Delayed Response of QM- and DA/DAPI-Fluorescence in C-Heterochromatin of the Small Japanese Field Mouse, Apodemus argenteus

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Delayed Response of QM- and DA/DAPI-Fluorescence in C-Heterochromatin of the Small Japanese Field Mouse, *Apodemus argenteus*

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ABSTRACT—The small Japanese field mouse *Apodemus argenteus* has the diploid chromosome number of 46, carrying rather large centromeric C-heterochromatin in most of the 44 autosomes and a large amount of C-heterochromatin in the sex chromosomes: the largest subtelocentric X was heterochromatic in almost two-fifth (whole short arm and proximal part of the long arm) of its entire length and the medium-sized acrocentric Y was totally heterochromatic. The C-heterochromatin (C-positive) areas, other than those of the Y and smallest three pairs, had a unique property of "delayed QM-fluorescence", which has not been reported to-date, showing dull QM-fluorescence immediately after exposure to blue light (BL), but gradually turning to bright fluorescence in a few minutes. The fluorescence intensity gradually decreased after attaining its peak, and finally became extinct. A similar pattern of fluorescence was also obtained in DA/DAPI-stained X chromosome C-heterochromatin, but not in autosomal C-heterochromatin. No such dull-to-bright transition of QM-fluorescence could be obtained by CMA_3 staining, for which the C-positive areas were apparently negative even after overexposure to BL. These facts indicate that the C-positive areas of *A. argenteus* showing dull-to-bright transition of QM-fluorescence contain A-T rich DNA. The delayed QM-fluorescence was found only in *A. argenteus*, in thirteen mammalian species so-far examined. Furthermore, this unique property of QM-fluorescence could be artificially altered to non-delayed ordinary type of fluorescence by sequentially pretreating the fixed chromosomes with hydrochloride and barium hydroxide solutions. The cytological implication of the delayed fluorescence in the C-heterochromatin of *A. argenteus* is briefly discussed.

INTRODUCTION

A fluorescent alkylating agent, quinacrine mustard (QM), specifically binds to A-T base pairs of DNA by intercalation (Sumner, 1990). Thus, chromosomal regions showing bright QM fluorescence contain DNA rich in adenine and thymine residues (A-T rich DNA). This relation has been confirmed in a variety of animal and plant species (Barsacchi-Pilone *et al.*, 1986; Cionini *et al.*, 1985; Jalal *et al.*, 1974; Lau *et al.*, 1977; Ranganath *et al.*, 1982; Schmid *et al.*, 1979; Sumner, 1990). However, there were exceptions to this generalization for which no persuasive explanation has been made so far. For example, the centromeric regions of *Mus musculus* chromosomes are all heterochromatic, containing a large amount of A-T rich DNA, yet the centromeric regions show dimmer fluorescence for QM staining than the rest of the chromosome (Nesbitt and Francke, 1973). Fluorescent chromosome analyses have been done on the assumption that the abundantly fluorochrome-bound areas of chromosomes show the highest intensity of fluorescence immediately after exposure to BL under microscope irrespective of the kind of fluorochromes as well as species and sex, decrease fluorescence intensity with the time of exposure to BL and finally become extinct.

It has been thought that the decline in the intensity of fluorescence after exposure to BL is an inevitable attribute of the fluorochrome-bound chromosomes irrespective of animal or plant origin and of euchromatic or heterochromatic aspects. In fact, this decline of fluorescence intensity has been the largest weak point of fluorescent analysis of chromosomes, and researchers have struggled against how to retard the decline of fluorescence during the course of microscopic observation. However, the authors found unexpectedly on the occasion of Q-banding analysis that C-heterochromatin of the small Japanese field mouse *Apodemus argenteus* showed just reverse pattern of fluorescence for the QM staining; the fluorescence condition in the C-positive areas transformed from dull fluorescence to bright fluorescence with the lapse of time after exposure to BL (Obara, 1994; Obara and Sasaki, 1995). This unusual property of fluorescence was tentatively termed "delayed fluorescence" in this report. As far as the authors are aware, no such delayed QM-fluorescence has been referred to so far.

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In this short report, the unique pattern of QM-fluorescence in the *A. argenteus* C-heterochromatin is examined in detail in comparison with the fluorescent behavior after the double staining with DNA binding A-T specific antibiotic distamycin A (DA) and the A-T specific fluorochrome 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) or after the fluorescent staining with G-C specific antibiotic chromomycin A_3 (CMA_3), and the technical development of artificial switch of delayed fluorescence to non-delayed ordinary fluorescence is also described briefly.

MATERIALS AND METHODS

A total of forty four specimens from young and adult of the small Japanese field mouse *Apodemus argenteus* were collected from 10 localities of Tohoku and Hokkaido districts (South Hakkoda, Mt. Iwaki, Shirakami mountains, Zatoishi and Nagamine of Aomori Prefecture, Yatate of Akita Prefecture, Atami-cho of Fukushima Prefecture and Hayakita-cho, Kamishihoro-cho and Shibecha-cho of Hokkaido).

