Polytene chromosomes in mouse trophoblast giant cells

SUSANNAH VARMUZA, VALERIE PRIDEAUX. RASHMI KOTHARY and JANET ROSSANT

Mount Sinai Hospital Research Institute and Department of Medical Genetics, University of Toronto, 600 University Avenue, Toronto, Ontario, Canada, M5G 1X5

Summary

Mouse trophoblast giant cells undergo successive rounds of DNA replication resulting in amplification of the genome. It has been difficult to determine whether giant cell chromosomes are polyploid as in liver cells or polytene as in Dipteran salivary glands because the chromosomes do not condense. We have examined the pattern of hybridization of mouse giant cells with a variety of *in situ* chromosome markers to address this question. Hemizygous markers displayed one hybridization signal per nucleus in both diploid and giant cells, while homozygous markers displayed two signals per nucleus in both cell types. These patterns are consistent with cytological evidence indicating that giant cell chromosomes are polytene rather than polyploid. However, in contrast to the situation in Dipteran salivary glands, the two homologues do not appear to be closely associated. We conclude that the mechanism of giant cell DNA amplification involves multiple rounds of DNA replication in the absence of both karyokinesis and cytokinesis, and that sister chromatids, but not homologous chromosomes, remain closely associated during this process.

Key words: mouse, trophoblast, giant cells. polytene chromosome, DNA replication.

Introduction

The first differentiated tissue to arise in the mouse embryo is the trophectoderm. Trophectoderm cells form a monolayer surrounding the inner cell mass (ICM) and the blastocoelic cavity at the blastocyst stage of development; those cells directly overlying the ICM are designated polar trophectoderm, while those away from the ICM are termed mural trophectoderm. These latter cells are believed to be involved in implantation of the blastocyst into the uterine epithelium (Gardner, 1972). Polar trophectoderm continues to proliferate and forms extraembryonic ectoderm and the ectoplacental cone, which later contribute to the fetal placenta (Rossant & Papaioannou, 1977). Mural trophectoderm cells cease mitosis, but not DNA replication, beginning at 4·5 days *post coitum*. These enlarged cells, called primary trophoblast giant cells, may endoreduplicate their genomes by up to 500-fold (Zybina, 1970; Zybina & Grishchenko, 1972; Barlow & Sherman, 1972). Secondary giant cells develop later at the periphery of the ectoplacental cone and are phenotypically indistinguishable from primary giant cells.

The high ploidy levels achieved by these cells make them unique among mammalian cell types. The increase in C value is accomplished by DNA replication in the apparent absence of mitosis and chromosome condensation. Occasional polyploid mitoses have been observed in trophoblast cells (Zybina & Grischenko, 1970; Ilgren, 1980), but the frequency of such events is insufficient to account for the increased ploidy values. Several possible alternative modes of replication can be envisaged. Multiple rounds of DNA synthesis without either karyokinesis or cytokinesis may result in chromosome structures in which (i) all homologous strands are closely associated, (ii) sister chromatids of individual chromosomes, but not of homologues, are closely associated, or (iii) all chromatids are unassociated. The first case closely resembles the mode of replication of polytene chromosomes in Dipteran insects, while the last case is similar, but not identical, to liver cell polyploidization, where karyokinesis still occurs (reviewed in Brodsky & Uryvaeva, 1985). Various cytological studies have purported to show that giant cell chromosomes are polytene (Barlow & Sherman. 1974; Snow & Ansell, 1974; Zybina, 1977), although

condensed polytene chromosomes are not observed normally in the interphase nuclei of trophoblast giant cells. It has also been reported that single Barr bodies occur in rabbit trophoblast giant cells (Zybina et al. 1973).

The advent of sensitive in situ DNA–DNA hybridization techniques allows resolution of the problem of the mode of endoreduplication in trophoblast giant cells. We have utilized in situ hybridization to several chromosome markers to show that the number of hybridization signals does not increase with ploidy levels in trophoblast giant cells. Moreover, the number of signals per nucleus approximates the number of marked chromosomes per diploid complement. These results suggest that sister chromatids, but not homologous chromosomes, are closely associated throughout the development of trophoblast giant cells.

Materials and methods

Mouse strains

For studies utilizing hybridization to endogenous DNA sequences, outbred CD-1 mice (Charles River, St Constant, Quebec) were used. Two transgenic mouse strains were also used. Cecilia Lo derived a transgenic mouse line, line 85, by DNA iontophoretic introduction of a mouse β-globin plasmid into a hybrid C57BL/6/C3H mouse zygote (Lo, 1986). It contains 1000 tandemly repeated copies of the plasmid inserted at the telomere of chromosome 3 (Lo, 1986 and C. Lo, personal communication). We obtained a homozygous male from Lo and have crossed the marker on to the outbred CD-1 strain where it is now maintained as a homozygous outbred line, Tg-MβG-1. A second transgenic line, Tg-HSZ-4, was produced in this laboratory and contains 16 tandem copies of a mouse hsp68–E. coli lacZ fusion gene fragment on a CD-1 background (R. Kothary, M. Perry, S. Clapoff, L. Moran & J. Rossant, unpublished data). The insert is located near the centromere of a large chromosome, possibly chromosome 1.

Plasmids

A plasmid containing five tandem repeats of the mouse major satellite DNA sequence was prepared by inserting AvaII fragments from the satellite peak isolated from a caesium chloride density gradient into pTZ19R (C. Davis, unpublished data). pMβδ2, a derivative of the plasmid inserted into Tg-MβG-1 from which a mouse repetitive sequence has been removed, was a kind gift of C. Lo (University of Pennsylvania). pCH126 containing the E. coli lacZ gene was used for in situ detection of the Tg-HSZ-4 insert. A cDNA clone, pH2-IIa, which cross-reacts with all Class I MHC genes, was kindly provided by L. Hood (Steinmetz et al. 1981).

