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# Chromatin organization in the homogeneously staining regions of a methotrexate-resistant mouse cell line: interspersion of inactive and active chromatin domains distinguished by acetylation of histone H4

**Linda Nicol* and Peter Jeppesen†**

MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland, UK

*Present address: MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh, EH3 9EW, UK
†Author for correspondence (e-mail: peter.jeppesen@hgu.mrc.ac.uk)

## SUMMARY

**We have analyzed the organization of the homogeneously staining regions (HSRs) in chromosomes from a methotrexate-resistant mouse melanoma cell line. Fluorescence in situ hybridization techniques were used to localize satellite DNA sequences and the amplified copies of the dihydrofolate reductase (DHFR) gene that confer drug-resistance, in combination with immunofluorescence using antibody probes to differentiate chromatin structure. We show that the major DNA species contained in the HSRs is mouse major satellite, confirming previous reports, and that this is interspersed with DHFR DNA in an alternating tandem array that can be resolved at the cytological level. Mouse minor satellite DNA, which is normally located at centromeres, is also distributed along the HSRs, but does not appear to interfere with centromere function. The blocks of major satellite DNA are coincident with chromatin domains that are labelled by an autoantibody that recognizes a mammalian homologue of *Drosophila* heterochromatin-associated protein 1, shown previously to be confined to centric heterochromatin in mouse. An antiserum that specifically recognizes acetylated histone H4, a marker for active chromatin, fails to bind to the satellite DNA domains, but labels the intervening segments containing DHFR DNA. We can find no evidence for the spreading of the inactive chromatin domains into adjacent active chromatin, even after extended passaging of cells in the absence of methotrexate selection.**

Key words: Chromatin, HSR (homogeneously staining region), Methotrexate resistance, Heterochromatin, Histone H4 acetylation

## INTRODUCTION

It has been shown that one of the mechanisms by which cells can become resistant to the anti-folate drug methotrexate (MTX) is to increase the intracellular activity of the target enzyme, dihydrofolate reductase (DHFR). This occurs in patients receiving the drug as anti-cancer therapy (Bertino et al., 1963; Carman et al., 1984; Trent et al., 1984; Horns et al., 1984) and in cell lines which have been selected for resistance to MTX (Nunberg et al., 1978; Bostock et al., 1979; Dolnick et al., 1979). Some resistant cells (usually those that have been developed by in vitro step-wise selection for resistance) can grow in MTX concentrations greater than 10,000 times the concentration required to kill sensitive cells and the intracellular activity of DHFR may be increased as much as 1,000-fold. The increased DHFR production results from increased levels of DHFR mRNA (Chang and Littlefield, 1976; Kellems et al., 1976), which reflects amplification of DHFR gene copies (Alt et al., 1978).

A common cytological characteristic of MTX-resistant cells is the presence of large marker chromosomes that contain homogeneously staining regions (HSRs) (Biedler and Spengler, 1976; Biedler et al., 1980; Bostock et al., 1979). The presence of amplified DHFR genes within the HSRs of a number of MTX-resistant cell lines has been demonstrated by in situ hybridization (Nunberg et al., 1978; Dolnick et al., 1979). However, DNA measurements of HSRs from MTX-resistant mouse and Chinese hamster cells demonstrate an excess of DNA over that expected if only the DHFR gene sequence had been amplified during selection (Nunberg et al., 1978; Bostock et al., 1979; Schimke et al., 1979). In situ hybridization has shown that satellite DNA sequences are a major component of the repeating units within the HSRs of MTX-resistant mouse melanoma cells (Bostock and Clark, 1980).

We present here results obtained from a cytological analysis of the organization of DNA sequences and chromatin domains in the HSRs of the MTX-resistant mouse melanoma line PG19T3:$MTX_R.10^{-4}$ M (Bostock et al., 1979). By combining primed in situ hybridization (PRINS) with fluorescence in situ hybridization (FISH), we show that DHFR gene sequences are distributed throughout the HSRs, and are interspersed with

both major and minor mouse satellite DNA sequence families. Using immunofluorescence, we also show that a 26 kDa heterochromatin-associated protein, p26, immunologically related to heterochromatin protein 1 of *Drosophila* (Nicol and Jeppesen, 1994), is coincident with the major satellite sequences.

We have also investigated the acetylation of histone H4 within the HSRs, using immunofluorescence with an antiserum specific for acetylated histone H4. This approach has previously been shown to identify regions of potentially active chromatin in metaphase chromosomes (Jeppesen et al., 1992; Jeppesen and Turner, 1993). Antibody bound non-uniformly over the HSRs, where underacetylated, inactive chromatin domains, coincident with p26 localizaton and major satellite DNA sequences, are interspersed with acetylated, active chromatin domains containing the amplified DHFR gene copies.

## MATERIALS AND METHODS

### Cell culture

PG19T3 mouse melanoma cells, resistant to $10^{-4}$ M methotrexate (MTX) (Bostock et al., 1979), were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% (v/v) foetal calf serum. Two independent cell lines were maintained: one in the presence of $10^{-4}$ M MTX (Sigma), the other in the absence of MTX selection. No mycoplasma contamination was detected in 4′,6-diamidino-2-phenylindole (DAPI) stained cell preparations.

