EcoRI methylase was also performed. Results obtained by checking pUC19 clones (data not shown) show that only one of 20 molecules was digested by EcoR I. This frequency is consistent with the theoretical prediction (146/4096 = circa. 4%).

Second, by using CM-Sephadex (CM-25) to deplete H1/H5 at 0.35 M NaCl instead of 0.65 M NaCl as used in the experiments of Satchwell et al., possible translocation of nucleosomes during isolation was prevented.

Third, overdigestion is thought to be able to produce a sequence preference in remaining nucleosomes, because of the preference of micrococcal nuclease to cut at AT sites. To prevent such an overdigestion problem, a pilot digestion panel was carried out to select the optimum time for the digestion. Data from the gel analysis has shown that there was very little overdigested product. This is confirmed by the fact that there is no significant difference in base composition between whole chicken DNA and the cloned fragments.

The relative distribution of dinucleotide occurance of these molecules was analyzed with the FREQS program. The major periodicities of each dinucleotide obtained from the FFT computations as depicted in Figure III-4 are summarized in Table III-1. The results reported here are consistent with the other reports (Uberbacher et al., 1988; Trifonov, 1980; Satchwell et al., 1986). The small difference (less than 0.5 bp) was probably due to the different parts of dyad and different groups of DNA used for the analysis (also see Results section). Although no simple, definitive DNA bending pattern can be found to explicitly determine nucleosome positions, it is of interest to note that periodicity of some degenerate groupings of dinucleotides and of DNA bending correspond in nucleosomal DNA sequences (see Table III-1). From the above results, it seems likely that nucleosome positioning on DNA may depend on the existance of periodic regularities in the DNA bending. Even though these properties of periodicity of dinucleotides may be a general characteristic of any genomic DNA (Trifonov, 1980; Uberbacher et al., 1988), these studies emphasize that information manifest in local sequence dependent properties could affect the differential propensity for positioning of nucleosomes. DNA bending, which is the property particularly addressed here, seems

very likely to represent at least one of the major sequence directed structural constraints on the ability of any particular stretch of DNA to form nucleosomes.

The possible three dimensional structures of these 35 nucleosomal DNA molecules were also studied using a DNA structure prediction program, BIOCAD, developed by Dr. Pui Shing Ho. Preliminary results from these programs show that these molecules could be divided into two approximately equal groups representing two kinds of structures. One group has only one predominent bend, the other group has two. This does not conflict with the evidence from the other programs used in this analysis. Since these programs do not yet take into account the variable helix twist of different dinucleotides, these results are considered preliminary, but certainly warrant further examination.

The results in Table III-2, from the WRAP program which finds the maximum positive and negative bending over all possible (0-360) rotational degrees of freedom, show that each molecule in the cloned set is capable of theoretically wrapping around the histone octomer at least 1.1 turns to 1.5 turns (380.8 degree to 532.1 degree) without extra energy from DNA-histone interaction. The consequence is that all such interaction will add to the negative free energy of nucleosome formation. This datum is very interesting and provides evidence to show that the inherent DNA bending may play a significant role in nucleosome positioning.

After testing a number of molecules, it was found that the range of maximum bending for those DNA molecules which readily form nucleosome structure appears to lie in the range 258 to 532 degrees. Too much rigidity will prevent the nucleosome formation. The results from the XBEND

program (see Figure 7 and Table 3 for the summation) show that by this means it is possible to predict the nucleosome positioning on any extended range of DNA. One interesting finding from doing these analysis is that the dyad of nucleosome positioning is located at the least maximum bending position (see the arrow in Figure III-7). This evidence implies that too much bendability at certain position can inhibit the nucleosome positioning, as well as too little bendibility.

Considering the process of chromatin reconstitution, the results suggest that histone octamers will start to associate with those sequences of DNA which permit maximum bending. Although newly associated nucleosomes may be prone to slide, a possible place to stop nucleosome sliding is adjacent to a site which is too rigid to allow further moving. The wide range of maximum bending reported here may imply that the nucleosome positioning is dynamic and will be able to shift over the majority of DNA which falls between these extremes. This could allow for a plasticity or chromatin structure modulated by the presence of other proteins and adjacent nucleosomes.

Indeed, the nucleosome structure has been reported to be dynamic and to vary under different physiological conditions (see review in CHAPTER 1). In this research, one "house-keeping" gene (TK gene) and another tissue-specific gene (beta-globin gene) were used to investigate the nucleosomal spacing on these gene sequences (either located inside of the coding sequence in the case of TK, or in the 5' flanking region with beta-globin).

The results from this research indicate that the nucleosome spacing is most likely to be cell type-dependent, rather than gene dependent. These *in vivo* results are consistent with those from an *in vitro*

reconstitution experiment reported by Stein and Mitchell (1988). They suggested that H1 variants, such as H5 in this case, could cause different nucleosomal spacings, a result also seen in the data reported here. In Figure III-1, the repeating length of the chromatin from myoblast cells was shown to be similar to the length from RBC after H1/H5 depletion. More experiments are needed to see how relative proportion of H1/H5 affect the chromatin structure.

Although the precise determinants of chromatin structure are not resolved by the research reported here, the information obtained from these studies should provide further insight into how a nucleosome is organized and how that organization may affect the gene transcriptional activity.

Figure III-1. Nucleosome spacing determination on the chicken TK coding sequence.

Nuclei of chicken RBC and myoblast cells were partially digested with micrococcal nuclease, DNA was purified and 15 µg was loaded on 2% agarose gel. M: Hind III digested lamda DNA marker; A, B, C, D, E, N: sample from RBC; F, G, H, I, J: sample from myoblast; A, F: for 5 min digestion; B, G: for 20 min digestion; C, H: for 2 hours digestion; E, J: Pst I redigestion; D, I: Hind III redigestion; N: nucleosomal DNA.

Figure III-1

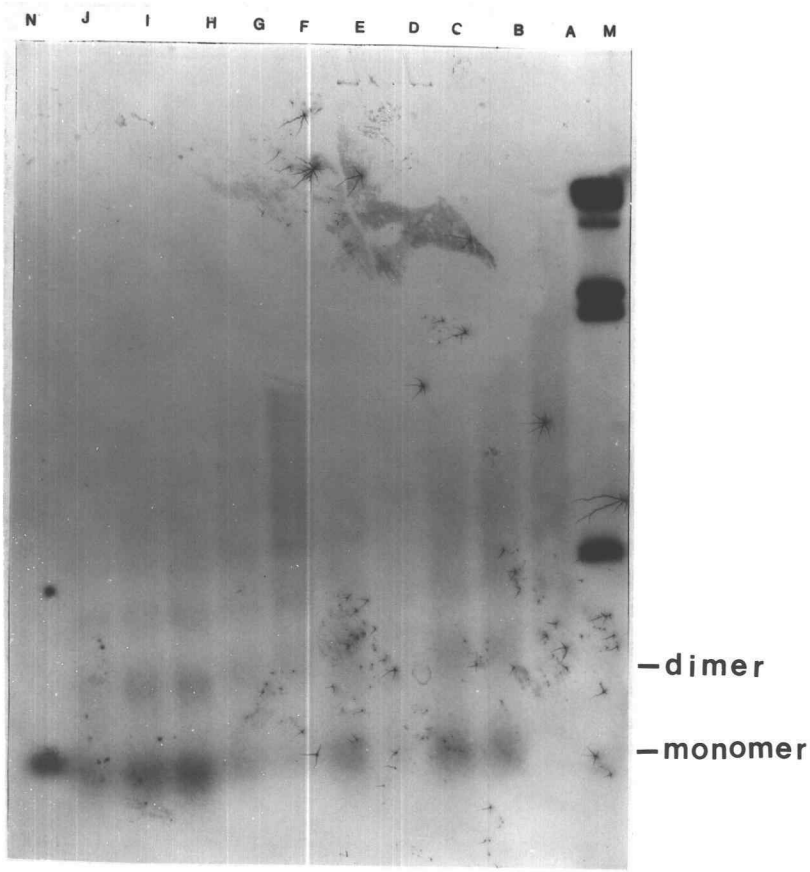


Figure III-2. Nucleosome spacing in the chicken beta-globin gene 5' flanking region.

DNA prepared as in Figure III-1 and 50 μg was loaded on the gel. A: myoblast with 10 min digestion; B, C, D, E, F, G: from RBC and with 5, 10, 15, 20, 25, 30 min digestion; H: RBC micrococcal nuclease trimmed DNA after H1/H5 depletion.A-G redigested with BstN I.

Figure III-2

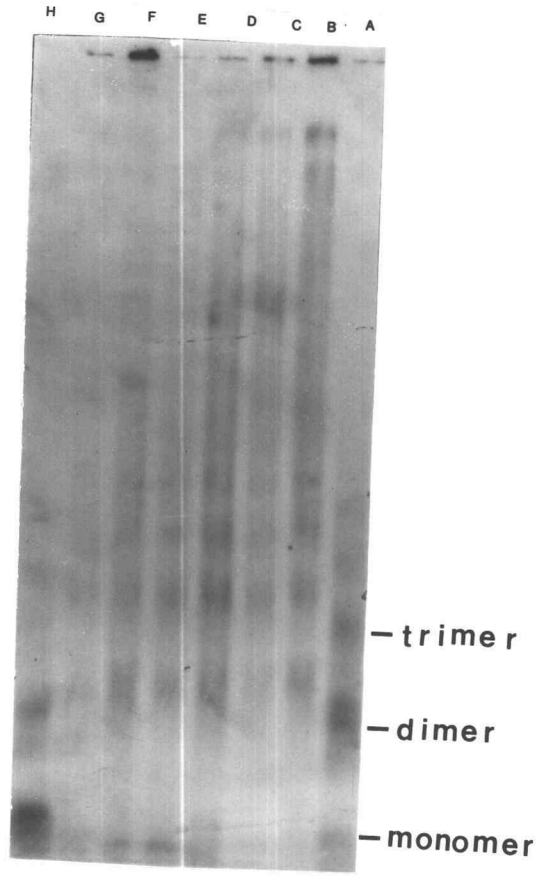


Figure III-3. Results of the analysis from GENALIGN program for sequence homology search.

Figure III-3

```
Sun 20 Aug 89 13:20:33-PST

Solution Parameters:

    Nucleic Alphabet    = Identity
    Output line length = 80
    Compress            = Off
    Histogram           = Off
    Randomization       = Off

    AMINO-Res-length    = 2
    DELetion-weight     = 1.00
    LEngth-factor       = 0
    Matching-weight     = 1.00
    NUCLEIC-Res-length = 4
    SPread-factor       = 50

Clustered order of selected sequences:

    19. RBC-23              (1-146)
    27. NK-7                (1-146)
    34. NK-31               (1-146)
    28. NK-14               (1-146)
    33. NK-29               (1-146)
    17. N35                 (1-146)
    24. NK-1                (1-146)
    11. N12                 (1-146)
     6. RBC-2               (1-146)
    32. NK-27               (1-146)
    10. N11                 (1-146)
     3. N1-3                (1-146)
    22. RBC-15              (1-146)
    14. N22                 (1-146)
     5. C1-D                (1-146)
    26. NK-4                (1-146)
    16. N29                 (1-146)
     8. RBC-11              (1-146)
    13. N16                 (1-146)
    20. RBC-34              (1-146)
    21. RBC-12              (1-146)
     7. RBC-9               (1-146)
    31. NK-25               (1-146)
    23. RBC-32              (1-146)
    18. RBC-17              (1-146)
    15. N27                 (1-146)
     2. N1-2                (1-146)
    29. NK-16               (1-146)
    30. NK-21               (1-146)
    25. NK-3                (1-146)
    35. NK-33               (1-146)
     4. C4-1                (1-146)
     9. N5                  (1-146)
     1. N1-1                (1-146)
    12. N13                 (1-146)

Region Alignment: (listed in Clustered order)

 19    1 cCtcTctcTccTGcTactgtAgacaCcAAaAacTacCagatgcacacaTcctagAaaGgaggAGAGca
 27    1 tCctTtcTTtTTGtTCTaAcAttttCaAAtAcaTtaCtacctgtgctgTgtagAAGtGccCAAGAGaG
 34    1 AaggaaaTgCTgTGgCTgAtAgaAagcAAaAgGgCTggtgGaTgTgacaTggAAAGcagaCAgtgctG
 28    1 AtctgtccTCTcTGaaGcCacagAtatATGAaGcCTTAcaGcTcTagAcTacACAGttagCtAccaaT
 33    1 AgAgcCatTtaaAcCcGGCggtttCTggTGcTcGGTTAagtGCaTtTAaTtTgCtcgagCagAGgTGT
```

Figure III-3 (cont)

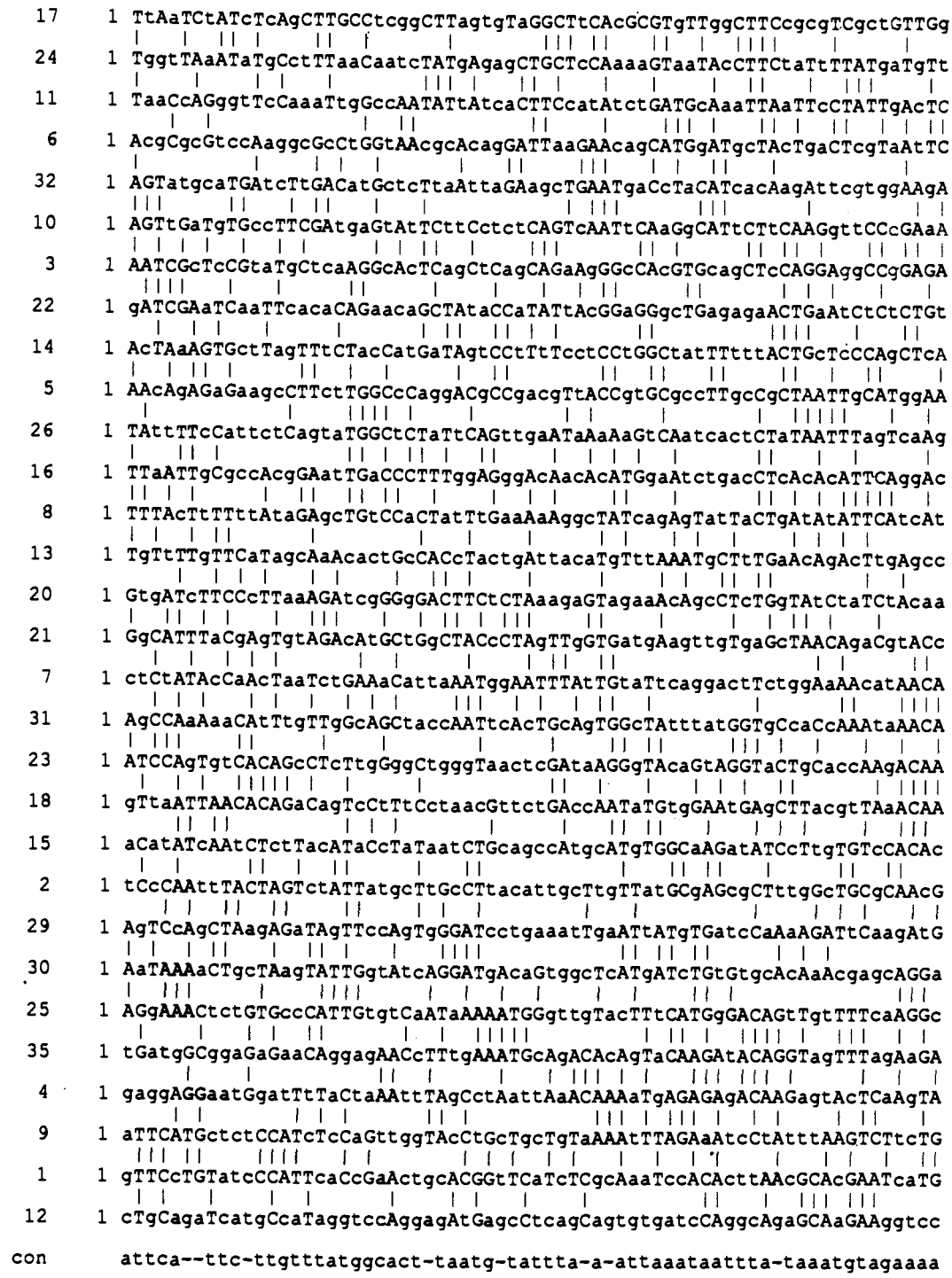


Figure III-3 (cont)

