Assembly of additional heterochromatin distinct from centromere-kinetochore chromatin is required for de novo formation of human artificial chromosome

Hiroshi Nakashima1,2,3, Megumi Nakano1,*, Ryoko Ohnishi1, Yasushi Hiraoka4, Yasufumi Kaneda2, Akio Sugino1,3 and Hiroshi Masumoto1,*,‡

1Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan
2Division of Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
3Laboratories for Biomolecular Networks, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan
4Kansai Advanced Research Center, National Institute of Information and Communications Technology, 588-2 Iwaoka, Iwaoka-cho, Nishi-ku, Kobe 651-2492, Japan
*Present address: Laboratory of Biosystems and Cancer, National Cancer Institute, National Institutes of Health, Bldg. 37, Rm 5040, 9000 Rockville Pike, Bethesda, MD 20892, USA
‡Author for correspondence (e-mail: masumoth@mail.nih.gov)

Accepted 20 September 2005
Journal of Cell Science 118, 5885-5898 Published by The Company of Biologists 2005
doi:10.1242/jcs.02702

Summary
Alpha-satellite (alphoid) DNA is necessary for de novo formation of human artificial chromosomes (HACs) in human cultured cells. To investigate the relationship among centromeric, transcriptionally permissive and non-permissive chromatin assemblies on de novo HAC formation, we constructed bacterial artificial chromosome (BAC)-based linear HAC vectors whose left vector arms are occupied by genes coding genes with or without a functional promoter in addition to a common marker gene on the right arm. Although HACs were successfully generated from the vectors with promoter-less constructs on the left arm in HT1080 cells, we failed to generate a stable HAC from the vectors with a functional promoter on the left arm. Despite this failure in HAC formation, centromere components (CENP-A, CENP-B and CENP-C) assembled at the integration sites correlating with a transcriptionally active state of both marker genes on the vector arms. However, on the stable HAC, chromatin immunoprecipitation analysis showed that HP1α and trimethyl histone H3-K9 were enriched at the non-transcribing left vector arm. A transcriptionally active state on both vector arms is not compatible with heterochromatin formation on the introduced BAC DNA, suggesting that epigenetic assembly of heterochromatin is distinct from centromere chromatin assembly and is required for the establishment of a stable artificial chromosome.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/118/24/5885/DC1

Key words: Centromere, Heterochromatin, HAC, Transcription, Epigenetics

Introduction
Faithful chromosome segregation is the crucial event during cell division. The centromere is a highly specialized area of the chromosome that controls this process. A proteinaceous structure, the kinetochore, forms at the outer surfaces of the centromere on a mitotic chromosome, and is responsible for microtubule attachment, chromosome movement and regulation of the synchronous resolution of sister chromatid cohesion (Cleveland et al., 2003; Maiato et al., 2004). A group of proteins (CENP-A, CENP-C, CENP-I/hMis6, hMis12 and others) associated with the centromere-kinetochore through cell cycles are conserved from yeast to humans (Kitagawa and Hieter, 2001; Obuse et al., 2004; Cheeseman et al., 2004). Although the dependency of the components for assembly is not completely conserved among these species, one of the essential steps common to many species for centromere assembly appears to be the deposition of CENP-A, a centromere-specific histone H3 variant (Sullivan et al., 1994; Howman et al., 2000; Oegema et al., 2001; Van Hooser et al., 2001; Goshima et al., 2003). Therefore, CENP-A chromatin assembly is believed to be fundamental in specifying the position of the centromere and in its function.

By contrast, centromeric DNA organization varies widely among species (Murphy and Karpen, 1998; Choo, 2001). It is not known why functional centromeres are established and maintained on evolutionarily diverse sequences, however, these structures are formed and maintained on species-specific centromere DNA not only in humans but also in mouse, rice, maize, and budding and fission yeasts (Henikoff et al., 2001). In all normal human chromosomes, including the sex chromosomes X and Y, the centromere components (including CENP-A) assemble on alpha-satellite (alphoid) DNA loci composed of tandem repeats of AT-rich 171bp monomer units (Willard and Wayne, 1987; Ikeno et al., 1994; Haaf and Ward, 1994; Heller et al., 1996; Mills et al., 1999; Ando et al., 2002). Recently, several groups have succeeded in generating human
artificial chromosomes (HAC) with functional centromeres that depend on type I alphoid arrays composed of highly homogenous higher-order repeating units (Harrington et al., 1997; Ikeno et al., 1998; Masumoto et al., 1998; Henning et al., 1999; Ebersole et al., 2000; Mejia et al., 2001; Grimes et al., 2002). HAC formation assay has demonstrated that type I alphoid DNA and the CENP-B box, a 17 bp CENP-B binding motif found in type I alphoid DNA (Masumoto et al., 1989b), are necessary for CENP-A chromatin and functional centromere assembly (Ohzeki et al., 2002).

However, several lines of evidence also support the importance of epigenetic mechanisms. On stable di-centric chromosomes, caused by chromosomal rearrangements, most functional centromere proteins do not assemble on the inactive centromere despite the presence of human centromere-specific alphoid DNA and CENP-B (Sullivan et al., 2001; Cleveland et al., 2003). More strikingly, on rearranged chromosome fragments from patients with mostly congenital abnormalities, a rare phenomenon may occur whereby a functional centromere, the ‘neocentromere’, forms and is maintained in the complete absence of alphoid DNA (Saffery et al., 2003). However, introduced alphoid yeast artificial chromosome (YAC) and/or bacterial artificial chromosome (BAC) DNA generates stable HACs in some transfected cell lines, whereas in the remainder of the transfectants it is integrated into host chromosomes and inactivated. It has not been established to date how epigenetic mechanisms lead to the different fates of the input DNA, active centromere assembly or centromere inactivation. CENP-A chromatin and centromere components, however, can be reassembled specifically on the ectopic alphoid YAC integration sites that correlate with transcriptional activation on the marker gene on the YAC sites (Nakano et al., 2003). The result indicates that the suppressed state on ectopic alphoid loci can be reversed by the epigenetic change of the adjacent chromatin to a transcriptionally active state. A lack of a unique marker sequence in centromeres of native chromosomes makes the analyses of the relationship between transcription and the centromere more difficult in higher eukaryotes. However, the presence of transcribed genes on CENP-A-assembled centromeric domains is found in a human neocentromere, and in a native centromere on rice chromosome 8 with relatively few copies of satellite DNA (CenO) (Saffery et al., 2003; Nagaki et al., 2004). In human and Drosophila, CENP-A-containing centromere-chromatin domains are found to be enriched with dimethyl Lys4 of histone H3, which represents a transcriptionally permissive chromatin state (Sullivan and Karpen, 2004). This evidence suggests that the centromere is organized as a flexible and permissive chromatin for transcription and, therefore, different from the silent state usually associated with heterochromatin.

Chromosomes also need to maintain sister chromatid cohesion until the completion of metaphase for proper chromosome segregation. Centromeric cohesion is associated with heterochromatin at the pericentromeric regions. In fission yeast, cohesin plays a physical role in the cohesion of replicated chromatids. Cohesin is associated with heterochromatin protein HP1 homolog (Swi6) and, thus, is recruited to centromeric heterochromatin sites (Bernard et al., 2001; Nonaka et al., 2002). In higher eukaryotes, the disruption of heterochromatin causes the loss of proper cohesion and missegregation of chromosomes (Peters et al., 2001; Valdeolmillos et al., 2004; Guenatri et al., 2004). Nevertheless, the relationships among heterochromatin-cohesion function, centromere-chromatin assembly and the presence of transcriptional genes in higher eukaryotic chromosomes are still unclear.