Preparation of trophoblast cells

Primary giant cells were prepared by culturing blastocysts, flushed from the uteri of 3-5-day pregnant mice, into

multichambered culture slides (Lab-Tek, Miles Scientific) in α-MEM (Gibco) supplemented with 10% fetal bovine serum, 100 i.u. ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. Cultures were maintained for 4–6 days in humidified air plus 5% CO_2 at 37°C, during which time giant cells grew out from the hatched blastocysts. Secondary giant cells were cultured from ectoplacental cones which had been dissected from 7-5-day postimplantation embryos. The cells were grown as described above, except that culture time was reduced to 2–4 days. Giant cell nuclei from 10-5-day embryos were isolated by teasing apart the decidual cell layer and the trophoblast giant cell layer in a dish containing phosphate-buffered saline. The nuclei, which fell to the bottom of the dish, were collected in a drawn Pasteur pipette, applied directly to a poly-L-lysine-coated glass microscope slide and allowed to air dry. Liver cells were prepared by simply touching a poly-L-lysine-coated slide with a freshly cut piece of liver. The blotted cells were allowed to air dry.

In situ hybridization with biotinylated probes

Cells attached to microscope slides were fixed for 30 min or more in 3:1 ethanol:acetic acid, followed by two rinses in absolute ethanol. After air drying, the DNA was denatured in 70% formamide plus 2 × SSC at 70°C for 5 min. The denatured slides were quenched by immersion in cold 70% ethanol, dehydrated through an ethanol series and air dried.

Plasmid DNA was nick translated with biotinylated dUTP as described (Rossant et al. 1986). The hybridization solution, consisting of 2-4 μg ml^{-1} denatured biotinylated DNA, 200 μg ml^{-1} sheared denatured herring sperm DNA, 5 × SSC and 10% dextran sulphate, was layered over the cells under a coverslip and hybridized overnight at 60°C. Slides were washed twice at room temperature in 2 × SSC, once at room temperature in 0·1 × SSC and once at 50°C in 0·1 × SSC.

Biotin was detected by binding of streptavidin conjugated with horseradish peroxidase (HRP) (Detek-1-hrp, Enzo Biochem), followed by staining with 3,3′-diaminobenzidine (DAB) to detect HRP activity (Rossant et al. 1986). In some cases, the DAB staining was silver enhanced (DAB enhancement kit, Amersham). Following staining for HRP, cells were counterstained with haematoxylin and eosin, dehydrated and mounted with Permount.

Results

In situ hybridization to satellite DNA

The first chromosome marker examined in this study was the major satellite sequence which is associated with the centromeres of all mouse chromosomes. Primary and secondary giant cells displayed essentially identical patterns when they were hybridized with a cloned satellite sequence. Discrete hybridization signals of varying sizes could be seen on all interphase nuclei (Fig. 1A,C,E). The number of signals per nucleus varied, but was distributed normally around a mean which approximated the haploid
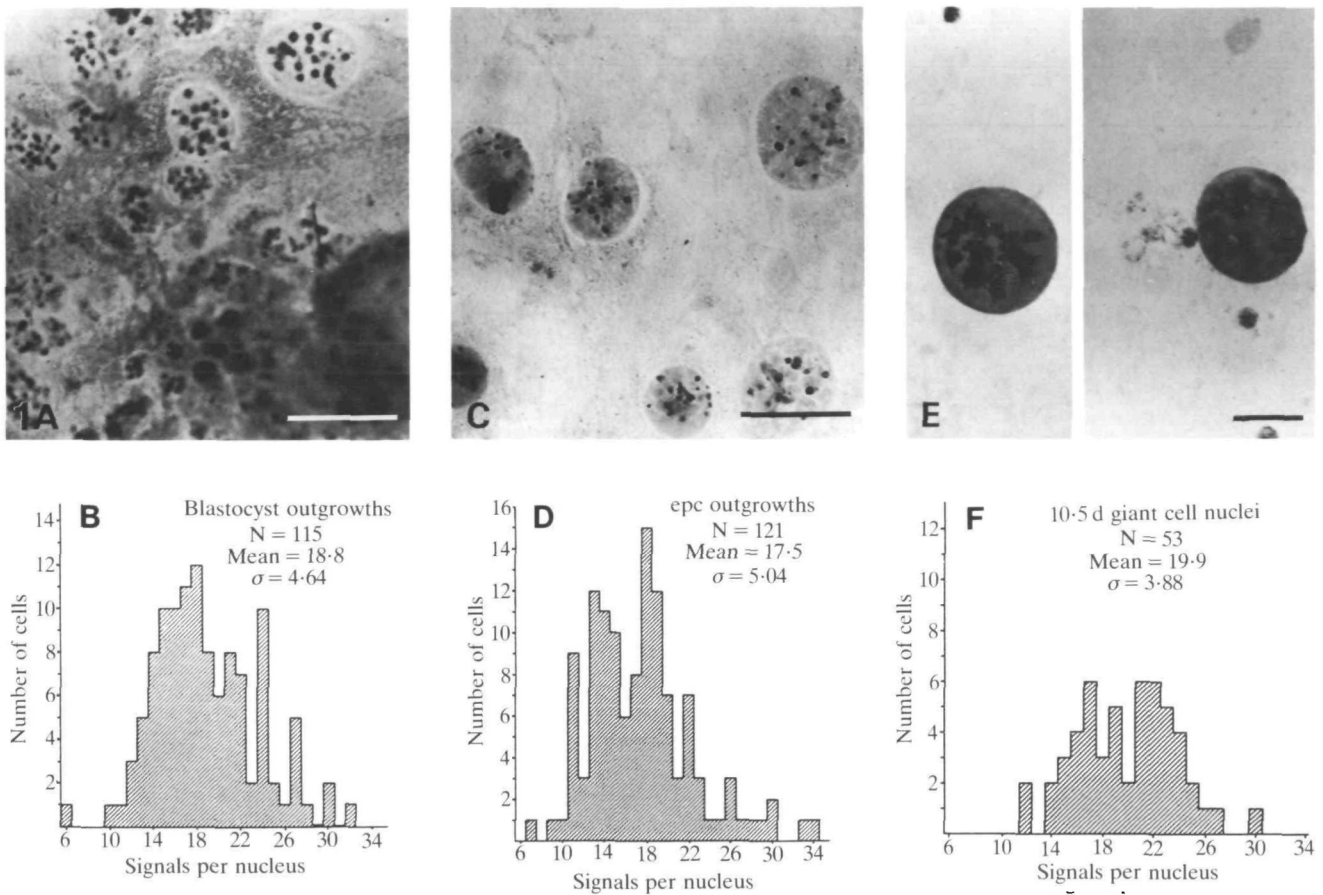
Fig. 1. Satellite DNA sequence distribution in giant cell nuclei. Cells were fixed and hybridized with biotinylated pTZ19K. Photomicrographs of *in situ* hybridizations to giant cells from blastocyst outgrowths (A), ectoplacental cone outgrowths (C), and 10·5-day conceptuses (E) show multiple hybridization signals in each nucleus. The bars in each photomicrograph represent 50 μm. Histograms of the number of hybridization signals per nucleus are shown for blastocyst outgrowths (B), ectoplacental cone outgrowths (D), and isolated 10·5-day giant cell nuclei (F). Photomicrographs of blastocyst outgrowths and ectoplacental cone outgrowths which had been hybridized with biotinylated satellite DNA were used to score the number of hybridization signals per nucleus. Only clearly distinguishable nuclei were scored. Giant cell nuclei from 10·5-day conceptuses were scored directly under the microscope for the number of hybridization signals per nucleus.