Chromosome preparations were made from bone marrow cells after colchicine treatment by intraperitoneal injection (0.1 ml of 12.5 μg colchicine/ml) for half an hour. Air-dried chromosomes were stained with 50 μg/ml solution of quinacrine mustard (QM) or 200 μg/ml solution of chromomycin A_3 (CMA_3), or sequentially stained with 25 μg/ml solution of distamycin A (DA) and 0.3 μg/ml solution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The QM, CMA_3 and DA/DAPI staining were done according to Caspersson *et al.* (1971), Amemiya and Gold (1987) and Haaf *et al.* (1986), respectively. After chromosome preparation the rest of the cell suspension in Carnoy's fixative was preserved at -20°C, which served as a source for chromosome preparations, when necessary. For G- and C-band staining, the ASG method of Sumner *et al.* (1971) and the BSG method of Sumner (1972) were adopted, respectively.

Fluorescence analysis was made with an Olympus Biological System Microscope BH-2 equipped with epifluorescence (BH2-RFC; USH-102D lamp). A B435 exciter filter was used for the QM and CMA_3 staining, and a BV405 exciter filter for the DA/DAPI staining. Microphotographs were taken using an UVFL × 100 or UVFL × 40 objective and Technical Pan 2415 film developed in Kodak D19. Exposure time ranged from 20 to 90 sec depending on the purpose of the experiment.

To examine the effects of HCl, NaCl and $Ba(OH)_2$ on the fluorescence conditions, air-dried chromosome preparations were pretreated with 2 M NaCl (2-4 hr at room temperature), 0.2 N HCl (1-24 hr at room temperature), 5% $Ba(OH)_2$ (7-10 min at 50°C) and with the combination of 0.2 N HCl (1 hr at room temperature) and 5% $Ba(OH)_2$ (7 min at 50°C) prior to the QM staining.

The fluorescence behavior of C-heterochromatin with QM and CMA_3 was checked with bone marrow cells from at least two specimens of thirteen mammalian species including inbred mouse and rat strains: Insectivora; *Chimarrogale himalayica* and *Urotrichus talpoides*, Chiroptera; *Pipistrellus abramus*, Rodentia; *Clethrionomys rutilus mikado*, *Microtus montebelli*, *Apodemus speciosus*, *A. argenteus argenteus*, *A. argenteus hokkaidi*, *A. peninsulae giliacus*, *Rattus norvegicus* (Fischer rat), *Mus musculus* (BALB/c mouse), and Carnivora; *Mustela nivalis*, *M. namiyei* and *M. sibirica itatsi*.

RESULTS

Karyotypic profiles of A. argenteus

The small Japanese field mouse *A. argenteus* has the diploid number of 46 chromosomes, consisting of 20 pairs of gradually decreasing acrocentrics, 2 pairs of medium and small metacentrics (M1 and M2) and the sex chromosomes X and Y. All the autosomes have rather large centromeric C-heterochromatin, and the largest subtelocentric X chromosome carries a large amount of C-heterochromatin (tentatively termed as C-block) which makes-up almost two-fifth of its entire length (Fig. 1, upper). In well spread metaphases, some small interstitial and telomeric C-bands were also detected on the long arm of the X chromosome. The Y chromosome was a medium-sized acrocentric element, carrying deeply stained centromeric and a little lighter whole arm C-heterochromatin, although Yoshida *et al.* (1975) considered the entire Y chromosome to be deeply stained after C-banding. All the chromosomes were identified by their G-banding pattern (Fig. 1, lower). It is known that mammalian chromosome areas that are C-positive such as centromeric, interstitial and telomeric C-bands are negative by G-band staining. However, the C-positive areas of *A. argenteus* are apparently positive by G-band staining. Typical heteromorphism was observed in the X chromosomes of one female specimen from Hayakita-cho, Hokkaido. Details of this heteromorphism will be reported elsewhere.