To investigate how centromere chromatin assembly and heterochromatin acquisition are affected epigenetically by transcriptional activities from linked genes on transfected naked DNA and the consequence for de novo artificial chromosome forming efficiency, we analyzed the fate of transfected linear alphoid BAC constructs containing a transcriptional unit with or without a functional promoter proximate to the insert alphoid DNA in addition to a common marker gene on the right arm. De novo stable HAC formation was only observed in transformed cell lines with the alphoid BAC bearing the non-functional promoter. Lower and variegated assembly of centromere components at integration sites was observed with the alphoid BACs bearing the functional promoter. Furthermore, chromatin immunoprecipitation (ChIP) analysis showed that heterochromatin protein HP1αs and trimethyl histone H3-K9 were enriched on the non-transcriptional BAC arm on the stable HAC. These results suggest that interference in stable HAC formation with alphoid BAC constructs arise from the interruption of heterochromatin acquisition on the vector arm by the presence of an additional transcriptional unit on the arm.

**Materials and Methods**

**DNA construction**

The 7C5-basic BAC was constructed as follows. EcoRI-PavII fragment (SV40-bsr cassette) with SV40 early promoter (including the enhancer element) and bsr gene from pSV2bsr (Kaken, Japan) was inserted into the SalI site of pBlueScript II (Stratagene) after end-filling reactions. An XhoI and SalI fragment containing the SV40-bsr cassette from the resultant plasmid was then inserted in the XhoI site of pBeloBAC11 (BAC-SVbvsr). Two 1.1 kb human telomeric DNA fragments derived from pMeanA (Ikeno et al., 1998) inverted and cloned into a plasmid (pTel1.1) with ampR gene in the center, was cut with SalI and SpeI, end-filled, and inserted into the Hpal site of BAC-SVbvsr DNA (BACTSVbvsr). A 7 kb NorI fragment of the alphoid DNA from α7CShtETEL YAC (Ikeno et al., 1998) was inserted into the NorI site of the BACTSVbvsr vector to produce the 7C5-basic BAC.

The `loxP` insertion plasmid vectors were assembled as follows. A synthetic `loxP` sequence was cloned between the SacI and NorI sites of pBlueScript II. Into the Smal and EcoRV digestion site of the resulting plasmid, an `Adal` fragment of kanamycin resistance gene (KanR) from pET30a (Novagen) was inserted (ploxK). A blunt-ended HindIII and SalI fragment containing βgeo gene excised from pcDNA3βgeo (for βgeo insertion vector), a blunt-ended SalI fragment containing CMV promoter (including the enhancer element) and βgeo gene excised from pcDNA3βgeo (for CMV-βgeo insertion vectors with different orientations), or a blunt-ended SalI fragment containing SV promoter and βgeo gene excised from pcDNA3βgeo (for SV-βgeo insertion vector), was inserted into the blunt-ended BamHI site of ploxK. The DNA fragment of 1.2 kb insulator element (chs4) at 5' end of the chicken β-globin locus (Ge:1763620), was amplified from chicken genomic DNA by PCR (primer set: Hs4-F/Hs4-R), inserted into the Smal site of pBlueScript II, and then sequenced. To insert this `EcoRV` sites, a blunt-ended SalI fragment containing CMV promoter and βgeo gene excised from pcDNA3βgeo was inserted (for INS insertion vector).

Each HAC vector (7CS-SV, 7CS-SV/CMV, 7CS-SV/CMV Vrev, 7CS-SV/SV and 7CS-INS BAC) was assembled as follows: A part of
plasmid vector containing replication origin was removed from each insertion vector with XhoI and Sall digestion and self-ligation. This self-ligated DNA and 7C5-basic BAC DNA were inserted into theloxP sites by Cre recombinase (Clontech).

Cell culture and BAC transfection
HT1080 human fibrosarcoma cells were grown in DME supplemented with 10% (vol/vol) FBS (fetal bovine serum). BAC DNA was purified using a QIAGEN column. Linear BAC DNA was prepared by I-SceI digestion, pulsed-field gel electrophoresis (PFGE) separation, β-agargase treatment (NEB) and dialysis in STE buffer (10 mM Tris, pH 8.0, 1 mM EDTA and 50 mM NaCl). 100 ng of linear BAC DNA was transfected into 80% confluent HT1080 cells in a 3.5-cm dish with lipofectamine (Invitrogen). Blasticidin S hydrochloride (BS, Kaken, 4 μg/ml) and/or G418 (Wako) 400 μg/ml were used for selections. Stably transformed HT 1080 cell lines generated with 7C5-SV BAC DNA (S026, S013), 7C5-SV/CMV (K031) and 7C5-INS (IN010, IN011) were used for analyses.

HAC formation assay
Standard techniques were employed for FISH and simultaneous indirect immunostaining (Masumoto et al., 1989a, Masumoto et al., 1998). Used probes were as follows: p11-4 DNA (Ikeno et al., 1994) for the α21-I alphoid loci, BACSVbsr DNA for introduced BAC DNA and CMV insertion plasmid DNA for 7CSSV/CMV BAC specific DNA. Used antibodies were anti-CENP-A (mAN1), anti-CENP-B (2D8D8), anti-CENP-C (CGp2) (Nakano et al., 2003), anti-HP1α (Upstate), anti-aurora B kinase (BD) and anti-GFP (polyclonal, 2D8D8), anti-CENP-C (CGp2) (Nakano et al., 2003), anti-HP1α (Upstate), and CMV insertion plasmid DNA for 7C5-SV/CMV BAC (Ikeno et al., 1994). No host chromosomal DNA was detected in 50-100 metaphase cells that were scored at day 0 (N0) or at day 60 (N60) without selection.

Reverse transcriptase (RT)-PCR
Total RNA was isolated from culture cells using RNeasy Mini Kit (QIAGEN). First-strand cDNAs were synthesized by M-MLV reverse transcriptase (Takara). PCR was performed using 1:40 v/v transcriptase (Takara) using 1 μg total RNA of cDNA reaction and ExTaq (Takara). Amplified DNA was analyzed by agarose-gel electrophoresis and/or reverse transcriptase followed by moderate (25 cycles) or a high (30 cycles) PCR amplification with specific primers for the BAC constructs. The level of transcription from the bsr gene on all of the BAC constructs was high. A few transcripts that extended into the insert alphoid DNA were detected with the higher number of PCR amplification cycles in all BAC transfected cells. The transcripts from the βgeo gene on the left BAC vector arm were detected only in the cells transfected with the BAC constructs containing the CMV promoter. Insertion of the insulator decreased the transcriptional range downstream of βgeo on 7C5-INS BAC drastically compared with that on 7C5-SV/CMV BAC. It is conceivable that transcriptional elongation has a tendency to pause with an insulator even in human cells (Recillas-Targa et al., 2002). The transient expression levels tested on these similar βgeo plasmid DNAs – that differ only in the promoter sequences – showed 10× higher expression with the CMV promoter than those with the SV40 promoter in HT1080 cells as determined by β-galactosidase activity (Fig. 1C).

