Binding of microtubule protein to DNA and chromatin: possibility of simultaneous linkage of microtubule to nucleic acid and assembly of the microtubule structure

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ABSTRACT

    Microtubule protein binds to DNA through microtubule asso-
ciated polypeptides (MAPs). Among MAPs there is one high molecu-
lar weight polypeptide (MAP$_2$) which interacts with DNA fundamen-
tally through certain polynucleotide sequences. This interaction
is not affected by the presence of histones and other chromoso-
mal proteins. DNA can associate to assembled microtubules and
when a determinate DNA/protein ratio is reached the nucleic acid
behaves as a microtubule associated molecule. The nucleic acid
fragments which preferentially bind to microtubules have been
isolated and characterized. These fragments contain DNA regions
enriched in repetitive sequences that hybridize preferentially to
the pericentromeric zone of metaphase chromosomes. These results
give further support to the model of interaction microtubule-
chromosome based upon the mediator function of the microtubule
associated proteins.

INTRODUCTION

    During mitosis the eukaryotic chromosome interacts with

the microtubules through its pericentromeric region. It has been

shown that the chromosome can promote the assembly of microtubu-

les in vitro and that polymerization of microtubules is compati-

ble with its linkage to the chromosome (1,2). The kinetochore is

the site for attachment and, presumptive, assembly of microtubu-

les in the eukaryotic chromosome (3). The kinetochore has been

morphologically characterized but very little is known about its

nature, although it could contain nucleic acid and protein, like

the rest of the chromosome. Recent studies indicate that : a)

DNA could be involved in the chromosome-microtubule interaction

(4), b) high molecular weight microtubule associated polypepti-

des bind to DNA (5), and c) those polypeptides bind preferential-

ly to DNA regions found near the centromere (6). However DNA in-

hibits microtubule polymerization (4,7) although this effect

could depend on the sequence of the nucleic acid tested (4). The
se results suggest that DNA is involved in the microtubule-chro-
mosome interaction but do not explain the initiation of microtu-
bule assembly in the presence of DNA. To interpret the nuclea-
tion of microtubule polymerization on chromosomes, it is necessa
ry to hypothesize one or both of the following explanations :
a) the existence of a DNA sequence which does not decrease the
ability of microtubular protein for assembly; b) the existence
of another kinetochore component, different from DNA, which pro-
motes microtubule polymerization. In this report we explore
these possibilities.

METHODS

Preparation and fractionation of microtubule proteins

        Microtubule proteins were purified from porcine brain by
three cycles of assembly-disassembly by the procedure of Shelans
ki et al (8). Depolymerized microtubules were chromatographed on
DEAE-Sephadex according to Murphy et al (9) under the conditions
previously described (4), to obtain HMW polypeptides, tau frac-
tion and tubulin.
        Microtubule associated protein (MAP$_2$) was obtained as pre-
viously described (10) following the method of Herzog and Weber
(11).

DNA preparation

        Radioactive DNA was isolated from 3T6 mouse fibroblasts
cells after labelling exponentially growing cultures with 3.5
µCi/ml of ^{32}P-phosphate or 2 µCi/ml of ^{3}H-thymidine (20 Ci/mmol)
for 50 h at 37°C. The cells were lysed as previously described
(5) and the DNA was purified by the procedure of Botchan et al.
(12). The isolated DNA was chromatographed on a Sepharose-4B co-
lumn equilibrated in 15 mM NaCl, 1.5 mM Na citrate, to isolate
high molecular weight fraction. The purified ^{32}P-DNA was resis-
tant to alkali or protease treatment but sensitive to DNase di-
gestion.
        To test the affinity of microtubule protein for different
mouse DNA sequences, high molecular weight ^{32}P-DNA was digested
with EcoRI restriction nuclease (100 µg/ml) in 100 mM Tris

(pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, for 3 hours at 37°C. Digested DNA was electrophoresed in 0.3% agarose gels in an horizontal slab gel system as described by McDonell et al (13). 217 μg of mouse DNA were electrophoresed in a gel 12 cm long for 1 hour at 20 V followed by 12 hours at 50 V. The agarose gel was fractionated into fragments of 4 mm and the DNA was extracted by three cycles of freezing and thawing in 0.5 ml of 20 mM Tris (pH 7.0), 1 mM EDTA. The extracted DNA was sheared by passing it through a thin hypodermic needle (as indicated below) to obtain a homogeneous size distribution. The recovery was followed by measuring Cerenkov radiation.

