FACTORS FOR GIEMSA-BAND FORMATION IN AIR-DRIED MAMMALIAN CHROMOSOMES

SUSUMU TAKAYAMA

Biological Laboratory, Faculty of Science, Kwansei Gakuin University, Uegahara, Nishinomiya 662

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Since the advent of the new banding techniques to identify individual chromosomes, a great number of studies have been reported, but the mechanism of band formation is still a matter of controversy. Some authors have regarded band formation as a direct result of a denaturation-reassociation process of repetitive DNA (Drets and Shaw 1971; Schedl 1971; Sumner et al. 1971). On the other hand, it has been reported that proteolytic enzymes such as pronase and trypsin (Dutrillaux et al. 1971; Seabright 1971, 1972) and protein denaturants such as urea, mercaptoethanol and SDS (Kato and Yosida 1972; Shiraishi and Yosida 1971, 1972; Yosida and Sagai 1972), can also produce bands very effectively. It is interesting to note that the banding patterns obtained from these different techniques seem to be virtually identical to each other. Kato and Moriwaki (1972) tested dozens of reagents for their band producing abilities and assumed that the solubilization or extraction of chromosomal proteins must be the primary cause of the appearance of the banded structure.

It seems that since the metaphase chromosomes of eukaryotes mainly consist of DNA and proteins, studying the mechanisms involved in band formation should provide some clue as to the molecular architecture of metaphase chromosomes. From this point of view, the present author has examined in detail the conditions for band formation in mammalian chromosomes.

MATERIALS AND METHODS

HeLa cells and FL cells were employed for the present study. After a colcemid treatment (0.1 μg/ml for 1 h), cells were harvested by trypsinization, treated with 0.075 M KCl solution for 7 min, fixed in ethanol-acetic acid (3:1) and air dried.

In order to identify the effects of experimental conditions on chromosomes as finely as possible, most slides were used immediately after air drying and directly stained with the Giemsa staining solutions without any pretreatment. The staining solutions were prepared by diluting Giemsa-stain with phosphate buffered solutions (NaH₂PO₄ : Na₂HPO₄, KH₂PO₄ : K₂HPO₄), single solutions of other various salts, or Tris-HCl buffered solutions.

Prior to the staining, some slide were refixed with 10 % formalin, 5 % glutaraldehyde or a saturated aqueous solution of picric acid for 2 to 5 minutes. Some of these refixed slides were followed by a brief pretreatment with 0.02 % trypsin solution.
RESULTS

Giemsa solutions diluted with deionized water never revealed any banded structures regardless of their dye concentrations or time of staining. On the other hand, when Giemsa solutions were appropriately prepared with phosphate salts or Tris-buffer, Giemsa staining alone resulted in formation of banded structures to various degrees in fresh slides unless the concentrations of Giemsa were too high. However, even if the Giemsa concentration was adequate (e.g., 1/60) and the time of staining was sufficient (at least 10 min.), the band producing ability decreased with the reduction of the pH value of the staining solution. A phosphate buffered staining solution (1/10 M) with pH value lower than 6 revealed hardly any banded structure, and a solution prepared with 1/10 M NaH₂PO₄ alone (pH 4.8) never produced any band irrespective of the dye concentration or time of staining. However, despite the pH value of 7, the staining solution prepared with considerably diluted phosphate buffered solution (e.g., 1/100 M) failed to produce bands. Potassium phosphate buffered Giemsa solutions gave virtually the same results as sodium phosphate Giemsa did. Staining with Tris buffer Giemsa (1/10 M, pH 7) solution alone also resulted in the production of bands but this solution appeared somewhat less effective than the phosphate buffered Giemsa.

In order to ascertain the significance of pH value as a condition for band induction, the band forming abilities of 9 sorts of aqueous solutions were examined, all of which have pH values higher than 6 and have been reported to be negative for band induction (Kato and Moriwaki 1972). Fresh slides were directly stained for 20 minutes or more with Giemsa staining solutions prepared from these saline solutions. The results are shown in Table 1. Band induction was observed after staining with these different sorts of Giemsa solutions, except for calcium chloride. Although the reason for failure to stain is unknown, it may be possible that the Ca cations combine more predominantly