number of chromosomes (Fig. 1B,D,F). The signals in 10·5-day giant cell nuclei (Fig. 1E) were noticeably larger than those on giant cells derived from ectoplacental cone (Fig. 1C) or blastocyst outgrowths (Fig. 1A).

In situ *hybridization to Tg-MβG-1 cells*

The observation that giant cells contain a haploid number of centromeric hybridization signals might be construed as evidence of polyteny in these cells, perhaps even involving association of homologous chromosomes. However, a similar pattern could arise by random association of centromeric sequences in the interphase nuclei of trophoblast giant cells. *In situ* hybridization to the major satellite sequence could not distinguish these possibilities because the sequence is found on all chromosomes. Therefore, we examined in detail the hybridization patttern of a

chromosome-specific marker, namely the mouse β-globin plasmid inserted at the telomere of chromosome 3 in Tg-MβG-1.

Control hybridizations of biotinylated pMβδ2 to liver cells from hemizygous or homozygous Tg-MβG-1 mice established that Tg-MβG-1 contained a suitable marker with which to distinguish polyploidy and polyteny *in situ* (Fig. 2A,B). Liver spreads contain cells with 2C, 4C and 8C ploidy levels of DNA, derived by karyokinesis without cytokinesis. The number of signals observed per cell generally exhibited the expected variation with ploidy values. Most hemizygous cells contained one (2C), two (4C) or four (8C) hybridization signals, while homozygous cells contained two (2C), four (4C) or, less frequently, eight (8C) signals (Fig. 2C). Intermediate numbers of signals were occasionally observed especially in homozygous cells. This was probably due

to overlap of signals within the nucleus. *In situ* hybridization can thus directly determine the ploidy level of a polyploid cell without the necessity of determining total DNA content.

Primary and secondary giant cells from hemizygous or homozygous transgenic embryos were hybridized with pMβδ2 and scored for the number of hybridization signals per cell. Unlike liver cells, the number of hybridization signals did not increase with ploidy levels in these cells. Most hemizygous Tg-MβG-1 giant cells contained one discrete hybridization signal (Fig. 3A,C,E), while homozygous cells contained two signals (Fig. 3B,D,F). Direct microspectrophotometric analysis of ploidy levels was not compatible with the *in situ* hybridization technique, but previous studies have shown that blastocyst and ectoplacental cone outgrowths contain cells with ploidy levels up to 16C (Barlow & Sherman, 1972; Rossant & Ofer, 1977). Thus, polytenic association of chromatids must occur in the region of the Tg-MβG-1 insert in giant cells outgrown in tissue culture.

Hybridization to 10·5-day giant cell nuclei obtained directly from embryos *in vivo* revealed that polytenic association of chromatids was a consistent feature of giant cells even at very high ploidy levels (Fig. 3E,F). These nuclei can become as large as 100 μm in diameter and may contain DNA levels of up to 512C (Barlow & Sherman, 1972). However, only one (hemizygous) or two (homozygous) hybridization signals were observed per cell. The fortuitous association of the giant cell nucleus in Fig. 3F with a group of diploid cells illustrates clearly the difference in size of the nuclei and the intensity of the hybridization signals between the diploid and giant cells. The difference is also clear in a sectioned 7·5-day conceptus (Fig. 6).

