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Original investigations

Specific staining of human chromosomes in Chinese hamster × man hybrid cell lines demonstrates interphase chromosome territories

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Summary. In spite of Carl Rabl's (1885) and Theodor Boveri's (1909) early hypothesis that chromosomes occupy discrete territories or domains within the interphase nucleus, evidence in favor pf this hypothesis has been limited and indirect so far in higher plants and animals. The alternative possibility that the chromatin fiber of single chromosomes might be extended throughout the major part of even the whole interphase nucleus has been considered for many years. In the latter case, chromosomes would only exist as discrete chromatin bodies during mitosis but not during interphase. Both possibilities are compatible with Boveri's well established paradigm of chromosome individuality. Here we show that an active human X chromosome contained as the only human chromosome in a Chinese hamster × man hybrid cell line can be visualized both in metaphse plates and in interphase nuclei after in situ hybridization with either ^{3}H- or biotin-labeled human genomic DNA. We demonstrate that this chromosome is organized as a distinct chromatin body throughout interphase. In addition, evidence for the territorial organization of human chromosomes is also presented for another hybrid cell line containing several autosomes and the human X chromosome. These findings are discussed in the context of our present knowledge of the organization and topography of interphase chromosomes. General applications of a strategy aimed at specific staining of individual chromosomes in experimental and clinical cytogenetics are briefly considered.

Introduction

The hypothesis that interphase chromosomes are not diffusely extended throughout the interphase nucleus but occupy rather compact territories has first been put forward in the classical papers of Rabl (1885) and Boveri (1909). While the chromosomes of some algae and protozoa remain distinctly visible during interphase (DuPraw 1970; Grell 1973), evidence for this hypothesis in higher plants and animals has remained limited and mainly indirect (Stack et al. 1977; Murray and Davies 1979). So far a direct observation of interphase chromosome territories has been limited to interphase polytene nuclei (Sedat and Manuelidis 1978; Agard and Sedat 1983). In fact, Wischnitzer (1973) reviewing the scanty results of electron microscopy studies came to the opposite conclusion that these studies had "established that discrete interphase chromosomes are absent". The reasons for this uncertainty are twofold. First, the total length of the DNA molecules which have to be compacted within a diploid mammalian nucleus of some 10 to 20 μm diameter is roughly 2×10^{6} μm. Even when this DNA is packed to a thick chromatin fiber (Finch and Klug 1976; Hozier et al. 1977) assuming a packaging factor of 25 to 40-fold each individual chromosome is composed of a chromatin fiber of several hundred to several thousand μm. Obviously, models ranging from very compact interphase chromosome domains to a dispersed arrangement of individual chromosomes throughout the whole interphase nucleus (e.g. Comings 1968; Vogel and Schroeder 1974) are compatible with these data. Second, the methodology to recognize the distribution of euchromatic regions of *uncondensed* individual chromosomes unequivocally during interphase was lacking so far.

Recent evidence for interphase chromosome territories stems from laser-UV-microirradiation experiments. Small subnuclear regions of fibroblastoid Chinese hamster cells were subjected to laser-UV-microirradiation in G1 and pulse-labeled with ^{3}H-thymidine in order to detect unscheduled DNA synthesis (UDS) in the microirradiated chromatin (Zorn et al. 1979; Cremer et al. 1982). When these cells reached the subsequent mitosis, chromosome preparations were made in situ and chromosomes were investigated for UDS-labeling after autoradiography. In all cases label was found concentrated on a few chromosomes (Zorn et al. 1979; Cremer et al. 1982). The same result was obtained when a small site of the interphase nucleus was microirradiated at S-phase and the microirradiated chromatin was visualized in the subsequent metaphase by indirect immunofluorescence microscopy using antibodies specific for UV-irradiated DNA (Hens et al. 1983). Recently it has been demonstrated that the number of chromosomes in which sister chromatid exchanges (SCEs) can be induced by laser-UV-microirradiation depends on the size of the nuclear area subjected to the laser-UV-microbeam (Raith et al. 1984).

These data strongly support the concept of interphase chromosome territories in cultivated Chinese hamster cells. Here, we show that human chromosomes can be visualized directly both in mitotic cells and interphase nuclei of Chinese hamster × man hybrid cells after in situ hybridization either with ^{3}H- or biotin-labeled genomic human DNA, and demonstrate that human chromosomes in hybrid cell nuclei are organized in distinct domains throughout the whole cell cycle.