We then confirmed whether the transcription from promoters worked appropriately on these three kinds of HAC vector constructs in HT1080 cells (Fig. 1D). The transcripts from the HAC vector DNA at 24 hours after transfection were analyzed by reverse transcription, followed by moderate (25 cycles) or a high (30 cycles) PCR amplification with specific primers for the BAC constructs. The level of transcription from the bsr gene on all of the BAC constructs was high. A few transcripts that extended into the insert alphoid DNA were detected with the higher number of PCR amplification cycles in all BAC transfected cells. The transcripts from the βgeo gene on the left BAC vector arm were detected only in the cells transfected with the BAC constructs containing the CMV promoter. Insertion of the insulator decreased the transcriptional range downstream of βgeo on 7C5-INS BAC drastically compared with that on 7C5-SV/CMV BAC. It is conceivable that transcriptional elongation has a tendency to pause with an insulator chHS4 sequence on the 7C5-INS BAC (Zhao and Dean, 2004). These HAC vectors contain different transcriptional activities on the left vector arm.

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were carried out according to the method from the previous description (Nakano et al., 2003). Cells were cross-linked in 1% formaldehyde for 10 minutes. Immunoprecipitated DNA with anti-CENP-A (mAN1), anti-trimethyl histone H3-K9 (Upstate) and anti-GFP (monoclonal, Roche), and the soluble chromatin (as input) were quantitated by real-time PCR (Nakano et al., 2003). ChIP assays using anti-acetylated histone H3 (Upstate) were performed according to the manufacturer’s instructions.

Results
Construction of HAC vectors with varied transcription activities on the left vector arm
Stable human artificial chromosomes (HACs) are composed of a multimerized structure of an input alphoid YAC DNA if linear DNA molecules that end with telomere sequences are used (Ikeno et al., 1998). To investigate how transcriptional activity and resulting chromatin structures affect de novo centromere-chromatin assembly and HAC formation, we first constructed the basic alphoid BAC (7C5-basic BAC). It contains 70 kb of type-I alphoid (α21-I) array, inverted human telomeric repeats placed at both ends of the molecule by I-SceI digestion, a bsr gene driven by the SV40 early promoter (including the enhancer element) on the right side of the alphoid array and a loxP sequence on the left side (Fig. 1A). Next, we obtained three derivative HAC vectors by the Cre-mediated loxP recombination system (Fig. 1B). Although all HAC vectors have the βgeo coding gene, the βgeo gene on the 7C5-SV BAC has no promoter. The 7C5-SV/CMV BAC has the βgeo gene driven by a CMV promoter (including the enhancer element), and the 7C5-INS BAC has a CMV-βgeo gene flanked by 1.2 kb sequences of chicken β-globin 5′HS4 region (chHS4) which is known to function as an insulator even in human cells (Recillas-Targa et al., 2002). The transient expression levels tested on these similar βgeo plasmid DNAs – that differ only in the promoter sequences – showed 10× higher expression with the CMV promoter than those with the SV40 promoter in HT1080 cells as determined by β-galactosidase activity (Fig. 1C).

De novo HAC-forming efficiency was affected by the multiple insertion of active genes
To examine efficiency of de novo HAC formation, the HAC vector DNAs (7C5-SV, 7C5-SV/CMV and 7C5-INS BAC; Fig. 1B) were introduced into HT1080 cells. Then, the obtained blasticidin S (BS)- or G418-resistant cell lines were analyzed by fluorescence in situ hybridization (FISH) with an α21-I 11-mer alphoid DNA probe and a BAC DNA probe (Table 1). Five out of 30 analyzed cell lines (four of 21 analyzed cell lines in the second experiment) generated with the 7C5-SV BAC DNA transfection contained one copy of a minichromosome (HAC) signal with both probes in more than 50% of metaphase spreads (Fig. 2A), similar to the HAC formation observed with the YAC-based α21-I alphoid constructs (Ikeno et al., 1998; Masumoto et al., 1998). No host chromosomal DNA was
Fig. 1. Structures of HAC vector constructs and transcriptional activities. (A) 7C5-basic BAC DNA contained an alphoid repeat (~70 kb) sequence, a mammalian selectable maker gene (SV40-bsr), human inverted telomeres (each ~1.1 kb), a loxP site, and a chloramphenicol resistance (CmR) and ampicillin resistance (AmpR) gene. Insertion vectors contained a kanamycin resistance (KanR) gene, sequences of interest (SOI) and a loxP site, from which the replication origin had been removed (see Materials and Methods). (B) HAC vector constructs were linearized with I-SceI endonuclease. Each HAC vector had an insertion of different DNA at the loxP site on the left arm of 7C5-basic BAC: 7C5-SV BAC lacks the promoter of βgeo gene. 7C5-SV/CMV BAC contains the βgeo gene with CMV promoter. 7C5-INS BAC possesses the chicken β-globin insulator sequences (cHS4) flanking both sides of the CMV-βgeo cassette. 7C5-SV/CMVrev BAC is identical to 7C5-SV/CMV BAC but contains the CMV-βgeo cassette in reversed orientation. (C) β-Galactosidase activity of HT1080 cells was analyzed 24 hours after transfection of either SV-βgeo or CMV-βgeo plasmid DNA, or after transfection of pBluescript II as a control. Luciferase activity of co-transfected luciferase expression vector (pRL-CMV, wako) was used for normalization. Data are the averages of three independent experiments. Error bar shows the s.e.m. (D) RT-PCR was performed 24 hours after transfection of each HAC vector DNA (7C5-SV, 7C5-SV/CMV or 7C5-INS BAC). PCR was carried out with 25 or 30 cycles against reverse transcribed cDNAs with specific primers (from top: β-actin, bsr, bsr-pA, right junction, left junction, kanR, βgeo-pA, βgeo1). Control reactions were performed against mock-transcribed cDNAs without reverse transcriptase (–RT).

Table 1. Chromosomal events in stably transformed cell lines

| Input DNA | Selection drug | Analyzed cell number | HAC | Chromosomal integration events* | No signal† |
|-----------|----------------|----------------------|-----|---------------------------------|-----------|
| 7C5-SV (exp.1) | BS | 30 | 5 (16.7%) | 11 (36.7%) | 14 (46.7%) |
| (exp.2) | BS | 21 | 4 (19.0%) | 17 (81.0%) | 0 |
| 7C5-SV/CMV | BS | 29 | 0 | 26 (89.7%) | 3 (10.3%) |
| | G418 | 31 | 0 | 21 (67.7%) | 10 (32.3%) |
| 7C5-INS | BS | 26 | 0 | 18 (69.2%) | 8 (30.8%) |
| | G418 | 31 | 0 | 21 (67.7%) | 10 (32.3%) |
| 7C5 mix‡ | BS, G418 | 19 | 3 (15.8%) | 16 (84.2%) | 0 |
| 7C5-SV/CMVREV | BS | 29 | 0 | 26 (89.7%) | 3 (10.3%) |

Cell lines were classified according to the predominant fate (>50%) of transfected input DNA as measured by FISH analysis using α21-I probe and BAC probe (n=20). A Fisher’s exact probability test of the predominant chromosomal events between 7C5-SV BAC and other cell lines with introduced HAC-deficient vector DNAs shows P<0.05.