To isolate mouse DNA fragments showing higher affinity for microtubule protein, high molecular weight DNA was sheared by passing it through a 20 gauge needle about 10 times. The obtained DNA fragments have an average molar mass of 3×10^6, as determined by sedimentation in sucrose gradient. This fragmented DNA was added to a depolymerized microtubule protein solution and three cycles of assembly-disassembly were done in the presence of DNA. After the third cycle, depolymerized microtubule protein was chromatographed on a Sepharose 4B column equilibrated in 0.1 M MES (pH 6.4), 0.5 mM $MgCl_2$, 2 mM EGTA. The excluded material was mixed with a 40-fold excess of $\emptyset$29 phage DNA and filtered through a nitrocellulose filter (100 μg of protein per 2.5 cm diameter filter) as described below. DNA was extracted from the filter by addition of 1% sodium dodecyl sulfate, 5 mM EDTA, and it was deproteinized by two phenol extractions. After sonication, to obtain shorter DNA fragments, this material was used for Cot analysis.

Fragments with a higher affinity for MAP_2 were obtained by binding, in a mass ratio one : one (see 4), the purified polypeptide to whole labelled mouse DNA, previously sheared by sonication. Mouse DNA fragments bound to MAP_2 were isolated by filtration on nitrocellulose membranes and prepared for Cot analysis as indicated above.

Chromatin preparation

3T6 cell nuclei were prepared by the method of Kumar and Lindberg (14). Nuclei were resuspended in 10 mM Tris (pH 7.4),

10 mM NaCl, 3 mM MgCl$_2$ and 1 mM CaCl$_2$, at a DNA concentration of
1.5 mg/ml (30 A$_{260}$ units/ml) and digested for 30 min at 37°C
with micrococcal nuclease (Worthington, 10 units/ml). The reac-
tion was terminated by chilling the tubes on ice followed by
centrifugation at 1000 x g in the Sorvall HB-4 rotor. The preci-
pitate was resuspended by gentle homogenization in 0.22 mM EDTA,
pH 7.5, lysed and centrifuged again at 16.000 x g for 10 min.
The supernatant was then centrifuged in a 5 to 20% sucrose gra-
dients, containing 10 mM Tris (pH 7.5), 0.7 mM EDTA. Centrifuga-
tion was at 30.000 rpm for 13 hours in the SW40 rotor (15). The
gradient fractions, corresponding to hexanucleosomes, were used
as chromatin in the binding assays.

Binding of microtubule proteins to DNA and to chromatin
        Complex formation was measured using nitrocellulose fil-
ters according to Jones and Berg (16) in the conditions previous-
ly described (5).

DNA reassociation analysis
        ^{32}P-DNA was sonicated and afterwards it was denatured and
reassociated in 0.57 M NaCl, 1 mM EDTA, 0.05% SDS and 10 mM Tris
(pH 7.5) at 65°C. The renaturation reactions were stopped by im-
mersing the reaction mixtures into dry ice-acetone. The samples
were then added to a column of hydroxylapatite (BioRad) at 60°C.
Single stranded DNA was eluted by washing the column with 0.12 M
phosphate buffer (pH 7.0) and double stranded DNA by washing
with 0.4 M of the same buffer. The amount of eluted DNA was de-
termined by measuring Cerenkov radiation.

Nucleic acid hybridization to the DNA of metaphase chromosomes
        In situ hybridization was done essentially following the
procedures of Pardue and Gall (17) and Jones (18). Exponentially
growing 3T6 cells were treated for 10 h with 0.2 µg/ml of vin-
blastine. Mitotic cells were collected by treatment with trypsin
and gentle centrifugation. Afterwards the cells were fixed in
cold 50% acetic acid for 30 min and squashes were made from a
drop of a dense cell suspension. The cover slips were flipped
off after freezing the squashes in liquid nitrogen and the pre-
paration was maintained in 95% ethanol for 10 min, air dried and

stored at 4°C.