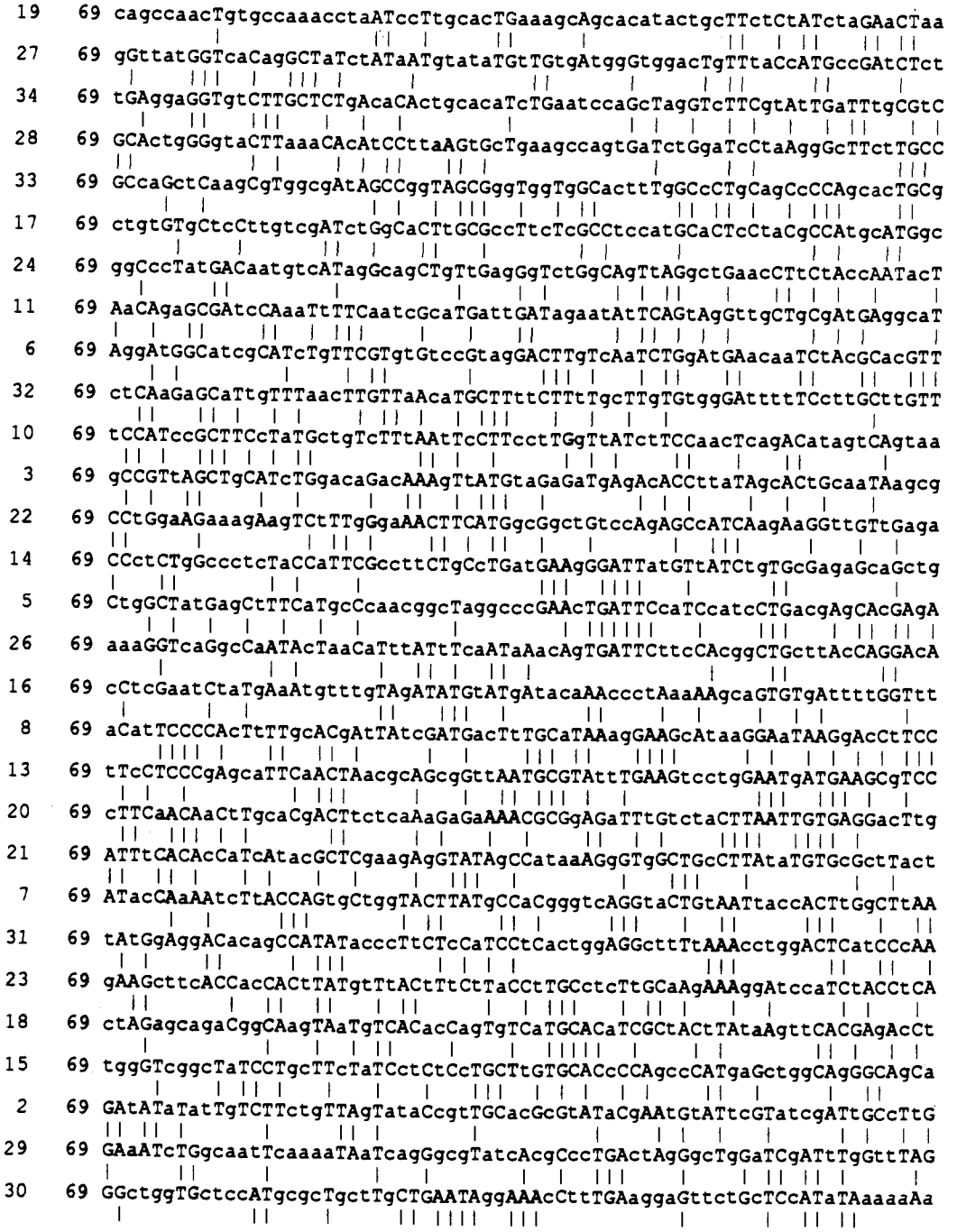
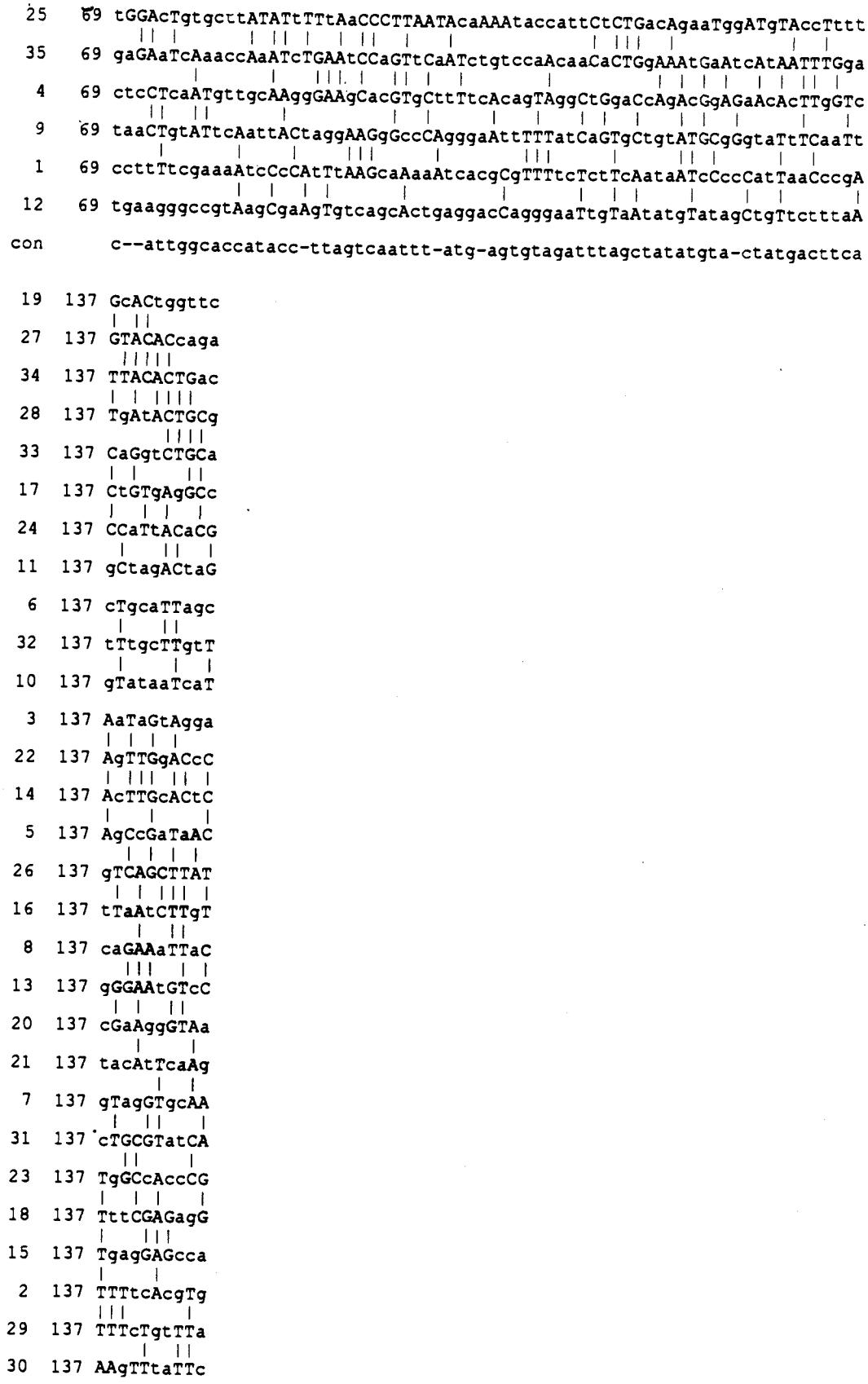


Figure III-3 (cont)



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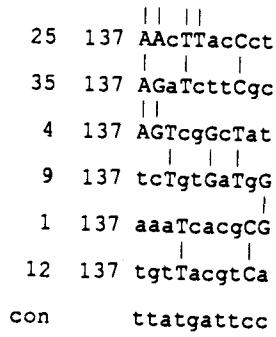

Figure III-3 (cont)

Alignment score = 4526.00

Scoring matrix:

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2			40	37	36	39	45	38	46	42	39	36
3				40	42	36	38	36	33	51	35	33
4					34	34	33	35	42	32	40	37
5						37	35	28	30	39	38	31
6							38	38	39	39	45	36
7								38	35	37	36	39
8									40	41	35	34
9										31	41	39
10											41	32
11												40
12												
13												
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30												
31												
32												

Figure III-3 (cont)

	13	14	15	16	17	18	19	20	21	22	23	24
1	31	37	36	27	39	33	34	35	29	39	43	38
2	46	44	44	41	36	33	36	42	38	37	47	35
3	29	40	34	33	31	31	38	36	32	46	33	30
4	36	33	38	42	28	41	39	39	34	35	35	39
5	32	44	36	36	33	35	34	28	43	38	39	36
6	36	43	32	43	41	44	31	34	36	37	44	39
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13		43	39	36	37	36	42	48	43	30	36	34
14			39	35	51	37	37	40	39	49	43	34
15				41	43	46	35	37	30	38	36	29
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20									54	36	38	29
21										39	30	36
22											36	38
23												30
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25												
26												
27												
28												
29												
30												
31												
32												
33												
34												
35												

Figure III-3 (cont)

	25	26	27	28	29	30	31	32	33	34	35
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5	40	48	29	27	34	40	40	38	35	42	31
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7	25	38	32	40	39	38	52	39	27	34	41
8	38	43	30	40	35	39	28	39	39	35	41
9	34	46	33	44	47	42	27	39	33	39	33

31
32
33
34
35

Figure III-3 (cont)

10	35	49	31	39	41	40	38	43	37	35	37
11	38	48	36	33	41	34	37	40	29	37	38
12	39	32	35	39	46	34	42	40	36	40	34
13	42	37	35	39	45	40	38	35	43	31	34
14	39	38	45	33	35	43	48	44	35	38	33
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17	38	30	39	39	33	32	37	45	50	38	35
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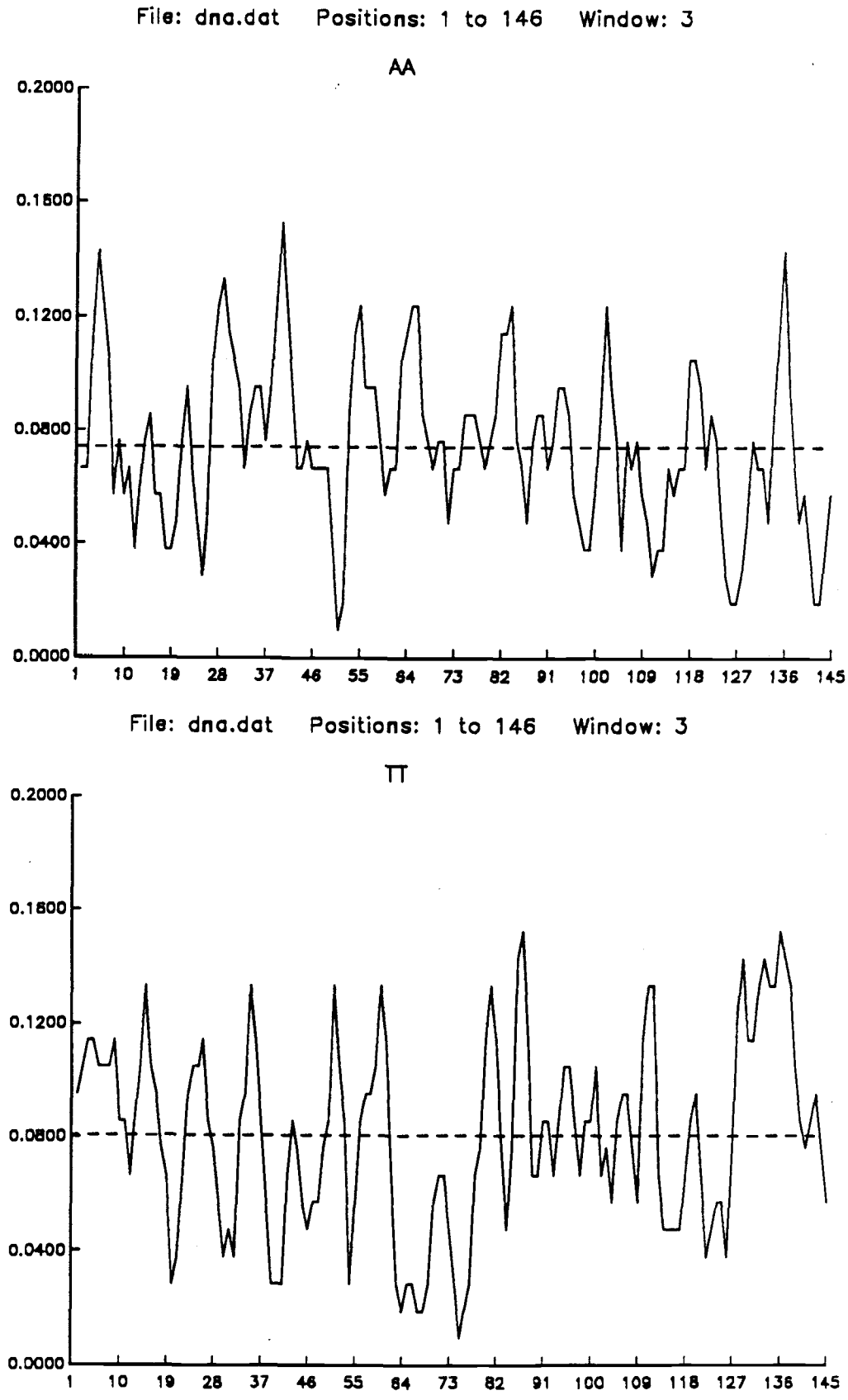
24	45	46	38	36	30	38	24	42	44	36	37
25		41	37	38	45	44	37	36	35	37	49
26			37	35	39	39	34	36	31	32	44
27				33	33	38	42	42	32	39	34
28					42	31	29	34	36	46	42
29						45	40	40	39	45	38
30							41	36	41	38	32
31								34	31	47	33
32									40	33	39
33										34	34
34											34
35											

Figure III-4. Dinucleotide distribution on the random picked nucleosomal DNA.

The composition of each dinucleotide was calculated with a offset of 1 bp to take account the possible base shifting of the DNA sequence. In each Figure, X axis is the position and Y axis is the dinucleotide content. Dinucleotides on the top panel and the bottom panel are complementary.

Figure III-4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0.1200
0.0800
0.0400
0.0000
1 10 19 28 37 46 55 64 73 82 91 100 109 118 127 136 145
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TT
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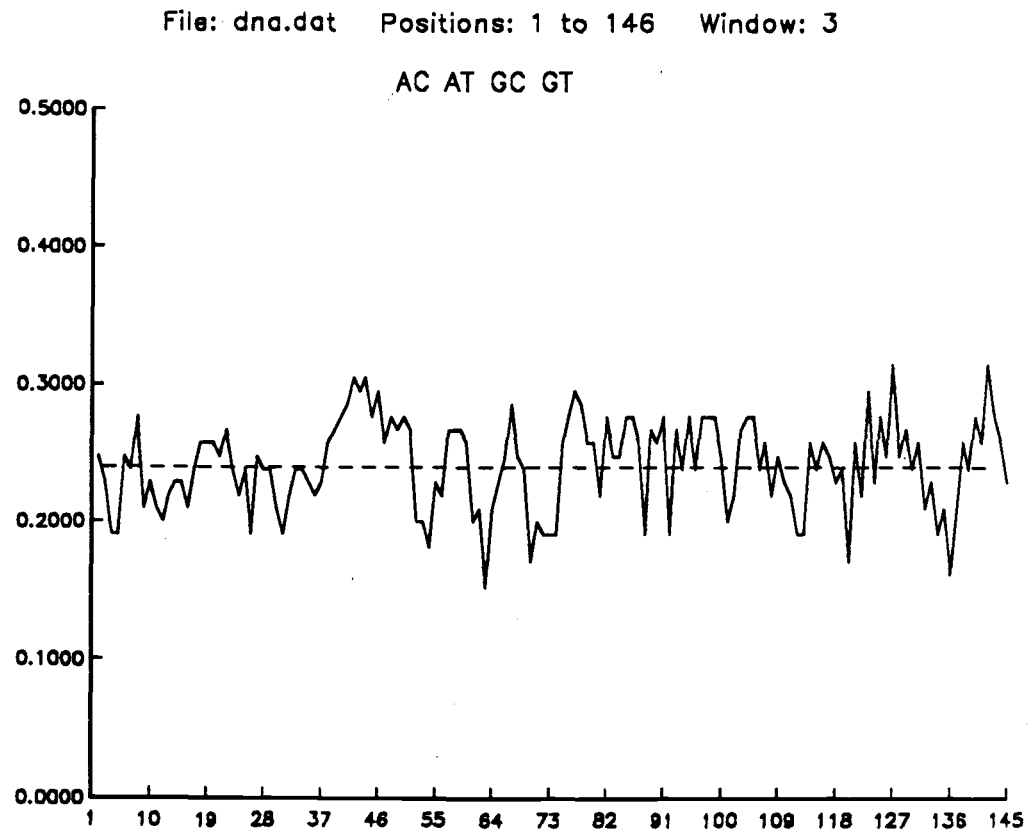
0.1200
0.0800
0.0400
0.0000
1 10 19 28 37 46 55 64 73 82 91 100 109 118 127 136 145

Figure III-4 (cont)



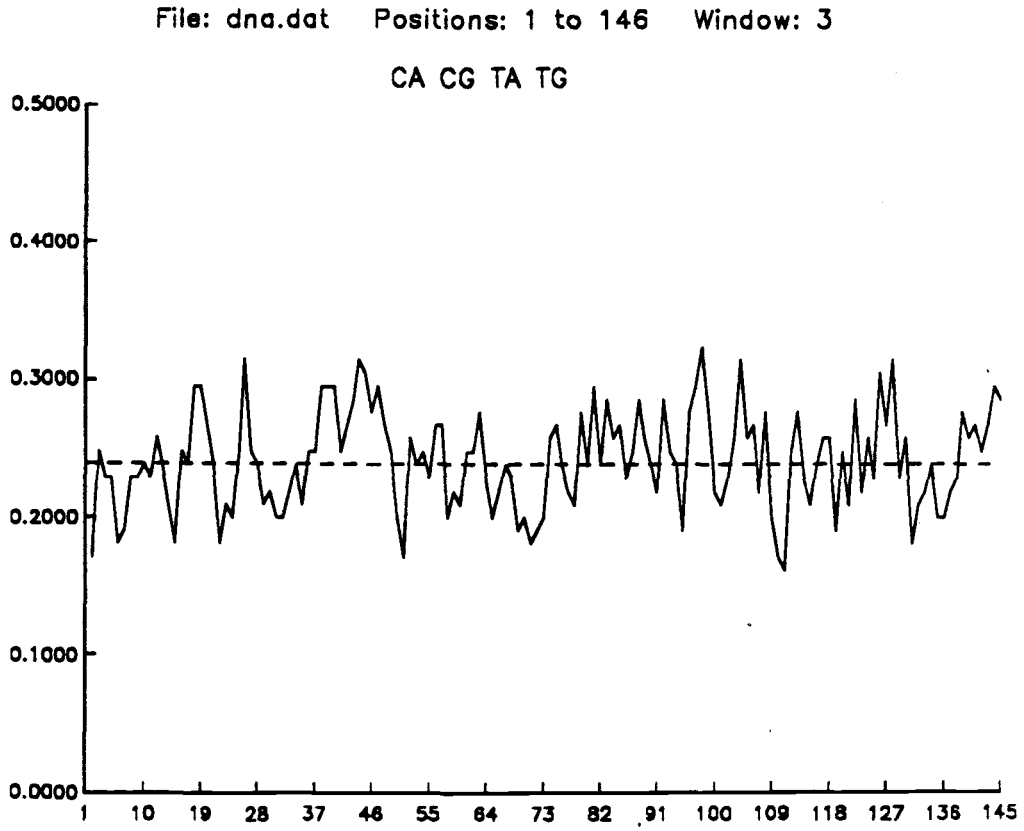
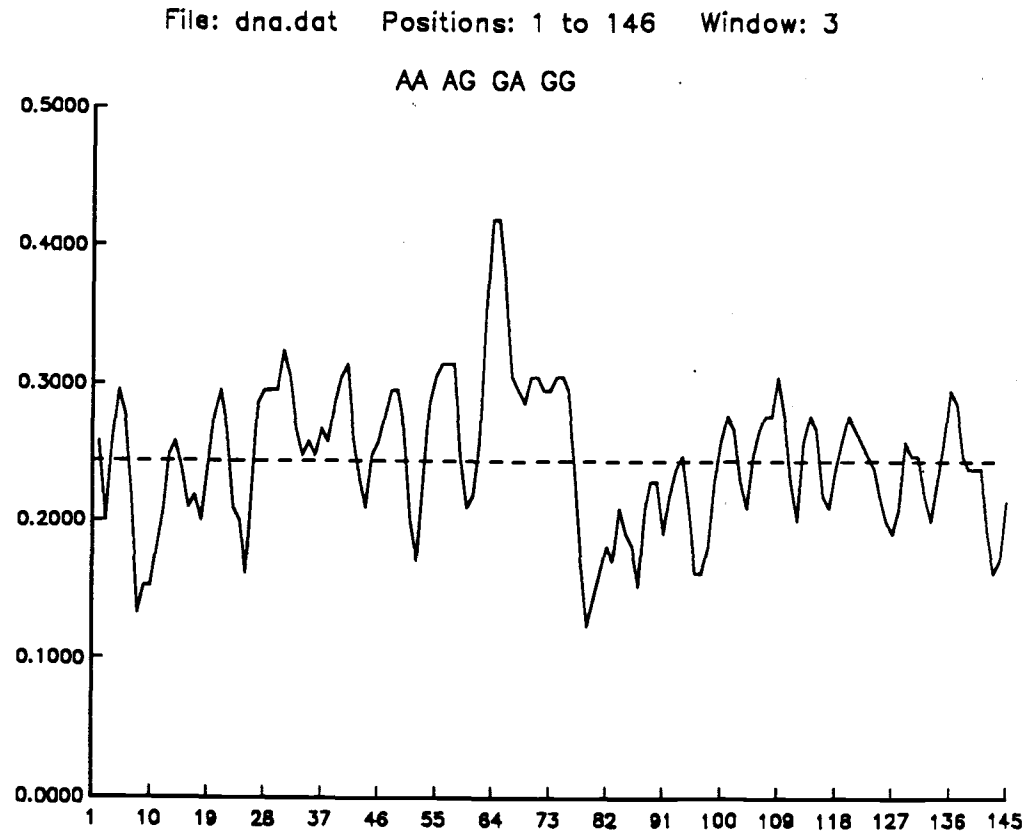
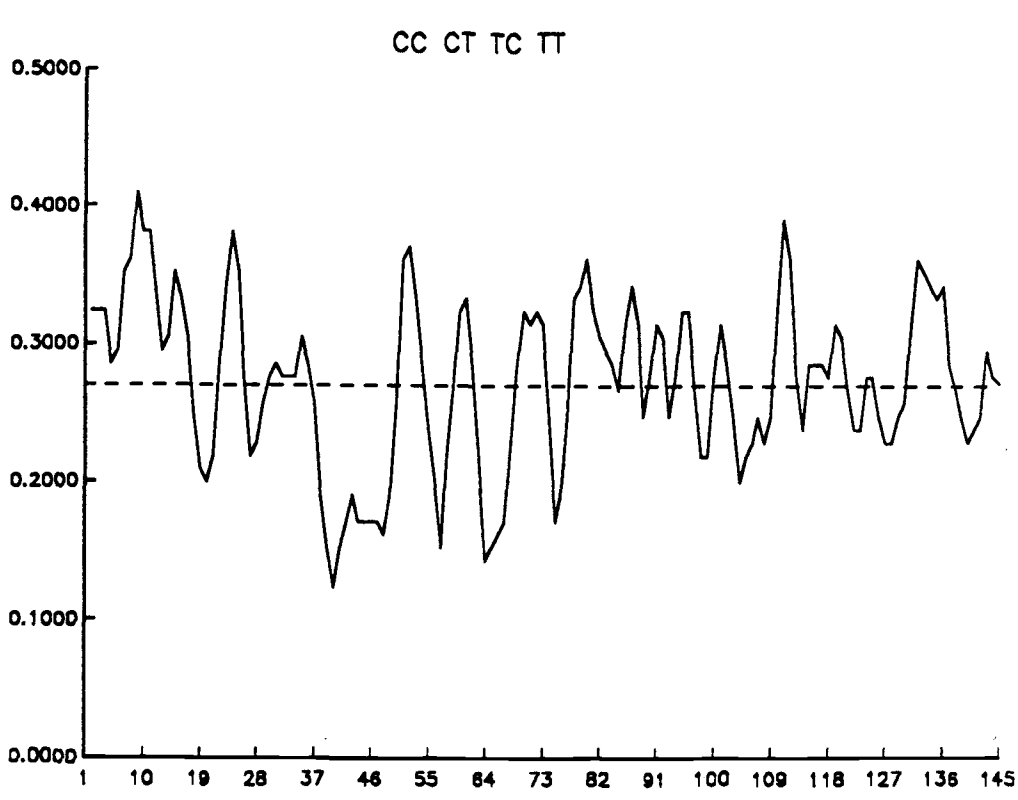


