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CLONING AND SEQUENCING OF THE ECO RII MONOMER FRAGMENT
OF SATELLITE DNA FROM THE MOUSE Mus musculus

by

Timothy Patrick Boyle

A Thesis
Submitted to the
Faculty of the Graduate College
in partial fulfillment of the
requirements for the
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Department of Biological Sciences

Western Michigan University
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CLONING AND SEQUENCING OF THE ECO RII MONOMER FRAGMENT
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Timothy Patrick Boyle, M.S.

Western Michigan University, 1989

Total mouse satellite DNA was isolated in cesium chloride gradients in the presence of the dye Hoechst 33258. This satellite fraction was digested with Bst N1, an isoschizomer of the restriction enzyme Eco RII, and the 234 base pair monomer fragment was cloned into the plasmid vector pBS. Dideoxynucleotide sequencing of the cloned DNA yielded significant homology with the published consensus sequence of mouse satellite DNA (92%).

ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my wife, Pierina, who exercised great patience and support during its production, from beginning to end.

I would also like to thank those individuals who offered guidance and assistance toward this project.

Timothy Patrick Boyle

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Ann Arbor, MI 48106

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CHAPTER I

INTRODUCTION

The mouse Mus musculus used in the specific locus test (MSLT) was used to show that ionizing radiation, the anticancer pharmaceutical product cyclophosphamide, and the laboratory chemical ethyl nitrosourea induce heritable mutations. These results strongly suggest that these and similar agents may also induce mutations in humans. The MSLT has two short-comings, however: (1) it can not be modified as an eventual test in humans, and (2) it requires 10^4 - 10^5 mice for the evaluation for mutagenicity of a single test agent.

The research group of G. Ficsor and L. C. Ginsberg which I joined as a graduate student at Western Michigan University chose as its long-term research objective the development of a mammalian test which can be modified for use in humans, and which when performed in mice requires far fewer mice than the MSLT.

When DNA is subjected to centrifugation in the presence of cesium chloride solutions, it forms a band in the centrifuge tube where the centrifugal force is cancelled by the bouyant density of the cesium chloride solution. The mouse Mus musculus was discovered to possess a subpopulation of DNA sequences when total DNA was subjected to density gradient centrifugation (Kit, 1961). Waring and Britten, 1966, and McLauren and Walker, 1966 discovered that this satellite band represented a highly repetitive sequence of the mouse

genome through its reassociation after having been denatured. This reassociation rate is termed a C_0T value. It was found that the mouse satellite DNA (msDNA) existed in perhaps one million copies per genome through its C_0T value.

The goal of this research was to confirm the nucleotide sequence of mouse satellite DNA (msDNA). This was to be done by first cloning msDNA, and then sequencing the cloned DNA. This information was judged to be a contribution toward the long-term objective of developing a mouse mutagen test system which was better than the MSLT.

CHAPTER II

REVIEW OF THE LITERATURE

Satellite DNA is the name given to a sub-population of DNA which denotes its relative position to main band DNA after nuclear DNA has been centrifuged in cesium chloride gradients. Main band DNA makes up the bulk of the DNA seen within the gradient, but in some species, a faint band of DNA can be detected above or below the main band. Hence the name satellite is given to these bands. Mouse satellite DNA was first discovered by Kit (1961), and he suggested that its position in the gradient was a function of its base pair composition. Waring and Britten (1966) found that when this mouse satellite DNA was heat denatured and allowed to reassociate, it did so at a much faster rate than any other type of DNA measured under identical conditions. Since the rate of reassociation is a measure of the number of different nucleotide sequences in a given sample of DNA, the concentration of each kind of sequence in that sample will determine the rate (Waring and Britten, 1966). These authors went on to show that mouse satellite DNA exists as multiple tandem repeats of a segment 300 to 400 nucleotides long that occur over one million times in the genome, since satellite DNA makes up approximately 10 percent of the total DNA.

Flamm and associates (1967) took note of the reassociation kinetics of satellite DNA and devised a method for the isolation of

the complementary strands of this species of DNA using an alkaline CsCl gradient. They found that the complementary strands of satellite DNA formed discrete bands in the alkaline gradient, and they labelled them the H (heavy) strand and the L (light) strand. The separation of complementary strands seen by these investigators suggested that the ratio of purines to pyrimidines was different in each strand. An analysis of the base composition of each strand proved this assumption. They found the purine to pyrimidine ratio was 0.5 in the H strand and 1.8 in the L strand. The cause of this difference was an inordinately high number of adenine residues in the L strand; this, of course, was compensated by a high number of thymine residues in the H strand.

In an attempt to characterize and find a possible function(s) for mouse sDNA, Pardue and Gall (1970), and Manuelidis and Manuelidis (1976) found through in situ hybridization experiments that mouse satellite DNA is localized at the centromeres of all the chromosomes--the heterochromatin--with the possible exception of the male Y chromosome. Satellite DNA may exist elsewhere in the chromosome, but not in sufficient quantity to be detected via in situ hybridization. This contention was supported by Stambrook (1981), who showed through hybridization experiments that satellite-like sequences are also interspersed with main band sequences. Pardue and Gall (1970) suggested that satellite DNA may function as the site of microtubule binding, but their experimental results failed to support this contention.

Biro and associates (1975) undertook the task to determine the

sequence analysis of satellite DNA to determine if within the previously defined 300-400 base pair repeat there existed one or more smaller repeat units. They analyzed pyrimidine tracts and cRNA preparations to arrive at their conclusions. Also, these investigators performed phosphodiesterase sequencing of pyrimidine tracts to derive some of the component oligonucleotide sequences of the larger satellite repeat. Their results indicated that mouse satellite DNA has evolved from a short nonamer, d(GA₅TGA), or a closely related sequence. These authors suggested that this original oligonucleotide sequence diverged to the other related sequences which they found to exist in high frequency in their cRNA transcripts: d(GA₄CTGA), d(GA₄TGA), and d(GA₃TG). These four sequences then went through a series of multiplication events to become the present mouse satellite DNA unit.

Southern (1975) used restriction enzyme analysis of mouse satellite DNA to detect more recent evolutionary changes in the structure of the repeating unit, and to develop an overall scheme of the evolutionary development of satellite DNA. This scheme begins with the 9 to 18 "starter sequence" indicated by Biro et al. (1975). This sequence then undergoes four stages of multiplication followed by approximately 10% or less divergence after each multiplication step. The lengths of the DNA segments at each stage of multiplication were 9 to 18, 36 to 72, 110 to 130, and 220 to 260 base pairs. Given that there is about 1% divergence per million years for repeated DNA in rodents (Rice, 1972), Southern concluded that mouse satellite DNA was approximately 20 million years old. In

addition, from his restriction enzyme analyses, Southern (1975) found a unique Hae III site in some of the satellite units. This site produced restriction fragments of 0.5 and 1.5 times the usual fragment length of satellite DNA. He concluded that these 1/2-mers and 1 1/2-mers were the result of divergence and unequal crossing-over, and that these events have been the most recent in the evolutionary history of mouse satellite DNA.