Characterization of QM-, DA/DAPI- and CMA_3-fluorescence in the C-positive areas

Figure 2 shows the transition of QM-fluorescence in the metaphase chromosomes of *A. argenteus* following exposure to BL (435 nm) under a microscope. Immediately after exposure to BL, the QM staining induced, excluding the C-positive areas, a typical Q-banding pattern which essentially corresponds to the G-banding pattern (Fig. 2, left). The C-band areas including the C-block seemed to be all negative for the QM staining except for the Y chromosome (not present in Fig. 2) and the smallest pairs, in which centromeric C-heterochromatin was rather brightly fluorescing from the beginning of exposure and gradually turned pale. After 1.5 minutes of exposure, these Q-negative areas of the same plate increased their fluorescence intensity, and in contrast in the remaining (euchromatin) areas it decreased to some extent (Fig. 2, middle). Up to this stage, the X chromosome fluoresced with a rather homogeneous appearance along its entire length. Five minutes later all these Q-negative (C-positive) areas turned into bright fluorescence and, in reverse, the remaining areas turned into dull fluorescence (Fig. 2, right). This unique property of fluorescence was demonstrable even under varied conditions such as different pH's (pH 4.5, 7.0, 9.0) of the staining solution, the condition of chromatin condensation (long-stretched less condensed prometaphase chromosomes or short and thick highly condensed chromosomes), aging (1 day, 1 week, 1 month) after chromosome preparation or sex and age. Thus, the property of delayed QM-fluorescence could be considered to be of intrinsic to *A. argenteus*, but not the product of technical causes. Furthermore, this unique pattern of transition of QM-fluorescence was detected only in *A. argenteus*, of the thirteen species of mammals examined (Table 1). The time lapse analysis of QM-fluorescence revealed that the C-positive areas of twelve species other than

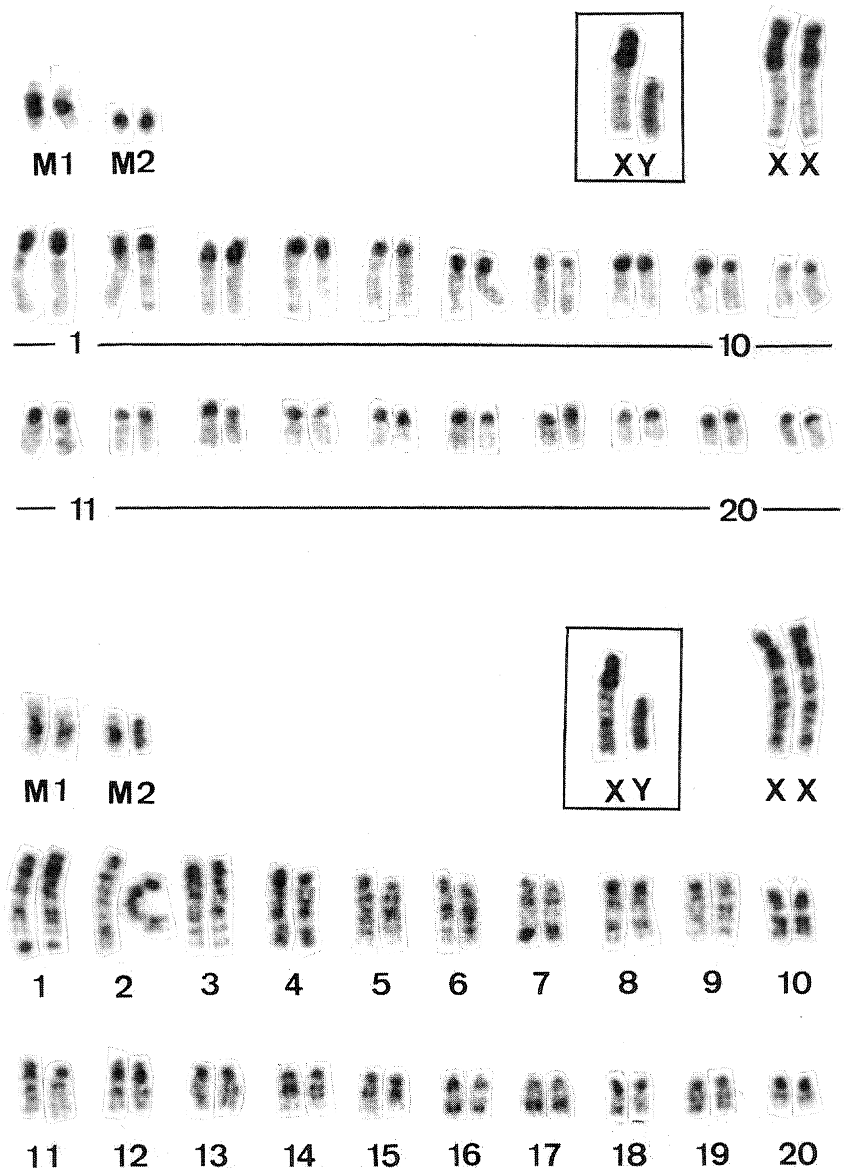

Fig. 1. C-banded (upper) and G-banded (lower) karyotypes of *Apodemus argenteus* (93Aah-2). X and Y in squares are the sex chromosomes from a male specimen (93Aah-3).

A. argenteus showed dull fluorescence for the QM staining even with overexposure to BL. The C-positive areas of *A. argenteus* and *Mus musculus* were apparently negative for the CMA_3-staining irrespective of the exposure time to BL, and those of the remaining species showed bright CMA_3-fluorescence immediately after exposure to BL.