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Materials and methods

Cell culture

The Chinese hamster × man hybrid cell line 29-11B containing an active human X chromosome as the only free human chromosome was kindly provided by Dr. Uta Francke (Yale). This cell line was cultivated in HAT-medium as described by Littlefield (1964). The Chinese hamster × man hybrid cell line A1 Wbf2 containing the human chromosomes 11, 17, and X as free chromosomes, was kindly provided by Dr. P. Pearson (Leiden) and cultivated in minimal essential medium (MEM) with 10% fetal calf serum. For in situ hybridization experiments unsynchronized cultures grown on glass-slides were fixed with acetic acid/methanol (1:3). In other experiments metaphase chromosome preparations were prepared according to standard procedures.

DNA-labeling

Nick-translation of human genomic DNA with ^{3}H-dTTP (100 Ci) mmol; New England Nuclear Co; 1 Ci = 3.7×10^{10} becquerels) was carried out as described previously (Rappold et al. 1984a). For nick-translation of human DNA with biotin-11-dUTP (Langer et al. 1981) a nick-translation reagent system was used from Bethesda research laboratories (BRL; No. 9507) according to the protocol provided by the supplier.

DNA-preparation

Human genomic DNA of a healthy female adult was isolated according to the method of Kunkel et al. (1977).

In situ hybridization experiments

In situ hybridization of ^{3}H-labeled human genomic DNA to metaphase preparations of the hybrid cell line 29-11B and autoradiography were carried out as described by Rappold et al. (1984a). In situ hybridization of the biotin-labeled human genomic DNA to hybrid cells grown on glass slides or to metaphase preparations was performed as follows. DNA in cell nuclei and chromosome spreads was denatured as described by Rappold et al. (1984a). The hybridization mixture consisted of 40% formamide, 4 × SSC, 2 × Denhardt's solution, 10% dextran sulfate, 10 m*M* $NaPO_4$, 100 ng/ml denatured (95°C, 5 min) biotin-labeled human genomic DNA, and 200 μg/ml salmon DNA. Hybridization was allowed to proceed at 37°C in a moist chamber over night. Thereafter slides were washed in formamide/2 × SSC (1:1) and 0.1 × SSC each at 37°C for 30 min.

Visualization of biotin-labeled DNA

For visualization of biotin-labeled DNA after in situ hybridization the BRL DNA detection system (No. 8239A) was used. For this purpose the procedure described by Leary et al. (1983) for the detection of biotin-labeled DNA sequences in Southern or dot-blot hybridizations was modified for the use in in situ hybridization experiments. Briefly, slides were rinsed in AP 7.5 buffer (0.1 *M* Tris/HCl (pH 7.5); 0.1 *M* NaCl; 2 m*M* $MgCl_2$; 0.05 % (v/v) Triton X-100) and covered with 3% bovine serum albumin (BSA) in AP 7.5 buffer for 20 min. After rinsing in AP 7.5 buffer the slides were covered with a freshly prepared solution of streptavidin (2 μg/ml) in AP 7.5 buffer for 30 min, followed by three washes in AP 7.5 buffer, each 3 min. Due to the high affinity of streptavidin to biotin a streptavidin-biotin complex is formed at the sites where the biotin-labeled DNA probe had hybridized to chromosomal DNA. The slides were then incubated with poly (AP), a biotinylated polymer of calf intestinal alkaline phosphatase (1 μg/ml AP 7.5 buffer) for 30 min and washed twice (3 min each) in AP 7.5 buffer, followed by three washes (3 min each) in AP 9.5 buffer (0.1 *M* Tris/HCl (pH 9.5); 0.1 *M* NaCl; 5 m*M* $MgCl_2$). Thereafter the slides were incubated in a freshly prepared dye solution (about 2 ml per slide) within a sealed polypropylene bag. This dye solution contained 0.33 mg/ml nitro-blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in AP 9.5 buffer and was prepared as described in the instruction manual for the BRL DNA detection system. A purple precipitate was allowed to develop in the dark for about 4 h at the sites where streptavidin-poly (AP) complexes had formed with biotin-labeled DNA sequences. Thereafter slides were rinsed in 20 m*M* Tris/HCl (pH 7.5) containing 5 m*M* EDTA and air dried. Finally, cells and metaphase spreads were stained with Giemsa or DAPI (Hens et al. 1983).

