DISTRIBUTION OF PROTEIN SULPHHYDRYS AND DISULPHIDES IN FIXED MAMMALIAN CHROMOSOMES, AND THEIR RELATIONSHIP TO BANDING

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SUMMARY

Fixed mammalian mitotic chromosomes, when stained with a variety of sensitive, sulphhydryl-specific fluorochromes directly or following reduction of disulphide groups, show uniform fluorescence. Thus sulphhydryl and disulphide groups are uniformly distributed along the length of the chromosomes, and do not show patterns related to chromosome bands. Performance of G- or C-banding procedures oxidizes sulphhydryls to disulphides, but does not produce an inhomogeneous distribution of these groups. Cross-linking sulphhydryl groups has no effect on G-, C- or Q-banding of chromosomes. Thus there appears to be no connection between chromosome bands and the distribution or state of oxidation of sulphur in chromosomal proteins.

INTRODUCTION

Ten years ago I suggested, on the basis of indirect evidence, that positive G-bands in chromosomes might be relatively rich in protein disulphide (SS) cross-links, while negative G-bands might have a greater proportion of their protein sulphur in the form of sulphhydryl (SH) groups (Sumner, 1974). At that time it was not possible to test this hypothesis by direct cytochemical observations. Nevertheless, the hypothesis was consistent with the idea that positive G-bands represent regions of chromosomes that condense earlier in mitotic prophase, and with evidence that disulphide cross-links occur at a higher concentration in condensed chromatin. For a brief review of these concepts, see Sumner (1983).

In recent years several very sensitive fluorochromes specific for sulphhydryl groups have become commercially available, so the opportunity was taken to examine cytochemically the distribution of SH and SS groups on mammalian chromosomes. In addition, the effect of routine chromosome banding procedures on these groups was studied. Because the results obtained did not support the original hypothesis that positive G-bands are rich in SS groups, some of the original experiments were repeated using different reagents. Since one of the important pieces of evidence for the hypothesis was that cross-linking of chromosomal SH groups prevented G-banding, different reagents that produce cross-linking of SH groups were tested to assess their effect on chromosome banding. A preliminary account of some of the experiments described in this paper has already been published (Sumner, 1983).
MATERIALS AND METHODS

Chromosome preparations

Cells from cultures of either mouse A9 cells or human lymphocytes stimulated with phytohaemagglutinin were treated with hypotonic (0.075 M) potassium chloride, fixed with methanol/acetic acid (3:1, v/v) and chromosome preparations made by spreading on glass slides according to conventional techniques.

Fluorescent staining of SH and SS

The fluorescent sulphydryl reagents used, their sources, the solutions in which they were used, and their reaction mechanisms are listed in Table 1. Chromosome preparations were stained for 1 h with these reagents, followed by 1 h in the appropriate solvent to wash out non-specifically bound fluorochrome. The only exceptions were DMS and DMSDS (see Table 1), which were used for 24 h since the shorter staining time produced negligible fluorescence, and monobromobimane (MBB), which was used for 45 min.

N-ethyl maleimide (NEM; 0·03 g/50 ml) was used to block staining of SH groups (Sippel, 1973), and tri-n-butyl phosphine (TBP; 0·25%) to reduce SS to SH (Sippel, 1978). Both substances were dissolved in 2-propanol/phosphate buffer pH 6 (1:1, v/v) and treatment was for 1 h, followed by a wash in the same buffer for 5 min. Using these two reagents, total protein sulphur (SH+SS) could be demonstrated by reducing chromosome preparations with TBP, while SS alone could be demonstrated using NEM (to block SH groups), followed by TBP (to reduce SS to SH).