Figure III-4 (cont)





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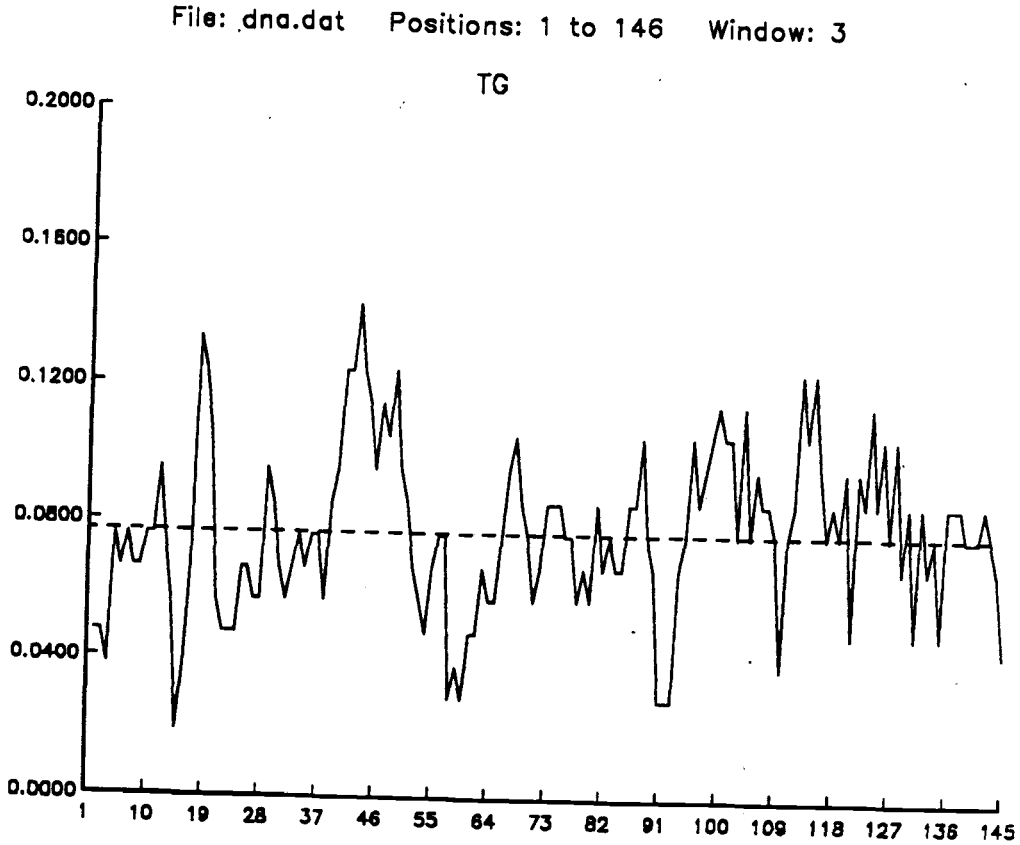

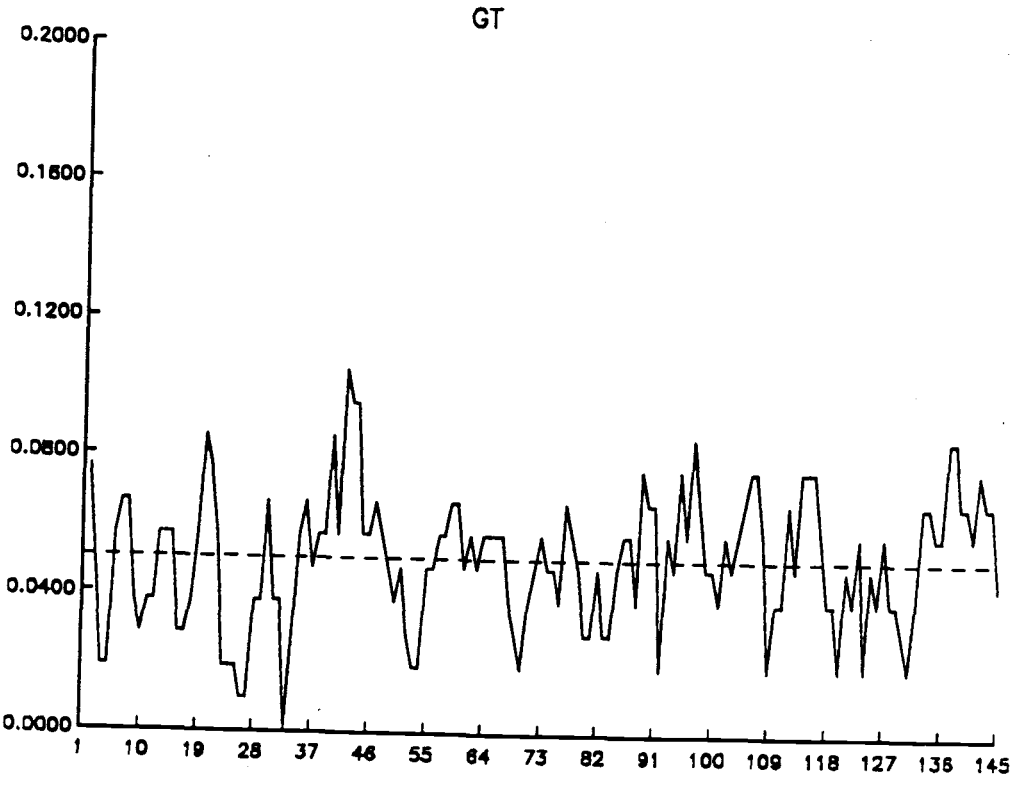

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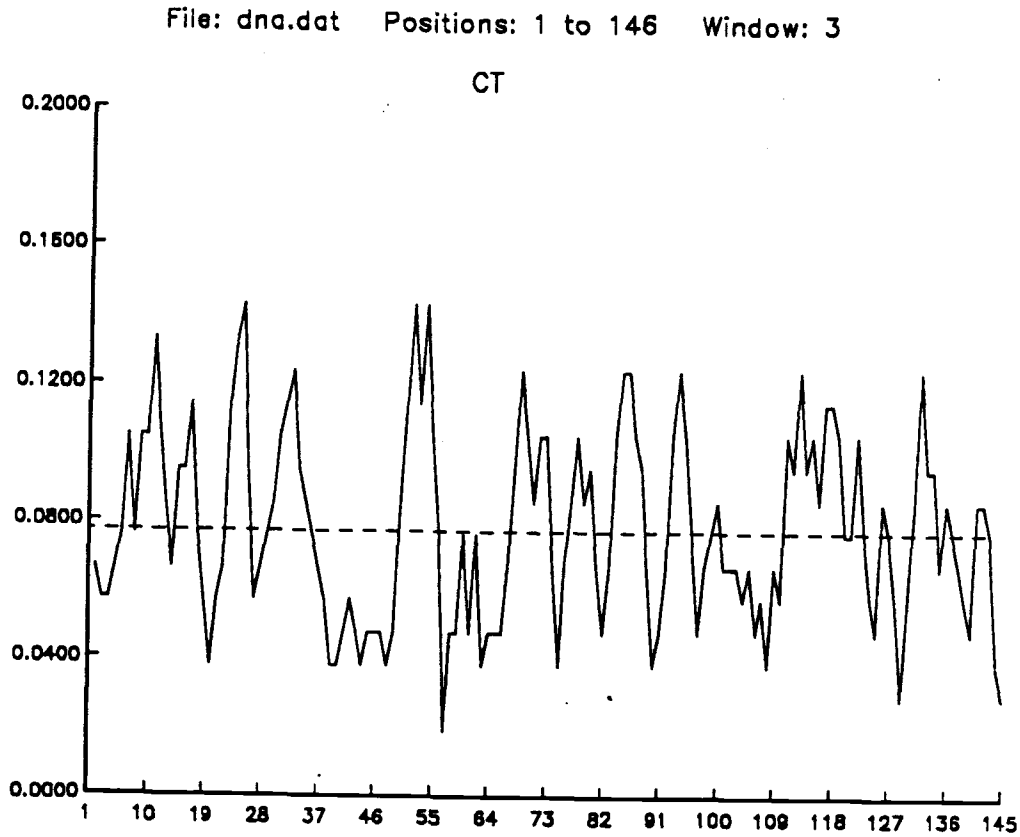

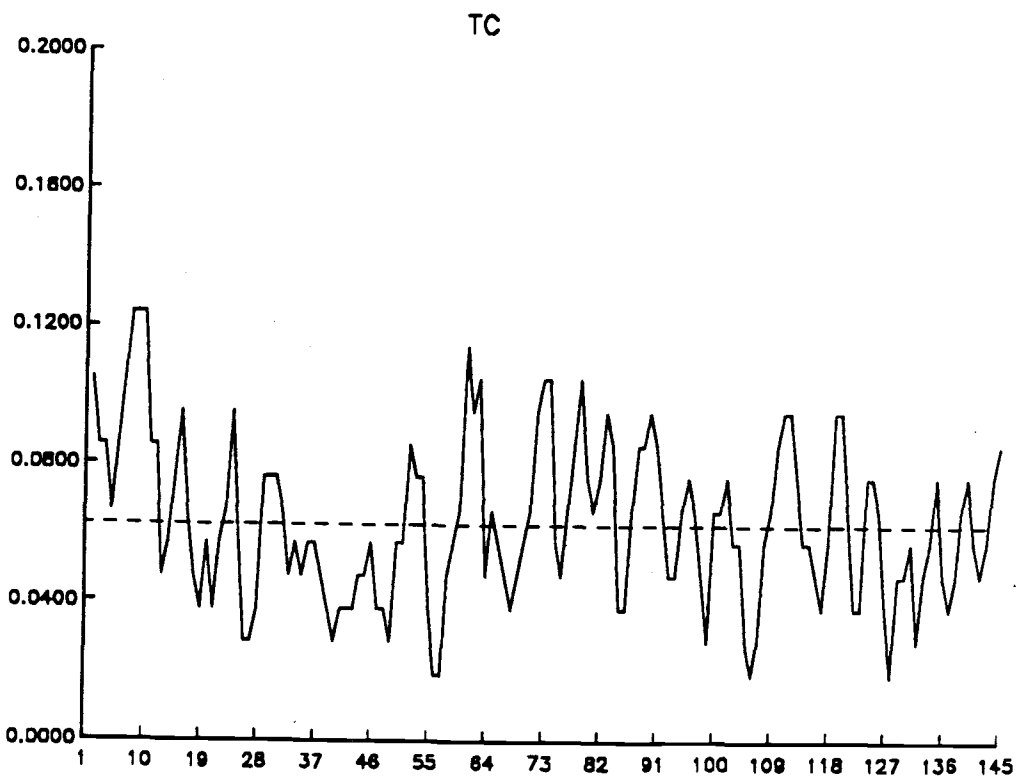

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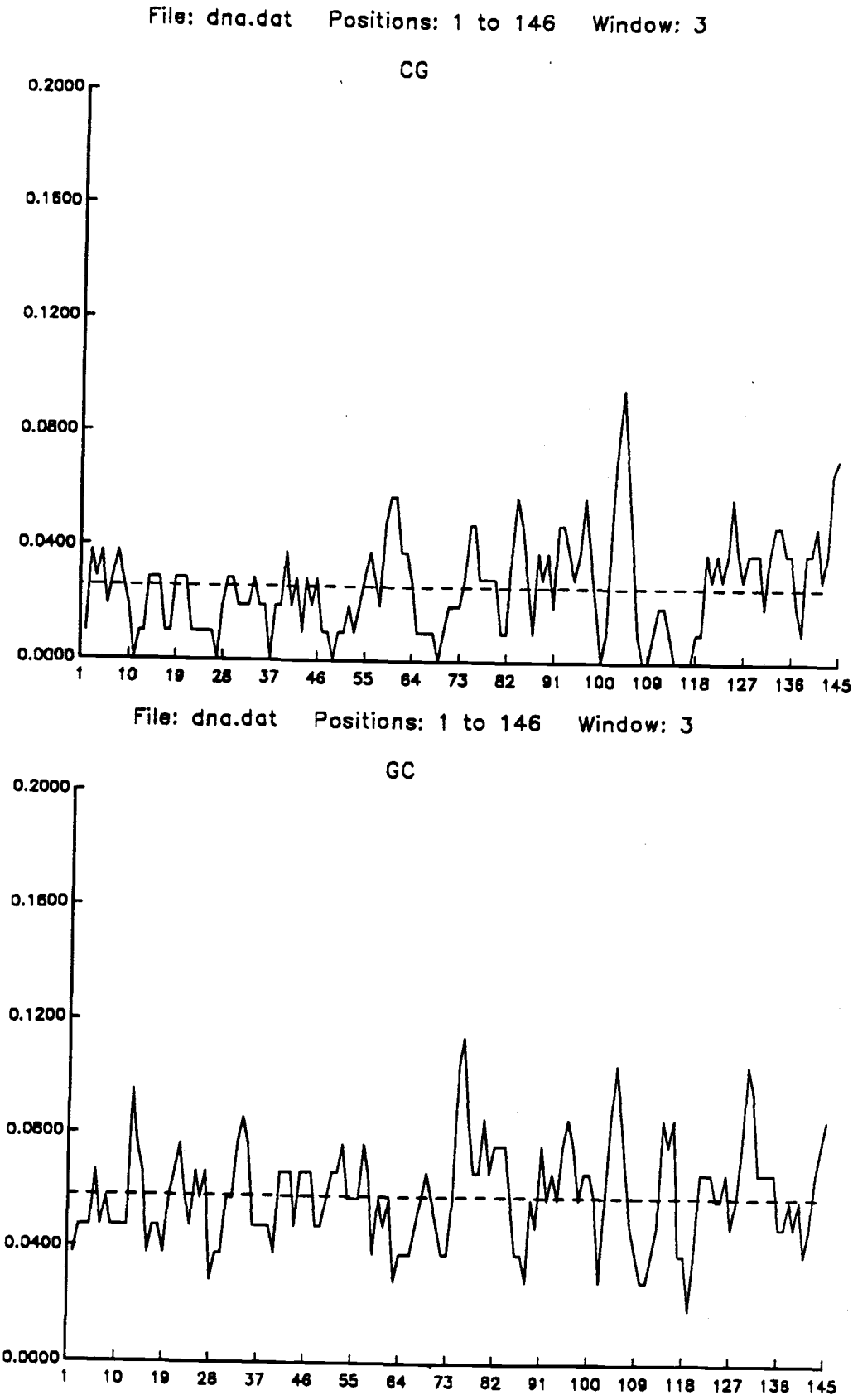

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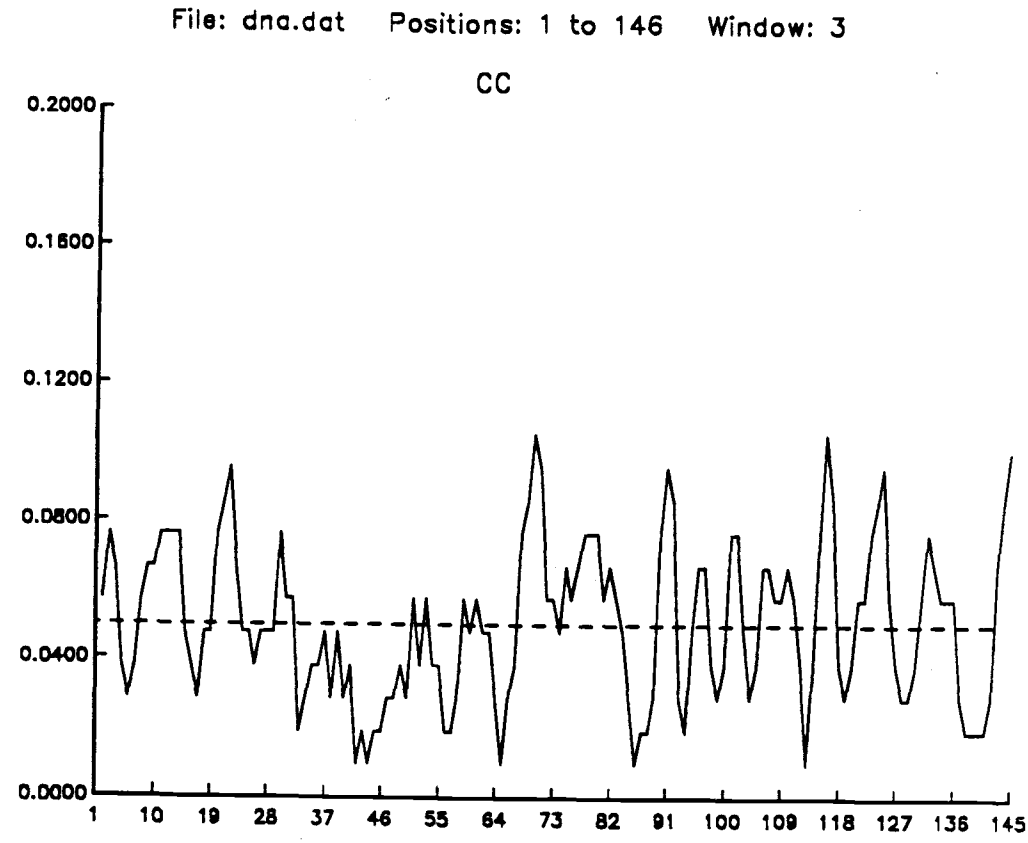