Horz and Zachau (1977) also characterized mouse satellite DNA using restriction nucleases. They observed two similar digestion patterns from all the enzymes tested. The first was termed type A. This was the pattern seen from digests with Eco RII (Bst NI). Fragments generated by this enzyme were about 245 base pairs, or some multiple of this, or even half multiples (viz., multiples of about 120 base pairs). The other digestion pattern recognized by these authors was termed type B. This pattern was essentially the same as type A, except the multimers were the more predominant species, instead of the 245 base pair segment seen in type A digestions. These authors claimed type B pattern cleavage sites are clustered on various parts of the satellite, whereas type A cleavage sites are found on virtually 80% of the satellite DNA. Evolution of satellite DNA, according to these researchers, could be the result of saltatory amplification steps that are confined to certain regions of the satellite sequence. If this region contained a type B site, this would be accordingly amplified. They also state that local amplification and divergence schemes could account for the fragments seen which are fractions of the 245 base pair monomer, as opposed to

the unequal crossing-over scheme of Southern (1975). Contingent with their hypothesis for the evolution of satellite DNA, these researchers imagine that different chromosomes could have slightly different satellite segments which could have some functional aspect specific for that chromosome.

Shmookler-Reis and Biro (1978) were able to clone a 240 base pair fragment of mouse satellite DNA into a lambda phage vector and sequenced it via the dideoxy chain termination method of Sanger (1975). This sequence, along with cRNA-fingerprint analyses and S1 nuclease mapping, led these authors to conclude that satellite DNA could have evolved from spontaneous unequal recombinations and "drift." These researchers assumed that if unequal recombinations occur relatively frequently compared to mutations, they will tend to produce a homogenous repeated DNA. They termed this process "random walk fixation," and argued that the evolutionary age of satellite DNA could not be accurately determined, as Southern had estimated (1975). To further their hypothesis of satellite DNA evolution, these authors could not detect any intermediate repeats of DNA between 15 and 60 base pairs from their cloned sequence.

Horz and Altenburger (1981) and Manuelidis (1981) each succeeded in sequencing the monomer fragment of mouse satellite DNA. Both groups of researchers digested the satellite with Eco RII and performed Maxam and Gilbert chemical sequencing on the isolated monomer. Each group arrived at a consensus sequence that consisted of 234 base pairs, and their sequences were identical. Each of the authors determined a subrepeat of the satellite which consisted of 58

to 60 base pairs. Horz and Altenburger derived three ancestral sequences from their consensus sequence: GA₅TGA, GA₆CT, and GA₅CGT. Both Horz and Altenburger and Manuelidis cited the evolutionary scheme of Southern (1975) as the most likely method that produced the current 234 base pair monomer sequence which their experiments yielded.

After the discovery of the unambiguous sequence of the monomer unit of mouse satellite DNA, researchers turned their attention to the function of this unusual segment of DNA. Some repeated DNAs are functional genes. Among these are the ribosomal RNA genes, transfer RNA genes, and the globin, actin, and immunoglobulin gene families, as well as others (for a review, see Long and Dawid, 1980). Some repeated DNA sequences in the mouse also produce RNA transcripts. These are the B1, B2, MIF-1, R, and the EC1 mouse repetitive DNA families (Bennett et al., 1984). Yet the major satellite DNA from the mouse Mus musculus does not produce any RNA transcript. Previously, from localization experiments, researchers thought satellite DNA functioned in chromosome recognition during meiotic pairing and aggregation of functionally related sequences of nonhomologous chromosomes during interphase, or that satellite DNA helped to regulate recombinant frequencies during meiosis (Berridge, 1986).

Zhang and Horz (1984), and Linxweiler and Horz (1985) determined nucleosome positioning on the satellite monomer unit through nuclease treatment of DNA and subsequent isolation of satellite DNA via denaturation and reassociation. They found sixteen dominant

nucleosome positions, and these positions correlated with the internal 9 base pair repeat. Thus, the histone octamers that bind DNA do so in a sequence dependent fashion in satellite DNA. However, given the divergent nature of the 9 base pair repeat, this sequence dependency is not absolute. The authors stopped short of saying that the function, or one of the main functions, of satellite DNA is in the folding organization of chromatin. Neuer-Nitsche et al. (1988) discovered a new satellite sequence which they called satellite II. Through denaturation experiments designed to separate the DNA from the nuclear polypeptides, they determined this satellite fraction functions in the nuclear matrix as attachment points. Lica et al. (1986), using electron microscopy, determined that satellite DNA is required for maintenance of contact between sister chromatids. Avila et al. (1983) showed through digestion protection experiments that the microtubule-associated protein MAP₂ binds to satellite DNA.

In a number of methylation studies, satellite DNA was found to be more methylated than either moderately repetitive sequences or single copy sequences (Gama-Sosa et al., 1983), and satellite DNA was also found to exist in sperm in a state of methylation far less than in somatic cells (Adams et al., 1983, Ponzetto-Zimmerman and Wolgemuth, 1984, and Feinstein et al., 1985). It is known that extensive methylation is correlated with nonexpression of genes (Goodenough, 1984). As the satellite DNA is not actually a gene and therefore not expressed, the heavy methylation pattern follows.

Mouse satellite DNA has been extensively studied, and it now seems apparent that this fraction of the genomic DNA plays an

important part in the large scale organization of chromosomes within the nucleus. In particular, mouse satellite seems most suited to condensation of the chromosomes and to chromatid pairing, both during and after replication events.

CHAPTER III

MATERIALS AND METHODS

Figure 1 shows a flow chart of the basic road-map followed in the cloning and sequencing of mouse satellite DNA (msDNA). Briefly, genomic DNA was isolated from the testes of mice, and msDNA was isolated via ultracentrifugation of the genomic DNA. The sDNA was digested to its monomer fragment and cloned into the plasmid pBS (Stratagene, La Jolla, CA). Dideoxy sequencing was performed on the cloned msDNA. Most protocols followed, and buffers and solutions, were from Maniatis, *et al.* (1982).

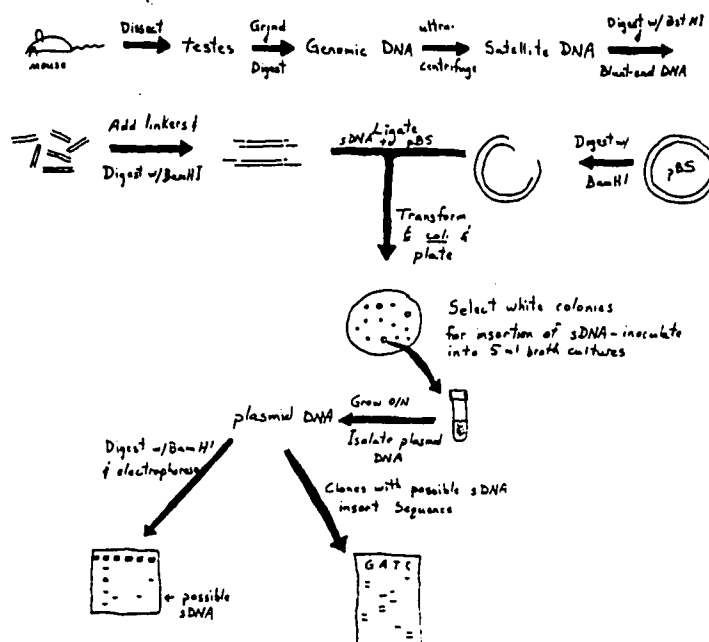


Figure 1. Flow chart protocol for cloning and sequencing msDNA.