Results

Figures 1–5 show metaphase plates and interphase nuclei obtained from the hybrid cell lines 29-11B and A1 Wbf2 respectively after in situ hybridization with human total genomic DNA. Figure 1 shows the autoradiograph of 29-11B cells obtained in an experiment with ^{3}H-labeled human DNA, while Fig. 2 shows a 29-11B metaphase plate obtained in an experiment with biotinylated human DNA. Note that the human X chromosome can be distinctively labeled throughout its whole length by both these procedures, while the Chinese hamster chromosomes appear completely unlabeled. There is no indication of any translocation of human chromosome material to Chinese hamster chromosomes in this cell line. While most mitotic cells showed a single human X chromosome, two human X chromosomes were occasionally observed in polyploid cells.

Figure 3 shows two 29-11B hybrid cells grown on a glass slide after in situ hybridization with biotinylated human DNA. The human X chromosome can be seen as a distinct nuclear domain in each of the two interphase nuclei. Figure 3a shows the local accumulation of the purple dye precipitate which was formed by the poly (AP) complex at the site of in situ hybridization of the biotin-labeled genomic human DNA to the human X chromosomal DNA (see Materials and methods). Figure 3b shows the same nuclei as seen in a Zeiss fluorescence microscope after counterstaining with DAPI. Fluorescence is spared in each nucleus at the site of the human X chromosome territory due to the accumulation of the dye precipitate which interferes with the excitation of the fluorescent light at these particular areas. Figure 4a, b shows a typical area from a growing culture of unsynchronized 29-11B cells at lower magnification. A localized site of hybridization of human genomic DNA can be seen in each nucleus indicating that all nuclei in a population of cells at different stages of the cell cycle maintain the active human X chromosome as a distinct chromatin body. Note that the human X chromosome is