The C-block of *A. argenteus* showed dull-to-bright transition of fluorescence with the DA/DAPI-staining, but the autosomal C-heterochromatin remained dull-fluorescent without turning into bright fluorescence (Fig. 3). The C-positive areas were apparently CMA_3-negative in the autosomes as well as in the C-block, and the dull-to-bright transition of fluorescence could not be observed in the CMA_3-stained metaphases, even though the exposure time was extended up to ten minutes (Fig. 4). Thus, so far as the initial stage of exposure to BL is concerned, these C-positive areas were apparently dull fluorescent for any of the QM, DA/DAPI and CMA_3 staining. The differential staining characteristics of the X, Y, M1 and M2 chromosomes, all of which can be easily identified on the basis of their morphology and staining patterns, are summarized in Fig. 5. The Y chromosome heterochromatin showed, contrary to that of the X chromosome, an ordinary pattern of fluorescence with the QM and DA/DAPI staining, being characterized by bright fluorescence from the beginning of exposure to BL and gradual decline of fluorescence. The Y chromosome brightly fluoresced with the CMA_3 staining, though the intensity seemed to be slightly weaker than that with QM or DA/DAPI. The M1

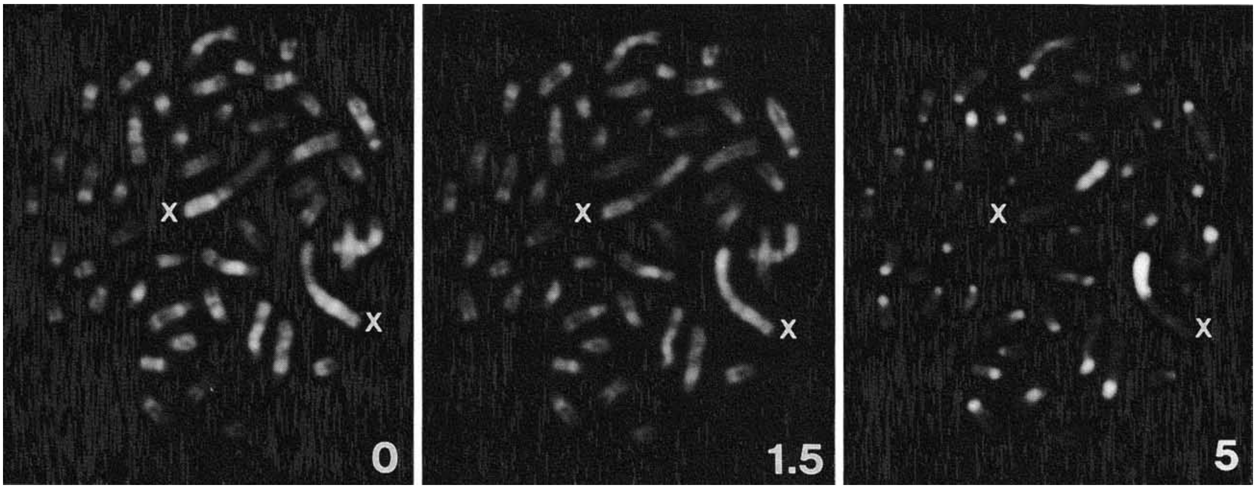

Fig. 2. Sequentially photographed QM-fluorescence profiles of the same metaphase from a female specimen (93Aaa-1). X: X chromosome, 0: initial stage of BL illumination, 1.5: one and half minutes after BL illumination, 5: five minutes after BL illumination.

Table 1. QM- and CMA_3-fluorescence in the C-positive areas of thirteen species of mammals examined

Species	C-heterochromatin areas (C-bands)		
	CMA_3	QM	Bases
Insectivora			
Chimarrogale himalayica	+	–	GC-rich
Urotrichus talpoides	+	–	GC-rich
Chiroptera			
Pipistrellus abramus	+	–	GC-rich
Rodentia			
Clethrionomys rutilus mikado	+	–	GC-rich
Microtus montebelli	+	–	GC-rich
Apodemus speciosus	+	–	GC-rich
Apodemus peninsulae giliacus	+	–	GC-rich
Apodemus argenteus argenteus	–	– → +	AT-rich
Apodemus argenteus hokkaidi	–	– → +	AT-rich
Rattus norvegicus (Fischer rat)	+	–	GC-rich
Mus musculus (BALB/c mouse)	–	–	AT-rich
Carnivora			
Mustela nivalis	+	–	GC-rich
Mustela namiyei	+	–	GC-rich
Mustela sibirica itatsi	+	–	GC-rich

+: bright fluorescence
–: dull fluorescence
Bases: Base pair composition estimated from QM- and CMA_3-fluorescence.

chromosome C-heterochromatin showed essentially the same fluorescence behavior with the X chromosome C-heterochromatin with the QM, DA/DAPI and CMA_3 staining, but the M2 chromosome C-heterochromatin seemed to be different in the fluorescence behavior for both the QM and DA/DAPI staining from the X and M1 chromosome C-heterochromatin, though these areas were all pale with the CMA_3 staining.

