Difference in constitutive heterochromatin behaviour between human amniocytes and lymphocytes detected by a sequential in situ exonuclease III digestion-random primer extension procedure

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Abstract

Fixed chromosomes from human amniotic fluid cells and peripheral blood lymphocytes were digested in situ with exonuclease III and the single stranded DNA obtained was used as template for an in situ random primer extension. Under these conditions an R banding pattern, more evident in lymphocytes than in amniocytes, was obtained. Nevertheless, constitutive heterochromatin of chromosomes 1, 16, Yq, and mainly the pericentromeric region of chromosome 9 was far more intensely labelled in amniocytes than in lymphocytes. Fluorescence in situ hybridisation with a specific classical satellite DNA probe, showed that this differential labelling was dependent on a greater sensitivity of chromosome 9 constitutive heterochromatin to exonuclease III digestion in amniocytes than in lymphocytes, thus indicating qualitative differences in this region between both human cellular materials.

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Chromosome banding procedures are powerful tools in clinical cytogenetics since they reproduce specific longitudinal differentiation in each single human chromosome. Additionally, they can show interesting data on chromosome structure and function.¹ Recently, molecular approaches, such as digestion with restriction endonucleases or nick translation, have been successfully applied in situ on fixed chromosomes, resulting in several specific banding patterns.^{2,3}

There are three general rules that apply to most of the banding procedures. (1) All banding techniques applied to human chromosomes result in a G banding (intercalary heterochromatin), an R banding (euchromatin), or a C or C-like banding (constitutive heterochromatin) pattern. (2) C banding selective for certain human chromosomes, or an overlap of G with several C banding patterns, can simultaneously be obtained. However, conventional C banded preparations using the barium hydroxide technique sometimes show a residual R banding.⁴ Simultaneous euchromatin and specific constitutive hetero-

chromatin banding for certain human chromosomes is not easily obtained. (3) The banding pattern resulting from a specific procedure is quite consistent across all human cellular types.

In the present paper we present a modified in situ random primer extension procedure on exonuclease III treated chromosomes to show that there are exceptions to items (2) and (3) mentioned above. In fact, in the model we have tested, R bands coexist with labelled constitutive heterochromatin in chromosomes 1, 9, 16, and Yq. Moreover, this is shown to be specific to human amniocytes, human lymphocytes producing a different banding pattern.

Materials and methods

CULTURES AND METAPHASE PREPARATION

Human amniotic fluid cells from four persons were obtained by amniocentesis, performed at 14 to 16 weeks' gestation, and cultured at 37°C in the dark with 10ml Ham's F10 medium supplemented with 10% fetal calf serum, antibiotics, L-glutamine, hepes-buffer pH 7.2, and ultrosor G. After a week of incubation, the amniocytes were subcultured, and harvested two days later by the trypsin suspension method or mechanically detached.

Human peripheral blood lymphocytes were obtained from four normal persons, ranging from newborn to 35 years old. Cultures were set up for 72 hours at 37°C with 4 ml TC199 medium supplemented with 10% fetal calf serum, antibiotics, and phytohaemagglutinin.

Both amniocytes and lymphocytes were arrested at metaphase for three hours with 0.5 µg/ml colchicine, treated for 10 minutes with 0.075 mol/l KCl at 37°C, fixed three times in methanol:acetic acid (3:1), and spread on opposite sides of the same glass slide by the air dry method.

EXONUCLEASE III (EXO III) DIGESTION-RANDOM PRIMER EXTENSION PROCEDURE

Recently spread chromosomes were incubated at 37°C for 20 minutes in a moist chamber with 150 units of *E coli* Exo III (Stratagene) in 50 µl of incubation buffer. Slides were thoroughly washed in 2 × SSC, pH 7, dehydrated in cold 70, 90, 100% ethanol baths, and air dried.

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In situ random primer extension was performed using a multiprime DNA labelling kit (Amersham) in which dTTP had been replaced with biotinylated-16-dUTP (Boehringer Mannheim). The reaction was carried out with 30 µl of the labelling mixture in a moist chamber at 37°C for 75 minutes. The slides were washed twice with 2 × SSC, pH 7, for three minutes and once in PBD buffer. After a five minute incubation with a blocking solution from a chromosome in situ hybridisation kit (Oncor, Inc), the slides were incubated for 20 minutes in a moist chamber at 37°C with 60 µl of fluorescein labelled avidin solution (Oncor, Inc). Finally, they were washed in PBD buffer three times for two minutes each, mounted in an antifade solution, and visualised under epifluorescence.