For the hybridization procedure chromosomal DNA was dena-
tured by addition of 0.07 N NaOH at room temperature for 5 min.
The same denaturing treatment was followed for mouse ^{3}H-DNA
(0.1 mg/ml, 10^7 cpm/ml) isolated as indicated above for Cot ana-
lysis. Hybrid formation was performed at 65°C in a moist chamb-
ber humidified with 0.3 M NaCl, 0.03 M Na citrate. 0.1 ml of DNA
solution was added to the chromosome preparation. After incuba-
tion, the cover slips were washed and dehydrated and afterwards
they were covered with Kodak AR10 stripping film for radioauto-
graphy. Before being stored at 4°C the covered slides were expo-
sed to vapours of hydrogen peroxide to erradicate background.

Other methods

The size distribution of DNA molecules was analyzed elec-
trophoretically on 1% agarose gels as described (13). Fragments
of Ø29 DNA (kindly supplied by Dr. C. Escarmís) prepared by di-
gestion with EcoRI restriction endonuclease, were run in paral-
lel as size markers. Proteins were analyzed by sodium dodecyl
sulfate/polyacrylamide gel electrophoresis according to Laemmli
(19). Protein determination was accomplished using the method
of Lowry et al (20).

RESULTS

Binding of microtubule associated proteins (MAPs) to chromatin

In previous reports (4-6) we have described the interac-
tion of MAPs with DNA, suggesting that it could explain the in-
teraction between chromosome and microtubule. However the chro-
mosome is composed of proteins and DNA, therefore, chromosomal
proteins could also be implicated in the chromosome-microtubule
interaction. To test the influence of chromosomal proteins in
the binding of MAPs to DNA we have obtained sheared mouse chroma-
tin by digestion of nuclei with micrococcal nuclease, and tested
its binding to MAPs. Figure 1a shows the sedimentation profile
of the mouse nuclei digest, on a sucrose gradient. The size of
the DNA from the different gradient fractions and the protein
composition of sheared chromatin were determined by electropho-
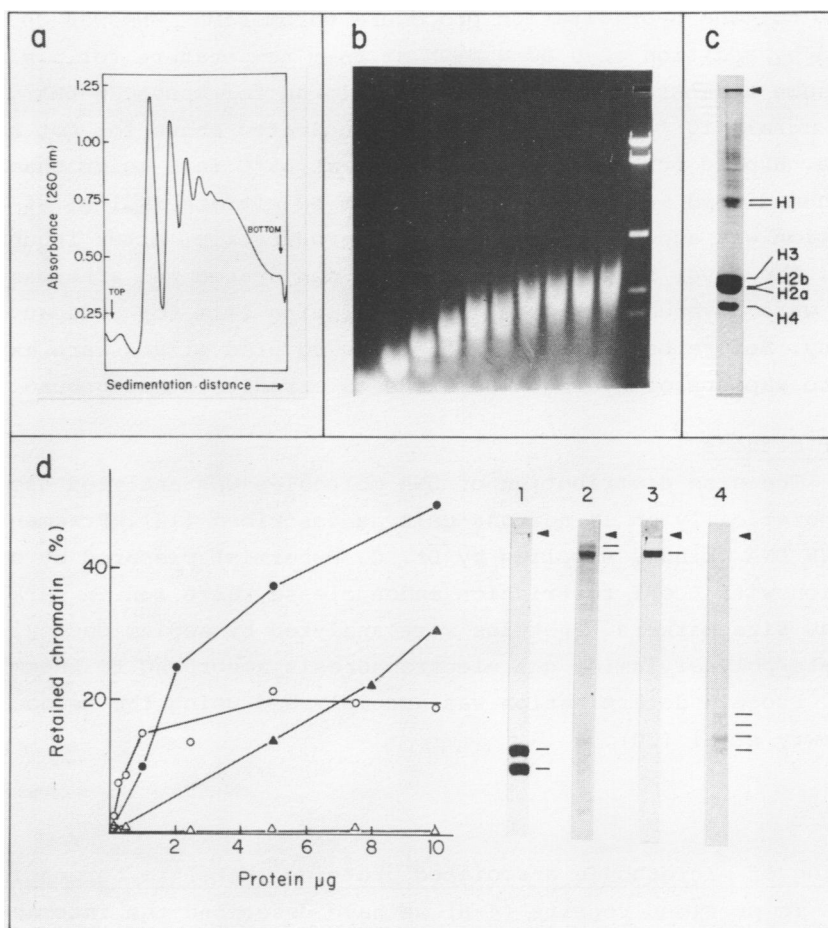resis (Fig. 1b and 1c). Fractions corresponding to mono, di, ..

