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Unusual chromosome architecture and behaviour at an HSR

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Abstract. Amplification of sequences within mammalian chromosomes is often accompanied by the formation of homogeneously staining regions (HSRs). The arrangement of DNA sequences within such amplicons has been investigated, but little is known about the chromosome structure or behaviour of these unusual regions. We have analysed the metaphase chromosome structure of the dihydrofolate reductase (DHFR) amplicon of CHOC400 cells. The chromatin in this region contains hyperacetylated nucleosomes yet, at the same time, appears to be densely packed like heterochromatin. The region does not bind heterochromatin proteins. We show that the dense packing of the region is restricted to DNA located close to the chromosome core/scaffold. In contrast, levels of the chromosome scaffold protein topoisomerase II at HSRs are the same as those found at other euchromatic locations. Metaphase chromosome condensation of the HSR is shown to be sensitive to topoisomerase II inhibitors, and sister chromatids often appear to remain attached within the HSRs at metaphase. We suggest that these features underlie anaphase bridging and the aberrant interphase structure of the HSR. The DHFR amplicon is widely used as a model system to study mammalian DNA replication. We conclude that the higher-order chromosome structure of this amplicon is unusual and suggest that caution needs to be exercised in extrapolating data from HSRs to normal chromosomal loci.

Introduction

At amplified dihydrofolate reductase (DHFR) loci in CHOC400 cells, 243 kb of DNA containing the DHFR gene has been amplified to ~1000 copies at several sites (Milbrandt et al. 1981; Hamlin et al. 1994). The resulting homogeneously staining regions (HSRs) contradict normal cytogenetic patterns by combining some superficial features of active chromatin with those of heterochromatin. They stain brightly with 4′,6-diamino-2-phenylindole (DAPI) and appear to be highly condensed. While these are properties usually equated with transcriptionally silent, late-replicating heterochromatin, the DHFR locus is actively transcribed and replicates early in S-phase of CHOC400 cells (Dijkwel and Hamlin 1992).

Since the DHFR amplicon is used as a model system for the study of chromosomal DNA replication, a process contingent upon chromosome structure, it is important to understand the basis for the unusual cytological appearance of the DHFR HSR. Here we show that the basic chromatin structure of the HSR is typical of that expected of a transcriptionally active locus but that aspects of the higher levels of chromosome architecture are unusual.

By swelling and extracting metaphase chromosomes, we show that there is an intensity of DAPI staining close to the axial chromosome core at HSRs, which more closely resembles that of the centromeric regions than that of other regions of the chromosome arms. In contrast, the levels of topoisomerase II at the HSRs are indistinguishable from the rest of the chromosome arms and lower than levels at the centromeres. We suggest that there might be an imbalance between the concentrations of DNA and topoisomerase II within the metaphase structure of HSRs. Since topoisomerase II activity is essential for correct chromosome condensation and segregation, this might compromise the behaviour of the HSR during mitosis. Indeed, we find that inhibiting topoisomerase II perturbs mitotic condensation at the HSR more readily than at other regions of the CHO genome. We also see aberrant association of sister chromatids at the HSRs and a high level of lagging chromosome regions carrying HSRs at anaphase. These results suggest that chromosome condensation and/or chromatid cohesion at

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the DHFR amplicon is atypical. We discuss how these features of HSR chromosome structure could contribute to the generation of amplicon structure, and suggest that HSRs should not be considered as general models for mammalian chromosome structure/behaviour.

Materials and methods

Cell culture

The CHOC400 cells were grown in DMEM supplemented with nonessential amino acids (GIBCO/BRL), 5% fetal calf serum at 37°C and were periodically exposed to selection for DHFR with 1 mg/ml methotrexate. To isolate metaphase chromosomes, subconfluent flasks of CHOC400 cells were incubated with Colcemid (0.1 µg/ml) for 16 h and harvested by mitotic shake-off. For anaphase/telophase chromosomes, mitotic cells, obtained by shake-off, were incubated at 37°C in prewarmed medium for 5 to 15 min before harvest. To inhibit topoisomerase II activity, cells were incubated for 1 h with 0.1 µg/ml Colcemid together with 0, 50 or 100 µm amsacrine (AMSA) before harvest (Sumner 1995). Cells were swollen in 8 mM trisodium citrate, 33 mM KCl before fixation in 3:1 methanol:acetic acid (MAA).

Metaphase chromosome isolation, swelling and extraction

Isolated mitotic chromosomes were prepared by a modification of the method detailed in Bickmore and Oghene (1996). Cells were harvested, washed in phosphate-buffered saline (PBS) and resuspended at 2×10^6 cells/ml in hypotonic buffer for 10 min. Cells were then resuspended in cold polyamine (PA) buffer (15 mM TRIS-HCl, 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.1 mM $CuSO_4$, pH 7.2) at 8×10^6 cells/ml. After centrifugation at 260 *g* for 5 min, the pellet was resuspended at 10^7 cells/ml in cold PA buffer containing 1 mg/ml digitonin, and vortexed for 30 s to release the mitotic chromosomes. Nuclei were spun out at 260 *g* for 5 min and the supernatant was layered onto a 30%–60% sucrose step gradient made up in: 5 mM PIPES, pH 7.2, 5 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA and spun at 4000 *g* for 15 min at 4°C. Chromosomes were collected at the 40%–50% and 50%–60% interfaces and stored at –70°C.