Isolation of Genomic DNA

Genomic DNA was isolated from the testes of the mouse Mus musculus. Male HAM/ICR mice were sacrificed by cervical dislocation, and the testes were quickly removed and lanced with a thin needle projecting from the underside of a flat piece of cork. The testes were then immediately placed into liquid nitrogen to freeze the tissue and prevent any nuclease damage to the DNA. After two or three minutes in the liquid nitrogen, the testes were removed and placed into a shallow dish which was equilibrated at -70° C using dry ice. A mortar and pestle, previously equilibrated in dry ice, was then used to grind the frozen tissue to a fine powder. The powdered tissue was then resuspended in 1.2 ml digestion buffer per 100 mg tissue (digestion buffer is 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml proteinase K). Proteinase K must be added fresh with each use. The tissue suspension was then incubated with shaking at 50° C for 12 to 18 hours in a tightly capped polypropylene Falcon tube, type 2059. After the digestion period, there may be a sludge of undigestible cell components and possibly small pieces of tissue too large for the overnight digestion to completely degrade. The viscous solution was extracted with an equal volume of phenol:chloroform (note chloroform is 24:1 chloroform:isoamyl alcohol) and centrifuged ten minutes in a table-top centrifuge at 5,000 rpm (5000 x g) in a swinging bucket rotor fitting a Sorvall RT6000B centrifuge. The upper aqueous phase was transferred to a fresh Falcon 2059 tube. Care must be taken to avoid transferring the white interface and the lower organic phase.

The equal-volumed phenol:chloroform extraction was repeated until the interface between the phases was gone. The final aqueous layer was transferred to a clean polypropylene centrifuge tube, and 1/2 volume of 7.5 M ammonium acetate and two original volumes of 100% ethanol were added. The resulting solution was mixed and the tube centrifuged in a table-top centrifuge (Sorvall RT6000B) at 5000 rpm for 5 minutes. The supernatant was carefully decanted and the pellet washed with 70% ethanol and centrifuged as before. The ethanol was again decanted and the pellet was dried in a vacuum dessicator for 5 minutes. The pellet was resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) until it dissolved completely, about 1 ml. Warming the solution to 65° C for a few hours facilitated solubilization of the DNA. Five microliters of the resulting genomic DNA solution, diluted to one milliliter, was analyzed at absorbances of 260 and 280 nm in a spectrophotometer to determine the concentration. At such a dilution (0.005), the absorbance at 260 nm times 10 will yield mg/ml. The concentration of the prepared genomic DNA was then adjusted to be 1 mg/ml.

Isolation of Satellite DNA

Satellite DNA was isolated from genomic DNA by isopycnic centrifugation in the presence of the DNA-binding dye Hoechst 33258, at a final concentration between 0.6 and 1.3 ug dye/ug DNA. CsCl gradients (12 ml each) were made to a density of 1.68 g/ml ($n=1.3975$) by dissolving 10.65 g of CsCl in 9.1 ml TE, pH 8.0 (note that this 9.1 ml consists of: (1) the volume of nuclear DNA used, (2) the

volume of Hoechst 33258 used, and (3) TE buffer to 9.1 ml). Typically, 100 ug of nuclear DNA is added to each tube; thus, a yield of about 10 ug per tube can be expected, as satellite DNA comprises approximately 10% of total genomic DNA in the mouse *Mus musculus*. A stock solution of Hoechst 33258 should be made up at 1 ug/ul, thus, if 100 ug of nuclear DNA are used, 100 ul of Hoechst 33258 will be needed. When all the components have been added to each ultracentrifuge tube fitting a Beckman 70.1 Ti rotor, the dye was allowed to interact with the DNA for ten minutes at room temperature. The tubes were balanced using a CsCl solution, density 1.68 g/ml, sealed, and centrifuged for 17 hours at 55,000 rpm in a Beckman ultracentrifuge (model L8). After ultracentrifugation, the tubes were carefully removed from the rotor and the tops cut off with scissors, being cautious not to disturb the gradient. The position of the DNA bands was monitored with a long-wave ultraviolet light. The upper satellite band was removed with a self-filling plastic pipet lowered into the tube. As the upper band was removed, the separation between the satellite and main bands diminished, so a quantitative recovery of the satellite band was forfeited for a lesser main-band contaminated recovery. All fractions of satellite DNA were combined into one Falcon 2059 polypropylene tube. The dye was extensively extracted by adding an equal volume of isopropyl alcohol saturated with 5 M NaCl, capping the tube and shaking it in a horizontal position, and centrifuging to separate the aqueous and organic phases in a tabletop centrifuge (Sorvall). The upper organic phase was discarded each time and a fresh aliquot of isopropanol with NaCl

added, and the extraction continued until no dye was evident, as determined via ultraviolet illumination of the solution. The volume of the satellite DNA solution was measured, and one-tenth volume of 3 M NaCl was added and mixed by thorough shaking. The solution was transferred to a 30 ml polypropylene tube and three volumes of sterile distilled water were added. Finally, an equal volume of isopropanol was added and the solution mixed well and stored at -20° C overnight. The following day, the tube was spun in an SS-34 rotor fitting a Sorvall RC-5C centrifuge at 18,000 rpm (39000 x g) for 30 minutes. The supernatant was carefully decanted and the pellet rinsed with 20 ml of 70% ethanol (room temperature), vortexed, and spun as before. The ethanol was poured off and the tube inverted on a paper towel to allow remaining alcohol to drain away. The tube was covered well with parafilm, a few holes were poked in the parafilm with a pin, and the tube put in a vacuum dessicator for five minutes or until the pellet was dry. The pellet was resuspended in 2 ml of TE, pH 8.0. Five microliters of this solution was used to determine the 260/280 absorbance as described earlier. This solution of crude satellite DNA was stored at -20° C.

Bst N1 Digestion of Satellite DNA

Digestion of mouse satellite DNA was accomplished using the enzyme Bst N1 (Biolabs, Beverly, MA) . This enzyme recognizes the sequence 5'-CC(A/T)GG-3'. To identify the crude satellite preparation as satellite DNA, a 10 ul aliquot of the DNA solution was digested in a total volume of 30 ul. Other components of the

digestion were 3 ul of 10X buffer, and 1 ul of Bst N1 (10 U/ul) and sterile distilled water to 30 ul. Digestion was allowed to proceed for 2 hours at 65° C. After digestion, 3 ul of loading buffer (20% Ficoll, 20 mM EDTA, pH 7.5, 0.05% Orange G) were added and the sample loaded into the well of a 1.6% agarose gel (GTG agarose, FMC). Electrophoresis was carried out at 100 volts until the dye was near the bottom edge of the gel (approximately 1.5 hours).

From the digestion pattern, it appeared that the putative satellite DNA was indeed satellite DNA. Two 100 ul aliquots of the satellite DNA were digested with Bst N1 (New England Biolabs, Beverly MA) by adding 12 ul of 10X Bst N1 digestion buffer (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 8 ul of Bst N1 (10 U/ul). Thus the final volume of the reaction was 120 ul. The reaction was allowed to proceed for four hours. After digestion, one tube was used for an end-filling reaction while the other served for a mung bean nuclease reaction.

Reactions Producing Blunt-end DNA

The end-filling reaction was carried out as follows: to the 120 ul reaction mix were added 6 ul of a 2 mM solution of dNTPs, 15 ul of 10X Nick Translation buffer (1X = 50 mM Tris-Cl, pH 7.2, 10 mM MgSO₄, 50 ug/ml bovine serum albumin), and 9 ul of sterile water. Ten units of the Klenow fragment of DNA polymerase I (Biolabs, Beverly, MA,) was added and the components were mixed and the reaction was allowed to proceed for 30 minutes at 22° C in a water bath. The reaction was stopped by heating the tube to 70° C for five minutes. Fifteen

microliters of 3 M NaCl was added and mixed, and the solution was extracted once with an equal volume of phenol:chloroform (1:1), and then once with chloroform (chloroform:isoamyl alcohol, 24:1). The aqueous layer containing the digested, blunt-end satellite DNA was precipitated with 400 microliters of 95% ethanol at -20° C overnight.