### Antibodies and immunofluorescence

The antibodies used were: CP, a CREST patient auto-immune serum which, as well as containing antibodies against centromeric antigens, also labels mammalian centric heterochromatin (Nicol and Jeppesen, 1994), and R5/12, a rabbit antiserum that recognizes histone H4 acetylated at Lys-12 (Turner and Fellows, 1989).

Metaphase cell preparation, cytocentrifugation and immunolabelling were all carried out as described previously (Jeppesen et al., 1992; Nicol and Jeppesen, 1994). For these experiments cells were diluted to ~$1.5\times10^5$ per ml and 500 µl samples centrifuged onto slides. Both CP and R5/12 were diluted 1/100 in 120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1% (v/v) Triton X-100 (KCM; Gooderham and Jeppesen, 1983), containing 10% (v/v) normal goat serum (NGS). Secondary antibodies were: FITC-goat anti-human IgG (Sigma) for CP or FITC-goat anti-rabbit IgG (Sigma) for R5/12, diluted 1/20 in KCM + 10% NGS.

### Primed in situ hybridization (PRINS)

The method used was based on that of Koch et al. (1989) as modified by Mitchell et al. (1992). Oligonucleotides were prepared using Applied Biosystems DNA Synthesizer Model 381A. Oligonucleotide 204 (5′-CACTTTAGGACGTGAAATATGGCGAGGAAAACTGA-3′) hybridizes to mouse major satellite DNA (Horz and Altenburger, 1981). Oligonucleotide 220 (5′-TCCCGTTTCCAACGAATG-3′) hybridizes to mouse minor satellite DNA and is the consensus murine CENP-B box sequence (Masumoto et al., 1989) with the addition of a G at the 3′ end.

PRINS hybridization was carried out on immunolabelled cell spreads and similarly prepared slides in which the immunolabelling was omitted, fixed in 4% formaldehyde (Jeppesen et al., 1992). Partial reversal of formaldehyde crosslinking was found previously to increase the efficiency of the PRINS labelling procedure (Mitchell et al., 1992). This was achieved by immersion of slides in 0.1 N NaOH (30 seconds), followed by brief washes in 10 mM Tris-HCl, pH 7.5, and distilled water. Slides were denatured in 70% formamide/2× SSC for 4 minutes at 70°C, dehydrated through an ethanol series, air dried and then preheated to the annealing temperature before application of the probe. The probe mixes contained 300 pmol of the oligonucleotides, and digoxygenin-11-dUTP (Boehringer Mannheim GmbH) replaced thymidine triphosphate in the nucleotide triphosphate mix. They were preheated to the annealing temperature for 10 minutes, Taq polymerase (Boehringer Mannheim GmbH) added and the probe applied to slides. Annealing temperature was 53°C for 10 minutes, and extension temperature was 64°C for 20 minutes. Slides were then immersed for 5 minutes each in 2× SSC containing 500 mM NaCl, 50 mM EDTA, and 0.05% (v/v) Triton X-100, followed by 2× SSC/0.05% Triton X-100 + 0.6% non-fat milk. Rhodamine conjugated anti-digoxygenin (Fab fragments; Boehringer Mannheim GmbH) was applied to slides in blocking solution at 1:100 dilution from the manufacturer's recommended stock concentration. Slides were incubated at room temperature for 1 hour, washed in 2× SSC/0.05% Triton X-100, then distilled water, and air-dried. Hoechst 33258 was used to counterstain DNA. Images were recorded either on a Leitz Ortholux microscope with HBO 50 W mercury vapour lamp using Kodak Tmax 400 film or on a Zeiss Axioplan fluorescence microscope with 100 W mercury source and photometric CCD camera.

### Combined PRINS and fluorescence in situ hybridization (FISH)

This was carried out on methanol/acetic acid (3:1) fixed spreads, using oligonucleotide 204 to incorporate digoxygenin-11-dUTP at the mouse major satellite by PRINS, followed by FISH with a biotinylated DHFR probe. The probe was prepared from plasmid pK14A (Brinton and Heintz, 1995), which has a 1.7 kb DHFR cDNA insert. This fragment was cut from the plasmid and labelled with biotin-16-dUTP (Boehringer Mannheim GmbH) by nick translation (Langer-Safer et al., 1982). Slides were taken through RNase treatment and ethanol dehydration as described for FISH (Fantes et al., 1992). Chromosomal DNA was denatured by immersing in 1 M NaCl, 30 mM NaOH, at 4°C for 45 minutes, neutralized with 10 mM Tris-HCl, pH 7.5, and air dried (Mitchell et al., 1993). The PRINS reaction was carried out up to the end of the extension stage at 64°C as described above. The slides were washed in 2×SSC at 37°C for 15 minutes, then 50 ng of biotinylated DHFR probe was applied and incubated overnight at 37°C (Fantes et al., 1992). Antibodies were applied as described previously for FISH (Fantes et al., 1992) and PRINS, but in this case the first antibody step was a combination of Avidin-FITC (Vector labs) to detect the biotin labelled FISH probe and rhodamine anti-digoxygenin to detect the PRINS hybridization. Another two antibody steps to amplify the FISH signal (Fantes et al., 1992) were then carried out. Slides were mounted in Vectashield mounting medium (Vector labs) containing 1 µg/ml DAPI and analysed on the Zeiss microscope system described above.