Artificial switch from delayed fluorescence to non-delayed ordinary fluorescence

In order to shed light on the causality of this unique property of fluorescence, chromosomes were pretreated, prior to the fluorescent staining, with 2 M NaCl or 0.2 N HCl, by which histone-phosphate bindings loosen and histone proteins are depleted from chromosomes (Comings, 1978; Comings and Avelino, 1974; Okada, 1985), or with 5% $Ba(OH)_2$ and by which DNA molecules in chromosomes are denatured for the most part (Sumner, 1990). All of these pretreatments had little

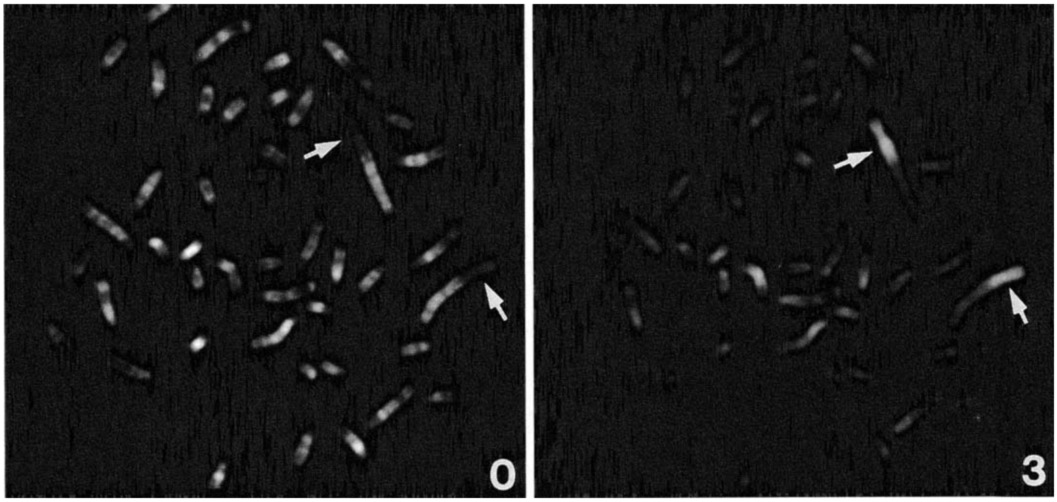

Fig. 3. Sequentially photographed DA/DAPI-fluorescence profiles of the same metaphase from a female specimen (93Aah-1). Arrows indicate the C-block areas of the X chromosomes. 0: initial stage of BL illumination, 3: three minutes after BL illumination.

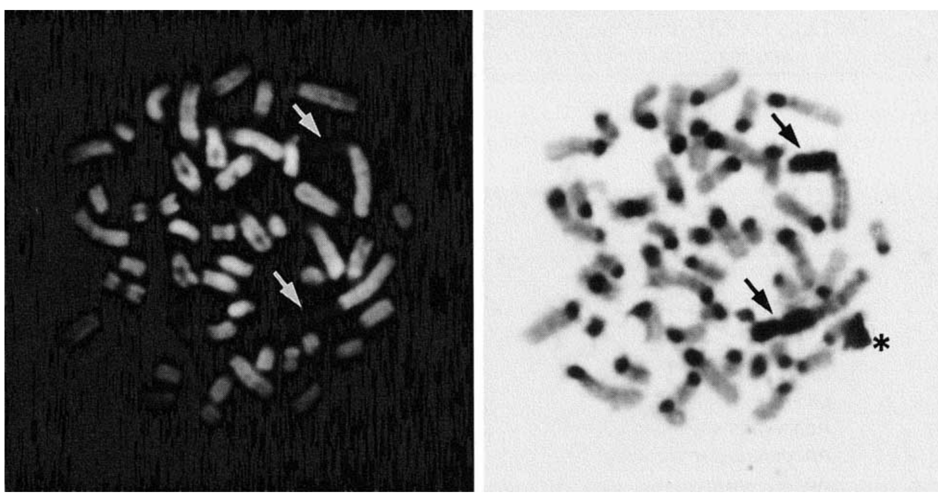

Fig. 4. CMA_3-stained (left) and C-banded (right) profiles of the same metaphase from a female specimen (93Aah-1). Arrows indicate C-block areas of the X chromosomes. Asterisk is a debris which adhered during processing of C-banding.

influence on the property of the dull-to-bright transition with QM-fluorescence, as the C-positive areas gradually turned, even after these pretreatments, from dull fluorescence to bright fluorescence in the same way as the controls, though the transition time seemed to be shortened to a certain degree in the barium-treated metaphases (Fig. 6). However, the sequential combined pretreatment with 0.2 N HCl and 5% $Ba(OH)_2$ resulted in drastic alteration in the fluorescence property of C-heterochromatin: the C-positive areas brightly fluoresced without delay from the beginning of exposure to BL after the treatment, though the euchromatin areas (C-negative areas) showed a dim and homogeneous pattern of fluorescence along chromosomes without any distinguishable Q-bands (Fig. 7). After the double treatment the Y chromosome was rather pale in contrast with the bright fluorescence in the C-block and other C-band areas.

