The inactive X chromosome in the human female is enriched in 5-methylcytosine to an unusual degree and appears to contain more of this modified nucleotide than the remainder of the genome

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Abstract

By employing a procedure that combines ELISA and photoacoustic spectroscopy, we have examined the content of 5-methylcytosine (m^5C) in DNA of individuals who differed from one another in the number of X chromosomes in their genomes. The results show that the human inactive X chromosome (Xi) contains very high amounts of this modified nucleotide. We estimate that in the 46,XX female there is more m^5C in Xi ($\sim 3.6 \times 10^7$) than in all the remaining chromosomes put together ($\sim 2.1 \times 10^7$). Our results also suggest that nearly one-fifth of all cytosines in Xi are methylated and that, in addition to CpG methylation, there is extensive non-CpG methylation as well.

Introduction

The hypothesis that methylation of cytosine residues in DNA is a key step in the maintenance of the inactive state of the mammalian X chromosome (Holliday and Pugh 1975; Riggs 1975) has found support from several types of experiment (Liskay and Evans 1980; Mohandas *et al*. 1981). But there are no data on the amount of 5-methylcytosine (m^5C) in the inactive X chromosome (Xi). A combination of ELISA and photoacoustic spectroscopy (PAS) provides a means of estimating the genomic content of m^5C irrespective of the sequence context in which the modified nucleotide occurs (Achwal and Chandra 1982; Achwal *et al*. 1984). We have previously used this method to measure the very small amounts of m^5C present in *Drosophila melanogaster* DNA (Achwal *et al*. 1984) and these observations have been confirmed by direct sequencing (Lyko *et al*. 2000) and dinucleotide analysis (Gowher *et al*. 2000). In this paper we report on

experiments aimed at estimating the content of m^5C in the human inactive X chromosome by application of the ELISA–PAS method to samples of genomic DNA from individuals whose karyotypes differed from one another in the number of X chromosomes (45,XO; 46,XX; 47,XXX and 48,XXXX).

Materials and methods

DNA was isolated from lymphocytes or cultured fibroblasts of seven women and from cultured fibroblasts of a female foetus. Details of these sources are given below.

(i) 45,AAXO (Turner syndrome patient #1); female, Indian; lymphocytes

(ii) 45,AAXO (Turner patient #2); female, Indian; lymphocytes

(iii) 45,AAXO (GM 775); fibroblast culture derived from a Caucasian Turner patient; at the time of isolation of DNA, these cells were in serial passage number 15

(iv) 46,AAXX (MRC-9, ATCC CCL 212); fibroblast-like cell line derived from normal lung tissue of a

15-week-old female foetus of Caucasian parents; this line was reported, at the time of this study, to stably maintain a diploid karyotype with a stemline number of 46 chromosomes (passage number 28)

(v) 46,AAXX; female, Indian; lymphocytes

(vi) 46,AAXX; female, Indian; lymphocytes

(vii) 47,AAXXX (GM 254); fibroblast culture derived from a Caucasian female; no chromosomal mosaicism was reported (passage number 10)

(viii) 48,AAXXXX (GM 1415); fibroblast culture derived from a Caucasian female; the culture was reported to have 4% polyploid cells (passage number 9).

Peripheral blood from individuals (i), (ii), (v) and (vi) was kindly provided by Dr Manorama Thomas, St John's Medical College, Bangalore. The fibroblast cell lines GM 775, GM 254 and GM 1415 were obtained from the Human Mutant Cell Repository (Camden, USA). The diploid cell line MRC-9, ATCC CCL 212 was obtained from the National Institute of Virology, Pune, India. There was no evidence of chromosomal mosaicism among the sampled cells in these eight cases. 'Unmethylated' DNA from bacteriophage lambda grown in dcm^-, dam^- *E. coli* served as negative control and DNA from the mealybug *Planococcus lilacinus*, which has approximately 1.8 mol% m^5C in its genome (Deobagkar *et al.* 1982), served as positive control. DNA from the bacteriophage ϕX174, which contains one m^5C residue in its genome of 5386 nucleotides (Sanger *et al.* 1978), was used for calibration. The methods by which the polyclonal anti-m^5C antibody used in this study was prepared and characterized (K_a of 1.56×10^9 M^{-1}) have been described earlier (Achwal and Chandra 1982; Achwal *et al.* 1984). Identical amounts of DNA (six nanograms from each individual) were spotted on nitrocellulose membrane (BA 85 from S&S or an equivalent product from Advanced Microdevices, Ambala, India), treated with anti-m^5C antibody, and processed as described (Achwal and Chandra 1982). The m^5C-antibody–DNA complex was treated with biotin-avidin-peroxidase and stained by using 0.1% DABT and 0.02% H_2O_2. The staining intensity was measured at 520 nm in a photoacoustic spectrometer. This instrument, built by Ganguly and Rao (1981), had been fitted with a 200 W tungsten lamp. The chopping frequency was 23 Hz. The PA signal intensity of each spot of DNA was measured thrice and the average value was calculated.

The numbers of cytosine residues and CpG dinucleotides in the X chromosome were calculated from the human genome sequence database (ftp://ftp.ensembl.org/pub/current_human/data/golden_path/).