Cross-linking of protein sulphydryls

Two reagents were used to produce cross-linking of sulphydryl groups: sodium tetrathionate (Haest, Kamp, Plasa & Deuticke, 1977; Kaufmann, Coffey & Shaper, 1981) and diamide (Kosower, Kosower & Wertheim, 1969; Haest et al. 1977). Sodium tetrathionate was obtained from Fluka, and slides were treated in one or other of the following ways: 0·05% in Tris-HCl buffer (pH 7·4) for 1, 3 or 24 h at 4 °C, or 0·05, 0·5 or 5% (saturated) in the same buffer for 24 h at room temperature (approximately 22 °C), or 0·05 or 0·5% in 70% (v/v) ethanol for 24 h at room temperature. Diamide (diazine-dicarboxylic acid bis-(N,N-dimethylamide)) was obtained from Sigma, and slides were treated with a 0·1% solution in phosphate buffer (pH 7·2) for 1 or 24 h at room temperature. Control slides were treated with buffer or ethanol as appropriate.

Two methods were used to assay the efficacy of cross-linking with these two reagents. The first assay involved staining chromosome preparations with any convenient SH-specific fluorochrome after treatment with the cross-linking agent, and also after subsequent reduction with TBP. Inhibition of specific fluorescence of SH groups, reversible by TBP reduction, was taken to indicate the probable formation of SS cross-links. The second, ‘cytological’ assay measured differences in dry mass and area of fixed diploid human lymphocyte nuclei that had been treated with the reagents under study, and then either treated with a 1% solution of sodium dodecyl sulphate (SDS) for 1 h, or left untreated. Measurements of dry mass and area were made with a Vickers M86 Integrating Microdensitometer and Interferometer. For measurement of area, nuclei were heavily stained with Giemsa, but were, of course, left unstained for dry mass measurements. The occurrence of cross-linking was deduced from a reduction of swelling of nuclei and a smaller loss of dry mass in the SDS solution compared to control slides.

Chromosome banding

Standard techniques were used for chromosome banding, as follows:

G-banding: the ASG technique (Sumner, Evans & Buckland, 1971).

C-banding: the BSG technique (Sumner, 1972).

Q-banding: 0·4% quinacrine in acetate buffer (pH 4·5) for 30 min, followed by mounting in the same buffer.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Source</th>
<th>Solution</th>
<th>Reaction mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin-5-iodoacetamide</td>
<td>E5I</td>
<td>Molecular Probes</td>
<td>3·6 mg/50 ml P-P* (pH9)</td>
<td>Alkylation</td>
<td>Curtis &amp; Cowden (1980)</td>
</tr>
<tr>
<td>Eosin-5-maleimide</td>
<td>E5M</td>
<td>Molecular Probes</td>
<td>2 mg/50 ml P-P (pH6)</td>
<td>Alkylation</td>
<td>Curtis &amp; Cowden (1980)</td>
</tr>
<tr>
<td>Fluorescein-5-maleimide</td>
<td>F5M</td>
<td>Molecular Probes</td>
<td>2 mg/50 ml P-P (pH6)</td>
<td>Alkylation</td>
<td>Curtis &amp; Cowden (1980)</td>
</tr>
<tr>
<td>N-(7-dimethylamino-4-methylcoumarinyl)maleimide</td>
<td>DACM</td>
<td>Polysciences</td>
<td>2 mg/50 ml P-P (pH6)</td>
<td>Alkylation</td>
<td>Curtis &amp; Cowden (1980)</td>
</tr>
<tr>
<td>3-(4-Maleimidylphenyl)-7-diethylamino-4-methylcoumarin</td>
<td>CPM</td>
<td>Molecular Probes</td>
<td>2 mg/50 ml P-P (pH6)</td>
<td>Alkylation</td>
<td>Sippel (1981)</td>
</tr>
<tr>
<td>Monobromobimane</td>
<td>MBB</td>
<td>Calbiochem</td>
<td>0·02 g dissolved in 1 ml acetone and added to 100 ml phosphate/saline at pH7</td>
<td>Alkylation</td>
<td>Gainer &amp; Kosower (1980)</td>
</tr>
<tr>
<td>Ammonium 4-chloro-7-sulphobenzofurazan</td>
<td>SBF-Cl</td>
<td>Pierce</td>
<td>0·01 g/40 ml borate (pH8) + 1 mm-Na2 EDTA</td>
<td>Alkylation</td>
<td>Andrews, Ghosh, Ternai &amp; Whitehouse (1982)</td>
</tr>
<tr>
<td>Didansylcystine</td>
<td>DDC</td>
<td>Molecular Probes</td>
<td>0·04 g/40 ml phosphate (pH7-2)</td>
<td>SS/SH exchange</td>
<td>Lazarides &amp; Granger (1978)</td>
</tr>
<tr>
<td>Difluorescein disulphide</td>
<td>DFDS</td>
<td>Polysciences</td>
<td>10 mg/40 ml phosphate (pH7-2)</td>
<td>SS/SH exchange</td>
<td>Wingender &amp; Arellano (1982)</td>
</tr>
<tr>
<td>4, 4'-Dimaleimidyl-stilbene</td>
<td>DMS</td>
<td>Molecular Probes</td>
<td>3 mg/30 ml acetone</td>
<td>Alkylation; fluorescent when cross-linking SH in hydrophobic sites</td>
<td></td>
</tr>
<tr>
<td>4-4'-Dimaleimidyl-stilbene-2, 2'-disulphonic acid</td>
<td>DMSDS</td>
<td>Molecular Probes</td>
<td>4 mg/40 ml P-P (pH6)</td>
<td>As DMS, but cross-linking hydrophilic sites</td>
<td></td>
</tr>
</tbody>
</table>