Fig. 1. Binding of microtubule protein to chromatin. ^{32}P-or-^{3}H-labelled mouse cell nuclei, at a DNA concentration of 1,5 mg/ml (30 A_{260} units/ml), were digested with micrococcal nuclease (10 units/ml). The solubilized chromatin fragments were centrifuged as indicated in Methods. The sedimentation pattern is shown (a). The size of the DNA from the different gradient peaks of Fig. 1a, was analyzed electrophoretically on 1% agarose gels (b). EcoRI digestion fragments from phage Ø29 DNA were used as size markers. Protein composition (c) of the fractions from the gradient was analysed by electrophoresis on 12% acrylamide gels as described (22). Fig. 1d shows the binding of tubulin (△——△), HMWs (●——●), MAP$_2$ (o——o) and tau factor (▲——▲) to sheared chromatin (0.5 µg of DNA) characterized as indicate in the top of this figure. Electrophoresis gels indicate the polypeptide composition of the preparations of tubulin (1), HMWs (2), MAP$_2$ (3) and tau factor (4) used in the binding assay.

and heptanucleosomes were filtered on nitrocellulose membranes
to test whether or not they are quantitatively retained. Our re-
sults indicate a 20% retention for every fraction tested, which
allowed us to use the nitrocellulose binding assay to analyze
the interaction of sheared chromatin with MAPs. Our previous re-
sults suggested that high molecular weight MAPs, essentially
MAP_2, showed a preferential binding to specific DNA sequences(4)
while tau factor did not. Similar results were obtained when
the binding to chromatin of HMWs, MAP_2, tau factor and tubulin
was tested (Fig. 1d). This figure suggests a preferential bin-
ding of MAP_2 to certain regions of sheared chromatin, an unspe-
cific binding of tau factor to the whole chromatin and the ab-
sence of binding of tubulin to chromatin. From the results of
Figure 1 we conclude that chromosomal proteins do not substantia
lly alter the interaction of MAPs with DNA. In a previous report
(4) we have focused our study on the interaction of high molecu-
lar weight MAPs (HMWs) to DNA, because it appears to be prefe-
rential for certain sequences, as also confirmed above. Our re-
sults have indicated that such DNA regions could be enriched in
satellite DNA (6) when mouse DNA was used. However from such re-
sults it was difficult to conclude whether satellite regions
contains, exclusively, the DNA sequences whose interaction with
HMWs is preferential.

HMWs bind preferentially to certain DNA sequences

To test whether there is one or several, non clustered,
DNA sequences in mouse DNA to which high molecular weight micro-
tubule associated polypeptides bind preferentially, we have di-
gested high molecular weight mouse DNA with EcoRI restriction
nuclease and have fractionated the resultant fragments by elec-
trophoresis. Botchan et al (12) have previously shown that EcoRI
digested mouse DNA results in different sized fragments, and
that the larger ones (about 10 millions of molar mass) are enri-
ched in satellite DNA. Figure 2 shows a size distribution for
DNA fragments similar to that reported by Botchan et al. for
EcoRI digested mouse DNA. We have extracted the DNA from diffe-
rent gel electrophoresis fractions and used it to test its bin-
ding to HMW polypeptides, tau factor and tubulin. The result in