Occasionally, twice the expected number of discrete signals were observed per giant cell (summarized in Fig. 4), presumably resulting from either a breakdown in the polytenic association, or a polyploid mitotic division. The sporadic appearance of nondiscrete (fuzzy) hybridization signals in giant cells grown *in vitro* is less easy to explain (see arrows Fig. 3C). However, since the frequency of these signals varied from experiment to experiment, we suspect it was an *in vitro* artefact perhaps relating more to the penetration of the DAB stain into the cells than the state of the DNA. Such signals were never observed in giant cells collected *in vivo* or in sections of intact conceptuses (Fig. 6). It should be noted that the mouse β-globin sequences in the probe did not detect the endogenous mouse globin genes in giant cells from nontransgenic CD-1 controls, indicating that there may be a minimum target size for hybridization with this technique.
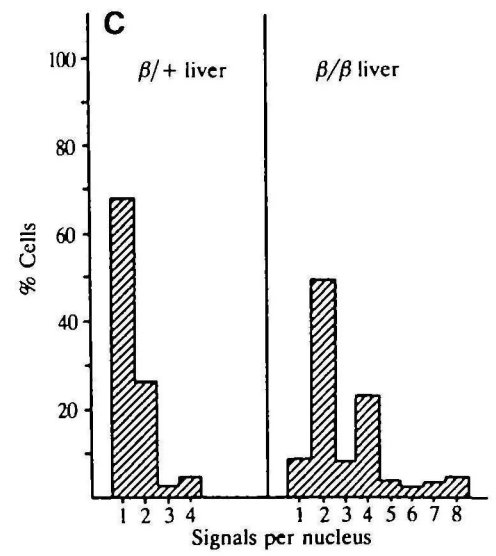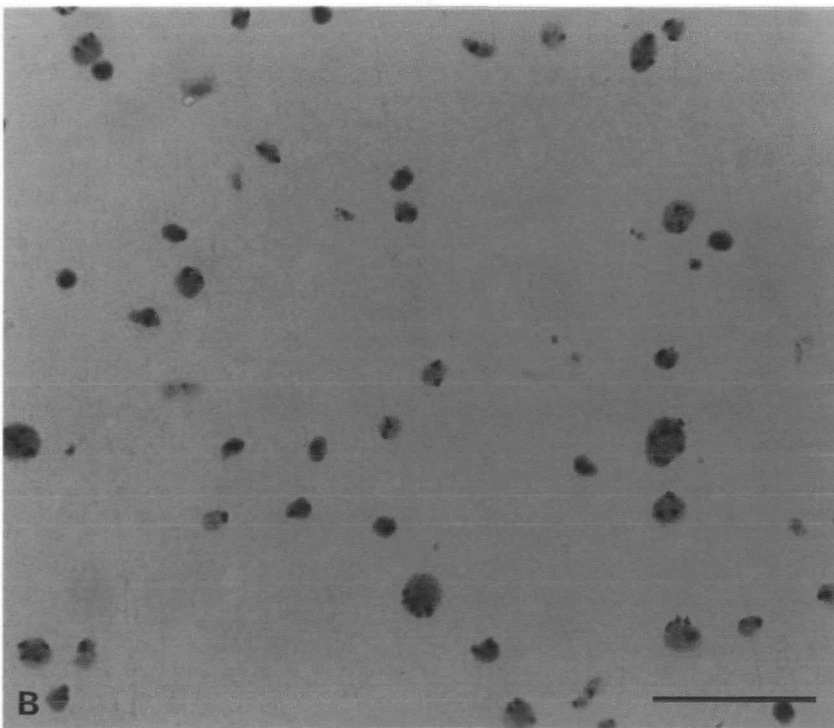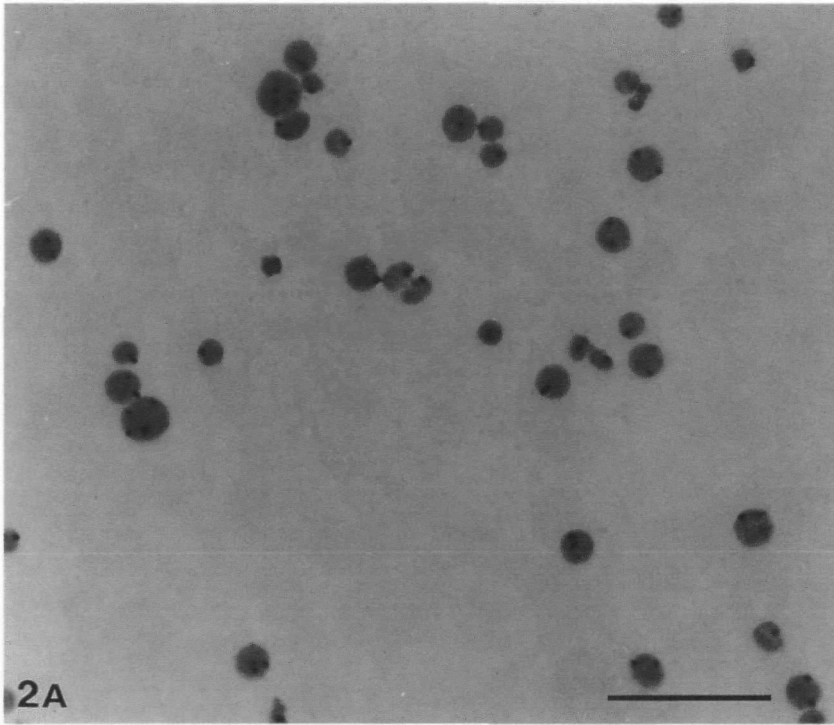
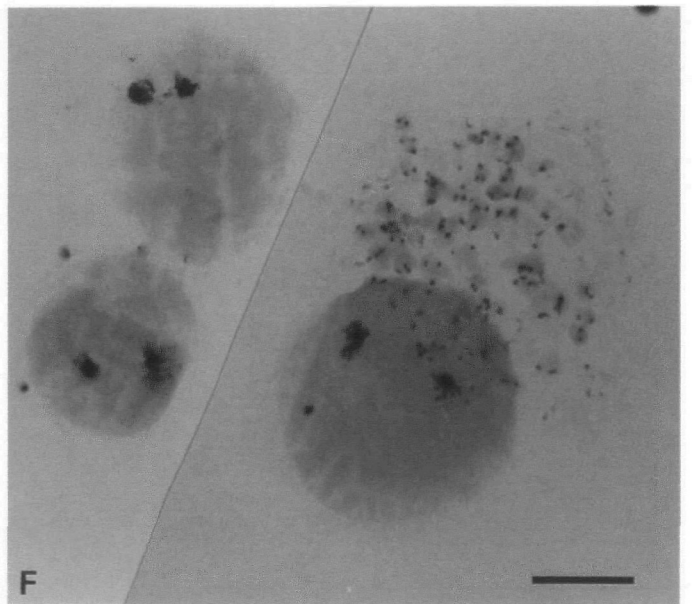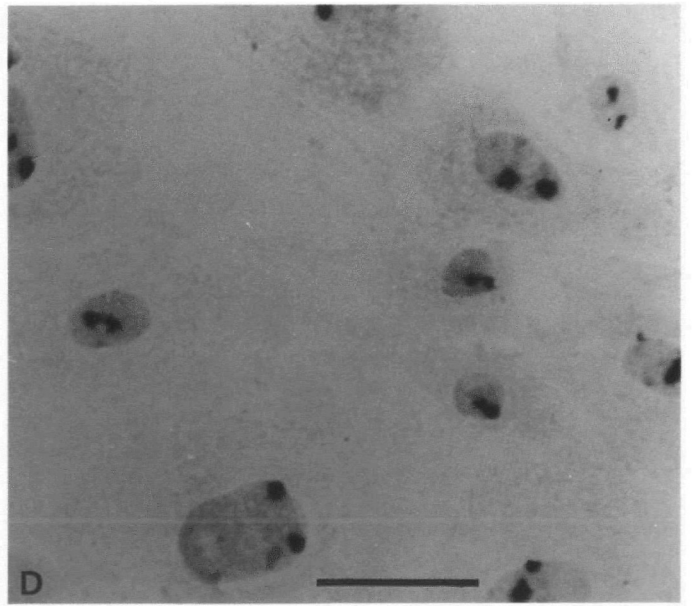## In situ *hybridization to Tg-HSZ-4 and H-2*