Chromosomes were settled onto slides, then gently immersed for 10 min in extraction buffer EB (10 mM TRIS-HCl, 10 mM EDTA, 0.1% Nonidet P40, 20 µg/ml phenylmethylsulphonyl fluoride, 1 µg/ml aprotonin, 0.1 mM $CuSO_4$, pH 8.0) (Paulson and Laemmli 1977). Slides were then moved to baths of EB containing increasing concentrations of NaCl (0.5 and 1.0 M). After swelling and extraction, slides were fixed twice in MAA.

Immunofluorescence and fluorescence in situ hybridisation (FISH)

Unfixed metaphase spreads were obtained as described previously (Sullivan and Schwartz 1995). Metaphase chromosomes were obtained by mitotic shake-off, swollen in 75 mM KCl, or 66 mM KCl, 8 mM sodium citrate, for 15 min and 8×10^4 cells/ml were centrifuged at high acceleration on to SuperFrost/Plus slides (Life Science International) using a Shandon Cytospin 3 for 10 min at 2000 rpm. For immunofluorescence with monoclonal antibody IF6, directed against topoisomerase IIα (anti-topo IIα), slides were placed in –20°C methanol for 20 min, air-dried and rehydrated in PBS, 0.1% BSA (PBSA) (Gimenez-Abian et al. 1995; Sumner 1996). For polyclonal serum R41 recognising acetylated lysine 5 of histone H4 (Keohane et al. 1996), for monoclonal antibody 353 recognising M31 (Wreggett et al. 1994), and for antisera 670 and 674 recognising MeCP2 (Nan et al. 1998), cells were swollen in KCM (120 mM KCl, 20 mM NaCl, 10 mM TRIS-HCl, pH 8.0, 0.5 mM EDTA, 0.1% Triton X100) at room temperature for 10 min prior to addition of primary antibodies. Anti-mouse, anti-rat, and anti-rabbit secondary antibodies conjugated with fluorescein or Texas Red were diluted at 1:200 in PBSA or KCM. Slides subsequently used for FISH were soaked in PBS or KCM to remove coverslips then fixed and processed as previously described (Sullivan and Schwartz 1995).

Twenty-five nanograms each of DHFR cosmids (Looney and Hamlin 1987), labelled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP, were used per hybridisation. Signal from repetitive DNA was suppressed with hamster CotI DNA. Hybridisation and detection were as previously described (Bickmore and Oghene 1996).

Image analysis

Slides were counterstained with 2 µg/ml DAPI and examined on an epifluorescence microscope using a Chroma 81000 filter set. Images were captured with a Photometrics cooled CCD camera and analysed with Smartcapture software supplied by Digital Scientific/Vysis.