The satellite DNA in the second tube was digested with mung bean nuclease as follows: to the 120 ul reaction mix were added 14 ul of 10X mung bean nuclease buffer (1X = 30 mM NaAc, pH4.6, 250 mM NaCl, 1 mM ZnCl₂, 5% glycerol) and 6 ul (6 units) of mung bean nuclease (Pharmacia, Uppsala, Sweden). The reaction was done at 30° C for 30 minutes. The DNA was extracted and precipitated as above.

Ligation to Linkers

After the end-filling and mung bean nuclease reactions had been ethanol precipitated, the pellets were resuspended in 10 microliters of TE, pH 8.0. For the sake of further reactions, it was assumed the yield of DNA was 3 micrograms in each tube. Since satellite DNA is 234 base pairs, then its molecular weight is 234 bp x 660 daltons/bp = 154440 daltons. For successful ligation of linkers to DNA, a minimum linker excess of 100 fold is required (Struhl, 1987, in Current Protocols in Molecular Biology). This is 12.5 micrograms of a 10-mer linker for 3 micrograms of satellite DNA.

Bam H1 linkers were obtained as a lyophilized pellet from Stratagene (La Jolla, CA). The pellet of linkers was resuspended at a concentration of 1 ug/ul in TE, pH 8.0. To assure proper register of the two strands of the linkers, the linkers were placed in a 90° C

water bath for 5 minutes. After this, the linkers were removed and placed on the lab bench and allowed to equilibrate to room temperature for about 30 minutes. Finally, the linkers were placed into an ice bath. At this time the linkers were stored at -20°C until used in a ligation. When thawing, the linkers were not allowed to come above room temperature. This was accomplished by thawing the linkers briefly by hand then keeping them on ice.

The satellite DNA which had been blunt-ended by either the end-filling reaction or mung bean nuclease was ligated to the treated linkers in a microfuge tube. To the 10 μl of satellite DNA, 12 μl of linkers, 1 μl of 10 mM ATP, 4 μl of sterile water, and 3 μl of 10X ligation buffer were added. The 10X buffer was purchased from International Biotechnologies, Inc. (IBI), catalog number 29185; components were 250 mM Tris-Cl, pH 7.8, 100 mM MgCl_2 , 4 mM ATP, 40 mM beta-mercaptoethanol. One microliter of T4 DNA ligase (IBI, catalog number 29182) was added, and the components were vortexed briefly and collected at the bottom of the microfuge tube by a short 2-3 second centrifugation in an Eppendorf microcentrifuge. The tube was placed in a room temperature water bath (22°C) overnight for the ligation reaction to occur. The next day, 3.5 μl of 10X digestion buffer (1X = 10 mM Tris-Cl, pH 7.5, 5 mM MgCl_2 , 100 mM NaCl, 1 mM beta-mercaptoethanol) and 2 μl of Bam H1 (Biolabs, 25 U/ μl) enzyme were added directly to the ligation tubes, and the DNA was digested at 37°C for 3 hours. The reaction was stopped by the addition of 2 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA). The volume of the reaction was brought to 100 μl with sterile water, and the solution

was extracted with 100 μ l of phenol:chloroform (1:1). After the addition of the phenol:chloroform, the mixture was vortexed for ten seconds and centrifuged in a microfuge for 2 minutes. To separate the unligated linkers from the satellite DNA, the upper aqueous layer was applied to a Sepharose CL-4B column (poured in a Bio-Rad Econocolumn, catalog number 737-1010, 1.0 x 10.0 cm) equilibrated with TE, pH 7.5, 0.3 M NaCl. The DNA was washed into the column with 0.4 ml of TE, pH 7.5, 0.3 M NaCl, and twelve 0.4 ml fractions were collected. Fifteen microliters of each fraction were electrophoresed on 1.6% agarose (FMC Bioproducts, Rockland, ME). The fractions containing satellite DNA were combined in a 3 ml polypropylene tube and concentrated with butanol: an equal volume of butanol was added, the tube was vortexed for 5 seconds, and the phases separated in a tabletop centrifuge at 5000 rpm for 2 minutes. When the volume of the aqueous solution was 300-400 μ l, the solution was transferred to a 1.5 ml microfuge tube, one-tenth volume of 3 M NaCl, and two volumes of ethanol were added. The components were mixed by vortexing for ten seconds and the tubes were placed at -20° C overnight. The tubes were centrifuged for 30 minutes in a microfuge, the supernatant was carefully poured off, and 1 ml of 70% ethanol was added to the tube. The pellet was vortexed a few seconds and centrifuged for 15 minutes in a microfuge. The supernatant was again carefully poured off and the tube was inverted on a paper towel for a few minutes. The pellet was dried for 5 minutes in a vacuum dessicator and resuspended in 10 μ l of TE, pH 8.0. This solution represented the completed insert to be cloned: satellite DNA with

digested Bam H1 linkers. The concentration of this DNA was approximately 0.2 ug/ul.

Linearization and Dephosphorylation of Vector

The vector used for cloning was the plasmid pBS (Stratagene, La Jolla, CA). Two ug of pBS were digested with 25 units of Bam H1 in a 20 ul reaction. Components were added to a microfuge tube in this order: 15 ul sterile water, 2 ul 10X buffer (1X = 10 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 1 mM beta-mercaptoethanol), 2 ul pBS DNA (2 ug), 1 ul of Bam H1 (25 U/ul). The tube was vortexed 2-3 seconds and the solution collected in the bottom of the tube via a short 3 second centrifugation in a microcentrifuge. After 2.5 hours of digestion at 37° C, the vector was treated with calf intestinal alkaline phosphatase (CIAP, Stratagene, catalog number 600015) to dephosphorylate the 5-prime ends of the linearized DNA molecule. A 2.5 ul aliquot of 10X CIAP buffer (1X = 20 mM Tris-Cl, pH 8.0, 1 mM MgCl₂, 1 mM ZnCl₂) and two ul of CIAP (26.4 U/ul) were added and the reaction allowed to proceed for 30 minutes at 37° C. The reaction was stopped by heating the tube and its contents to 75° C for five minutes. The volume of the solution was made to 100 ul with sterile water, and the solution was extracted once with phenol:chloroform as described before. The aqueous phase was transferred to a fresh microcentrifuge tube, and one-tenth volume of 3 M NaCl and two volumes of ethanol were added and mixed. The tube was placed in dry ice for 15 minutes, then centrifuged for 20 minutes in a microcentrifuge at top speed (12000 rpm). The supernatant was gently

poured off and 1 ml of 70% ethanol was added to the tube. The tube was vortexed briefly to wash the pellet, and then centrifuged as before. After the 70% ethanol wash was carefully decanted, the tube was inverted on a paper towel for 2-3 minutes to allow excess fluid to drain away. The pellet was dried in a vacuum dessicator for 5 minutes and resuspended in 20 ul TE, pH 8.0. The concentration of the pBS vector was approximately 0.1 ug/ul.

Cloning of Satellite DNA into Plasmid Vector

From the previous reactions, there exists linear dephosphorylated pBS with cohesive Bam HI ends and satellite DNA with cohesive Bam HI linkers. A ligation reaction was set up to clone the modified satellite DNA into the pBS vector in a final volume of 30 ul in a 1.5 ml microfuge tube. The components were added as follows: 14 ul sterile water, 2 ul 10X ligation buffer (IBI, catalog number 29185), 1 ul 10 mM ATP, 2 ul modified satellite DNA (400 ng), 1 ul linear, dephosphorylated pBS (100 ng), and 1 ul T4 DNA ligase (IBI, catalog number 29182, 2 U/ul). The components of the reaction were mixed by a gentle vortexing for 2-3 seconds and then collected in the bottom of the tube by a 3 second centrifugation. The reaction was carried out overnight at 15° C in a water bath. The following day, 3 ul of the ligation reaction was used to transform E. coli strain XL1-Blue according to the procedure from the supplier (Stratagene, CA).