## RESULTS

### Stability of the HSRs

After several months passaging in the absence of selection the total amount of HSR material in the PG19T3:$MTX_R$ cells used in these studies was reduced and the ability of the cells to grow in $10^{-4}$ M MTX was diminished. The number of chromosomes containing HSRs appeared to stabilize at two or three per cell. Even with continued MTX selection a gradual change in the size and distribution of HSR blocks was apparent after extended culture. The total amount of HSR material remained roughly the same, contained in four or five chromosomes per cell, but there was a tendency for a redistribution from the larger to the smaller chromosomes. However, the sub-structure of the HSRs appeared to be identical cytologically, irrespec-

tive of MTX selection or passage number. The cells represented in this report have from three to five chromosomes containing HSRs, depending on when the experiment was carried out and whether they are from cells cultured in the presence or absence of MTX. A typical early passage cell with five HSR-containing chromosomes is shown in Fig. 1E, in which the HSRs exhibit their characteristically bright Hoechst fluorescence.

### Mouse satellite DNA

The distribution of mouse satellite DNA sequences in the chromosomes of MTX-resistant PG19T3 cells was investigated by PRINS (Koch et al., 1989). Fig. 1A shows that the labelling obtained using primer oligonucleotide 204, which hybridizes to mouse major satellite DNA (Pardue and Gall, 1969; Jones, 1970), is confined to pericentromeric heterochromatin and the HSRs, which are all strongly labelled, indicating high concentrations of major satellite DNA. The labelling within the HSRs has a striated appearance as if composed of bands of major satellite, rather than being one large block. Fig. 1C confirms the location of the HSRs by Hoechst staining. This result agrees with that of Bostock and Clark (1980), who used $^{125}I$ in situ hybridization to show that the HSRs were largely composed of satellite DNA sequences.