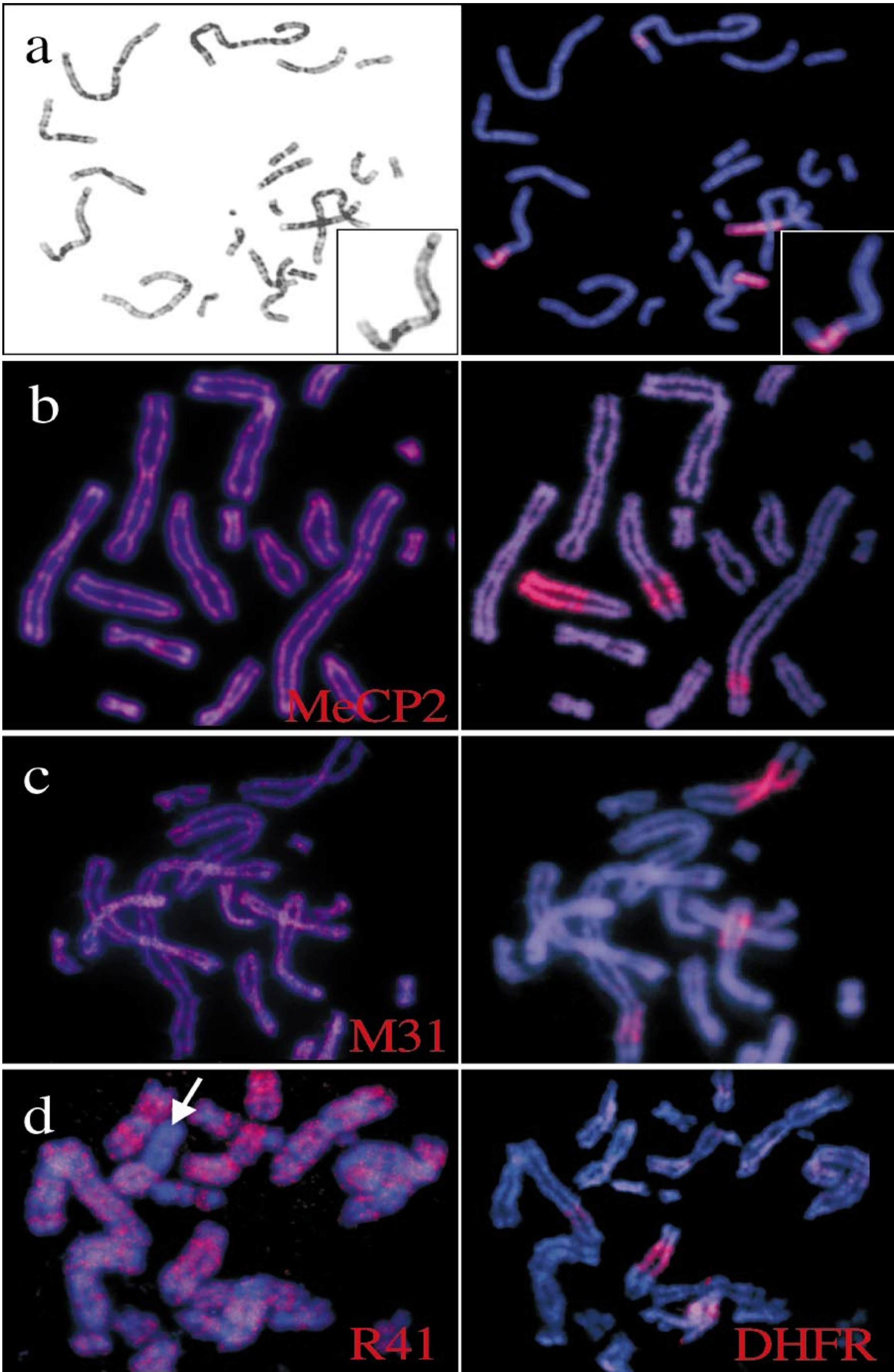
Results

Cytogenetics of the DHFR amplicon

The DHFR amplicons in CHOC400 cells are distributed between a major locus in a marker chromosome (that may have derived from chromosome 6) and two minor loci, distal 1q and the long arm of a derivative of chromosome 4 (Milbrandt et al. 1981; Trask and Hamlin 1989). In some cells we also detect an additional locus on a small chromosome that is almost entirely composed of amplified sequences. All of these HSRs stain intensely with DAPI (Fig. 1a).

Other DAPI-bright areas of the CHO genome include the heterochromatic parts of the X chromosome and the centromeres of CHO chromosomes (Ray and Mohandas 1976). CHOC400 chromosomes were analysed by immunofluorescence with antibodies against MeCP2 and M31, two proteins that concentrate in the heterochromatic regions of rodent chromosomes (Wreggett et al. 1994)

Fig. 1a–d. Cytogenetic analysis of dihydrofolate reductase (DHFR) homogeneously staining regions (HSRs). **a** Hybridisation of CHOC400 chromosomes with DHFR probes (*red*). *Left-hand panel* shows 4′,6-diamidino-2-phenylindole (DAPI) staining in black and white. *Right-hand panel* shows hybridisation signals on DAPI-stained (*blue*) chromosomes. *Insets to right* show a single HSR-bearing acrocentric chromosome at higher magnification. The intense DAPI staining of centromeric heterochromatin and the HSRs can be seen. **b–d** Immunofluorescence analysis of CHOC400 chromosomes with antisera directed against: **b** the methyl-CpG binding protein MeCP2, **c** HP1 homologue M31, and **d** acetyl-lysine 5 of histone H4. In the *left-hand panels*, antibody signals are pseudo-coloured and shown in *red*, on DAPI-stained chromosomes (*blue*). *Right-hand panels* show subsequent fluorescence in situ hybridisation (FISH) analysis with DHFR probes (*red*) to identify the HSRs. Note the absence of anti-acetyl-lysine 5 staining at the centromeres and the long arm of the X chromosome (*arrowed*) in **d** (Arrighi et al. 1974)


