Sequential staining of euchromatic and heterochromatic regions of the human Y chromosome

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SUMMARY A sequential silver-Giemsa (SG) procedure is presented, initially to stain the p11 and q11 euchromatic bands and subsequently the q12 heterochromatic band of the human Y chromosome. A three sub-band division of the q11 band can be identified.

The same technique differentially stains the secondary constriction of chromosome 9 as well as most of the other satellite III DNA regions of the human karyotype.

Several staining procedures, such as Q banding, G11 banding, C banding, and more recently with DAPI-distamycin A,¹ are useful in the study of the q12 band (constitutive heterochromatin of the long arms) of the human Y chromosome.

Unlike the results obtained by the C banding technique, the silver nitrate procedure of Howell and Denton² selectively leaves unstained the secondary constrictions of chromosomes 1, 9, and 16 and the q12 band of the Y chromosome.

Another silver procedure (AS III), which preferentially stains the secondary constriction of chromosome 9, does not make any particular region of the Y chromosome stand out and it remains lightly stained.³

None of the methods in use at present can preferentially stain the p11 and q11 bands (euchromatin) of the Y chromosome, thus preventing fine structural analysis of these regions.

Presented here is a silver-Giemsa (SG) sequential procedure to stain consecutively the euchromatic and then the heterochromatic regions of the human Y chromosome. The SG method allows accurate structural analysis of the Y chromosome and identifies the internal distribution of the chromatin within the q11 band.

Material and methods

CULTURES

A total of 2×10^6 human peripheral blood lymphocytes was grown for 70 hours at 37°C in 4 ml TC 199 supplemented with 15% fetal calf serum, antibiotics, 2 mmol/l L-glutamine, and 3 µg/ml phytohaemagglutinin. During the last 3 hours of incubation,

0.5 µg/ml colchicine was added to the cultures. Then the lymphocytes were treated with 0.075 mol/l KCl for 10 minutes, twice fixed in 4 ml 1:3 acetic methanol, dropped on slides, and air dried.

PRETREATMENT WITH SALTS

One- to 7-day-old slides were rehydrated for 30 minutes in 0.5 to $2 \times$ SSC. (Similar results were obtained with incubations in $0.5 \times$ SSC at 55°C for 1 hour.) Alkaline pretreatment increases the differential staining results.

SILVER STAINING

Immediately after the pretreatment, slides were immersed three times in distilled water and dried with Watman paper, and 0.10 ml each of A and B reagents were pipetted onto the slides. Reagent A: ammoniacal silver carbonate (2.5 g AgNO₃, 4.5 ml 50% methanol, 3 ml NH₃, 30 mg Na₂CO₃). Reagent B: formaldehyde solution (0.4 ml formaldehyde, 9.6 ml 50% methanol). Cover slides were immediately applied and staining was monitored by phase contrast microscope. Adequate staining usually took 8 to 14 minutes at room temperature. Reduced staining periods or heavier dying can be obtained by incubating the slides for 2 to 5 minutes at 37°C. Once stained, the cover slides were removed under running tap water. Dried slides can be examined directly or mounted with Hystomount.

To avoid silver precipitates during the staining procedure, particular care should be given to (1) careful removal of all the 0.5 to $2 \times$ SSC droplets remaining after rehydration and before the silver staining step; and (2) exactly the same amount of A and B reagents must be added, one onto the other. The cover slide should be applied immediately to avoid air contact.

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Staining twice with the A and B reagents, as recommended by Howell and Denton³ in their system, increases the rate of silver binding to the chromosome proteins, and in our hands it resulted in well-stained slides without disturbing background. This silver reaction⁴ is in some respects a simplified and widely modified version of Howell and Denton's procedure.²

Reagent B is stable for more than a month at room temperature. Reagent A should be stored in a glass tube covered with black photographic paper. It can be used for several weeks if kept at -10°C and filtered (Millipore filter 0.22 μm) before use.

REMOVAL OF SILVER STAIN

After photographic recording of selected metaphases, the silver dye was removed by immersing the slides for 60 seconds in 10% hydrogen peroxide.

C BANDING

For sequential staining, chromosomes were treated for 10 minutes with 1 N HCl, then with 5% barium hydroxide for 30 to 45 minutes at 55°C , and finally incubated for 60 minutes in $2 \times \text{SSC}$ at 60°C . Staining with 5% Giemsa was performed for 30 to 45 minutes. The metaphases recorded were relocated and photographed.

Three hundred metaphases from three normal men were examined.

Results and discussion

The sequential staining for the Y chromosome presented here consists of a three-step procedure. In the first, the euchromatin of the Y is preferentially stained by a silver salt reaction. After photographic recording of the selected metaphases, the silver dye is removed (second step) and during the third step the heterochromatic region (band q12) of the same chromosome is stained by a modified C banding method.

FIRST SG STEP

Once the metaphases had been pretreated with 0.5 or $2 \times \text{SSC}$ and stained with the silver A and B solutions, the chromosomes appeared well defined and had a light yellow colour. Heavy metallic staining in the form of black-brown spots were noticed on the secondary constriction of chromosome 9, on the short arms of chromosomes 14, 15, 21, and 22, occasionally on the whole of chromosome 19, and on the p11-q11 bands of the Y chromosome (figure). These chromosomal positions were confirmed by R banding methods (not shown).

