行政院國家科學委員會專題研究計畫　期中進度報告

哺乳類動物著絲點 DNA 的分子結構及功能之研究(2/3)

計畫類別： 個別型計畫
計畫編號： NSC92-2320-B-040-048-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 中山醫學大學生命科學系

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報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中 華 民 國 93 年 5 月 28 日

中文摘要：

關鍵詞：著絲點、衛星 DNA、著絲點蛋白、著絲點蛋白鍵結特異序列

著絲點在有絲分裂及減數分裂的過程中扮演了相當重要的角色，它負責將姐妹染色分體平均分配至兩個子代細胞中有關。而功能不正常或失去功能的著絲點通常會造成染色體套數不完整的現象，也就是所謂的 Aneuploid。在細胞遺傳學上的研究報告指出 aneuploidy 和自發性的流產，先天性的染色體疾病，如唐氏症和透納氏症，甚至與某些癌症的發生有關。近年來，有許多實驗室為了改良基因治療載體，於是致力於研究人類人造染色體的建構上。但想要了解 Aneuploid 形成的機制及成功地建構人類人造染色體為基因治療的載體，就必須先透澈著絲點的功能及構造。

著絲點在染色體上是以一種收縮的結構型態顯現，而其分子結構的基本元素包括鹼基序列及特殊的蛋白質結構名 Kinetochore(是以一種三層板狀的結構所構成，最外層是和紡錘絲連結，最內層是相連於著絲點 DNA，中間是一層狹窄的透明區)。就目前所知，在高等的真核生物內，存在著高度複雜性的著絲點 DNA 族群，其中已確定的著絲點 DNA 主要是由重複性的衛星 DNA 所組成，舉例來說，阿爾發衛星 DNA 是最主要著絲點 DNA 且存在每個人類染色體著絲點上，而且阿爾發衛星 DNA 的某些單元體內具有 17 個鹼基對的 CENP-B 鍵結序列。因此曾被認為是支配人類染色體著絲點功能的衛星 DNA。可惜的是，後來在某些異常染色體 (marker chromosomes)上發現具有功能的新生著絲點上並沒有阿爾發衛星 DNA 的存在，而且近來研發的人造染色體構築中，若只是以重複的阿爾發衛星 DNA 作為著絲點 DNA 的基本架構元素，通常無法穩定存在轉殖細胞中。從低等到高等的生物體，著絲點的衛星 DNA 往往差異相當大，且在不同種族甚至同一種不同個體間也都不同；然而，著絲點的蛋白質的功能及構造卻保留相當大的一致性。正因如此，使得著絲點的構造及功能更加複雜且不易了解。

印度山羌的染色體是獨特的，因為它擁有最少的染色體數目（2N=6♀,7♂）、最大的染色體 及不尋常的 X+3 複合著絲點. 在先前的研究中, 我們已選殖到三種印度山羌衛星 DNA (分別為 I、II 和 IV) ，在原位螢光雜交及 3-D 影像建構的結果中，顯示衛星 II DNA 及衛星 IV DNA 是以相同的螺旋方式圍繞在著絲點上，圍繞二圈而在 X+3 複合著絲點上則圍繞四圈，每一圈約由六個原位螢光訊號所形成；而 Kinetochore 仍是以兩條平行對稱的方式排列在衛星 DNA 的外側，每條約有四個免疫螢光訊號在 X+3 的複合著絲點上。此一結果說明了衛星 DNA 的螺旋結構可能是為了將著絲點染色質絲表現在染色體的外側，使其能夠支配 kinetochore 的蛋白質的包裝，且和紡錘絲作用。然而這並不足以說明這兩種衛星 DNA 可和 kinetochore 上的蛋白質直接鍵結。因此我們利用染色質絲免疫沉澱法直接找到 kinetochore 蛋白質的鍵結 DNA 片段，再以此一 DNA 當作探針搜尋 BAC 基因庫以找到完整的 kinetochore 鍵結序列。首先我們已成功取得 kinetochore 上建構蛋白質的鍵結 DNA 片段，至於基因庫的部分, 我們已得到 6700 個 BAC 菌株, 涵蓋 0.1 個基因組, 目前仍繼續建構中。

英文摘要。

Keyword: centromere, satellite DNA element, centromeric proteins (CENPs), kinetochore binding DNA.