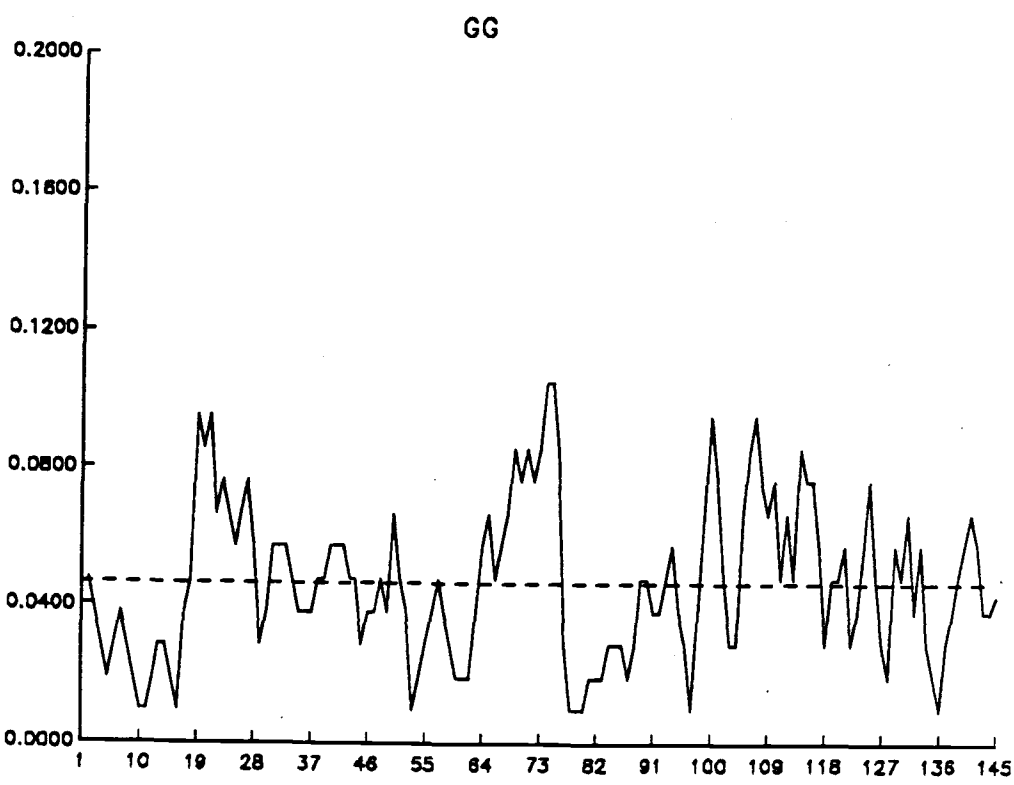

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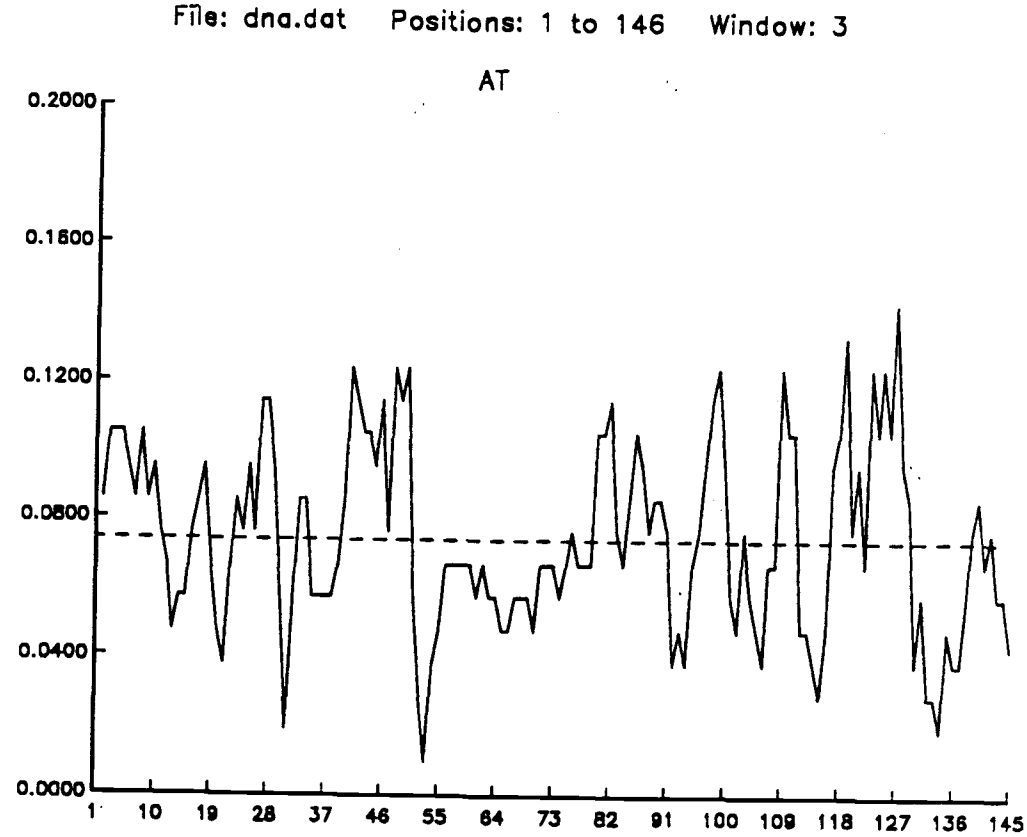

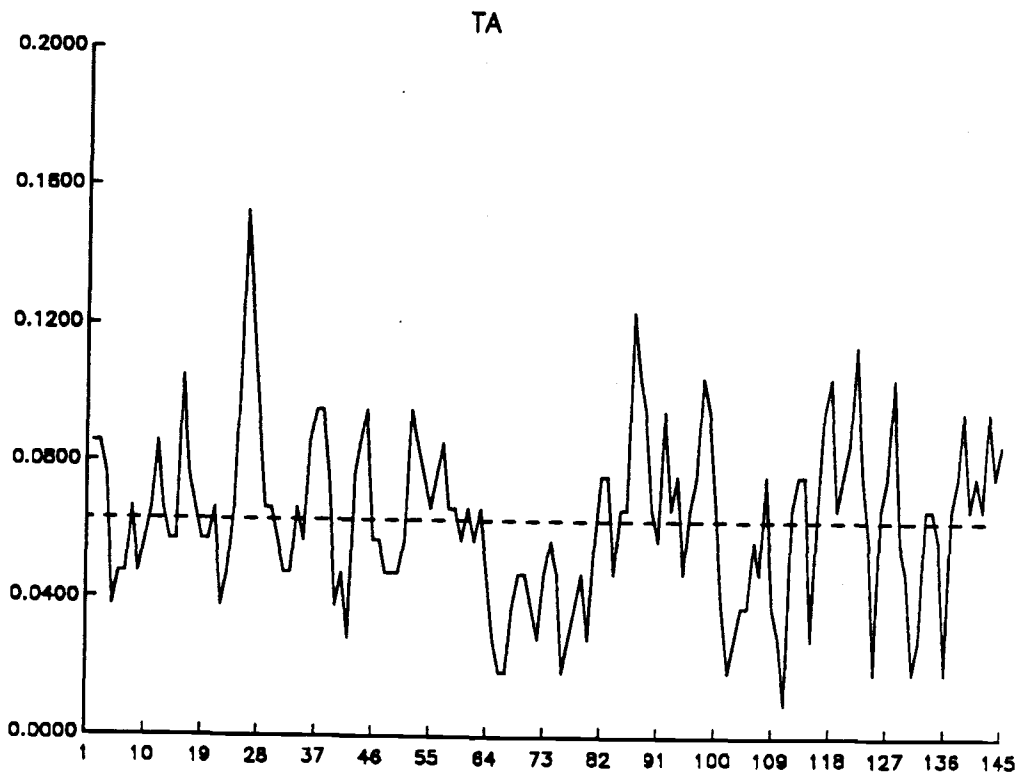

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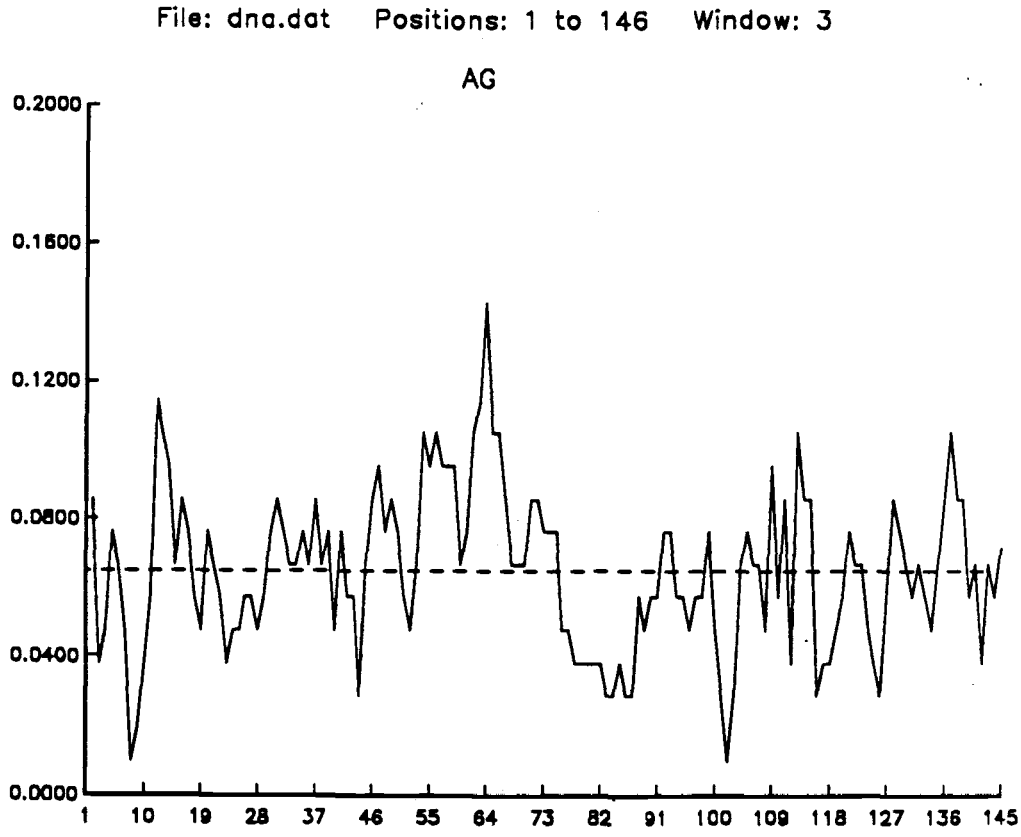

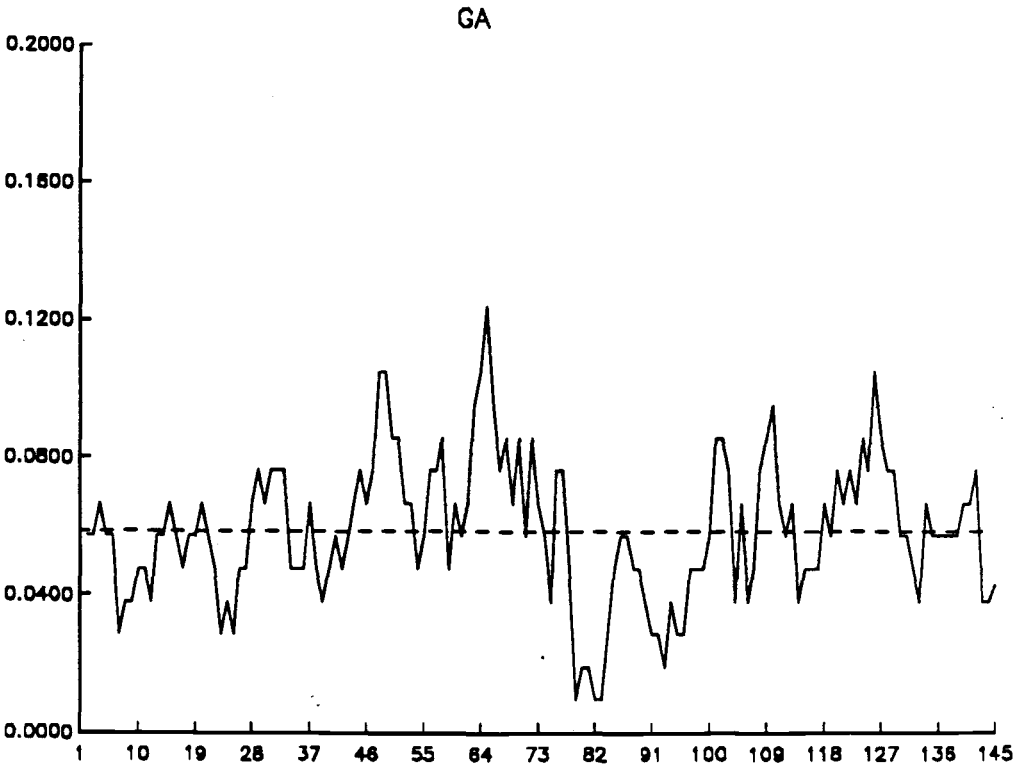

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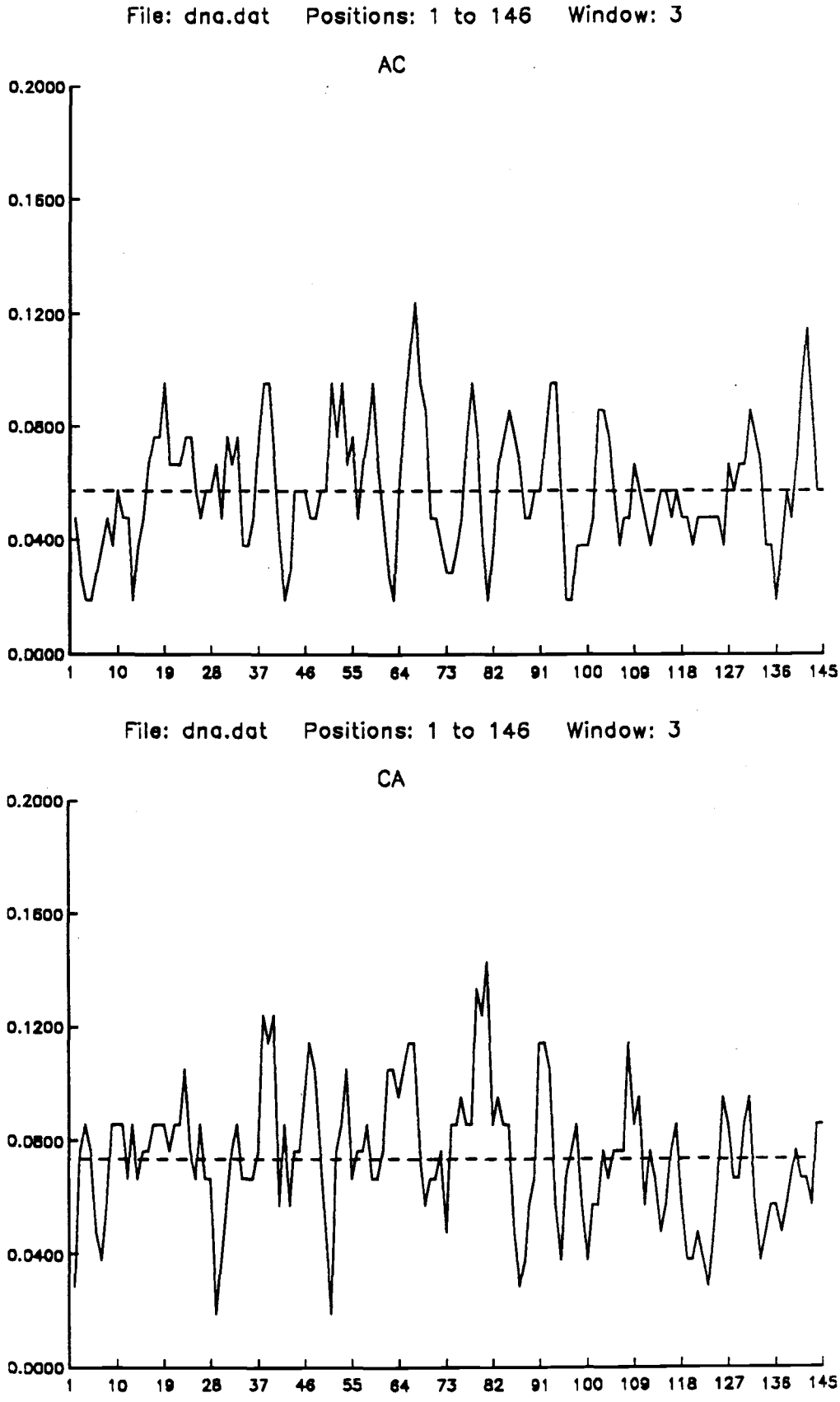
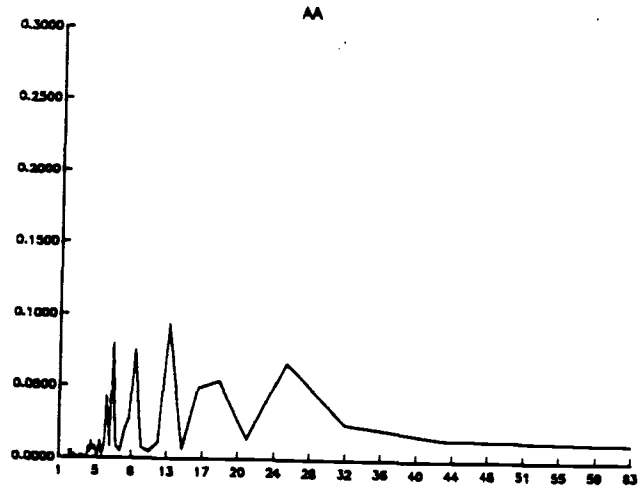


Figure III-5. The periodicity of each dinucleotide on the nucleosomal DNA molecules.

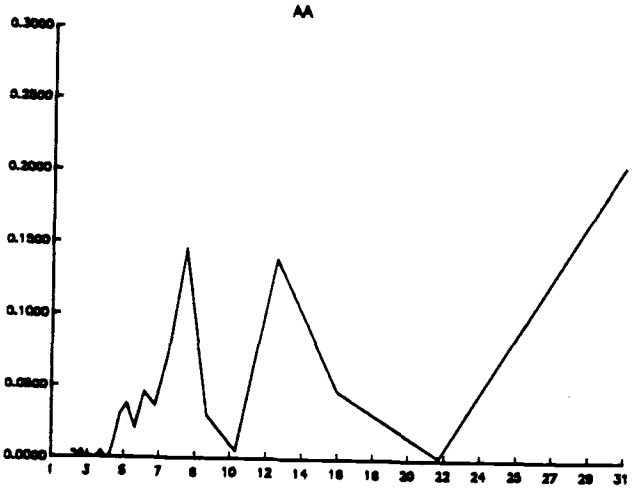
Fast Fourier transformation was used to determine the periodicity of each dinucleotide on the whole molecules (top panel), the left part (central panel) and the right part of molecules (bottom panel).