Three control experiments were performed: (1) the chromosomes were not digested with Exo III; (2) the Klenow fragment of DNA polymerase I from *E coli* was not added to the reaction mix; and (3) primers were not added to the reaction mixture.

FLUORESCENCE IN SITU HYBRIDISATION (FISH)

After Exo III digestion, some dehydrated slides were incubated at 37°C with 30 µl of a classical satellite DNA probe specific for chromosome 9, D9Z1 locus, biotin labelled (Oncor, Inc). This probe had been previously denatured in the hybridisation mix at 70°C for 10 minutes.

Figure 1 Metaphase spreads from human amniotic fluid cells (A,B) and from a human lymphocyte (C) processed by Exo III digestion-random primer extension. Amniocyte chromosomes show a faint R banding pattern coexisting with highly labelled constitutive heterochromatin, mainly of chromosome 9 (arrows) and of chromosomes 1, 16, and Yq. However, lymphocyte chromosomes show only a sharp R banding pattern.

Control slides were denatured in 70% formamide/2 × SSC at 70°C for three minutes, dehydrated in an increasing ethanol series, and incubated with the same probe. All slides were washed in 50% formamide/2 × SSC, pH 7, at 43°C for 15 minutes and in 2 × SSC, pH 7, at 37°C for eight minutes. The bound probe was detected by treatment with two layers of fluorescein labelled avidin, intercalating a layer of biotinylated goat anti-avidin. Finally, chromosomes were counterstained with propidium iodide, mounted in an antifade solution, and visualised with a Zeiss photomicroscope.

Results and discussion

Exo III catalyses the removal of nucleotides from double stranded DNA, beginning with a free 3' hydroxyl end or nick.⁵ When used in situ on methanol-acetic acid fixed and spread air dried chromosomes, this enzyme removes nucleotides at spontaneously occurring nicks and breaks in the DNA. This results in extensive single stranded DNA motifs that are capable of acting as targets for in situ hybridisation of biotinylated DNA probes.⁶ Consequently, these single stranded DNA areas can also hybridise with oligonucleotides of random sequences, which can then be extended by the Klenow fragment of *E coli* DNA polymerase I.⁷ Incorporation of biotinylated nucleotides by the polymerase could be finally detected under fluorescence microscopy using a fluorescein tagged streptavidin. When using this method, all recently spread mitoses from amniotic fluid cells showed a faint R banding pattern (fig 1 A,B). Surprisingly, this euchromatin banding coexisted with highly labelled pericentromeric constitutive heterochromatin of chromosome 9. Constitutive heterochromatin of chromosomes 1, 16, and Yq was also highlighted, though less intensely than in chromosome 9 (fig 1 A,B). Nevertheless, when chromosomes from human peripheral blood lymphocytes were similarly processed on the same slide, only a highly contrasted R banding pattern was obtained (fig 1C). These R bands are similar to those obtained on thermally denatured human lymphocyte chromosomes when they are in situ random primed and extended.⁸

Our control experiments with lymphocyte chromosomes showed that both the Exo III digestion and the Klenow polymerisation were necessary for the differential R labelling, although it was very faint. Initially, it appears that random primers were not essential but this differential labelling was greatly strengthened when they were added. Possibly the euchromatic bands would be more sensitive to Exo III digestion, giving more single stranded DNA areas which are then filled by the Klenow polymerase, mainly after the hybridisation of random primers. The differential sensitivity between R and G bands to Exo III could be explained in terms of a distinct chromosome organisation in both regions. For example, DNase I sensitivity studies show different behaviour of both chromosome domains⁹ and the nick translation experiments using DNase I and restriction endonucleases sensitive to methyl-

ation of CpG doublets¹⁰ also support this point of view. In this case, short or long treatments of nucleases modulate the extraction of DNA on the chromosomes, and while short treatments produce R bands, long treatments give rise to G bands. This clearly indicates that while the chromatin on R bands is very sensitive to nuclease activity, longer treatments remove the DNA of more R bands and leaves the DNA housed in the G bands targeted for *in situ* nick translation. Obviously, sensitivity to Exo III digestion is not the only factor contributing to the final labelling. In fact, we have found that centromeric areas of lymphocyte chromosomes are highly digested by Exo III and they are unlabelled when *in situ* random primer extension is performed after Exo III digestion (fig 1C). Possibly local organisation and the presence of specific primers in the labelling mix in a sufficient quantity to hybridise in the respective target areas obtained after Exo III digestion, may be important factors in the final labelling.