* P-P is 2-propanol/phosphate buffer in a 1:1 mixture at the pH stated.
† Muller & Crothers (1975).
RESULTS

Distribution of SH and SS groups on chromosomes

All the fluorescent SH reagents tested gave essentially similar results (Fig. 1). Both SH groups (demonstrated by direct staining) and SS groups (shown by the NEM/TBP/staining sequence) were uniformly distributed on both human (including...
prophase) and mouse chromosomes, and patterns corresponding to either G- or C-bands were not seen (with one exception mentioned below). Treatment with NEM before staining reduced the fluorescence of chromosomes to negligible levels, but did not abolish fluorescence completely.

Some SH-specific fluorochromes were better than others, and some produced minor variations in staining pattern. The brightest fluorescence was given by F5M, DACM, CPM and MBB, with CPM perhaps the best of these. E5M produced only moderate fluorescence, and E5I gave fluorescence too weak for practical use. DDC gave moderate fluorescence, but characteristically the chromosomes appeared as bright outlines with dimmer interiors, which were, however, still uniform. A similar phenomenon was sometimes seen with DFDS, which produced brighter fluorescence, and occasionally revealed centromeric dots. Of the straightforward SH reagents, SBF-CI produced the least satisfactory but most intriguing results. In an initial experiment, this fluorochrome produced a pattern apparently similar to G-banding, which, however, faded so rapidly that it could not be recorded photographically. This observation could not be repeated. In subsequent experiments, a variety of mountants was used in an attempt to increase the brightness and stability of the fluorescence; these are listed in Table 2. Only DPX permitted a level of fluorescence to be produced that was usable, and the chromosomes were quite uniform.

The two fluorochromes that cross-link SH groups, DMS and DMSDS, also produced uniform fluorescence on chromosomes. Both required 24 h staining to give a usable level of fluorescence. DMS, which is expected to cross-link SH groups in hydrophobic sites, gave a low level of fluorescence when slides were mounted in DPX and illuminated with blue light. Ultraviolet illumination, or mounting in phosphate buffer at pH 7.2, resulted in negligible fluorescence. The absorption spectrum of the DMS staining solution has a narrow peak at 327 nm (Fig. 2A), which would not result in efficient excitation of fluorescence in a microscope; this may account for the low level of fluorescence found on stained chromosomes. DMSDS, which should cross-link SH groups in hydrophilic sites, gave a higher level of fluorescence than DMS, which was also improved by mounting in DPX and illuminating with blue light. The absorption of the DMSDS staining solution extends into the blue region of the spectrum when fresh (Fig. 2B), and this fact alone may account for the higher level of fluorescence obtained with DMSDS than with DMS. Both DMS and DMSDS appeared to produce a moderate level of cross-linking in nuclei (Fig. 3).