Centromere plays a pivotal role during mitosis and meiosis. Malfunctioned centromere would result in aneuploidy that causes aberrant chromosome number in the complement. Epidemically reported that aneuploidy is associated with disorders such as spontaneous abortion, birth defect (e.g. Down syndrome, Klinfelter syndrome, Tuner syndrome, etc.) and some neoplasia. Recently, several laboratories have endowed in the construction of human artificial chromosomes with centromeric DNA for gene therapy intervention. In order to unveil the enigma of aneuploidy and to construct successfully artificial chromosomes for gene therapy intervention, it is an important and necessary to understanding the molecular architecture and composition of centromere.

Several detailed analyses have demonstrated that the centromere contains various centromeric DNA and an exquisite and dramatic proteinaceous structure, the kinetochore, which in turn interacts with the spindle microtubules. Satellite DNA is a predominant and ubiquitous centromeric DNA in mammals. Alphoid satellite DNA, the major centromeric satellite DNA of human chromosomes, was considered a sufficient component for a functional centromere in the construction of human artificial minichromosomes. It was not questioned until alphoid satellite DNA was not found in some mitotically stable markers with functional centromere. Moreover, taken the available data from yeast to man, the centromere sequence and sequence organization have diverged significantly, even amongst different chromosomes of a single organism; however, overall centromere and kinetochore components might be significantly more conserved than thought previously. The centromeric DNAs found so far are quite complex so that it becomes even less clear about the structure and function of the mammalian centromeres. In order to understand completely the centromere structure and function in mammals, it will require detailed sequence analysis of centromeric DNA and further characterization of the 3-D spatial organization among centromeric DNAs.

The chromosomes of the Indian muntjac (*Muntiacus muntjak vaginalis*) is unique among mammals due to their low diploid number (2N=6♀,7♂), giant size, and unusual large centromeres. Particularly, the centromere of X+3 chromosome appears having a compound kinetochore. We had isolated three cervid centromeric satellite DNA elements (satellite I, II and IV) from this muntjac species. In the simultaneous 3D-FISH and immunofluorescence study, the CENP immunofluorescence signals parallels along with centromere as well as both satellite II and IV signals are organized into a spiral structure. The spiral structure may be to present centromeric chromatin to the exterior of the chromosome, where it can mediate kinetochore assembly and interactions with the spindle. However, it is no enough to prove that the satellite II or IV can directly bind with kinetochore protein. Therefore, we would uncover the whole DNAs that exactly associate with kinetochore protein. First, we have successfully use chromatin immunoprecipitation to isolate the DNA fragments that associate with kineotchore protein. Subsequently, we would use the isolated DNA fragments to be a probe to screen the BAC library. We have gotten 6700 BAC clones to having 0.1 coverage. The BAC library is still under construction.

Introduction:

Centromere is a specialized and primarily constricted structure of eukaryotic chromosome. This constricted structure is presented as 30nm parallel fibers in several electron microscopic studies of mammalian centromere (Rattner and Lin 1985; Wanner and Formanek 2000). Early cytogenetic staining showed that the centromeric chromatin is packed into the constitutive heterochromatin (Heitz. 1928). Later, ultrastructure analyses had shown that a trilaminar disc plate-like structure, designated as the kinetochore, parallels the lateral surface of the centromeric heterochromatin block (Brinkley and Stubblefield 1966; Rieder 1982; Rattner 1986 and 1987; McEwen et al 1993). The trilaminar structure is composed of an electron-dense inner plate that is connected with the centromeric heterochromatin DNAs, an electron-translucent interzone and an electron-dense outer plate which surface is a fibrous corona where the microtubules of the mitotic spindle attach (Pepper and Brinkley 1977; Mitchison and Kirschner 1985; Rattner 1987; Cooke et al. 1993). Electron microscopic analyses of serial sections showed that the caffeine-induced detached kinetochores appear as fragments derived from whole kinetochores. A repeat subunit model is suggested to interpret the organization of a centromere-kinetochore complex (Zinkowski et al.1991). Thanks to the available anticentromere antibody from the patient with CREST disorders (**c**alcinosis, **R**aynaud's phenomenon, **e**sophageal dysmotility, **s**clerodactyly, **t**elangiectasia) variant of scleroderma (Moroi et al. 1980; Brenner et al. 1981; Earnshaw and Rothfield 1985) or with watermelon-stomach disease (He et al. 1998), proteinaceous kinetochore could be stained and identified by immunofluorescence microscopic analysis with this antibody. Subsequently, simultaneous immunofluorescence and fluorescence in situ hybridization study further supported the model that the subunits of stretched kinetchores arrange in a linear array and a repetitive pattern along a centromeric DNA fiber consisting of tandemly repeated subunits interspersed by DNA linkers (Zinkowski et al.1991).

In view of molecular building blocks, centromere is known to compose of centromeric DNA associated with a unique proteinaceous structure, the kinetochore (Rieder 1982). In human centromere, more than 20 different proteins found are divided into two groups: one is the constitutive centromere protein and the other is the transient centromere protein. The constitutive centromeric proteins, such as CENP-A, B, C, G, H and I, are responsible for the structure of centromere and for the assembly of kinetochore. The transient centromere/kinetochore proteins, such as CENP-E, F and INCENP, are essential for promoting spindle microtubules capture, proper chromosome congregation, sister chromatid cohesion, and appropriate movement of chromosomes to opposite poles. The constitutive centromeric proteins appear to be needed to fulfill the intricate and precise dynamic function of the centromere-kinetochore complex when various transient kinetochore proteins are recruited during different stages of the mitosis (Choo 2000; Pidoux and Allshire 2000; Tyler-Smith and Floridia 2000). Specifically, CREST antiserum was known to interact with the inner kinetochore region by recognizing three human "constitutive" centromere proteins CENPs-A, -B, and –C that are located in this region (Brenner et al. 1981; Earnshaw and Rothfield 1985). Additionally, human anti-CREST serum is also found to label centromeric regions of other species as well (Saffery et al. 1999; Hoopen et al. 2000) thus suggesting that the constitutive centromeric proteins are functional and evolutionary conservation.

From low to high eukaryotes, there are many centromeric DNAs found and characterized. In the budding yeast (*Saccharomyces cerevisiae*), the centormere occupies a 125-bp DNA region, comprising three elements (CDEI, CDEII, and CDEIII) with well characterized function and conferring mitotic stability after it is inserted into plasmid vector (Clarke and Carbon 1980; Clarke 1990; Schulman and Bloom 1991; Hegemann and Fleig 1993; Pluta et al. 1995). The *Drosophila* centromere appears to be composed entirely of repeated DNA. *Neurospora crassa* centromeric DNA contains repeated sequences in the manner similar to that of *Drosophila*. The repeated