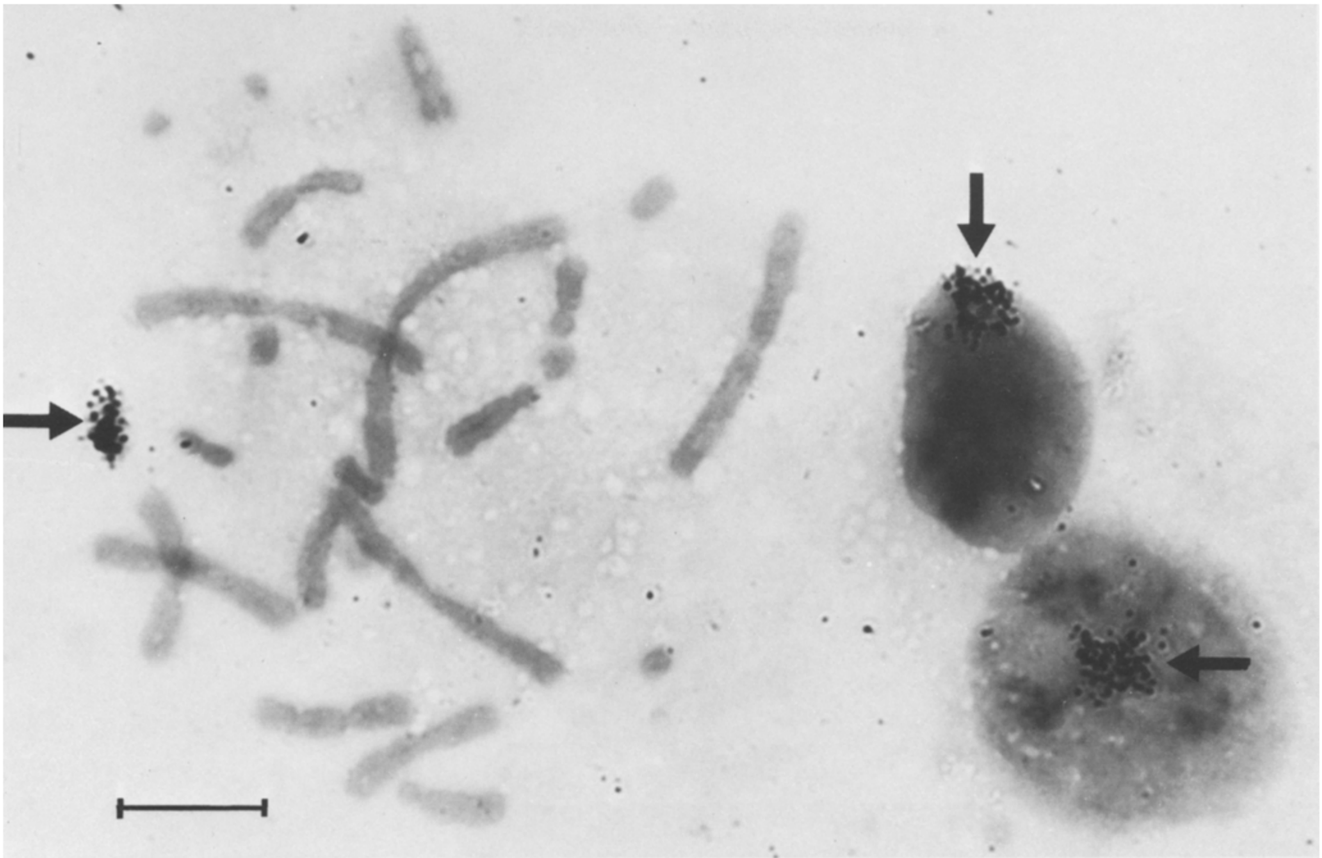

Fig. 1. This autoradiograph shows a metaphase plate and two interphase nuclei of the hybrid cell line 29-11B after in situ hybridization with ^{3}H-labeled human genomic DNA. A free human X chromosome which is the only human chromosome contained in this cell line is heavily labeled in the metaphase plate *(arrow)*. No label is found over the Chinese hamster chromosome complement. Interphase nuclei show a heavily labeled area *(arrow)* indicating that the X chromosome is organized within a chromosomal domain. *Bar* indicates 10 μm

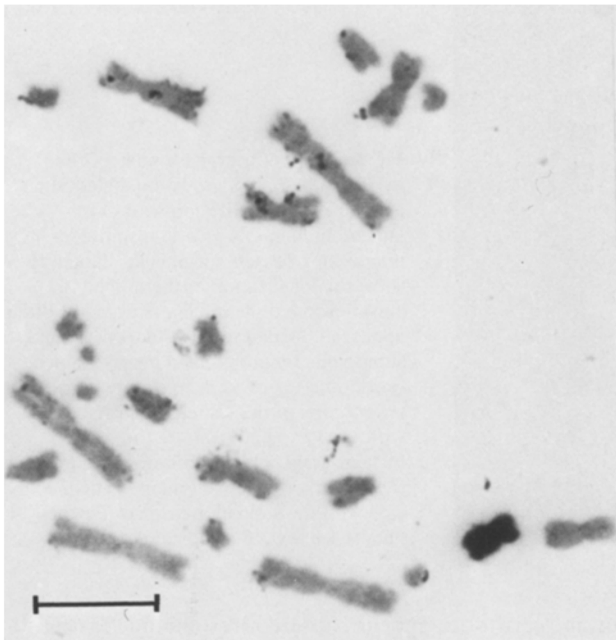

Fig. 2. Metaphase plate from the hybrid cell line 29-11B after in situ hybridization with biotin-labeled human genomic DNA. Note specific dark staining of the human X chromosome (see Materials and methods). Chinese hamster chromosomes are slightly stained with Giemsa. *Bar* indicates 10 μm

situated at the nuclear edge in some cases, while it appears in the middle of the nuclear area in other cases. The shape of territories as viewed from above varied from rather circular to more elongated structures. Interestingly, in many 29-11B interphase nuclei staining of the human X chromosome suggested two closely adjacent and intensely stained sites of major hybridization separated by a weakly stained region (Fig. 3a, *arrows*). The structural significance of this finding with regard to the interphase organization of this chromosome is presently not clear. In a few cases, two clearly separated X chromosome domains could be distinguished in a 29-11B hybrid cell nucleus.