*Chromosomal integration events show numbers of cell lines integrated into host chromosomal DNA.
†Cell lines classified as no signal contain no detectable signal of BAC probe.
‡7C5 mix DNA indicates a mixed DNA of 7C5-SV/CMV BAC and 7C5-basic BAC DNA in the ratio of 0.1:1 before transfection.
detected on the 7C5-SV BAC derived HACs (supplementary material Fig. S1). By contrast, all cell lines obtained with the 7C5-SV/CMV BAC and the 7C5-INS BAC DNA showed integration signals into host chromosomes in a majority of the spreads. The result indicates that HAC formation efficiency of these BAC constructs containing the transcriptional activity on the left vector arm is significantly decreased compared with that of 7C5-SV BAC DNA (Table 1, P<0.05). Then we analyzed the cell lines obtained with the 7C5-SV/CMVrev BAC DNA, where the transcriptional direction of the CMV-geo gene was reversed (Fig. 1B). However, no de novo stable HAC formation was observed (Table 1). Therefore, when the HAC vector had been designed to permit transcripts for either directions on the left vector arm, we could not obtain de novo any stable HAC cell lines, whether we used identical alphoid DNA competent for centromere-kinetochore assembly or decreased the transcriptional activities by inserting the insulator.

Low level of assembly of centromere proteins at ectopic integrated sites

To examine whether centromere proteins assemble on the input DNAs, we analyzed cell lines that contained a HAC or a host chromosome integration site by immunostaining and simultaneous FISH. In the S026 cell line, which contains a HAC composed of multimerized 7C5-SV BAC DNA (33 copies of the input BAC construct; Table 2), centromere-kinetochore components CENP-A, CENP-B and CENP-C signals were detected on all HACs analyzed (Fig. 2A, Table 2). The HAC was also very stable for an extended periods without the selective drug BS, with R=-0.0011 during 60 days of culture. We analyzed the cell lines in which introduced BAC DNAs had been integrated into a host chromosome (S013 cell line with 7C5-SV BAC DNA, K031 with 7C5-SV/CMV and IN010 and IN011 with 7C5-INS). Although CENP-B signals of weaker levels were detected in 60-100% of cells at the ectopic integration site of multimerized BAC DNAs, lower and variegated CENP-A and CENP-C signals were detected in about 6-26% of the cells (Fig. 2B, Table 2). We did not find any correlation among the levels of the variegated assembly at the ectopic integration sites, the total size of the alphoid DNA, which varied in different cell lines from 0.6 to 3.5 Mbp (multimers of 8-51 copies of the input DNA), and the differences in BAC constructs (Table 2). Although we could not obtain stable HAC de novo, the ability to assemble centromere-kinetochore components is not completely lost from these HAC formation-deficient alphoid BAC constructs.
Reactivation as a centromere at the ectopic integration site of HAC formation-deficient BAC correlated with transcriptional activity

Our previous study showed that CENP-A chromatin and centromere components can be reassembled specifically on the ectopic alphoid YAC integration sites that correlated with transcriptional activation on the marker gene on the YAC sites (Nakano et al., 2003). To confirm that the transcription from the additional marker gene on the BAC left vector arm is not directly conflicting with the reassembly of CENPs at the integrated site of HAC formation-deficient alphoid BACs, we analyzed K031 cells, which survived under BS and G418 double-selective conditions for 30 days, by indirect immunostaining and simultaneous FISH. The numbers of BAC integrated sites that show CENP-A, CENP-B and CENP-C signals increased to 62-98% in K031 cells with double selective conditions from the low assemblies of CENP-A and CENP-C (6-26%) in BS single selected K031 cells (Table 2). And a stable episomal chromosome fragment containing the BAC integration site (defined as a reformed minichromosome) with CENP signals was frequently observed (45.9±9.6%, Fig. 2C, a loss rate R=0.0027 for further 60 days of culture without the selective drugs). These reformed minichromosomes were accompanied by various sizes of DAPI-stained host chromosome fragments acquired from the integration site following the breakage event (Fig. 2C). Therefore, functional centromere-kinetochore structure can still be assembled at the HAC-formation-deficient alphoid BAC integration site.

Table 2. CENPs assemble at alphoid BAC sites

| Introduced DNA | Cell line | Initial fate | Selection drug | Copy†/ % of cells with signals for CENPs‡ | % of cells with extra-chromosomes |
|---------------|-----------|--------------|----------------|------------------------------------------|----------------------------------|
| 7C5-SV        | S026      | HAC          | BS             | 100* 100* 100* 88                        |                                  |
|               | S013      | arm int.     | BS             | 15.2 100 7.4 0                           |                                  |
| 7C5-SV/CMV    | K031      | arm int.     | BS, G418       | 26 60 6.6 0                             | 0                                |
| 7C5-INS       | IN010     | tel int.     | BS             | 73.5/100* 98/100* 62.2/100* 45.9 (9.6)  |                                  |
|               | IN011     | tel int.     | BS             | 20 100 18 1.5 (1.3)                      |                                  |

†Copy number of transformed BAC DNA was estimated from quantification of bsr gene locus by real-time PCR on the basis of the 7C5HT1-2 cell line (Nakano et al., 2003). Total size of alphoid DNA composed of multimerized input DNA was calculated from copy number as being equivalent to 70 kb of inserted alphoid DNA.

‡Percentage of centromere-kinetochore component (CENP) was assessed based on colocalized signals from indirect immunostaining of CENP and FISH analysis of BAC DNA probe (n>40). *, indicates the proportion of CENP assembly on extra-chromosomes. Number brackets indicate s.e.m. (n=3).

Fig. 3. Levels of transcription and acetylated-histone H3 on HAC or integrated sites. (A) RT-PCR analysis of the transformed cell lines (S026, S013 and K031) was performed as described in Fig. 1D. The schematic map of 7C5-SV/CMV BAC indicates PCR probe sites. The primers used are, from top: β-actin, bsr, bsr-pA, right junction, left junction, kanR, βgeo-pA, βgeo1. (B) ChIP and real-time PCR analysis of alphoid BAC using antibody against acetylated histone H3 and normal IgG (as a control). Recovery rate of immunoprecipitated DNA against the input DNA is shown in the histogram, error bars give the s.e.m. (n=3). As controls, an endogenous promoter region of the CENP-B gene (P_{CENP-B}) and the pericentromeric satellite 2 repeat loci (sat2) were used.
The level of transcription from the bsr gene analyzed by RT-PCR in S026, S013 and K031cell lines was high because of BS selection. Transcription from the βgeo gene was suppressed in K031 cells under the BS single selective condition [Fig. 3A, lane of K031(BS)]. By contrast, transcription levels from the βgeo gene were increased and extended to the inserted alphoid sequence in the 7C5-SV/CMV BAC under BS-G418 double selection conditions [Fig. 3A, lane of K031 (BS+G418)].

We then analyzed the chromatin structure by chromatin immunoprecipitation (ChIP) with an anti-acetylated histone H3 antibody. The precipitated DNA samples were quantitated by real-time PCR with specific primers for alphoid BAC constructs. As shown in Fig. 3B, immunoprecipitates of acetylated histone H3 enriched transcriptionally active regions such as the endogenous promoter of the CENP-B gene (11.2-15.3%, controls with normal IgG were less than 0.04%), but did not enrich satellite 2 (sat2) control sequences (0.08-0.10%), which are located at pericentromeric heterochromatin regions of chromosome 1 and 16 (Espada et al., 2004). Acetylated histone H3 was abundant in the bsr gene (12.4-16.5%) on the right arm of the introduced alphoid BAC constructs in all cell lines. Under double selection conditions, acetylated histone H3 was enriched by the βgeo gene (5.5%) in K031 cells, whereas less acetylated histone H3 was observed in the single drug selection (BS) of K031 cells and other cell lines (0.56-1.28%). In all cell lines, the levels of acetylated histone H3 correlated very well with the transcriptional activities of the BAC arms and were very low on the left and the right alphoid junctions, especially on α21-1 alphoid DNA (0.09-0.17%), which is located in the centromere of the HAC or the integration sites of the BAC DNA, as well as at the centromere region of human chromosome 13 and 21.