### Table 2. Mountants used for SBF-CI fluorescence

<table>
<thead>
<tr>
<th>Mountant</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Phosphate buffer (pH 7.2)</td>
<td>—</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.2) containing 1% sodium dithionite</td>
<td>Gill (1979)</td>
</tr>
<tr>
<td>Citifluor/PBS</td>
<td>Davidson &amp; Goodwin (1983)</td>
</tr>
<tr>
<td>Citifluor/glycerol</td>
<td>Davidson &amp; Goodwin (1983)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>—</td>
</tr>
<tr>
<td>Immersion oil (Leitz, fluorescence-free)</td>
<td>—</td>
</tr>
<tr>
<td>DPX (B.D.H.)</td>
<td>—</td>
</tr>
</tbody>
</table>
Fig. 2. A. Absorption spectrum of DMS (0.05 mg/ml) in acetone. B. Absorption spectrum of DMSDS (0.05 mg/ml) in 2-propanol/phosphate buffer (pH 6). (—) Fresh solution; (—) solution 24-h-old. For all spectra the light-path was 5 mm.

Effect of chromosome banding procedures on SH groups in chromosomes

Chromosome preparations that had been treated with G-banding or C-banding procedures (except for Giemsa staining) showed greatly reduced levels of fluorescence with MBB or DACM. The remaining fluorescence tended to be concentrated round the outlines of the chromosomes, but there was no sign of either G- or C-banding. Treatment of G- or C-banded chromosomes with TBP to reduce SS groups to SH restored strong fluorescence; again this tended to be concentrated at the outlines of the chromosomes, and occasionally centromeric dots could be seen. No banding pattern was visible after TBP reduction of banded chromosomes and staining with SH-specific fluorochromes.
**SH and SS distribution in chromosomes**

![Graph showing SH and SS distribution in chromosomes](image)

Fig. 3. Results of the cytological cross-linking assay for DMSDS and DMS. The lengths of the bars are proportional to the ratios for dry mass and area of nuclei treated with 1% SDS to untreated nuclei, for each cross-linking treatment and the corresponding controls. Values nearest to 1 for both dry mass and area indicate least change and therefore the greatest degree of cross-linking.

**Effect of SH cross-linking on chromosome banding**

Prior treatment of chromosome preparations with sodium tetrathionate or diamide, under any of the conditions used, had no effect on G-banding (Fig. 4), C-banding or Q-banding of chromosomes. Glutaraldehyde, a known powerful cross-linking agent, prevents G-banding of chromosomes (Fig. 4D).

Treatment of chromosomes with sodium tetrathionate or diamide greatly reduced the level of fluorescent staining with MBB or DACM; bright fluorescence was restored by reduction with TBP after tetrathionate or diamide treatment.

Typical results of the cytological assay for cross-linking are illustrated in Fig. 5. It must be pointed out that the degree of loss of dry mass and that of swelling of nuclei varied considerably from one batch of slides of lymphocyte nuclei to another, depending, no doubt, on differences in fixation and on the age of the slides. Nevertheless, comparison with control slides treated only with the buffers used to dissolve the sodium tetrathionate or the diamide showed that these two cross-linking reagents reduced both the swelling of nuclei and the loss of dry mass. Both these losses were also reduced in nuclei treated with buffer alone when compared with untreated nuclei. These effects were, however, relatively small compared to the effect of glutaraldehyde, which largely prevents loss of dry mass and swelling of nuclei. Thus sodium tetrathionate and diamide appear to introduce a relatively low level of SS cross-links into nuclei and chromosomes in preparations fixed in methanol/acetic acid.