In order to quantitate the relative size of the human X chromosome territory, camera lucida drawings of randomly selected samples of interphase nuclei ($n = 100$) from growing 29-11B cultures were made and both the total nuclear area and the area covered by the dye precipitate (in the case of in situ hybridization with biotinylated human DNA) or silver grains (in the case of in situ hybridization with ^{3}H-labeled human DNA) were determined by planimetry. The relative area covered by the human X chromosome in each of the two samples was calculated as a percentage of the total nuclear area. It was 9.2 ± 4.0% (SD) in the first sample (biotin-labeled human DNA) and 9.0 ± 3.4% in the second sample (^{3}H-labeled human DNA). The range of the relative size of the X chromosome territory was similar in samples of small, medium sized, and large nuclei.

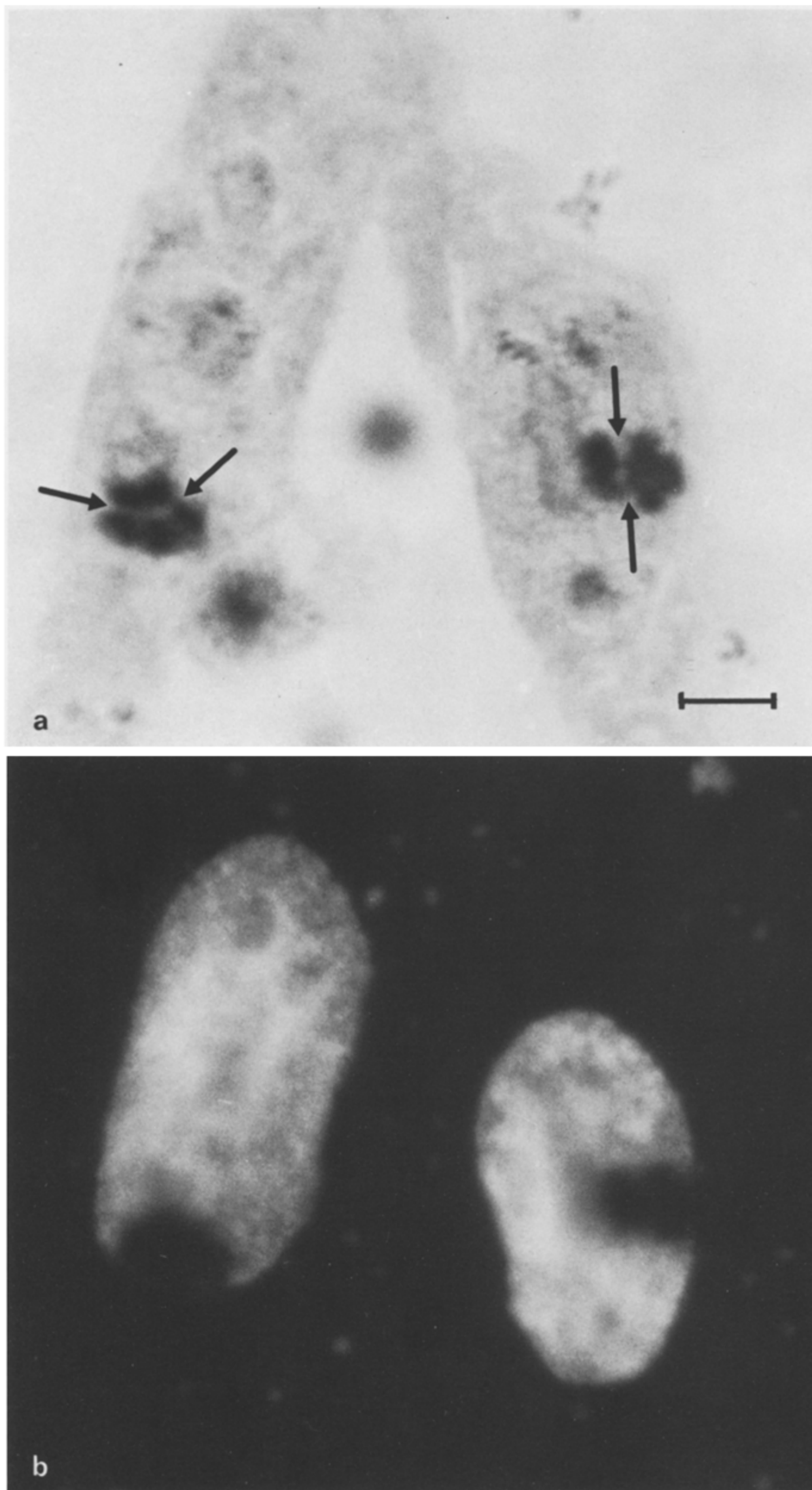

Fig. 3a, b. Two interphase nuclei from hybrid cells 29-11B grown on a glass slide after in situ hybridization with biotin-labeled human DNA. **a** Transmitted light microscopy of cells specifically stained to show the localization of the human X chromosome domain. These domains often appeared divided into two darkly stained subregions separated by a lightly stained borderline *(arrows)*. **b** Epifluorescence microscopy of the same cells poststained with DAPI to demonstrate the whole nuclear areas. Note nonfluorescent areas in the brightly fluorescent nuclei, which coincide with the stained areas in **a**. Further details see text. *Bar* indicates 5 μm