Figure III-5

File: dna.dat Positions 10 to 137. FFT frequency domain. Window: 3



File: dna.dat Positions 1 to 64. FFT frequency domain. Window: 3



File: dna.dat Positions 83 to 148. FFT frequency domain. Window: 3

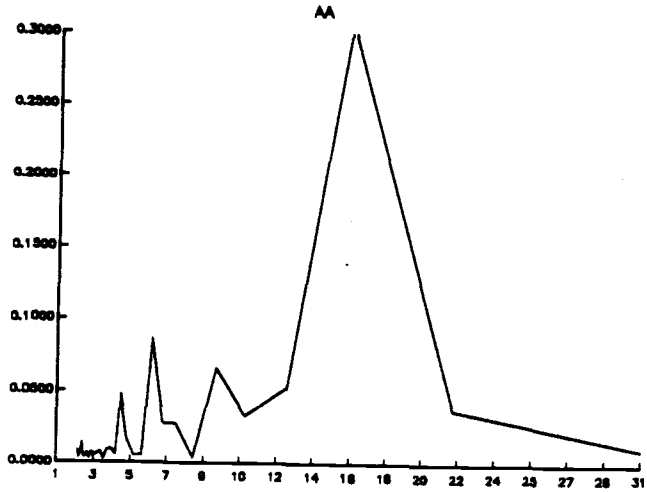
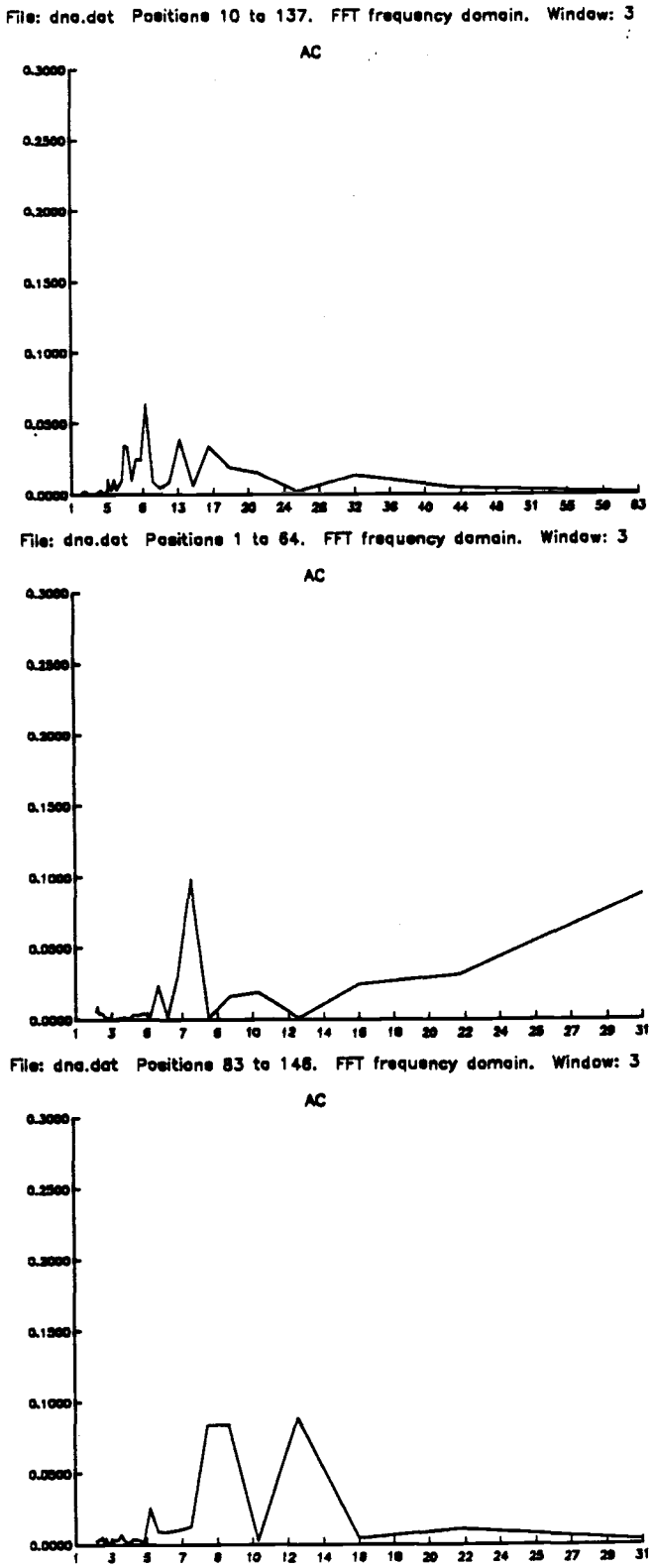


Figure III-5 (cont)



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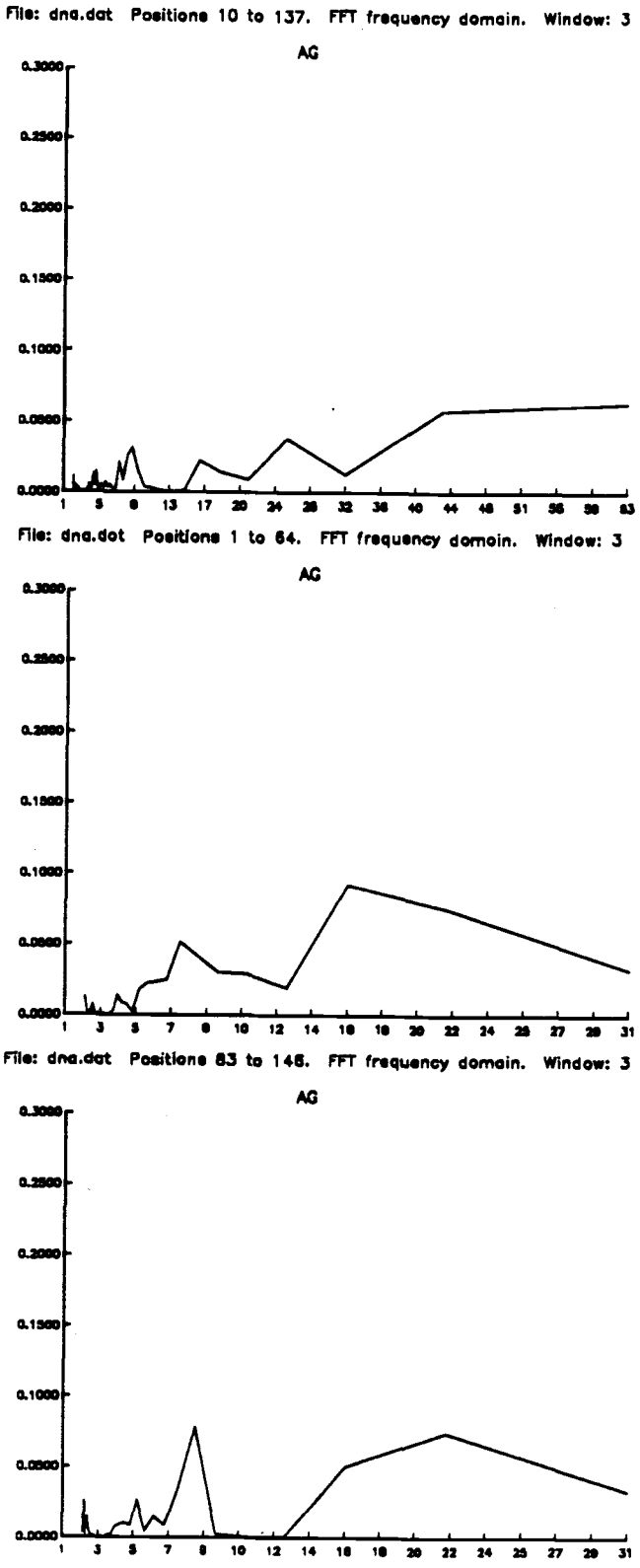

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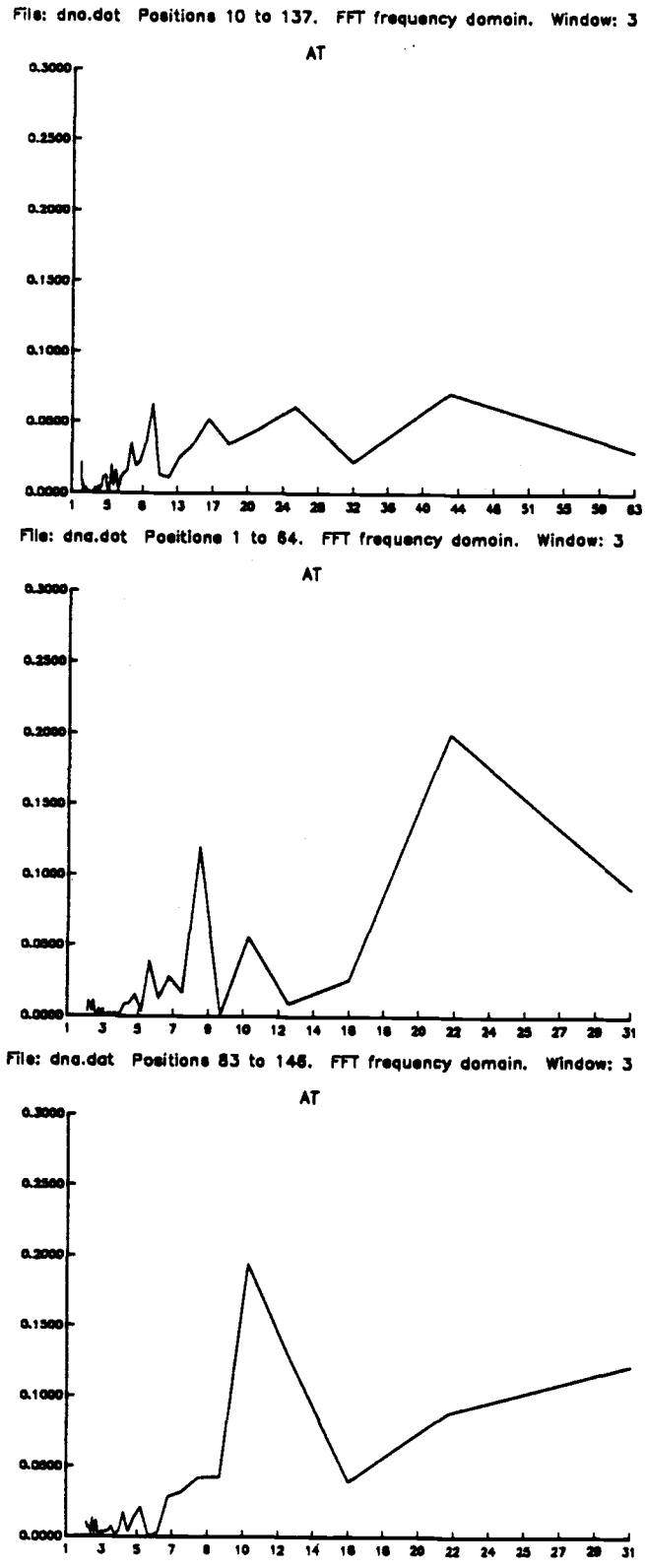

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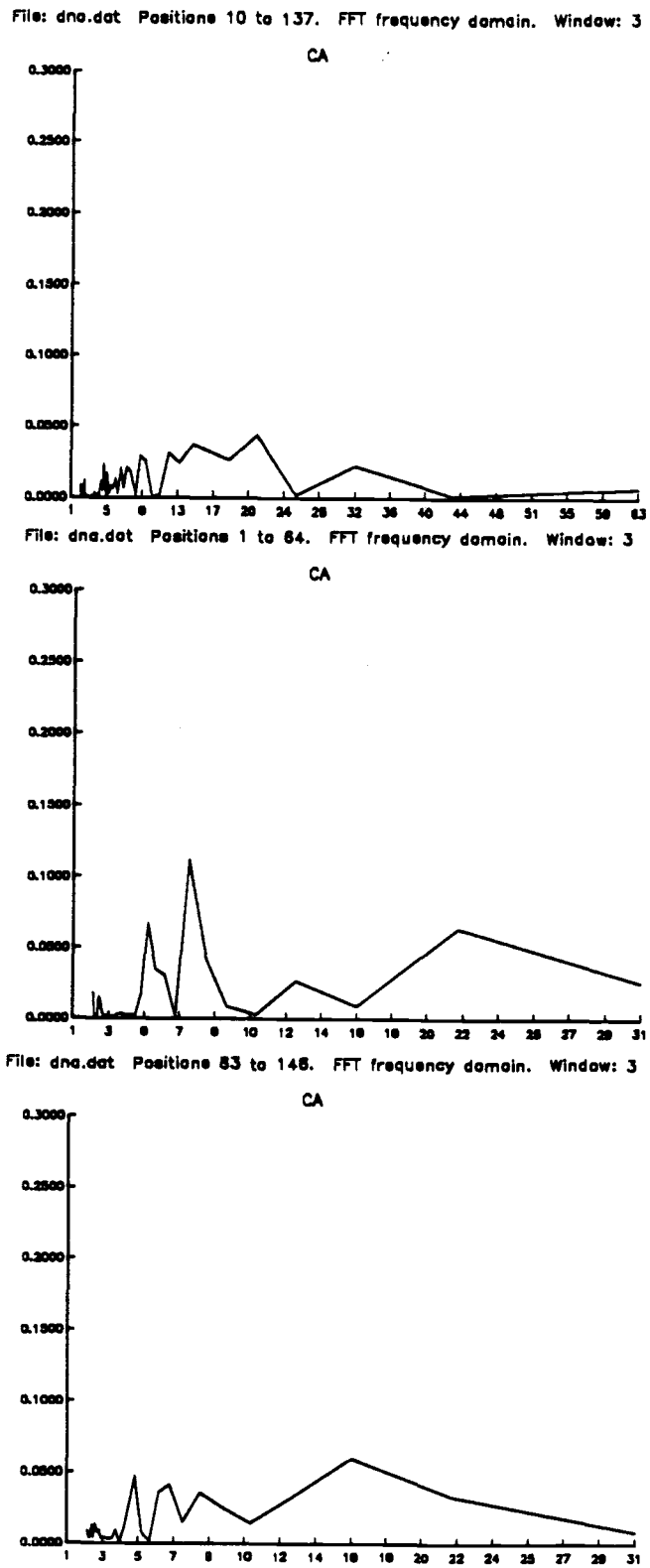

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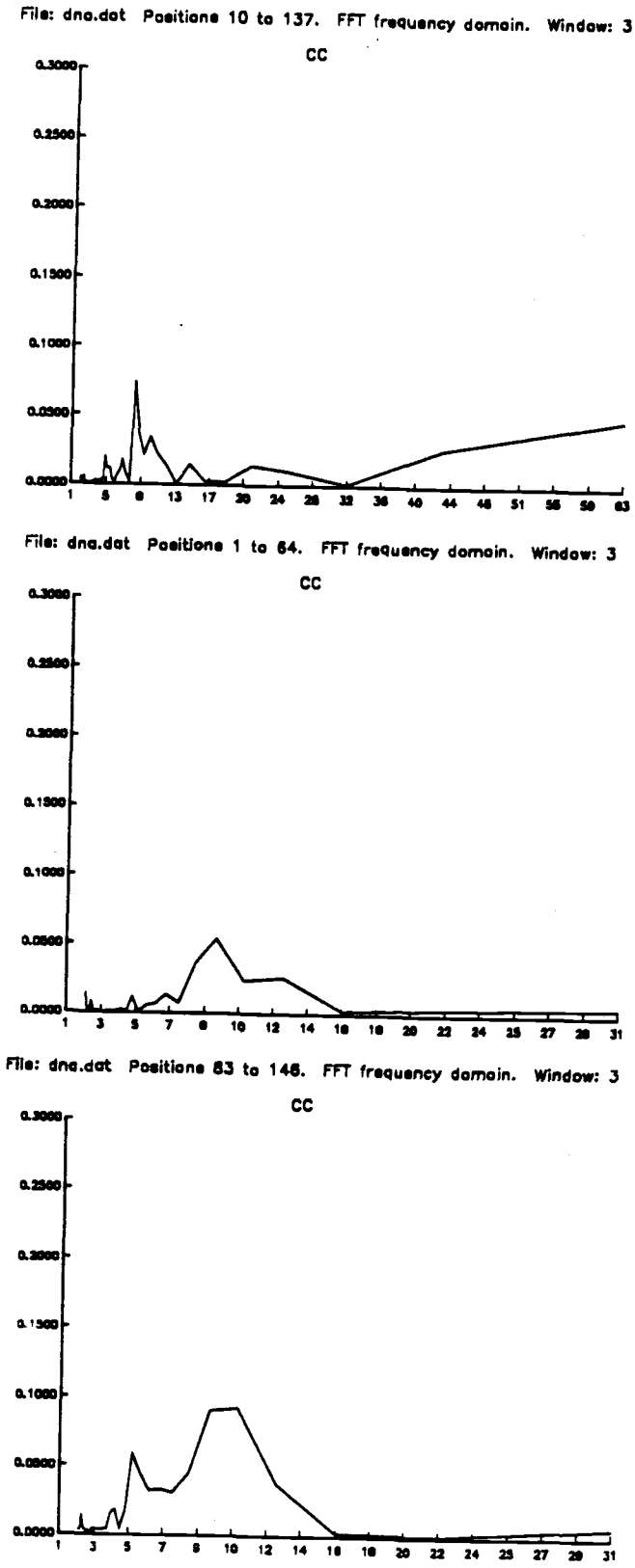
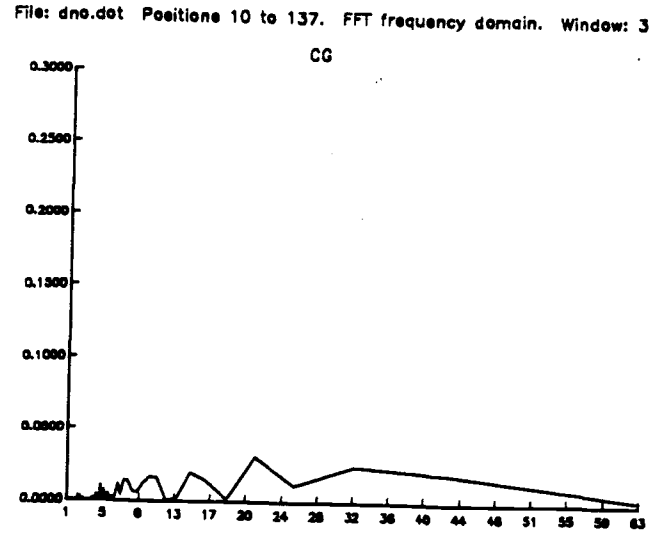
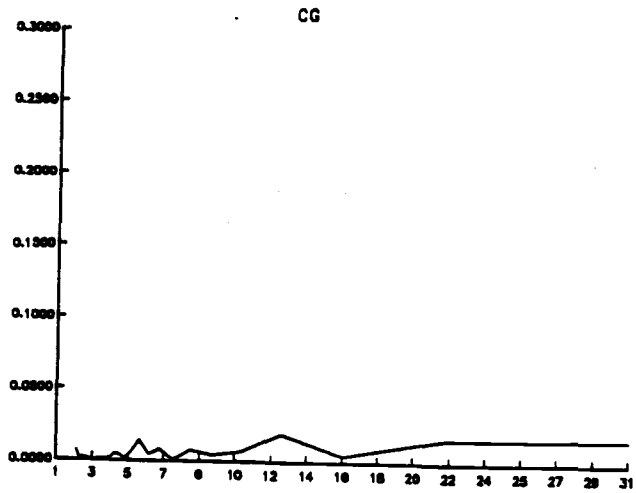


Figure III-5 (cont)