Transformation of Bacterial Cells with Chimeric Plasmid

E. coli strain XL1-Blue (Stratagene) cells were stored frozen at -70°C until used. Cells were thawed on ice and gently mixed by hand. For each transformation, 100 μl of cells were aliquoted into pre-chilled 15 ml Falcon 2059 polypropylene tubes. A fresh solution of a 1:10 dilution of B-mercaptoethanol was made in sterile water, and a 1.7 μl aliquot of this was added to each 100 μl of cells. The cells were swirled gently and kept on ice for 10 minutes, gently swirling every 2 minutes. Three microliters of the ligation reaction was added to each tube of cells and again swirled gently. The cells were kept on ice for 30 minutes, then the tubes were immersed in a 42°C water bath for 45 seconds. The tubes were returned to ice for 2 minutes, and 0.9 ml of preheated (42°C) SOC medium (per liter: 20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.5 g NaCl, 1 ml 2 M glucose) was added and the cells were incubated at 37°C with shaking for one hour. Two hundred microliters of transformation mixture was spread onto 150 x 25 mm plates with LB agar (per liter: 10g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, 15 g Bacto-agar) containing 100 $\mu\text{g/ml}$ ampicillin, 1 mM isopropylbeta-D-thiogalactopyranoside (IPTG), and 40 $\mu\text{g/ml}$ X-gal (5-bromo-4-chloro-3-indolylbeta-D-galactoside). The plates were allowed to dry at room temperature for 10 minutes, and then placed in a 37°C incubator overnight. White colonies were further analyzed for the insertion of satellite DNA via the mini-preparation alkaline lysis method of

Birnboim and Doly, as presented in Molecular Cloning, A Laboratory Manual (Maniatis et al., 1982).

Preparation of Plasmid DNA for Detection of Insertion
of Satellite DNA (Birnboim and Doly, 1979)

A few representative white colonies were inoculated into 5 ml of LB broth (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl) containing 100 ug/ml ampicillin and grown overnight at 37° C with shaking. The next day, 1 ml of culture was placed in a 1.5 ml microfuge tube and centrifuged at 12000 rpm for one minute. The supernatant was aspirated off, being careful not to disturb the pellet. The pellet was resuspended by vortexing in 100 ul of 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0, 4 mg/ml lysozyme (powdered lysozyme must be added to the solution just before use) and the tubes were left at room temperature for 5 minutes. A 200 ul aliquot of a fresh solution of 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS) was added, the top of the tube was closed, and the contents mixed by rapidly inverting the tube a few times. The tubes were then placed on ice for 5 minutes. Each tube then received a 150 ul aliquot of an ice-cold solution of potassium acetate, pH 4.8. The tube was closed, vortexed in an inverted position for 10 seconds, and placed on ice for 5 minutes. (The potassium acetate was made by combining 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml sterile water.) The tube was centrifuged for 5 minutes in a microfuge at top speed (12000 rpm), and the supernatant was transferred to a fresh 1.5 ml tube. A 400 ul aliquot of

phenol:chloroform (1:1) was added and the contents mixed by vortexing for 10 seconds. After centrifuging for 2 minutes in a microfuge, the supernatant was transferred to a fresh tube. Two volumes of room temperature ethanol were added and the tube vortexed well. The tubes were left at room temperature for 10 minutes, then centrifuged for 5 minutes in a microfuge. The supernatant was decanted, and 1 ml of 70% ethanol was added. The tube was vortexed briefly and recentrifuged as before. The supernatant was again decanted, the tubes inverted on a paper towel to allow residual fluid to drain away, and then the tubes were dried in a vacuum dessicator for 5 minutes. The pellet was resuspended in 50 μ l TE, pH 8.0, and one microliter of 10 mg/ml RNase A was added. A 10 μ l aliquot of this plasmid preparation was removed to a fresh tube, and 1.2 μ l of 10X Bam HI digestion buffer (10 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM beta-mercaptoethanol) and 1 μ l of Bam HI (25 U/ μ l) were added. The reaction was incubated at 37° C for 2 hours. Two μ l of loading buffer (40% sucrose, 0.25% bromophenol blue) was added and the digested solution was run on a 1.6% agarose gel to identify clones which had the satellite DNA insert. Those clones which contained an insert of approximately 240 base pairs were sequenced via the dideoxy chain termination method of Sanger.

Sequencing

Satellite DNA clones were sequenced via the dideoxynucleotide chain termination method of Sanger, Miklen, and Coulson (1977). A sequencing kit from United States Biochemicals, utilizing the

Sequenase enzyme, was used to sequence those clones which contained a 240 base pair insert. Sequencing of the satellite DNA was performed on plasmid DNA which had been isolated by the alkaline lysis mini-preparation method of Birnboim and Doly (1979), by a modification of the method of Zagursky et al. (1985) and Chen and Seeberg (1985).

To 8 ul sterile water in a 1.5 ml microfuge tube, 2 ul of plasmid DNA (1-2 ug) obtained from the alkaline mini-prep method were added. Two microliters of a 2 N NaOH, 2 mM EDTA, pH 8.0 solution were added and the components mixed well. The DNA was incubated at room temperature for 5 minutes. A 5 ul aliquot of the M13 -20 primer, concentration 50 ng/ul, was added to the tube, and then 3 ul of 3 M NaAc and 75 ul of 100% ethanol were added. The tube was gently vortexed 2-3 seconds and placed in dry ice for 20 minutes. The DNA was pelleted by centrifugation at top speed in a microfuge, and the supernatant was carefully decanted. The DNA pellet (not always visible) was rinsed with 400 ul of 70% ethanol and centrifuged as before. After the 70% ethanol supernatant wash was decanted, the tube was placed in a vacuum dessicator for 5 minutes. The DNA pellet, which is not necessarily visible, was resuspended in 8 ul of sterile water. This solution is referred to as the Primer-Template. From this point, the sequencing protocol for Sequenase (United States Biochemicals), Version 2.0 was followed.

To the 8 ul of denatured plasmid DNA was added 2 ul of 5X sequencing buffer (1X = 40 mM Tris-Cl, pH 7.5, 20 mM MgCl₂, 50 mM NaCl). For each clone sequenced, four 1.5 ml tubes were needed, labelled G, A, T, and C. To each of these tubes 2.5 ul of

dideoxynucleotide termination mix was added. (Each mix was 50 mM NaCl, 80 μ M in dNTPs, and 8 μ M in the specific ddNTP for dGTP labelling. For dITP labelling, each mix was 50 mM NaCl, 160 μ M dITP, 80 μ M for three of the dNTPs, and 8 μ M for the appropriate ddNTP.) For the tube labelled G, 2.5 μ l Termination Mix ddGTP was added. Similarly, for the tubes labelled A, T, and C, the appropriate Termination Mix was added. These tubes were capped and placed on ice. The Sequenase sequencing kit is supplied with two Labelling Mixes, dGTP (7.5 μ M in dGTP, dCTP, dTTP) and dITP (15 μ M dITP, 7.5 μ M dCTP and dTTP), as 5X concentrates. The Sequenase enzyme is supplied at 12 units/ μ l; it is diluted to 1.5 U/ μ l with enzyme dilution buffer (10 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol (DTT), .5 mg/ml BSA). After the Labelling Mix and Sequenase were diluted, the remaining components were added to the Primer-Template in the following order: 1 μ l of 0.1 M DTT, 2 μ l of diluted Labelling Mix, 1 μ l of [35 S]dATP, and 2 μ l of diluted Sequenase, Version 2.0. These components were mixed thoroughly with a pipet tip and incubated at room temperature for 2-5 minutes. This solution is now referred to as the Labelling Reaction. During this pre-incubation, the Termination Mixtures were removed from the ice and placed in a 37 $^{\circ}$ C water bath. To each of the four Termination Mixtures was added 3.5 μ l of the Labelling Reaction. The components were mixed well and incubation was continued for 5 more minutes at 37 $^{\circ}$ C. The reactions were stopped by the addition of 4 μ l of Stop Solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Before loading 3 μ l aliquots onto 8% acrylamide sequencing gels, the

samples were heated to 75° C for 2 minutes, centrifuged in a microfuge for 8 seconds at top speed, and placed on ice.