a
b
MeCP2
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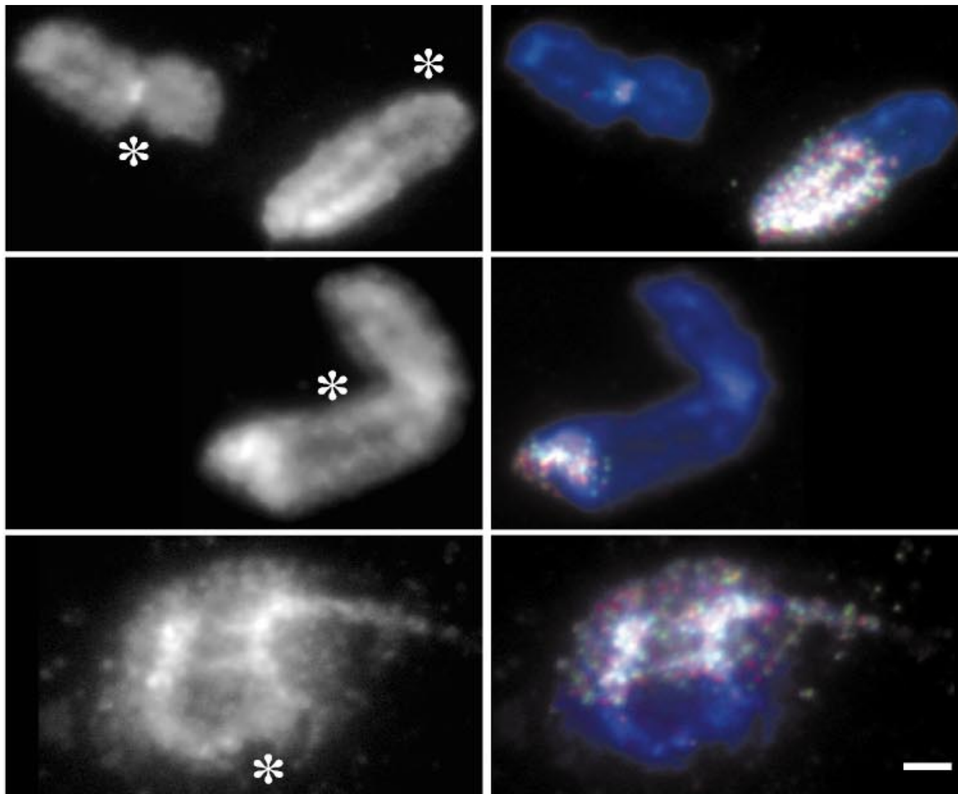
R41
DHFR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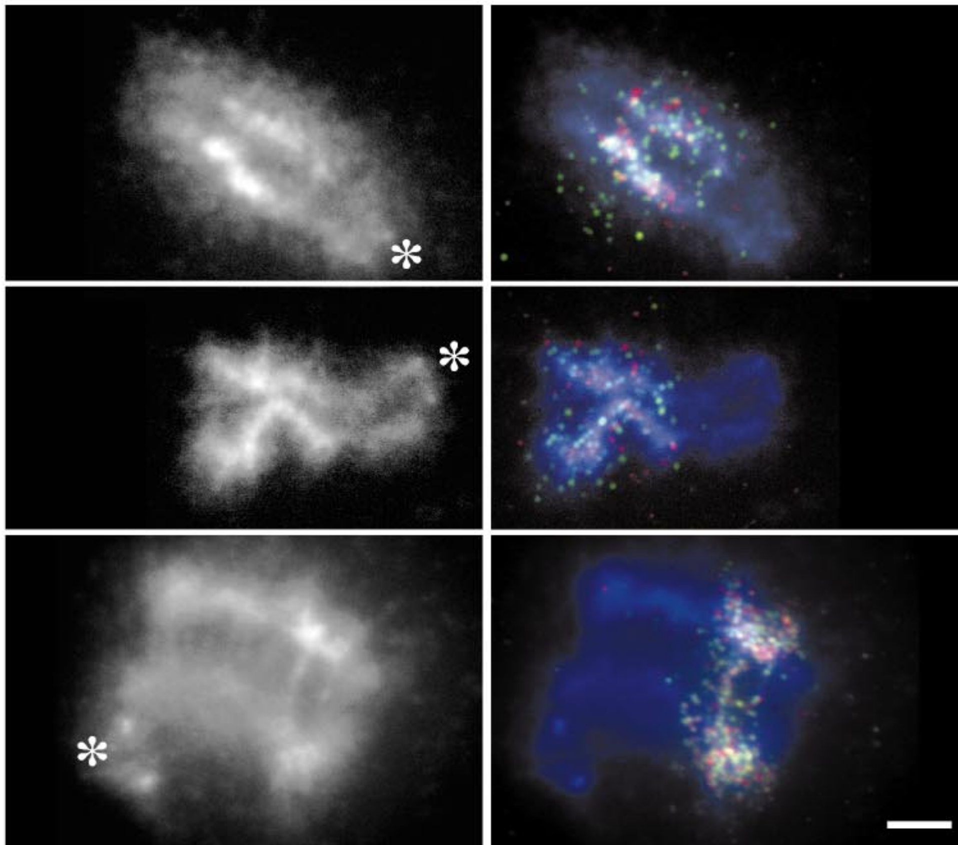
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and also at other sites (Nan et al. 1997). The MeCP2 signal was concentrated over the centromeric, and some telomeric, regions of the CHOC400 karyotype; signal was also seen over other interstitial sites (Fig. 1b). However, FISH revealed that there was no concentration of MeCP2 at HSRs. The M31 signal was more generally distributed over chromosome arms (Fig. 1c), but again there was no specific concentration over HSRs.