Thus, consistent with our previous finding, an open chromatine structure that allows a transcriptionally active and hyper-acetylated histone H3 state proximate to the inserted alphoid DNA is coupled with the reassembly of CENPs, even on the HAC-formation-deficient alphoid DNA at the ectopically integrated site, and does not inhibit the formation of a centromere-kinetochore structure.

The transcription-capable structure itself conflicts with assembly required for the HAC
To analyze further the antagonism between HAC formation and the addition of the CMV promoter on the left vector arm, we co-transfected 7C5-SV/CMV BAC DNA and 7C5-basic BAC DNA into HT1080 cells and selected with BS and G418. De novo HAC-forming-activity was recovered in up to 16% in analyzed cell lines (3 out of 19 analysed cells with 7C5 mix, see Table 1). The signals specific for the 7C5 SV/CMV BAC DNA were detected only on those HAC that overlapped with extrachromosomal signals detected by a BAC probe or alphoid probe (see supplementary material Fig. S2A). Thus, the conflict can be overcome by a further recruitment of CMV-promoter-less alphoid BACs without acquiring other chromosomal fragments. The existence of an additional transcription-capable structure on the left arm might conflict with an unknown structural assembly required for stable artificial chromosomes other than the centromere-kinetochore.
tagged HP1α was enriched on the BAC left arm region (L2, L3 and L4 probes, 5.3-7.5-fold) similar to the positive control of satellite2 DNA (8.1-fold). Interestingly, α21-I alphoid DNA was also slightly enriched (2.7-fold), regardless of CENP-A abundance, indicating that some parts of the α21-I alphoid array were covered by heterochromatin. Therefore, the localization pattern of HP1α shown by ChIP analysis is consistent with the assembly of triMet H3-K9 on the promoter-less left vector arm of the stable HAC (Fig. 4A,B).

**HP1α and aurora B kinase at the BAC integration sites**

We next analyzed the distribution of HP1α at the BAC integration sites in metaphase-arrested cells. Interestingly, an HP1α signal was not detected at the BAC-integrated site on the chromosome arm region in K031 cells, but was detectable at the canonical centromere region on the host chromosome (Fig. 5C). However, we detected the small HP1α signal on the reformed minichromosomes in K031 cells by double selection (Fig. 5D). The mitotic localization of HP1α is not consistent with the distribution of triMet H3-K9 at the BAC integrated sites on the chromosome arm or on the reformed minichromosome (Fig. 4A).

Therefore, we confirmed the localization of aurora B kinase in metaphase-arrested cells. Aurora B kinase behaves as a chromosomal passenger protein (CPP), forming a complex with INCENP, survivin and the recently identified Borealin (Gassmann et al., 2004). INCENP associates with pericentromeric heterochromatin and this pericentromeric localization depends on cohesin function early in mitosis (Ainsztein et al., 1998; Sonoda et al., 2001). As the results of immunostaining and simultaneous FISH analysis using anti-aurora B kinase antibody and BAC probe show, we could observe the localization of aurora B kinase on the de novo HAC and reformed minichromosome with an activated centromere from the alphoid BAC integration site (Fig. 5D and data not shown). However, we could not detect aurora B kinase signal at the integrated loci on the chromosome arm region, even though the signal was detectable at the canonical centromere region on the host chromosome in K031 cells (Fig. 5C). Therefore, at a minimum, a pericentromeric heterochromatin-cohesion-related protein (HP1α) and an inner centromere protein (aurora B kinase) were assembled on the HAC as well as the reformed minichromosome that correlates with the centromere function, but were not distinctively enriched on the alphoid BAC integration site of the metaphase chromosome arm, which does not have the centromere function.

Taken together, all these results strongly support the view
that heterochromatin assembly on the vector arm of the introduced alphoid BAC-YAC construct is incompatible with the insertion of a transcriptionally active CMV promoter (including the enhancer element), and crucial for de novo HAC formation after transfection and centromere-chromatin assembly on the \( \alpha 21-I \) alphoid array.

**Discussion**

Kinetochore formation does not conflict with a transcriptionally permissive chromatin proximate to alphoid DNA

The centromere domains of humans and *D. melanogaster* are marked by an interspersed structure with dimethylated histone H3-K4 clusters and CENP-A clusters (Sullivan and Karpen, 2004). This recent observation supports the repeat-subunit model of centromere structure proposed by Zinkowski et al. (Zinkowski et al., 1991) and is consistent with a transcriptionally permissive state of centromere chromatin. Transcriptionally active genes within a functional centromere also have been found in a human neocentromere and the centromere of rice chromosome 8 (Saffery et al., 2003; Nagaki et al., 2004). These observations implicate a coexistence of transcriptional competence and kinetochore function within the same centromere domain, perhaps because the centromere is the repeat-subunit structure and these two events function during different phases of the cell cycle; transcription during interphase and kinetochore assembly during mitosis.

HACs are composed of a repeated chromatin structure on an input alphoid YAC-BAC multimer. However, we could not generate any stable de novo HAC when HAC vectors, containing the centromere-competent \( \alpha 21-I \) alphoid DNA, permitted transcription from the left vector arm in addition to the right arm. Our previous study had shown that transcriptional activity does not interfere with the assembly of centromere chromatin on the Type-I alphoid YAC-BAC multimer (Nakano et al., 2003). Moreover, a structural change of chromatin – from a suppressed state to a transcriptionally active state – at the marker genes at the ectopic integration site of the alphoid YACs, using a short-term treatment with the...
histone deacetylase inhibitor (TSA) or a long-term culture under drug selection, facilitates the reassembly of centromere components including CENP-A to the nearby inserted alphoid DNA. Present data also showed that, the reassembly of centromere components at the ectopically integrated site of even a HAC-formation-deficient α21-I alphoid BAC correlated with the transcriptional activation at the vector arm region (Fig. 2C, Fig. 3). Therefore, the active transcriptional state at the vector arms proximate to the insert alphoid DNA does not inhibit the assembly of centromere components directly (Fig. 6 model B).