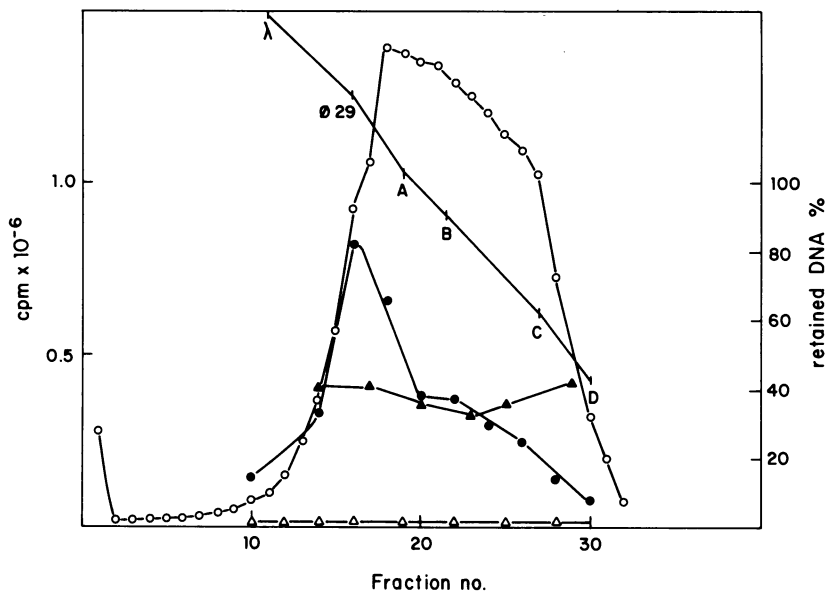Fig. 2. Length distribution of the DNA fragments obtained by digestion of mouse ^{32}P-DNA with EcoRI, after electrophoresis in agarose gels. 217 µg of ^{32}P-labelled mouse DNA were digested with EcoRI nuclease for 3 h at 37°C and the digested mixture layered onto a 0,3% agarose gel and electrophoresed, as indicated in Methods. DNAs of phages λ, Ø29 and EcoRI Ø29 DNA fragments were used as markers of molar mass. The agarose gel was cut into 4 mm slices and the DNA extracted from the gel. A fraction of the extracted DNA was used to determine radioactivity (o—o) and the rest was sheared by passing it through a 20 gauge needle to be used for binding experiments. The binding test of (●——●) HMW polypeptides, (▲——▲) tau factor and (△——△) tubulin to DNA fragments, was done in 0.02 M Tris-HCl (pH 7.0), 0.05 M NaCl, using a constant amount of protein (0.3 µg for HMW and 5 µg for tau factor and tubulin) and DNA (0.1 µg) per assay.

Figure 2 indicates a preferential binding of HMW polypeptides, or MAP$_2$ alone (not shown), to the slow migrating DNA fragments, a similar binding of the tau factor to the different DNA fractions and the absence of binding of tubulin to DNA. These results confirm the previous report of a preferential binding of HMW polypeptides to certain eukaryotic DNA regions and support the hypothesis that HMW polypeptides (6), possibly MAP$_2$, (4), could be the bridge between the chromosome and the microtubule

in mitosis, through their binding to centromeric DNA.

Partial isolation and characterization of mouse DNA sequences
showing a preferential affinity to microtubule protein.

We and others have indicated (4,7) that the addition of
DNA to microtubule protein results in a decrease of its ability
to polymerize. This has been interpreted as a trapping of MAPs
by the nucleic acid, which results in an increase of the criti-
cal concentration that tubulin requires to polymerize. It is not
known whether after addition of DNA, the nucleic acid remains
associated to the assembled microtubules or not. To test it we
have polymerized microtubules in the presence of labelled mouse
DNA through successive cycles. About 43%, 14% and 6% of the total
labelled mouse DNA added is found in the pellet of the first, se-
cond and third polymerization cycle respectively. The high pro-
portion of DNA found in the first pellet could be due to a pre-
ferential but not exclusive binding of HMWs to certain DNA se-
quences, as previously shown in Figure 2, and to the existence
in the microtubule solution of other proteins as, for example,
tau factor, which bind without any specificity to the whole DNA
(see Figure 2 and (4)).