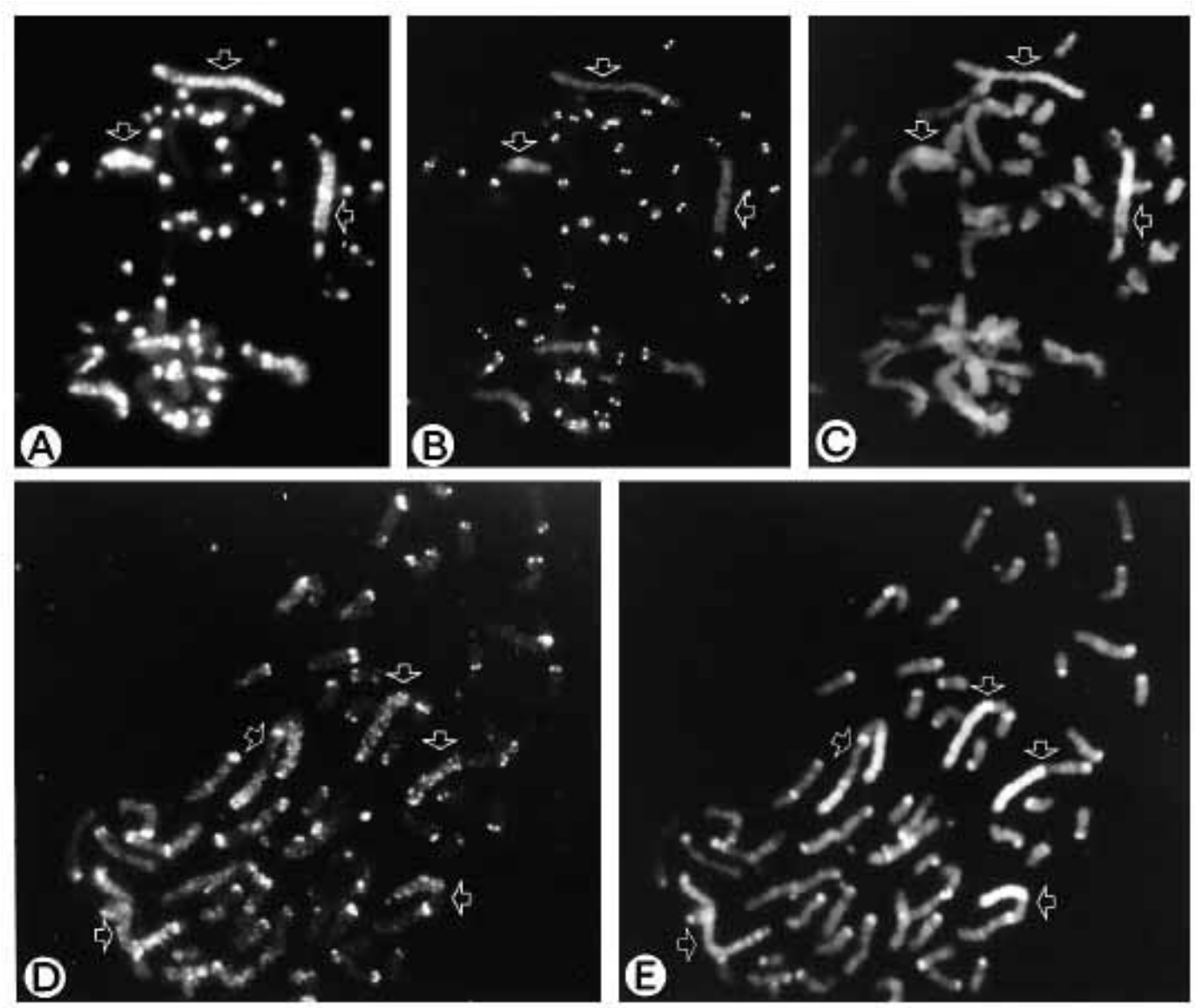


**Fig. 1.** (A-C) Co-localization of mouse major satellite DNA and centric heterochromatin in metaphase chromosomes of MTX-resistant PG19T3 cells demonstrated by combined PRINS and immunofluorescence. (A) PRINS labelling using oligonucleotide 204, showing localization of major satellite sequences in pericentric heterochromatin and striated labelling of HSRs. (B) The same chromosomes immunolabelled with CREST serum CP: in addition to labelling centromeres (double-dots), CP binds to an antigen, p26, that is located in pericentromeric heterochromatin, and which is also distributed along the HSRs. (C) Hoechst 33258 fluorescence of same field of view. The arrows point to large HSRs present in three chromosomes. (D,E) Localization of mouse minor satellite DNA in MTX-resistant PG19T3 chromosomes. (D) PRINS labelling using oligonucleotide 220: all chromosomes show minor satellite sequences at centromeres (double-dots); there is also punctate labelling along the HSRs. (E) Hoechst 33258 fluorescence of the same metaphase, showing HSRs as brightly fluorescent regions. Five HSR-containing chromosomes are identified by arrows. The cells in this figure were cultured in the absence of MTX.

Fig. 1D demonstrates the labelling pattern observed when oligonucleotide 220, which hybridizes to mouse minor satellite DNA (Pietras et al., 1983; Wong and Rattner, 1988), was the reaction primer for PRINS. The HSRs are again labelled, but this time the labelling is much less intense throughout the HSRs and appears to take the form of discrete dots. This is generally weaker than the labelling of centromeric minor satellite DNA. Fig. 1E shows the corresponding Hoechst stained spread confirming the location of HSRs.

The structure of the HSRs was also investigated using a CREST autoimmune serum, CP, which we have previously shown contains antibodies against a 26 kDa antigen that is localized in the pericentromeric heterochromatin of mouse and human chromosomes (Nicol and Jeppesen, 1994). This antigen, which we called p26, appears to be highly conserved among mammals, is antigenically related to heterochromatin-associated protein 1 of *Drosophila* (James and Elgin, 1986), and is probably identical to the chromodomain proteins M31 and HSM1 identified in mice and humans respectively (Singh et al., 1991; Wreggett et al., 1994). Immunolabelling of PG19T3:$MTX_R$.$10^{-4}$ M metaphase chromosomes with serum CP (Fig. 1B) shows the intense 'double dot' fluorescence at centromeres typical of CREST sera, and, in addition, a slightly weaker labelling of the adjacent centric heterochromatin that contains p26. Fig. 1B shows that the HSRs are also labelled with an intensity similar to centric heterochromatin, from which we deduce that the HSRs contain p26. Wreggett et al. (1994) also obtained labelling of the HSRs in PG19T3:$MTX_R$.$10^{-4}$ M cells using a monoclonal antibody raised against M31. In the double labelling experiment shown in Fig. 1A,B, p26 immunofluorescence and major satellite PRINS labelling are coincident. Although less pronounced than is seen in the PRINS labelling of major satellite sequences, there is an indication of a similar periodicity in the immunofluorescence of the HSRs with CP serum, consistent with co-localization. Bostock and Clark (1980) reported that there was a 66% increase in mouse satellite DNA content in PG19T3:$MTX_R$.$10^{-4}$ M cells compared with the parent line. Commensurate with this, we estimate from protein blotting experiments using CP serum that there is an approximate doubling in the amount of p26 in nuclear extracts from MTX-resistant PG19T3 cells (data not included). We have not determined if the increased p26 activity in these cells is due to increased transcription, or to amplification, of the p26 gene.