All these observations do not conflict with the idea that a transcriptionally permissive chromatin state could also allow assembly of centromere chromatin composed of CENP-A nucleosomes. In addition, overexpression of CENP-A leads to mislocalization of the proteins across entire chromosome arms, especially euchromatic loci (Van Hooser et al., 2001). This indicates that CENP-A loading on chromatin might be

![Fig. 6. Models of chromatin assembly and structural organization on input alphoid BACs. Chromatin assemblies and transcripts on one unit of multimerized alphoid BAC DNA (7C5-SV BAC in A, 7C5-SV/CMV BAC in B–D) are shown as hypothetical models. Chromatin states supported by RT-PCR and ChIP analyses are indicated by colored lines. Arrows below the vector maps show transcription level (width) and length. Even though the ChIP analysis represents the sum of chromatin structures formed on multimerized alphoid BAC units, our ChIP data show a tendency for distinct chromatin structures to correspond to the sequence structures of the alphoid BAC constructs, implying that the HAC is maintained as punctuated blocks of chromatin structures. (A) Open chromatin or euchromatin (green) at the transcriptional gene on the right arm enhances the assembly of centromere chromatin (red) on the inserted alphoid DNA. In addition, when heterochromatin assembly (blue) occurs at the left arm and at a part of alphoid repeats (Fig. 4), functional HAC is generated and stably maintained. (B) If the heterochromatin-formation domain on the left arm is replaced with euchromatin by inserting a transcriptional gene unit, centromere-kinetochore components still can assemble on the alphoid array, but cohesion and inner centromere functions are absent. As a result, the unstable structure of the extra-chromosome is lost or integrated into a host chromosome. (C) Otherwise, at the integration site of a host chromosome, a silencing effect may be induced and/or heterochromatin spreads as indicated by triMet H3-K9 (Fig. 4A) into both non-selective marker gene and the insert alphoid DNA; consequently, centromere assembly on the alphoid DNA would be inactivated. (D) On the ectopic integration sites of multiple alphoid BAC DNAs, however, a chromatin opening also occurs stochastically in a part of the multiple array as indicated with the variegate assembly of the CENPs at the sites. If the open chromatin is selected again by the double selection as described in B, the chromatin opening and the functional assembly of kinetochore components on the ectopic alphoid array are accelerated and causes the reformation of minichromosomes accompanied by chromosome breakage events and with a part of the host chromosome fragment as a donor of heterochromatin-cohesion.
regulated by the expression level of CENP-A and/or by the cell cycle (Shelby et al., 2000), and might occur on permissive chromatin but be displaced from histone H3 nucleosomes by some loading factors.

Our ChIP experiments, however, showed that CENP-A chromatin localizes almost exclusively at the inserted alphoid DNA on HACs but only rarely spreads across the BAC-YAC vector arm DNAs, including the transcribing marker genes (Fig. 4A) (Nakano et al., 2003). CENP-A chromatin preferentially assembled on AT-rich (~60% AT) synthetic alphoid repeats that contain CENP-B boxes, but not on GC-rich (~40% AT) synthetic repeats based on a pBR322 vector fragment (Ohzeki et al., 2002). Therefore, these results suggest that the transcriptionally active chromatin state of marker genes increases the deposition activity of CENP-A nucleosomes on adjacent alphoid repeats but not on these GC-rich transcribing genes and vector DNA themselves under normal cellular condition (Masumoto et al., 2004).

The role of heterochromatin and the inner side of the centromere for de novo HAC formation

Heterochromatin protein HP1α assembly on HACs was recently observed on both BAC-based (Grimes et al., 2004) and YAC-based stable HACs (our unpublished results). Our data show that triMet H3-K9 and HP1α localize on the stable HAC and are enriched at the left arm of 7C5-SV BAC DNA (Fig. 4). This region includes the βgeo coding gene without a functional promoter. By contrast, HAC-formation-deficient derivative alphoid BACs (7C5-SV/CMV and 7C5-INS) include a transcriptional βgeo gene with the CMV promoter. We also failed to generate de novo stable HACs from the other derivatives on which the CMV promoter of the βgeo gene was replaced by a SV40 promoter (data not shown) or the orientation of the CMV driven βgeo gene was reversed (Table 1). However, the association of transcriptional activities is not inhibitory for the assembly of the functional centromere as discussed above. On the other hand, heterochromatin structures are incompatible with the transcriptional activation of the left arm by an additional promoter and even by the insolation of this region.

Why is a heterochromatin structure required for de novo HAC formation and maintenance? In many eukaryotes, the centromere is embedded in heterochromatin, which contributes to faithful chromosome segregation and genome stability. HP1α and triMet H3-K9 are enriched at the pericentric regions on mitotic chromosomes in human and mouse (Fig. 5) (Hayakawa et al., 2003; Peters et al., 2003; Guenatri et al., 2004). In fission yeast, Swi6 (a HP1 homologue) is physically associated with a cohesin protein Rad21, whose complex is essential for sister-chromatid cohesion and faithful chromosome segregation, and thus the association may explain the assembly of cohesin to the pericentromeric heterochromatin region (Bernard et al., 2001; Nonaka et al., 2002). In higher eukaryotes, centromeric heterochromatin domains and cohesion domains also overlap on mitotic chromosomes and the disturbance of the heterochromatic structure causes the loss of proper chromatin cohesion (Peters et al., 2001; Valdeolmillos et al., 2004; Guenatri et al., 2004). Moreover, the cohesin complex does not only comprise sister-chromatid cohesion, but also plays a role in the accumulation of chromosome passenger proteins (CPP) to the inner centromeric region (Sonoda et al., 2001; Vagnarelli and Earnshaw, 2001; Vass et al., 2003). Aurora B kinase, one of the CPP components, dynamically and widely regulates mitotic events, kinetochore function and cytokinesis, and is also involved in cohesion releasing from chromosome arms and accumulating at inner centromere regions until just before onset of anaphase. Therefore, the accumulation of aurora B kinase is an important functional marker for the inner side of the centromere, which connects the kinetochore and heterochromatin-cohesion functions (Carmena and Earnshaw, 2003; Maiato et al., 2004). Real-time observation of HACs in living mitotic cells showed that HACs are accurately aligned at the spindle equator, and the sister chromatids of the HAC are resolved with the same timing as natural chromosome separation synchronized with mitotic cell-cycle progression (in preparation). Thus, de novo HACs also retain the normal mechanism controlling sister chromatid cohesion and resolution. Indeed, our data show that HP1α and aurora B kinase localize to HACs in metaphase-arrested cells and on reformed minichromosomes that contain an activated centromere from the alphoid BAC integration site, but not on the ectopically integrated and inactivated site of the alphoid BAC on the chromosome arm region (Figs 4, 5). HP1α and CPP might be more dynamically regulated on the mitotic chromosomes. Correspondingly, heterochromatin structure is also acquired close to the kinetochore domain at neocentromeres (Saffery et al., 2000; Aagaard et al., 2000). These results strongly suggest that acquisition of heterochromatin structure is also necessary for introduced naked DNAs – coincident with the assembly of a kinetochore structure during an early stage of stable HAC formation – and, thus, the introduced DNA needs to supply appropriate sequences not only for the kinetochore-forming domain but also for the heterochromatin domain (Fig. 6 model A).

Potential influence of transcriptional activity upon epigenetic chromatin assembly and HAC formation

Treatment of mouse cells with RNase causes release of HP1 and disturbance of the chromatin state of the pericentromere (Maison et al., 2002). In fission yeast, small interfering RNAs (siRNA) – generated by the cleaving double stranded RNA (dsRNA) by Dicer – is found to be associated with epigenetic chromatin modification and the establishment of heterochromatin (Hall et al., 2002; Volpe et al., 2002). A conditional loss-of-function mutant of Dicer in a chicken cell line showed premature sister chromatid separation and mitotic defects without an abnormal assembly of CENP-A and C (Fukagawa et al., 2004). On the other hand, a Dicer-deficient mouse embryonic stem (ES) cell line showed defective differentiation and centromere silencing, but maintained ES morphology, cell viability and chromosome stability (Kanellopoulou et al., 2005), suggesting the existence of another mechanism for maintaining chromosomal stability in ES cells, in addition to Dicer-RNAi.