Figure 3 shows the ratio of DNA to protein found in the
pellet fraction after successive polymerization cycles. The DNA/
protein ratio decreased gradually until a plateau was reached.
The figure shows that, when the nucleic acid is in a mass ratio
to protein of about 1/150, the nucleic acid behaves as a micro-
tubule associated molecule, pelleting at a constant ratio to mi-
crotubule protein. On the other hand, the decrease in the amount
of DNA associated to microtubules after several polymerization
cycles could suggest an increase in the specificity of the bound
nucleic acid. We have isolated the protein-bound DNA to charac-
terize it. Microtubules assembled in the presence of DNA (3 cy-
cles, Figure 3), were depolymerized and the nucleic acid-protein
mixture was filtered through nitrocellulose membranes to measu-
re the amount of DNA associated to protein. About 85% of the nu-
cleic acid was retained on the filter, indicating that DNA is
bound to protein in that proportion. This result was confirmed
when the protein-DNA mixture was chromatographed on a Sepharose
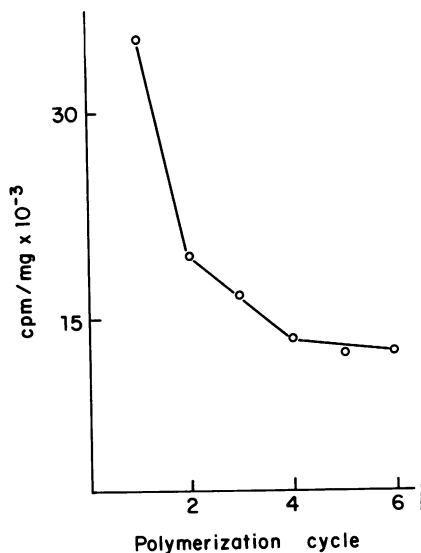
Fig. 3. Amount of mouse DNA found in microtubules pellets after sucessive polymerization-depolymerization cycles. a) 15.5 mg of microtubule protein was mixed with 0.26 mg of ^{32}P-labelled mouse DNA (1500 cpm/µg). The mixture was polymerized-depolymerized for several times. After each polymerization step the pellet was resuspended and the protein content and radioactivity associated was determined. From these data the DNA/protein ratio in each pellet was calculated. The fraction of the genome bound to microtubule pellet was 43%, 14%, 6%, 4.5%, 3.9% and 3.2% after the first, second, third, fourth, fifth and sixth polymerization cycles respectively.

4B column. The nucleic acid, along with protein, was excluded from the column, while DNA was included in the column, in the absence of protein (not shown).

To determine whether or not the interaction microtubule protein-DNA takes place through certain DNA fragments, we mixed the excluded material from the Sepharose 4B chromatography with a large excess of cold Ø29 phage DNA, to compete with the labelled mouse DNA for the microtubule protein. The mixture was filtered through nitrocellulose membranes and the labelled DNA, retained on the filter, characterized on the basis of its reassociation kinetics

On the other hand, we have isolated the sonicated mouse

DNA bound to purified MAP$_2$ by filtration on nitrocellulose mem-
branes. This DNA was also characterized by its reassociation ki-
netics. Figure 4 shows the reassociation analysis of the mouse
DNA fragments bound to microtubule protein, the sonicated mouse
DNA bound to MAP$_2$ and the whole mouse DNA used as a control. In
the figure we can see an enrichment in repetitive sequences of
the DNA fragments that interact with microtubule protein or
MAP$_2$, as compared with the total mouse DNA.

The result of Figure 4 suggests that microtubule protein
interact preferentially with repeated sequences. We have locali-
zed these repeated sequences by in situ hybridization on meta-
phase chromosomes. Figure 5 indicates that these sequences are
preferentially localized on the centromeric region of the meta-
phase chromosome. We have analysed the chromosomes containing
silver grains and have observed that, from 844 chromosomes coun-
ted, 506 contained silver grains over the pericentromeric regi-
on. Preliminary data suggests a similar distribution when the
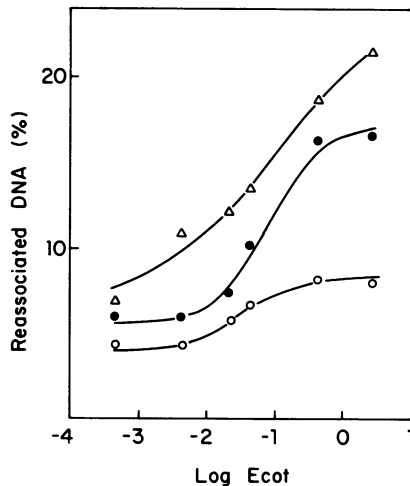sonicated DNA bound to MAP$_2$ was used.