## DHFR gene sequences

Localization of the amplified DHFR genes within the HSRs of MTX-resistant PG19T3 cells was confirmed by FISH on methanol: acetic acid fixed spreads, using a biotin labelled probe. All the HSRs were found to contain DHFR gene sequences (data not shown). These results were confirmed and extended by a double label PRINS and FISH experiment, where the major satellite oligonucleotide 204 was used to incorporate digoxygenin-11-dUTP by PRINS, and biotin-16-dUTP labelled DHFR probe was used simultaneously to detect the DHFR DNA. A prometaphase cell labelled in this way is illustrated in Fig. 2. The DHFR gene sequences are labelled by FITC (green, Fig. 2A) and the major satellite by rhodamine (red, Fig. 2B). DAPI counterstaining of the chromosomes is shown in blue in Fig. 2C. The green, red and blue images (Fig. 2A-C) are superimposed in Fig. 2D. FISH labelling of DHFR genes (appearing white in Fig. 2D) has a more particulate appearance than the homogeneous signal produced by the PRINS technique. The DHFR genes are clearly located within

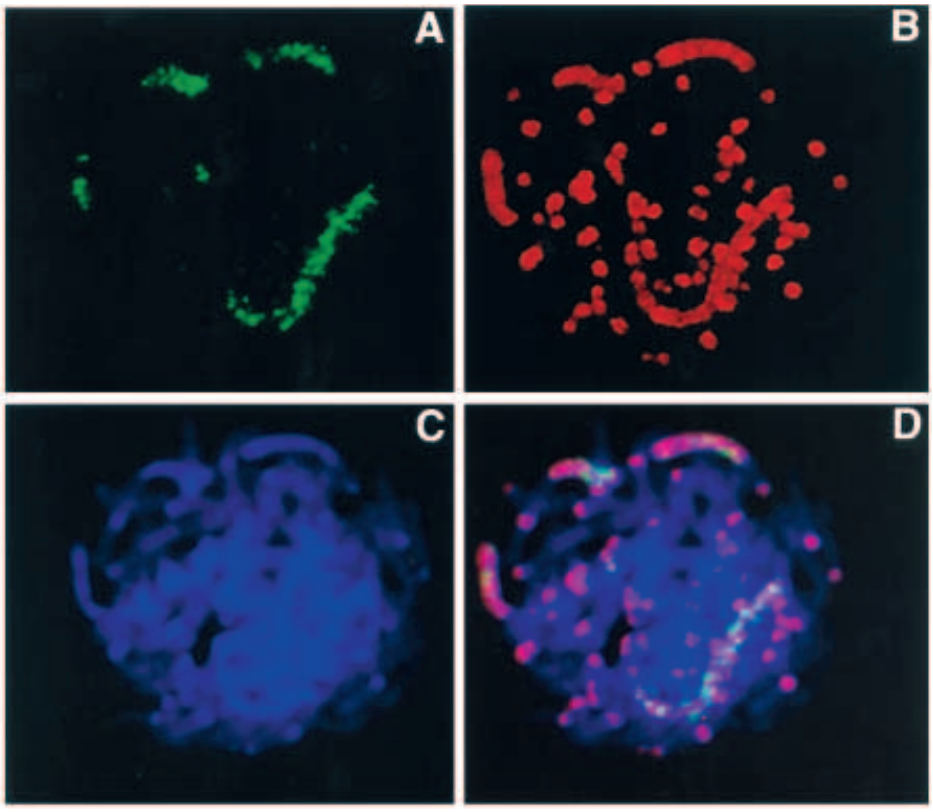


**Fig. 2.** Interspersion of DHFR genes and major satellite DNA in HSRs, shown by combined in situ hybridisation and PRINS in a prometaphase cell. (A) Localization of DHFR genes by FISH using a biotinylated cDNA probe. (B) Major satellite labelled by PRINS using oligonucleotide 204. (C) DAPI fluorescence of the same chromosomes. (D) Images (A-C) superimposed: the DHFR signal appears white, interspersed in the pink major satellite HSR arrays. Cells were cultured in the presence of $10^{-4}$ M MTX.