Results and discussion

Although each spot contained the same amount of DNA, it was clear from visual inspection itself that the staining intensity increased with the number of X chromosomes in the genome, in the order: AAXO < AAXX < AAXXX < AAXXXX (figure 1). The contrast between XO DNA and XX DNA was particularly clear. The staining intensities of these spots as recorded by PAS are given in table 1. The relationship between the number of X chromosomes in the genome and m^5C content was linear (figure 2). Since X chromosomes in excess of one are inactive, these data indicate that the m^5C content of Xi is very high. The amount of m^5C in the inactive X chromosome was estimated in the following manner. The PA signal intensity of XO DNA was 0.27 ± 0.05 units (table 1). Measured against the ϕX174 calibration plot, this value corresponds to ~ 56.25 ng of ϕX174 DNA (figure 3). This amount of ϕX174 DNA is expected to contain 1.98×10^{10} m^5C residues because there is a single m^5C residue in the 5386 nucleotides that constitute the genome of this phage. Since 6 ng of XO DNA would contain roughly 932 copies of the human genome, we estimate that the 45,XO genome contains approximately 2.1×10^7 m^5C residues (table 1).

The signal intensity of 6 ng of XX DNA was more than twice the signal intensity of the same amount of XO DNA (table 1). It required 142.5 ng of ϕX174 DNA to give a signal equivalent to that of 6 ng of human XX DNA (figure 3). This amount of ϕX174 DNA is expected to contain about 0.49×10^{11} residues of m^5C. On the assumption that in 6 ng of human 46,XX DNA there are roughly 910 copies of the genome, we estimate that there are approximately 5.4×10^7 m^5C residues in the genome of the human XX female.

The average PA signal intensity attributable to each inactive X was calculated by dividing the sum of the pairwise signal intensity differences (XX – XO), (XXX – XO) and (XXXX-XO) by six, the number of inactive X chromosomes in these four genomes. This value turns out to be 0.445 (table 1), which is very similar to that
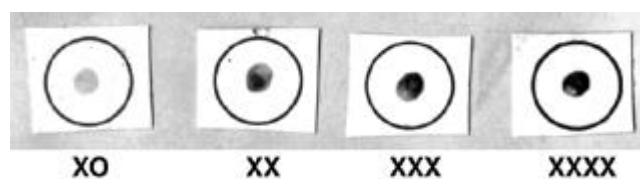

Figure 1. Staining intensities of samples of genomic DNA from individuals who differed from one another in the number of X chromosomes. Identical amounts (6 ng) of DNA were spotted on nitrocellulose membrane, treated with anti-m^5C antibody, and stained by avidin-peroxidase. All DNA samples were processed simultaneously on the same nitrocellulose membrane and the individual spots of DNA were then cut out for measurement of staining intensity in a photoacoustic spectrometer. As noted under Materials and methods, a 200 W tungsten filament lamp was used in the photoacoustic spectrometer. It may be possible to improve the sensitivity of this method substantially by the use of a 1000 W xenon arc lamp.

Table 1. Estimation of m^{5}C content by ELISA and photoacoustic spectroscopy.

Karyotype and genome size[a]	Photoacoustic units[b]	No. of m^{5}C residues[c]	m^{5}C content (mol%)[c]	No. of Cs in Xi(s)[d]	No. of CpGs in Xi(s)[d]
45AAXO 6.44 × 10^9	0.27 ± 0.05	2.05 × 10^7	0.38	–	–
46AAXX 6.6 × 10^9	0.7 ± 0.05	5.46 × 10^7	0.83*	0.16 × 10^9	1.14 × 10^6
47AAXXX 6.76 × 10^9	1.18 ± 0.05	9.49 × 10^7	1.41	0.32 × 10^9	2.28 × 10^6
48AAXXXX 6.92 × 10^9	1.6 ± 0.05	13.1 × 10^7	1.89	0.48 × 10^9	3.42 × 10^6
Average value per Xi	0.445	3.64 × 10^7	0.53	–	–

(a) A = one set of autosomes; X = one X chromosome. The genome size (number of nucleotides) was calculated from the human genome database (ftp://ftp.ensembl.org/pub/current_human/data/golden_path/). It is expected that in the XX, XXX and XXXX genomes the amount of autosomal and active-X DNA is the same as in the XO genome whereas both the amount and the proportion of inactive-X DNA would increase with genome size.

(b) The sign '±' refers to the extent of variation among (i) individual measurements in the XXX and XXXX cases and (ii) the averages of the measurements from different individuals in the XO and XX cases. Within the XO and XX chromosomal classes, the PA signal intensities of lymphocyte DNA and fibroblast DNA were similar.

(c) The calibration plot of the relationship between PA signal intensity and amount of φX174 DNA (figure 3) was used to obtain these values. The procedure followed is described under Results and discussion.

(d) Calculated from total X-chromosome sequence data (ftp://ftp.ensembl.org/pub/current_human/data/golden_path/) by using the EMBOSS program (Rice *et al.* 2000).