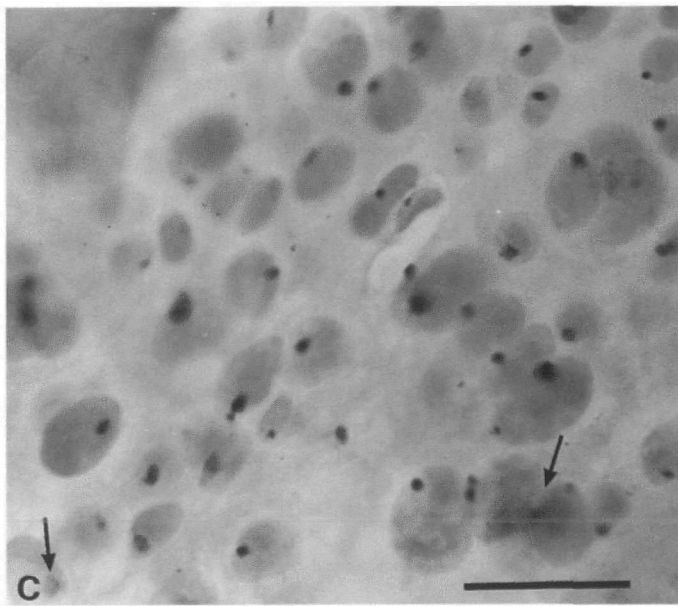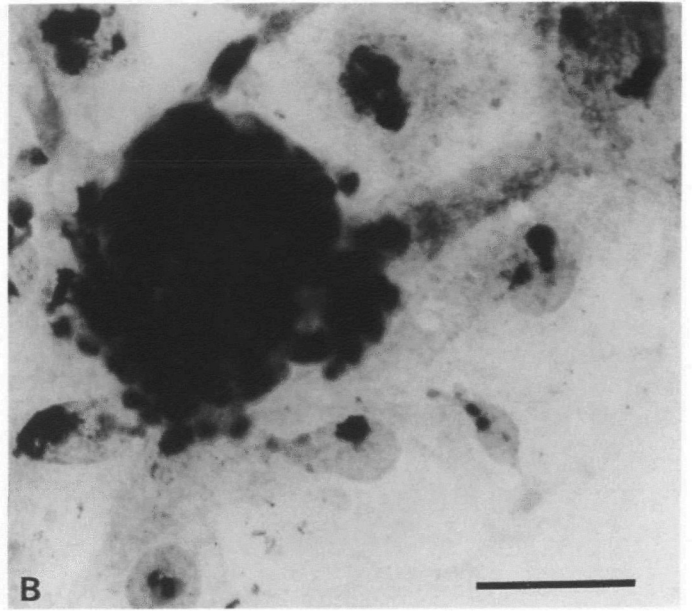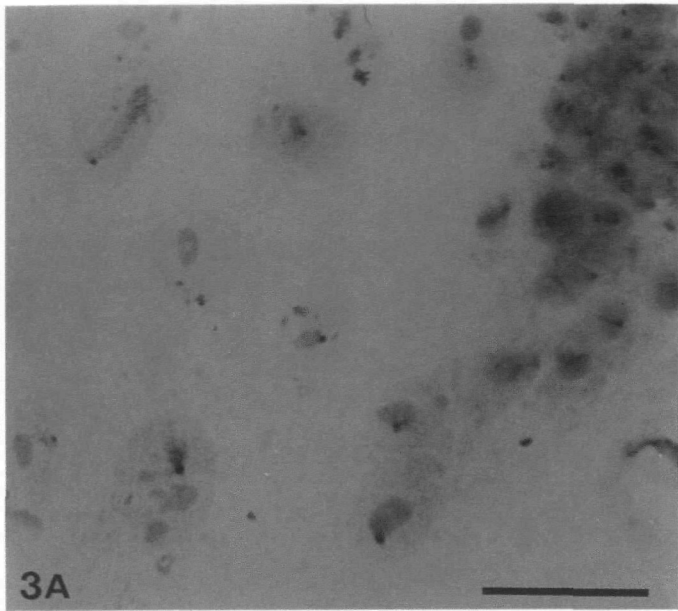
In order to confirm that polyteny was not a property of the telomeric Tg-MβG-1 insert only, we performed a limited series of hybridizations to two other markers. Hybridizations were performed to giant cells from hemizygous Tg-HSZ-4 and CD-1 giant cells using an *E. coli lacZ* or a class I MHC probe respectively. Since the target sizes for hybridization in these cells were smaller than in Tg-MBG-1, DAB-stained slides were silver enhanced to increase sensitivity. Giant cell nuclei isolated from ·10·5-day embryos were examined since these have higher ploidy levels than cells cultured *in vitro* and should not be subject to any culture artefacts. Hemizygous Tg-HSZ-4 cells contained one hybridization signal as was also observed in diploid amnion tissue on the same slide (Fig. 5A,B). Hybridization to the endogenous H-2 complex on chromosome 17 revealed two clear signals per giant cell (Fig. 5C), even though the level of sensitivity was not sufficient to detect any signal in diploid cells.