sequences of flies appear as the remnants of transposable elements and are interspersed between different types of simple repetitive sequence (Cambareri et al. 1998). Mammalian centromeres typically encompass several million bases of DNA (Willard 1990; Tyler-Smith and Willard 1993; Farr et al. 1995; Sun et al. 1997; Harushima et al. 1998) and are consisted predominantly of tandem repetitive DNAs (e.g. satellite DNAs). Major and minor satellite DNA families are ubiquitous in the centromere of mouse (*Mus musculus*) (Horz and Altenburger 1981; Manuelidis 1981; Wong et al. 1990; Garagna et al.1993). There are eight different satellite DNAs well characterized in calf centromeric hetereochromatin region (Singer, 1982). In cervid species, three satellite DNA families I, II and III are found to localize in centromeric heterochromatin region (Bongerberger et al. 1985; Lin et al. 1991; Lee et al. 1997b; Qureshi et al. 1995; Vafa et al. 1999; Li et al. 2000a, b) and most recently, a novel satellite IV DNA family has been identified in several deer species (Li et al. 2002). In human, satellite DNA families (e.g. classical satellite DNAs, alpha satellite DNAs, beta satellite DNAs, gamma satellite DNAs etc.) are the major components of the centromeric DNAs along with some interspersed repetitive DNAs (e.g. SINEs and LINEs). Specifically, unlike other repeated DNA such as telomeric or rDNA, these identified and characterized repetitive centromeric DNAs appear quite complexly organized and display a high degree of variation among species (Lee et al. 1997c). Interestingly, a functional conserved centromeric domain is made up of the diverse repetitive centromeric DNAs and highly conserved kinetochore proteins. Some of repetitive centromeric DNAs from various species are identified to be associated with a specific kinetochore protein. Alphoid satellite DNA, the most predominant human centromeric DNA family, is present in every centromeric heterochromatin region of the human chromosome complement and is capable of binding with CENP-B (Pluta et al. 1992; Haff et al. 1994); therefore, it is thought of as the preferred substrates for kinetochore assembly (Ikeno et al. 1994). The cervid satellite II DNA could be immunoprecipitated with anti-CENP-A serum and also presents in every centromere of deer species studied; thus suggesting this given satellite DNA may be a candidate functional centromeric DNAs in cervid species (Vafa et al. 1999). *Mus musculus* minor satellite DNAs contains the 17 bp CENP-B binding box (ATTCGTTGGAAACGGGA) similar to that of alphoid satellite DNAs and is associated with CENP-B proteins in centromere region by simultaneously immunofluorescence and FISH analysis (Broccoli et al. 1990; Mitchell 1996). More recent studies on the construction of human artificial chromosomes (HACs) each containing an array of the human α- satellite DNA together with the genomic and telomeric DNA elements indicated that the HACs could acquire mitotic stability in cell lines (Harrington et al. 1997; Ikeno et al. 1998; Schueler et al. 2001). Together with these findings, the ubiquitous presence of repetitive sequences at the centromeres of higher eukaryotes led to the suggestion that sequence repetitiveness are needed for centromere function. Moreover, it has recently been proposed that the conservation of centromere functions with diverse repetitive centromeric DNAs could be the result of being able to form higher order structures, such as a secondary or tertiary structure. Such a higher order structure could bind to key centromeric proteins, or serve as targets for critical DNA modification, rather than on particular DNA sequence itself (Copenhaver and Preuss 1999). The requirement of at least some repetitive DNAs for centromere function has not been called into question until the recent observation of stable human marker chromosomes with functional “neocentromeres” that appeared to lack of any repetitive DNA (Voullaire et al. 1993; Depinet et al. 1997; Vance et al. 1997; Barry et al. 1999). Therefore, whether centromeric satellite DNA has a definitive functional role may be still debatable (Choo 2000; Henikoff et al. 2001). On the other hand, it also could not completely rule out the possibility that a conserved sequence nucleates centromere formation in higher eukaryotes**,** unless a complete DNA sequence analysis in any higher eukaryotic centromere had been accomplished. One should reconsider the idea whether the centromere function is dependent on a specific DNA sequence itself or a high-order structure of sequence. Even though, to certain extend, α-satellite sequences could be sufficiently responsible for the construction of human artificial chromosomes (Harrington et al. 1997; Ikeno et al. 1998;

Warburton and Cooke, 1997; Henning et al. 1999; Schueler et al. 2001), one should pay attention to the fact that stable human marker chromosomes with "neocentromere" lack any repetitive DNA (Voullaire et al. 1993; Depinet et al. 1997; Vance et al. 1997; Barry et al. 1999). Therefore, it is still controversial whether the α-satellite DNA sequence itself is essential for a functional centromere. Additionally, the diverse centromeric DNAs and conserved centromeric proteins have been found to be in the centromere (Sunkel and Coelho1995).