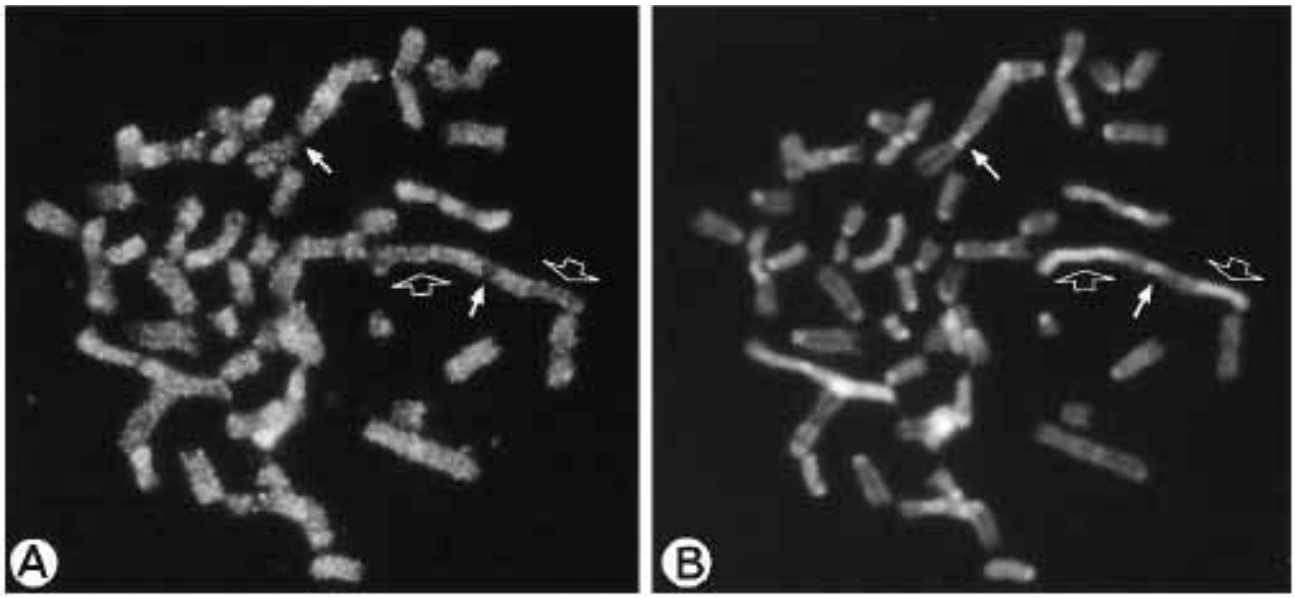


**Fig. 3.** Distribution of histone H4 acetylation in metaphase chromosomes of MTX-resistant PG19T3 cells determined by immunofluorescence. (A) Chromosomes immunolabelled with anti-acetylated H4 antiserum R5/12. (B) DAPI fluorescence of the same metaphase. Large arrows indicate two HSRs in a large chromosome, and small arrows point to the the centromere in this and another chromosome for comparison. Immunofluorescence in the HSRs is clearly above the level of centric heterochromatin, but less than the euchromatic region. Cells were cultured in the absence of MTX.

the region defined by the major satellite probe. Several regions show prominent DHFR labelling at gaps in the major satellite signal, indicating an interspersion of DHFR genes and satellite DNA.

## Histone H4 acetylation

The distribution of potentially active chromatin within the HSRs was investigated by labelling cell spreads with rabbit antiserum R5/12, which recognizes histone H4 acetylated at lysine-12 and binds preferentially to multiply acetylated H4 isoforms (Turner and Fellows, 1989). Unlike pericentromeric heterochromatin, whose level of H4 acetylation is not detectable by immunofluorescence with R5/12 (Jeppesen et al., 1992; Jeppesen and Turner, 1993), the HSRs showed positive labelling throughout their length using this antiserum with, once again, an indication of bands of fluorescence (Fig. 3A). In contrast, centric heterochromatin in both the HSR- and non-HSR-chromosomes shown in Fig. 3A is unlabelled. The overall intensity of anti-acetylated histone H4 immunofluorescence within the HSRs appeared lower than that in other regions of the chromosome arms, as clearly shown on the large chromosome arrowed in Fig. 3A. This is consistent with a major proportion of the HSR material corresponding to transcriptionally inactive satellite DNA sequences, as shown by PRINS labelling, and histone H4 acetylation being confined to potentially active chromatin domains containing the DHFR genes. Interspersion of DHFR DNA with major satellite DNA (Fig. 2D) effectively dilutes the concentration of active chromatin in the HSRs compared with a typically acetylated euchromatic region.

To confirm the interspersion of major satellite and potentially active chromatin domains, cell spreads were first labelled with R5/12, after which the major satellite was detected by PRINS. Fig. 4 illustrates typical HSR-chromosomes labelled in this way. In Fig. 4A, R5/12 labelling (green) shows acetylated histone H4 present along the chromosome arms, but virtually absent in centromeric regions. The HSRs are generally less intensely immunofluorescent than euchromatin,

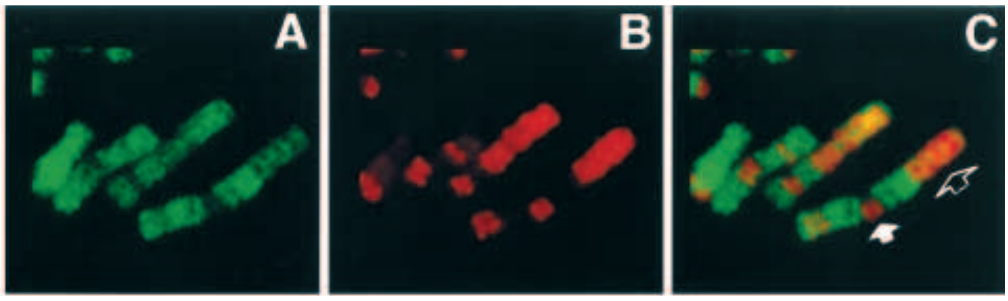


**Fig. 4.** Interspersion of active chromatin and major satellite domains in HSRs shown by combined immunofluorescence and PRINS. (A) Part of metaphase showing immunolabelling of acetylated histone H4 by antiserum R5/12. (B) PRINS labelling of major satellite DNA in the same chromosomes using oligonucleotide 204. (C) Images A and B superimposed. In the HSR indicated by the open arrow, domains of histone H4 acetylation appear yellow, and are clearly interspersed with major satellite DNA sequences (red). The regular striated appearance of anti-acetylated H4 immunolabelling in the HSR is evident in (A). In contrast, the centromere indicated by the filled arrow shows an overall lack of H4 acetylation. Cells were cultured in the absence of MTX.