Discussion

Trophoblast giant cells in mammalian embryos endoreduplicate their genomes and become enlarged as a consequence. Until recently, the mechanism by which giant cells amplify their genomes could only be inferred from indirect evidence and inconclusive cytological observation. We have shown, using four different chromosome markers which are detectable *in situ*, that polytenic association of sister chromatids occurs in trophoblast giant cells despite the absence of visible condensed polytene chromosomes. Giant cells from three different stages of embryogenesis and with varying ploidy levels were analysed in our study. All produced essentially the same pattern of hybridization with three of the four markers used. In embryos containing a single marked chromosome per diploid complement (two hemizygous transgenic mouse lines), only one hybridization signal was observed in giant cell nuclei; embryos containing two marked chromosomes per diploid complement (the homozygous transgenic Tg-MβG-1 and endogenous H2 sequences) displayed only two signals per giant cell

Fig. 2. Distribution of a telomeric marker in polyploid liver cells. Photomicrographs of liver cells from hemizygous (A) and homozygous (B) Tg-MβG-1 mice, which were hybridized with biotinylated pMβδ2, reveal multiple signals in polyploid cells. Bars represent 50 μm. The number of hybridization signals visualized in all nuclei in several randomly chosen fields were scored and presented in histogram form (C). The number of cells scored was 327 for hemizygous (β/+) liver and 408 for homozygous (β/β) liver.

2A

B

C

$\beta/+$ liver β/β liver

% Cells

100
80
60
40
20

1 2 3 4   1 2 3 4 5 6 7 8

Signals per nucleus

3A   B

C   D

E   F

nucleus. In contrast, polyploid liver cells from hemizygous and homozygous Tg-MβG-1 mice contained multiple signals.

The major satellite sequence, which is located at the centromeres of all mouse chromosomes, produced a different pattern. Giant cells contained approximately 18 hybridization signals, which is slightly fewer than the haploid number of mouse chromosomes (20). Although this observation is consistent with polytenic association of both sister chromatids and homologous chromosomes at the centromeres, more convincing evidence would be provided by chromosome-specific centromeric markers. Other interphase nuclei display haploid numbers of 'chromocentres' after staining with Hoechst stain (Hilwig & Gropp, 1972), or hybridization with the satellite marker (Manuelidis *et al.* 1982; Manuelidis, 1984), suggesting that association of centromeres is a general property of interphase nuclei.

We have not demonstrated directly association of sister chromatids for all regions of the genome. However, the three sequences we have used map to different chromosomes and to different chromosomal regions. Thus, it seems reasonable to conclude that the general mechanism of giant cell formation involves successive rounds of DNA replication in the absence of either karyokinesis or cytokinesis, perhaps because of impaired mitotic functions. Sister chromatids remain closely associated, but homologous chromosomes do not associate with each other. The close association of sister chromatids may reflect steric intertwining of strands subsequent to asynchronous rounds of DNA replication, or it may reflect a more concerted function, such as specific chromatid association mediated by a synaptonemal complex-like

structure. The observation that occasional nuclei contained multiple signals tends to support the first possibility. Interphase-like association of centromeres continues, even though the centromeres may also be endoreduplicated. Some evidence has been presented to support the notion that interphase chromosomes associate with each other in a nonrandom fashion (Hadlaczky *et al.* 1986; Manuelidis, 1984). It may prove useful in future to examine the
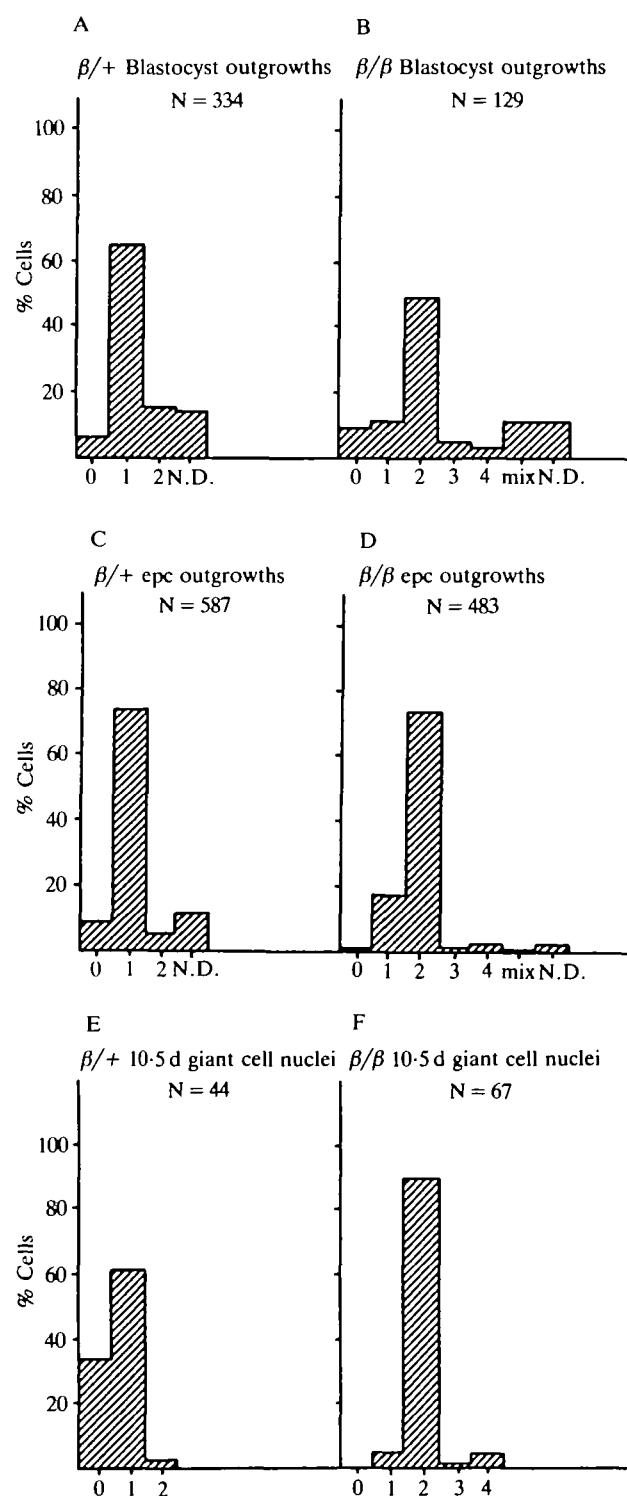