**Discussion**

Three conclusions can be drawn from the results described in this paper. Firstly, the distribution of SH and SS groups on fixed mammalian prophase and metaphase...
Fig. 4. Fixed human metaphase chromosome preparations, G-banded using the ASG technique. a. ASG with no pretreatment. b. 0·5 % Sodium tetrathionate in Tris-HCl buffer (pH 7·5), 24 h, followed by ASG. c. Tris-HCl (pH 7·5), 24 h, followed by ASG (control for tetrathionate treatment). d. 2·5 % Glutaraldehyde in phosphate buffer (pH 7·2), 6 h, followed by ASG.
chromosomes is uniform. Secondly, chromosome banding procedures oxidize SH groups to SS cross-links, but do not cause any changes in the distribution of SH and SS, which remains uniform. Thirdly, cross-linking of SH groups produces relatively little stabilization of chromosome structure and has no obvious effect on the patterns produced by standard chromosome banding procedures.

It was the major prediction of the hypothesis put forward by Sumner (1974) that SH and SS groups would be non-uniformly distributed along chromosomes in a pattern corresponding to G-banding. This idea was consistent with a number of observations showing that treatment of chromosomes, either in cell cultures or after fixation, with reagents that attack SS or SH groups can have a marked effect on
chromosome banding (Zakharov et al. 1973; Kato & Yosida, 1972; Utakoji, 1973a, b; Ronne, 1979). It must nevertheless be pointed out that the precise mechanism of action of such reagents in affecting banding has not been established, and such observations merely suggest that in some way SH and SS groups are involved in those aspects of chromosome structure that can be revealed by banding techniques. As mentioned in the Introduction, the hypothesis is also consistent with the idea that positive G-bands are centres of chromosome condensation, and that condensed chromatin is richer in SS cross-links (Sumner, 1983). In spite of this variety of indirect evidence, the observations described here on the uniformity of distribution of SH and SS groups on chromosomes make the original hypothesis untenable; there is no relationship between banding patterns and SH and SS distribution. The distributions of these groups remain uniform both in the earlier (prophase) and later (metaphase) stages of chromosome condensation, and they remain uniform during banding procedures (although SH groups are oxidized to SS). Using reagents that only fluoresce when cross-linking SH groups (DMS and DMSDS), the distribution of cross-linkable sites, whether hydrophobic or hydrophilic, also appeared to be uniform. It should be noted that Buys and his colleagues illustrated uniform distribution of SH groups along chromosomes, except for brighter fluorescence in the nucleolar organizing regions, although their studies were not specifically intended to show this point (Buys & Osinga, 1980; Buys & Stienstra, 1980).

In the original experiments described by Sumner (1974), cross-linking of SH groups was reported to prevent G-banding of chromosomes, the whole chromosome being darkly stained with Giemsa. This observation was one of the main pieces of evidence for the hypothesis put forward in that paper, but was not consistent with a uniform distribution of SH and SS groups on chromosomes. The experiments with sodium tetrathionate and diamide reported in the present paper show in fact that cross-linking of SH has no effect on G-, C- or Q-banding of chromosomes. Furthermore, the cross-linking assays carried out suggest that although most available SH groups are effectively cross-linked, the number of cross-links introduced is relatively small and insufficient to stabilize chromosome structure to any great extent. These results agree with the finding by Sumner (1974) that p-phenylene dimaleimide had no effect on banding. It has been shown that extensive cross-linking, for example with glutaraldehyde, does prevent chromosome banding (Hancock & Sumner, 1982). The effect of mercurial reagents in preventing banding (Sumner, 1974) might therefore be attributable to their more extensive, non-specific fixative action (Pearse, 1980; Horobin, 1982); it should also be noted that mercurial compounds can react with nucleic acids (Horobin & Flemming, 1982; Takeuchi & Maeda, 1976). Reactions of mercurial compounds with chromosomes are therefore likely to be complex and not attributable solely to reaction with SH groups.

It must be concluded that there is no evidence from the results described in this paper that the SH and SS groups of chromosomal proteins are in any way involved in chromosome banding. Although cross-linking of SH to form disulphide cross-links may be involved in chromosome condensation, and different types of chromosome bands may be a reflection of condensation processes, the present work provides no
evidence for a connection between the two. Similarly, there is no evidence on how reagents that are believed to react with SH and SS groups might produce banding patterns on chromosomes.

REFERENCES


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