Preparation of the Gel Sandwich and Electrophoresis

Glass gel plates (39.5 x 33 cm) were washed thoroughly with detergent and rinsed with copious amounts of distilled water. The plates were then rinsed with ethanol and allowed to dry. The surfaces of the plates which would serve as the inner surfaces of the gel sandwich were wiped well with a few Kimwipes soaked in 5% dimethyldichlorosilane in chloroform. After the chloroform had evaporated, the plates were laid together with 0.4 mm spacers along the long axis and between the silanized faces. The bottom and edges of the gel were sealed well with yellow 3MM tape. The gel was now ready to be poured. When pouring the gel, the sandwich was held at approximately a 45 degree angle and the acrylamide solution was slowly poured between the plates, allowing the solution to run down along the spacer and fill the sandwich space the lower corner to the opposite upper corner. This method helps to eliminate air bubbles in the gel. When the sandwich was full, the gel was laid down with a rubber stopper under the top edge of the glass plates and the gel comb (0.4 mm, 64 well) was inserted between the two plates. The gel comb was a shark's tooth comb, and thus the smooth edge was inserted between the plates; when running the gel, the comb was turned around such that the teeth formed wells when they contacted the gel.

Eight percent acrylamide gels were made by combining 31.7 ml high purity water, 50 g urea, 10 ml of 10X TBE (1X = 0.09 M Tris-

borate, 0.09 M boric acid, 0.0025 M EDTA), and 20 ml 40% acrylamide and heating the mixture at 55° C until all components had dissolved. (Forty percent acrylamide is 38 g acrylamide and 2 g bis-acrylamide in a total volume of 100 ml high purity water. To dissolve, this mixture is warmed to 55° C. This solution is stable for about 3 months at 4° C.) The acrylamide mixture was filtered through a Corning 0.22 um filter and cooled on ice to 15° C. Thirty-eight microliters of TEMED (N,N,N',N'-tetramethylethylenediamine, Boehringer Mannheim Biochemicals, catalog number 100139) and 0.83 ml of 10% ammonium persulfate were added to the acrylamide mixture and quickly swirled to mix. The acrylamide solution was then pipetted into a glass gel sandwich which had previously been thoroughly washed and the inner surfaces coated with 5% dichlorodimethylsilane. The gel was allowed to polymerize for 45-60 minutes, and then the tape was removed from the glass plates and the gel sandwich was put into the electrophoresis apparatus (BRL, model S2). TBE buffer was poured into both the upper and lower buffer chambers. Any air bubbles which may have formed on the lower edge of the gel sandwich were removed by running a gloved finger along the bottom of the gel. The electrodes were hooked up, making sure the anode was at the loading well side. The power unit was turned on and the gel was pre-run for 45-60 minutes at 90 watts, 45 mAmps. The voltage level for these parameters was 2000-2300. After the samples were heated and centrifuged, the power was shut off and 3 ul aliquots of the sequencing reaction mixtures were loaded into the wells. The typical loading pattern was GATC. The power was turned on and the samples

electrophoresed until the second blue dye reached the bottom edge of the gel. The power was shut off, and a second loading of each sample was applied to the gel. These samples were electrophoresed until the leading dye reached the edge of the gel.

After electrophoresis was complete, the power was turned off and the gel sandwich removed from the apparatus and blotted on plastic-backed bench paper. The gel comb was removed and the gel was laid down on the lab bench. Using a spatula, the glass plates were gently pried apart. At this point, care must be taken to watch which plate the gel is sticking to; the sandwich may have to be flipped over to avoid ripping the gel. The free glass plate was removed entirely and set aside. Two sheets of Whatman 3MM filter paper were laid on the now-exposed gel. Starting from the center of the gel and working out toward the edges, the filter paper was smoothed out over the gel using the hands. The filter paper was then carefully lifted from one corner, and with the gel sticking to it, the filter paper was rolled back and over such that the gel was now uppermost. The gel was quickly covered with Saran wrap and the excess paper was trimmed from around the gel. The gel was placed in a gel-drying apparatus (Hoefer Scientific Instr., San Francisco, model SE1150) with vacuum and heated for at least 3 hours at 80° C. The gel was removed from the drying apparatus and the Saran wrap was carefully peeled off the gel. The gel was finally exposed to Kodak XAR-5 film overnight at -70° C with an intensifying screen.

The film was developed in Kodak developer for 2-5 minutes until the bands appeared dark. The film was rinsed in water for one

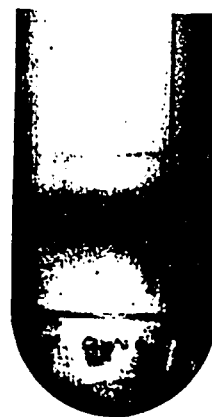
minute, and fixed in Kodak fixer until the background was clear, about 2-5 minutes. The film was rinsed 5 minutes in water and hung to dry in a 37° C warm room. The sequences were read visually.

CHAPTER IV

RESULTS

The isolation of genomic DNA yielded about 1.5 mg DNA per gram of tissue. When the genomic DNA was subjected to isopycnic ultracentrifugation in the presence of the dye Hoechst 33258, the satellite band typically formed about 1 centimeter above the upper edge of the main band DNA, as can be seen in Figure 2. This was much less than the 3.8 to 4.2 centimeter separation observed by Manuelidis (1977). Although this author did not measure the separation from main band peak to satellite band peak, this would only have added 0.5 to 1.0 centimeter, and this 2 centimeter separation still would be less than that reported by Manuelidis (1977). Nevertheless, the separation was more than sufficient to allow for recovery of the satellite band only, without having contamination from the main band.

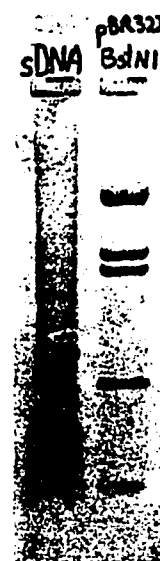
Figure 2. Isolation of satellite DNA in cesium chloride gradients. The lower band is main-band DNA, the upper, satellite DNA. CsCl gradients ($d=1.68$ g/ml) were centrifuged as described in the text.



Upon digestion of the satellite DNA with the enzyme Bst N1 and subsequent electrophoresis in 1.6% agarose gels, the putative

satellite DNA was observed to produce bands that were multimers of approximately 240 base pairs, although the bands at 240 and 480 consisted of nearly 95% of the total satellite DNA (Figure 3). This evidence led credence to the assumption that the DNA was in fact satellite DNA. To further prove this assumption, the Bst N1 digest was cloned into plasmid pBS and sequenced.