<table>
<thead>
<tr>
<th>Salts</th>
<th>Conc.</th>
<th>pH</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>KCl</td>
<td>1/10 M</td>
<td>6.5</td>
<td>+</td>
</tr>
<tr>
<td>NaCl</td>
<td>&quot;</td>
<td>6.8</td>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>LiCl</td>
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<tr>
<td>Li₂CO₃</td>
<td>&quot;</td>
<td>11.4</td>
<td>−</td>
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<tr>
<td>&quot;</td>
<td>1/80 M</td>
<td>11.0</td>
<td>+</td>
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<tr>
<td>KHCO₃</td>
<td>1/10 M</td>
<td>8.5</td>
<td>+</td>
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<td>KSCN</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>CH₃COONH₄</td>
<td>&quot;</td>
<td>6.9</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Band inducing ability of Giemsa staining solutions diluted with various salt solutions in the proportion of 1/60.
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with phosphate groups of DNA to form an insoluble compound than Giemsa does. When 1/10 M lithium carbonate was used, chromosome staining was very poor and no bands were found. It seems that the inability to induce bands might be the result of a loss of chromosomal materials through the quite strong alkaline effect of this saline solution. Use of the solution diluted to 1/80 M successfully showed bands. The results mentioned above suggest that it is one of the most important conditions for band formation to expose slides to a solution whose pH value is higher than about 6, while the sort of the solution and the ionic strength of the salt solution also seem to play some significant roles on the phenomenon. It is possible that these factors may cause the structural lesion of chromosomal proteins which in turn induce the occurrence of the banded structure.

As briefly mentioned before, too high concentrations of Giemsa stain in the staining solutions appeared to prevent band induction. Twenty-minute staining with the Giemsa solution prepared with 1/10 M Na2HPO4 solution alone (pH 9.2) in the proportion of 1/8 stained chromosomes very clearly but without bands. To the contrary, twenty-minute staining with the same staining solution, but much diluted (1/60 in dye concentration), revealed clear banded structures. Staining with an even more diluted Giemsa solution resulted in band formation after a longer time of staining. Chromosomes which had once been stained uniformly with the concentrated alkaline Giemsa solution were decolorized by washing the slide in absolute ethanol and then restained in the diluted alkaline Giemsa for 20 minutes. Banded structures were observed as clearly as if the slide had been stained at first with the effectively diluted alkaline Giemsa (Figs. 1 and 2).

It was found that, in general, prestaining with deionized water-Giemsa markedly prevented the chromosomes from revealing bands even after exposing them to the treatments effective for band formation (Figs. 3 and 4). These results seemed to reveal that Giemsa-dye itself acted as a rather preventing factor for band formation. Since it was assumed that Giemsa bands might represent the chromosomal DNA stained with basic dye components of Giemsa stain, toluidine blue, a single basic dye, was adopted to reveal bands. Twenty-minute staining with a phosphate buffered toluidine blue solution (pH 7.0, 0.04 % in dye concentration) showed bands as clearly as the effective Giemsa staining solution did, whereas chromosomes once stained heavily with deionized water-toluidine blue became markedly insensitive to the band inducing treatments unless the stained chromosomes were decolorized prior to the treatments.

If band formations are initiated from some changes in chromosomal proteins, protein fixatives can inhibit induction of bands. Such effects were tested using 10 % formalin, 5 % glutaraldehyde and a saturated aqueous solution of picric acid. Slides were refixed with one of these fixatives for 2 to 5 minutes, rinsed in tap water for 5 minutes, and then stained with a phosphate buffered Giemsa solution (1/10 M, pH 7.0, 1/60 dilution) for 20 minutes with or without pretreatment of trypsin (0.02 %, for 2 seconds). Slides refixed with formalin or glutaraldehyde for 5 minutes no longer revealed any banded structure after the application of the band-inducing procedures, but a picric acid solution appeared somewhat less effective for preventing band formation. The results clearly suggest that the chromosomal proteins are not thoroughly
Figs. 1 and 2. These chromosomes were stained immediately after air drying. 1. Chromosomes stained for 20 min. with Giemsa solution diluted with 1/10 M Na₂HPO₄ into 1/8 (pH 9.2), showing no bands. 2. The same chromosomes restained with the diluted alkaline Giemsa solution for 20 min. after decolorization by washing in ethanol, showing clear bands.

Figs. 3 and 4. These chromosomes were treated 4 days after air drying. 3. These chromosomes were first stained with a deionized water-Giemsa solution (1/30 dilution) for 5 min., and then restained with phosphate buffered Giemsa solution (pH 7.0, 1/30 dilution) for 5 min. after a trypsin treatment (0.02%, 2 sec), showing no bands. 4. The same chromosomes were, then, decolorized by washing in ethanol, and restained with the phosphate buffered Giemsa following the trypsin treatment as mentioned above. This time clear bands were shown.
fixed with ethanol-acetic acid, and that they, therefore, can be readily affected through the band-inducing procedures.