As previously described, the constitutive heterochromatin of chromosome 9, and that of chromosomes 1, 16, and Yq to a lesser extent, was far more labelled in amniotic fluid cells than in lymphocytes. In this case, this result was strongly related to a differential sensitivity to Exo III digestion. Thus, this hypothesis was confirmed by FISH of a classical satellite DNA probe specific for chromosome 9, D9Z1 locus. After Exo III digestion, the intensity of the hybridisation signal of the probe was clearly weak in lymphocyte chromosomes in all mi-

tosomes examined, while it was as intense as thermally denatured chromosomes in the case of amniocyte chromosomes (fig 2). FISH performed on undenatured or undigested chromosomes failed to show hybridisation signals. Then the pericentromeric constitutive heterochromatin of chromosome 9 from amniocytes was more fully digested by Exo III than in lymphocytes, resulting in more single stranded DNA areas, thus allowing a more efficient *in situ* hybridisation of the probe.

The differential result after Exo III digestion and random priming or FISH could be dependent on culture preparation conditions. Nevertheless, it is not so since other conventional banding procedures resulted in similar patterns in both cellular materials and both were processed simultaneously on the same glass slide. Overall, Exo III clearly detects a qualitative difference in the same regions of constitutive heterochromatin between amniocytes and lymphocytes, reflecting a different organisation of these sequences, possibly mediated through interactions with cell specific DNA associated proteins. It could be assumed that DNA fractions contained in the constitutive heterochromatin of both types of chromosomes do not show sequence differences that could affect Exo III activity. According to our results it seems more likely that the DNA from lymphocytes would show a more compact organisation inside the constitutive heterochromatin domains of chromosomes 1, 9, 16, and Yq, possibly dependent on a greater local concentration of specific proteins, which

Figure 2 FISH with a classical satellite DNA probe specific for human chromosome 9 (arrows), on Exo III digested amniocyte (A) and lymphocyte (B) chromosomes and on conventionally thermal denatured chromosomes (C). Exo III digestion of amniocyte chromosomes results in sufficient single stranded DNA in pericentromeric heterochromatin of chromosome 9 to allow hybridisation of the probe as intense as that in thermally denatured chromosomes. Otherwise, this pericentromeric heterochromatin is not digested as efficiently in lymphocyte chromosomes, thus showing a less intense hybridisation signal. Picture B has been overexposed to collect the weak fluorescein signal.

would obstruct the accessibility of Exo III. It is well known that these particular DNA fractions are highly methylated and they show themselves to be undercondensed after treatment with the demethylating agent 5-azacytidine, in the case of human lymphocytes. On the other hand, human amniotic fluid cells have been shown to be insensitive to 5-azacytidine treatment.¹¹ The highly methylated DNA domains in human lymphocyte chromosomes may bind specific proteins which could stabilise their high order structures.^{12,13} The absence of such proteins or their replacement by analogues would explain our results and those obtained by Schmid *et al*¹¹ after treatment of amniotic fluid cells with 5-azacytidine. In fact, Haaf *et al*¹⁴ reported a non-histone protein which is cell type specifically associated with constitutive heterochromatin. Although it remains to be shown directly, indirect evidence suggests that these types of proteins, or others which are not homogeneously distributed throughout the chromosomes (for example, those in the High Mobility Group¹⁵), may interfere differentially with DNA digestion by certain nucleases. For example, it is known that isoschizomers used under the same conditions of digestion may produce a different banding pattern on the same chromosome type, that is, the cleavage efficiency of a DNA molecule immersed in a proteinaceous matrix is not solely dependent on the characteristics of the DNA molecule.¹⁶ Taking into account the presumed heterogeneity of amniotic fluid cells, further investigation is required to assess whether this differential behaviour of constitutive heterochromatin domains is cell specific or represents a process in tissue differentiation or embryonic-fetal development.

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