and exhibit a regular banded appearance. Fig. 4B shows in red the localization of major satellite DNA sequences at the centromeres and throughout the HSRs. Fig. 4C combines the two previous images, and demonstrates that domains of acetylated histone H4 alternate with major satellite within the HSRs. The similarity in distribution of H4 acetylation and DHFR DNA (Fig. 2), supports the conclusion that the DHFR genes are located in active chromatin domains, which are distinguished by acetylation of histone H4. These are interspersed with under-acetylated, inactive chromatin domains containing satellite DNA sequences. Attempts to demonstrate directly the co-localization of R5/12 binding and DHFR DNA by double-labelling proved inconclusive owing to a reduction in the efficiency of FISH when applied to immunolabelled chromosomes.

## DISCUSSION

The mouse melanoma PG19T3:$MTX_R$.$10^{-4}$ M cell line was selected for its ability to grow in $10^{-4}$ M MTX, resistance to the drug being due to as much as a 1,000-fold increase in activity of the enzyme dihydrofolate reductase (Bostock et al., 1979). We have analyzed the HSRs evident in the karyotype of these cells by new cytogenetic techniques that were not available to those authors, with particular emphasis on chromatin organization. Bostock and Clark (1980) showed that mouse satellite DNA was the main constituent of the HSRs, in which they also observed a cytologically resolvable periodic sub-structure. Using FISH to localize DHFR gene sequences and PRINS to label satellite DNA, we show that the periodicity is a consequence of the interspersion of DHFR gene repeats with larger blocks of mouse major satellite DNA throughout the HSRs of these cells (Fig. 2). This is the first direct evidence by in situ hybridization that amplified DHFR genes are present in the HSRs of this particular MTX-resistant cell line. Bostock and Clark (1980) estimated that satellite DNA comprised roughly 60% of the HSRs, and that the mean size of the amplicon or amplified repeat unit (satellite plus coding sequences) was about 3 Mb, assuming one copy of the DHFR gene per repeat. Experience suggests that the limit of resolution at the cytological level on metaphase chromosomes is in the range 1-3 Mb. The striations that we observe within the HSRs using all of the labelling procedures described here vary slightly in spacing and appear to merge in places, confirming an average repeat unit size of approximately 3 Mb. This organization is common to large HSRs with 20 or more resolvable repeats (e.g. Fig. 1A), and to shorter HSRs from cells maintained in long-term culture in the absence of MTX selection, containing fewer resolvable striations (e.g. Fig. 4). Thus, about 1 Mb of non-satellite DNA could be accommodated per repeat. This amount is considerably in excess of the size of the DHFR gene, which has been reported to be approximately 31 kb (Crouse et al., 1982). Genes that are co-amplified along with DHFR have been identified in MTX-resistant Chinese hamster cells (Mitchell et al., 1986; Foreman and Hamlin, 1989), but it is not known if this is also true in the PG19T3:$MTX_R$.$10^{-4}$ M mouse line.

The amplicon size in PG19T3:$MTX_R$.$10^{-4}$ M cells is much larger than has been reported for other MTX-resistant cell lines (e.g. Nunberg et al., 1978; Dolnick et al., 1979; Heintz and Hamlin, 1982). Probably as a consequence, the HSRs constitute a much larger proportion of total cellular DNA (as much as 10%; Bostock et al., 1979). The large number of HSR-containing chromosomes in PG19T3:$MTX_R$.$10^{-4}$ M cells compared to other MTX-resistant lines is probably explained by the need to accommodate this amount of amplified chromatin in chromosomes capable of functioning normally (e.g. during segregation at mitosis). The largest chromosome in this cell line, which is almost entirely composed of HSR material (R1; Bostock et al., 1979), appears to be unstable in long-term culture, even when the cells are kept under constant MTX selection: this chromosome may well be above the optimum size. While under selection, cells need to maintain the copy number of their amplified DHFR genes, but further chromosomal rearrangements to reduce chromosome size may facilitate HSR propagation through the cell cycle. This might explain the apparent instability of the karyotype in this cell line, even with MTX selection.

Our studies also show that a small but significant fraction of the HSRs consists of interspersed mouse minor satellite DNA, amplified along with the major satellite and DHFR gene sequences. Oligonucleotide 220, the primer used for detecting minor satellite sequences by PRINS, is the consensus DNA sequence of the murine ‘CENP-B box’, which is the binding site for CENP-B, a protein found at the centromeres of human and mouse chromosomes (Masumoto et al., 1989; Pluta et al., 1992). We have shown that the HSR-containing chromosomes are relatively stable in short term culture. This would not be the case if the CENP-B box sequences distributed along the HSRs resulted in additional functional centromeres, which would interfere with chromosome segregation at mitosis. (The karyotypic instability in long-term culture that we report is probably a function of chromosome size, as discussed above.) The presence of CENP-B box sequences at sites removed from the functional centromere has been observed previously in DBA/2 mice, which have amplified minor satellite arrays on chromosome 1 (Mitchell et al., 1993).