Fig. 3. Distribution of a telomeric marker in giant cells of different ploidy. Giant cells from hemizygous (A,C,E) and homozygous (B,D,F) Tg-MβG-1 embryos were hybridized with biotinylated pMβδ2 DNA. In two cases (B,D), the DAB signal was enhanced as described. The different ploidy levels were represented by blastocyst outgrowths (A,B), ectoplacental cone outgrowths (C,D), and isolated 10·5-day giant cell nuclei (E,F). Bars represent 50 μm. The arrows in C indicate examples of diffuse hybridization. Note that one giant cell nucleus in F lies adjacent to a cluster of diploid cells.

Fig. 4. Histograms of the number of hybridization signals in giant cells from hemizygous and homozygous Tg-MβG-1 embryos. Hemizygous (A,C,E) and homozygous (B,D,F) giant cells from blastocyst outgrowths (A,B), ectoplacental cone outgrowths (C,D), and isolated giant cell nuclei (E,F) which had been hybridized with biotinylated pMβδ2 DNA were scored for the number of signals per nucleus. All nuclei in several randomly chosen fields were scored under the microscope, and the number of hybridization signals per nucleus were tabulated in histogram form.

distribution of chromosome-specific markers such as those described here, which would unambiguously demonstrate whether specific chromosomes are associated in interphase cells.

Other investigators have proposed a complicated series of endomitotic and endoreduplicative rounds of chromosome duplication to account for the accumulation of DNA in rodent trophoblast giant cells (Zybina & Grischenko, 1970; Ilgren, 1980). Our data do not support the idea that initial amplification is accomplished by endomitosis leading to polyploid cells. Indeed, the polyploid metaphases observed by Ilgren (1980) are far too few to account for the degree of DNA amplification which occurs in the giant cell population. We suspect that the few polyploid metaphases observed by Ilgren (1980) and by Zybina & Grischenko (1970) may have arisen from extraembryonic ectoderm rather than trophoblast giant cells.

Recently, Brower (1987) reported similar findings to ours from a limited study using in situ hybridization of a cDNA clone of α-1 antitrypsin to giant cells. She was unable to detect any signal in giant cells with less than 8C, however, and she reported that 45 % of giant cell nuclei from 11-day conceptuses displayed dispersed staining. We did not observe such dispersed signals in giant cell nuclei of similar age. Because in situ hybridization to the transgenic inserts was sensitive enough to detect signals in diploid cells, we are confident that multiple signals in giant cell nuclei would have been readily detectable with these probes had they occurred.

Polyploidy and polyteny are widespread throughout the biological world (reviewed in Brodsky & Uryvaeva, 1985). The functional significance of polyploidy is unclear, but polyteny may afford a cell the opportunity to differentially amplify important sequences. This has been demonstrated to be the case for Drosophila chorion genes in polyploid ovarian follicle cells (Kalfayan et al. 1986; Kafatos et al. 1986). No differential amplification of specific sequences has yet been observed in mouse trophoblast giant cells.

Sherman et al. (1972) measured the relative amount of satellite sequence versus unique sequence DNA in giant cells by comparing their hybridization kinetics and found no difference between giant cells and diploid cells, indicating that satellite sequences are amplified to the same degree as unique sequences. This is contrary to the situation in Dipteran salivary glands where satellite sequences are under-replicated (Gall et al. 1971). Further analysis of the state of replication of trophoblast-specific genes may shed light in this area.
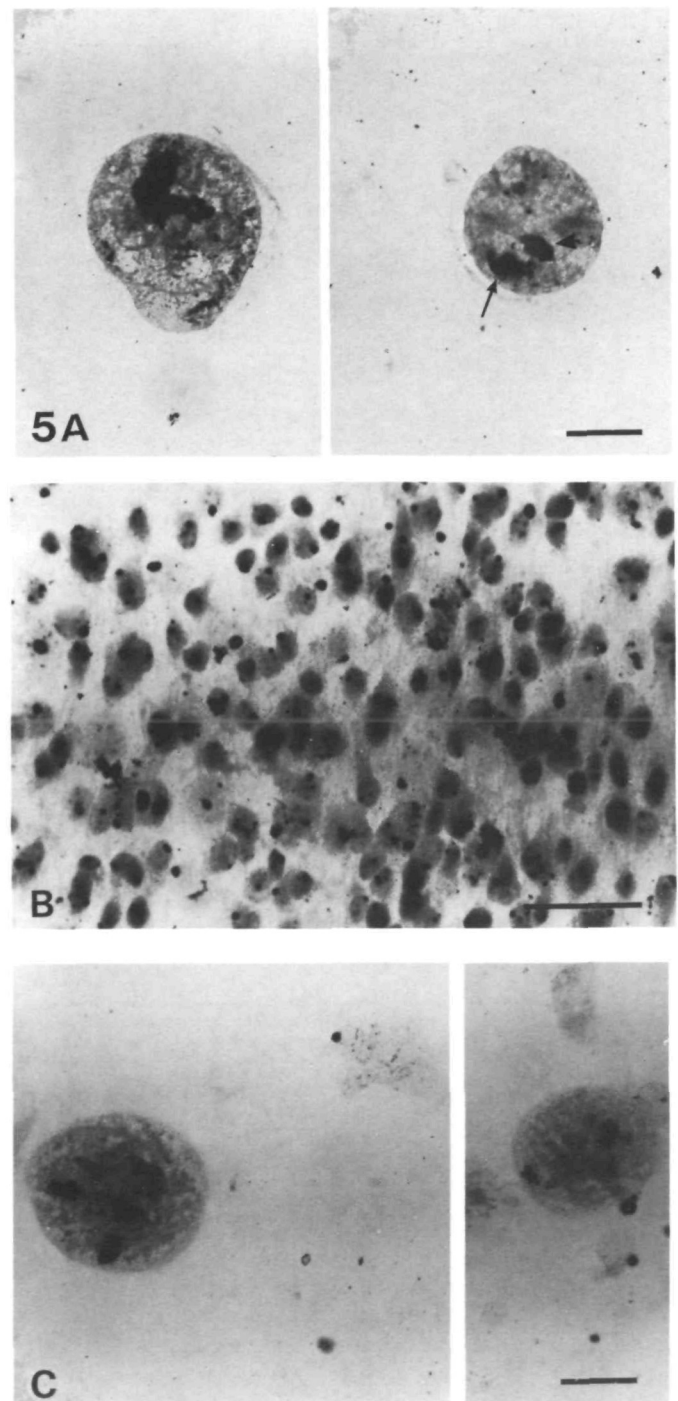