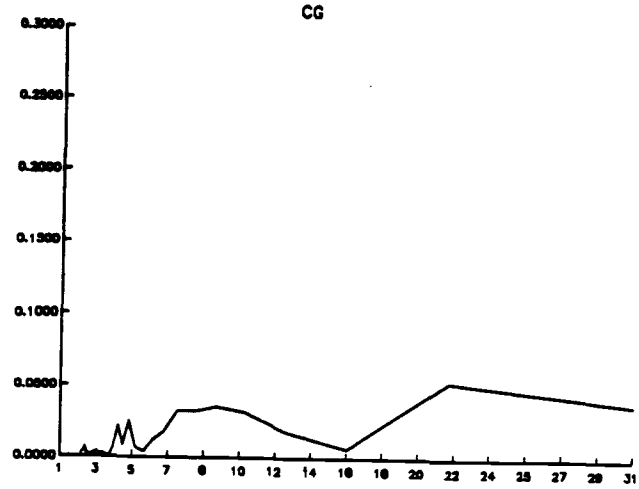
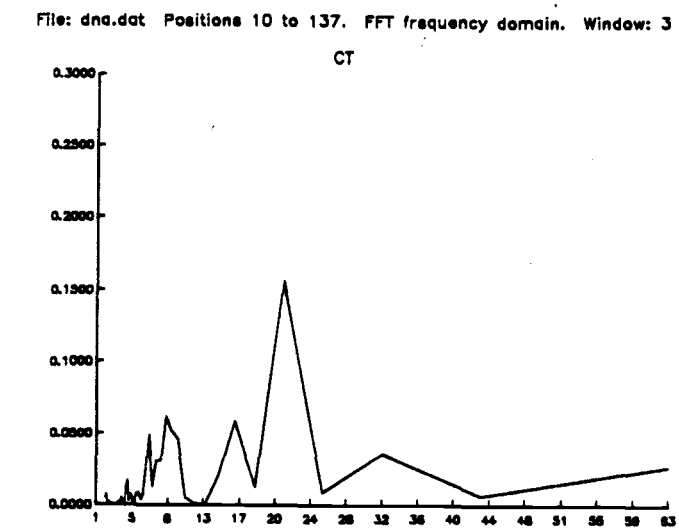
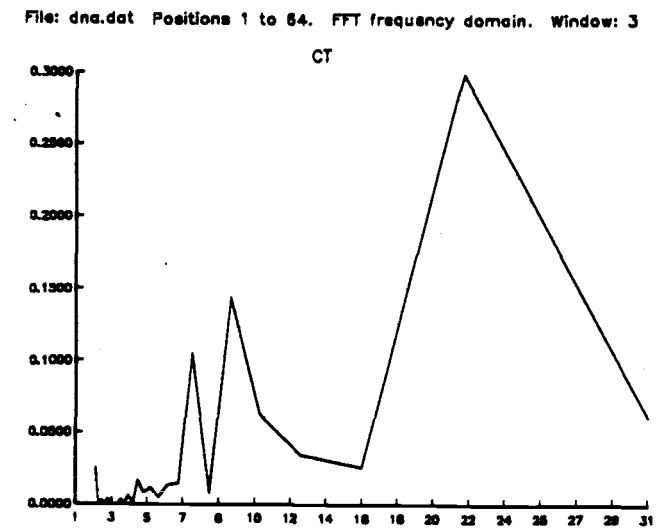
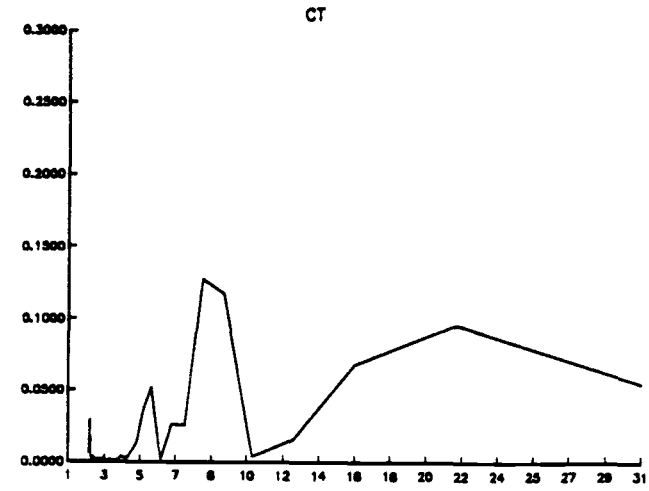


Figure III-5 (cont)







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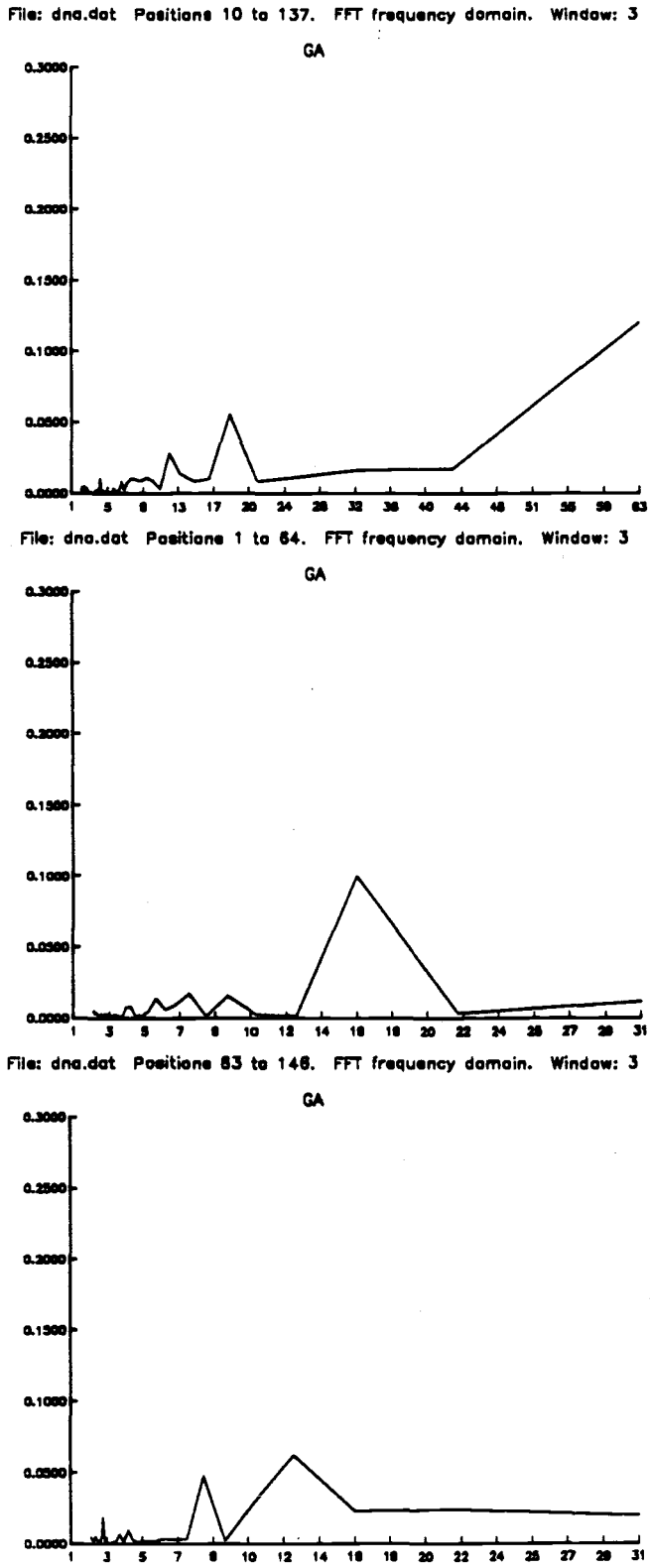

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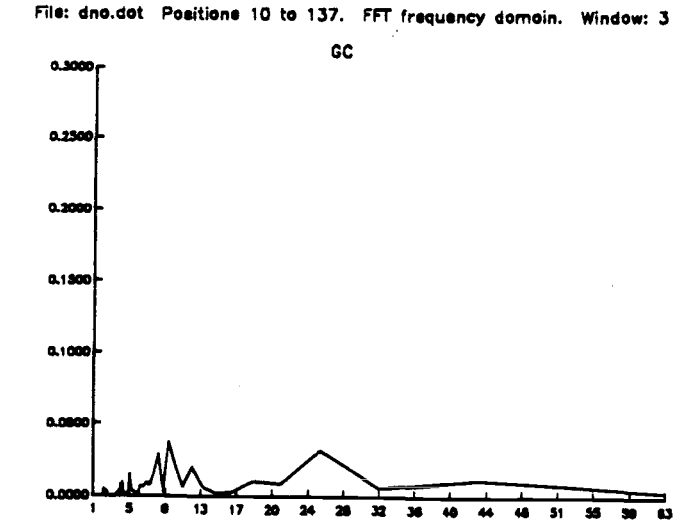

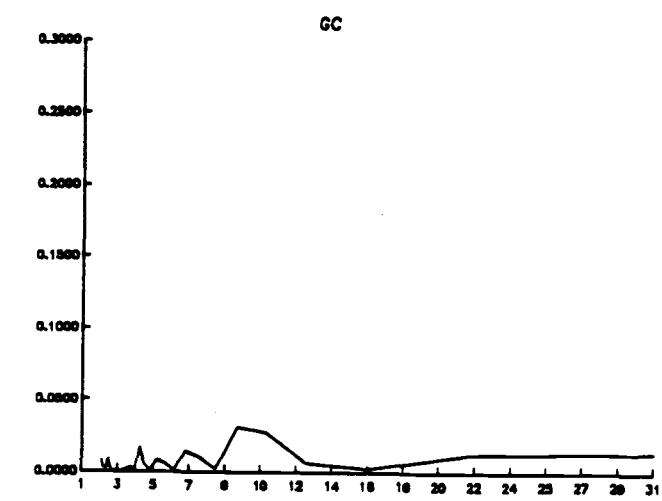

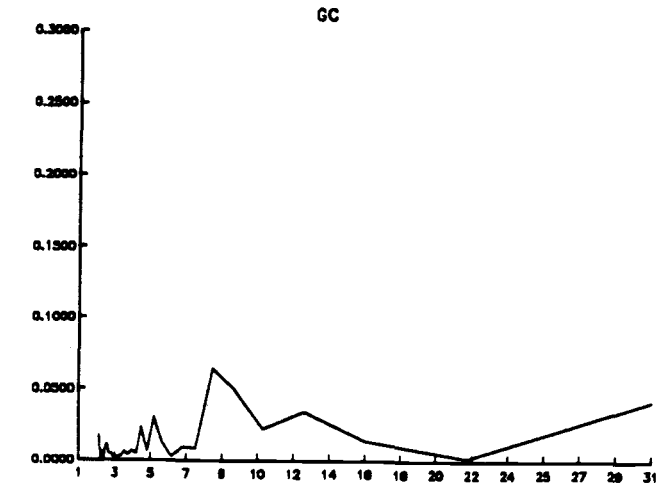

Figure III-5 (cont)

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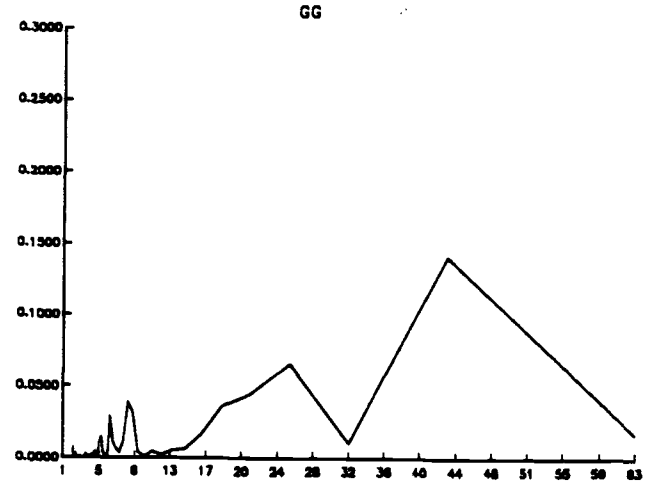

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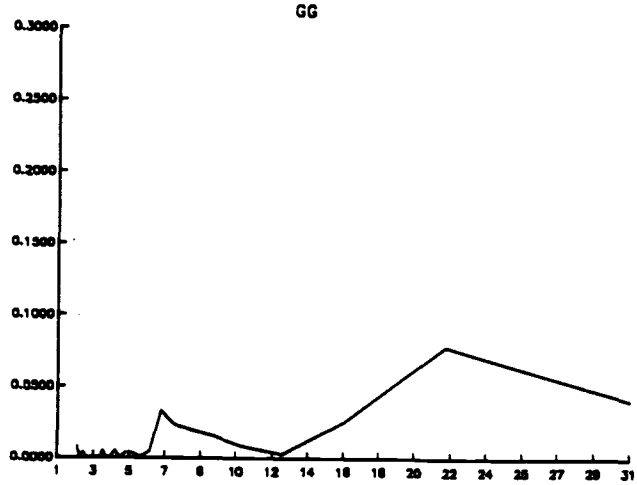
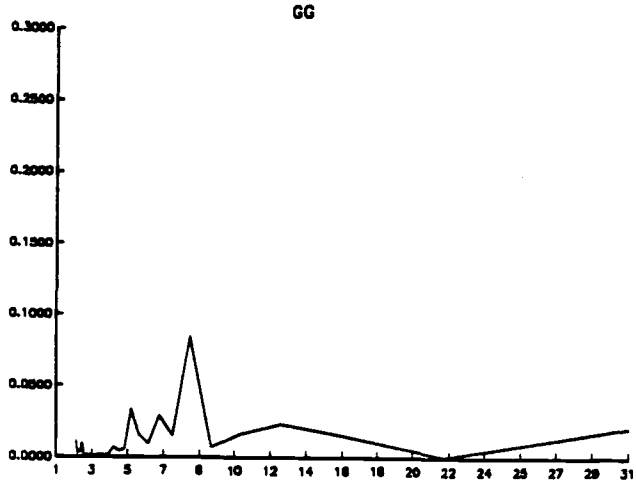


File: dno.dat Positions 83 to 148. FFT frequency domain. Window: 3



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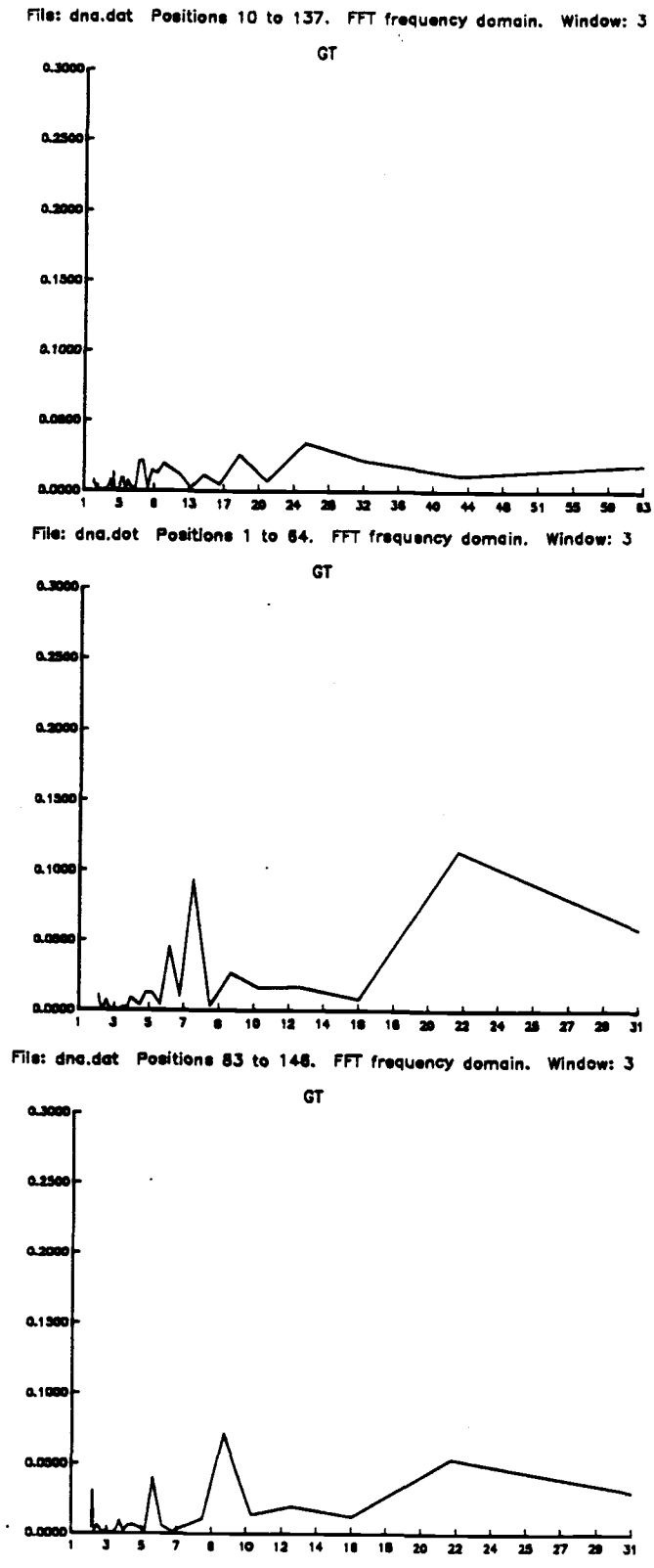

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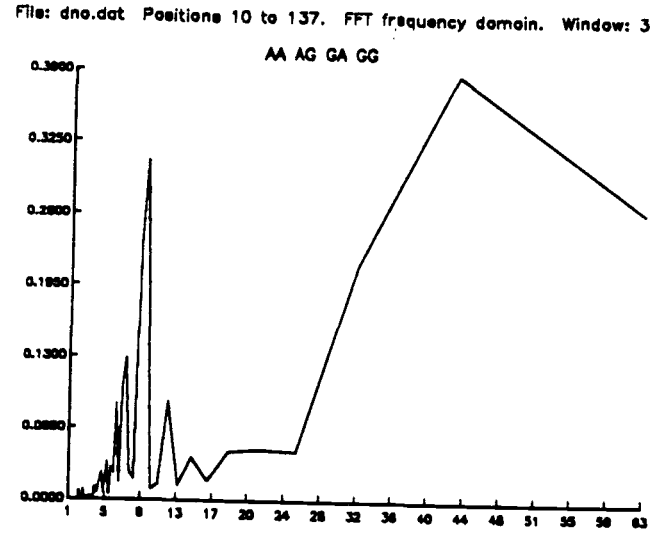

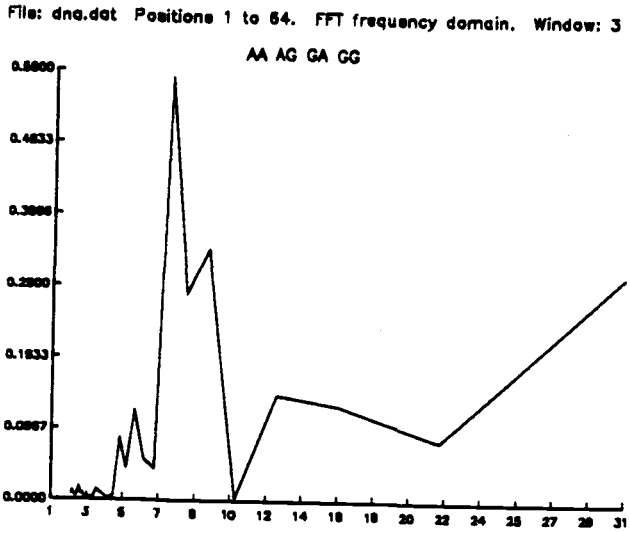

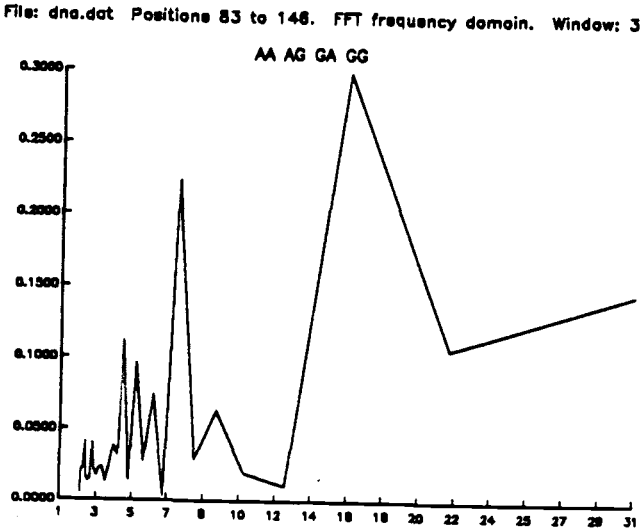

Figure III-5 (cont)