The subdivision of the HSRs of MTX-resistant PG19T3 cells by DNA content into alternating non-coding satellite sequences and potentially transcribable DHFR gene sequences is paralleled by the analysis of HSR chromatin organization with antibodies capable of distinguishing protein markers for different chromatin types. Using rabbit antiserum R5/12, which recognizes histone H4 when acetylated at lysine-12 and binds preferentially to the more highly acetylated isoforms (Turner and Fellows, 1989), we show that the mouse major satellite sequences are coincident with underacetylation of histone H4, a distinguishing characteristic of both constitutive and facultative heterochromatin in mammals (Jeppesen et al., 1992; Jeppesen and Turner, 1993). We have also used human autoantibodies to demonstrate that an antigen p26, shown previously to be a mammalian homologue of *Drosophila* HP1 and exclusively associated with pericentromeric heterochromatin in mouse and human chromosomes (Nicol and Jeppesen, 1994), co-localizes with the major satellite sequences in the HSRs of PG19T3:$MTX_R$.$10^{-4}$ M cells. Thus, the mouse major satellite sequences, which form the predominant component of the HSRs, seem to be organized in heterochromatin-like domains, giving the HSRs an overall heterochromatic appearance (Bostock et al., 1979; Bostock and Clark, 1980).

In contrast, the DHFR gene sequences in the HSRs of these

cells are associated with chromatin in which histone H4 is highly acetylated. Acetylation of core histones has long been associated with transcriptional activity (reviewed by Csordas, 1990; Turner, 1991). In chick erythrocytes it has been shown by immunoprecipitation of acetylated nucleosomes that transcribed and potentially transcribable ('poised') globin genes are enriched in the acetylated fraction, whereas the non-transcribed ovalbumin gene is not (Hebbes et al., 1988, 1992). Using immunofluorescence with rabbit antisera recognizing site-specific acetylation of histone H4 to label unfixed mammalian cells in situ, we have demonstrated that H4 acetylation in potentially transcribable euchromatic domains persists through to metaphase, and provides a cytogenetic marker of potential genetic activity (Jeppesen and Turner, 1993). The similar distributions of histone H4 acetylation and DHFR gene sequences in the HSRs of PG19T3:$MTX_R$.$10^{-4}$ M cells is entirely consistent with our previous results, and provides further support for the link between histone acetylation and potentially transcribable, or active, chromatin. The active domains may contain other transcribed sequences in addition to DHFR genes, but so far none has been identified.

It is interesting to note that, despite being embedded in satellite DNA, DHFR genes are stably expressed in the HSRs of MTX-resistant PG19T3 cells. In thymidine kinase (tk) deficient strains of mouse L cells transfected with the herpes *tk* gene, revertants were unstable if *tk* DNA became integrated into satellite DNA (Butner and Lo, 1986; Talarico et al., 1988). This was interpreted as *tk* gene repression through the spreading of heterochromatinization, analogous to position-effect variegation (PEV) in *Drosophila* (Spofford, 1976; Henikoff, 1990). Mutations in the gene for HP1 suppress PEV (Eissenberg et al., 1990), indicating a functional role for HP1 in the formation of heterochromatin. In the HSRs of MTX-resistant PG19T3 cells, we show that coding DNA containing DHFR genes alternates with satellite DNA that is associated with a mouse homologue of HP1. Gene repression may spread over 1 Mb in *Drosophila* (Henikoff, 1990), which, if repeated in the HSRs of MTX-resistant PG19T3 cells, would be sufficient to inactivate the 1 Mb of non-satellite DNA estimated to be present in an average amplicon. However, there is no evidence for instability of DHFR expression in PG19T3:$MTX_R$.$10^{-4}$ M cells, which retain a high level of DHFR activity even after extended passage without MTX selection (Bostock et al., 1979), when reduced DHFR expression could be tolerated. In this study too, we have followed MTX-resistant PG19T3 cells over many months of culture in the absence of MTX without observing any change in the overall density or distribution of H4 acetylation in the HSRs that might suggest a reduction in active chromatin content. The apparent difference between the instability of the *tk* gene transfectants referred to above and the stability of DHFR expression in the HSRs of MTX-resistant PG19T3 cells may indicate specific barriers isolating the active chromatin domains within the HSRs of these chromosomes. Alternatively, mammalian chromosomes may be more resistant to the spread of heterochromatinization than those of *Drosophila*, and although the few kilobases corresponding to a herpes *tk* gene might be susceptible to repression, the estimated 1 Mb blocks of non-satellite DNA containing the DHFR genes in the HSRs of PG19T3:$MTX_R$.$10^{-4}$ M cells are too large to be significantly affected by the spread of inactivation.

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