The DAPI-bright regions of mammalian chromosomes generally lack staining with antibodies against histone H4 acetylated at lysine 5 (Keohane et al. 1996). In contrast, combined FISH and immunofluorescence showed that the DHFR amplicons were labelled at least as strongly by anti-acetyl lysine 5 as other euchromatic regions of the karyotype (Fig. 1d). We conclude that the HSRs in CHO400 cells are packed into a tight structure that reflects aspects of higher-order chromosome architecture, since at the chromatin level the region is not hypoacetylated and is not associated with heterochromatin proteins.

DNA and topoisomerase II within the core of HSRs

Because of the level of condensation within metaphase chromosomes, it is hard to unravel details of their structure. However, swelling and extracting metaphase chromosomes reveals some aspects of their internal organisation, specifically the central chromosome axial core surrounded by a halo of DNA loops (Bickmore and Oghene 1996). When extracted CHOC400 chromosomes were hybridised to DHFR probes (Fig. 2), axial cores at the HSRs were more intensely stained with DAPI than other places on the chromosome arms and even appeared to exceed the level of staining at centromeres (marked by asterisks in Fig. 2). The intensity of DNA staining at HSRs, centromeres and chromosome arms was compared along 5 pixel width lines along the chromosome core and 1–2 μm away in the surrounding halo on chromosomes extracted with 0.5, 0.8 and 1 M NaCl. At these respective salt concentrations, staining of the core was 2.3±0.4, 1.9±0.2 and 1.8±0.3 times more intense at the HSRs than in the chromosome arms (n=20), while in the surrounding halo it was not elevated to the same extent (1.5±0.2, 1.3±0.1, 1.2±0.1). At all salt concentrations, the DAPI staining of the chromosome core of HSRs was even 1.5±0.1 times more intense than at the centromere. These data suggest that metaphase structure within HSRs is unusual and fundamentally different from that of normal euchromatic regions.

Topoisomerase IIα is a major scaffold protein located at the axial core of metaphase chromosomes (Earnshaw and Heck 1985; Adachi et al. 1989). It is most concentrated at centromeric heterochromatin and at other heterochromatic sites of mammalian chromosomes (Rattner et al. 1996; Sumner 1996). Immunofluorescence of CHOC400 chromosomes with an antibody against topoisomerase IIα (Gimenez-Abian et al. 1995), together with FISH for DHFR probes, revealed levels of topoisomerase IIα at HSRs that are indistinguishable from those along the rest of the chromosome arms (Fig. 3a). However, topoisomerase II levels at HSRs are lower than those at centromeres.

Chromosome condensation at HSRs is sensitive to topoisomerase II inhibitors

Topoisomerase II activity is required for metaphase chromosome condensation (Downes et al. 1994). An imbalance between the concentration of enzyme and substrate DNA within HSRs may present problems in chromosome condensation. Abnormal chromosome condensation occurs in the presence of topoisomerase II inhibitors (Downes et al. 1994). In one-quarter of CHOC400 metaphase cells (n=31) formed in the presence of 50 or 100 μm AMSA, some chromosomes of the karyotype had either a pulverised appearance or regions of thin and extended chromatin. Hybridisation with DHFR probes confirmed that such sites were most often the amplicons (Fig. 3b). Hence, mitotic condensation at HSRs appears to be more sensitive to the inhibition of topoisomerase II than other regions of the CHOC400 genome.

Inappropriate sister chromatid cohesion and chromosome segregation at HSRs

Topoisomerase II activity is also required to decatenate sister chromatids after DNA replication to prevent them being torn apart in anaphase (DiNardo et al. 1984; Uemura et al. 1987; Shamu and Murray 1992; Buchenau et al. 1993; Sumner 1995). Insufficient topoisomerase II activity may result in incomplete decatenation of sister chromatids within the HSRs and this, in turn, may manifest as anaphase bridging (Gimenez-Abian et al. 1995).

In extracted CHOC400 chromosomes we noted that, although there were distinct sister chromatid cores at sites of HSRs, there was also often apparent adhesion between the axial cores at these sites (middle panels of Fig. 2). In more extreme cases, there were prominent strands of DHFR-hybridising DNA connecting sister chromatids (bottom panels of Fig. 2). Such close association of sister chromatid cores is usually only seen at centromeres (asterisks in Fig. 2) (Bickmore and Oghene 1996).

