An extra band within the human 9qh+ region that behaves like the surrounding constitutive heterochromatin

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Abstract

An extra variant G band in a human 9qh+ region was analysed in normally condensed and 5-azacytidine undercondensed chromosomes. Fluorescence in situ hybridisation showed that specific, classical, alphoid and beta satellite DNA was not present. Nevertheless, this extra band behaves like the surrounding heterochromatin because (1) its chromatin fibres showed condensation inhibition after 5-azacytidine treatment, as confirmed by electron microscopy, and (2) it was not affected by in situ digestion with the restriction endonucleases *AluI* and *Sau3A*. These results suggest that this variant band may correspond to euchromatin that has become inactivated by a position effect.

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An extra euchromatic G band within the large heterochromatic pericentromeric region in the long arm of human chromosome 9 is a well recognised normal variant.¹⁻⁴ Around 1.4% of persons possess this euchromatic heteromorphism, which is inherited in almost all cases.^{5,6} However, this extra band is not easily detected in all cells of a carrier, because of its small size.

Most studies have described chromosomes from one or a few persons or families, as ascertained by conventional banding procedures. Although this extra band is thought to be non-heterochromatic, there is no information on its ultrastructural organisation and functional behaviour in relation to chromatin folding. Furthermore, a more precise biochemical characterisation would be of interest in understanding its integrated structure and function. Here we analyse the sensitivity of this 9qh variant region to 5-azacytidine, a cytosine base analogue which produces chromatin undercondensation under certain experimental conditions. Electron microscopy of whole mounted chromosomes showed the chromatin fibre organisation of this area. Selective in situ digestion with restriction endonucleases and fluorescence in situ hybridisation (FISH) with classical alphoid and beta satellite DNA probes specific for chromosome 9 were also performed. The results suggest that this extra band is non-heterochromatic, although its structural and functional behaviour appears similar to that of surrounding heterochromatin.

Materials and methods

CULTURES AND RESTRICTION ENDONUCLEASE DIGESTION

Human peripheral lymphocyte cultures from a clinically healthy male with an extra band in the 9qh region were set up using standard techniques. He was referred because his wife had a fetus with multiple physical malformations which miscarried at 15 weeks' gestation. Undercondensation of constitutive heterochromatin regions of chromosomes 1, 9, 15, 16, and Y was induced by a 5-azacytidine treatment (3.5×10^{-7} mol/l) during the last seven hours before harvesting.⁷ Chromosome preparation and GTG banding were performed by standard procedures. In situ digestion of metaphase spreads with the restriction endonucleases *AluI* and *Sau3A* (Boehringer Mannheim) was performed in both 5-azacytidine treated and untreated cultures. The slides were incubated overnight in a moist chamber at 37°C with 100 µl of the appropriate incubation buffer, and 30 units of each enzyme. Finally, they were washed in phosphate buffer and stained with 3% Giemsa in phosphate buffer. Around fifty mitoses were assessed in each procedure.

ELECTRON MICROSCOPY

The procedure for whole mounted chromosomes has been described previously.⁸ In brief, after hypotonic treatment, the cells were centrifuged at 1200 rpm for five minutes. The undisturbed pellet was covered with 3 ml of isolation buffer (1% Triton X-100, 1% citric acid, and 6 mmol/l MgCl₂) for 30 minutes. Chromosomes were isolated by gently syringing seven to ten times through a No 22 gauge needle. The chromosome suspension was centrifuged at 2000 rpm for five minutes onto Formvar coated electron microscope grids. The grids were immersed into successive 50, 75, and two 100% ethanol baths for five minutes each. Finally they were immersed twice for five minutes in baths of 100% amyl acetate, air dried, and examined with a Zeiss 109 Turbo electron microscope operated at 50 kV.

FISH

Slides were treated with RNase for one hour at 37°C, washed in $2 \times$ SSC, dehydrated in an ethanol series, and denatured in 70% formamide/ $2 \times$ SSC at 70°C for three minutes. After cold 70, 80, 90, and 100% ethanol baths, the slides were air dried.

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Biotinylated DNA probes specific for chromosome 9 were obtained from Oncor Inc. A D9Z1 locus probe (classical satellite DNA), a D9Z5 locus probe (beta satellite DNA), and an alphoid probe, included in a cocktail of human alpha sequences also available from Oncor Inc, were denatured in the hybridisation mix at 70°C for 10 minutes and spread on denatured chromosomes. Hybridisation conditions and stringency washes were performed according to the recommended schedules. Hybridisation signals were detected by fluorescein labelled avidin. Finally, chromosomes were counterstained with propidium iodide and examined under epifluorescence using a dual bandpass filter.