In our RT-PCR experiment, transcripts from both the left arm and the right arm sides continue into the alphoid array in the 7C5-SV/CMV BAC during transient transfection (Fig. 1D). Moreover, a suppression of transcriptional activity from the CMV promoter towards the alphoid array was observed in the
cell line with the integration of the 7C5-SV/CMV BAC DNA. It is conceivable that the homologous alphoid repeats transcribed from both vector arms are potential targets of Dicer and thus contribute to the suppression of centromere chromatin assembly on the insert alphoid DNA (Fig. 6, model C). However, our results do not strongly support an explanation, only a potential dsRNA-mediated mechanism on alphoid DNA, because we failed to generate de novo HAC from 7C5-SV/CMV<sub>geo</sub> BAC (in which the orientation of the CMV driven βgeo gene was reversed) precluding the presence of dsRNA on the alphoid DNA. It, therefore, appears that a transcribing structure on the left arm itself conflicts with HAC formation (Fig. 6 model B). In addition, we observed reformed minichromosomes accompanied by an activated centromere from integrated loci of the 7C5-SV/CMV BAC DNA (Fig. 2C) and HAC formation from the 7C5 mix (Table 1, supplementary material Fig. S2), despite being under BS and G418 double selection and requiring transcription on the left arm of the 7C5-SV/CMV BAC DNA. However, in these cases, a missing structure for heterochromatin-cohesion on the left vector arm is still present (Table 1, Fig. S1), indicating that the alphoid DNA is a potential target for dsRNA-mediated suppression of transgene expression in vivo. Therefore, these chromosomes were not maintained stably only under de novo assembly of heterochromatin-cohesion on this site. These results are consistent with the hypothesis that the alphoid DNA is a potential target for dsRNA-mediated suppression of transgene expression in vivo.

From these results we conclude that the transcriptionally active chromatin on the left arm of input-DNA conflicts with de novo assembly of heterochromatin-cohesion on this site. Therefore, these chromosomes were not maintained stably only by forming a de novo centromere-chromatin structure, assembled on the inserted alphoid DNA (Fig. 6 model B). Thus, de novo HAC formation might arise from a delicate balance on the introduced naked alphoid BAC-YAC DNA multimer between transcriptionally permissive chromatin for centromere assembly and heterochromatinization (Fig. 6 model A).

At this moment, we do not know how heterochromatin structure is assembled preferentially at the left vector arm. The promoter-less structure of the left arm limits chromatin opening at the site and its GC-rich (GC content of 52-57% and also CpG-rich) structure might be a good candidate for targets of CpG methylation and methylated CpG-binding proteins (Rhee et al., 2002; Fujita et al., 2003; Espada et al., 2004; Jorgensen et al., 2004). CpG-rich vectors derived from bacterial DNA cause suppression of transgene expression in mammalian cells (Hodges et al., 2004; Chen et al., 2004). The interplay between such suppressive elements and factors may cooperatively affect the assembly of heterochromatin at the left arm. Recently, HP1α and HP1γ were found to co-purify with the centromere component hMis12, suggesting that pericentromeric heterochromatin is possibly linked to centromere function (Obuse et al., 2004). We could not distinguish the difference between the alphoid DNA on the HAC and the alphoid DNA on the centromere of chromosome 21; we found that trimethyl histone H3-K9 and HP1α were slightly enriched also at the α21-L alphoid array (Fig. 4), suggesting that alphoid DNA, outside the centromere core region, is epigenetically inactivated and utilized as a part of pericentromeric heterochromatin. It has also been found that the maize centromere core repeat array (CentC) is transcribed and these transcripts are bound to centromere CENP-A chromatin (Topp et al., 2004). However, it remains unclear whether these transcripts play a role in centromere function and how the borders of the centromere and heterochromatin domains are defined epigenetically. Thus, further investigations are required to elucidate functional relationships between the primary DNA sequences, centromere chromatin, heterochromatin and the epigenetic modification correlating with transcriptional activity.

We thank K. Yoda (Nagoya University) for producing the anti-CENP-A antibody, N. Nozaki (Kanagawa Dental College) for producing anti-CENP-A and -B antibodies, Y. Yamashita for HP1α analysis, and V. Laronov, T. Lopez and T. Ebersole (NCI/NIH) for critical reading. This work was supported by a grant-in-aid for Scientific Research on Priority Areas (B) and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Science, Sports and Culture of Japan, a grant-in-aid from National Institute of Information and Communications Technology of Japan and a grant-in-aid from the Cell Science Research Foundation to H.M. H.N. was a recipient of a JSPS (Japan Society for the Promotion of Science) Pre-doctoral Research Fellowship. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, USA.

References
Aagaard, L., Schmid, M., Warburton, P. and Jenuwein, T. (2000). Mitotic phosphorylation of SUV39H1, a novel component of active centromeres, coincides with transient accumulation at mammalian centromeres. J. Cell Sci. 113, 817-829.
Ainsztein, A. M., Kandels-Lewis, S. E., Mackay, A. M. and Earnshaw, W. C. (1998). INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1. J. Cell Biol. 143, 1763-1774.
Ando, S., Yang, H., Nozaki, N., Okazaki, T. and Yoda, K. (2002). CENP-A, -B, and -C chromatin complex that contains the I-type alpha-satellite array constitutes the prekinetochore in HeLa cells. Mol. Cell. Biol. 22, 2229-2241.
Bernard, P., Maure, J. F., Partridge, J. F., Genier, S., Javerzat, J. P. and Albshire, R. C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science 294, 2539-2542.
Carmena, M. and Earnshaw, W. C. (2003). The cellular geography of aurora kinases. Nat. Rev. Mol. Cell. Biol. 4, 842-854.
Cheeseman, I. M., Niessen, S., Anderson, S., Hyndman, F., Yates, J. R., 3rd, Oegema, K. and Desai, A. (2004). A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. Genes Dev. 18, 2255-2268.
Chen, Z. Y., He, C. Y., Meuse, L. and Kay, M. A. (2004). Silencing of episonal transgene expression by plasmid bacterial DNA elements in vivo. Gene Ther. 11, 856-864.
Choo, K. H. (2001). Domain organization at the centromere and neocentromere. Dev. Cell 1, 165-177.
Cleveland, D. W., Mao, Y. and Sullivan, K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 112, 407-421.
Ebersole, T. A., Ross, A., Clark, E., McGill, N., Schindelhauer, D., Cooke, H. and Grimes, B. (2000). Mammalian artificial chromosome formation from circular alphoid input DNA does not require telomere repeats. Hum. Mol. Genet. 9, 1623-1631.
Espada, J., Ballesta, E., Fraga, M. F., Villar-Garea, A., Juarranz, A., Stockert, J. C., Robertson, K. D., Fuks, F. and Esteller, M. (2004). Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. J. Biol. Chem. 279, 37175-37184.
Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., Chiba, T. and Nakao, M. (2003). Methyl-CpG binding
domain 1 (MBD1) interacts with the Suv39h1-H1P1 heterochromatin complex for DNA methylation-based transcriptional repression. J. Biol. Chem. 278, 24132-24138.

Fukagawa, T., Nomaguchi, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. and Oshimura, M. (2004). Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. 6, 784-791.

Gassmann, R., Carvalho, A., Henzing, A. J., Ruchaud, S., Hudson, D. F., Honda, R., Nigg, E. A., Gerloff, D. L. and Earnshaw, W. C. (2004). Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J. Cell Biol. 166, 179-191.