DISCUSSION

It has been the general understanding in fluorescent analyses of chromosomes that, aside from unusual behavior of fluorescence caused by technical causes, fluorescence intensity under a microscope is the strongest immediately after exposure to BL, gradually fading with time of exposure. Contrary to this inevitable fading of fluorescence, the fluorescence kinetics of QM-bound C-heterochromatin of *A. argenteus* is quite unusual in that the fluorescence intensity in most of the C-heterochromatin areas grows from "dull" to "bright" with the lapse of a few minutes after exposure to BL. This transition of QM-fluorescence could be retarded at will by controlling the dosage of BL with a focusing knob and an ND filter; the weaker the BL dosage becomes, the longer the time necessary for the transition to occur. After peaking, the

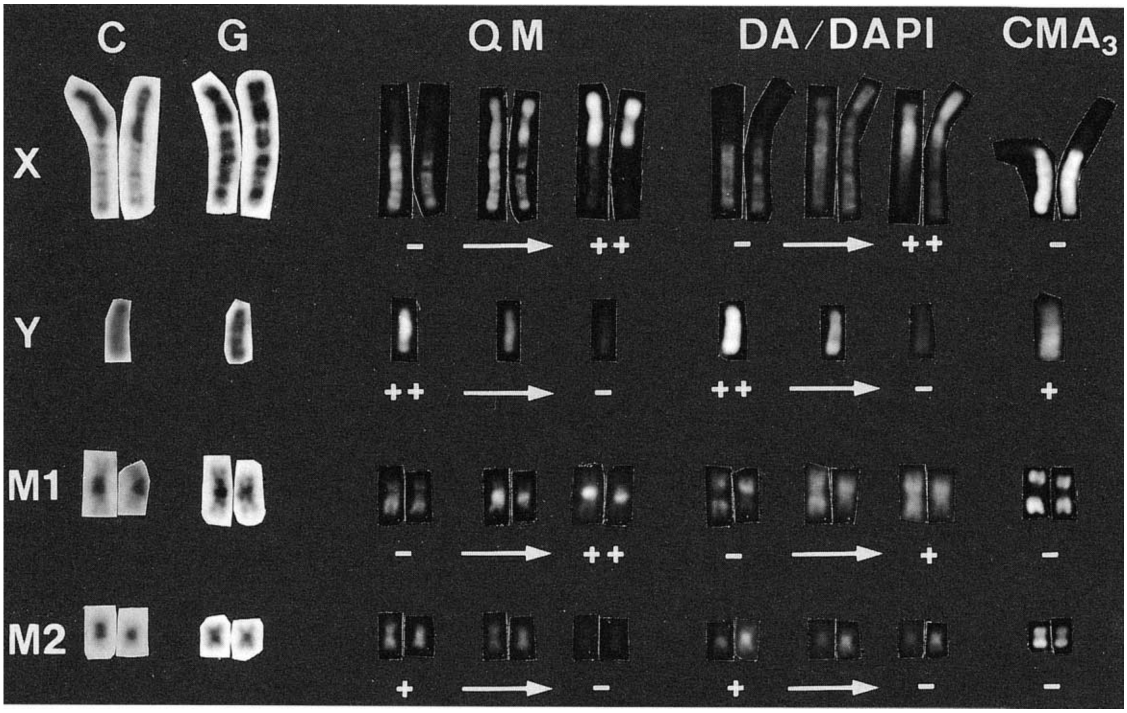

Fig. 5. Representative differential staining profiles of the X, Y, M1 and M2 chromosomes. From the left, C- and G-banded and QM-, DA/DAPI- and CMA_3-stained chromosomes. –: dull fluorescent, +: fluorescent, ++: bright fluorescent.