*In an earlier publication (Achwal *et al.* 1984), our estimate of the amount of cytosine methylation in human DNA on the basis of ELISA–PAS measurements contains an error. The PA signal value of human DNA in table 1 of that paper should have been 0.5 units and not 1.5 units; consequently, the mol% m^{5}C content as estimated from the corrected value turns out to be about 0.7, which is close to the value reported here.

obtained by dividing by three the sum of the pairwise differences (XX – XO), (XXX – XX) and (XXXX – XXX). From this value, following the same procedure as that outlined above for XO and XX DNA, we obtain ~ 3.64 × 10^7 as the number of m^{5}C residues in a single inactive X chromosome (figure 3 and table 1). This observation suggests that the amount of cytosine methylation in a single inactive X chromosome is higher than that in the entire 45,XO genome.

On the basis of dinucleotide analysis, it has been reported that a majority of m^{5}C residues in human DNA are not in the CpG dinucleotide (Woodcock *et al.* 1987), but more recent reports suggest that most methylated cytosines occur in CpG dinucleotides (reviewed in Cross and Bird 1995). Analysis of the human genome sequence database shows that the X chromosome contains ~1.6 × 10^8 cytosines and ~1.14 × 10^6 CpG dinucleotides (table 1). This observation, when viewed in light of our estimate of the number of m^{5}C residues in Xi, suggests that in the inactive X roughly one in every five cytosines is methylated, and that over 90% of the m^{5}C residues occur in dinucleotides other than CpG.

What relationship does such a high level of cytosine methylation have to the spread and maintenance of the X inactivation process? Answers to this question may be forthcoming if, by direct sequencing or other methods,
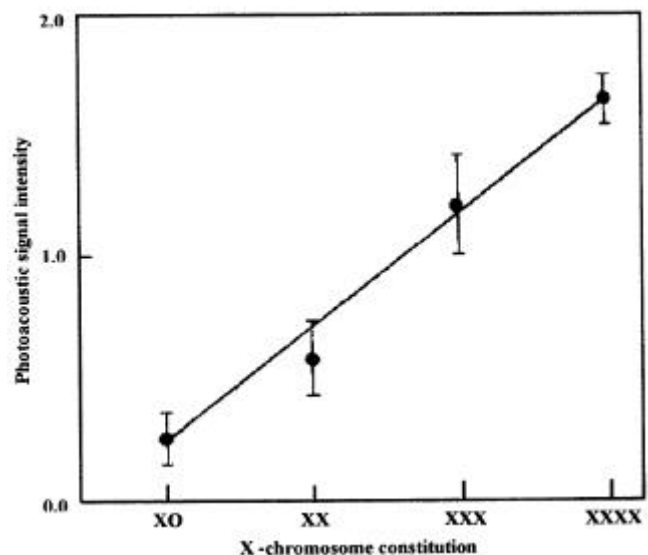

Figure 2. Photoacoustic signal intensities of the DNA samples [(PA signal intensity of spotted DNA) – (PA signal intensity of background)] plotted against the X-chromosome constitution of the individuals studied. The PA values used to prepare this plot are the same as those shown in table 1. See footnotes to table 1 for additional details. The average PA signal intensity of DNA samples from two 46,XY males was slightly higher (data not shown) than that of the XO samples, and significantly lower than that of the XX samples..
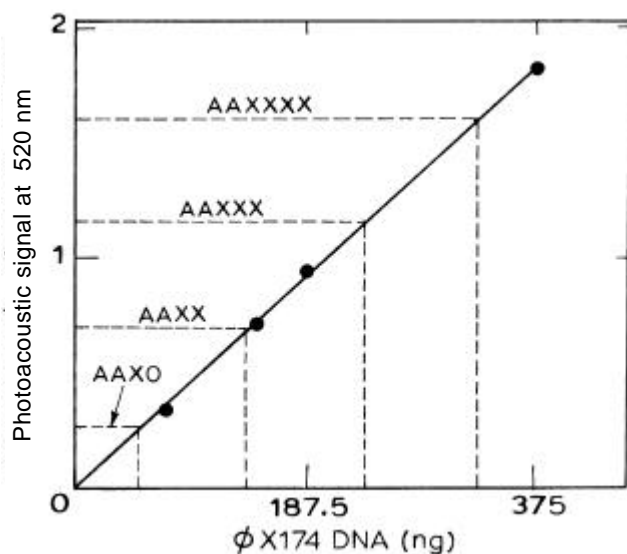
Figure 3. Intensity of photoacoustic signal ($I_{spot}-I_{background}$) plotted against increasing amounts of φX174 DNA (dark circles). Superimposed on the Y-axis are the PA values (figure 2 and table 1) of 6 ng of DNA from AAXO, AAXX, AAXXX and AAXXXX individuals (broken lines). For the PA values of each of the human DNA examples, the amount of φX174 DNA that gave an equivalent PA signal was noted (X-axis). These data were then used to estimate the amount of m⁵C in the human DNA samples as described under Results and discussion.

comparisons could be made between the distribution of m⁵C residues in homologous sequences in active and inactive X chromosomes and between regions of the X chromosome that are subject to inactivation and those that are not.

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