Figure 5 presents results obtained after in situ hybridization of the hybrid cell line A1 Wbf2 with biotinylated human genomic DNA. Besides three free human chromosomes, a chromosome derived from a translocation between a human chromosome and a Chinese hamster chromosome can clearly be seen in the metaphase plate shown in Fig. 5a. Human chromosome territories appear clustered in some nuclei (Fig. 5a, *right*) but more or less distributed in other nuclei (Fig. 5b–d). It is presently not clear whether or not the distribution of human chromosomes occurs at random in Chinese hamster × man hybrid cell nuclei. Further experiments are necessary in order to define the stage of the cell cycle of individual cells be-

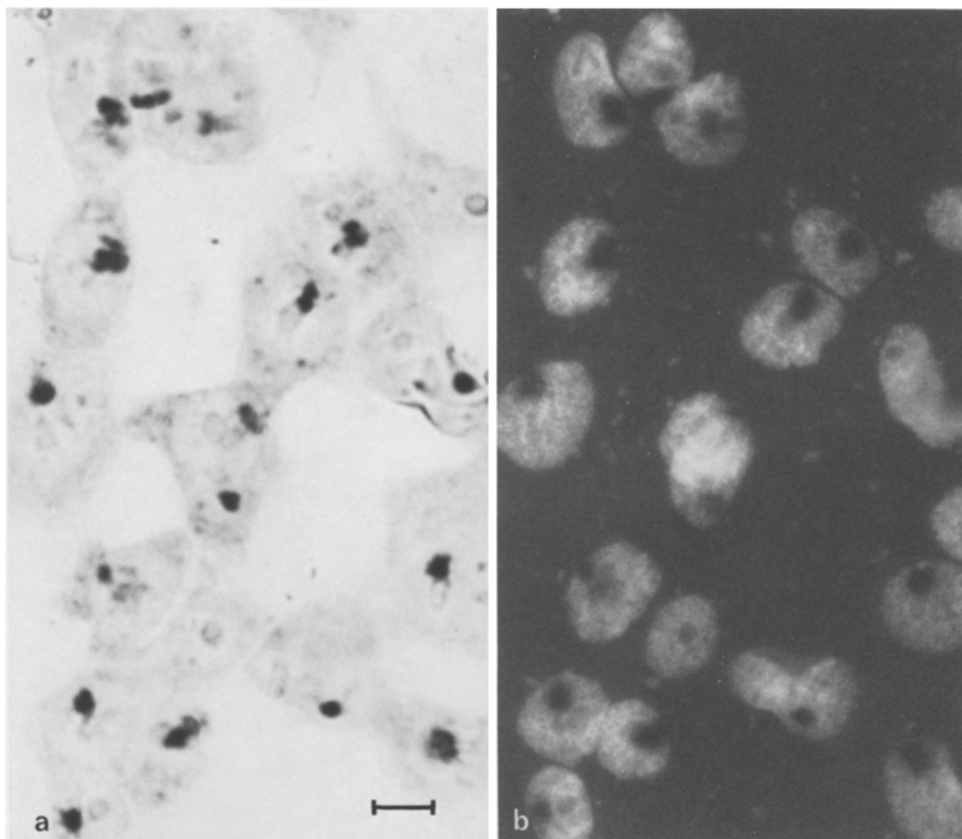

Fig. 4a, b. Representative area with interphase cells from the hybrid cell line 29-11B after in situ hybridization with biotin-labeled human DNA at smaller magnification. **a** Transmitted light microscopy of the cells showing the specifically stained human X chromosome; **b** epifluorescence microscopy of the same cells (compare Fig. 3). Note that each individual nucleus of this growing population of unsynchronized cells shows a distinct chromosomal domain. *Bar* indicates 10 μm

fore the question of possible changes of the relative size and distribution of chromosome domains during the cell cycle can be answered unequivocally.