Fig. 2. Morphology of the axial cores in CHOC400 chromosomes. CHOC400 metaphase chromosomes extracted with 0.5 M or 1 M NaCl and hybridised to probes from the amplicon. To the *left*, DAPI staining is shown in *black and white* with *asterisks* highlighting the location of the centromeres. On the *right*, DAPI staining is shown in *blue* and hybridisation signals of probes from the DHFR amplicon are in *green* and *red*. Note the more intense DAPI staining of the axial core at the site of the DHFR locus, as compared with other chromosomal regions. The surrounding halos are of near normal DAPI-staining intensity. In some chromosomes (*centre rows*) association between the sister chromatids persists at the site of amplification. In some amplicons (*bottom rows*), there are visible interconnecting strands of hybridising DNA between sister chromatids. *Bar* represents 2 μm

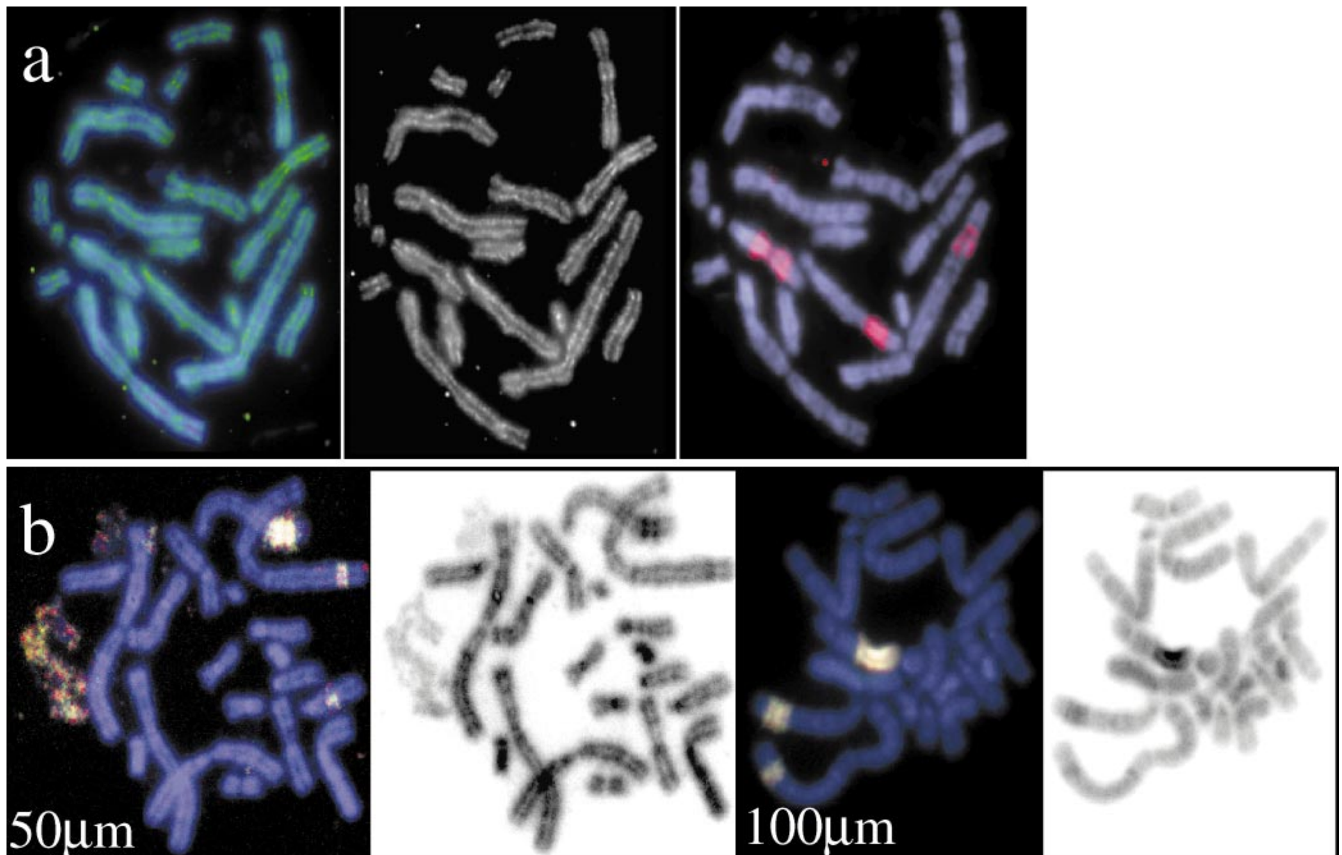

Fig. 3a, b. Activity and localisation of topoisomerase IIα at HSRs. **a** Immunofluorescence of CHOC400 chromosomes with anti-topoisomerase IIα. On the *left*, antibody staining (*green*) is shown running along the axes of chromosomes counter-stained with DAPI (*blue*). In the *central panel*, only the antibody signal is shown. To the *right*, the position of HSRs is shown byhybridisation to DHFR cosmids (*red*) after immunofluorescence. **b** CHOC400 metaphase chromosomes condensed in the presence of 50 and 100 μm amsacrine (AMSA). At each concentration, the DAPI staining of the chromosomes is shown in *black and white* on the *right*. On the *left*, FISH signals of probes (*red* and *green*) from the amplified unit are shown on chromosomes counter-stained with DAPI (*blue*)