The chromosomes of the Indian muntjac (*Muntiacus muntjak vaginalis*) is unique among mammals due to their low diploid number (2N=6♀,7♂), giant size, and unusual large centromeres. The centromere of X+3 chromosome appears having a compound kinetochore. In our study, we had isolated three cervid centromeric satellite DNA elements (satellite I, II and IV) from this muntjac species. The satellite II and IV co-localize in centromere by FISH (fluorescence in situ hybridization) detection as well as they associate with kinetochore by simultaneous immunofluorescence and FISH study. Additionally, the 3D- FISH and immunofluorescence were carried out to show the satellite II and IV signals are organized into a spiral structure on metaphase chromosomes. It is suggested that the purpose of the spiral structure may be to present centromeric chromatin to the exterior of the chromosome, where it can mediate kinetochore assembly and interactions with the spindle. However, there is no direct evidence to prove that the satellite II and IV both bind to kinetochore proteins. To explore this question, we would isolate the kinetochore binding DNA fragments as a probe to screen the whole kinetochore binding DNA from BAC library.

Method:

To isolate the whole kinetochore binding DNA, we would screen BAC library using kinetochore binding DNA fragment as a probe that is isolated from chromatin immunoprecipitation. First, we would establish BAC library for Indian muntjac. Second, we would isolate the kinetochore binding DNA fragment by chromatin immunoprecipitation. The BAC library has been under construction. Briefly, the 10^6 cells were harvested and embedded in low melting agarose. The embedded cells were digested with proteinase K and *Not*I to get the digested DNA without nick. After pulse field gel electrophoresis, elute DNAs from the agarose with 100kb range. Clone the eluted DNAs into pCUGIBAC2 vector and transform into competent cells. The chromatin immunoprecipitation were referred to Lo et al. (2001) with briefly modified. The nuclei were isolated by dissolving cell membrane with 0.4% NP-40 in buffer A (10mM Tris-HCl, 10mM KCl, 0.1mM EDTA) and were sonicated. The sonicated nuclei were incubated with specific anti-ACA serum for overnight at 4oC. The immunocomplex were captured by agarose protein A for overnight at 4oC. Finally, the DNA fragments associated with kinetochore protein were purified by phenol/chloroform.

Results and Discussion:

一: A novel satellite DNA family generated by microdissection of Y-chromosome of Indian muntjac (*Muntiacus muntjac vaginalis*)

In the present study, we isolated a DNA probe, designated MMV-0.23 using a chromosome microdissection technique (Taguchi et al. 1997) from the Y-chromosome of Indian muntjac and showed it was highly specific for the Y-heterochromatin and the pericentric region of the chromosome 3 of the species. The probe was obtained by screening microclones constructed from second DOP-PCR products initially generated from 15 whole Y chromosome with cervid satellite I, II and IV (Li et al. 2002). Two microclones (IM-Y4-52 and IM-Y5-7) negative for all three satellite DNAs were identified. The size of the clones is 339- and 426-bp respectively, and each contains a ~230-bp element with 71.3% sequence homology between the elements. Southern blot analysis showed repetitive nature of clones but lack of typical A-type pattern based on the restriction enzymes used (appendix: Fig.1). No similar sequence of the clones have been deposited in the

GenBank data base suggesting that they could belong to a new cervid satellite DNA family with monomer size ~230-bp. Both clones hybridized specifically to the heterochromatin portion of the Y chromosome and the pericentric region of the chromosome 3 of the male Indian muntjac as demonstrated by FISH (appendix: Fig.2). The solely present of MMV-0.23 in the Y-chromosome and in the pericentric region of chromosome 3 could provide a mechanism for the formation X+3 chromosome (by close association of the X-Y and chromosome 3) of the Indian muntjac.