Discussion

The three-dimensional arrangement of chromosomes in interphase nuclei has been a matter of much speculation and controversy (Vogel and Schroeder 1974; Comings 1980; Avivi and Feldman 1980; Sperling and Luedtke 1981; Feldman and Avivi 1984; Cremer et al. 1984; Bennett 1984; Therman and Denniston 1984). However, in spite of Rabl's (1885) and Boveri's (1909) early suggestion of chromosomal domains, even the relatively simple question of how single chromosomes are arranged in interphase nuclei has not been answered definitely so far. The present data contribute to a growing body of evidence in favor of a territorial organization of interphase chromosomes (see Introduction). This evidence fits well into the concept of an interphase nucleus where each individual chromosome is organized into a number of loops which possibly possess specific attachment sites to a nuclear matrix (Hancock and Boulikas 1982; Lebkowski and Laemmli 1982). Two possible drawbacks of the present experiments have to be considered.

First, fixation of the cells with acetic acid/methanol might have produced chromosome territories as fixation artefacts. However, for the following reason it appears very unlikely that fixation should have led to an alteration of the chromatin arrangement of such a profound nature. In previous experiments we have laser-UV-microirradiated selected subnuclear regions of individual interphase nuclei of Chinese hamster cells at several (2–4) sites. Thereafter the cells were fixed with acetic acid/methanol and the microirradiated chromatin was visualized by indirect immunofluorescence microscopy using antibodies specific for pyrimidine dimers in the DNA. We have found that the relative arrangement of the microirradiated chromatin in the living cells remained practically unchanged after the fixation procedure (Baumann 1983; Cremer et al. 1984, and our unpublished data). A second possible drawback of the present experiments concerns the organization of human chromosomes in hybrid cell nuclei, which is not necessarily the same as in normal human nuclei. It should be noted that the human X chromosome present in the hybrid cell line 29-11B was an active one, since human hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) is necessary for the survival of the hybrid cells in HAT-medium (Littlefield 1964). Furthermore, laser-UV-microbeam experiments performed with cultivated Chinese hamster cells strongly suggest that Chinese hamster chromosomes are also organized as distinct chromosomal domains (see Introduction).

In situ hybridization techniques using DNA probes such as centromeric and ribosomal DNA probes which hybridize to repetitive DNA sequences contained in specific chromosomal subregions, have already become a potent tool to study the chromosome topography in interphase nuclei (Manuelidis et al. 1982; Manuelidis 1984; Rappold et al. 1984b). The rapid development of chromosome sorting and the establishment of DNA-sequences libraries from specific chromosomes should provide an avenue in the foreseeable future to use pools of single or low copy sequences specific for individual chromosomes or parts thereof and thus generalize the present approach to study the chromosome topography in any interphase nucleus for which suitable DNA probes exist.