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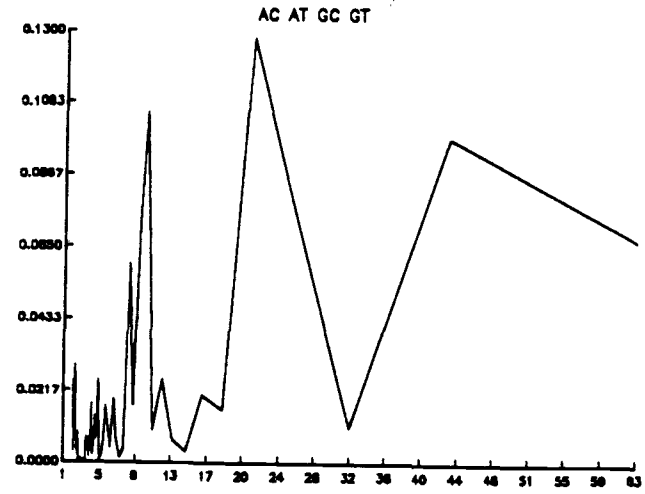

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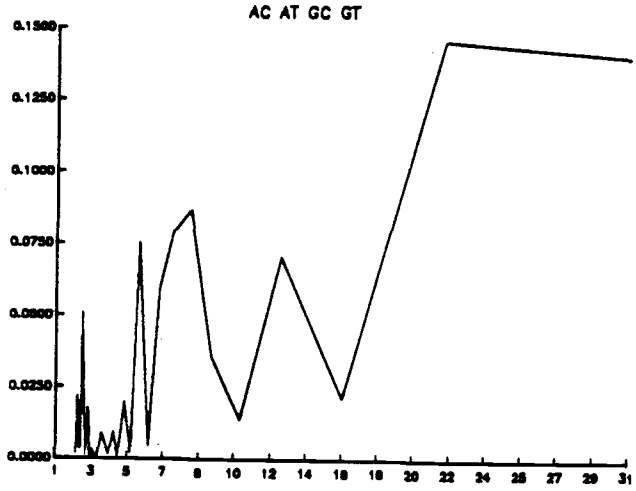

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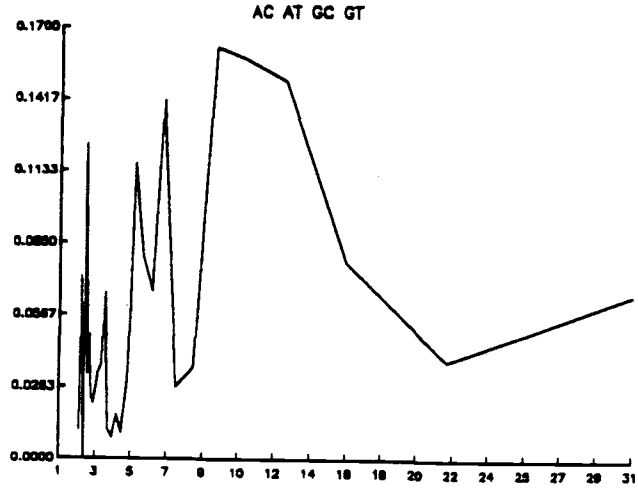

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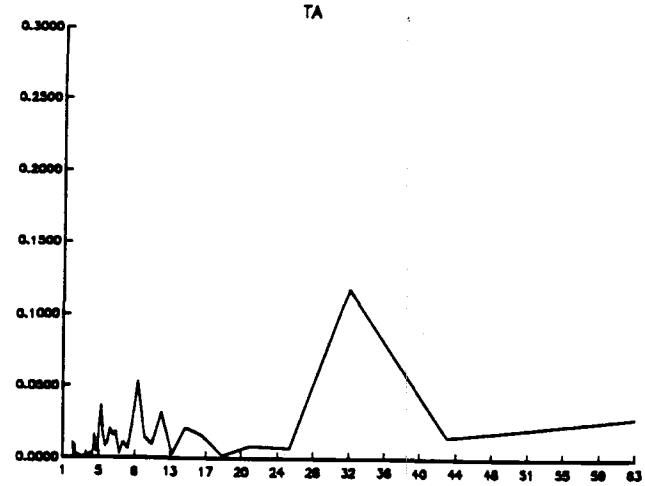

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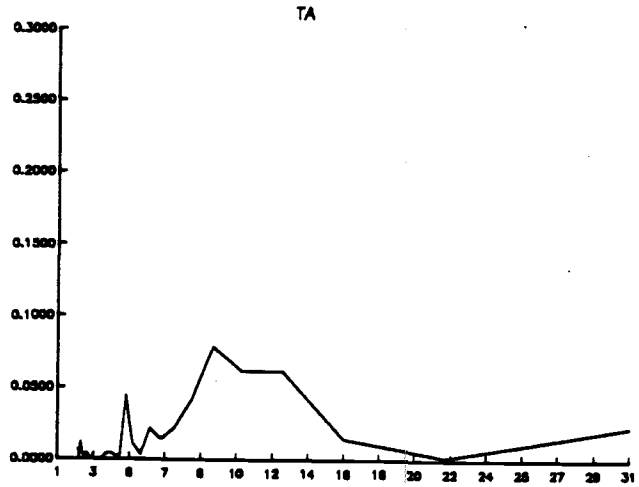

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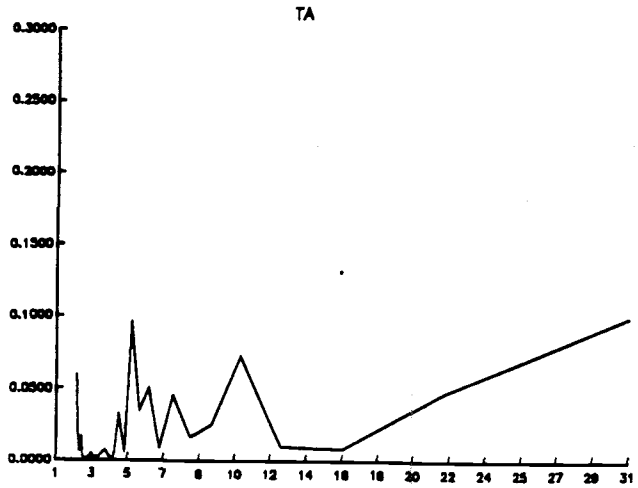

Figure III-5 (cont)

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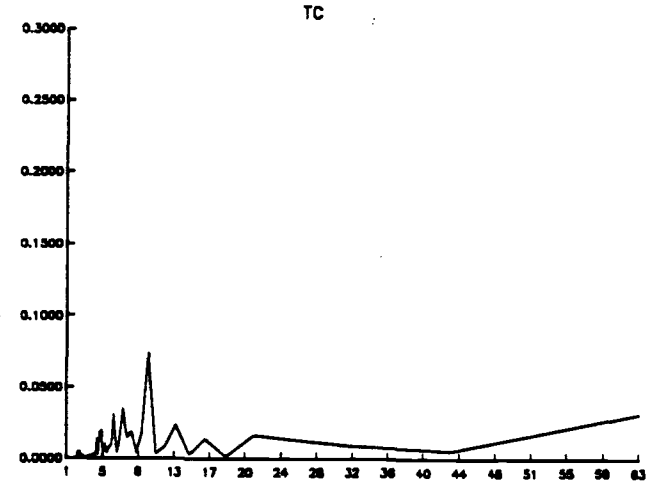

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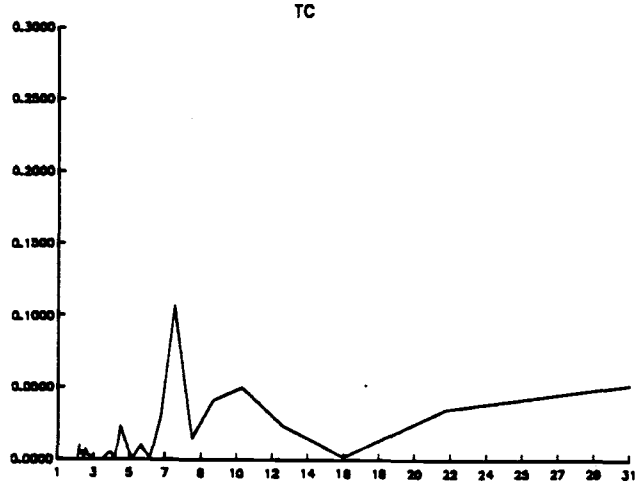

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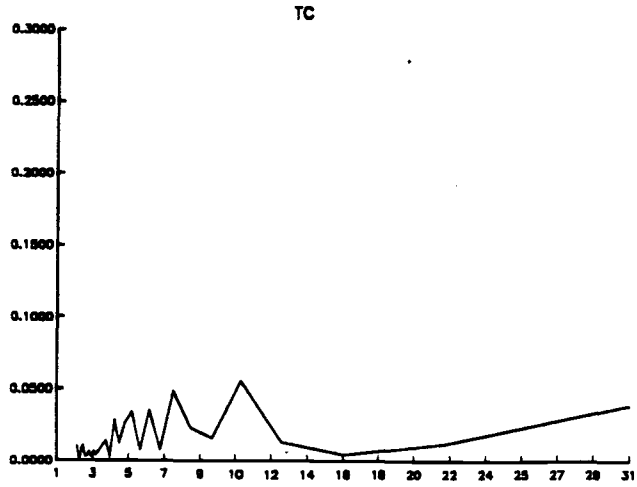

Figure III-5 (cont)

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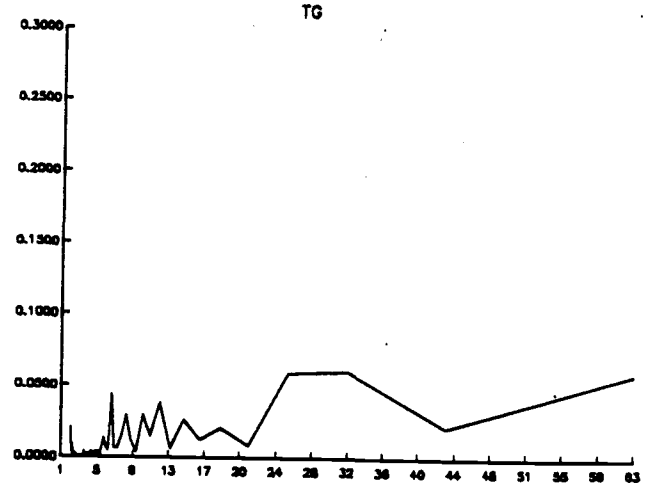

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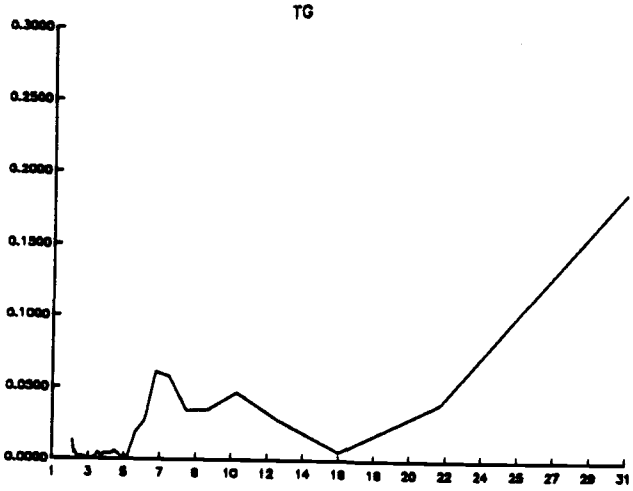

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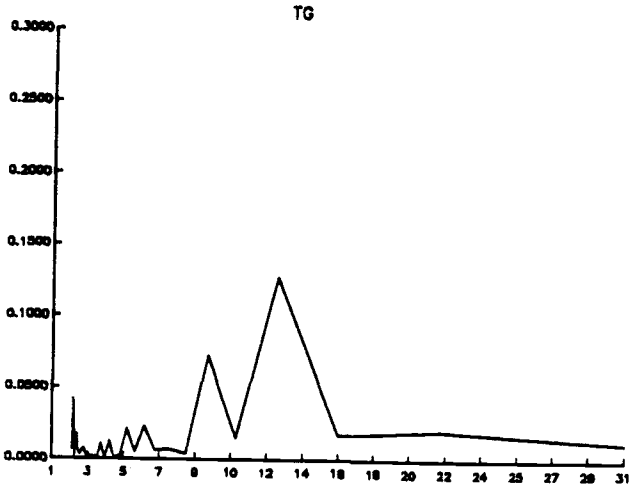

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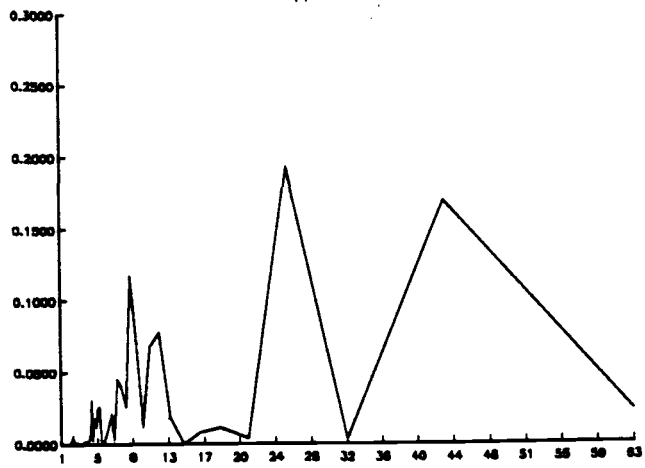

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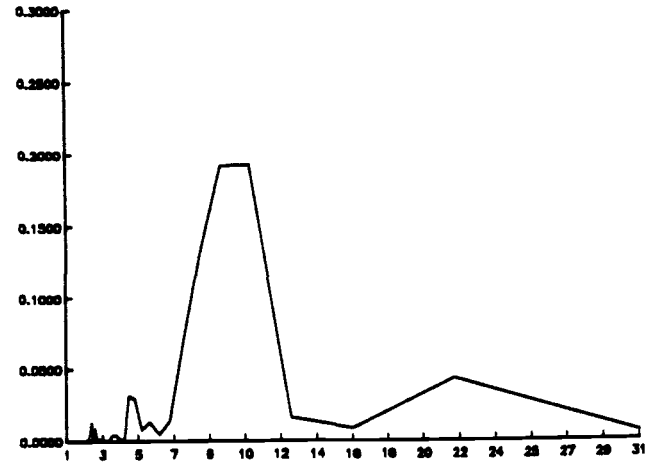

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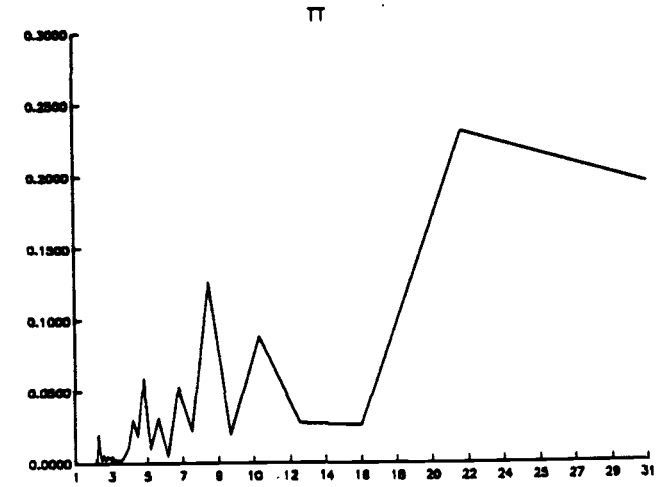

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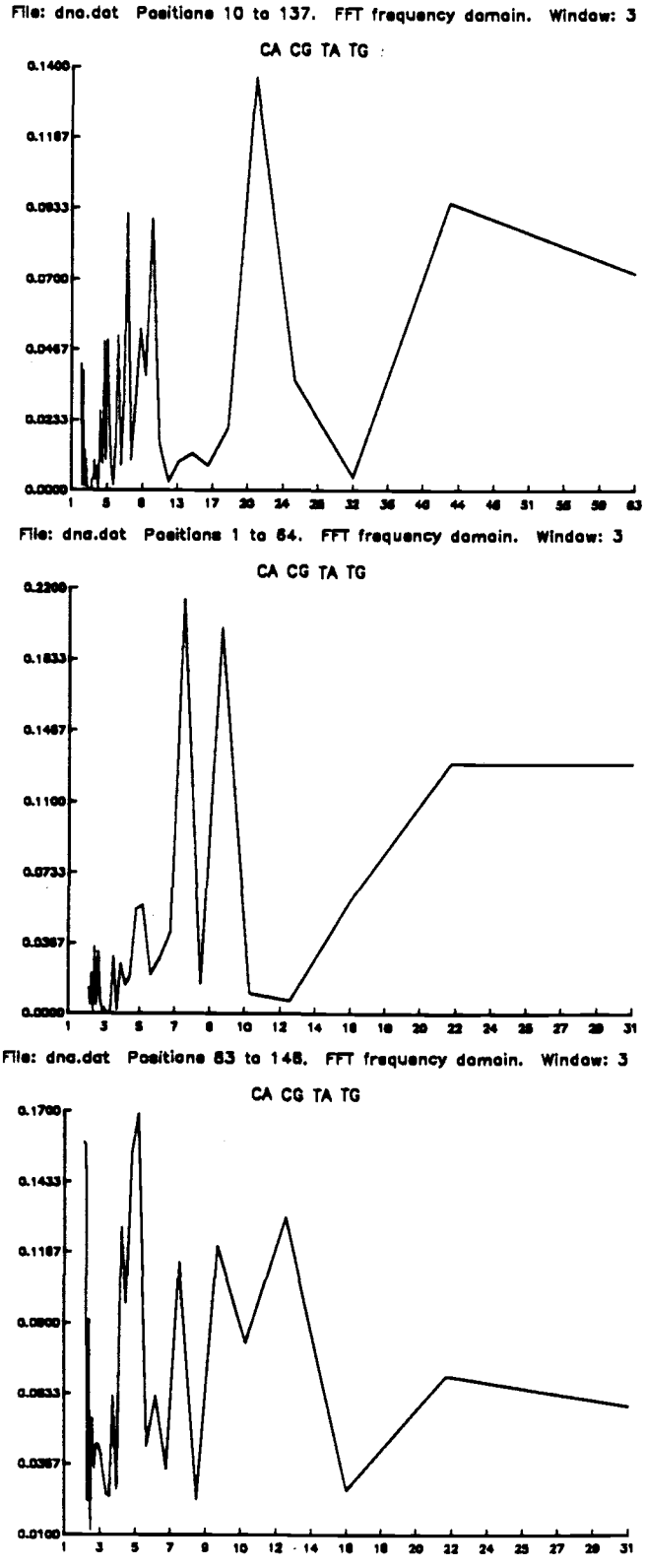
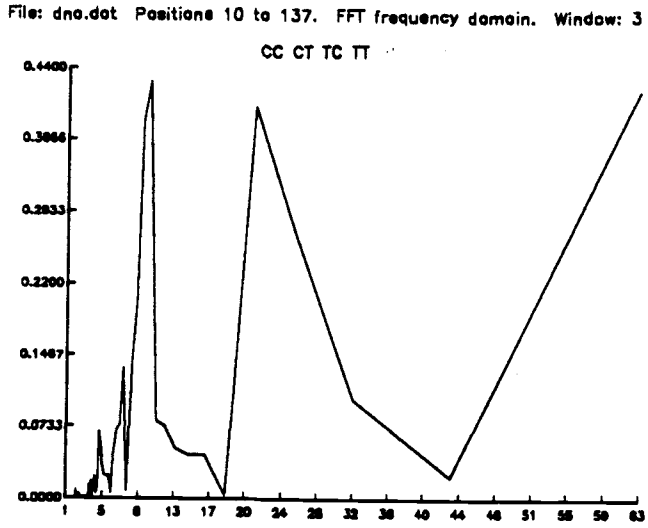
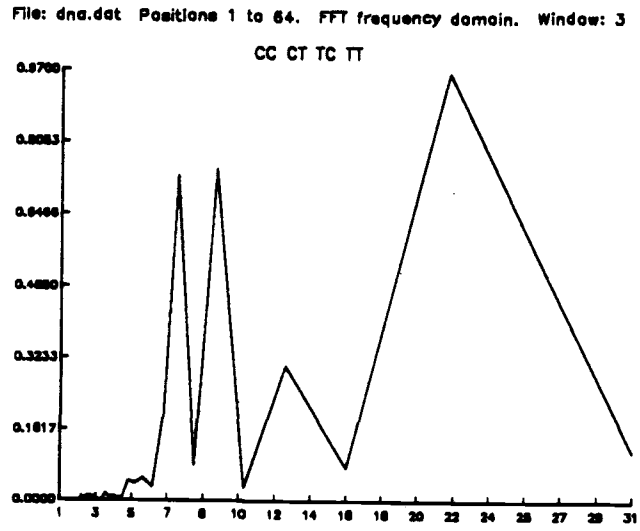