Fig. 4. Kinetics of renaturation of the DNA which preferentia-
lly binds to MAP$_2$. Total mouse DNA fragments (o——o), DNA frag-
ments which remains associated to microtubule protein after se-
veral polymerization cycles (●——●) and DNA fragments wich pre-
ferentially bind to MAP$_2$ (▲——▲) were denatured and reassocia-
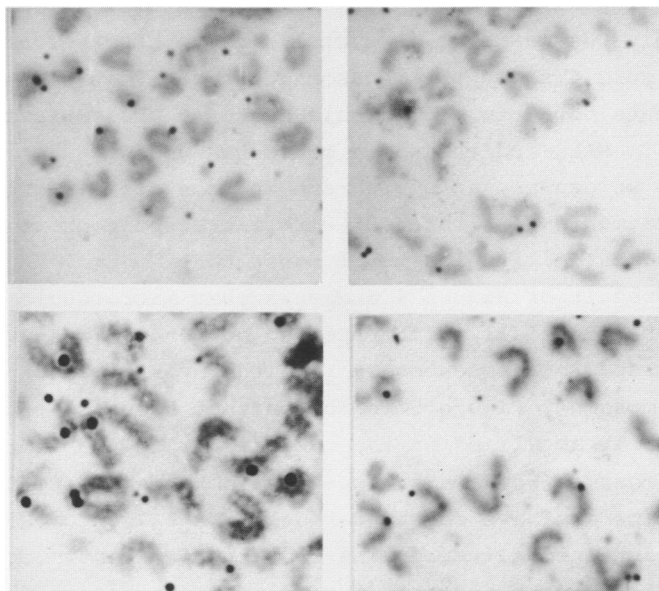ted as indicated in Methods.

Fig. 5.   Autorradiograph of spread mouse metaphase chromosome
preparation after cytological hybridization with ^{3}H-labelled
mouse DNA fragments isolated as indicated in Methods. Nucleic
acid reassociation was done until a Cot/2 of about0.8 molx s/
liter, (10 μg of DNA and 72 hours of incubation time). This
value of Cot/2 is above the calculated Cot/2 for a homogeneous
nucleic acid fragment with an average molar mass of 3 x 10^6,
size of purified DNA fragments.

DISCUSSION

        Our results indicate a preferential binding of isolated

high molecular weight polypeptides or purified MAP$_2$, to certain

regions of mouse DNA. These DNA fragments are enriched in repe-

titive sequences localized, primarily, in the centromeric region

of the chromosome. Binding of HMW polypeptides and MAP$_2$ protein

to sheared chromatin was found to be similar to that of DNA pre-

viously described (4-6), indicating that some chromosomal poly-

peptides, like histones, do not affect the interaction of HMW

polypeptides or MAP$_2$ to DNA. These results support the previous

model for chromosome-microtubule interaction involving the di-

rect binding of HMW polypeptides, possibly MAP$_2$, to the DNA pre-

sent in the kinetochore (4-6). Such a model, that implicates a

direct binding of microtubule protein to DNA, is not consistent with two observated facts : 1) the inhibitory effect of DNA on microtubule polymerization (4,7) and 2) the microtubule assembly on chromosomes (1,2). To overcome this apparent contradiction we suggest one or several possibilities : a) The implication of some chromosomal proteins such as histones (21), in the polymerization process without interfering in the DNA-microtubule protein binding, b) the existence of certain DNA sequences showing little or no effect on the inhibition of microtubule polymerization (4), c) the existence of enough DNA to allow the interaction with the microtubule but small enough to avoid microtubule polymerization, as suggested in Figure 3, d) if MAP_2 or related proteins are involved in the DNA-microtubule binding process, and also in reducing the critical concentration that tubulin requires to polymerize, these proteins could contain two or more DNA binding domains. One of them could be responsible for the binding to tubulin, and the addition of nucleic acid would result in a competitive inhibition. The other protein domain(s) would bind only to DNA, so that the nucleic acid could bind to it without interfering with the binding of the protein and tubulin.

In this report we have explored some of these possibilities. Our results indicate that histones do not interfere the binding of microtubule protein to DNA. Histones could not be implicated alone in the polymerization of microtubules on chromosomes, since such assembly is site specific and histones are localized along the whole chromosome. Possibilities b), c) and d) could be related if the domain of the microtubule protein (MAP_2), which interact preferentially with certain DNA sequences, is not the same as the protein domain which binds to tubulin. Preliminary results in our laboratory suggest the existence of such different protein domains in MAP_2. This could explain the apparent contradictory effects of DNA on microtubule binding and on microtubule polymerization. Moreover, the results showed in this report indicate the possibility of interaction of microtubules to certain DNA regions enriched in repetitive DNA sequences and localized preferentially in centromeric zones.

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