Results and discussion

5-azacytidine is a base analogue that inhibits DNA methylation at cytosine residues. When present in lymphocyte culture medium during the last few hours before harvesting, it induces a selective inhibition of condensation of the highly methylated chromatin fibres corresponding to the late replicating heterochromatin of human chromosomes 1, 9, 15, 16, and Y.⁷ In our case, after the 5-azacytidine treatment, the chromosome 9 containing the larger quantities of constitutive heterochromatin showed a

greater level of undercondensation than its homologue, within the same mitotic cell (fig 1A). The chromosome 9 with the greatest amount of constitutive pericentromeric heterochromatin possesses a small extra G band within this heterochromatic block (fig 1B). The extra G band appears to be maintained inside the undercondensed area, but its normal condensation is inhibited, thus achieving the same intensity of condensation as that of the surrounding heterochromatin (fig 1C). Electron microscopy of this chromosome confirmed the generally homogeneous undercondensation of the whole pericentromeric heterochromatic region (fig 1G). Nevertheless, a less electron dense region, corresponding to the extra band, is present in the middle of this area, in which the chromatin is arranged in longitudinal fibres. This is an expected consequence of inhibition folding.⁹ It is remarkable because, under our experimental conditions, 5-azacytidine would not be expected to be incorporated into this euchromatic band unless it replicated late, as is the case in the inactive contiguous heterochromatin. This less electron dense area clearly corresponds to the extra band because it has not been visualised under electron microscopy on other normal undercondensed 9qh regions.¹⁰ Furthermore, it correlates with the less stained region observed in the middle of the undercondensed area after Giemsa staining (fig 1A).

In situ digestion with certain restriction endonucleases, like *AluI*, results in a C like banding pattern, but only in certain human chromosomes.¹¹ Thus, constitutive heterochromatin banding in chromosome 9 by *AluI* appears as a consequence of the cleavage of specific target DNA sequences present in the rest of the chromosome material, followed by removal of the fragments. In our experience *AluI* digestion produces sharper and cleaner images than with conventional C banding and yields better resolution with very little distortion in chromosome morphology.¹² Strikingly, *AluI* can detect euchromatic regions within the pericentromeric heterochromatin in mouse chromosomes, which are otherwise undetected by conventional C banding.¹³ However, after *AluI* digestion, the variant heterochromatic area of our chromosome 9 appears as a homogeneous block, without any internal differentiation, even at the high resolution afforded by undercondensed chromosomes in all mitoses examined (fig 1D, E). Another restriction enzyme, *Sau3A*, which induces highly specific banding of constitutive heterochromatin of chromosome 9, also failed to digest the euchromatic band (fig 1F).

FISH, using a classical satellite DNA probe, D9Z1 locus, specifically delineated the pericentromeric heterochromatin. Differences in the size of both chromosomes are partially explained in terms of differences in the amount of this chromosome domain (fig 2A). Nevertheless, a gap in the hybridisation signal is clearly visible within the target region, mainly after the 5-azacytidine treatment. This region corresponds to the extra G band (fig 2A, B, C). A similar lack of staining has been reported when the satellite III area is shown by a silver-Ag

Figure 1 Metaphases showing a chromosome 9qh+ with a variant band (arrow) and its homologue (arrowhead) from 5-azacytidine treated and untreated cultures. (A) Giemsa staining. A less stained area is shown in the middle of the undercondensed 9qh+ region. (B, C) G banding. After 5-azacytidine treatment, the extra band appears undercondensed (C). After *AluI* incubation, no digestion is detected within the heterochromatic area of the variant chromosome, either in condensed (E) or 5-azacytidine undercondensed chromosomes (D). In situ digestion with *Sau3A* resulted in similar images of this chromosome 9 (F). (G) Electron micrograph of a whole mounted 5-azacytidine treated chromosome 9 variant, showing a roughly homogeneous undercondensation of the qh region. The area between the arrows, corresponding to the extra G band, shows less electron density and fewer longitudinal fibres. The area between the arrow heads identifies the highly condensed centromere.

Figure 2 FISH of satellite DNA probes specific for chromosome 9 on the variant chromosome (arrow) and its homologue (arrowhead). FISH of a D9Z1 locus probe delineates a non-classical satellite DNA domain within the hybridised pericentromeric area of the variant chromosome, either in condensed (A) or undercondensed chromosomes (B, C). Note that alpha satellite DNA probe appears hybridised on a compact domain close to the short arms (D). Specific beta satellite DNA probe is localised in this same region.

procedure that detects certain specific proteins or DNA-protein interactions.⁵ The extra band might be composed of other satellite DNA sequences, but neither the alpha nor the beta satellite DNA domains of chromosome 9 are susceptible to undercondensation by 5-azacytidine. Furthermore their location by FISH is restricted to the centromere, close to the short arms (fig 2D).^{14 15}

Therefore, the extra band is composed of chromatin fibres whose DNA does not correspond to the constitutive heterochromatin within which it is located. Nevertheless, this extra band shows similar behaviour to that of pericentromeric heterochromatin, because it is homogeneously undercondensed by the 5-azacytidine treatment and is not digested by the restriction enzymes *AluI* and *Sau3A*. The target sequences of this area might be integrated in a conformation of chromatin inaccessible to cleavage or extraction, as is the case for the contiguous heterochromatin. This conformation might be dependent on factors that inactivate the whole region structurally and functionally. Thus, the immersed band would be processed by a position effect in the same way as the constitutive heterochromatin. The response to 5-azacytidine suggests that DNA methylation is one possible factor involved in the inactivation.

These results support the hypothesis that the variant band may represent inserted or amplified inactivated euchromatin^{16 17} rather than altered condensation (“euchromatisation”) of a block of heterochromatin.

A similar study on an extra G band within the human chromosome 9 constitutive heterochromatin has recently been published by Verma *et al.*¹⁸ Their results and conclusions are in general agreement with our findings. Nevertheless, in their case the extra band was digested by *AluI*, and alphoid and beta satellite DNA probes hybridised at two positions on the aberrant chromosome, indicating its dicentric nature. This discrepancy could be dependent on a different origin or nature of these two variant bands, as suggested by the authors.

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