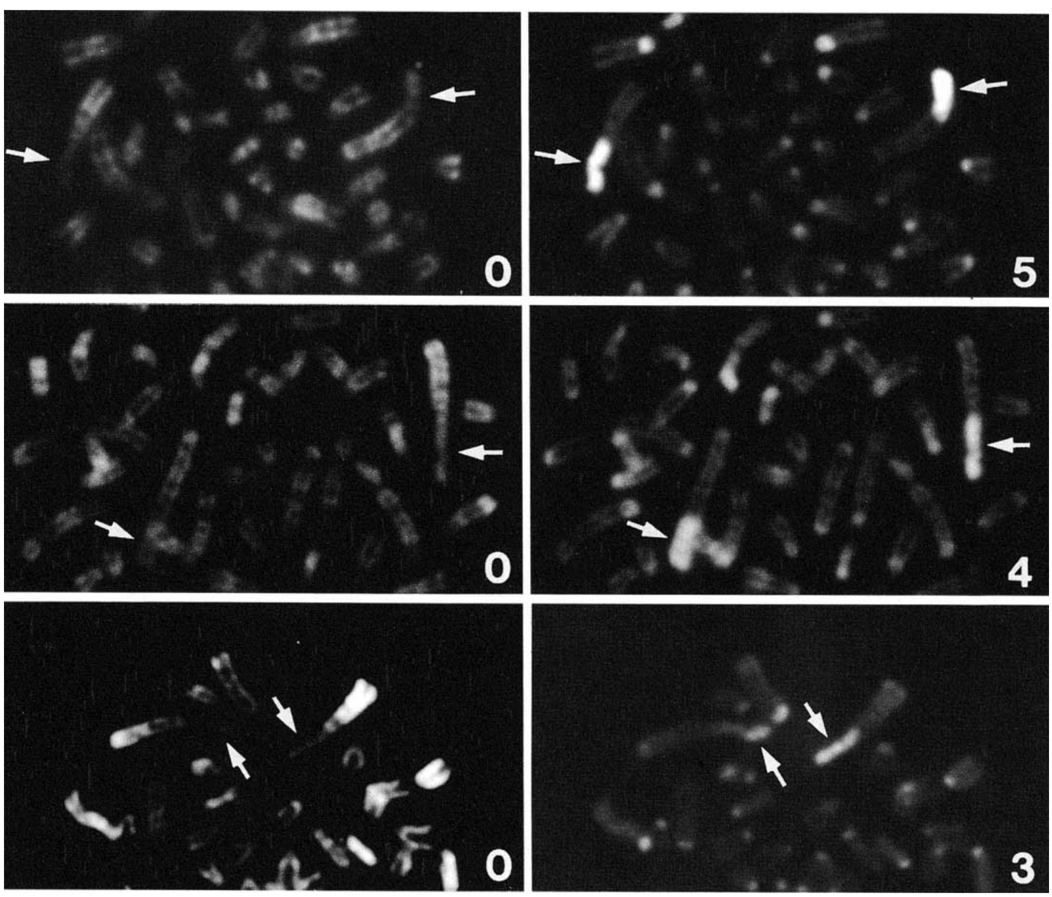

Fig. 6. Sequentially photographed QM-stained metaphases after single pretreatment with 2 M NaCl (upper), 0.2 N HCl (middle) or 5% $Ba(OH)_2$ (lower). Left: initial stage of BL illumination, Right: three to five minutes after BL illumination. Arrows indicate the C-block areas of the X chromosomes.

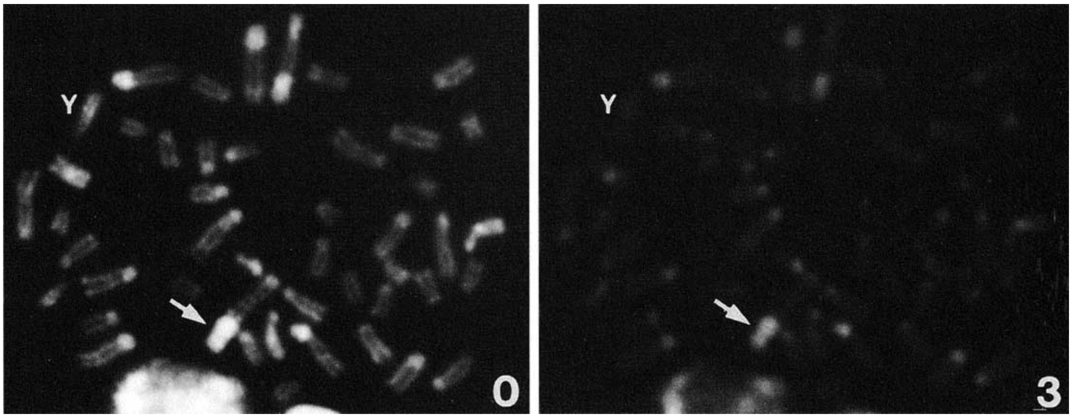

Fig. 7. Sequentially photographed QM-stained metaphases after double pretreatment. Left: initial stage of BL illumination, Right: three minutes after BL illumination. Y: Y chromosome. Arrows indicate the C-block areas of the X chromosomes.

fluorescence intensity attenuated sooner or later with an ordinary fluorescence behavior shown by gradual fading. Therefore, this phenomenon of fluorescence transition may reflect delayed reaction of intercalated QM molecules to BL. The dull-to-bright transition of QM-fluorescence was ascertained in all 44 specimens collected from 10 different localities of Hokkaido and Tohoku districts. No significant difference in fluorescence behavior was detected between two subspecies, *A. argenteus hokkaidi* from Hokkaido and *A. argenteus argenteus* from Tohoku district. Therefore, the delayed response of QM-fluorescence can be regarded as a specific cytogenetic character irrespective of collecting localities or subspecies.