Figure 3. Mouse satellite DNA Bst N1 digest. The molecular weight marker is pBR322 Bst N1; fragment sizes are 1857, 1060, 929, 383, and 121 bp. The stDNA appears as a band approximately 240 bp long. The 1.5-, 2-, and 4-mers can also be detected.



Since the putative satellite DNA could not be cloned directly into pBS, because the ends generated by the Bst N1 digest were not compatible with any ends available in the polylinker cloning site of the plasmid, the DNA had to be modified to accomodate easier cloning. Thus, the satellite DNA was blunt-ended by either an end-filling reaction or mung bean nuclease reaction, and Bam H1 linkers were then ligated to the satellite DNA fragment. This introduced Bam H1 site

was digested with enzyme to produce the Bam H1 cohesive ends for cloning into pBS. Before the modified satellite DNA with Bam H1 linkers could be cloned into pBS, however, it had to be separated from linkers which had not been ligated to any satellite DNA molecules. This was accomplished by running the post-ligation solution of satellite DNA and Bam H1 linkers through a Sepharose CL-4B column. This column merely separated the DNA species on the basis of size. The end-filling and mung bean nuclease treated satellite DNAs were run through separate columns; the results of these columns can be seen in Figure 4, a & b, respectively.

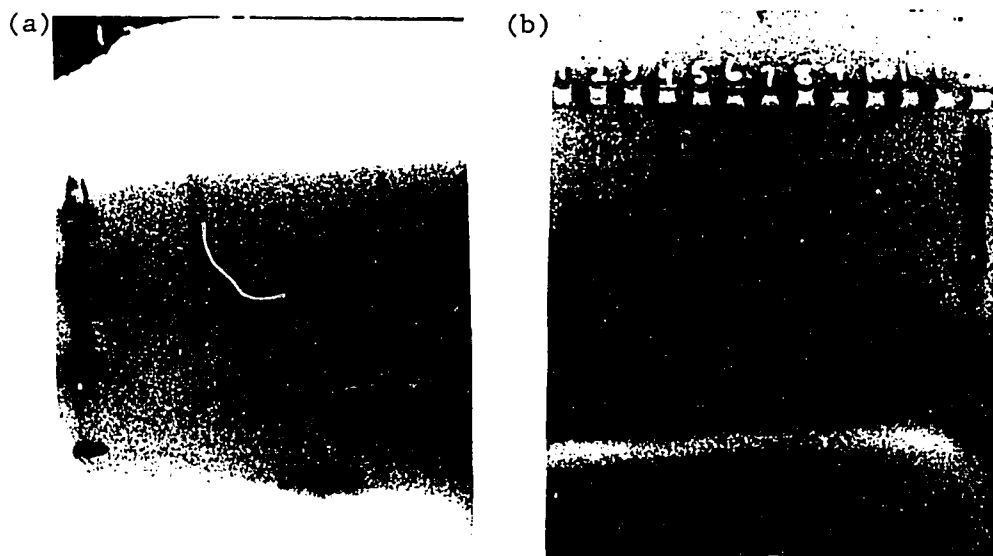


Figure 4. Separation of blunt-ended satellite DNA on Sepharose CL-4B. (a). End-filling reaction. Fractions 4-6 contain satellite DNA only, without unligated linkers. Fractions 7-9 contain unligated linkers with satellite DNA. (b). Mung bean nuclease reaction. Fraction 5 contains satellite DNA only. Fractions 6 & 7 contain unligated linkers with satellite DNA.

As can be seen in Figure 4a, from the end-filling reaction, the satellite DNA with linkers was eluted in fractions 4-8. Fraction 4

had some larger molecular weight species in it, and thus this fraction was discarded. Fractions 5 and 6 contained the desired satellite DNA with linkers, and therefore these fractions were used for the cloning step. Although fractions 7 and 8 also had satellite DNA, they contained a large amount of unligated linkers. These fractions were discarded also.

Figure 4b shows the contents of the fractions from the mung bean nuclease reaction. It can be seen that the satellite DNA with linkers was eluted in fractions 5-7. Fraction 5 was the only fraction which did not also elute unligated linkers, so fractions 6 and 7 were discarded and fraction 5 was used for the cloning step.

After cloning the satellite DNA with Bam H1 linkers into the Bam H1 site of pBS and subsequent transformation of *E. coli* strain XL1-Blue, a number of potential colonies were observed by their white pigmentation. Three dozen representative white colonies were then grown in 5 ml cultures so that plasmid DNA could be obtained via the alkaline lysis protocol.

The analysis of the mini-preparations of the putative pBS-satellite DNA plasmids can be seen in Figure 5. It is evident from the figure that many of the putative clones indeed have an insert which is approximately 240 base pairs in length. Initially, four of the most likely satellite inserts were sequenced via the dideoxy method. Only one of these inserts gave promising results from the sequence, and so this clone was subjected to further sequencing. The clone was sequenced four times in the forward direction using dGTP, twice in the reverse direction using dGTP, twice in the forward

direction using dITP, and once in the reverse direction using dITP. The results of all of these sequencing runs were collated and a consensus sequence derived (Figure 6). This consensus was compared to the consensus sequence from Manuelidis (which is identical to that of Wolfram and Horz). This chimeric plasmid with the satellite DNA monomer fragment was given the name pMSAT.

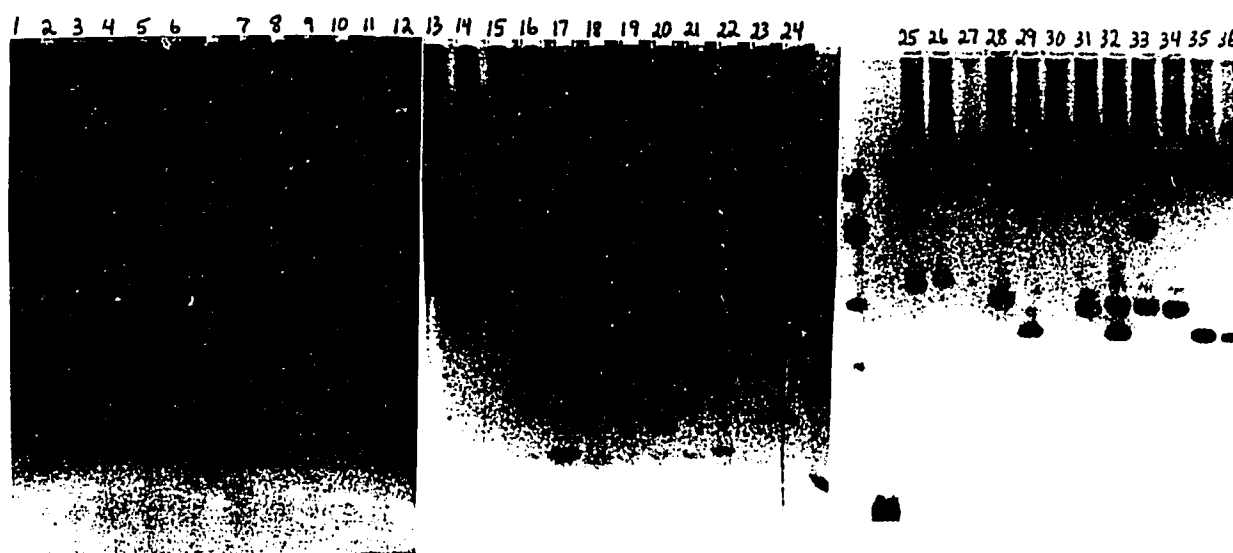


Figure 5. Analysis of mini-preparations of plasmid DNA. Plasmid DNA was isolated as described in the text. The molecular weight marker is pBR322 Bst N1. Its fragment sizes are 1857, 1060, 929, 383, and 121 bp. Clones which appear to harbor the stDNA insert are: 1, 2, 3, 5, 10, 16, 18, 20, 24, 29, 32, 35, and 36. Clones 5, 10, 16, and 24 were originally sequenced. Clone 16 appeared the most promising and was subjected to more extensive sequencing.