Figure III-5 (cont)





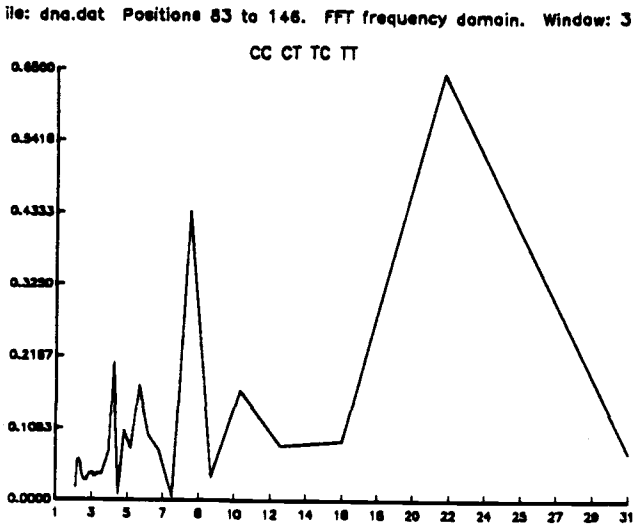


Figure III-6. The sequencing gel of one of the nucleosomal DNA clones. (# 31= NK25, sequence see Figure III-3)
a: use reverse primer; b: use forwarding primer.

Figure III-6

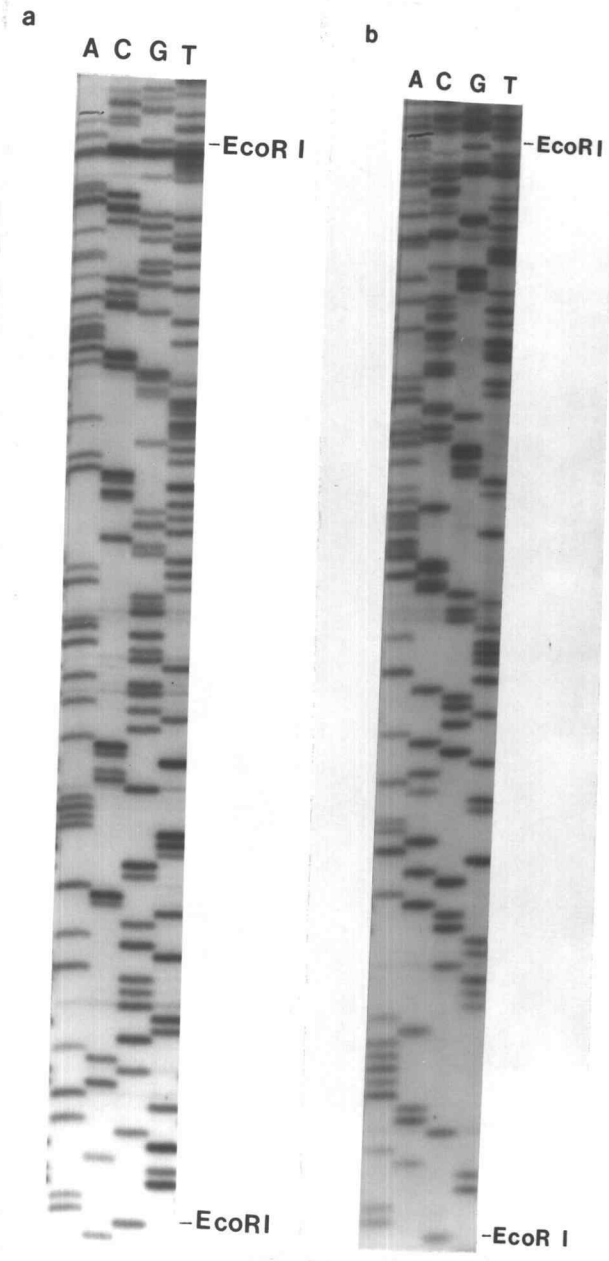
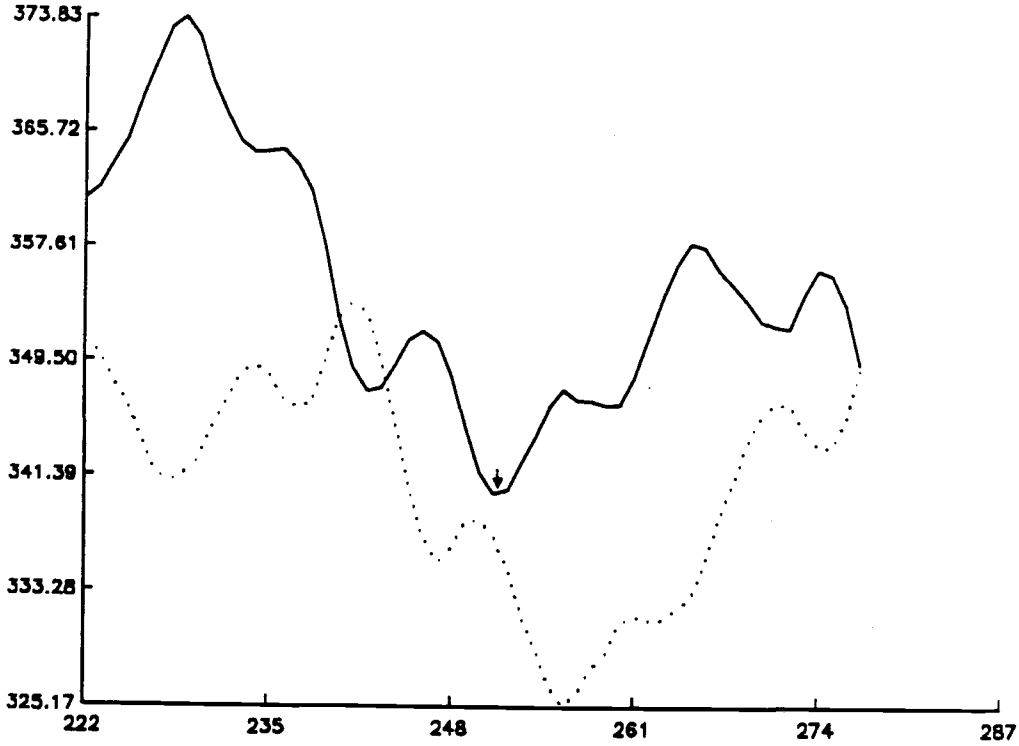


Figure III-7. The prediction of nucleosome positioning from XBEND program.

Solid line: positive maximum bending; dotted line: negative maximum bending; arrow shows dyad position; X axis stands for position; Y axis stands for calculated maximum bending of that fragment.

Figure III-7

File cbg.dat. Fragment 145. Window 11. Rotation 0.0
Positions 222 to 278.



File jeff1.dat. Fragment 145. Window 11. Rotation 0.0
Positions 73 to 189.

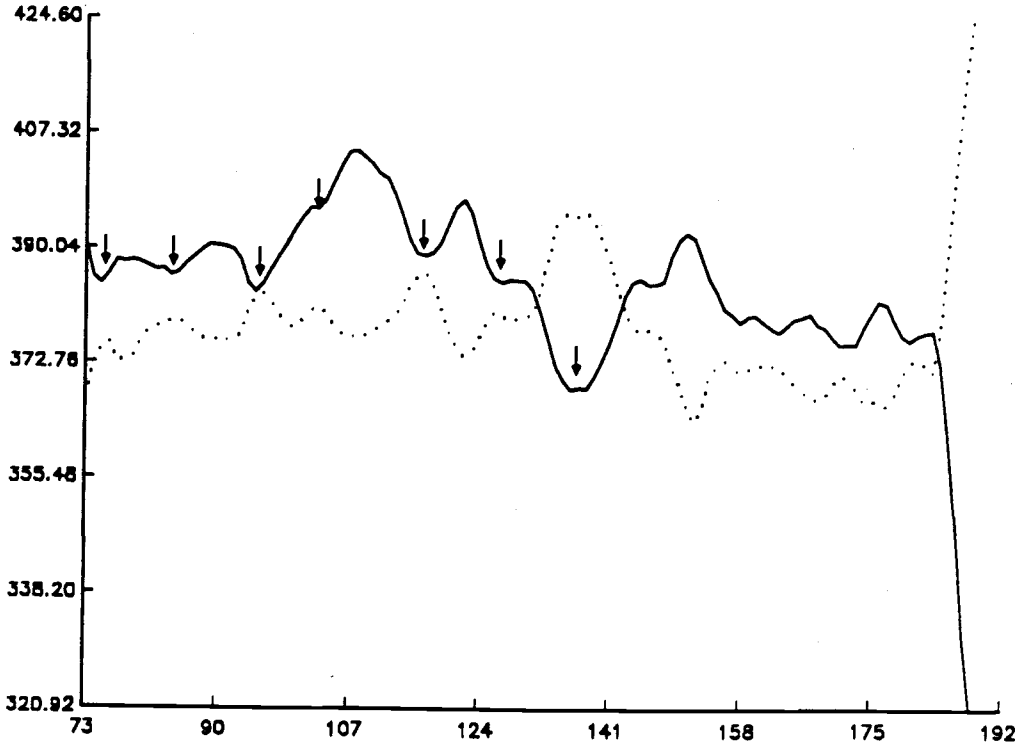
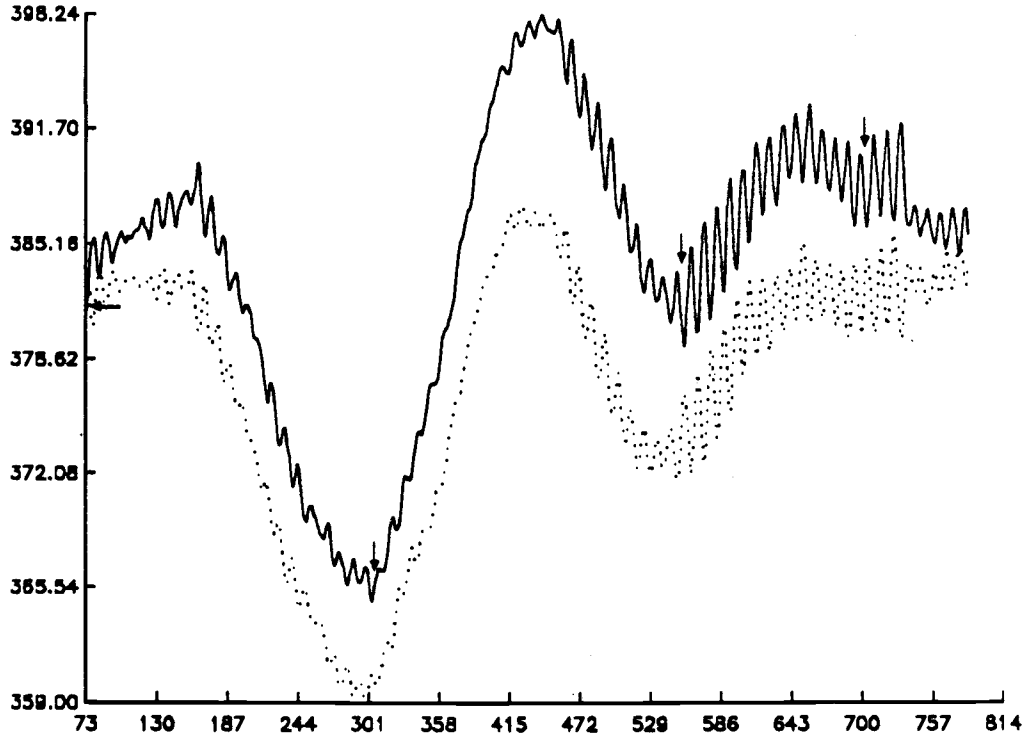


Figure III-7 (cont)

File drew.dat. Fragment 145. Window 101. Rotation 90.0
Positions 73 to 787.



File rep.dat. Fragment 145. Window 11. Rotation 90.0
Positions 73 to 189.

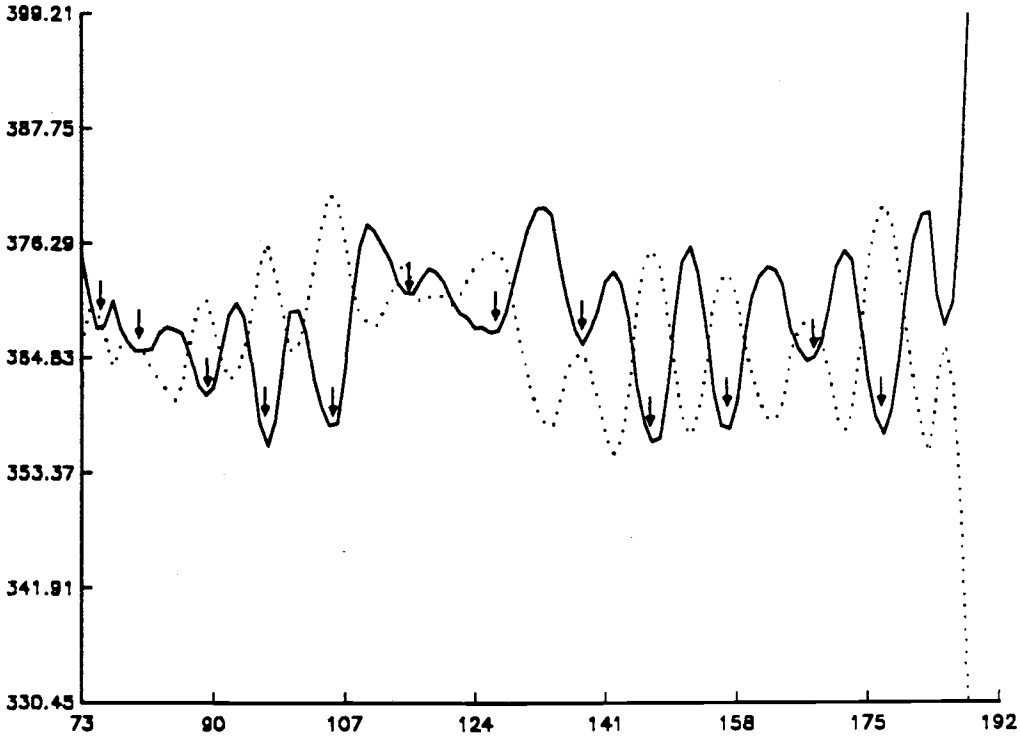


Table III-1. The periodicity of each dinucleotide and bendability among DNA molecules.

Major period of bending	Dinucleotides which have the same period
6.1	AA, GG, TA, TC
6.4	CA, TG, TT
6.7	AA, AC, CC, CT, TA
7.1	AG, CA, TC, TT
7.5	AT, GA, GT, TA
8.0	CC, GG, GC, TC, TG
8.5	AG, CA, CT, GT, TT
9.1	AA, AC, GA, GC, TA
9.9	GT, CG, CC, TC, TG
10.7	AC, AT, CC, TA, TC, TT
11.6	GA, GC, CA, TA, TG, TT
12.8	AA, TC
14.2	CC, CA, CT, GT, TA, TG
16.0	AT, CT, TC
18.3	AA, GA, GC, GT, TG, TT
21.3	CA, CC, CG, CT, TC
25.6	AA, AG, AT, GC, GG, GT
32.0	AC, CA, CG, CT, TA, TG
42.7	AT, GC, GG, TT
64.0	AG, CC, CT, GA, GG, GT, TA, TC

Table III-2. The results from WRAP program.

Table of maximum THEORETICAL bends by sequence.

Seq	Min	Plane	Max	Plane
1	-491.52	120.00	491.52	300.00
2	-416.11	120.00	416.11	300.00
3	-477.44	180.00	477.44	0.00
4	-490.11	80.00	490.11	260.00
5	-407.71	300.00	407.71	120.00
6	-488.81	80.00	488.81	260.00
7	-380.83	340.00	380.83	160.00
8	-506.04	260.00	506.04	80.00
9	-420.01	180.00	420.01	0.00
10	-457.18	140.00	457.18	320.00
11	-397.80	200.00	397.80	20.00
12	-460.53	160.00	460.53	340.00
13	-483.32	340.00	483.32	160.00
14	-466.05	80.00	466.05	260.00
15	-458.12	320.00	458.12	140.00
16	-392.99	40.00	392.99	220.00
17	-445.83	280.00	445.83	100.00
18	-497.77	180.00	497.77	0.00
19	-463.33	140.00	463.33	320.00
20	-532.08	20.00	532.08	200.00
21	-464.73	0.00	464.73	180.00
22	-504.12	100.00	504.12	280.00
23	-461.79	200.00	461.79	20.00
24	-481.17	340.00	481.17	160.00
25	-400.55	20.00	400.55	200.00
26	-492.53	280.00	492.53	100.00
27	-440.40	200.00	440.40	20.00
28	-459.31	260.00	459.31	80.00
29	-456.51	80.00	456.51	260.00
30	-416.10	340.00	416.10	160.00
31	-405.51	300.00	405.51	120.00
32	-503.66	280.00	503.66	100.00
33	-443.65	240.00	443.65	60.00
34	-445.05	20.00	445.05	200.00
35	-466.47	20.00	466.47	200.00

Table III-3. The results summerized from the XBEND prediction (Figure 7) comparing with the published data.

Name of the gene	Experimental dyad	Prediction from XBEND	Reference
Chicken beta globin 5' flanking region	251	251	Kefalas 1988
Lytechinus variegatus 5S rRNA gene	73, 83, 93 103, 113	74, 84, 94, 103 117, 126, 136	Jeff Hanson personal communication
Mouse satellite DNA	84, 94, 104 114, 124, 131 138, 149, 168	75, 80, 88, 96, 105 115, 125, 138, 147 156, 167	Linxweiler 1985
Frog X. borealis 5S rRNA gene	70, 200 330-332, 420 580, 747-751	73, 303, 554 705	Drew 1987

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