Cytogenetically, the Indian muntjac is the most fascinating mammalian species with only 6 chromosomes in female and 7 chromosomes in male. Extensive tandem fusions and few centric fusions occurred in the ancestor species with 2n=70 resemble the *Mazama gouazoubira* and *Hydropotes inermis*was karyotype were thought to have resulting the present-day Indian muntjac karyotype (Hsu et al. 1975; Neitzel 1987; Lee et al. 1993; Fronicke and Scherthan 1997; Li et al. 2000; Hartmann and Scherthan 2004). The different chromosome number between male and female of the species is due to the fact that the X-chromosome is translocated onto an autosome 3 by centric fusion forming the X+3 chromosome and therefore the female have two X+3 chromosomes. The male on the other hand has one X+3 chromosome, an “unfused chromosome 3” and a Y-chromosome, thus having one more chromosome than the female. Satellite DNA appeared to play an important role in karyotypic evolution of the mammalian species by promoting chromosomal rearrangement (Slamovits and Rossi 2002).

二: Isolation of kinetochore binding DNA from Indian muntjac by chromatin immunoprecipitation.

In this study, we have been constructing a BAC library of Indian muntjac. There are 6700 BAC clones having been analyzed. The insert size is 100kb. The coverage size is 0.1. We would keep constructing this BAC library. We are planning to get 2 coverage sizes. On the other hand, we also isolated the centromere binding DNA and the CENP-I binding DNA by chromatin immunoprecipitation using ACA(anti-centromere antibody) and anti-CENP-I antibody, respectively. The chromosome localization of the isolated centromere binding DNA was identified by FISH (appendix: Fig. 3). The FISH result shows that most of the isolated centromere binding DNA localized at kinetochore. It is similar to kinetochore signals which were observed by ACA immunofluorescence parallel along the centromere. It is different to the satellite II and IV signals where located on the centromere region. This suggests that the isolated centromere protein associated DNA should include the real centromere binding DNA. We will use this isolated DNA fragment to establish a mini-library for obtaining the real and functional centromeric DNA which binds with kinetochore protein. In the meantime, we will use this isolated DNA fragment as a probe to screen the constructed BAC library for obtaining the whole functional centromeric DNA sequence. In 1999, Vafa et al. had isolated the cervid satellite II DNA using the similar way that isolated DNA from immunoprecipitated complex with anti-CENP-A serum. Moreover, Lo et al. (2001) also identify the 330kb CENP-A binding DNA in human genome by the chromatin immunoprecipiation. Here we use the different anti-centromeric protein antibodies to immunoprecipitate the kinetochore binding DNA. We estimate there are significant centromeric DNAs isolated in this study.

Reference:

Barry et al. (1999) *Hum Mol Genet* **8**:217-227.
Blower et al. (2002) *Dev Cell* **2**: 319-330
Bongerberger et al. (1985) *Eur J Biochem* **148**:55-59.
Brenner et al. (1981) *J Cell Biol* **91**:95-102.
Brinkley, B.R. and Stubblefield, E. (1966) *Chromosoma* **19**:28-43.
Broccoli et al.(1990) *Cytogenet. Cell Genet*. **54**:182-186.
Cambareri et al. (1998) *Mol Cell Biol* **18**:5465-5477.