Goshima, G., Kiyomitsu, T., Yoda, K. and Yanagida, M. (2003). Human centromere protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway. J. Cell Biol. 160, 25-39.

Grimes, B. R., Rhoades, A. A. and Willard, H. F. (2002). Alpha-satellite DNA and vector composition influence rates of human artificial chromosome formation. Mol. Ther. 5, 798-805.

Grimes, B. R., Babcock, J., Rudd, M. K., Chadwick, B. and Willard, H. F. (2004). Assembly and characterization of heterochromatin and euchromatin on human artificial chromosomes. Genome Biol. 5, R89.

Guenatri, M., Bailly, D., Maisonn, C. and Almouzni, G. (2004). Mouse centric and pericentric satellite repeats form distinct functional domains. J. Cell Biol. 166, 493-505.

Haaf, T. and Ward, D. C. (1994). Structural analysis of alpha-satellite DNA and centromere proteins using extended chromatin and chromosomes. Hum. Mol. Genet. 3, 697-709.

Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A. and Grewal, S. I. (2002). Establishment and maintenance of a heterochromatin domain. Science 297, 2322-2327.

Harrington, J. J., Van Bokkelen, G., Mays, R. W., Gustashaw, K. and Willard, H. F. (1997). Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. Nat. Genet. 15, 345-355.

Hayakawa, T., Haraguchi, T., Masumoto, H. and Hiraoka, Y. (2003). Cell cycle behavior of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. J. Cell Sci. 116, 3327-3338.

Heller, R., Brown, K. E., Burgdorf, C. and Brown, W. R. (1996). Mini-chromosomes derived from the human Y chromosome by telomere directed chromosome breakage. Proc. Natl. Acad. Sci. USA 93, 7125-7130.

Henikoff, S., Ahmad, K. and Malik, H. S. (2001). The centromere paradox: stable inheritance with rapidly evolving DNA. Science 293, 1098-1102.

Henning, K. A., Novotny, E. A., Compton, S. T., Guan, X. Y., Liu, P. P. and Ashlock, M. A. (1999). Human artificial chromosomes generated by modification of a yeast artificial chromosome containing both human alpha satellite and single-copy DNA sequences. Proc. Natl. Acad. Sci. USA 96, 592-597.

Hodges, B. L., Taylor, K. M., Joseph, M. F., Bourgeois, S. A. and Scheule, R. D. (2004). Long-term transgene expression from plasmid DNA gene therapy vectors is negatively affected by CpG dinucleotides. J. Cell Biol. 160, R89.

Ikeno, M., Masumoto, H. and Hiraoka, Y. (1999). Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. Exp. Cell Res. 181, 181-196.

Ikeno, M., Nakano, M., Okazaki, T. and Koide, S. (1989a). Alphoid satellite DNA interacts with the Suv39h1-HP1 heterochromatic protein complex. J. Cell Biol. 109, 1963-1973.

Ikeno, M., Ikeno, M., Nakano, M., Okazaki, T., Grimes, B., Cooke, H. and Suzuki, N. (1998). Assay of centromere function using a human artificial chromosome. Chromosoma 107, 406-416.

Ikeno, M., Nakano, M. and Ohezki, J. (2004). The role of CENP-B and alpha-satellite DNA: de novo assembly and epigenetic maintenance of human centromeres. Chromosome Res. 12, 543-556.

Jenuwein, T., Livingston, D. M. and Rajewsky, K. (2001). Partitioning and plasticity of repressive histone methylation domains in mammalian chromatin. Mol. Cell 12, 1209-1226.

Kielczewski, F., Malik, K. C., Pikaart, M. J., Burgess-Beusse, B., Bell, A. C., Litt, M. D., West, A. G., Gromer, S. and Lengauer, C. (2002). Position-effect protection and enhancer blocking by the beta-cell-globin insulator is separable activities. Proc. Natl. Acad. Sci. USA 99, 6883-6888.

Kohlmaier, A. et al. (2003). Partitioning and plasticity of repressive histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell 107, 323-337.

Koshima, K., Ikeda, K., Baillie, D., Peters, A. H., Quivy, J. P., Roche, D., Taddei, A., Lachner, M., Jenuwein, T. and Almouzni, G. (2002). Higher-order specific pericentric euchromatin in somatic cells involves a distinct pattern of histone modification and an RNA component. Nat. Genet. 30, 329-334.

Kuhn, A. L., Ganesan, S., Drapkin, R., Lachner, M., Jenuwein, T. and Almouzni, G. (2002). Higher-order specific pericentric euchromatin in somatic cells involves a distinct pattern of histone modification and an RNA component. Nat. Genet. 30, 329-334.

Lachner, M., Jenuwein, T. and Almouzni, G. (2002). Higher-order specific pericentric euchromatin in somatic cells involves a distinct pattern of histone modification and an RNA component. Nat. Genet. 30, 329-334.
at kinetochores is uncoupled from DNA replication. *J. Cell Biol.* **151**, 1113-1118.

Sonoda, E., Matsusaka, T., Morrison, C., Vagnarelli, P., Hoshi, O., Ushiki, T., Nojima, K., Fukagawa, T., Waizenegger, I. C., Peters, J. M. et al. (2001). Sccl/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. *Dev. Cell* **1**, 759-770.

Sullivan, B. A. and Karpen, G. H. (2004). Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nat. Struct. Mol. Biol.* **11**, 1076-1083.

Sullivan, B. A., Blower, M. D. and Karpen, G. H. (2001). Determining centromere identity: cyclical stories and forking paths. *Nat. Rev. Genet.* **2**, 584-596.

Sullivan, K. F., Hechenberger, M. and Masri, K. (1994). Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J. Cell Biol.* **127**, 581-592.

Topp, C. N., Zhong, C. X. and Dawe, R. K. (2004). Centromere-encoded RNAs are integral components of the maize kinetochore. *Proc. Natl. Acad. Sci. USA* **101**, 15986-15991.

Vagnarelli, P. B. and Earnshaw, W. C. (2001). INCENP loss from an inactive centromere correlates with the loss of sister chromatid cohesion. *Chromosoma* **110**, 393-401.

Valdeolmillos, A., Rufas, J. S., Suja, J. A., Vass, S., Heck, M. M., Martinez, A. C. and Barbero, J. L. (2004). Drosophila cohesins DSA1 and Drah21 persist and colocalize along the centromeric heterochromatin during mitosis. *Biol. Cell* **96**, 457-462.

Van Hooser, A. A., Ouspenski, I. I., Gregson, H. C., Starr, D. A., Yen, T. J., Goldberg, M. L., Yokomori, K., Earnshaw, W. C., Sullivan, K. F. and Brinkley, B. R. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. *J. Cell Sci.* **114**, 3529-3542.

Vass, S., Cotterill, S., Valdeolmillos, A. M., Barbero, J. L., Lin, E., Warren, W. D. and Heck, M. M. (2003). Depletion of Drad21/Sccl in Drosophila cells leads to instability of the cohesin complex and disruption of mitotic progression. *Curr. Biol.* **13**, 208-218.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. and Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833-1837.

Willard, H. F. and Waye, J. S. (1987). Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet.* **3**, 192-198.

Zhao, H. and Dean, A. (2004). An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene. *Nucleic Acids Res.* **32**, 4903-4919.

Zinkowski, R. P., Meyne, J. and Brinkley, B. R. (1991). The centromere-kinetochore complex: a repeat subunit model. *J. Cell Biol.* **113**, 1091-1110.