We also observed that ~10% of anaphase CHOC400 cells had amplified regions lagging towards the mid-region of dividing chromosome masses (Fig. 4a). A failure of these lagging regions to become correctly incorporated into the forming nucleus is supported by the presence of telophase cells with interconnecting strands of DHFR-hybridising DNA (Fig. 4b). The subsequent fate of lagging HSRs may vary. They may become broken off and indeed micronuclei that contain material hybridising to DHFR probes are seen in CHOC400 cultures (Fig. 4c) (Toledo et al. 1992). The organisation of DHFR amplicons that do become incorporated into apparently intact interphase nuclei is also unusual. Hybridisation signal is often seen within bulges or blebs protruding from the nucleus (Fig. 4d). Such blebs may be precursors to micronucleus formation (Miele et al. 1989), as has also been suggested for amplified sequences present in double minutes (DMs) (Shimizu et al. 1996, 1998). Acentric DMs appear to sort to the nuclear periphery during S-phase, and are then selectively eliminated from the nucleus by micronucleation (Shimizu et al. 1998). Nuclear blebs at other HSRs have been suggested to result from a destabilisation of nuclear organisation and chromatid breakage (Miele et al. 1989; Toledo et al. 1992). However, we suggest that in CHOC400 cells blebs and micronuclei arise as cells exit mitosis and form a new nuclear membrane around lagging chromosomes that carry HSRs.

Discussion

The HSRs of CHOC400 cells are compact and stain intensely with DAPI (Fig. 1a), characteristics normally associated with condensed and transcriptionally silent heterochromatin. However, DHFR genes within the HSRs are transcribed, since the cells grow under strong selection for DHFR expression, and the HSR DNA replicates early in S-phase (Dijkwel and Hamlin 1992). The presence of active genes and early replication are two characteristics of R-band regions of mammalian chromosomes. R-bands stain poorly with DAPI, have a less condensed chromatin structure than G- or C-bands (Yokota et al. 1997), and are associated with hyperacetylated histones (Jeppessen and Turner 1993). Unlike other DAPI-bright areas of the CHO karyotype, we have shown that