The secondary constrictions of chromosomes 1

and 16 remained completely unstained, and the q12 band of the Y chromosome was only lightly dyed (figure a, h, i). In accordance with reported results of the G11 procedure,⁵ division of the chromosome 9 heterochromatin into two subunits in each chromosome arm, as well as polymorphisms in this region (not shown), could occasionally be observed (figure b). Although the short arms of chromosomes 14, 15, 21, and 22 were heavily stained, the stalks and satellites remained lightly dyed (figure c).

The AS III procedure of Howell and Denton,³ as well as the G11 technique of Bobrow *et al.*,⁵ preferentially stains the secondary constriction of chromosome 9. This last method also heavily stains areas of chromosome 1 heterochromatin, the long arms of chromosomes 5, 10, and 20, the short arms of chromosomes 7, 17, and the D and G group chromosomes, and the heterochromatin of the Y chromosome. However, Howell and Denton² reported another silver method which leaves the secondary constrictions of chromosomes 1, 9, 16, and the distal Yq unstained.

With the silver technique presented here, results are intermediate between the previously mentioned G11 method and Howell and Denton's methods, and show a roughly coincidental staining affinity with the loci assigned to satellite III DNA.⁶

Different dying results using silver salts are the result of the dissimilar conditions of pH, ionic strength, and pretreatments which modulate the staining behaviour of both the silver dye and chromosome proteins, consequently defining the regions where the metal deposits occur.

Although silver salts have been reported to react with histones,^{7,8} recent reports indicate that some non-histone proteins are the chromosomal molecules responsible for the staining reaction with silver nitrates and carbonates.^{2,4,9,10}

SECOND SG STEP

After photographic recording of selected metaphases, to make a careful analysis of the short and long arms of the Y chromosome, the silver was removed by a 60 second immersion of the slides in 10% hydrogen peroxide.

THIRD SG STEP

Immediately after removing the silver, a modified version of the barium hydroxide C banding procedure (see methods) was used to stain the heterochromatic regions of the Y and other chromosomes. The particularly hard C banding treatment used in this third step was designed to loosen the rigid chromosome structure produced by protein-DNA and protein-protein cross links induced by formaldehyde during the first silver staining step.^{11,12} This

FIGURE Three step sequential staining of the euchromatin and heterochromatin of the human Y chromosome using a silver-Giemsa procedure (SG).

(a) First step. Human lymphocyte metaphase stained by ammoniacal silver carbonate after a $0.5 \times$ SSC pretreatment. The secondary constriction of chromosome 9, short arms of 14, 15, 21, and 22, and the q11 band of the Y, are mainly stained. In the interphase nucleus, two spots corresponding to chromosome 9 secondary constrictions can be observed. The inset shows a high magnification of the Y chromosome (arrow). Notice the two positively stained bands of the chromatin corresponding to the q11 region of the Paris G band classification.

(b) A two spot distribution of the heterochromatin of the secondary constriction in each arm of a chromosome 9.

(c) While short arms of acrocentric chromosomes appear heavily stained, satellites and stalks show the same silver affinity as the rest of the chromosome.

(d, e, f, g) Results of the three step sequential stain of the Y. In the first SG step the silver carbonate mainly stained the euchromatin of the chromosomes (d, f). After removal of silver with hydrogen peroxide (second SG step) the heterochromatin of the same chromosomes (e, g) was shown by a Giemsa C banding procedure (third SG step).

(h, i) During the first SG step the silver carbonate deposits on the Y followed two kinds of pattern distribution: (h) all the euchromatin is stained; (i) in addition to the p11 area, two bands within the q11 region mainly appear stained.

treatment was essential in order to obtain a partial chromosome protein extraction and the subsequent identification of the more resistant heterochromatic areas by Giemsa staining.

With this three step sequential procedure the consecutive analysis of the p11–q11 bands (euchromatin), and then the q12 band (constitutive heterochromatin) of the human Y chromosome is possible (figure d, e, f, g).

Two kinds of silver distribution could be observed in the q11 band of the Y chromosome. In the three normal men tested, almost half of the metaphases in each subject showed a two spot pattern of the Y. The silver settled heavily, close to the centromere and at the end of the q11 band, just in the junction between the q11 and q12 bands. The area between these spots remained lightly stained (figure i and inset of a). In the rest of the metaphases all the q11 band of the Y chromosome appeared heavily stained.

A spontaneous chromomeric distribution of the chromatin fibres along the chromosome arms was postulated by Bahr *et al*¹³ as the structures responsible for the bands. In fact, Bahr and Larsen¹⁴ showed a good correlation between chromomere positions and spontaneous (Q bands) or induced (trypsin G bands) bands in human chromosomes.

Using electron micrographs of whole-mount human chromosomes, Bahr and Larsen¹⁴ described several chromomeres along the arms of the human Y chromosome.

In our proposed two spot pattern, mainly observed in early metaphase Y chromosomes, the chromatin fibres are distributed in two chromomeres, as opposed to one spot Y chromosomes with a more condensed structure, where the two chromomeric areas would possibly fuse together. Yunis *et al*¹⁵ have shown a clear sub-banding structure of the q11 and q12 regions in G banded prophase Y chromosomes.

The two silver spot pattern described here could indicate a three sub-band organisation of the actual q11 band of the human Y chromosome.

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