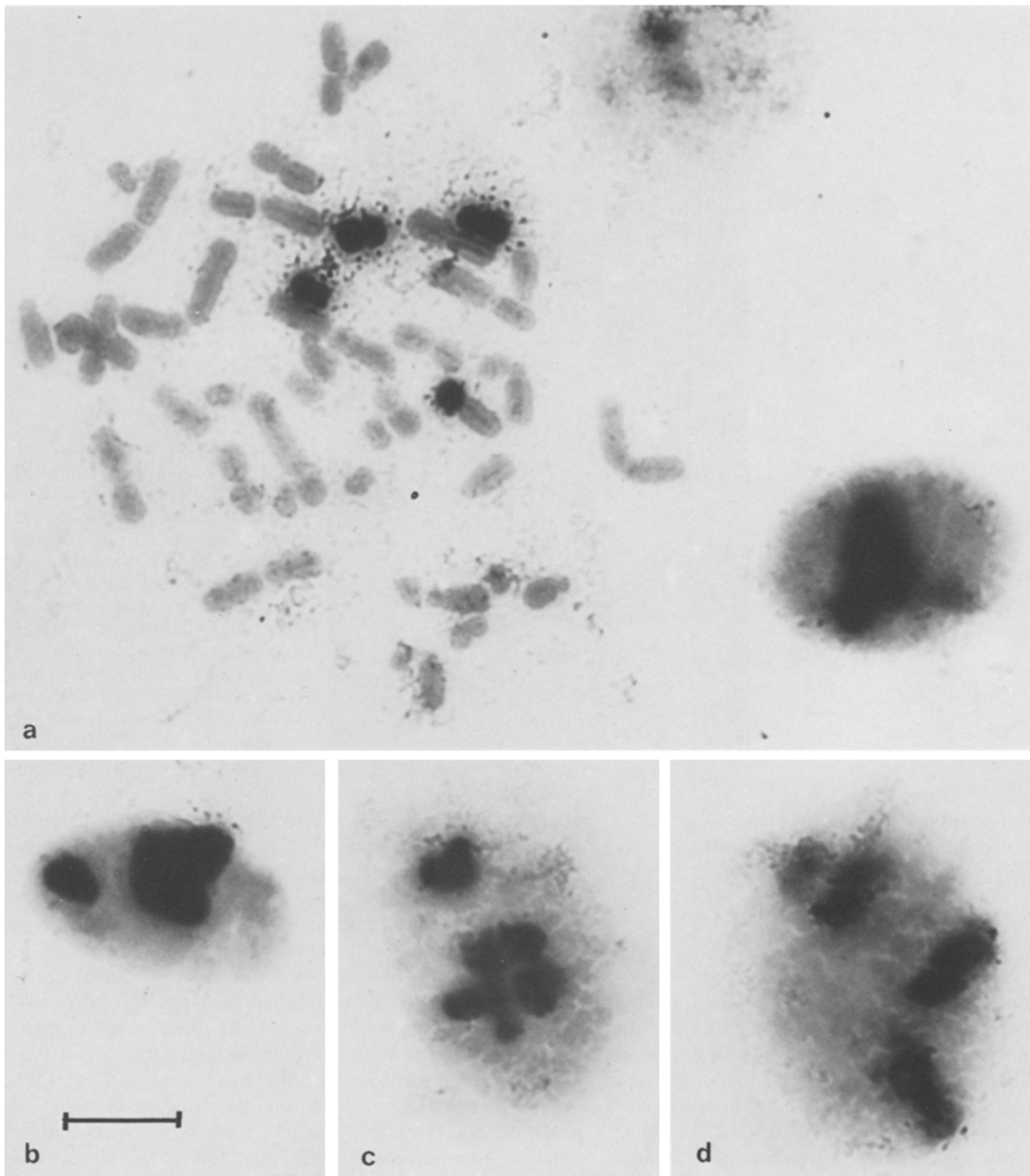

Fig. 5a-d. Metaphase plate (**a**) and interphase nuclei from hybrid cell line A1 Wbf2 after in situ hybridization with biotin-labeled human genomic DNA. **a** This shows a specific dark staining of the three free human chromosomes (presumably 11, 17, and X) contained in the metaphase plate plus a translocation chromosome containing a small human chromosome fragment *(arrow)*. Chinese hamster chromosomes are slightly stained with Giemsa. **a** *(right side),* **b–d** These show examples of interphase nuclei with a distinct territorial organization of the human chromosomes. While in some cases the human interphase chromosomes appeared clustered as shown in **a**, distribution of these chromosomes at several sites was also observed (**b–d**). *Bar* indicates 10 µm

Cytogenetic analysis of hybrid cell lines is considerably facilitated by this procedure especially with regard to the detection of translocations between chromosomes from different species. If chromosome suspensions of these cell lines could be hybridized with biotinylated human genomic DNA, free human chromosomes and translocation chromosomes could be labeled with fluorescent dyes by immunocytochemical procedures and subsequently used for chromosome sorting (J. W. Gray, personal communication). Finally, a generalized approach for the detection of specific human chromosomes or subregions thereof could be used to detect numerical aberrations of these chromosomes, as well as duplications or deficiencies of particular chromosomal subregions directly in the interphase nucleus. For example, using a cloned DNA sequence from repetitive pericentric DNA which hybridizes specifically to chromosome 18 (Devilee et al. 1986), we were able to detect three specifically labeled sites in interphase nuclei of lymphocytes and fibroblasts obtained from

probands with trisomy 18, while two labeled sites were observed in interphase nuclei from normal probands (unpublished data).

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