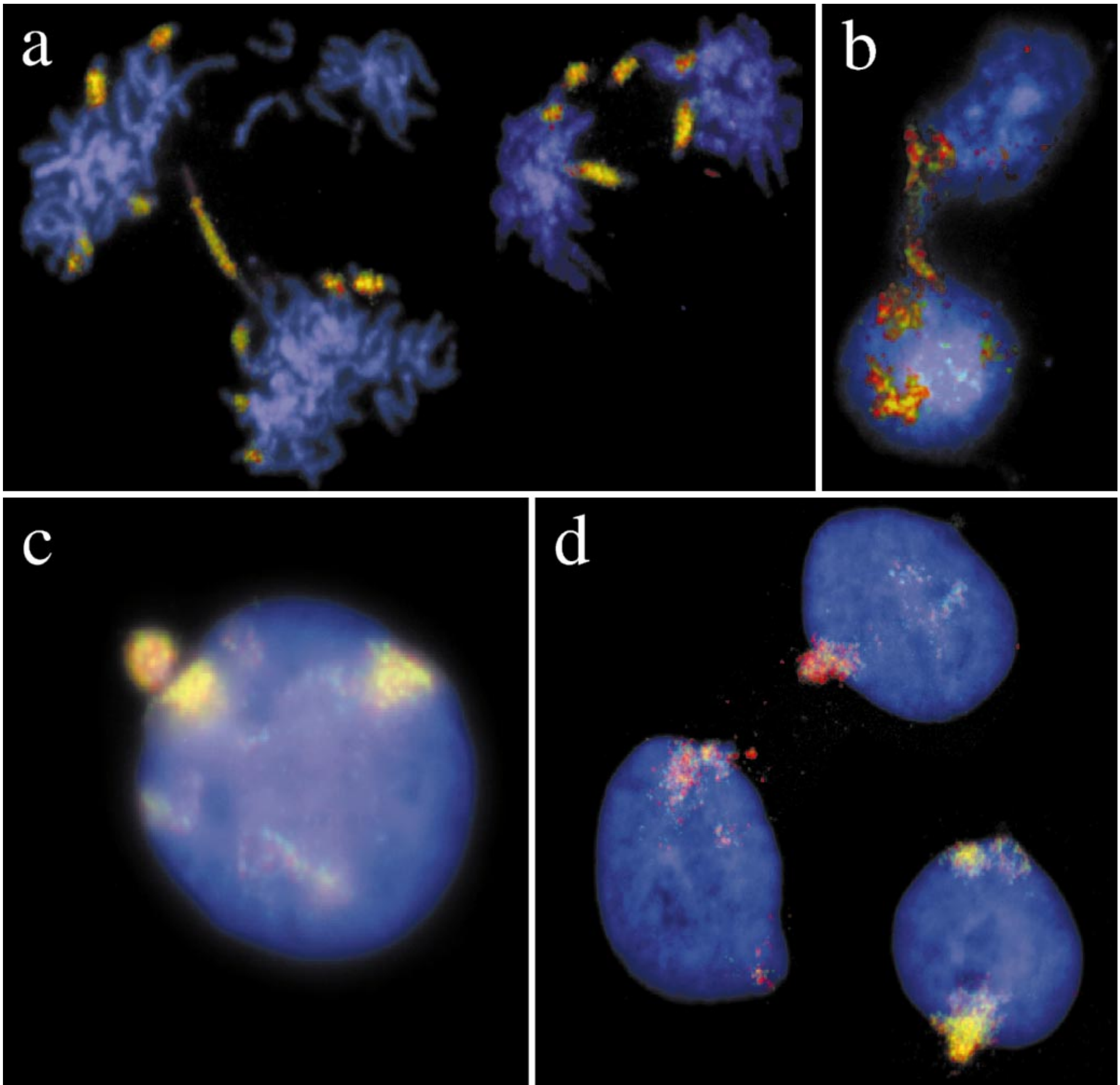

Fig. 4a–d. Anaphase bridges, micronuclei and nuclear blebs at the DHFR HSR. **a** Two CHOC400 anaphase figures with anaphase bridges involving amplicons. Chromosomes were stained with DAPI (*blue*) and hybridised with DHFR cosmids (*green* and *red*). **b** Telophase cells treated as in **a**. Most of the CHO chromosomes are decondensing and forming into separate daughter nuclei; however, strands of DNA hybridising to DHFR probes remain connecting the two chromosome sets. **c** Micronucleus hybridising to DHFR probes (*green* and *red*), adjacent to an interphase nucleus. **d** CHOC400 nuclei (*blue*) hybridised with DHFR probes (*green* and *red*). Deformities/ protrusions from the nuclear periphery contain DNA from the DHFR amplicon

the CHOC400 HSRs are also packaged into acetylated nucleosomes (Fig. 1d), and they do not preferentially bind heterochromatin proteins (Fig. 1b, c). Therefore at the most basic levels of chromatin packaging and condensation, the HSRs of CHOC400 cells have typical features of active genomic regions, i.e they contain transcribed genes, replicate early, and have acetylated histones. We suggest that intense DAPI staining of these regions reflects aspects of their higher-order chromosome architecture.

In support of this, we have demonstrated unusually dense packing of DNA within HSRs close to the axial core of the metaphase chromosome (Fig. 2). We show that this elevated DNA concentration, in the vicinity of the axial core, is not accompanied by a corresponding increase in the levels of topoisomerase IIα detectable by

immunofluorescence (Fig. 3a). Correct mitotic condensation of HSRs is readily perturbed by AMSA, an inhibitor of topoisomerase II enzymatic activity (Fig. 3b). We suggest that an inappropriate higher-order chromosome structure results in problems in the decatenation and/or the release of sister chromatid attachments at HSRs (Figs. 2, 4) (Biggins and Murray 1998). This may manifest as anaphase bridging (Fig. 4a, b) and as HSR-containing micronuclei and interphase nuclear blebs (Fig. 4c, d).

Breaks at fragile sites are proposed to initiate sister chromatid fusion, leading to breakage-fusion-bridge (BFB) cycles at early stages of amplification (Trask and Hamlin 1989; Ma et al. 1993; Coquelle et al. 1997). Continuing rounds of BFB may be perpetuated through the inappropriate attachments between sister chromatids and the consequent anaphase bridging that we have reported (Fig. 4) (Miele et al. 1989; Warburton and Cooke 1997).

The CHOC400 DHFR amplicon has been used to analyse the factors that influence replication origin choice. In vivo, cleavage sites of metaphase scaffold-associated toposiomerase II co-map with known origins of replication (Razin et al. 1993) and some other mammalian replication origins are located in the vicinity of the chromosome core (Bickmore and Oghene 1996). It has been suggested that higher-order levels of chromosome folding may have a role in restricting the position of potential origins of replication in the vertebrate genome (De Pamphilis 1999). Our findings, presented here, question how appropriate the CHOC400 DHFR amplicon is as a general model for normal chromosome structure and function. We suggest that extrapolation of conclusions gained from studying the CHOC400 HSR to normal chromosomal regions must be exercised with caution. It is possible that the unusual structure of the DHFR HSR that we have described is inherent in the organisation of the single-copy DHFR locus from which it arose, but which is not amenable to analysis at the cytological level because of its small size. It will therefore be important to analyse these levels of chromosome structure and behaviour for other HRSs/amplicons, including those generated experimentally for the explicit purpose of studying chromosome structure and dynamics (Li et al. 1998).

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