DISCUSSION

It is evident from the present study that pretreatment prior to Giemsa staining is not necessarily indispensable for inducing bands but rather plays a promoting role. Some authors have already reported that Giemsa staining alone produces bands in air-dried chromosomes (Patil et al. 1971; Kato and Moriwaki 1972; Nombela and Murcia 1972), and in those cases, the Giemsa solutions used have been prepared with phosphate buffer solution at about pH 7 or higher. It should be noted that all the pretreatments for producing bands so far reported by other authors have also been followed by staining with Giemsa solutions prepared with buffer solution at about pH 7. Considering these situations, it seems that the pH value of the solution to which chromosomes are exposed is one of the important factors. In addition, the present experiment shows that the sort of the solution as well as its ionic strength have also some significance to band production. It is interesting to note that Dev et al. (1972) have reported that a common factor in the Giemsa-banding method is the pretreatment fixed metaphase chromosomes with a solution which is deficient in the divalent cation, Ca$^{2+}$ and Mg$^{2+}$.

It has been reported that proteolytic enzymes (Dutrillaux et al. 1971; Seabright 1971, 1972) as well as various protein denaturants (Kato and Moriwaki 1972) act as potent band inducing agents. The present study showed that refixing slides with protein fixatives such as formalin or glutaraldehyde markedly prevented occurrence of bands. Formalin can combine with a number of different functional groups of tissue proteins to form bridging links between them (Pearse 1961). The finding, therefore, strongly suggests that the disruptive lesion of chromosomal proteins would be the primary cause of the band induction. It is generally accepted that association of histones with DNA occurs mainly through ionic interaction between the phosphate groups of the DNA and the amino groups of the protein, and according to Bradbury et al. (1967) hydrogen bonding and hydrophobic interactions are also involved. It is likely that histones play a structural role and involved in the coiling of DNA into the compact form of the chromosome (Cole 1962; Richards and Pardon, 1970; Luie and Dixon 1973). According to Huberman and Attardi (1966), human chromosomes include about twice as much proteins at metaphase as at interphase. It seems likely that such increased proteins which consist of both histones and non-histon proteins play a role on maintenance of a chromosomal configuration at metaphase. The present study showed that chromosomal proteins were not thoroughly fixed with acetic acid-ethanol. It has been noted that despite the fixing effect by precipitating the nucleoproteins, the acid fixatives progressively break the bonds between the nucleic acids and the proteins, and therefore, a long sojourn in Carnoy causes extraction of DNA (Pearse 1961). It is possible, therefore, that the salt solutions at appropriate concentrations with pH values higher than 6 which are used in Giemsa solutions, may severely affect the chromosomal proteins to disrupt the interaction of the DNA and the proteins. Thus, the DNA molecules may easily become to give rise to some distortion in their arrange-
ments under the condition provided by the salt solution with pH value higher than 6. This resulting distortion may appear to be the formation of bands. If so, the emergence of constant banding pattern may be a reflection of the characteristic localization of some special base sequences along the chromatid. Compositional heterogeneity of DNA along the human chromosomes has been studied by Hsu et al. (1972) and Chapelle et al. (1973).

On the other hand, it seems that the maine component of Giemsa-stain is a basic dye which tends to be cationic, and therefore, dye itself has a strong affinity to combine with phosphate groups of DNA molecules. When chromosomes are exposed to such a basic dye, the chromosomal DNA would combine with the dye molecules, thus the stained chromosomes would become more stable than unstained chromosomes and consequently, they would become resistant to band induction. In fact, in the present study, those chromosomes once stained with deionized water-Giemsa or -toluidine blue became reluctant to show bands. It can be said, therefore, that the dye concentration of the staining solution is also critical to produce bands.

**SUMMARY**

The conditions for Giemsa-band formation in air-dried chromosomes of HeLa cells and FL cells were examined in detail. When chromosome preparations were stained immediately after air drying, Giemsa staining alone, if the staining solutions were prepared with saline solutions of which pH values were higher than 6, produced bands. It seems that a variety of pretreatments including trypsin treatment so far reported are not essential conditions for band formation but rather promoting ones. Brief refixations with formalin, glutaraldehyde or picric acid solution prior to band inducing procedures markedly prevented band formation. Basic dye itself appeared to act to prevent band formation, since chromosomes once stained with Giemsa or toluidine blue became extremely reluctant to show bands. It seems very likely that the structural lesion of chromosomal porteins would be an indispensable triggar to induce banded structures.

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**LITERATURE CITED**


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