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GGAAC ATGGCGAAAA AACTGAAAAT CACAGAAAAT GAGTAATACC CATTITAGTA
CGTGAAATAT GGCGAGGAAA ACGGAAAAAG TTTGAAAATT TAGAAATGTC CACTGTAGGA
CATGAAATAT GGCAAGAAAT ATGAAAATCA TGGAAAATGA GAAACATCCA CTTTACGACT
TGAAAAATGA CGAAATCAGA GAAAAACGTG GAAAATGAGA AATGCACACT GTAGGACCTC

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Figure 6. Consensus sequence of pMSAT. The satellite DNA was sequenced nine separate times by the Sanger dideoxy method as described in the text. The consensus of these nine sequences is shown below.

The sequence of the cloned satellite DNA can be seen in Figure 6. Since the Bst N1 site has been partially cleaved out from the cloning process, the sequence has been reorganized to compare it directly to the consensus sequence of Manuelidis. This comparison can be seen in Figure 7. As can be seen, the cloned satellite has very strong homology to the consensus sequence. Only 19 of the 234 bases are different, giving a percent homology of 91.9. It is thus possible to conclude that the segment of mouse DNA cloned into the plasmid pBS (producing plasmid pMSAT) in these experiments is indeed mouse satellite DNA.

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CCTGGAATAT GGCGAGAAAA CTGAAAATCA CGGAAAATGA GAAATACACA CTTTAGGACG
|           |           |           |           |           |
CCTGGAACAT GGCGAAAAAA CTGAAAATCA CAGAAAATGA GTAATACCCA TTTTAGTACG

TGAAATATGG CGAGGAAAAAC TGAAAAAGGT GGAAAATTTA GAAATGTCCA CTGTAGGACG
|           |           |           |           |           |
TGAAATATGG CGAGGAAAAAC GGAAAAAGTT TGAAAATTTA GAAATGTCCA CTGTAGGACA

TGGAATATGG CAAGAAAACT GAAAATCATG GAAAATGAGA AACATCCACT TGACGACTTG
|           ||          |           |           |           |
TGAAATATGG CAAGAAATAT GAAAATCATG GAAAATGAGA AACATCCACT TTACGACTTG

AAAAATGACG AAATCACTAA AAAACGTGAA AAATGAGAAA TGCACACTGA AGGA
|           |||         |           |           |           |
AAAAATGACG AAATCAGAGA AAAACGTGGA AAATGAGAAA TGCACACTGT AGGA

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Figure 7. COMPARISON OF pMSAT AND THE CONSENSUS SEQUENCE OF MOUSE SATELLITE DNA. The Upper sequence is the consensus sequence of Horz & Altenburger and Manuelidis, the lower sequence is the satellite DNA cloned into pBS. Hashed bars between bases signal differences between the two sequences.

CHAPTER V

DISCUSSION

The results of these experiments were successful in their intent to clone and sequence mouse satellite DNA (msDNA). At each step in the isolation process of mouse satellite DNA, the expected DNA was obtained. As can be seen in Figure 2, whole satellite DNA was isolated as per the method of Manuelidis (1977). In the presence of the dye Hoechst 33258, mouse satellite DNA forms a smaller band above the main band of DNA when centrifuged to equilibrium in a cesium chloride gradient. When this whole fraction of satellite DNA is subsequently digested with the enzyme Bst N1 (Eco RII), the monomer fragment of approximately 240 base pairs (bp) is obtained. This can be seen in Figure 3. The satellite DNA also can be seen to exist in fragments that are multiples of 1.5 and 2 times the 240 bp monomer. Thus, this gives credence to the statement that the DNA in question is indeed mouse satellite DNA.

To successfully clone the msDNA into the polycloning site of the plasmid pBS, it had to be modified such that the msDNA could be cleaved out of the plasmid when potential clones were being analyzed for insertion of msDNA, since the msDNA did not possess a restriction site that was unique to the plasmid's polycloning site. The answer to this problem was to add Bam H1 linkers to the msDNA. Since the Bst N1 digestion of msDNA left a 5' overhang on the molecule, the DNA

had to be blunt-ended before the linkers were ligated to the msDNA. Two methods of producing blunt-ended DNA were used. These were end-filling reactions using the polymerase activity of the Klenow fragment of DNA polymerase I, and the single-stranded digesting reactions of mung bean nuclease. After the msDNA had been blunt-ended by either of these reactions, Bam HI linkers were ligated to the msDNA and then the linkers were subsequently digested with Bam HI. To separate unligated linkers from the msDNA with linkers, the DNAs were run through a Sepharose CL-4B column. Fractions were collected and analyzed for the presence of fragments which were approximately 240 bp. As can be seen in Figure 4 (a) and (b), the modified msDNA was found in fractions 4-6. It appears from the figures that the end-filling reaction (Fig. 4a) produced a much cleaner msDNA without unligated linkers. The best fractions (#5 & 6 for end-filling and #5 for mung bean nuclease) were used for the ligation reaction into pBS. After ligating the msDNA into pBS, the ligation products were used to transform E. coli strain XL1-Blue.

Analysis of some of the representative white recombinant bacterial colonies yielded a dozen possible clones with the msDNA insert. These can be seen in Figure 5, lanes 1, 2, 3, 5, 16, 20, 21, 24, 29, 32, 35, 36. Four of these putative msDNA clones were sequenced, and clone 16 appeared to have the highest homology with the published sequence of msDNA (Horz and Altenburger, 1981, and Manuelidis, 1981). Therefore, clone 16 was sequenced eight more times. The consensus sequence arrived at from the nine sequencing runs of clone 16 had a 92% homology with the published sequence.

Figure 7 shows a comparison between the published sequence and the sequence obtained from this research. There are 19 bp which are different in clone 16, hereafter referred to as pMSAT. Of the 19 variations from the published sequence, 7 of these are transitions (a purine to purine or pyrimidine to pyrimidine alteration) while 12 are transversions (a purine to pyrimidine or vice versa).

Although these differences from the published sequence are not significant to not call the pMSAT sequence satellite DNA, there may be a few reasons for the discrepancies. The published sequences of mouse satellite DNA (Horz and Altenburger, 1981, and Manuelidis, 1981) were derived from the Maxam-Gilbert sequencing technique, which in and of itself yields a consensus at each base, and the satellite DNA cloned represents only one sample of a large population of satellite sequences. Thus, any differences in the sample would stand out against an averaging of all the other base positions. Two, during the time the clone existed in the host *E. coli*, replication errors could have occurred which resulted in the altering of some of the bases from their original murine status. Three, this represents a slightly different form of mouse satellite DNA. Four, previous reports of the sequence of mouse satellite DNA (Horz & Altenburger, 1981, and Manuelidis, 1981) are incorrect. Five, the variation in the sequence is caused by strain differences in mice.

Therefore, it is concluded that the aim of this research was successfully accomplished. The monomer fragment of mouse satellite DNA (msDNA), a 234 bp segment, was cloned into the plasmid pBS to

produce the chimeric plasmid pMSAT, and the fragment was sequenced showing that it is msDNA.

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