Choo, K.H.A. (2000) *Trends Cell Biol* **10**:182-188.
Clarke, L. (1990) *Trends Genet*. **6**:150-154.
Clarke, L. and Carbon, J. (1985) *Ann Rev Genet* **19**:29-56.
Cooke et al. (1993) *J. Cell Biol.* **120**:1083-1091.
Copenhaver, G.P. and Preuss, D. (1999) *Curr Opin Plant Biol* **2**:104-108.
Depinet et al. (1997) *Hum Mol Genet* **6**:1195-1204
Earnshaw, W.C. and Rothfield, N.F. (1985) *Chromosoma* **91**:313-321.
Farr et al.(1995) *EMBO J* **14**:5444-5454.
Fronicke and Scherthan (1997) *Chromosome Res* **5**:254-261
Garagna et al.(1993) *Cytogenet Cell Genet* **64**:247-255.
Haaf and Ward (1994) *Hum Mol Genet* **3**:697-709.
Harrington et al. (1997) *Nat Genet* **15**:345-355.
Hartmann and Scherthan (2004) *Chromosoma* **112**:213-220
Harushima et al.(1998) *Genetics* **148**:479-494.
He et al. (1998) *Chromosoma* **107**:189-197.
Hegemann and Fleig (1993) *Bioessays* **15**:451-460.
Heitz (1928) *Ber Dtsch Bot Ges* **47**:274.
Henikoff et al. (2001) *Science* **293**:1098-1102
Horz and Altenburger (1981) *Nucleic Acids Res* **9**:683-96
Hsu et al. (1975) *Cytogenet Cell Genet* **15**:41-49
Ikeno et al. (1994) *Hum Mol Genet* **3**:1245-1257
Ikeno et al. (1998) *Nature Biotechnol*. **16**:431-439.
Lee et al. (1993) *Cytogenet Cell genet* **63**:156-159
Lee et al. (1997a) *J Mol Evol* **44**:327-335.
Lee et al. (1997b) *Hum Genet* **100**:291-304.
Li et al. (2000b) *Chromosome Res* **8**:363-373
Li et al. (2002) *Chromosoma* **111**:176-183
Li et al. (2000a) *Cytogenet Cell Genet* **89**:192-198.
Lin et al. (1991) *Chromosoma* **102**:333-339.
Lo et al. (2001) *EMBO J.* **20**:2087-96.
Manuelidis (1981) *FEBS Lett* **129**:25-28
Mcewen et al. (1993) *J. Cell Biol.* **120**:301-312.
Mitchell(1996) *Mutation Res*. **372**:153-162.
Mitchison and Kirschner (1985) *J. Cell Biol.* **101**:755-765.
Moroi et al. (1980) *Proc Natl Acad Sci USA* **77**:1627-1631
Neitzel (1987) In:Obe G, Basler A (eds) Cytogenetics, Springer, pp 91-112
Pepper and Brinkley (1977) *Chromosoma* **60**:223-235.
Pidoux and Allshire (2000) *Curr Opin Cell Biol*. **12**:308-319.
Pluta et al. (1992) *J Cell Biol* **116**:1081-1093.
Pluta et al. (1995) *Science* **270**:5191-1594.
Slamovits and Rossi (2002) *J Neotrop Mammal* 9:297-308
Taguchi et al. (1997) *Genes Chrom Cancer* 20:208-212
Vafa et a. (1999) *Chromosoma* 108:367-374

Appendix:

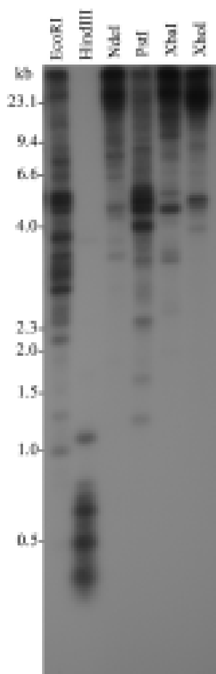

Fig 1: Southern blot analysis of a novel satellite DNA (IM Y 4-52) derived from microdissected Y chromosome. Different digested genomic DNA of male Indian munjac hybridized with microclone probe (IM Y 4-52).

Fig 2: FISH analysis of a novel satellite DNA(IM Y 4-52) derived from microdissected Y chromosome.

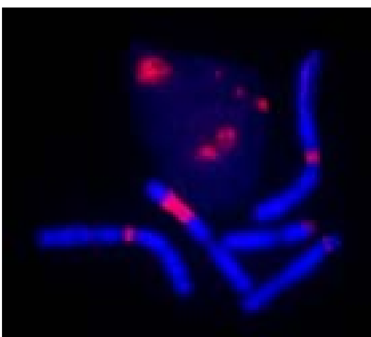

Fig 3: FISH analysis of kinetochore binding DNA which is immunoprecipitated by anti-ACA serum.