The M1 chromosomes and all autosomal acrocentrics other than two smallest pairs showed, as noted above, dull-to-bright transition of QM-fluorescence in their centromeric C-heterochromatin, but markedly differed in DA/DAPI-fluorescence from the X chromosome C-heterochromatin (Figs. 3 and 5). This may reflect the difference in the binding mode of QM and DA/DAPI to C-heterochromatin DNA, that is "intercalation" in QM (Sumner, 1990) or "preferential groove binding" in DA/DAPI (Schweizer, 1980; Schweizer *et al.*, 1978), if the QM-intercalation is inducible in all C-heterochromatin, and in the DA/DAPI staining DA selectively binds to C-heterochromatin DNA of acrocentric autosomes and DAPI selectively binds to C-heterochromatin DNA of X, Y, M1 and M2 chromosomes, respectively. The Y and M2 chromosomes were also different in their fluorescent patterns from that of the X, M1 and autosomal acrocentrics mentioned above. The centromeric C-heterochromatin of the smallest two pairs of acrocentrics showed M2 type of fluorescence for either the QM or DA/DAPI staining. From these observations the following five types of C-heterochromatin could be classified, so far as the fluorochromes examined are concerned, in the *A. argenteus* chromosomes: (1) the X type C-heterochromatin which shows delayed QM- and DA/DAPI-fluorescence and dull CMA_3-fluorescence, (2) the M1 type C-heterochromatin which shows delayed QM-fluorescence, delayed but moderate DA/DAPI-fluorescence and dull CMA_3-fluorescence, (3) the M2 type C-heterochromatin which shows non-delayed QM- and DA/DAPI-fluorescence and dull CMA_3-fluorescence, (4) the acrocentric type C-heterochromatin which shows delayed QM-fluorescence, dull DA/DAPI-fluorescence and dull CMA_3-fluorescence and (5) the Y type C-heterochromatin which shows non-delayed bright QM- and DA/DAPI-fluorescence and rather bright CMA_3-fluorescence.

The C-positive areas of the *A. argenteus* chromosomes showing dull-to-bright transition of QM-fluorescence can be considered, according to the Sumner's view (1990), to contain A-T rich DNA as those of *Mus musculus* chromosomes, and this is well supported by the finding that these areas were apparently CMA_3-negative in either species. In spite of such similarities (AT-richness and CMA_3-negativity), these two murid species showed quite a different response to QM-fluorescence: in the former C-heterochromatin showed delayed QM-fluorescence and in the latter no sign of delayed response was seen. It may be worth noticing that in either species the response of C-heterochromatin could be changed for QM-fluorescence from delayed fluorescence in *A. argenteus* or dull fluorescence in *Mus musculus* to non-delayed bright fluorescence by sequentially pretreating fixed chromosomes with 0.2 N hydrochloride and 5% barium hydroxide. In none of the single pretreatments by 0.2 N hydrochloride, 2 M sodium chloride or 5% barium hydroxide, did the BL illumination exert an influence, at least immediately after exposure to BL, on the DNA-bound QM molecules up to visually detectable levels of fading in fluorescence. In all probability the transformation from delayed type of QM-fluorescence to non-delayed type is caused by structural alteration of C-heterochromatin by the sequential combined pretreatment. It may be possible that the unique structural organization of C-heterochromatin, probably resulting from excessive chromatin packaging, was altered by the combined pretreatment so as to sustain directly the action of the light energy to the DNA-bound QM molecules. The combined pretreatment made such transformation possible also in the mouse C-heterochromatin: the combined pretreatment could induce bright QM-fluorescence in these C-positive areas immediately after exposure to BL.

Neither the precise mechanism of delayed QM-fluorescence, nor the exact nature of such unusual C-heterochromatin, is yet understood. It may be tempting to speculate, in the light of the photooxidation view presented by Ferrucci and Mezzanotte (1982), that the QM-stained C-heterochromatin of *A. argenteus* is prone in itself to be gradually photooxidized with continued illumination of BL under a microscope, thereby interspersed guanine residues are photolytically destroyed, and in consequence increasing the QM-fluorescence intensity. In fact, we recently found in our preliminary experiment that the methylene blue-mediated photooxidation for the fixed *A. argenteus* chromosomes could induce bright fluorescence on the QM-bound C-heterochromatin areas immediately after exposure to BL. Molecular cytogenetic analyses such as FISH analysis with the DNA probes from the *A. argenteus* C-heterochromatin and restriction enzyme banding analysis may also be quite informative for the mechanical solution of this delayed fluorescence phenomenon. These studies are now in progress in our laboratory.

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