Fig. 5. Distribution of a nontelomeric hemizygous marker and of an endogenous marker in 10·5-day giant cell nuclei. Giant cell nuclei (A) and amnion epithelium (B) from 10·5-day hemizygous Tg-HSZ-4 embryos were hybridized with biotinylated pCH126 DNA. The amnion epithelium served as a diploid control. Giant cell nuclei from 10·5-day CD-1 embryos were hybridized with biotinylated pH2-IIa DNA (C). All samples were treated with the DAB enhance procedure. The pH2-IIa clone recognized endogenous H2 sequences in giant cells, but not in diploid cells (data not shown). In Fig. 5A the arrow indicates the hybridization signal while the arrowhead is pointing at heterochromatin. These two features are distinguishable in colour reproductions but appear similar in monochromatic reproduction.

Drosophila polytene chromosomes have proved to be invaluable tools in the mapping and cloning of genes. A similarly abundant source of material for mapping and cloning mouse genes would be useful. Clear demonstration of polyteny in trophoblast giant cells gives new impetus to the search for a means of condensing these chromosomes and visualizing them more readily. *In vitro* extracts of frog oocytes showing maturation-promoting activity might be applicable (Lohka & Maller, 1985; Miake-Lye & Kirschner, 1985).

We have demonstrated here that transgenic inserts can be used as *in situ* chromosome markers to address questions related to the dynamics of the genome.
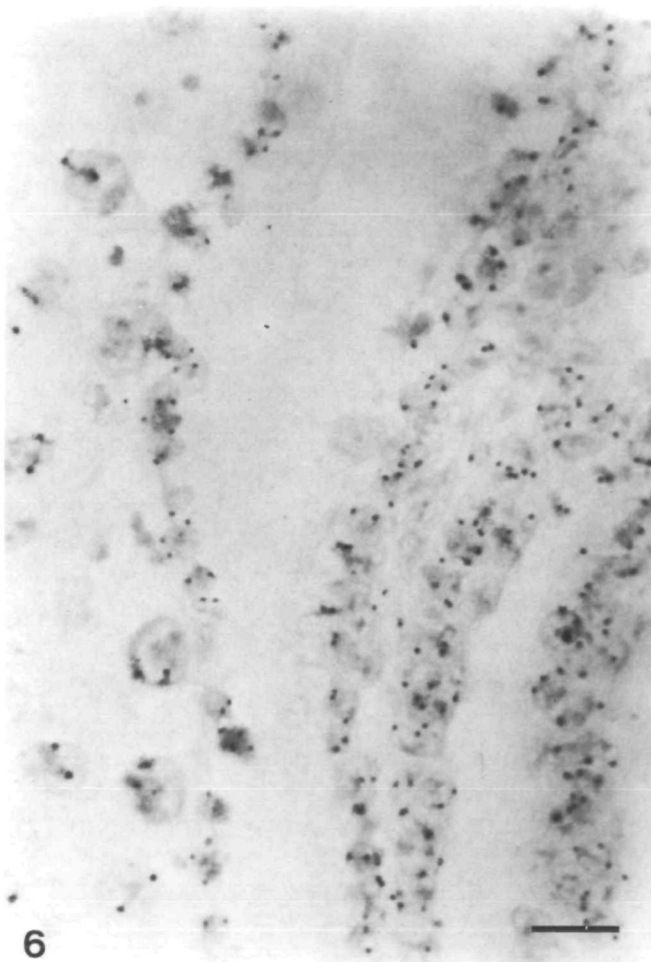
They may also be used effectively as ubiquitous cell autonomous markers in experiments involving analysis of cell distribution in chimaeric mice. Previous studies employing differential *in situ* hybridization to satellite sequences in two different species of mouse were limited by interspecific incompatibilities (Rossant & Frels, 1980; Rossant *et al.* 1983). Transgenic mice, which can be made congenic with the nontransgenic partner, eliminate such barriers to experimental design. We are currently using the Tg-MβG-1 strain in a variety of chimaera studies.

Trangenic inserts may also act as closely linked *in situ* markers of known mutations in future chimaera studies. At present, this approach is limited only by the paucity of transgenic mice with large detectable inserts at interesting chromosomal locations. As the number of transgenic lines increases in various laboratories, the possibilities of this application will surely expand.

Fig. 6. Distribution of a telomeric marker in sectioned giant cells. Homozygous 7·5-day Tg-MβG-1 embryos were fixed in 3:1 ethanol:acetic acid, embedded in ester wax and sectioned to a thickness of 7 μm. Sections were hybridized with biotinylated pM$\beta\delta$2 DNA and counterstained with haematoxylin and eosin. Many giant cell nuclei appear to contain only one signal, and some contain none. This is a result of cutting the marker out of the plane of the section. Note that the signals in adjacent diploid cells are smaller than those in the giant cells. The bar represents 50 μm.

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